STUDIES ON THE FUNCTIONS OF NOCICEPTIVE AFFERENTS IN THE SKIN AND THEIR MICROVASCULAR INTERACTIONS.

A Thesis submitted to University of Adelaide
for the Degree of Doctor of Medicine

by

Roderick Alan Westerman

March, 1994.

Department of Physiology,
Monash University,
Clayton, Vic 3168

iii. ABSTRACT OF THESIS

[Reference nos. cited in abstract are candidate's bibliography of 29 papers]

Physical, chemical, and neurophysiological processes responsible for fabric-evoked discomfort, itch and prickle sensation, and skin rash have been defined[1,2,3,4,5,6]. Microneurographic recording from identified sensory fibres during itching in human subjects evoked by histamine iontophoresis characterized neural and sensory correlates [3,4]. Noxious transcutaneous electrical stimulation (TENS) evoked prickle, or pain, neurogenic inflammation and rash[3,4,5]. Algorithms defined fabric properties most likely to generate discomfort, with predictive value for the textile industry.

Laser Doppler velocimetry (LDV) measured neurogenic inflammation evoked by transcutaneous electrical skin stimulation and iontophoresis of vasoactive compounds in human skin[7,8,10,11,12,15]. Cutaneous electrical axon reflex and microvascular responses are reduced in human diabetes mellitus[9,12] with endothelial function also impaired in streptozotocin diabetic rats[13,15]. The impairment depended on the duration of diabetes, being partly reversed by insulin treatment[13,15].

Primary sensory nerves in skin have nicotinic acetylcholine receptors[16,17].Systemic nicotine exposure for 7-14 days enhances cutaneous axon reflexes[16,18];maternal nicotine exposure, whether gestational or lactational, enhances cutaneous axon reflexes in neonatal rat[18]. Sensory denervation impairs cutaneous microvascular function and significantly prolongs blister healing time in rat hind paw[17,19].

On normal skin acute capsaicin evokes concentration-dependent inflammation and pain[22]. Repeated capsaicin-treatment (0.05% 3-4x daily for 5-6 days) desensitized skin for up to 4 weeks[22] and attenuated axon reflexes, but not autonomic responses[22]. In post-herpetic neuralgia repeated capsaicin variably desensitized affected dermatomes[20,21],affording >60% of patients worthwhile pain reduction[21] which did not correlate with the extent of desensitization measured by thermal threshold testing[20,21].

Simultaneous comparisons between infrared photoplethysmography or laser Doppler velocimetry (LDV), and absolute skin blood flow using radioactive-labelled microspheres, showed linear correlation[10] and functional compartmentalization of skin microvasculature[25]. Infrared laser wavelengths longer than 780nm penetrated epidermis more deeply[24].

In patients with diabetes mellitus, severity of autonomic neuropathy (measured by oesophageal and gastric emptying times) and cutaneous neurovascular dysfunction (electrical axon reflexes) was generally concordant[26]. Similarly, there was correspondence between the severity of the somatic sensory neuropathy (thermal perceptual thresholds) and autonomic neuropathy[26]. Vibration exposure and smoking may result in impaired neurovascular responses[27] and may be confounding factors for the assessment of neurovascular function[27]. These non-invasive techniques have potential as clinical measures of small nerve fibre and microvascular function in many types of neuropathic disorders[28,29], and for investigation of Raynaud's phenomena and conditions such as scleroderma[27].

(367 words)

iv. DECLARATION

The work described in this thesis was carried out in the Department of Physiology, Monash University; the International Diabetes Institute , Caulfield General Medical Centre; the Departments of Endocrinology and Medicine, Royal Adelaide Hospital; Department of Physiology, Heidelberg University (FRG); Department of Pharmacology, Medical University, Pecs (Hungary); CSIRO Division of Textile Industry, Belmont, (Geelong); and CSIRO Division of Animal Physiology, Prospect,(NSW). The reprints and photocopies of twenty nine original articles here are reports of research initiated and carried out by the candidate alone, as an equal collaborator, or in collaboration with graduate students conducting research under his direct supervision. Other papers of the candidate published during the period of this thesis but on topics not directly pertaining to this Thesis have not been mentioned.

The work has not been submitted to any other University or Institution for a higher degree, with the exception of portion of papers number 7,8,11,15,16, in collaboration with Amy M.Low who submitted this work to Monash University for the Degree of Ph.D.

To the candidates's knowledge and belief the thesis contains no material previously published or written by another person except where due reference is made in the text of the thesis.

The candidate is willing to make the thesis (or parts thereof) available for photocopying and loan if it is accepted for the award of the degree of Doctor of Medicine.

Signed:

Roderick Alan Westerman

v. ACKNOWLEDGEMENTS

The author is particularly indebted to Dr. Russell Garnsworthy for initially suggesting that physiological mechanisms of prickle sensations evoked by fabric should be examined critically, and for his subsequent collaboration and continuing friendship.

The encouragement, support and helpful discussions of Professors Manfred Zimmermann, and Hermann Handwerker in Heidelberg (FRG), and Janos Szolcsanyi in Pecs (Hungary) are gratefully acknowledged. The research in this thesis has been greatly facilitated by the enthusiastic assistance and collaboration of Paul Kenins, Walter Magerl, Robert Widdop, Amy Low, Ric Roberts, Carol Delaney, Alan Walker, Robert Mayfield, Andrew Hahn, and Richard Carr. The technical assistance of Ray Gully, Craig Hogan, Julie Hannaford, Brad Carter, Peter Kent, Julie Kiln and Kate Tuck was valuable and much appreciated.

The study of human diabetic neuropathy and the subsequent invaluable database of patients with diabetic neuropathy would not have commenced without the encouragement, imagination and support of Professor Paul Zimmet, Dr. Matt Cohen and the Diabetic Support Association, International Diabetes Institute, Caulfield General Medical Centre. To all these I am pleased to acknowledge my debt of gratitude.

Most academics make unceasing demands on their family, and the author is no exception. This work would not have been possible without the constant support, love, understanding and encouragement of my wife Manci, and children Michael, Susan and David. Later in these studies, my grandchildren Nicholas and Laura Westerman, and Katherine and Douglas Stott also generously gave their love each in their own way.

vi.

ESSAY

"Studies of nociceptive afferents in the skin and their microvascular interactions: A review."

This essay reviews cutaneous sensory receptors and their nerves. Attention will focus on nociceptors and mechanisms involved in nociception, pain sensations, neurogenic inflammation and nocifensor (motor) functions of primary sensory nerves. Mechanisms of prickle, itch and capsaicin desensitization of nociceptive afferents are also considered. Clinical tests of neurovascular function are discussed including skin blood flow measurements, iontophoresis of vasoactive substances, and microvascular function. Measurement of sensory nerve and microvascular dysfunction in diabetes mellitus and other neuropathic states are also reviewed.

1. Cutaneous nerves and sensory receptors

Human cutaneous nerves contain many myelinated nerve fibres ranging in diameter from about 1-16μm and about five times as many non-myelinated fibres which are less than 2μm in diameter. For example, the human sural nerve has approximately 8,500 myelinated and 43,000 unmyelinated fibres (Dyck, Gutrecht et al., 1968). The myelinated fibres, termed A fibres, are subdivided into Aα,β (6-16μm) and Aγ,δ (2-6μm) groups, while unmyelinated fibres are C-fibres. One should note that the terms Aβ and Aγ are controversial when used with skin nerves (Gasser, 1960). All fibres have their parent cell bodies in dorsal root ganglia, and terminate peripherally in the skin and subcutaneous structures (Lance & McLeod, 1981). The sensory receptors in the skin may be encapsulated nerve-endings such as Meissner's or Pacinian corpuscles, specialized nerve endings such as Merkel's discs, or simple free nerve endings. Specialized and encapsulated nerve endings are concentrated in body regions which are particularly sensitive, such as finger tips, lips, breast areolae and genitalia. Cutaneous receptors are slowly or rapidly adapting. Many exhibit stimulus specificity and via their myelinated afferent fibres convey information about light touch and pressure, joint position sense, vibration sense, temperature sense both cold and warm, prickle, itch and pain (Iggo, 1965). Some free nerve endings have a high mechanical threshold and respond to painful

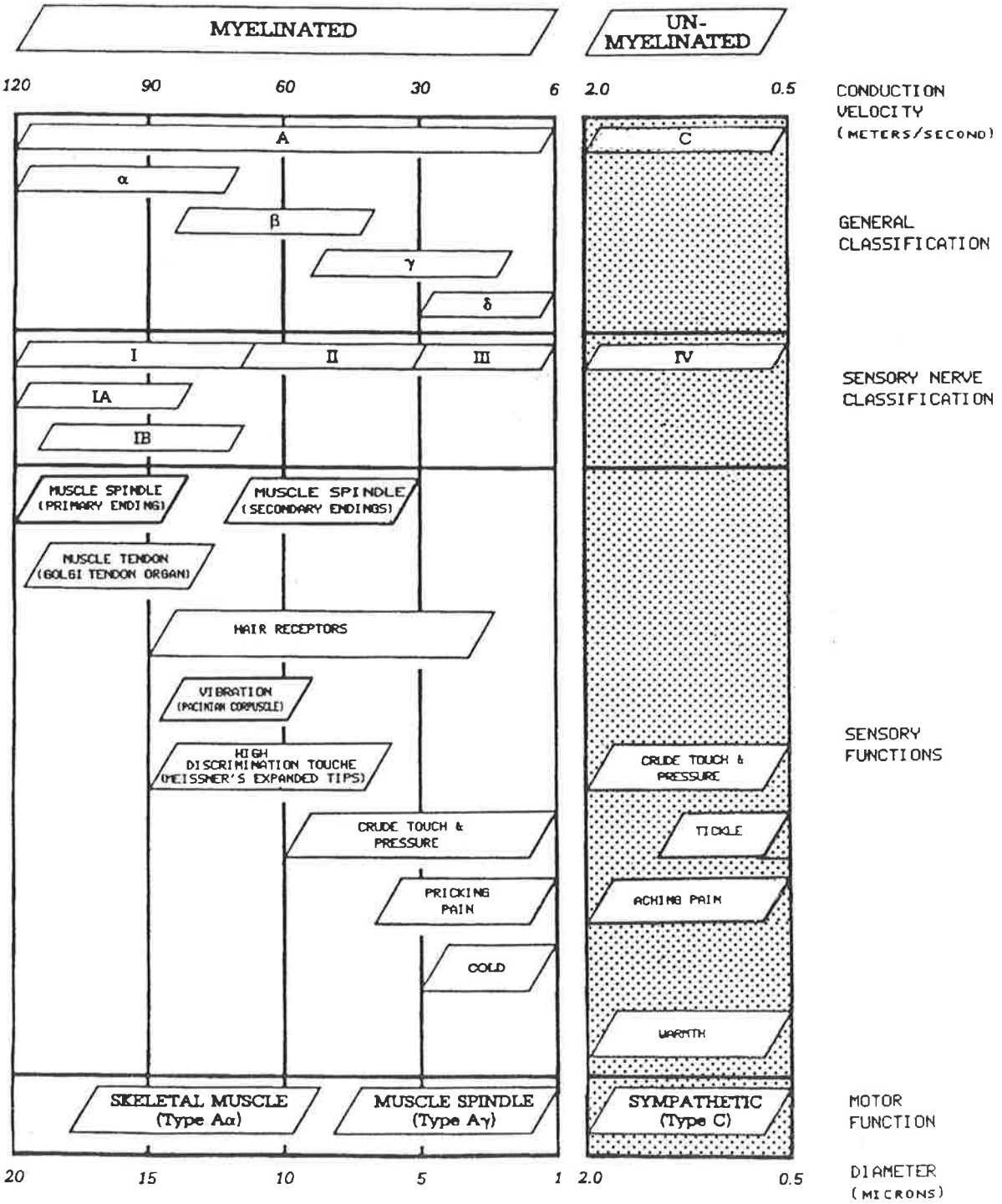


Figure 1. Physiological classification of peripheral nerve fibres. Unmyelinated (C-) fibres, shown in the stippled column at the right, convey sensations of pressure, prickle, aching pain, warmth and motor functions of neurogenic inflammation. Vasomotion and sweating are post-ganglionic autonomic motor functions. (Adapted with permission from Medelec, 1989)

stimuli only; others have a much lower threshold and are believed to play an important role in discriminative sensations (Bessou & Perl, 1970).

Figure 1 illustrates diagrammatically the various classes of nerve fibres and their functions.

The peripheral nervous mechanisms of pain involve both small myelinated Aδ and unmyelinated fibres and their nociceptors (Zotterman, 1939; 1959). It seems to be necessary to activate Aδ and C-fibres in order to evoke pain sensation (Clark et al., 1935; Collins et al., 1960), although these fibre groups also play an important role in the transmission of other sensory modalities. It has been suggested that Aδ fibres are responsible for the initial 'sharp' sensation of pain and C fibres for the persisting 'dull' sensation. The unmyelinated sensory C-fibres in skin are involved in warm and all the non-discriminative sensations of prickle, itch and pain (Douglas & Ritchie, 1959; Zotterman, 1972; Iggo, 1977). C-nociceptors also have an essential role in the perception of heat pain and the development of hyperalgesia that develops after cutaneous injury (LaMotte R H,et al., 1983).

2. Pain and other sensations

Nociception **is the process by which activity in particular sensory receptors, afferent pathways and their relays is able to alter awareness at a cortical or subcortical level to produce unpleasant or pain sensations and which trigger appropriate adaptive behaviour, often accompanied by affective responses. Usually, but not invariably, pain sensation is initiated by noxious stimulation of nociceptors.**

The presence of many different types of sensory receptors in skin (Iggo, 1965; 1977) poses the question of how all the various neuronal messages are selectively processed to facilitate passage of pertinent stimuli but retard the awareness of the background sensory activity (Lance & McLeod, 1981). Thus we may become largely unaware of the touch of clothing, the pressure of footwear or firm seats, and normal vegetative functions of viscera. Some of the processes which participate in this sensory selectivity include:

(1) Adaptation of sensory receptor end-organs to maintained stimuli (Douglas & Ritchie, 1959).

(2) Presynaptic inhibition of adjacent neurones by collaterals from active neurones. This occurs at many levels of the nervous system to ensure priority passage of signals, as distinct from noise (Eccles, 1964). The gate-control theory of pain (Melzack & Wall, 1962) arose from observations that activity in fibres from cutaneous mechanoreceptors may inhibit that in fibres from nociceptors. However, the original views have required modification to accomodate more recent findings (Schmidt, 1972; Handwerker et al, 1975; Nathan, 1976)

(3) Modulation of transmission through sensory nuclei is mediated by supraspinal descending paths (Handwerker et al, 1975) and from cortex by cortico-spinal pyramidal tract fibre collaterals to cuneate, gracile, trigeminal nuclei and ventrobasal thalamus (Phillips & Porter, 1977) which enables voluntary suppression of sensory input or involuntary suppression during movement.

(4) Alteration in the state of awareness at a cortical or subcortical level. The final perception is thus dependent on the activity of peripheral receptors, relay pathways, and complex cortical connexions, as well as the individual's emotional state (Nathan, 1977).

(5) Apart from the mechanisms already mentioned there are processes for pain control involving endogenous opioids (Hughes & Kosterlitz, 1977).

(6) Mechanisms of pain generation in pathological states or after nerve injury are considered elsewhere (Wall & Devor, 1985; Roberts & Fogelsong, 1986).

3. Nociception and neurogenic inflammation

The peripheral vasodilator (motor) effects of activity in nociceptive afferents were first described by Bayliss (1901) and later elegantly elaborated by Sir Thomas Lewis (1927). The protective functions of nociception involving the primary sensory neurone were first proposed as a damage-control system by Lewis (1931). In this review the original axon reflex of Bayliss (1901) and Lewis (1927) has been re-examined as part of a more general nocifensor or damage control system (Lembeck,1983) and the involvement of peptidergic nerves in this system has been considered (Hokfelt T, et al., 1980; Kenins et al, 1984). Nocifensor functions have been reviewed recently by Kumazawa (1990).

In addition to the original observations on skin, neurogenic inflammation and its accompaniments has been demonstrated in most tissues, including skeletal muscle (Hilton & Marshall, 1980), bladder and gastrointestinal mucosa (Szolcsanyi, 1988) and even

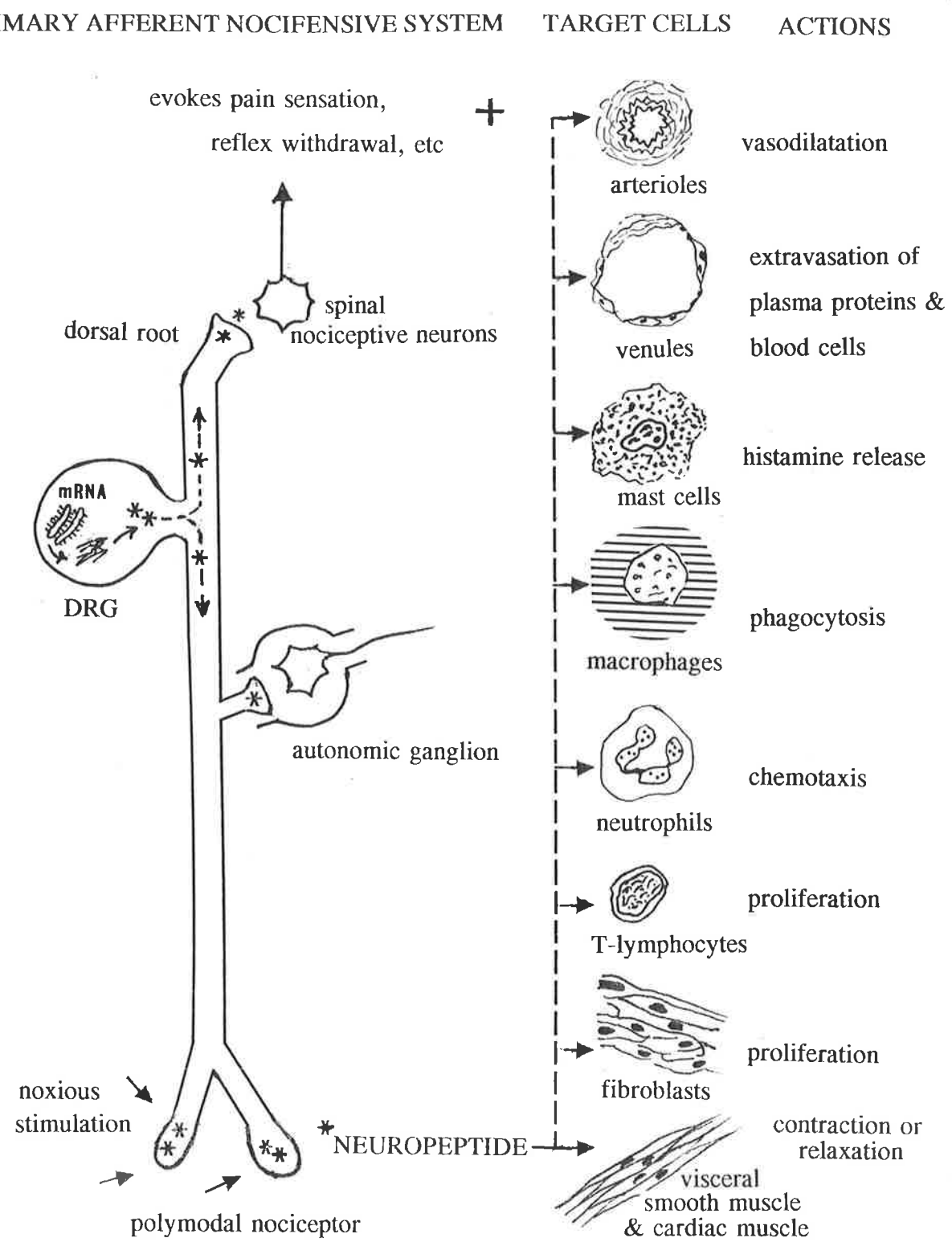


Figure 2. Nocifensor functions of primary afferent neurons. All the local regulatory effects illustrated here are mediated by neuropeptides which, together with their precursors, are synthesised, transported and released by primary sensory neurons.

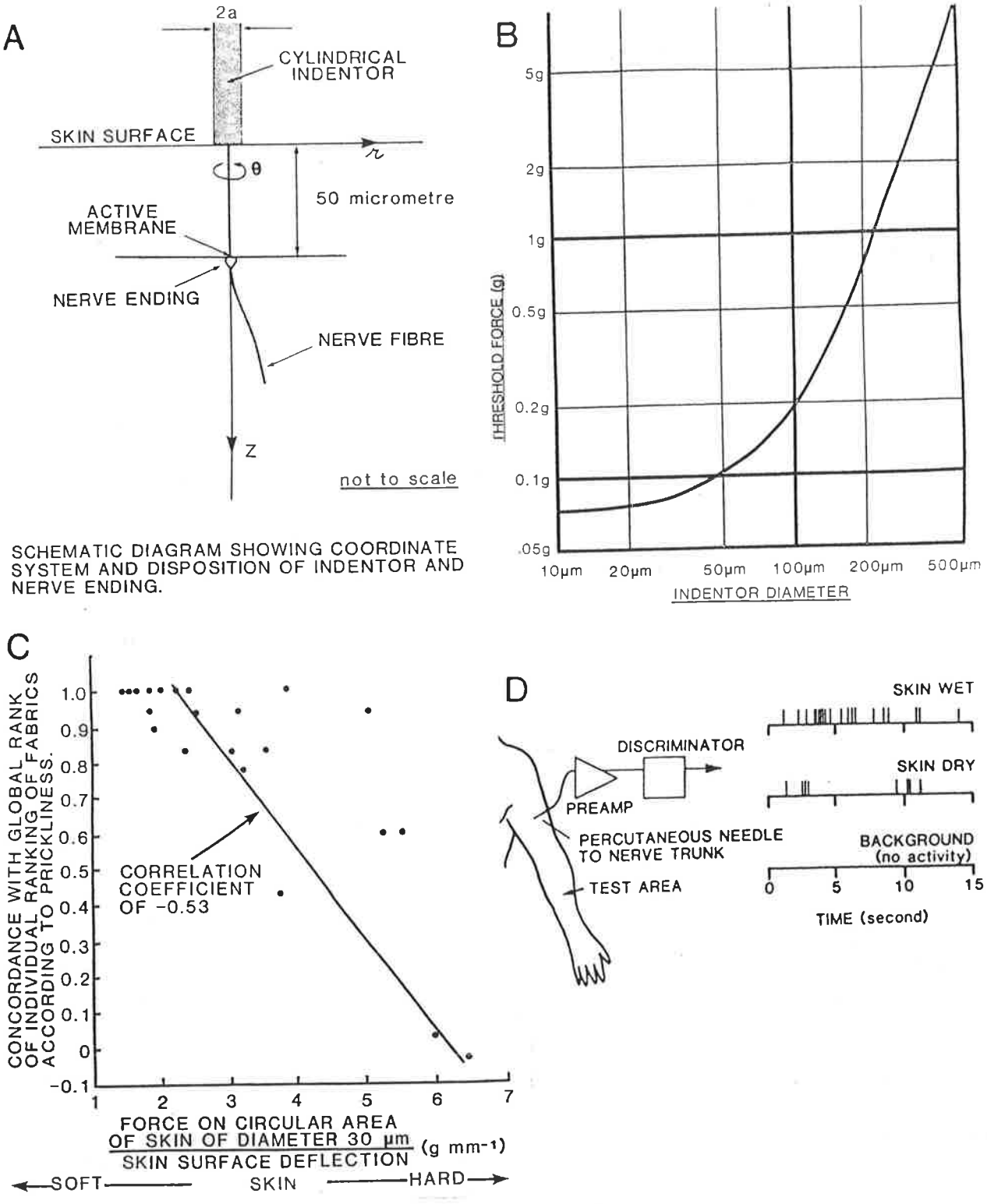


Figure 3. The number of high load bearing fibre ends in a fabric correlated best (r=1.000) with subjective assessment of prickle.

A: Diagram shows a cylindrical 'probe' of small diameter (eg 20um) applying a low load (eg 100mgf) which generates large enough shear forces to activate the receptor ending.

B: Computed threshold force vs cylindrical probe diameter.

C: Graph of ability to rank fabrics according to prickliness vs skin hardness (surface deflection of constant area/ unit force).

D: Microneurographic recording from identified C-polymodal or mechanoreceptors confirmed that their response to fabric pressures generated prickle sensation, and was enhanced by skin wetness.

operates to modulate blood flow in the pia-arachnoid at spinal dorsal root entry zones (Westerman, 1988a).

Figure 2 illustrates the skin nocifensor system in schematic summary.

4. Mechanisms of prickle and itch sensation

Certain fabrics with fibre ends, including wool and brushed nylon, when worn sometimes evoke discomfort and sensations of prickle or itch (Garnsworthy et al., 1985). The underlying physiological mechanisms and the physical, chemical, and neurophysiological processes responsible for fabric-evoked discomfort and skin rash have been explored by Westerman et al., 1984. The prevailing view of this fabric evoked discomfort and rash was that it occurred mainly in hypersensitive or allergic individuals, particularly those with any evidence of 'atopy' such as asthma, hayfever, eczema, allergic rhinitis, etc. Populations of atopic and non-atopic subjects identified by a consultant dermatologist were tested and compared; subjects with atopy who wear wool fabric are not significantly more likely to experience fabric-evoked skin discomfort or rash (Garnsworthy et al, 1985). The physical stimulus for itch and prickle sensations was identified by Garnsworthy and co-workers (1985, 1988a, 1988b) as mechanical excitation of polymodal nociceptors, together with slowly adapting type II mechanoreceptors. In the case of C-polymodal receptors an adequate mechanical stimulus may be as low as 150-200mg force applied by a von Frey hair. This confirmed findings by Adriaensen et al.(1983) about nociceptors with low threshold for mechanical stimuli. The key requirement was a minimum dimension and stiffness of the fibres comprising the fabric.

Figure 3 explains schematically the mechanical excitation of nociceptors to evoke prickle sensation.

Iontophoretic application of histamine was used as a controllable itch stimulus to enable measurement of itch sensation under various conditions (Magerl et al., 1990). Factors such as body region, gender and season were found to be significant sources of variability in response to the same chemical stimulus (Magerl et al, 1990). The technique used was a significant improvement on previous electrical (Tuckett, 1982) and chemical methods (Keele & Armstrong, 1964) of evoking itch and permitted microneurographic recordings

from identified sensory fibres during itching in human subjects (Handwerker et al., 1987). In this way the sensory correlates and the neural basis of the fabric-evoked sensations were identified using microneurography (Garnsworthy et al., 1988a). Understanding the neural mechanisms resulting in these less discriminative sensations, then led to the development of algorithms defining those properties of fabrics which are most likely to generate discomfort (Garnsworthy et al, 1985). This had considerable predictive value for the textile industry and the same techniques are still in use for this purpose.

Finally, the effects of transcutaneous electrical stimulation of fine epidermal nerve fibres were defined (Garnsworthy et al., 1988b). These techniques of transcutaneous electrical stimulation, iontophoretic application of vasoactive chemical stimuli to skin, and the sensitive, non-invasive techniques of laser Doppler flowmetry to measure relative changes in cutaneous microvascular flux appear to have potential value as clinical indicators of small nerve fibre and microvascular function. This will be further elaborated.

5. Capsaicin desensitization of nociceptive afferents

Capsaicin, (4-6-vanillyl nonen-amide), which is an octopeptide neurotoxin with specific effects on primary sensory fibres, has been studied for its potential as a topical desensitizing agent for post-herpetic neuralgia (Bernstein et al, 1986; Watson et al., 1988; Westerman et al., 1989). Acute effects of capsaicin on cutaneous axon reflex flare in man was studied: capsaicin actions on all small nerve fibres in human skin appeared confined to peptidergic sensory nerves, with no effects on cutaneous sympathetic nerves (Roberts et al., 1992). The first report on the treatment of post-herpetic neuralgia with topical capsaicin was by Bernstein et al.(1986). Subsequently, its action in reducing the cutaneous hyperalgesia and allodynia in post-herpetic neuralgia was demonstrated (Watson et al, 1988; Westerman et al., 1989; Roberts et al., 1991). Unlike Bernstein or Watson, Westerman et al. (1989) and Roberts et al.(1991, 1992) also measured warm and cold thermal thresholds in the affected dermatome using a microprocessor controlled Peltier device, the Medelec TTT. When carried out before and after topical treatment, this monitored the extent of skin nociceptor desensitization by the 4 times daily topical application of capsaicin.

Capsaicin has also been described as effective in alleviating burning sensations often present in amputation stumps (Rayner et al., 1989), post-mastectomy pain syndrome (Dini et al, 1993) and some painful diabetic polyneuropathy (Ross & Varipapa, 1989; Scheffler

et al., 1991), but these have not been examined further in the present study.

6. *Clinical tests of nociceptive functions*

Until recently, in clinical neurology and neurophysiology the functions of nociceptors and their sensory-motor afferent nerve fibres have been tested only semi-quantitatively. Pinprick acuity and the size of the flare induced by intradermal histamine injection, are the commonly used tests of pricking pain sensibility (Holmes, 1960) and of neurogenic inflammation (Wallace, 1970), respectively. Clinical testing of pinprick acuity is only semi-quantitative at best, but in the hands of skilled clinicians both tests have the advantage of being rapid and useful as screening tests.

To evoke pain, radiant heat has also been used as a stimulus (Hardy et al. 1952). More recently a Peltier heating device (Fruhstorfer et al., 1976a,b) and a mechanical device to produce pain have been used in threshold studies (Lynn & Perl, 1977). Chemical stimulation of nociceptors by substances producing pain and itch has been difficult to quantify (Keele & Armstrong, 1964). All these techniques are beset by the many difficulties attending subjective pain assessment (Le Quesne & Fowler, 1986).

Koltringer et al.(1988; 1989) has described a hyperthermal laser Doppler test of autonomic function in polyneuropathies, but this appears to measure a blood flux increase to a barely noxious warm stimulus where metabolic and local and reflex vascular changes also contribute to the magnitude of the final response and confound the interpretation.

7. *Skin blood flow measurements*

Blood flow changes in skin such as accompany neurogenic inflammation have been indirectly measured in chronic pain states using emission thermography (Roberts & Fogelsong, 1986). Because of the expense and complexity of the equipment required for precise measurement, and many difficulties in interpreting temperature asymmetries, this technique has been more useful in research (Westerman et al., 1990). More direct measures of skin blood flow include ^{133}Xenon clearance, microsphere flowmetry, optical plethysmography, electromagnetic flowmetry, oximetry, and laser Doppler velocimetry. Not all are applicable for clinical use, nor are all convenient and accurate (Westerman et al., 1988a).

The evaluation of laser Doppler velocimetry as a sensitive, non-invasive measure of cutaneous blood flux was undertaken in order to better define what this technique measures (Westerman et al., 1988a; Hales et al., 1989; 1992). In preliminary communications there was some evidence of the usefulness of this technique in various situations: the responses of peripheral skin to temperature change, exercise and the induction of hypnosis; responses to transdermal iontophoresis of vasoactive drugs (Westerman et al., 1988a); comparison of skin blood flux measured by different wavelengths of red light (Hales et al., 1989; 1992). Further, direct evidence was obtained for the existence of postganglionic sympathetic active vasodilator nerves (Westerman et al.,1986). The utility of skin blood flux measurement non-invasively during a fun run provided evidence of profound circulatory changes in exercise-induced hyperthermia (Hales et al., 1986). In using any non-invasive technique of blood flow, inevitably a number of assumptions are made in arriving at an end-measurement. In using laser Doppler velocimetry (LDV) one assumes that the measured flux is largely, if not totally derived from the movement of red blood cells in the most superficial skin vessels.

Although numerous papers have used this technique, there are no serious published attempts to define the vascular bed(s) examined by LDV. The recent collaborative study with Hales, Roberts et al.(1992) provides evidence that the laser Doppler velocimeter (633-810nm) or photoplethysmographic wavelength (845nm) used may affect the apparent blood flux derived from LDV and other similar techniques (Hales et al., 1989; 1992). Thus there is an important caveat attached to the use of these methods.

8. Iontophoresis of vasoactive substances.

The techniques used by both Le Quesne and Westerman employ a Periflux laser Doppler (with visible red helium-neon laser of 633nm) as a sensitive indicator of relative changes in cutaneous blood flux at the test site. Subsequently near infrared wavelengths of 780-810nm have also been used (Westerman et al., 1990 ; 1992b; 1992c). However, the chemically evoked axon reflex flare of Le Quesne requires a larger chamber, from which the neurogenic inflammation must spread across at least 3-4 nociceptive receptive fields. To achieve this, a rather large total iontophoretic charge must be passed (1mA for 5 minutes, ie. 300mC), which corresponds to about 10^{-5}mmol Ach. This is almost two orders of magnitude larger than used by Westerman et al (1988). Such a supramaximal total

charge (ACh dose) has the disadvantage that it can only demonstrate plateau or maximal responses, but not the more subtle information of threshold and dose-response shifts.

By contrast, for the modified iontophoretic tests of Westerman et al.(1988a), good dose-response curves were obtained with total iontophoretic charge transfer of 1,2,4,8,16 mC. The largest doses of ACh are 32mC, equivalent to about 10^{-6}mmol ACh (Westerman et al., 1988a). Various agonists and antagonists of acetylcholine (ACh) were also tested, including methacholine, carbaminoylcholine, nicotine and atropine, pirenzepine, hexamethonium, tubocurarine respectively (Westerman et al., 1988a; 1988c; Grunfeld et al., 1991; Carr et al., 1993). This led to confirmation that nicotinic not muscarinic receptors excite polymodal sensory receptors and nerves. The parameters and caveats associated with using transdermal iontophoresis of polar drugs as a micropharmacological tool were defined (Westerman et al., 1988a).

9. Neuropeptides and microvascular function

Excitation of primary sensory neurons results in release of neuropeptides (Kenins, 1981) and initiates the neurovascular inflammatory flare (Hagemark al., 1978; Foreman et al., 1983; Kumazawa, 1991). The subsequent events result from a cascade of nocifensor processes involving cellular, humoral and microvascular responses to noxious stimulation (Lembeck, 1983, 1988; Kenins et al, 1984; Kumazawa, 1991).

Because the skin flare depends on the cutaneous vascular reaction, dysfunction of any one of the serial components of the microvascular dilator cascade could result in a reduced axon reflex dilator response (Westerman et al., 1987, 1988a, 1988b, 1988c; Parkhouse et al., 1988a). For this reason it is necessary to measure the direct local reaction (Lewis, 1927) which is non-neurogenic. This reaction can be produced in a variety of ways, it is confined to the skin area stimulated, and will reveal any intrinsic vascular dysfunction such as may be associated with microangiopathy or occlusive vasculopathy. Le Quesne et al. used Pilocarpine or ACh iontophoresed for five minutes from the central well of their chamber immediately underneath the laser Doppler probe (1987, 1988a,b). By contrast, Westerman et al. measures the endothelium-dependent vasodilatation evoked by very small doses, eg. 1,2 or 4 mC of acetyl-beta-methacholine or acetylcholine iontophoretically applied directly onto the skin under the probe (Westerman et al., 1988a, 1990; Low &

Westerman, 1989). This dilator response is influenced only slightly by functioning nociceptive C-fibres and is reduced when the microvascular endothelium is impaired (Westerman et al., 1988a, 1990; Low & Westerman, 1989) .

The final test is of vascular smooth muscle reactivity. This involves iontophoretic application of a directly acting nitrodilator such as sodium nitrite or sodium nitroprusside and laser Doppler measurement of the vasodilator response induced (Westerman et al.,1990). It has been recently shown by Tare,Parkington et al.(1990) that these nitrodilators do not involve the endothelial response, but act on the microvascular smooth muscle to release nitric oxide which evokes vascular relaxation in a manner similar to that produced by endothelium releasing its relaxing factor EDRF.

Notable advances during this period were:
(i) to measure relative changes in blood flux of skin and other tissues sensitively using laser Doppler velocimetry (Westerman et al., 1986, 1988a; Magerl et al., 1987).

(ii) to develop the method of noxious transcutaneous electrical stimulation and iontophoretic application of vasoactive drugs as controllable stimuli to elicit neurogenic inflammatory flare (Westerman et al.,1986; 1987; 1988a; 1988b; 1988c).

(iii) to demonstrate the clinical applicability of these non-invasive measures of neurovascular function in patients with diabetes mellitus (Westerman et al., 1987; 1988a; 1988b; 1990; 1992).

(iv) these non-invasive techniques allowed serial measurements to be made in animal studies, carried out in parallel with the human measurements. This established the endothelium-dependence of the axon reflex flare (Low & Westerman,1989; Westerman et al.,1990). It also led to the demonstration of reversible neurovascular dysfunction in Streptozotocin diabetic rats at different stages of the disease (Westerman et al., 1988a, 1990; Low & Westerman,1989). This was shown using single insulin injections, and also longer term therapy. Here both of the aldose reductase inhibitors Sorbinil and Ponalrestat were effective.

(v) chronic nicotine treatment, either by repeated injections or implanted osmotic pumps,

has been shown to produce marked enhancement of cutaneous axon reflex flares evoked by ACh (Grunfeld et al, 1991, 1993; Westerman et al, 1994). This may reflect significant sensitization of nociceptive afferents, and may have important implications for both the sensory (pain perception) and motor (neurogenic inflammation, wound healing) functions of these peptidergic nerves (Westerman et al., 1994).

10. Measurement of small cutaneous sensory nerve and microvascular function.

The quantitative measurement of various peripheral sensory modalities has become increasingly important in improving diagnosis and management of peripheral neuropathy of various types. This section of the review mainly deals with the development and validation of techniques for measuring disturbances of small cutaneous sensory and autonomic nerves.

With advances in electrophysiology and in methods for sensitively measuring changes in superficial microvascular blood flow, there have been new tests developed as more sensitive clinical tools, and for research purposes. These methods embraced under the heading of quantitative testing (Consensus Statement,1988) include :

1. **Electrical axon reflex** (Westerman et al, 1986; 1987)
2. **Chemical axon reflex** (Westerman, 1988a; 1988b; Benarroch & Low, 1991)
3. **Thermal thresholds** for noxious heat and cold (Fruhstorfer et al, 1976a; 1976b)
4. **Mechanical algometry** (Keele, 1954; Jensen et al., 1986; Kohlloeffel et al., 1991)
5. **Sympathetic skin potential** (Shahani et al, 1985; Knezevic & Bajada, 1985; Solveni et al., 1987).

Axon reflex sweat testing in diabetics induced by acetylcholine (ACh) iontophoresis (Low et al.,1983) led Le Quesne to develop this as a measure of peripheral nociceptor C-fibre function (Parkhouse & Le Quesne, 1987; 1988a, 1988b). Contemporaneously, Westerman et al.(1986, 1987) and Magerl et al.(1987) developed the electrically evoked axon reflex flare, and a modified chemically evoked axon reflex (Westerman et al., 1988a, 1990, 1992a,b,c) as quantitative tests of nociceptor C-fibre function. This technique was based on the classical observations concerning antidromic vasodilatation (Bayliss, 1901) and neurogenic inflammation (Thomas Lewis, 1927, 1931, 1935; Chahl, 1988)). Westerman

and colleagues (1986; 1987; 1992c; Magerl et al, 1987) use noxious TENS at a current strength sufficient to excite C-afferent fibres and evoke an axon reflex flare.

To test the clinical usefulness of this technique, two groups of patients with diabetes mellitus, ie, with and without clinical signs of neuropathy, were compared with normal volunteers and a marked reduction of the neurogenic flare response was found (Westerman et al., 1987; 1988c). A similar reduction of neurogenic flare responses in fingers of subjects exposed for longer periods to hand-arm vibration, and the distal extremities of persons with hereditary sensory radicular neuropathy (Denny-Brown, 1951) was also discovered (Westerman et al., 1992a, 1992b). These non-invasive techniques are now in continuous use in the clinical testing of patients with Hand-Arm Vibration Syndrome at the Karolinska Sodersjukhuset (Lindblad, Personal Communication, 1992).
This technique provides a useful new test for detecting the presence of and measuring the extent of neuropathy involving the smallest fibres (Westerman et al., 1986, 1987, 1988, 1990). It also enables the prospective gathering of epidemiological data on the incidence of small fibre neuropathy in diabetes mellitus and other conditions (Westerman et al., 1986, 1987, 1990, 1992a).

11. Clinical applications of measuring neurovascular function

11.1 Sensory nerve and microvascular dysfunction in diabetes mellitus

In diabetes mellitus both small fibre neuropathy (Westerman et al., 1990b, 1992a), and microvasculopathy (Yue et al., 1990, 1992) or reduced microvascular reactivity (Flynn & Tooke, 1992) have been shown to occur. This could involve either the endothelium or smooth muscle of the microvessels (Westerman et al.,1990a). Furthermore, acute changes in sensory nerve function with changes in blood glucose and insulin levels have been demonstrated. Until recently, it has been difficult to measure neural function other than conduction velocity of the larger myelinated sensory and motor nerve fibres. Because of this, and the possible co-existence of microvascular dysfunction in diabetic neuropathy, the strategy of testing the serial elements of the axon reflex flare is particularly appropriate (Westerman et al., 1990, 1992a). Recently, newer treatments such as using aldose reductase inhibitors have the potential to reverse or significantly improve early neuropathy. For the measurement of small fibre function during prolonged trials of such treatment, the non-invasive techniques discussed in this essay are an important complement to the classical clinical neurophysiological methods of measuring large fibre function (Lance &

McLeod, 1981). Finally, these quantitative techniques have identified other pathophysiological mechanisms contributing to small sensory nerve fibre dysfunction (Delaney et al., 1994a,b).

Thus diabetes mellitus is the initial clinical area in which the quantitative measures of small cutaneous nerve fibres and microvascular function have been tested (Westerman et al., 1990, 1992a), but the value of measuring small fibre neuropathy in diabetes mellitus is just becoming recognized (Benarroch & Low, 1991). If these techniques are to be used to best advantage, confounding factors must be defined (Westerman et al., 1992a), and the test equipment and protocol must be further simplified.

11.2 Other neuropathies /neurovascular syndromes

Other clinical disorders in which measurement of neurovascular dysfunction has proved helpful include hereditary sensory radicular neuropathy (Westerman et al., 1992b), reflex sympathetic dystrophy (Westerman et al., 1992c), and Raynaud's syndromes (Westerman et al., 1992b), either primary or secondary.

However, notwithstanding the drawbacks of these techniques in their present high-technology form, they have already added a new dimension to the clinical assessment of neurovascular dysfunction.

3800 words

BIBLIOGRAPHY RELEVANT TO THE ESSAY.

Adriaensen H, Gybels J, Handwerker H O, van Hees J (1983) Response properties of thin myelinated (Aδ) fibres in human skin nerves. J Neurophysiol 49: 111-122.

Assal J-Ph, Liniger C. (Eds) Peripheral Neuropathies 1988: What is significantly new? Fidia Research Series, Volume 21. Liviana Press, Padova, 1989.

Bayliss W M (1901) On the origin from the spinal cord of the vasodilator fibres at the hind limb and on the nature of these fibres. J Physiol 26: 73-209.

Benarroch E E, Low P A (1991) The acetylcholine-induced flare response in evaluation of small fiber dysfunction. Ann Neurol 29(6): 590-595.

Bernstein J E, Bickers D R, Dahl M V, Roshal J Y (1987) Treatment of chronic postherpetic neuralgia with topical capsaicin. J Am Acad Dermatol 17: 93-96.

Bessou P, Perl E R (1970) Response of cutaneous sensory units with unmyelinated fibres to noxious stimuli. J Neurophysiol 33: 1025-1043.

Boulton A J (1988) The diabetic foot. Medical Clinics of North America 72: 1513-1530.

Carr R W, Delaney C A, Westerman R A, Roberts R G D.(1993) Denervation impairs cutaneous microvascular function and blister healing in the rat hindlimb. NeuroReport 4/5: 467-470.

Chahl L A (1988) Antidromic vasodilatation and neurogenic inflammation. Pharmacology & Therapeutics 37: 275-300.

Clark D, Hughes J, Gasser H S (1935) Afferent function in the group of nerve fibres of slowest conduction velocity. Am J Physiol 114: 69-76.

Collins W F, Nulsen F E, Randt C T (1960) Relation of peripheral nerve fibre size and sensation in man. Archs Neurol 3: 381-385.

Consensus Statement: Report and Recommendations of the San Antonio Conference on 8-10 Feb,1988; Diabetes 37: 100-104.

Delaney C A, Mouser J V, Westerman R A (1994a) Insulin sensitivity and sensory nerve function. Proc Aust Physiol Pharmacol Soc 25(1) 32P.

Delaney C A, Mouser J V, Westerman R A (1994b) Insulin sensitivity and sensory nerve function in non-diabetic human subjects. Neurosci Lett . Submitted.

Denny-Brown D (1951) Hereditary sensory radicular neuropathy. J Neurol Neurosurg Psychiat 14: 237-252.

Dini D, Bertelli G, Gozza A, Forno G G (1993) Treatment of the post-mastectomy pain syndrome with topical capsaicin. Pain 54: 223-226.

Douglas W W, Ritchie J M (1959) The sensory functions of non-myelinated afferent nerve fibres from the skin. In: Wolstenholme G E W and O'Connor M (Eds). Pain and itch : Nervous mechanisms. Ciba Foundation Study Group No.1 Churchill Ltd, London, 1959.

Dyck P J, Gutrecht J A, Bastron J A, Karnes W E, Dale A J D (1968) Histologic and teased fibre measurements of sural nerve in disorders of lower motor and primary sensory neurons. Mayo Clin Proc 43: 81-123.

Eccles J C (1964) The controls of sensory communication to the brain. Australas Ann Med 13: 102-113.

Fischer A A, Rim A, Chang C H (1986) Correlation between thermographic findings and somatosensory cortical evoked potentials in lumbosacral radiculopathies. hermology 2: 29-33.

Fitzgerald M (1983) Capsaicin and sensory neurones - a review. Pain 15: 109-130.

Flynn M D, Tooke J E (1992) Aetiology of diabetic foot ulceration: a role for the microcirculation? Diabetic Medicine 8: 320-329.

Foreman J C, Jordan C C (1983) Histamine release and vascular changes induced by neuropeptides. Agents Actions 13: 105-116.

Fruhstorfer H, Lindblom U, Schmidt W G (1976a) Method for quantitative estimation of thermal thresholds in patients. J Neurol Neurosurg Psychiat 39: 1071-1075.

Fruhstorfer H, Goldberg J M, Lindblom U, Schmidt W G (1976b) Temperature sensitivity and pain thresholds in patients with peripheral neuropathy. In: Zotterman Y, ed. Sensory functions of the skin in primates. Oxford: Pergamon, 1976; 507-517.

Garnsworthy R, Mayfield R, Gully R, Westerman R A, Kenins P. (1985) Mechanisms in cutaneous sensations of prickle and itch evoked by fabrics. Proc Int Wool & Textile Symp Vol 3: 190-199.

Garnsworthy R K, Gully R, Kenins P, Mayfield R J, Westerman R A (1988a). Identification of the physical stimulus and the neural basis of fabric-evoked prickle sensation. J Neurophysiol 59(4): 1083-1097.

Garnsworthy R K, Walker A, Gully R, Westerman R A (1988b) Transcutaneous electrical stimulation of epidermal nerve fibres. J Neurophysiol 59(4): 1116-1127.

Gasser H S (1960) Effects of the method of leading on the recording of the nerve fibre spectrum. J Gen Physiol 43: 927-940.

Greaves M W and Shuster S (Eds). Pharmacology of the skin I. Pharmacology of skin systems. Autocoids in normal and inflamed skin. Springer-Verlag, Heidelberg, 1989.

Grunfeld J, Tiedemann G J, Westerman R A (1991). Chronic nicotine exposure enhances cutaneous axon reflexes in the rat. NeuroReport 2: 421-424.

Grunfeld J A, Tiedemann G J, Westerman R A (1993). Maternal nicotine exposure enhances cutaneous axon reflexes in the neonatal rat. NeuroReport 4/6: 635-638.

Hagemark O, Hokfelt T, Pernow B (1978) Flare and itch induced by substance P in human

skin. J Invest Dermatol ; 1: 233-235.

Hales J R S, Westerman R A, Stephens F R N, Vaughan J D, Fawcett A A, Richards D A B, Richards C R B (1986) Lowered skin blood flow and erythrocyte sphering in collapsed fun-runners. Lancet, June 28, 1495-1496.

Hales J R S, Stephens F R N, Fawcett A A, Daniel K, Sheahan J, Westerman R A, James S B (1989). Observations on a new non-invasive monitor of skin blood flow. Clin Exper Physiol Pharmacol 16: 403-415.

Hales J R S, Roberts R G D, Westerman R A, Stephens F R N, Fawcett A A (1992) Evidence for skin microvascular compartmentalization by laser-Doppler and photoplethysmographic techniques. Int J Microcirc Clin Exper 12: 99-104.

Handwerker H O, Iggo A, Zimmermann M (1975) Segmental and supraspinal actions on dorsal horn neurons responding to noxious and non-noxious skin stimuli. Pain 1:147-165.

Handwerker H O, Magerl F W, Klemm F, Lang E, Westerman R A (1987). Neural correlates of itch sensations. In: The role of fine afferent nerve fibres and pain sensation. Eds. R. F. Schmidt, Schaible H J and Vahle-Hinze E VCH, Weinheim P 461-473.

Hardy J D, Wolff H G, Goodell H (1952) Pain sensations and reactions. Baltimore: Williams & Wilkins, 1952.

Hilton S M, Marshall J M. (1980) Dorsal root vasodilatation in cat skeletal muscle. J Physiol 299: 277-288.

Hokfelt T, Johansson, Ljungdahl, Lundberg J M, Schultzberg M (1980) Peptidergic neurones. Nature 284: 515-521.

Holmes G (1960) Introduction to clinical neurology. 2nd edition Edinburgh, Livingstone.

Hughes J, Kosterlitz H W (1977) Opioid peptides. Brit Med Bull 33: 157-161.

Iggo A (1965) The peripheral mechanisms of cutaneous sensation. In: Studies in physiology.. Ed D R Curtis, A K McIntyre pp92-100. New York, Springer.

Iggo A. (1977) Cutaneous and subcutaneous sense organs. Brit Med Bull 33: 97-102.

Jamal G A, Hansen S, Weir A I, Ballantyne J P (1987) The neurophysiologic investigation of small fibre neuropathies. Muscle and Nerve 10: 537-545.

Jansco N, Jansco-Gabor A, Szolcsanyi J (1967) Direct evidence for neurogenic inflammation and its prevention by denervation and by pretreatment with capsaicin. Brit J Pharmacol 31: 138-151.

Jensen K, Andersen H O, Olesen J, Lindblom U (1986) Pressure-pain threshold in human temporal region: evaluation of a new pressure algometer. Pain 25: 313-323.

Keele K D (1954) Pain-sensitivity tests: the pressure algometer. Lancet i: 247-255.

Keele C A, Armstrong D, Substances producing pain and itch. London: Edward Arnold, 1964.

Kenins P (1981) Identification of the unmyelinated sensory nerves which evoke plasma extravasation in response to antidromic stimulation. Neurosci Lett ; 25: 137-140.

Kenins P, Hurley J V, Bell C (1984) The role of substance P in the axon reflex of the rat. Brit J Dermatol 111: 551-559.

Kjartannson J, Dalsgaard C-J (1987) Calcitonin gene-related peptide increases survival of a musculocutaneous critical flap in the rat. Eur J Pharmacol 142: 355-358.

Knezevic W, Bajada S (1985) Peripheral autonomic surface potential: A quantitative technique for recording autonomic nerve function in man. Cli Exper Neurol 21:

Kohlloeffel L U E, Koltzenburg M, Handwerker H O (1991) A novel technique for the evaluation of mechanical pain and hyperalgesia. Pain 46: 81-87.

Koltringer P (1988) A new diagnosis for polyneuropathies: The hyperthermal laser Doppler flowmetry. Acta Med Austriaca 3: 87-91.

Koltringer P, Langsteger W, Lind P, Reisecker F, Eber O. (1989) A new measuring design for autonomic dysfunction of skin neuropathies: Hyperthermal laser Doppler flowmetry. Acta Neurol Scand 80: 589-592.

Kumazawa T (1990) Functions of the nociceptive primary neurons. Jap J Physiol 40: 1-14.

Lance J W, McLeod J G. A physiological approach to clinical neurology. Butterworths, London, Third edition, 1981.

LaMotte R H, Thalhammer J G, Robinson C J (1983) Peripheral neural correlates of the magnitude of cutaneous pain and hyperalgesia a comparison of neural events in monkey with sensory events in human. J Neurophysiol 50: 1-26.

Lembeck F. (1983) Sir Thomas Lewis's nocifensor system, histamine and substance P-containing primary afferent nerves. Trends Neurosci 6: 106-108.

Lembeck F (1988) The Ulf von Euler lecture. Substance P: from extract to excitement. Acta Physiol Scand 133: 435-454.

Lewis T. The blood vessels of the human skin and their responses. Shaw & Sons, London, 1927.

Lewis T. (1931) The nocifensor system of nerves and its reactions. Brit Med J 194: 431-435; 491-494.

Lewis T (1935) Experiments relating to cutaneous hyperalgesia and its spread through somatic nerves. Clin Sci 2: 373-423.

Le Quesne P M, Fowler C J (1986) A study of pain threshold in diabetics with neuropathic foot lesions. J Neurol Neurosurg Psychiatr 49: 1191-1194.

Low P A, Caskey P E, Tuck R R, Fealey R D, Dyck P J (1983) Quantitative sudomotor axon reflex test in normal and neuropathic subjects. Ann Neurol 14: 573-580.

Lynn B, Perl E R (1977) A comparison of four tests for assessing pain sensitivity of different subjects and test areas. Pain 3: 353-365.

Magerl F W, Westerman R A, Handwerker H O (1990). Properties of transdermal histamine iontophoresis: differential effects of season, gender and body region. J. Investigative Dermatol. 94(3): 347-352.

Magerl F W, Szolcsanyi J, Westerman R A, Handwerker H O (1987) Laser Doppler measurements of skin vasodilatation elicited by percutaneous electrical stimulation of nociceptors in humans. Neurosci Lett 82: 349-354.

Melzack R, Wall P D (1962) On the nature of cutaneous sensory mechanisms. Brain 85: 331-356.

Nathan P W (1976) The gate-control theory of pain. A critical review. Brain 99: 123-158.

Nathan P W (1977) Pain. Brit. Med. Bull. 33: 149-155.

Nilsson J, von Euler A, Dalsgaard C-J (1985) Stimulation of connective tissue cell growth by substance P and Neurokinin A. Nature 315: 61-62.

Parkhouse N, Le Quesne P M, Scurr J (1987) Reduced axon reflex flare in diabetics with neuropathic foot lesions. Br J Surg 74: 534.

Parkhouse N, Le Quesne P M (1988a) Quantitative objective assessment of peripheral nociceptive C-fibre function. J Neurol Neurosurg Psychiat 51: 28-34.

Parkhouse N, Le Quesne P M (1988b) Impaired neurogenic vascular response in patients with diabetes and neuropathic foot lesions. N Engl J Med 318: 1306-1309.

Phillips C G, Porter R. (1977) Corticospinal neurones. Their role in movement. p450.

London, Academic Press.

Rayner H C, Westerman R A, Roberts R G D et al.(1989) Relief of local stump pain by capsaicin cream. Lancet 2: 1276. (Letter).

Roberts R G D, Westerman R A (1991). Capsaicin: Hot stuff for pain relief. Current Therapeutics. 32: 43-49.

Roberts R G D, Westerman R A, Widdop R E, Kotzmann R R, Payne R (1992). Effect of capsaicin on cutaneous axon reflex vasodilatation in man. Agents and Actions. 35(1/2): 53-59.

Roberts W J, Fogelsong B A (1986) A neuronal basis for sympathetically maintained pain. Thermology 2: 2-6.

Ross D R, Varipapa R J (1989) Treatment of painful diabetic neuropathy with topical capsaicin. N Engl J med 321(7): 474.

Scheffler N M, Sheitel P L, Lipton M N (1991) Treatment of painful diabetic neuropathy with capsaicin 0.075%. J Amer Pod Med Assoc 81(6): 288-293.

Schmidt R F (1972) The gate control theory of pain: an unlikely hypothesis. In Pain Eds J P Payne et al. pp 124-127 Edinburgh, Churchill Livingstone.

Shahani B T, Halperin J J, Boulu P, Cohen J (1984) Sympathetic skin response: a method of assessing unmyelinated axon dysfunction in peripheral neuropathies. J Neurol Neurosurg Psychiat 47: 536-542.

Soliven B C, Maselli R A, Jaspan J B, Green A J, Graziano H, Petersen M, Spire P (1987) Sympathetic skin response in diabetic neuropathy. Muscle and Nerve 10: 711-716.

Shephard A P, Oberg P A (Eds). Laser-Doppler blood flowmetry. Kluwer Academic Publishers, Dordrecht, The Netherlands, 1990.

Szolcsanyi J (1988) Antidromic vasodilatation and neurogenic inflammation. Agents Actions 23: 4-11.

Tare M, Parkington H C, Coleman H A, Neild T O, Dusting G J (1990) Hyperpolarization and relaxation of arterial smooth muscle caused by nitric oxide derived from the endothelium. Nature ; 346 (6279): 69-71.

Tuckett R P (1982) Itch evoked by electrical stimulation of the skin. J Investigative Dermatol 79: 368-373.

Wall P D, Devor M (1978) Physiology of sensation after peripheral nerve injury, regeneration and neuroma formation. In: Physiology and Pathobiology of axons. Ed S G Waxman New York, Raven Press.

Wallace D C. Hereditary sensory radicular neuropathy. A family study. Australian Medical Association Mervyn Archdall Medical Monograph Number 8. Australasian Medical Publishing Co., Sydney, 1970.

Watson C P N, Evans R J, Watt V R (1988) Post-herpetic neuralgia and topical capsaicin. Pain 33: 333-340.

Westerman R A, Garnsworthy R K, Walker A, Gully R and Fergin P (1984). Aspects of human small cutaneous nerve fibre function. In: Antidromic vasodilatation and neurogenic inflammation. Ed. L. A. Chahl, F. Lembeck and J. Szolcsanyi, Hungarian Academy of Science, p 329-345.

Westerman R A, Low A, Pratt A, Hutchinson J S, Szolcsanyi J, Magerl F W, Handwerker H O, Kozak W M (1986). Electrically evoked skin vasodilatation: a quantitative test of nociceptor function in man. Clin. Exper. Neurol., 23: 81-89.

Westerman R A, Widdop R E, Hogan C, Zimmet P (1987). Non-invasive tests of neurovascular function: reduced axon reflex responses in diabetes mellitus. Neurosci. Lett. 81: 177-182.

Westerman R A, Widdop R E, Hannaford J, Low A M, Roberts R G D, Kent P, Sideris K, Yip T, Hales J R S, Stephens F R N (1988a). Laser Doppler velocimetry in the measurement of neurovascular function. Aust. Phys. Eng. Sci. Med. 11(2): 53-66.

Westerman R A, Szolcsanyi J, Magerl F W, Handwerker H O, Low A, Pratt A, Widdop R, Kozak W M (1988b). Vasodilator axon reflexes. In: Vasodilatation: vascular smooth muscle, peptides, autonomic nerves and endothelium. Ed. P. Vanhoutte, Raven Press, New York p 107-112.

Westerman R A, Widdop R E, Hannaford J, Hogan C, Roberts R G D, Zimmet P (1988c) Non-invasive tests of neurovascular function. Clin Exper Neurol 24: 129-137.

Westerman R A, Roberts R G D, Kotzmann R R, Westerman D A, Delaney C A, Widdop R E, Carter B (1989). Effects of topical capsaicin on normal skin and affected dermatomes in herpes zoster. Clin. Exper. Neurol. 25: 71-84.

Westerman R A, Widdop R E, Low A M, Delaney C A, Neild T O (1990a). Non-invasive tests of neurovascular function in human and experimental diabetes mellitus. In: Molinatti G, Bar R, Belfiore S and Porta M (Eds). Frontiers in Diabetes v.9, Basel; Karger 1990, p 127-138.

Westerman R A, Delaney C A, Ivamy-Phillips A, Horowitz M, Roberts A. (1990b) Concordance between different measures of small sensory and autonomic fibre neuropathy in diabetes mellitus. Clin. Exper. Neurol. 26: 51-63.

Westerman R A, Lindblad L-E, Wajnblum D, Roberts R G D. (1992a) Confounding factors in non-invasive tests of neurovascular function in diabetes mellitus. Clin. Exper. Neurol. 29: 149-160.

Westerman R A, Block A, Nunn A, Delaney C A, Hahn A, Dennett X, Carr R W (1992b) Hereditary sensory radicular neuropathy: defective neurogenic inflammation. Clin. Exper. Neurol. 29: 189-209.

Westerman R A, Pano I, Rabavilas A, Nunn A, Hahn A, Roberts R G D, Burry H (1992c)

Reflex sympathetic dystrophy: altered cutaneous axon reflex and autonomic responses. Clin. Exper. Neurol. 29: 210-233.

Westerman R A, Carr R W, Delaney C A, Morris M J, Roberts R G D. (1993) The role of nociceptive afferent nerves in blister healing. Clin Exper Neurol 30: 39-61.

White D M, Helme R D (1985) Release of substance P from peripheral nerve terminals following electrical stimulation of sciatic nerve. Brain Res 336: 27-31.

Wiles P G, Pearce S M, Rice P J S, Mitchell J M O (1991) Vibration perception threshold: influence of age, height, sex and smoking, and calculation of accurate centile values. Diabetic
Medicine 8: 157-161.

Wolstenholme G E W and O'Connor M (Eds). Pain and itch : Nervous mechanisms. Ciba Foundation Study Group No.1 Churchill Ltd, London, 1959.

Yue D, McLennan S V, Turtle J R (1992) pathogenesis of diabetic microangiopathy: The roles of endothelial cell and basement membrane abnormalities. Diabetic Medicine 9: 218-220.

Zotterman Y, (1939) Touch, pain and tickling: an electrophysiological investigation on cutaneous sensory nerves. J Physiol 95: 1-28.

Zotterman Y (1959) The peripheral nervous mechanism of pain: a brief overview. In: Wolstenholme G E W and O'Connor M (Eds). Pain and itch : Nervous mechanisms. Ciba Foundation Study Group No.1 Churchill Ltd, London, 1959.

Zotterman Y (1972) Pain and tickle. A brief review of electrophysiological studies of cutaneous nerves. In: Pain. J P Payne et al.(Eds) pp4-15 Edinburgh, Churchill Livingstone.

PUBLICATIONS LIST : PAPERS IN EDITORIAL JOURNALS, INVITED PAPERS AND REVIEWS

SECTION I: SMALL CUTANEOUS SENSORY NERVE FUNCTIONS: MECHANISMS OF PRICKLE AND ITCH SENSATION.

1. WESTERMAN R A, GARNSWORTHY R K, WALKER A, GULLY R and FERGIN P (1984). Aspects of human small cutaneous nerve fibre function. In: Antidromic vasodilatation and neurogenic inflammation. Ed. L. A. Chahl, F. Lembeck and J. Szolcsanyi, Hungarian Academy of Science, p 329-345.

2. GARNSWORTHY R, MAYFIELD R, GULLY R, WESTERMAN R A and KENINS P (1985). Mechanisms in cutaneous sensations of prickle and itch evoked by fabrics. Proc. Int. Wool & Textile Symp. Vol. III, p 190-199.

3. HANDWERKER H O, MAGERL F W, KLEMM F, LANG E and WESTERMAN R A (1987). Neural correlates of itch sensations. In: The role of fine afferent nerve fibres and pain sensation. Eds. R. F. Schmidt, Schaible H J and Vahle-Hinze E VCH, Weinheim P 461-473.

4. GARNSWORTHY R K, GULLY R, KENINS P, MAYFIELD R J and WESTERMAN R A (1988). Identification of the physical stimulus and the neural basis of fabric-evoked prickle sensation. J. Neurophysiol. 59(4): 1083-1097.

5. GARNSWORTHY R K, WALKER A, GULLY R and WESTERMAN R A (1988). Transcutaneous electrical stimulation of epidermal nerve fibres. J. Neurophysiol. 59(4): 1116-1127.

6. MAGERL F W, WESTERMAN R A and HANDWERKER H O (1990). Properties of transdermal histamine iontophoresis: differential effects of season, gender and body region. J. Investigative Dermatol. 94(3): 347-352.

SECTION II: MEASUREMENT OF SMALL CUTANEOUS SENSORY NERVE AND MICROVASCULAR FUNCTION.

II.1 STUDIES IN HUMANS.

7. WESTERMAN R A, LOW A, PRATT A, HUTCHINSON J S, SZOLCSANYI J, MAGERL F W, HANDWERKER H O and KOZAK W M (1986). Electrically evoked skin vasodilatation: a quantitative test of nociceptor function in man. Clin. Exper. Neurol., 23, 81-89.

8. MAGERL F W, SZOLCSANYI J, WESTERMAN R A and HANDWERKER H O (1987). Laser Doppler measurements of skin vasodilatation elicited by percutaneous electrical stimulation of nociceptors in humans. Neurosci. Lett. 82: 349-354.

9. WESTERMAN R A, WIDDOP R E, HOGAN C and ZIMMET P (1987). Non-invasive tests of neurovascular function: reduced axon reflex responses in diabetes mellitus. Neurosci. Lett. 81: 177-182.

10. WESTERMAN R A, WIDDOP R E, HANNAFORD J, LOW A M, ROBERTS R G D, KENT P, SIDERIS K, YIP T, HALES J R S and STEPHENS F R N (1988a). Laser Doppler velocimetry in the measurement of neurovascular function. Aust. Phys. Eng. Sci. Med. 11(2): 53-66.

11. WESTERMAN R A, SZOLCSANYI J, MAGERL F W, HANDWERKER H O, LOW A, PRATT A, WIDDOP R and KOZAK W M (1988b). Vasodilator axon reflexes. In: Vasodilatation: vascular smooth muscle, peptides, autonomic nerves and endothelium. Ed. P. Vanhoutte, Raven Press, New York p 107-112.

12. WESTERMAN R A, WIDDOP R E, HANNAFORD J, HOGAN C, ROBERTS R G D and ZIMMET P (1988c). Non-invasive tests of neurovascular function. Clin. Exper. Neurol. 24: 129-137.

II.2 ANIMAL STUDIES.

13. WESTERMAN R A, WIDDOP R E, LOW A M, HANNAFORD J, KOZAK W M and ZIMMET P (1988d). Non-invasive tests of neurovascular function: reduced axon reflex reflex response in diabetes mellitus of man and streptozotocin-induced diabetes of the rat. Diabetes Res. Clin. Practice. 5(1): 49-54.

14. LOW A M and WESTERMAN R A (1989). Neurogenic vasodilatation in the rat hairy skin measured using a laser Doppler flowmeter. Life Sciences, 45(1): 49-57.

15. WESTERMAN R A, WIDDOP R E, LOW A M, DELANEY C A and NEILD T O (1990). Non-invasive tests of neurovascular function in human and experimental diabetes mellitus. In: Molinatti G, Bar R, Belfiore S and Porta M (Eds). Frontiers in Diabetes v.9, Basel; Karger 1990, p 127-138.

16. GRUNFELD J, TIEDEMANN G J and WESTERMAN R A (1991). Chronic nicotine exposure enhances cutaneous axon reflexes in the rat. NeuroReport 2: 421-424.

17. CARR R W, DELANEY C A, WESTERMAN R A, ROBERTS R G D.(1993) Denervation impairs cutaneous microvascular function and blister healing in the rat hindlimb. NeuroReport. 4/5: 467-470.

18. GRUNFELD J A, TIEDEMANN G J and WESTERMAN R A (1993). Maternal nicotine exposure enhances cutaneous axon reflexes in the neonatal rat. NeuroReport. 4/6: 635-638.

19. WESTERMAN R A, CARR R W, DELANEY C A, MORRIS M J, ROBERTS R G D. (1993) The role of nociceptive afferent nerves in blister healing. Clin Exper Neurol 30: 39-61.

SECTION III: CLINICAL APPLICATIONS OF MEASURING NEUROVASCULAR FUNCTION

III.1 CAPSAICIN DESENSITISATION OF NOCICEPTIVE AFFERENTS.

20. WESTERMAN R A, ROBERTS R G D, KOTZMANN R R, WESTERMAN D A,

DELANEY C A, WIDDOP R E, CARTER B (1989). Effects of topical capsaicin on normal skin and affected dermatomes in herpes zoster. Clin. Exper. Neurol. 25: 71-84.

21. ROBERTS R G D and WESTERMAN R A (1991). Capsaicin: Hot stuff for pain relief. Current Therapeutics. 32: 43-49.

22. ROBERTS R G D, WESTERMAN R A, WIDDOP R E, KOTZMANN RR and PAYNE R (1992). Effect of capsaicin on cutaneous axon reflex vasodilatation in man. Agents and Actions. 35(1/2): 53-59.

III.2 SKIN BLOOD FLOW MEASUREMENTS.

23. HALES J R S, WESTERMAN R A, STEPHENS F R N, VAUGHAN J D, FAWCETT A A, RICHARDS D A B and RICHARDS C R B (1986). Lowered skin blood flow and erythrocyte sphering in collapsed fun-runners. Lancet, June 28, 1495-1496.

24. HALES J R S, STEPHENS F R N, FAWCETT A A, DANIEL K, SHEAHAN J, WESTERMAN R A and JAMES S B (1989). Observations on a new non-invasive monitor of skin. Clin. Exper. Physiol. Pharmacol. 16: 403-415.

25. HALES J R S, ROBERTS R G D, WESTERMAN R A, STEPHENS F R N, and FAWCETT A A (1992) Evidence for skin microvascular compartmentalization by laser-Doppler and photoplethysmographic techniques. Int. J. Microcirc. Clin. Exper. 12: 99-104.

III.3 DIABETES MELLITUS.

26. WESTERMAN R A, DELANEY C A, IVAMY-PHILLIPS A, HOROWITZ M and ROBERTS A. (1990) Concordance between different measures of small sensory and autonomic fibre neuropathy in diabetes mellitus. Clin. Exper. Neurol. 26: 51-63.

27. WESTERMAN R A, LINDBLAD L-E, WAJNBLUM D and ROBERTS R G D. (1992) Confounding factors in non-invasive tests of neurovascular function in diabetes mellitus. Clin. Exper. Neurol. 29: 149-160.

III.4 OTHER NEUROPATHIES.

28. WESTERMAN R A, BLOCK A, NUNN A, DELANEY C A, HAHN A, DENNETT X and CARR R W (1992) Hereditary sensory radicular neuropathy: defective neurogenic inflammation. Clin. Exper. Neurol. 29: 189-209.

29. WESTERMAN R A, PANO I, RABAVILAS A, NUNN A, HAHN A, ROBERTS R G D and BURRY H (1992) Reflex sympathetic dystrophy: altered cutaneous axon reflex and autonomic responses. Clin. Exper. Neurol. 29: 210-233.

SUMMARY OF THE FINDINGS REPORTED IN THE 29 PAPERS SUBMITTED FOR THE DEGREE OF DOCTOR OF MEDICINE.

SECTION I. SMALL CUTANEOUS SENSORY NERVE FUNCTIONS: MECHANISMS OF PRICKLE AND ITCH SENSATION.

1. Certain fabrics with fibre ends, including wool and brushed nylon when worn, sometimes evoke discomfort and sensations of prickle or itch. The physical, chemical, and neurophysiological processes responsible for fabric-evoked discomfort, itch and prickle sensation, and skin rash have been identified and defined[1,2,3,4,5,6].

2. Itch sensation was measured under various conditions using iontophoretic application of histamine as a controllable itch stimulus[2,3,6]. This is a significant improvement on previous electrical and chemical methods of evoking itch.

3. Microneurographic recording from identified sensory fibre during itching in human subjects was performed and sensory correlates obtained[3,4].

4. The neural basis of fabric-evoked sensations was identified using microneurography[4].

5. The effects of transcutaneous electrical stimulation of fine epidermal nerve fibres was defined in respect of sensation, neurogenic inflammation and rash[3,4,5].

6. This new understanding of the neural mechanisms leading to these less discriminative sensations, enabled definition of those fabric properties most likely to generate discomfort. This has predictive value for the textile industry and the techniques are still in use by CSIRO personnel for this purpose.

SECTION II: MEASUREMENT OF SMALL CUTANEOUS SENSORY NERVE AND MICROVASCULAR FUNCTION.

II.1 STUDIES IN HUMANS.

1. Transcutaneous electrical skin stimulation which excites nociceptor activity and laser

Doppler velocimetry were used to measure the neurogenic inflammatory function of primary sensory nerve fibre in human skin[7,8,12].

2. The electrical axon reflex dilator responses in skin, an index of the nocifensor actions of primary sensory nerves, are reduced in diabetes mellitus[9,12].

3. Laser Doppler velocimetry has been used to measure the relative changes in cutaneous microvascular blood flux after iontophoretic application of vasoactive chemical stimuli to epidermis[10], or noxious electrical transcutaneous stimulation[11].

4. These techniques have potential as clinical measures of small nerve fibre and microvascular function in many types of neuropathic disorders.

II.2 STUDIES IN ANIMALS.

1. Laser Doppler flowmetry, noxious electrical transcutaneous stimulation and iontophoretic application of vasoactive compounds were used to assess neurovascular function in the rat hind-paw[14,15].

2. Non-invasive tests of neurovascular function were used to demonstrate reduced axon reflex responses, and impaired microvascular endothelial function in streptozotocin diabetic rats[13,15]. The impairment was dependent on the duration of diabetes and partly reversible by insulin treatment[13,15].

3. The acetylcholine receptors on primary sensory nerves in rat skin have been identified as nicotinic cholinoceptors[16,17].

4. Exposure to systemically injected nicotine for periods of 7-14 days enhances cutaneous axon reflex responses in the rat[16,18].

5. Maternal nicotine exposure, whether gestational or lactational, enhances cutaneous axon reflexes in the neonatal rat[18].

6. Sensory denervation impairs cutaneous microvascular function and significantly prolongs

blister healing time in the rat hind paw[17,19].

7. An important role of nociceptive afferent nerves in epidermal repair has been confirmed by using nicotine exposure to enhance cutaneous axon reflexes, blister healing and substance P release evoked by nerve stimulation[19].

SECTION III: CLINICAL APPLICATIONS OF MEASURING NEUROVASCULAR FUNCTION.

III.1 CAPSAICIN DESENSITIZATION OF NOCICEPTIVE AFFERENTS.

1. Topical capsaicin applied to normal skin evokes cutaneous axon reflex dilatation, acute redness, heat, inflammation and pain which is capsaicin concentration-dependent[22].

2. Repeated topical capsaicin application (3-4x daily for 5-6 days) desensitized the treated skin for periods of up to 4 weeks[22]. In such capsaicin-desensitized skin, cutaneous axon reflex responses were greatly attenuated, but not autonomic or microvascular dilator or constrictor responses[22].

3. Affected dermatomes in post-herpetic neuralgia show allodynia, hyperalgesia and neuralgia. Prolonged thrice-daily application of capsaicin (0.05%) desensitized affected dermatomes to a variable extent[20,21]. Pain relief did not correlate with the extent of desensitization measured by thermal perceptual threshold testing[20,21].

4. Topical capsaicin cream proved to be a useful treatment for post-herpetic neuralgia with approximately 60% of patients reporting worthwhile pain reduction[21].

5. A weaker (0.025%) topical capsaicin cream proved significantly more efficacious than 0.05% cream for the reduction of acute varicella zoster pain in the first 6 weeks of the neurocutaneous eruption[21].

III.2 SKIN BLOOD FLOW MEASUREMENTS.

1. Fun-runners who developed exercise-induced hyperthermia (rectal temperature exceeding 40°C) were found to exhibit profound reduction of cutaneous microvascular

perfusion, and cessation of sweating[23], as well as red blood cell spher ing.

2. Laser Doppler flux (Periflux Pf1d) correlated linearly and significantly with cutaneous microvascular flow measured using radio-labelled microspheres in the skin of anaesthetized sheep[10].

3. In using photoplethysmographic or laser Doppler velocimetry techniques to measure cutaneous microvascular fluxes the emitted light wavelength was shown to determine the particular skin microvascular beds which dominated the recorded flux[24]. The infrared wavelengths longer than nm penetrated epidermis more deeply[24].

4. Simultaneous comparisons between infrared photo plethysmography and laser Doppler velocimetry, with absolute measurement of microvascular blood flow using radioactive labelled microspheres, provided evidence of functional compartmentalization of skin microvasculature[25].

III.3 DIABETES MELLITUS.

1. In patients with diabetes mellitus, there was general concordance between the severity of autonomic neuropathy measured by oesophageal and gastric emptying, and cutaneous neurovascular dysfunction assessed by axon reflex dilator responses evoked by transcutaneous noxious electrical stimulation and measured by laser Doppler velocimetry[26].

2. Similarly, there was general concordance between the severity of the somatic sensory neuropathy measured by warm and cold thermal perceptual threshold tests, and the autonomic neuropathy gauged from oesophageal and gastric emptying times[26].

3. Cutaneous neurovascular function was measured in the fingers of healthy volunteers, smokers, diabetic patients with neuropathy and workers exposed to repeated vibration with and without reported Raynaud's phenomena[27].

4. Vibration exposure, particularly in subjects reporting Raynaud's phenomena and smoking as well as diabetes may result in impaired neurovascular responses[27]. Thus these risk factors may be confounding factors for the assessment of neurovascular function in

diabetes mellitus[27].

5. These non-invasive tests of neurovascular function are proposed as screening test for workers with occupational vibration exposure, and for the investigation of Raynaud's phenomena and conditions such as scleroderma[27].

III.4 OTHER NEUROPATHIES.

1. Subjects with the autosomal dominant inherited condition called hereditary radicular sensory neuropathy (HSRN) exhibit clinical features corresponding to loss of primary sensory neurones, predominantly in the extremities[28].

2. Non-invasive tests of neurovascular function and clinical neurological examinations were performed on eight subjects with HSRN[28]. Neurogenic inflammation was abolished or severely impaired, but other vascular responses were largely unaffected[28]. These tests should be useful in screening offspring of HSRN-affected parents before clinical features appear.

3. Neurovascular function was tested non-invasively in patients with the chronic limb pain, loss of function, vascular, autonomic and dystrophic changes typical of reflex sympathetic dystrophy[29].

4. Microvascular responses, including cutaneous axon reflexes and responses to iontophoretic application of autonomic agonists, were significantly altered in affected skin[29]. Vascular responses were either enhanced or reduced , depending on the particular sequence of agonist pre-treatment applied to skin test sites[29].

5. Because of the complex neurovascular interactions between sympathetic and nociceptive sensory and sympathetic nerves in this condition, more research is required before neurovascular function tests become clinically useful in this intractable condition[29].

(1104 words)

29th IUPS, Satellite Symp., Australia
L.A. Chahl, J. Szolcsányi, F. Lembeck (eds)

ASPECTS OF HUMAN CUTANEOUS SMALL NERVE FUNCTION: SENSATIONS OF PRICKLE AND ITCH

R.A. WESTERMAN, R.K. GARNSWORTHY*, A. WALKER*, P. KENINS, R. GULLY* and P. FERGIN**

Department of Physiology, Monash University, Clayton, Victoria; *CSIRO Division of Textile Industry, Belmont, Victoria; **Dermatologist, Royal Children's Hospital, Parkville, Victoria, Australia

INTRODUCTION

Among the functions classically ascribed to small cutaneous nerve fibres are sensations of prickling and itch (Bishop, 1960; Sinclair, 1981), two of the most elusive forms of tactile discomfort experienced by man. The sensation we refer to as prickle is different to sensations of pricking and itching described by Sinclair (1981). Prickle is not a punctate sensation - both the stimulus and the percept are extended. Even though subjects have described the sensation of prickle as being like "gentle touches with fine pins" or "pricking with many fine needles", multiple pricking points will not evoke the sensation of prickle. It is most readily evoked by application of coarse fabrics to the skin, but can also be caused by some fine fabrics, arrays of closed wire loops and sandpaper. Although the finger pads are usually the most sensitive areas for touch sensations (Weber, 1835; Weinstein, 1968) prickle cannot be felt on glabrous skin. To evoke the sensation of prickle the stimulus surface must be gently moved, patted or pressed with varying pressure. The sensation of prickle is enhanced by warmth and sweating and is more rapidly detected by an experienced rather than naive subject, suggesting some element of learning. Apart from its intrinsic interest as a sensation, prickle has economic importance for the textile industry, and clinical importance to atopic patients who are reported to experience more frequent and severe wool fabric intolerance (Rajka, 1975; Hambly et al. 1978; Hanifin and Rajka, 1980).

Atopy is characterised by a personal or familial history of asthma, allergic rhinitis or atopic dermatitis (eczema) (Schnyder, 1960; Hanifin and Rajka, 1980). Many features of the disorder appear to be related to elevated blood and tissue histamine (Cormia, 1952; Arthur and Shelley, 1958). Itch provoked by garments frequently accompanies the disorder, and the symptom is assumed to be a result of mechanical stimulation of mediator-sensitised nerve endings within the skin. Although the neural basis for itch is at present unknown, erythema can be produced by stimulation of sensory polymodal nociceptor C-nerve fibres (Celander and Folkow, 1953; Kenins, 1981). Although these sensory units generally have a high threshold to mechanical stimulation, sensitization can result in a much lower threshold, so that touch can evoke a response (Bessou and Perl, 1969). It is possible that some fabrics either by mechanical or chemical means can sensitize nociceptors resulting in sensory discomfort or erythema.

For these reasons we have examined aspects of the sensations of prickle and itch which throw light on the underlying physiological mechanisms. These include :

1. the sensory nerve fibres which mediate prickle and itch sensations;

2. the physical dimensions of the adequate stimulus for prickle sensations; and

3. the relationships between prickle-sensitive individuals and atopy.

METHODS AND RESULTS

1. CUTANEOUS NERVE FIBRES WHICH MEDIATE SENSATIONS OF PRICKLE AND ITCH

In twelve consenting volunteers a total of 22 selective nerve blocks were performed using ischaemia (16) and local anaesthetic (6). Ischaemia and selective conduction block of large through progressively smaller nerve fibres was achieved by maintaining a sphygmomanometer cuff inflated on the upper arm at 270 mm Hg. The progress of the block was checked with known stimuli of vibration, static touch, light brushing, heat, cold, pain and prickle. The vibration stimulus consisted of a Bruel and Kjaer minishaker (frequency 100 Hz) with a $4mm^2$ probe and undamped displacement 500μm. In the order given above the remaining stimuli were : static touching with a hand-held $4mm^2$ probe, light brushing with a cotton wool wisp, touching with glass rod thermometers equilibrated to 45° C or 0°C, skin pinch with Spencer Wells forceps, and application of a 5 x 8cm rectangle of a standard coarse "prickly" fabric, respectively. These stimuli were repeated sequentially as rapidly as possible, the complete sequence occupying approximately 3 minutes and the subject's reported sensations recorded. Touch sensations usually began to fail rapidly after about 10 minutes and by 20-25 minutes all subjects reported that vibration and static touch were already lost. Most strikingly at this time there was vivid enhancement of prickle sensation evoked by fabric application (Fig. 1A). Prickle sensation disappears later at about the time that Aδ mediated cold sensation is confused or lost. At this same stage the C-fibre mediated sensations of heat and pain are diminishing. This sequence of dissociated sensory loss strongly suggests that the Aδ range of nerve fibres convey prickle sensation (Fig. 1A).

Local anaesthetic infiltration of the medial cutaneous nerve of forearm was used to produce selective blockade of the smallest nerve fibres. This was achieved by injecting 1% prilocaine (Cousins and Bridenbaugh, 1980) and the subject's sensations were recorded during recovery from local anaesthesia of the anteromedial aspect of the forearm. During recovery from prilocaine block of medial cutaneous nerve (Fig. 1B) return of prickle sensations on the forearm only followed some return of pain and thermal sensations. There was a variable enhancement of the magnitude of fabric-evoked prickle sensation at times ranging from 12-20 min after the first return of pain and thermal sensibility, and a distorted cold perception was often reported at this time.

Although the evidence so far obtained is consistent with Aδ mechanoreceptors mediating the sensation of prickle, this remains to be further tested by direct microneurographic recording using the techniques of Vallbo and Hagbarth (1968) and Torebjork and Wallin (1978).

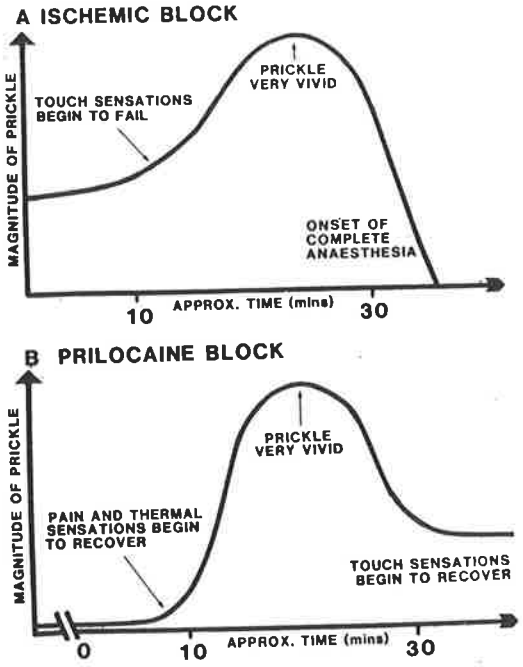


Fig. 1 A. Indicates approximate time course of loss of prickle and other sensations on forearm during ischaemic nerve conduction block at the upper arm.

B. Indicates recovery of prickle and other sensations on forearm during recovery from local anaesthesia of medial cutaneous nerve with 1% prilocaine.

2. PHYSICAL CHARACTERISTICS OF THE PRICKLE STIMULUS

Because the sensation of prickle is similar to that produced by numerous pricking points, most uninformed observers have assumed that prickle is caused by sharp ends indenting the skin. Although sharp ends such as shown in Fig. 2 are found in most fabrics they are completely unnecessary for the generation of prickle sensation. Surfaces consisting of fine wire loops with no protruding ends (Fig. 3) have evoked a sensation described by each of 49 subjects so far tested as "prickly" or "sharp". The percept of sharp ends experienced by these subjects is an illusion.

Coarse fabrics, sandpaper and wire loops all evoke a sensation of prickle. One factor common to all is the random irregularity of their surface topography. These stimuli are therefore likely to produce an extended random pattern of stresses in the skin with an approximate periodicity of 0.5 to 5 mm on the skin, and this might be the necessary

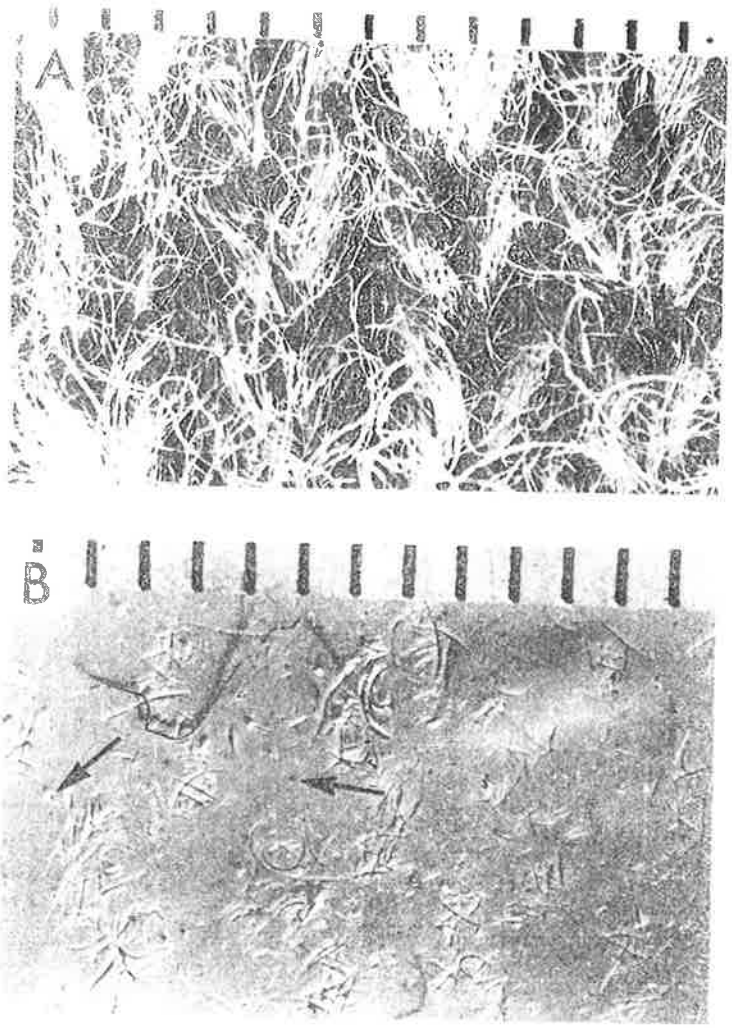


Fig. 2. Wool fabric and its negative impression.
A. Surface of wool fabric.
B. Impression of wool fabric on polished plasticine, load 1.5 Ncm^{-2}. Arrows indicate pits left by protruding fibre ends. The dark hair is a fibre pulled from fabric during impression. Scale divisions 1 mm.

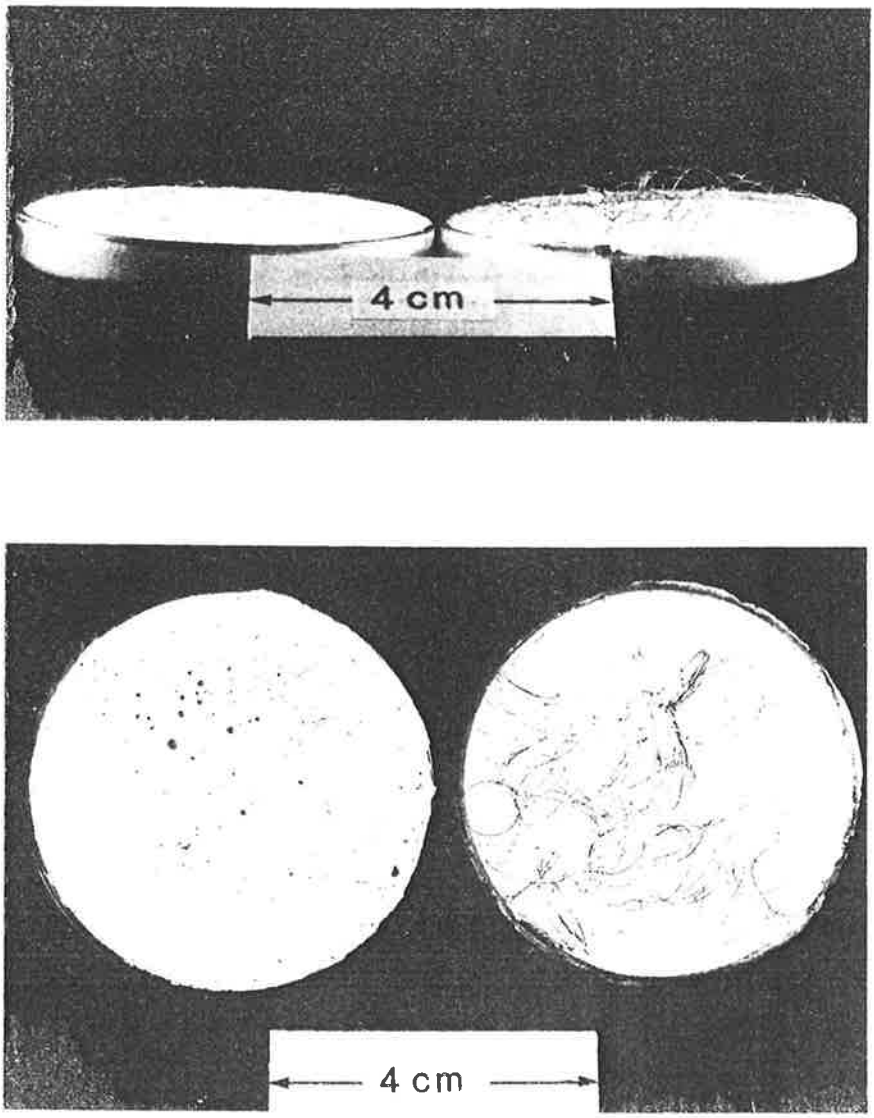


Fig. 3. Side views (upper) and vertical views (lower) of stainless steel wire loops embedded in plaster of paris with no protruding ends or roughness. At left, 35μm diameter wire; at right, 81μm diameter.

stimulus. More artificial surfaces are being made to test this hypothesis. However, none of these stimuli alone are sufficient, and the further conditions which must be satisfied are discussed below.

Prickle cannot be felt when the stimulus is moved across the skin. Subjects report that it feels rough but not prickly, suggesting that prickle is inhibited by activity in the large diameter rapidly-adapting cutaneous receptors. This is supported by the observation that a thin film of water on the skin enhances fabric-evoked prickle. The effect of water occurs even with fabrics that cannot absorb water or change their mechanical properties when wet. The effect occurs immediately after water is placed on the skin, and so is too prompt to be explained by a mechanical modification of the skin. It is possible that the effect of water is too slow, or prevents the movement of fabric hairs and loops, thereby reducing activity in the rapidly adapting cutaneous receptors. This effect of water-induced enhancement is reduced when activity in the large diameter sensory nerves is blocked by ischaemia.

Prickle cannot be felt on skin exposed to air cooler than about 15°C, but the sensation is enhanced if the subject is uncomfortably warm or sweating. Similar inhibitory convergences have been suggested by Kerr (1975) and by Kumazawa and Perl (1978).

These observations suggest that prickle depends on a balance between excitatory activity in Aδ mechanoreceptors caused by particular patterns of skin stress, and inhibitory activity in thermoreceptors and large diameter rapidly adapting mechanoreceptors.

3. ATOPY, PRICKLE AND ITCH

Atopy

We first identified and characterized the atopic status of a test population. These atopy assessments were always performed by a specialist dermatologist (P. Fergin), and 158 students, academic and technical staff ranging in age from 19 to 59 years were tested and classified as atopic, non-atopic or latent atopic individuals. Classification was based on the following criteria:

(a) history - personal or direct family history of asthma, eczema or hay fever (rhinitis);

(b) examination - any overt signs of asthma, hay fever or eczema (Hanifin and Rajka, 1980); any associated signs of atopy, e.g. Dennie-Morgan folds, hyperlinear palms, facial pallor, geographic tongue, etc.;

(c) intradermal skin testing (prick test) - for specific IgE activity was undertaken with common inhalant allergens including 7 mixed grass pollens (10,000 U/ml, CSL) and D. pterinissinus (10,000 U/ml, CSL), wool extract (1:1000, Hollister-Steer) with histamine (1:1000), and HS albumin-saline as positive and negative controls respectively.

The diameter of each wheal and erythema flare was measured and recorded. In addition, wool fabric was applied to the elbow flexures and any rashes, redness or vasomotor reactions were recorded.

Prickle

The physical characteristics of a stimulus that cause it to evoke prickle are unknown. To quantify this sensation we developed the following scale, using 6 experienced observers. Six fabrics (in 5 x 8cm rectangles) were selected, one that was smooth, soft and non-prickly to all observers and one that was coarse and prickly to each of the six. The four additional fabrics were selected such that they formed an ordered approximately equisectional scale as judged by our 6 experienced subjects (Gescheider, 1976). These fabrics were then numbered from 1 to 6 in order of increasing prickliness and tested on our 158 subjects.

Each subject was instructed to place a distinctly prickly fabric (more prickly than test fabric 6) on the inside of the forearm and without prompting asked to describe the sensation. Typically, words such as "sharp", "prickling" or "like fine pins" were used by the majority of our subjects. Those few (9) that did not give an accurate description of prickle were assumed to be attending to the other sensations evoked by the fabric and not prickle, and so were excluded from further testing. Two sets of the 6 test fabrics were then presented in order, face down to the subject, to find both an ascending and descending prickle threshold. Subjects were not informed that the second series of 6 fabrics was the reverse of the first series. Care was taken to ensure that there were no extraneous cues in the presentation or in the appearance of the test fabrics. The average of the ascending and descending thresholds, as a number from 1 to 6, was taken as the subject's threshold of prickle. This is justified as the fabrics were chosen to form an equisectional scale. If no prickle was felt for fabric 6 the subject was arbitarily assigned the number 7.

The subject was also asked to estimate the number of pricking points in the standard prickly fabric. 5 x 8cm rectangular photographs of random dot arrays (10, 30, 100, 300 and 1,000 dots) were presented as an aid to estimation.

Transcutaneous stimulation of small-diameter peripheral nerve fibres

From theoretical considerations discussed later, and from the work of others (Bishop, 1943; Edwards et al. 1976), we considered that it should be possible to assess human susceptibility to prickle and itch by transcutaneous electrical nerve stimulation (TENS). A constant current stimulator device was developed which was used to apply transcutaneous electrical stimuli of sufficiently low intensity to excite only the most superficial epidermal nerve fibres, presumably unmyelinated.

With the present TENS device (Fig. 6), a hand-held teflon probe contains a pair of etched tungsten needle electrodes 5 mm apart just contacting the skin surface, and delivers constant current through the skin for as long as the probe is held on the skin. For accessibility we have tested the anterior aspect of the forearm. However, it must be noted that different regions of human skin exhibit widely different sensory acuity as indicated by two point discrimination thresholds and other sensory discrimination tests (Weber, 1835; Weinstein, 1968).

A forced choice paradigm was used in which subjects had to choose between

probe "on" and "off" responses at the end of two immediately consecutive 15 second test intervals. The typical strategy most often used by subjects was to move the probe from place to place on the forearm, each contact lasting about one second. The first current producing 25% incorrect responses was scored as the threshold stimulus level.

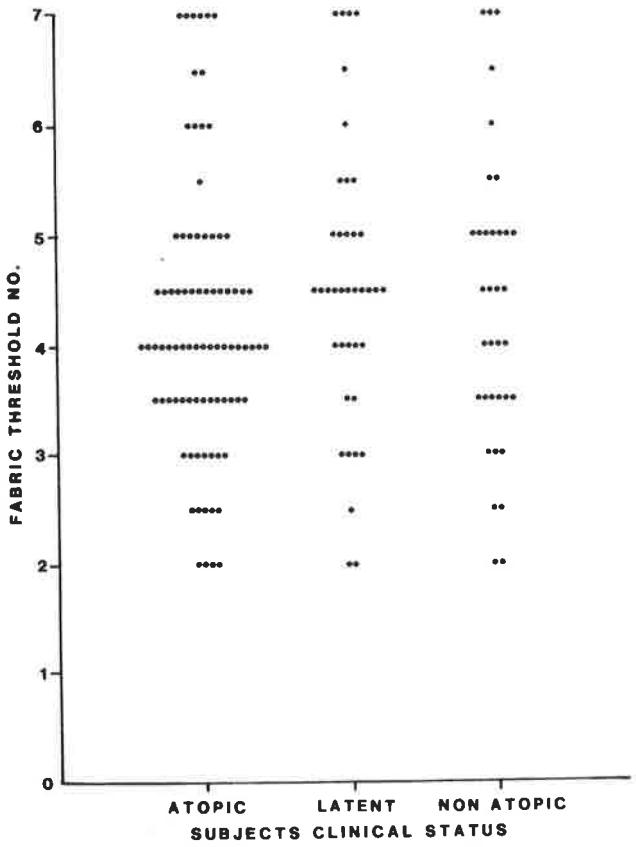


Fig. 4. Fabric-evoked prickle threshold vs. atopic status. The ordinate represents the average of the ascending and descending rank order for fabric-evoked prickle sensation threshold. There is no gross difference between the thresholds for atopic subjects compared to non-atopic subjects. However, there is a trend towards slightly lower average thresholds 4.21 (n=86) for atopic subjects compared with 4.54 (n=37) latent atopic or 4.36 (n=35) for non-atopic subjects.

Atopy and fabric-evoked cutaneous discomfort

Fig. 4 shows that there is no obvious difference between the averaged ascending and descending thresholds of prickle sensation evoked by the 6 graded. fabrics in atopic individuals compared with non-atopic.

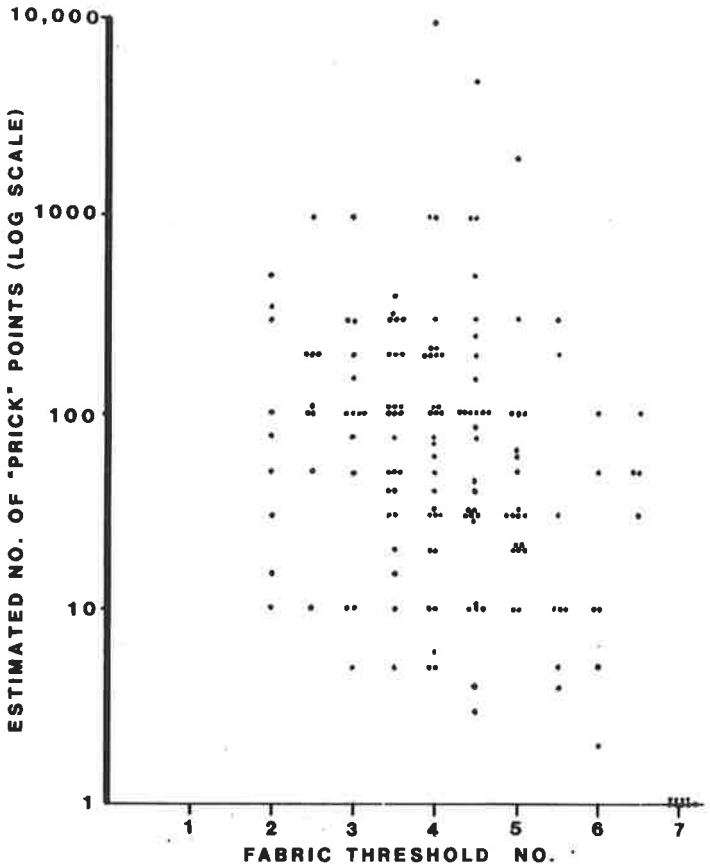


Fig. 5. Magnitude estimate vs. fabric-evoked prickle threshold. A threshold of 7 corresponds to a subject who did not experience a prickle sensation with the most prickly fabric. A logarithmic scale depicts the magnitude estimates in terms of "number of pricking points felt" when a standard fabric was applied and manipulated.

Nevertheless, there is a trend towards slightly lower averaged ranked fabric thresholds, 4.21, for the 86 atopic subjects compared with 4.54 for 37 latent atopic individuals and 4.36 for 35 non-atopic subjects (Fig. 4). There is no evidence that these subtle differences are statistically significant.

Fig. 5 shows magnitude estimates of the number of pricking points felt by subjects during the application and manipulation of a standard prickling fabric on the forearm. The estimates are plotted on the ordinate on a logarithmic scale, against fabric threshold number on the abscissa. Fabric number 1 was not perceived as prickly by any subject. Within the

individual response variability, a roughly linear relationship can be discerned between increasing magnitude estimate and decreasing fabric threshold number. This result suggests that subjects most likely to experience prickle sensation from finer fabrics also estimate larger numbers of prickling points, i.e. a more intense prickle sensation from the standard prickling fabric. Surprisingly there was not a close correlation between the number of short ends on a fabric surface as estimated from the polished plasticine negative image, and the intensity of prickle sensation it evokes. The results obtained using an artificial fabric from embedded wire loops (Fig. 3) in which there are absolutely no protruding ends support the conclusion that short fibre ends are not the only stimulus characteristic evoking prickle.

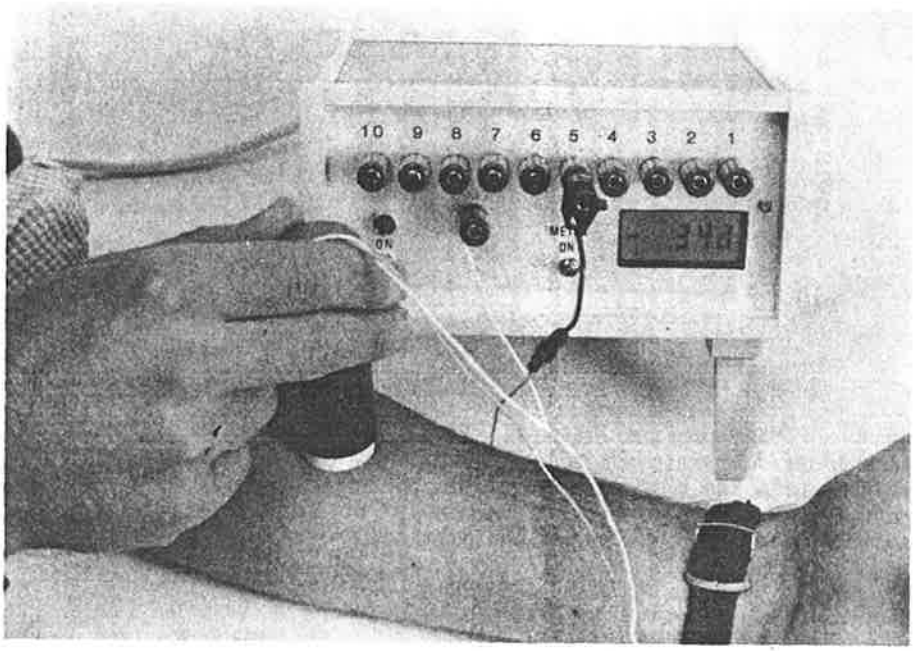


Fig. 6. Transcutaneous electrical nerve stimulator (TENS). PHotograph shows subject applying the teflon probe to the anterior aspect of forearm, the digital meter indicating current flow. During experimental testing of subjects a partition is placed to screen the instrument and experimenter from view. There are no auditory or visual cues to signal whether probe current is on or off during each series of forced-choice trials.

Temperature and mechanical masking effects on fabric-evoked cutaneous discomfort

The effect of temperature was measured by testing the sensation evoked by prickly fabrics applied to the forearm in a water bath in five prickle-susceptible subjects. If the subject is uncomfortably warm, prickle sensation is enhanced. If the temperature is below 15°C it is difficult to detect fabric-evoked prickle.

Two other experimental results provide evidence that activity in large rapidly-adapting cutaneous receptors could be inhibitory to prickle. First, prickle cannot be felt when the fabric is moved along the skin - subjects report that it feels rough but not prickly. Only very slow movement (approximately 1 cm sec^{-1} or less) allows prickle to be felt. Secondly, a thin layer of water on the skin enhances the sensation, and the effect of water is reduced when large diameter nerve fibres are blocked with ischaemia.

Selective electrical stimulation of cutaneous nerve fibres

This has been achieved with our TENS device at current intensities of 10^{-5} to 10^{-7} amperes. The threshold sensation most frequently evoked by the TENS probe applied to the anterior aspect of the forearm as in Fig. 1 is a fine pricking itch, and we believe this may qualitatively differ from the prickling sensation evoked by coarse fabrics. At greater current intensities distinct sharp pricking pain is felt, and at even greater intensities other sensations are reported which resemble those evoked by various mechanical stimuli (Gibson, 1968; Grimnes, 1983; Reilly and Larkin, 1983). An urgent need now is to identify by direct recording which types of epidermal sensory nerve fibres are excited by such transcutaneous electrical stimuli. We believe these to be small diameter intraepidermal C-fibres, some of which have been shown by immunofluorescence to contain substance P (Helme and Fletcher, 1982). Recruitment of nerve fibres as stimulus intensity is increased generally proceeds from largest to smallest. It is only possible to reverse this order if there is deliberate and precise positioning of stimulating electrodes near small fibres and remote from large fibres. With transcutaneous electrical stimulation of cutaneous nerves, geometric bias towards small nerve fibres is only possible if small fibres terminate most superficially within the skin. Transcutaneous electrical stimulation can preferentially engage the superficial C-fibres if needle surface-electrodes are used to ensure that the electrical current density within the skin falls rapidly with depth below the surface. Because of the insulating properties of the stratum corneum, the electrical current will channel its way through this layer and only then start spreading. This makes the geometric advantage of the small nerve fibres even greater since at least some terminate just beneath the stratum corneum.

Duration of the transcutaneous current pulse is also important if one wishes to preferentially stimulate small nerve fibres. Ranck (1981) shows that durations should be much greater than 10^{-3} second, and, in the present studies, durations of the order of 1 second have been used. With current pulses of this duration, current thresholds for neural stimulation are at the "rheobase" level (Ranck, 1981) and largely independent of duration. The distribution of electrical thresholds for our 158 subjects classified for atopic status is shown in Fig. 7. Of these, 84 were atopic (54%) and exhibited one or more of the features

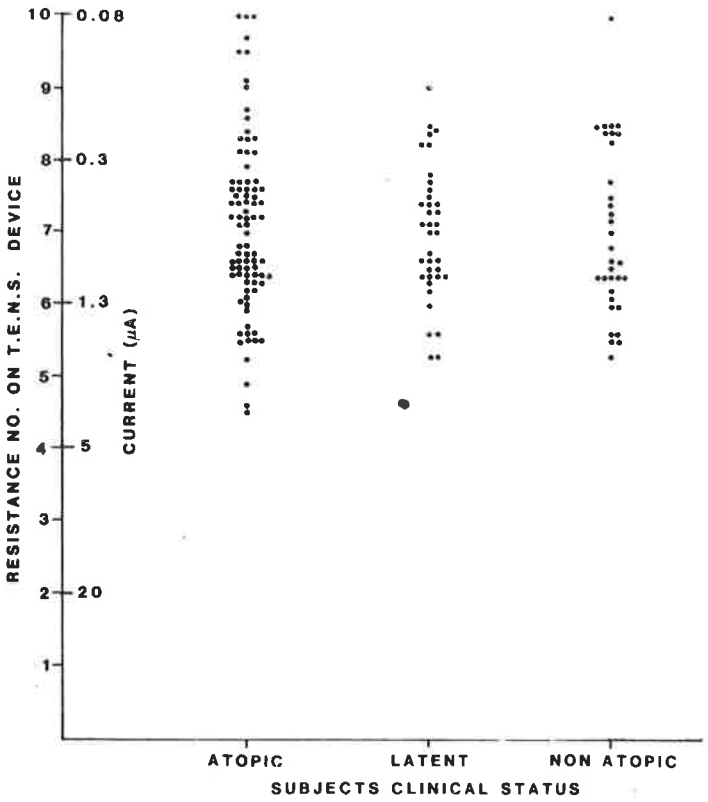


Fig. 7. TENS threshold vs. atopic status. The number of subjects in each category is indicated in brackets. No large difference in the mean threshold is seen between atopic and non-atopic subjects, nor in the number of subjects in each group developing a rash. The ascending series resistance numbers on TENS device correspond to increasingly small currents through the probe ranging from 1×10^{-5} amperes at resistance No. 1 to 1×10^{-8} amperes at resistance No. 10.

which make up the atopic diathesis (Rajka, 1975; Hanifin and Rajka, 1980), 37 were classified as latent atopics (23%) and 35 subjects with no evidence or family history of atopy were classed as non-atopic (22%). These figures are biased by selection of volunteers from a medical school and hospital staff population and do not reflect the prevalence of atopy in the community, which is 8.3% in Sweden (Hanifin and Rajka, 1980). The coincidence of skin erythema or rashes to wool fabric, or positive skin test to wool extract was slightly higher in the atopic group 7/86 (i.e. 9%) than in non-atopic subjects (2/35, i.e. 6%) and their threshold for electrically evoked cutaneous irritation was slightly less. Although the range of threshold currents was slightly greater for atopic than non-atopic subjects (Fig. 7), the number of subjects in each group was disparate.

GENERAL DISCUSSION

Our results do not confirm established opinions concerning the relationship between atopy and susceptibility to prickle and itch (Hanifin, 1982). There was no evidence to support the widely held belief that atopic individuals have a lowered threshold to fabric-induced discomfort. Nor did they have a lowered threshold to our electrical stimulus. However, subjects with a low threshold to fabric prickle estimate more pricking points and thus probably experience more intense prickle discomfort whether or not they are atopic.

Although the stimuli for evoking prickle and itch are unknown, short stiff fibre ends are not the only important parameter, as shown by our experiments with loops. Temperature also has an effect and there is evidence for mechanical masking. Itch sensation and its provocation by fabrics are often associated with sweating (Edwards et al. 1976). Substances with bradykinin-like activity appear both in sweat and in subdermal perfusates when sweat glands are stimulated by heat (Keele and Armstrong, 1960). Evidence that activity in large rapidly-adapting cutaneous receptors could be inhibitory to prickle is provided by the observation that prickle cannot be felt when the fabric is briskly moved along the skin. Furthermore, a thin layer of water on the skin enhances the sensation, and the effect of water is reduced when large diameter nerve fibres are blocked with ischaemia. Consistent with this it was noted that active sweating, in addition to lowering itch thresholds, may also increase prickle discomfort as soon as there is sufficient moisture on the skin.

Evidence to support $A\beta$ mediated inhibition from touch receptors comes from the work of Kumazawa and Perl (1978) who showed just such a system acting on the small $A\delta$ fibres. Our work with nerve block experiments supports the idea of prickle being mediated at least in part by $A\delta$ fibres, and from the study of Adriaensen et al. (1983) who measured the threshold of human $A\delta$ receptors, we would predict that the most likely candidates are the $A\delta$ mechanoreceptors because it is most unlikely that fabrics can generate the amount of force (22.5mN) required to excite $A\delta$ nociceptors.

The result of comparing our data on atopic and non-atopic subjects' thresholds to fabric, electrical skin stimulation and to magnitude estimates was unexpected. Although there is a widespread belief among dermatologists that there is an association between atopy, and prickle and itch susceptibility (Hambly et al. 1978; Hanifin and Rajka, 1980), this is not evident in our results. This suggests that atopy is not a necessary predisposing factor for fabric-evoked tactile discomfort, and that fabric-evoked prickle threshold is not a predictor of atopic status.

SUMMARY

Prickle and itch are often introspectively distinct sensations. The present study suggests that they are mediated by different kinds of small-diameter afferent nerve fibres. Sensory mechanisms and stimulus parameters for prickle have been examined and while some fabrics can cause prickle, for most people fabric-evoked itch may be restricted to those whose thresholds are lowered by atopy or sweating. It is hoped that these findings will lead to new fabric surface treatments for reducing tactile discomfort.

The measurement of fibre-evoked prickle thresholds and electrical thresholds and their correlation with the atopic status of the tested population suggests that atopy or allergy is not a necessary or major predisposing factor for fabric-evoked tactile discomfort. Conversely, fabric-evoked tactile discomfort thresholds do not indicate atopic status.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to the following individuals for their valuable technical assistance in this study : Jill Holloway, John Card, *Michael Cardamone, *Campbell Harvey, *Amanda Leaman, and to Drs. Robin Marks and Jim Butler for helpful critical discussion.

Supported by a grant from the Wool Research Trust of the Australian Wool Corporation and Monash Special Research Grant Committee.

*Audiovisual Services Alfred Hospital, Prahran, Victoria.

REFERENCES

Adriaensen, H., Gybels, J., Handwerker, H.O. and van Hees, J. (1983) Response properties of thin myelinated (Aδ) fibres in human skin nerves. J. Neurophysiol., 49, 111-122.

Arthur, R.P. and Shelley, W.B. (1958) The nature of itching in atopic dermatitis. Ann. Int. Med., 49, 900-908.

Bessou, E. and Perl, E.R. (1969) Response of cutaneous sensory units with unmyelinated fibres to noxious stimuli. J. Neurophysiol., 32, 1025-1043.

Bishop, G.H. (1943) Responses to electrical stimulation of single sensory units of skin. J. Neurophysiol., 6, 361-382.

Bishop, G.H. (1960) The relation of nerve fibre size to modality of sensation. In : Advances in biology of skin, W. Montagna (ed.), Pergamon Press: Oxford, Vol. 1, pp 88-98.

Celander, O. and Folkow, B. (1953) The nature of the distributions of afferent fibres provided with the axon reflex arrangement. Acta. Physiol. Scand., 29, 359-370.

Cormia, F.E. (1952) Experimental histamine pruritus. I., Influence of physical and psychological factors on threshold reactivity. J. Invest. Dermatol., 19, 21-34.

Cousins, M.J. and Bridenbaugh. P.O. (1980) Neural blockade in clinical anaesthesia and management of pain. J.P. Lippincott Co., Philadelphia.

Edwards, A.E, Shallow, W.V.R, Wright E.T. and Dignain, T.F. (1976) Pruritic skin disease, psychological stress and the itch sensation. Arch. Dermatol., 112, 339-343.

Gescheider G.A. (1976) Psychophysics method and theory. Lawrence

Erlbaum and Assoc., Hillsdale, New Jersey, p 102.

Gibson, R.H. (1968) Electrical stimulation of pain and touch. In: The skin senses, D.R. Kenshalo (ed.), Charles C. Thomas, Springfield, Illinois. pp 223-261.

Grimnes, S. (1983) Electrovibration, cutaneous sensation of microampere current. Acta. Physiol. Scand., 118, 19-25.

Hambly, E.M., Levia, L. and Wilkinson, D.S. (1978) Wool intolerance in atopic subjects. Contact Dermatitis, 4, 240-241.

Hanifin, J.M. and Rajka, G. (1980) Diagnostic features of atopic dermatitis. Acta. Derm. Venereol. (Stockh.), 92, 44-47.

Hanifin, J.M. (1982) Atopic dermatitis. J. Am. Acad. Dermatol., 6, (1), 1-13.

Helme, R.D. and Fletcher, J.L. (1982) Substance P in the trigeminal system at autopsy - evidence for a role in pain pathways in man. Clin. Exp. Neurol., 19, 37-44.

Keele, C.A. and Armstrong, D. (1960) Substances producing pain and itch. Edward Arnold Ltd., London. pp 288-304.

Kenins, P. (1981) Identification of the unmyelinated sensory nerves which evoke plasma extravasation in response to antidromic stimulation. Neurosci. Lett., 25, 137-140.

Kerr, F.W.L. (1975) Pain: a central inhibitory balance theory. Mayo Clin. Proc., 50, 685-690.

Kumazawa, T. and Perl, E.R. (1978) Excitation of marginal and substantia gelatinosa neurons in the primate spinal cord. J. Comp. Neurol., 177, 417-434.

Rajka, G. (1975) Atopic dermatitis, W.B. Saunders & Co. Ltd., London.

Ranck, J.B. (1981) Extracellular stimulation. Electrical stimulation research techniques, M.M. Patterson and R.P. Kesner, (eds.), Academic Press: New York. pp.1-36.

Reilly, J.P. and Larkin, W.D. (1983) Electrocutaneous stimulation with high voltage capacitive discharges. IEEE Trans. Biomed. Eng., BME30, 631-641.

Schnyder, U.W. (1960) Neurodermatitis, asthma, rhinitis, Karger: Basel, New York.

Sinclair, D. (1981) Mechanisms of cutaneous sensation, Oxford University Press: Oxford.

Torebjork, H.E. and Wallin, R.G. (1978) Skin receptors supplied by unmyelinated (C) fibres in man. In: Active touch, G. Gordon (ed.), Oxford Press. pp. 475-487.

Vallbo, A.B. and Hagbarth, K-E. (1968) Activity from skin

mechanoreceptors recorded percutaneously in awake human subjects. Exptl. Neurol., 21, 270-289.

Weber, E.H. (1835) Uber den Tastsinn. Archiv. fur Anat., Physiol. und Wiss. Med., pp. 152-159.

Weinstein, S. (1968) Intensive and extensive aspects of tactile sensitivity as a function of body part, sex, and laterality. In: The skin senses, D.R. Kenshalo (ed.), Illinois: Charles C. Thomas: Springfield, Illinois. pp. 195-222.

Discussion

Handwerker - Due to your excellent and stimulating talk I am now feeling prickly all over!

Lawson - It's the psychological effect that is the problem!

Westerman - You are quite right. I did mention the learning aspect. The question is where do we feel and where do the psychophysical phenomena occur? The psyche is very heavily involved - it is not just physics!

Handwerker - This makes it very difficult. You mentioned microneurography. Van Hees tried very hard to find the substrate of itching. First Van Hees tried intracutaneous injection of histamine, which didn't work because of pain from the pricking etc. His second approach was to use common European nettles because they contain acetylcholine and histamine and when applied to the skin they produce reddening and wheals.

Westerman - The other plant which may be used is cowage.

Handwerker - We had no cowage. It is an American plant, I think. We used nettles and in fact either nothing would happen or the subject would say this is not itching, it is burning. And this raises another difficulty. All of these sensations are very difficult to separate from each other. Van Hees found a few polymodal C fibres firing at low frequency with this stimulus of the nettles but it was not convincing because we know that low frequency firing of a few C fibres from polymodal nociceptors does not produce any sensation under other circumstances. So we must have a very powerful gating mechanism in our central nervous system for these sensations, which is much more effective than that for pain.

Westerman - I think some evidence to support that would be the effect of water which produces a most vivid enhancement of the sensation of prickle.

Handwerker - Yes. So finally we didn't find any conclusive evidence on which type of fibre transmits this kind of sensation.

The last point I want to mention is that of language. In fact there does not exist in German any word for "prickle". We use the same word as that used for "scratching".

MECHANISMS IN CUTANEOUS SENSATIONS OF PRICKLE AND ITCH EVOKED BY FABRICS

R. Garnsworthy*, R. Mayfield*, R. Gully*,
R. Westerman** and P. Kenins**

* CSIRO Division of Textile Industry,
Belmont, Victoria 3216, Australia
** Department of Physiology, Monash University,
Clayton, Victoria 3168, Australia

SYNOPSIS

Some fabrics evoke unpleasant pricking-itch-like sensations when worn against the skin. For wool fabrics, the irritation is shown to result from mechanical stimulation of pain nerve fibres that terminate just beneath the dry keratinous outermost layer of skin. The mechanical responsiveness of the nerve fibres changes with hydration of the layer and is of a form that indicates that the major irritant features of a wool fabric are protruding fibre ends capable of bearing loads of approximately 100 mg or more. A replication technique sensitive to these fibre ends has been developed and is being used for the objective measurement of fabric prickliness.

INTRODUCTION

Some fabrics evoke unpleasant prickle sensations when worn against the skin. Prickle evokes a desire to scratch and is often described as having pricking-itch-like qualities. The degree of discomfort depends strongly on the individual, and, in severe cases, can be followed by skin inflammation. With the current trend to casual wear, skin irritation from wool worn against the skin is of some concern.

Sensory physiologists have long ascribed sensations of pinprick and itch to nerve fibres that are involved in painful sensations, but, until now, prickle has received no such attention. Clinicians have thought it unlikely that pain nerve fibres could be mechanically excited by fabrics, and many have invoked immunological factors to help explain the skin irritation observed with some wools. Examples of this thinking exist in: the belief of allergy to wool, the use of commercial wool extracts for allergy testing, and the association of wool with atopy (a state of generally increased allergic sensitivity).

The lack of understanding of the physiological mechanisms of skin irritation from wool has also made it difficult to identify fabric surface features responsible for skin irritation. Fibre coarseness is the only feature so far identified with any certainty[1].

This paper describes measurements performed to elucidate the physiological mechanisms involved in prickle and in skin inflammation. The paper also shows how this information is being used in the development of objective test procedures for fabric prickliness, and in the definition of methods for controlling the skin-irritation problem. A short introduction to sensory physiology follows first to acquaint the reader with the subject.

BASIC PHYSIOLOGY OF SKIN NERVES

There are numerous sensations which can be experienced as a result of stimulating the skin, and three categories of sensory nerve are generally agreed to cover them all: the touch group of pressure and vibration, the thermal group of warmth and coolness, and the pain group[2]. In each case, there is a transducer, usually called a receptor, close to the surface of the skin, and a nerve fibre connecting the receptor more or less directly to the brain. In most cases, the structure of the receptor is unknown, but input-output characteristics are well documented.

The receptors of the touch group respond to mechanical displacement of the skin and of hairs on the skin. Some respond only to time-varying displacements, while others, in addition, respond to steady displacements. The transmission of information from the receptors to the brain is via large-diameter rapidly-conducting nerve fibres. The touch senses have excellent spatial and temporal discrimination, and are capable of exquisite sensitivity: vibrations with an amplitude of one micrometre can be detected[3]. The assessment of fabric texture depends on information gained by this group of senses.

The receptors of the thermal group maintain steady background levels of activity in their associated nerve fibres under ambient temperature conditions. There are two distinct kinds of receptor: "warm" receptors whose neural activity increases with increasing temperature, and "cold" receptors whose neural activity decreases with increasing temperature.

Information is transmitted from the receptors to the brain via small-diameter slowly-conducting nerve fibres. The group exhibits spatial summation, and temperature changes as small as 0.05°C can be detected if large areas of skin are involved[4].

The receptors of the pain group are responsible not only for sensations of frank pain, but also sensations of pinprick and itch. This group is the least well understood. Receptors respond variously to tissue damage and to potentially damaging mechanical, thermal and chemical stimuli. However, it is not known whether pain, itch and pinprick are the result of particular patterns of stimulation of the receptors, or whether there are separate receptors within the group for each sensation. Information is transmitted along small-diameter slowly-conducting nerve fibres, and is subject to considerable processing and filtering within the central nervous system. The group exhibits spatial and temporal summation, and a stimulus which is too mild to produce pain may do so if it is repeated in time or duplicated at nearby locations. Minor forms of skin irritation such as itch can be masked by simultaneous activity set up in the touch group of nerve fibres by, for example, scratching or rubbing[5].

In addition to the transmission of information to the central nervous system, some pain receptors also release chemicals including a peptide - Substance P - into the superficial skin layers[6]. Substance P is a potent dilator of blood capillaries, and quickly produces an inflammatory response called axon reflex.

PHYSIOLOGICAL EXPERIMENTS

Preliminary Observations on Prickle and Skin Inflammation

Unlike texture assessments, such as roughness, which can be made quickly, there is usually a delay of several seconds between the time when suitable fabric is placed on the skin and when prickle is felt. Prickle is an elusive sensation, and may fluctuate in intensity over a period of seconds. It is experienced most readily when suitable fabric is gently patted or pressed onto hairy skin, such as the forearm, but not on the glabrous skin of the fingers or palm. Prickle cannot be felt if: the fabric is rubbed or wiped over the skin, the skin is uncomfortably cold, or the skin contact area is smaller than about one one square centimetre. Moisture on the skin greatly increases the magnitude of the sensation.

In those rare instances when skin inflammation is observed, the response is consistent with axon reflex[7]. As seen earlier, this implies the excitation of pain nerves.

Nerve Group Mediating Prickle

Considerable progress towards the identification of nerve fibres mediating a particular sensation can be derived from nerve block experiments[2]. Two agents, pressure and local anaesthetic can be used, but only the former will be considered here.

Pressure blocks are produced either by a compression cuff around a limb or by direct pressure exerted on an accessible nerve trunk. In such blocks, the large-diameter nerve fibres fail before the small-diameter fibres, and by monitoring changes in sensation as the block proceeds it is possible to assign a particular sensation to one or other of the nerve groups. The procedure is controllable, reproducible, and completely reversible. Recovery from a block occurs rapidly.

In twelve volunteers a total of sixteen pressure-block experiments have been performed using, in each case, a blood-pressure cuff inflated on the upper arm at 270 mm Hg (Fig. 1). The progress of each block was checked on the forearm with known stimuli of vibration, static touch, light brushing, heat, cold, pain (forceps pinch) and prickly fabric. Touch sensations usually began to fail rapidly after about 10 minutes, and by 20-25 minutes all subjects reported that vibration and static touch were lost. Most strikingly at this time there was vivid enhancement of prickle (Fig. 1). Prickle sensation disappeared later at about the same time that sensations of heat, cold and pain were diminishing.

This sequence of dissociated sensory loss determines that small-diameter nerve fibres mediate prickle sensation, and that activity in large-diameter fibres may have an inhibitory affect. Small-diameter nerve fibres are involved in both thermal and painful sensations, but it is only the pain group that is known to mediate uncomfortable sensations which have qualities similar to that of prickle (for example, pinprick and itch). Thus, a reasonable working hypothesis based on the pressure-block experiments is that prickle sensation is mediated by nerve fibres of the

pain group. Prickle and skin inflammation are then both the result of pain-nerve excitation.

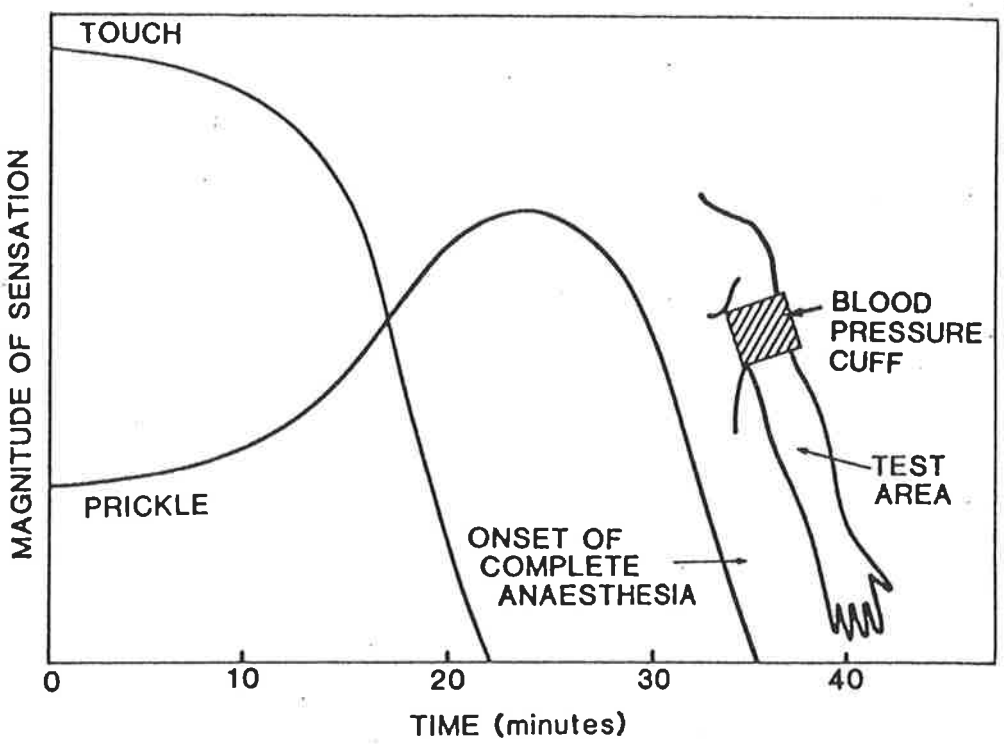


Fig. 1. Approximate time course of loss of prickle and touch sensations on forearm during nerve pressure block at the upper arm.

Confirmation of the working hypothesis has been sought in animal and human experiments in which electrical recordings have been made from nerve fibres of the pain group during manipulation of suitable fabric on the skin (Fig. 2). Figure 2 shows the electrical activity set up in a human pain nerve fibre during manipulation of prickly fabric on both dry and wet skin. The amplitude of the impulses carries no information, only their distribution in time. The presence of activity in pain nerve fibres during fabric manipulation, together with its increase when the skin is wet, matches the subjective observations and greatly strengthens the working hypothesis.

Excitation of touch receptors was also routinely observed during the electrical recordings, but in no case was any change in neural activity observed upon hydration of the skin.

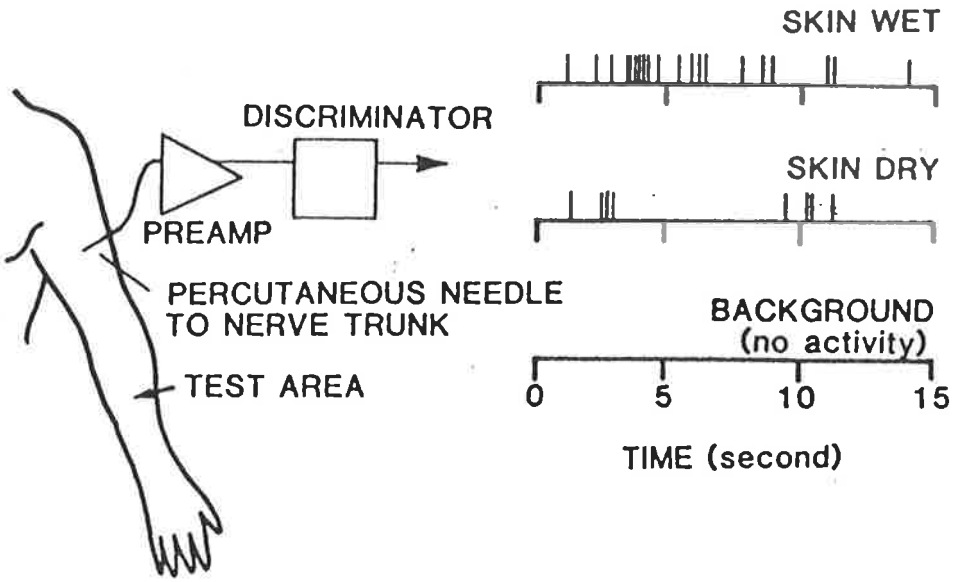


Fig. 2. Electrical recording from single pain nerve fibre during application of prickly fabric to the forearm.

Pain-Nerve Excitation

Measurement of mechanical excitation thresholds for pain receptors in animal skin has been performed using glass fibres of diameter similar to that of wool fibres. Thresholds of approximately 100 mg have been found for end contact. No change in threshold was observed when individual wool fibres were used, and it can be reasonably assumed that wool acts purely as a mechanical stimulus.

No such experiments have yet been performed with human subjects and recourse has been made to estimation of mechanical thresholds based on measurement of the depth within the skin of the pain receptors. A technique for measuring the depth on the forearm was developed using electrical stimulation from the skin surface[8]. The geometry chosen was one in which the stimulating current was made to channel through the dry keratinous outermost layer of skin (the stratum corneum) and then to spread uniformly and rapidly. This enabled the depth of the pain receptors beneath the stratum corneum to be derived. Considerable variation was found between individuals, the average value being approximately 5 μm. The average stratum corneum thickness on the forearm is 15 μm[9], and is thus the major component of the pain receptor depth in most individuals. Assuming a depth for the pain receptors beneath the skin surface of 20 μm, a continuum mechanics model of the skin was used[10] to compute mechanical

excitation thresholds as a function of diameter of a stimulating probe (Fig. 3). Even though the model makes use of a number of simplifying assumptions, there is general agreement with the animal work for probe diameters similar to that of wool fibres.

The above work suggests that the major prickle stimulus of a fabric will be protruding fibre ends capable of bearing loads of approximately 100 mg or more. Fibres which contact the skin over larger areas, such as by lying along the surface, could not support the very high forces then required to excite the pain receptors. Such fibres will only excite the much more sensitive receptors of the touch group.

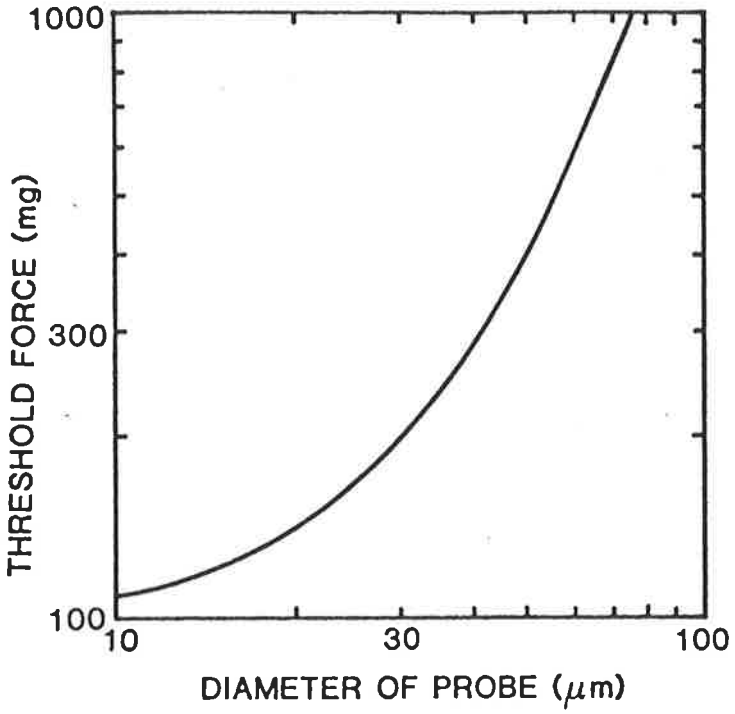


Fig. 3. Computed threshold force for pain nerve activity as a function of probe diameter.

TEXTILE EXPERIMENTS

Objective Measurement of Fabric Prickliness

A 'skin model' has been devised which provides a measure of the number of high-load bearing fibre ends on a fabric surface and their load distribution (Fig. 4). This model consists of a thin film of PTFE tape stretched across a microscope slide. The fabric sample is pressed against the PTFE film at a pressure of 4 gcm^{-2} which is considered to be near the upper limit of typical clothing pressures at which prickle is sensed.

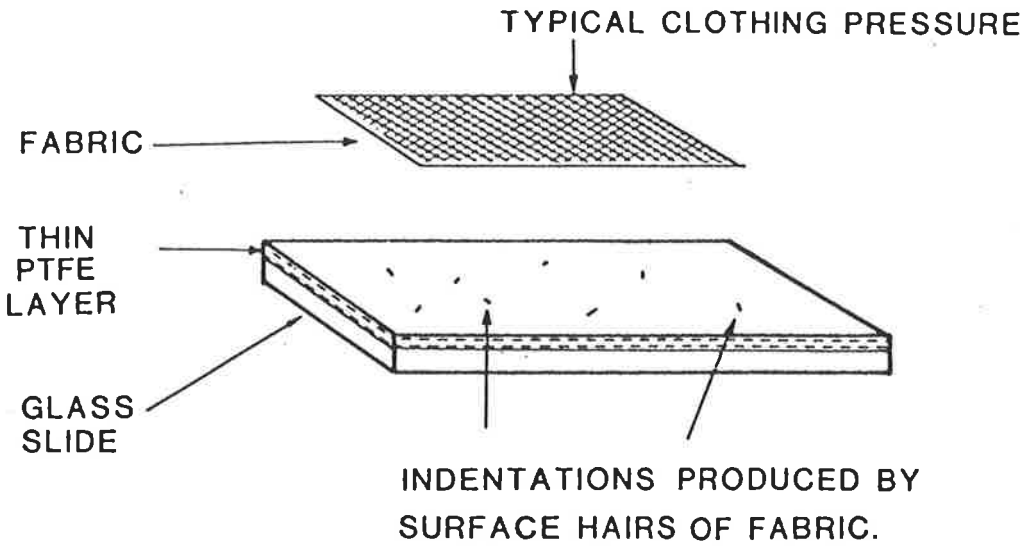


Fig. 4. Replication method used in the objective measurement of fabric prickliness.

Fibre ends supporting loads greater than 40 mg form an impression, as a crater, which remains because of the poor elasticity of PTFE. The form of the impression, as viewed under a microscope using alternately incident light inclined to the plane of the slide and transmitted light, provides an estimate of the load supported at the fibre end. By this procedure the high-load bearing ends are selectively identified and classified into four load groups (40-80, 80-120, 120-200, >200 mg). The method has been applied to a range of fabrics and the results correlated with subjectively assessed prickle.

Correlation coefficients of 0.15 and 0.77 were obtained between the mean subjective rankings of six fabrics in ascending order of prickle intensity by eighty judges and the density of fibre ends supporting loads >40 mg and >120 mg respectively. In another subjective test, mean magnitude estimates of the prickle intensity of seven fabrics by twenty-four judges were plotted against the density of fibre ends carrying loads >40 mg and >120 mg to give correlation coefficients of 0.52 and 0.76 respectively. The second of these plots is shown in Fig. 5.

Thus the density of fibre ends supporting loads of 120 mg or more correlated best with subjectively assessed prickle indicating these are the main prickle stimuli. This finding is in good agreement with the animal

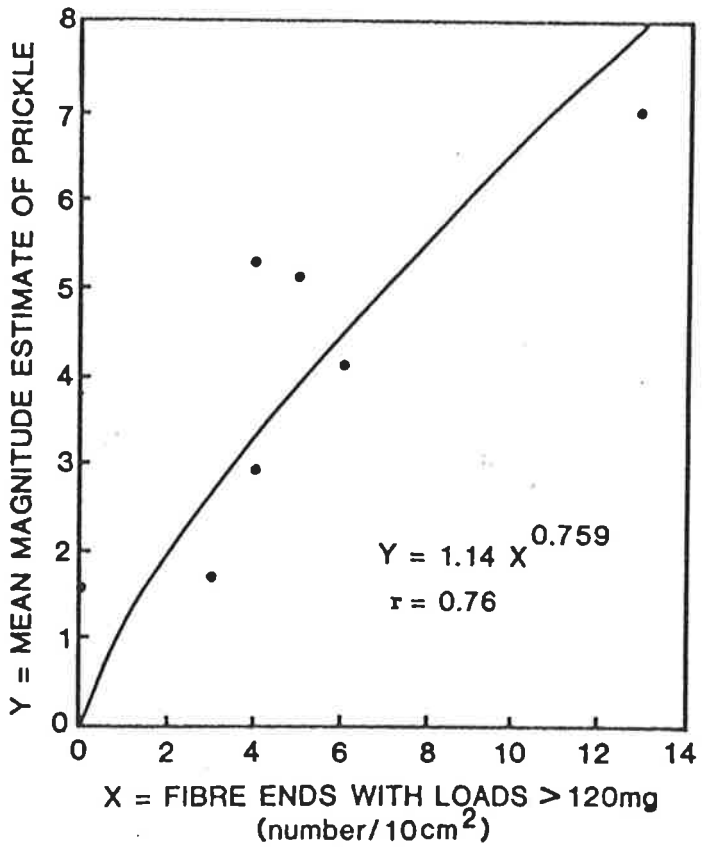


Fig. 5. Correlation of the mean magnitude estimate of prickle intensity of seven fabrics by twenty-four judges with the density of fibre ends supporting loads >120 mg. The psychophysical power law of S.S. Stevens was used to fit the data[11].

studies and the continuum mechanics model which suggest that threshold loads are in the order of 100 mg.

CONCLUSIONS

The physiological mechanisms involved in prickle and in skin inflammation have been identified. The major irritant features of a fabric are mechanical and comprise protruding fibre ends capable of bearing loads of approximately 100 mg or more. These fibre ends can be selectively counted using replication techniques. To increase the skin comfort of wool fabric it is imperative that irritant fibres be decreased in number, and that the skin be kept comfortably dry. Irritant fibre numbers might be decreased, for example, by decreasing fibre rigidity, by increasing fibre length, and by decreasing fibre-fibre interactions within the fabric. The skin can be kept reasonably dry by treating fibres chemically to give

hydrophilic surfaces, and by using fabrics with good heat and moisture transport. Work is in progress to evaluate these approaches.

REFERENCES

1. B.N. Hoschke, Objective Specification of Fabric Quality, Mechanical Properties and Performance, in "Proc. Aust.-Japan Symp. on Obj. Spec. Fabric Qual. Mech. Props.", S. Kawabata, R. Postle and M. Niwa, eds., The Textile Machinery Soc. of Japan, Osaka (1982).
2. D. Sinclair, "Mechanisms of Cutaneous Sensation", Oxford University Press, Oxford (1981).
3. W.H. Talbot, I. Darian-Smith, H.H. Kornhuber, and V.B. Mountcastle, the Sense of Flutter-Vibration: Comparison of the Human Capacity with Response Patterns of Mechanoreceptive Afferents from the Monkey Hand, J. Neurophysiol., 31, 301 (1968).
4. K.O. Johnson, I. Darian-Smith, C. Lamotte, B. Johnson and S. Oldfield, Coding of Incremental Changes in Skin Temperature by a Population of Warm Fibres in the Monkey: Correlation with Intensity Discrimination in Man, J. Neurophysiol., 42, 1332 (1979).
5. R. Melzack and P.D. Wall, Psychophysiology of Pain, International Anaesthesiology Clinics, 8, 3 (1970).
6. F. Lembeck, Mediators of Vasodilatation in the Skin, Brit. J. of Dermatology, 109, Suppl. 25, 1 (1983).
7. R.K. Garnsworthy and A. Walker, Axon Reflex and Inflammatory Responses of the Skin to Clothing, CSIRO Division of Textile Industry, Report No. GC 45 (1983).
8. R.K. Garnsworthy and R. Gully, Transcutaneous Electrical Stimulation and the Depth Within the Skin of Receptor Endings of Nerve Fibres Involved in Sensations of Prickle and Itch from Clothing, CSIRO Division of Textile Industry, Report No. GC41 (1982).
9. K.A. Holbrook and G.F. Odland, Regional Differences in the Thickness (Cell Layers) of the Human Stratum Corneum: An Ultrastructure Analysis, J. Invest. Dermatology, 62, 415 (1974).
10. R.K. Garnsworthy and A. Walker, The Importance of Skin Mechanical Properties to Sensory Reception in Sensations of Prickle and Itch from Clothing, CSIRO Division of Textile Industry, Report No. GC 42 (1983).
11. G.A. Gescheider, "Psychophysics: Method and Theory", Lawrence Erlbaum Associates, Inc., Hillsdale, New Jersey (1976).

ACKNOWLEDGEMENTS

The author sincerely thanks the Australian Wool Corporation and the South African Wool Board for granting the research project reported and the International Wool Secretariat, Ilkley, for providing the test samples.

The apparati and test methods used have been built and developed with grants from the Forschungskuratorium Gesamttextil, Frankfurt/M, funded by the Arbeitsgemeinschaft Industrieller Forschungsvereinigungen (AIF), Köln.

REFERENCES

1. K.H. Umbach, Textile transmission, Textile Asia 12, 75 (1981)
2. K.H. Umbach, Measuring the physiological properties of textiles for clothing, Melliand Textilber., English Edition, 747, June (1980)
3. K.H. Umbach, Comparative thermophysiological tests on blankets made from wool versus acrylic/cotton, Paper B-43 presented at 7th Int. Wool Text. Res. Conf., Tokyo (1985)
4. DIN 53 855 T 2, Determination of the thickness of textile fabrics, Oct. (1982)
5. K.H. Umbach, Biophysical evaluation of protective clothing by use of laboratory measurements and protective models, in : Proc. Int. Conf. on Med. and Biophys. Aspects of Protective Clothing, 226, Lyon (1983)
6. B. Holcombe, The thermal insulation performance of textile fabrics, Wool Science Review 60, 12 (1984)
7. K.H. Umbach, Verbesserung des Tragekomforts von Herrenoberbekleidung durch optimierte Kleidungsventilation, Hohensteiner Forschungsber., Jan. (1981)

oooooooooo

43 Quantitative Evaluation of Itch Sensation

H. O. Handwerker, W. Magerl, F. Klemm, E. Lang, and R. A. Westerman

n: Fine Afferent Nerve Fibers and Pain edited by R.F.Schmidt, H.-G.Schaible, and C.Vahle-Hinz. Weinheim(FRG),V.C.H.,"1987. pp 461-473

Introduction

The progress of sensory physiology in the last decades has led to a better understanding of the nervous apparatus subserving almost all sensory modalities of the skin. One notable exception, however, is the sensation of itch, for which a quantitative analysis is still lacking. It is even unknown whether itch is elicited by a specific subgroup of "itch" receptors or by a particular pattern of input from afferent nerve fibers subserving other sensations (e.g., pain).

A strong obstacle to a better understanding of this sensation is the lack of reliable stimuli which provoke itch sensations graded with stimulus strength. Thus a psychophysics of itch comparable to that worked out for pain sensations by Hardy et al. (1952) and by other laboratories is lacking. In pain research, psychophysical studies with rigidly controlled stimuli have been very useful in the search for and the characterization of the peripheral nervous elements mediating pain.

The aim of this study was threefold: (1) to find a quantifiable itch stimulus, (2) to establish the relationship between this stimulus and the itch sensations of healthy subjects, and (3) to find out which type of afferent nerve fibers might meet the requirements for a nervous apparatus mediating itch sensations.

Methods

Histamine was brought into the skin of healthy volunteers by iontophoresis. To this purpose 1% histamine (in the form of histamine dihydrochloride) was dissolved in a gel of 2.5% methylcellulose in aqua bidest. This jelly was placed in the cavity of an acrylic applicator having a diameter of 5 mm and a volume of 50 µl. A silver-silver chloride electrode in this applicator served for current delivery, a larger one (3 x 3 cm) in a sponge soaked with tyrode was used as reference. No air bubbles were allowed in the gel or between gel and skin, since they were found to interfere with the reliability of the stimulation.

Constant current pulses (10 s) from an isolated stimulator (WPI/305 B) were used. Current strength was varied from 0.01 to 3 mA for the application of different quantities of histamine. Stimulus strength is proportional to the product of current and time, i.e. to the charge.

These stimuli usually induced a wheal and a flare reaction developing slowly after termination of the stimulus. Both wheal and flare were marked with pens 10 min after each stimulus and redrawn on translucent paper for planimetric evaluation. To assess time courses and the relative increases in blood flow in the course of the flare reaction, a laser Doppler flowmeter (Periflux/PF1) was used.

Itch sensations evoked by these stimuli usually started within 20 s after termination of the stimulus and lasted for several minutes. They were assessed by the subjects on a horizontally placed visual analogue scale. The left and right ends of the scale were defined as

"threshold" and as "maximal imaginable itch" respectively. In some preliminary experiments a further mark at one third of the scale was defined as "itch strong enough to induce scratching." Ratings of the actual strength of itching were made by the subjects on acoustic signals at 10-s intervals for 10 min.

Differential blocks of superficial radial nerves were performed at the right forearm by two dangling weights of 5 kg via a sleeve pressing the nerve to the radius bone (Torebjörk and Hallin 1973). Two states of block were distinguished: one in which only the sensitivity to low von Frey hair (0.1 N force) stimulation was abolished in the skin field innervated by the nerve (block of Aβ fibers), and another one in which also the cold sensitivity failed (block of Aβ and Aδ fibers). In both states C fibers were still conducting, shown by the persisting sensitivity to pin pricks.

Six skin fields (20–50 cm^2) at the inner sides of the forearms of four subjects were desensitized with capsaicin. To this purpose the respective skin areas were painted first with dimethylsulfoxide (DMSO) followed by a solution of 1% capsaicin in 85% ethanol at intervals of 2–3 h. This treatment had to be repeated at least 10 times until capsaicin no longer provoked a burning sensation. (Method communicated by J. Szolcsanyi, who also served as a subject. The other subjects participating in the capsaicin experiments were coauthors of this study.)

Microneurography was performed following standard routines described elsewhere (Gybels et al. 1979).

Data evaluation was accomplished at the Computer Center of the University of Heidelberg using the Statistical Analysis Systems (SAS) package.

Following the declaration of Helsinki all subjects were informed about the risks of the respective experiment in which they participated and gave their consent.

Results

Correlations of Itch Sensations with Physiological Reactions to Histamine

In a pilot study on 48 subjects (27 male, 21 female) in which the stimuli were applied to the inner side of the forearm, the correlations between the stimulus size (charges), the radius of the wheal (RWH), the radius of the flare (RFL), and different parameters of the subjective itch responses were evaluated. The correlation coefficients between the two physiological parameters (RWH and RFL) vs the logarithm of charges were 0.88 each ($p < 0.0001$). The thresholds for both reactions were 0.04 mC (flare) and 0.05 mC (wheal). Figure 1 shows specimen records from one experiment. The figure indicates a strong correlation of the wheal size, the flare size, and the increase in blood flow (flux) to the subjective itch responses.

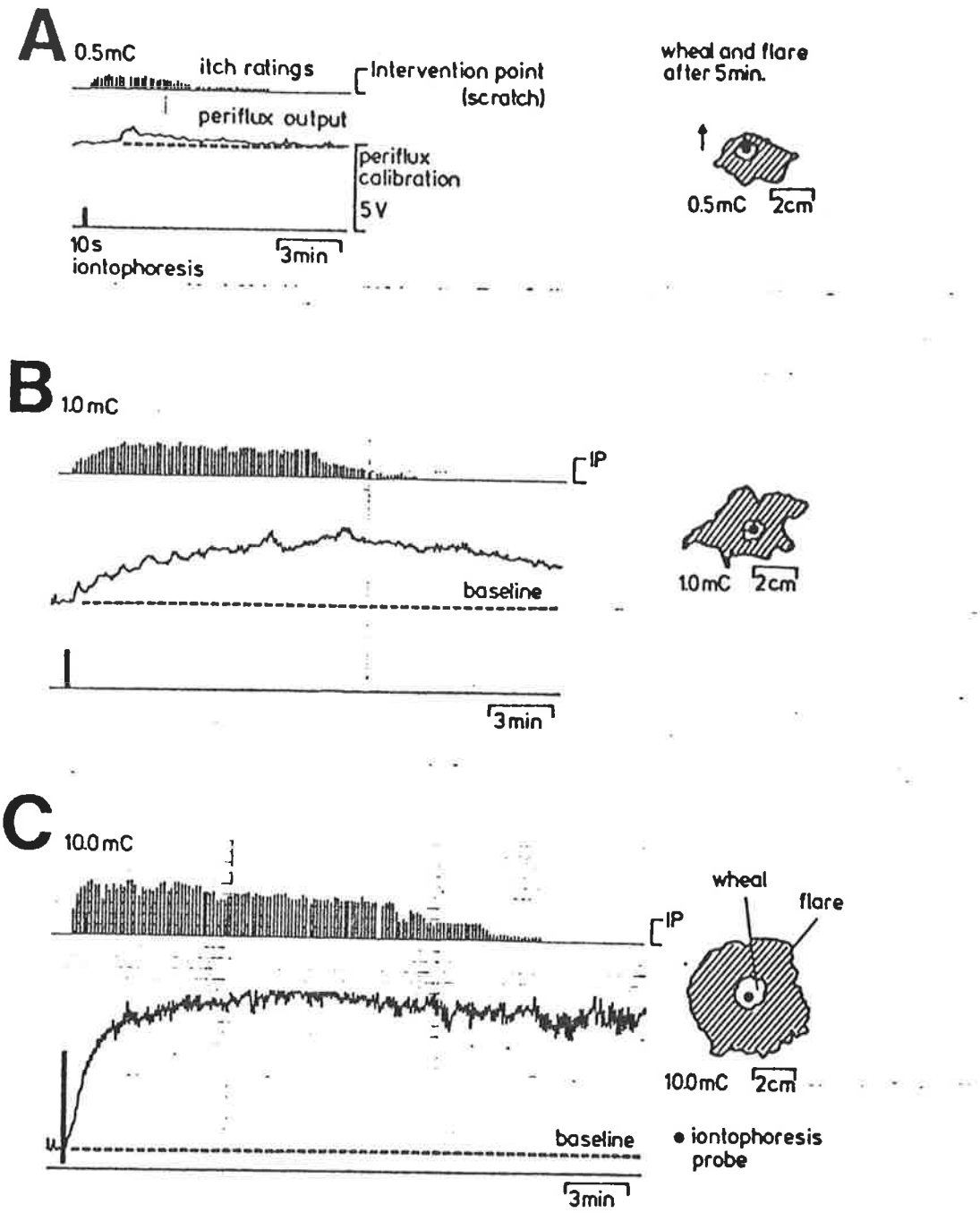


Fig. 1A–C. Specimen recordings from an experiment with histamine iontophoresis applied to the inner side of the forearm. Three different charges were applied to different skin sites. Upper traces: ratings of the intensity of itching given by the subject on a visual analogue scale at 10-s intervals. Middle traces: relative increases in blood flow in the flare region measured with a PERIFLUX laser Doppler flowmeter. Lower traces: duration and magnitude of current application. The sizes of the wheal and flare reactions are shown at the right side of the figure

In a more carefully designed study in 22 subjects (12 female, 10 male) these results were corroborated. Five stimulus levels (0, 0.156, 0.625, 2.5, and 10 mC) were applied in randomized order to the inner side of the forearm. As in the pilot study, the subjects were blind with respect to the stimulus magnitude. In this second study, again the correlation coefficients of the logarithms of the charges with RWH were 0.88. The respective correlations between the logarithms of the charges and mean fluxes were 0.72. The RWH parameter, for example, seems to be a better predictor of itching than the stimulus level. When itch responses were standardized on the basis of a first training stimulus of medium size, the correlation coefficient of RWH and itch was as high as 0.66 ($p < 0.0001$).

Time courses of the mean itch responses to a given charge are characterized by maxima occurring approximately 1 min after the end of the current application. The responses declined exponentially with time constants of 155–205 s.

Eight percent of the stimuli elicited no itch (9 of 110). These misses occurred at different stimulus levels.

The Influence of Nerve Blocks and of Capsaicin Pretreatment

Differential blocking of nerve fibers is one approach to identify those fibers which transfer the itch signals.

A pressure block of the superficial radial nerve was performed in 16 subjects (9 male, 7 female). The average values of the blood fluxes in the flare region and of the itch responses obtained in the course of this procedure are shown in Fig. 2.

None of the small changes in average responsiveness observed in the course of the blocking experiment were statistically significant. A first control stimulus delivered to the back of the right hand induced a somewhat smaller flux and an itch response which declined more slowly. Itch responses obtained during the blocking procedure were rather similar in the skin field at the right hand undergoing the blocking and in the contralateral field. We have also tested whether the itch sensations did change qualitatively in the course of the blocking. Some subjects (5 of 16) reported an increase of burning sensations under blocking conditions. It was, however, hard to discriminate whether this burning was induced by the pressure block itself or by the histamine stimuli, in particular since more prominent burning sensations in the course of the experiment were reported by those subjects who also exhibited block-induced aftersensations for some days. The majority of the subjects (11 of 16) had no block-induced aftersensations. When their data were analyzed separately, a small decrease of the itch response was found ($p < 0.05$) under total A-fiber blockade.

It is well established that a subgroup of afferent C fibers containing neuropeptides is characterized by a selective sensitivity to capsaicin (methyl-vanillyl-nonenamide) (for a review see Szolcsanyi 1985). We tried to block the receptive endings of capsaicin-sensitive C fibers by repetitively painting skin fields with this agent. It has been shown previously that C fibers sensitive to chemical stimuli can be temporarily blocked by this treatment. Painting was repeated until the substance no longer induced burning in the treated skin area, a stage reached after 2–3 days. Allowing some time for the subsidence of the signs of inflammation, this skin field showed a strongly increased threshold to heat stimuli, whereas the sensitivity

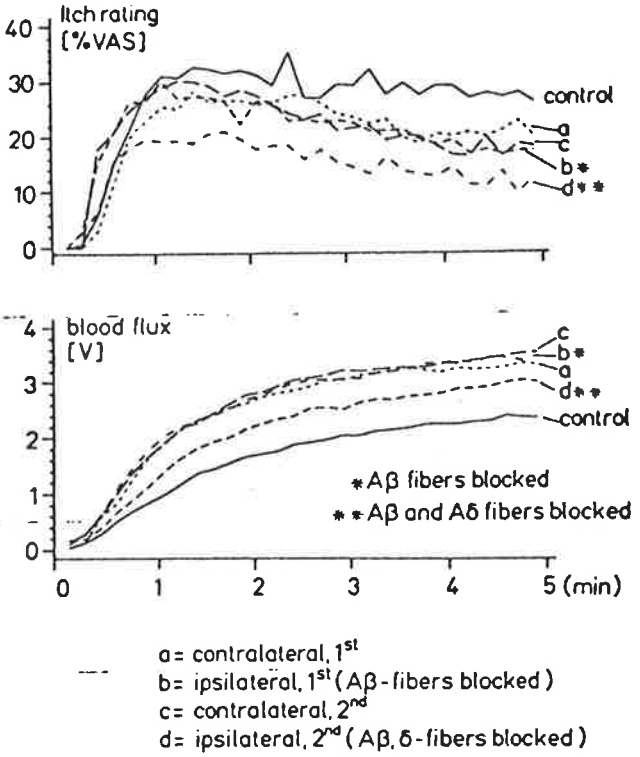


Fig. 2. Mean itch ratings (upper diagram) and mean blood flux measurements in the erythematous zone after histamine iontophoresis obtained in experiments with pressure blocks of the superficial radial nerve. VAS, visual analogue scale; V, Periflux reading in voltage

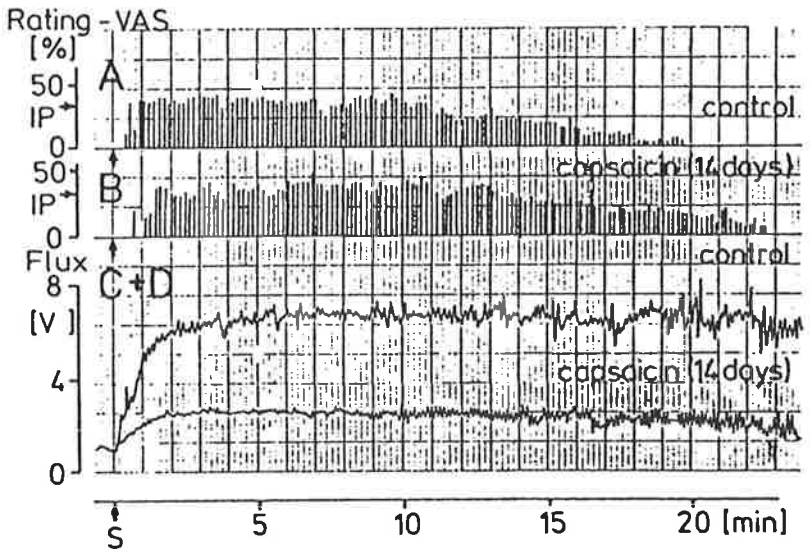


Fig. 3 A–D. Itch reaction and the respective flare reaction (blood flux measured with the Periflux) obtained from a skin site at the inner side of one forearm 2 weeks after capsaicin treatment (B, D) and from the contralateral untreated forearm (A, C).VAS, visual analogue scale; IP, intervention point (scratch); V, Periflux reading in voltages; S, stimulus (histamine)

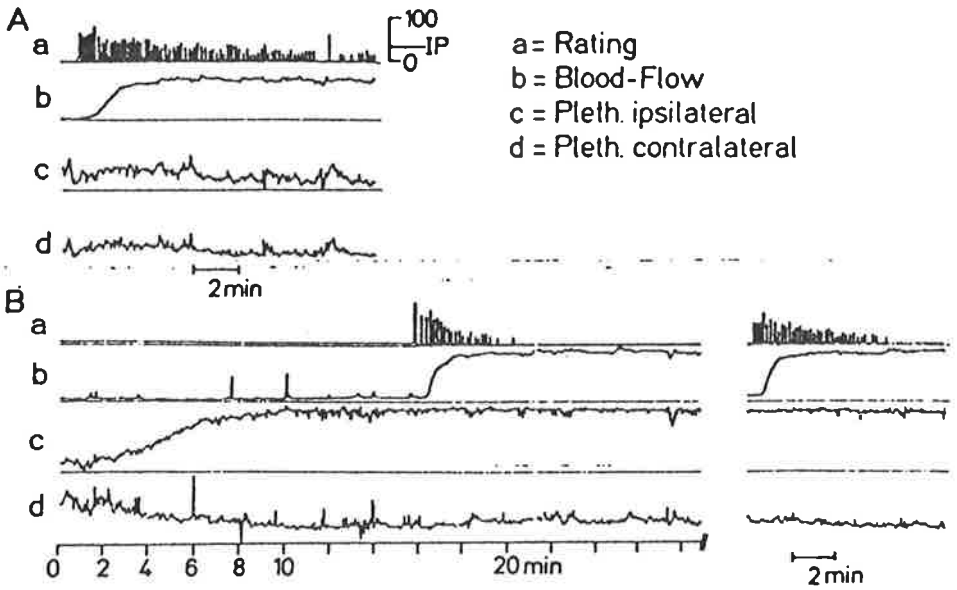


Fig. 4 A, B. Specimen recordings from an experiment in a patient undergoing a stellatum blockade. A Control recordings of itch and flux reactions. B Recordings obtained after development of the block which is documented by the increase in blood flow documented by finger photoplethysmography in trace C. a Ratings of itch intensity. IP, intervention point (scratch). b Blood fluxes through the skin affected by histamine iontophoresis measured with a laser Doppler flowmeter. c, d Finger plethysmograms from the middle fingers of both hands

to cold and to Frey hair stimulation was not altered. Vasodilatation following transcutaneous electrical stimulation strong enough to excite C fibers was reduced to 5 % of control values (Westerman et al. 1986). Histamine iontophoresis in a capsaicin-treated skin field no longer provoked itch sensations. The flare was also abolished, whereas the wheal reaction was unchanged. If the iontophoresis electrode was placed close to the border of the pretreated skin field (e.g., in a distance of 2–3 mm), a flare sometimes developed outside of the capsaicin-treated area. In these cases itch was felt by the subject, and it was localized in the flare region. Within 2 weeks after termination of the capsaicin treatment the itch response to histamine iontophoresis recovered completely. However, the flare response remained diminished for a longer period. Figure 3 shows an experiment conducted 2 weeks after capsaicin in a treated skin field and at the contralateral forearm.

According to occasional clinical observations, pathological itch states may be relieved by a block of the sympathetic chain. We were able to test three patients who had to undergo a blockade of the ganglion stellatum, which temporarily interrupted the sympathetic outflow to one arm. In none of these patients were we able to demonstrate a diminished itch response to histamine after blockade of the sympathetic innervation, which was proven by finger plethysmography revealing an increase in blood flow. Figure 4 shows an experiment with sympathetic blockade.

Microneurographic Recordings During Histamine Iontophoresis

A direct approach to the presumed "itch fibers" is by use of the microneurographic technique. We have tried to record impulse activity in different types of nerve fibers from the superficial radial nerves of volunteers and to apply histamine iontophoresis to the receptive fields. Ideally, one should be able to compare directly impulse patterns of afferent C fibers with subjective itch sensations, and thus to find the best candidates for the coding of itch.

We found, however, that under the conditions of microneurography itch is more rarely induced by histamine iontophoresis than under the conditions of a purely psychophysical experiment. We assume that this is due to the prolonged immobilization of the arm, which may induce an altered responsiveness of central sensory neurones. Furthermore, the manipulation of the nerve itself may induce a suppression of itch.

When recording from polymodal C fibers we were surprised that most of them did not respond to histamine. In some others we found a bursting discharge of low frequency which was hard to discriminate from background sympathetic discharges. Our sample of afferent C fibers (identified by the conduction delays to electrical stimulation in their receptive fields) is still too small to estimate the exact percentage of polymodal C fibers which show prolonged afterdischarges – albeit of low frequency – after histamine iontophoresis. The proportion of responding C fibers is, however, apparently less than 20 %. In a parallel study on anesthetized rats with a similar iontophoresis technique we found an even lower responsiveness of polymodal C fibers in this species. Ten "polymodal" C units and four C mechanoreceptors were tested. None of them responded to histamine with prolonged afterdischarges. Figure 5 shows the discharge pattern of a polymodal C fiber (recorded in man) to histamine iontophoresis in the receptive field. In the course of the recording additional sympathetic activity was recruited.

Another fiber group was much easier driven by histamine iontophoresis than the C fibers: the slowly adapting mechanoreceptors (SA) with fast-conducting myelinated fibers. Of 10 fibers tested, five showed regular (SA II units) or irregular (SA I units) discharges lasting for several minutes after termination of the iontophoresis. Figure 6 shows such a recording from an SA II fiber.

Discussion

Histamine iontophoresis as used in this study induced predominantly the sensation of itching. However, other sensory attributes, such as "burning" and "stinging," were also used by the subjects to characterize the stimuli. It is noteworthy that the incidence of sensory reports out of the semantic spectrum of pain (Mumford and Bowsher 1976) did not increase with higher current densities within the chosen range of stimuli. Thus, itching apparently does not turn to burning with stronger stimulation, in agreement with a report on electrically induced itching (Tuckett 1982).

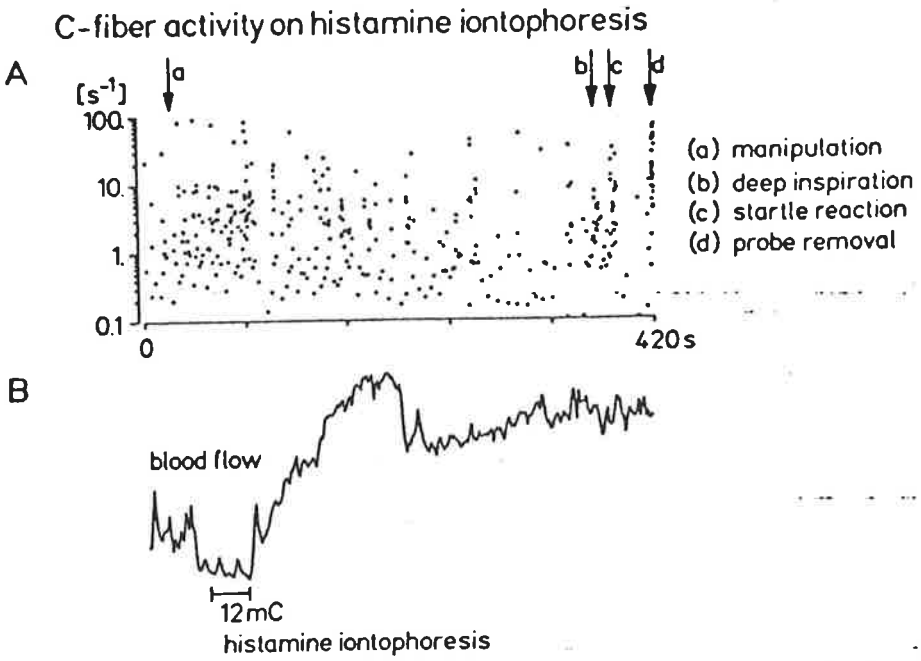


Fig. 5. Discharge patterns of a polymodal C-fiber unit after histamine iontophoresis, obtained in microneurography and the respective blood flux increases measured with the Periflux laser Doppler flowmeter. The recordings of an identified afferent polymodal C fiber were contaminated at the end of the recording by sympathetic activity (note tests b and c)

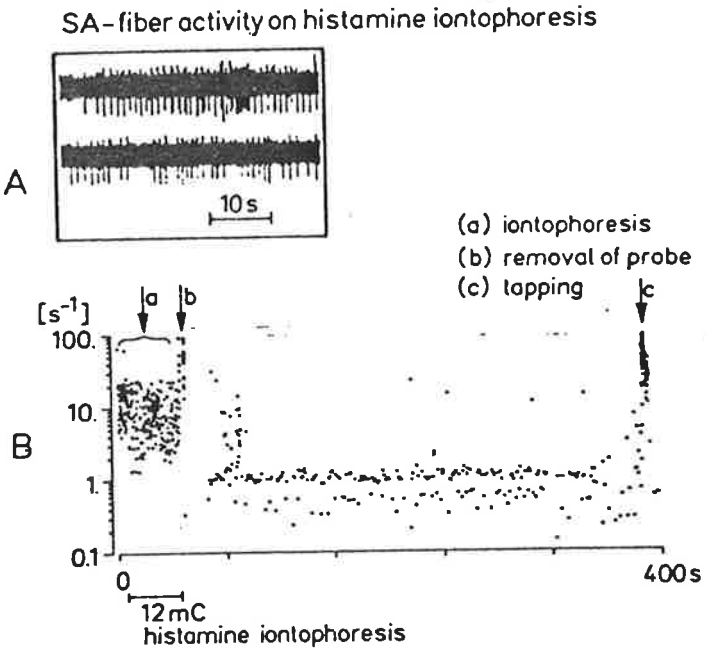


Fig. 6 A–C. Specimen recordings A of an SA II unit after histamine iontophoresis and B of the discharge pattern of an SA II unit during and after histamine iontophoresis obtained in a microneurographic experiment

The primary objective of this work was a quantitative analysis of itching with an appropriate stimulus method. We found that the subjects were well able to distinguish between different levels of itch in relation to the stimulus strength. The physiological reactions to the histamine iontophoresis, wheal and flare, turned out to be good predictors of the intensity of itching.

Another objective was to find the nervous elements mediating the sensations induced by the histamine iontophoresis. The results of our blocking experiments indicate that C fibers are the most relevant – if not the only – fiber group mediating the sense of itch. This is also indicated by the experiments of Bickford (1938) using ischemic and cold blocks of skin nerves. Furthermore the experiments with capsaicin painting of skin fields have shown that these C fibers are sensitive to this agent, a characteristic of peptidergic C fibers (Jancso et al. 1967, 1985; Lembeck and Gamse 1982). Among the different functional subgroups of C-fibers, in particular the "polymodal" C fibers are sensitive to capsaicin (Szolcsanyi 1985), i.e., those which are sensitive to strong mechanical stimulation, to heat, and to chemical stimulation (Bessou and Perl 1969). It has been shown in animal experiments that some of these fibers are sensitive to close arterial bolus injection of histamine (Fjällbrandt and Iggo 1961; Juan and Lembeck 1974). However, histamine is far from being the most effective substance for driving this fiber group. No group of exclusively chemosensitive fibers has yet been described in animal or in human experiments. One has to keep in mind, however, that search stimuli in single-fiber experiments are more often mechanical than chemical stimuli.

Though the indirect evidence of our blocking experiments indicates that polymodal C fibers are most probably the sensors for itch, little evidence was found in our microneurography experiments for an excitation of C fibers by histamine iontophoresis. Similar weak effects of histamine on polymodal C fibers have been observed previously in the cat (Fjällbrandt and Iggo 1961). We ourselves have seen no clear C fiber afterdischarges in the rat when a similar technique of histamine iontophoresis was used as in human psychophysics and microneurography.

Since most of the polymodal C fibers do not respond at all, it is rather unlikely that itch is mediated just by a weak excitation of the whole population of C fibers. It is much more likely that a small subgroup of the C fibers mediates itch. Most probably these "itch fibers" have their receptive endings in the most superficial layers of the epidermis (Shelley and Arthur 1957; Keele and Armstrong 1964), since a sufficiently high concentration of histamine is induced by the iontophoresis primarily in these superficial layers. It is well known that injection of histamine into deeper skin layers with a vaccinating pistol induces not itch, but pain (Lindahl 1961).

One side aspect of our microneurography experiments was the finding of the high sensitivity of myelinated SA fibers to histamine iontophoresis. It is unclear whether these SA fibers respond directly to the chemical stimulation or indirectly to the changed turgor of the skin. Since the impulses of these SA fibers were most probably blocked in our pressure block experiments, this part of the iontophoresis-induced input is lacking under blocking conditions. This apparently does not change the itch sensation to a greater extent. It is astonishing that this barrage of nervous impulses in a group of myelinated fibers seems to have so little impact on the itch sensation.

Acknowledgements. This work was supported by the Deutsche Forschungsgemeinschaft and by the Wilhelm-Sander-Stiftung. We wish to thank these institutions.

We wish to thank C. Forster, who participated in the microneurography experiments and was helpful in the statistical analysis, and Prof. Szolcsanyi for his help in the capsaicin experiments. Prof. Frosch kindly provided us with a laser Doppler flowmeter. We are grateful to D. Bechtle and M. Weinrich for typing the manuscript and to all subjects who volunteered for our experiments.

Note added in proof:

The results on the lack of flare and itching after histamine application in capsaicin pretreated skin has been confirmed recently by Toth-Kasa et al. (1986).

Toth-Kasa I, Jancso G, Bognar A, Husz S, Obal F (1986) Capsaicin prevents histamine-induced itching. Int J Clin Pharmacol Res 6 : 163–169

References

Bessou P, Perl ER (1969) Response of cutaneous sensory units with unmyelinated fibers to noxious stimuli. J Neurophysiol 32 : 1025–1043.

Bickford RG (1938) Experiments relating to the itch sensation, its peripheral mechanism, and central pathways. Clin Sci 3 : 377–386

Fjällbrant N, Iggo A (1961) The effect of histamine, 5-hydroxytryptamine and acetylcholine on cutaneous afferent fibers. J Physiol (Lond) 156 : 578–590

Gybels J, Handwerker HO, van Hees J (1979) A comparison between the discharges of human nociceptive nerve fibers and the subject's ratings of his sensations. J Physiol 292 : 193–206.

Hardy JD, Wolff HG, Goodell H (1952) Pain sensations and reactions. Williams & Wilkins, Baltimore

Juan H, Lembeck F (1974) Action of peptides and other algesic agents on paravascular pain receptors of the isolated perfused rabbit ear. Naunyn Schmiedebergs Arch Pharmacol 283 : 151–164

Keele CA, Armstrong D (1964) Substances producing pain and itch. Arnold, London

Jancso G, Obal F, Toth-Kasa M, Katona M, Husz S (1985) The modulation of cutaneous inflammatory reactions by peptide containing sensory nerves. Int J Tissue React 7 : 449–457

Jancso N, Jancso-Gabor A, Szolcsanyi J (1967) Direct evidence for neurogenic inflammation and its prevention by denervation and by pretreatment with capsaicin. Br J Pharmacol Chemother 31 : 138–151

Lembeck F, Gamse R (1982) Substance P in peripheral sensory processes. In: Substance P in the nervous system. Ciba Found Symp 91 : 35–49

LINDAHL O (1961) Experimental skin pain. Acta Physiol Scand 51 (Suppl 179)
MUMFORD JM, BOWSHER D (1976) Pain and protopathic sensibility. A review with particular reference to the teeth. Pain 2 : 223–243
SHELLEY WB, ARTHUR RD (1957) The neurohistology and neurophysiology of the itch sensation in man. AMA Arch Dermatol 76 : 296–323
SZOLCSANYI J (1985) Sensory receptors and the antinociceptive effects of capsaicin. In: HAKANSON R, SUNDLER F (eds) Tachykinin antagonists. Elsevier, Amsterdam, pp 45–56
TOREBJÖRK HE, HALLIN RG (1973) Perceptual changes accompanying controlled preferential blocking of A and C fiber responses in intact human skin nerves. Exp Brain Res 16 : 321–332
TUCKETT RP (1982) Itch evoked by electrical stimulation of the skin. J Invest Dermatol 79 : 368–373
WESTERMAN RA, LOW A, PRATT A, HUTCHINSON JS, SZOLCSANYI J, MAGERL W, HANDWERKER HO, KOZAK WM (1986) Electrically evoked skin vasodilatation: a quantitative test of nociceptor function in man. Clin Exp Neurol 23 : 81–89

JOURNAL OF NEUROPHYSIOLOGY
Vol. 59, No. 4, April 1988. Printed in U.S.A.

4

Transcutaneous Electrical Stimulation and the Sensation of Prickle

R. K. GARNSWORTHY, R. L. GULLY, P. KENINS, AND R. A. WESTERMAN

CSIRO Division of Textile Industry, Belmont 3216, Australia; and Department of Physiology, Monash University, Clayton, Victoria 3168, Australia

SUMMARY AND CONCLUSIONS

1. A high-voltage low-current transcutaneous electrical stimulating device was constructed and tested for its suitability to evaluate fabric-evoked prickle sensitivity in a population of 162 subjects. The initial sensation experienced by subjects with this device was the unpleasant sensation of prickle.

2. Single-unit recordings from the rabbit saphenous nerve established that at threshold most unmyelinated cutaneous receptors, both C low-threshold mechanoreceptive and polymodal nociceptive, were activated by the device.

3. Threshold measurements showed that there was no relationship of electrical threshold to atopic status, nor to fabric prickle threshold. It was concluded that our device preferentially excites unmyelinated afferents, but is not useful as a screening device for fabric intolerance.

INTRODUCTION

The use of electrical stimulation of the skin to investigate cutaneous sensation has been employed since the time of von Frey [for a brief review see Gibson (9)]. More recently, its possible role as a substitute sensation for visually and aurally handicapped people has been explored (2, 19). The range of sensations evoked by transcutaneous electrical stimulation have varied from tactile (touch, vibration, etc.) to pricking pain and itch; thermal sensations, however, have rarely been reported (1, 2, 4, 10, 14, 15, 17, 19, 20, 25). Often in these studies only very small changes in stimulus parameters are needed to convert tactile sensations to pain and vice versa.

Our interest in transcutaneous electrical stimulation concerned the development of an easily applied test for pricking pain sensitivity that could be used as a screening test to distinguish in a population of subjects those that are fabric intolerant. Although others have devised methods to measure cutaneous electrical threshold (14, 20), that of Bishop (4), who used the spark discharge from a capacitor to evoke sensations of pricking pain rather than touch as a first sensation, seemed an appropriate starting point. As recruitment of nerve fibers during electrical stimulation generally proceeds from largest diameter to smallest, and all available evidence indicates that, in man, pain is due to activation of thin fibers (Aδ for pricking pain and C fibers for burning pain) this reversal of expected recruitment order would seem to require some anatomical advantage of the smaller-diameter fibers in these experimental conditions.

This paper reports the development of a transcutaneous electrical stimulation device that preferentially evokes pricking sensations and its testing in animal experiments. A rationale for preferential stimulation of small nerve fibers is presented, with a mathematical model in the APPENDIX. The results of a psychophysical study of 162 subjects (classified for atopic status by a consultant dermatologist) tested by this device are also presented. A preliminary report of some of these results has already appeared (28).

METHODS

Human experiments

The transcutaneous electrical stimulation device (see Fig. 1) consists of a Teflon probe 2 cm in diameter containing a pair of etched tungsten needle electrodes, 5 mm apart. The needles have a tip

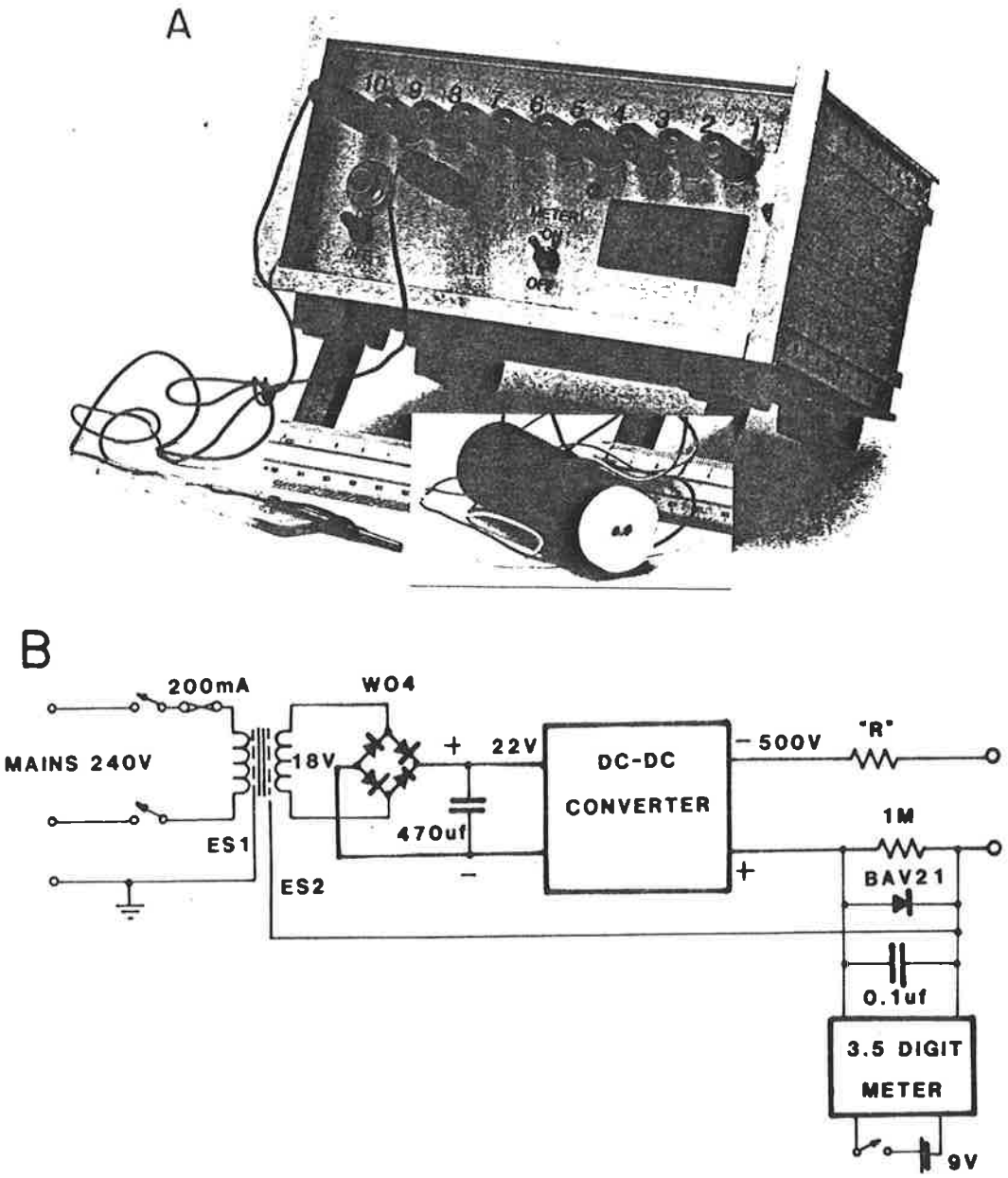


FIG. 1. *A*: TENS stimulator. Probe at the bottom left is the one used in animal experiments, insert at the bottom right is that used in human experiments. Adequate skin contact between the Teflon probe and the subject's forearm or animal's skin is attained when the meter indicates current flow. Note that during testing a screen hides the instrument from the subject's view and auditory cues are excluded. *B*: circuit diagram of TENS device. For DC-DC converter see Bird (3); 3.5 digit meter is Dick Smith model number K-3450.

radius of several micrometers and are arranged so that each point is just in contact with the skin surface when the probe itself rests on the skin. Electrical current is applied via the needles from a constant-current device of 500-V compliance (see circuit diagram Fig. 1*B*). The wires to the probe

are kept as short as possible to minimize introduction of stray capacitance effects. Ten resistors, "R" in Fig. 1*B*, in the range 12–6,400 MΩ were chosen such that at each successive step current was half its previous value. Currents applied were between 10^{-8} and 10^{-5} A, and were chosen by selecting the appropriate resistance.

Threshold of sensation on the forearm to applied current was estimated in each subject using a "forced choice" paradigm. The subject was placed behind a screen and asked to place the probe lightly on their forearm, with each contact lasting ~1–2 s. During each contact, current flowed through the skin with its density decreasing with increasing depth below the stratum corneum. The subject was allowed a brief familiarization time, then pairs of 15-s blocks of test times were allowed, current flowing only in one block of each pair. All extraneous cues were minimized or masked as far as possible, and the subject was asked to decide immediately after the second block of each pair in which block current flowed. Stimuli were varied in a pseudo-random manner and a graph was constructed for correct responses. The threshold value was found by interpolating from this graph to the 75% correct score. Because there is a widespread belief among dermatologists that atopic status confers on people a heightened sensitivity to cutaneous irritation, subjects were also classified as to their atopic status by a consultant dermatologist (Dr. P. Fergin). Standard clinical assessment of atopy was used, based on examination, clinical history (12), and response to prick testing with mixed grass pollens (10,000 U/ml; Commonwealth Serum Laboratories), Dermatophagoides pterinissinus (10,000 U/ml; Commonwealth Serum Laboratories), and wool extract (1:1,000, Hollister-Steer). Histamine (1:1,000) and human serum albumin—saline were used as positive and negative controls, respectively.

Animal experiments

Adult New Zealand white rabbits of either sex were anesthetized by a dose of urethane (0.5 g/ml) iv injected into the marginal ear vein. Once deep anesthesia had been achieved the trachea was cannulated and the left saphenous nerve was dissected free in the upper thigh, cut centrally, and prepared by standard nerve microdissection procedures to enable recording from single cutaneous sensory nerve afferents. Conventional stimulation and recording procedures were used. Single sensory afferents were classified, using conduction velocity (calculated from electrical stimuli applied 4 cm distally to the nerve trunk) and response to mechanical and thermal stimuli using published criteria (6), into the following groups: Aβ (hair receptors, QA field receptors, SAI and SAII), Aδ (hair and high threshold), and C (low-threshold mechanoreceptive and polymodal high threshold). Chemical stimuli were not used. Once classified they were then tested with a modified TENS probe (shown in Fig. 1) in which a negative polarity single central needle was placed inside an outer positive polarity brass barrel (3 mm diameter) mounted on an *X-Y* micromanipulator. For a recording to be accepted for analysis the receptor in question had to have the largest action potential in the nerve bundle under study and a signal-to-noise ratio of at least 5 to 1. Results were recorded on an FM tape recorder (Tandberg) and were analyzed "off line" using a BAK window discrimination to trigger a Digital computer with 6809 microprocessor to produce instantaneous frequency plots.

Once positioned over the receptive field the probe was lowered so that it just rested on the skin. This probe design was chosen for the animal experiments as it was a physically smaller system, and the use of a single needle allowed more accurate positioning of the active electrode on the receptive field. The design also led to a large reduction in stray capacitance effects from the probe touching the skin blocking the action potential recording amplifier.

Although the probe design was changed the essential element was retained in both—the method of applying current to the skin. The requirement is for a negatively charged point source of current with some return path to complete the circuit, both probe designs retain this essential feature using the same tungsten needles and height above the skin. In preliminary experiments on human subjects, it was found that varying probe shape or separation of the two needles changed the absolute threshold but did not alter the sensations once threshold had been exceeded. This result was as expected as the method of current application is the same and only the resistance in the current path to the return electrode is varied. Tests in human subjects confirmed that essentially the same pricking sensations were evoked by the animal probe as by the human probe.

RESULTS

Human experiments

In the human experiments the strategy employed by the subjects using the TENS probe was generally one of two types, but sometimes a combination. Some subjects would choose a particular spot on the forearm, which they claimed responded with a distinct prick and repeatedly tested the same spot. Others would tap over a large area of the forearm without any particular favorite area. Sometimes a particular spot or spots was favored, but surrounding areas also tested in any trial.

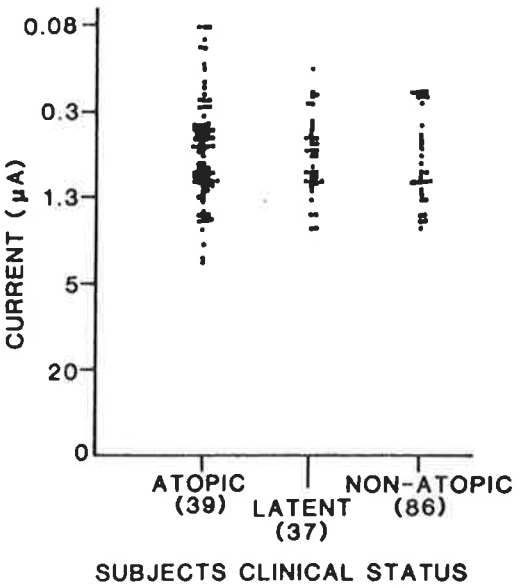


FIG. 2. TENS threshold vs. atopic status. A total of 162 subjects were tested and the numbers in each category are shown in brackets. Current at threshold from the TENS device is given on the ordinate. Note there are no obvious differences between atopic and nonatopic subjects.

All subjects reported that the initial sensation evoked by TENS was a pricking or itching sensation, localized to a small area under the probe. This sensation increased in intensity with increasing current, but only rarely were other sensations (usually tactile) reported. After-sensations of prickling or itching persisting for some seconds following probe removal were sometimes reported at higher current levels. At threshold, as determined by the forced-choice method, often no clear definable sensation (neither prick nor tactile) was present, even though the subject could accurately tell when current was flowing.

A graph plotting TENS threshold for 162 subjects separated into three groups (atopic, latent atopic, and nonatopic) is presented in Fig. 2. As can be seen, although there is a lower absolute range for atopic subjects, there is no significant difference in the means and standard deviations of these populations indicating that determining electrical threshold to our TENS device is not a useful method to define atopic status. Fabric prickle threshold, determined as previously

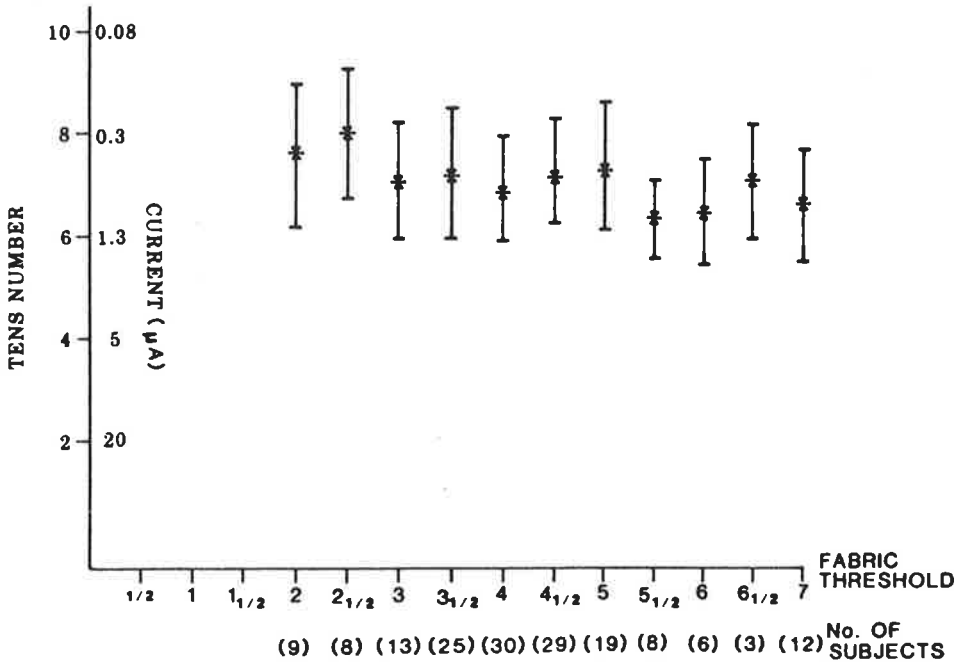


FIG. 3. Fabric-evoked prickle threshold vs. TENS threshold plotted on the ordinate (with current values also shown). Both geometric mean and standard deviations are plotted. No obvious differences can be seen between subjects with a fabric threshold of 2 (very sensitive) and those with a threshold of 7 (very insensitive).

TABLE 1. *Skin receptor response to TENS*

Receptor Type	No. Tested	No. Responding to TENS at 5 μA or Less
Aβ Hair	8	0
Field QA	4	0
SA I	5	0
SA II	5	0
Aδ Hair	8	0
High threshold	9	1
C mechanoreceptor	15	13
Polymodal	24	21

reported (28), can also be plotted against TENS threshold. As shown in Fig. 3 the mean and standard deviation of TENS threshold number is plotted against fabric threshold. For convenience a scale of corresponding current values (in microampere) is also supplied and it must be emphasized that the mean value is the geometric mean. As there is such a large variation from each mean it would appear that there is no relationship between TENS prickle threshold and fabric-evoked prickle threshold for those same 162 subjects. Thus TENS is not a useful means of identifying fabric intolerance.

Animal experiments

Table 1 shows the numbers and types of sensory units tested with the TENS device in this study. A value of 5 μA was chosen to construct this table as all human subjects had thresholds below this value. Further, most afferents responding to TENS did so at lower values, as shown in Figs. 6 and 7, but those not responding did not do so even at much higher currents. To avoid spurious results from large currents damaging the skin values above 5 μA were not routinely tested, but it seemed obvious that currents exciting C fibers were at least two orders of magnitude below those exciting other afferents. No Aβ-afferent of any type responded at current values within the threshold range for human subjects. With the exception of one high-threshold unit, no Aδ responded. This solitary Aδ had a low conduction velocity of 5.5 m/s but otherwise appeared to be a typical Aδ medium- to high-threshold unit. Most, though not all C units, both low and high threshold responded to TENS stimulation at current levels within the threshold range for our human subjects. There was no obvious reason as to why some units did not respond.

Responses of six different units to a current of 5 μA are shown in Fig. 4 for C mecha-

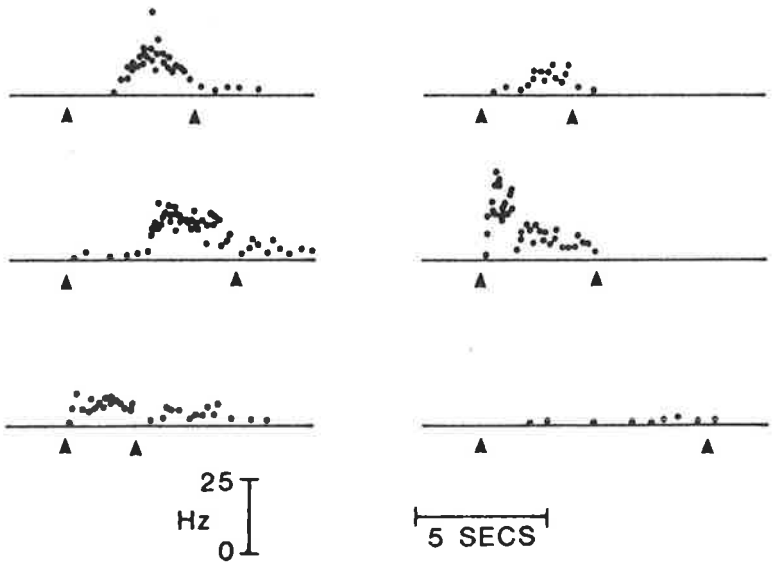


FIG. 4. TENS-evoked discharge in 6 low-threshold C mechanoreceptors recorded from the rabbit saphenous nerve to a current of 5 μA. Each trace is an instantaneous frequency plot, height of each *dot* above the line representing frequency of each action potential elicited relative to the previous one. *Arrows* mark the moment in time of turning "on" and "off" the TENS device. Bar scales represent 0–25 Hz and 5 s, respectively.

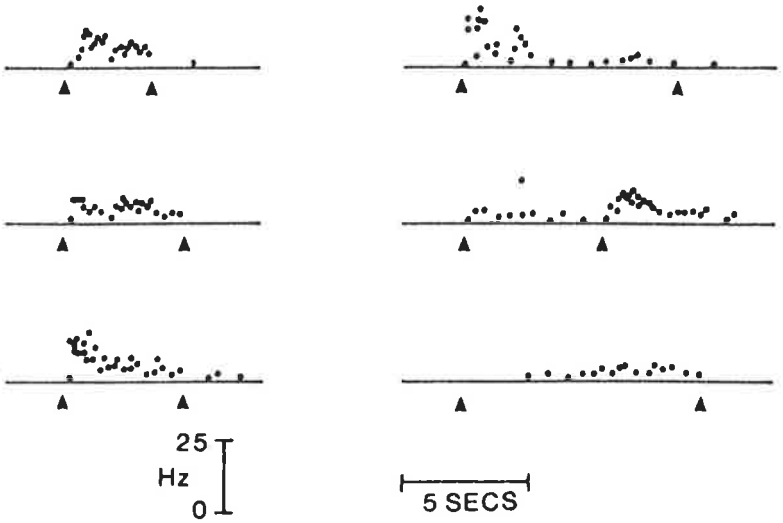


FIG. 5. TENS-evoked discharge in 6 polymodal C nociceptors to 5-μA current. Same display method as in Fig. 2.

noreceptive and Fig. 5 for C high-threshold polymodal receptors. The six units in each case were chosen to display the range of responses observed. Responses could be large or small and could begin after a delay or almost as soon as the current was turned on. Within the receptive field of a unit as defined by mechanical stimulation (hand-held von Frey hairs) the response was punctate, areas of maximum sensitivity to TENS corresponding to areas of maximum mechanical sensitivity. Outside the points of maximum sensitivity a response that was smaller and/or occurred with a longer delay could be evoked, usually at a higher current. The response sometimes persisted beyond the stimulus and 'off' responses were observed.

These effects may be related to the phenomenon known as "anodal break stimulation" (16, 22), as relatively long current pulses were used and the position of the cathode relative to a nerve ending in the skin, especially as there may be a number of such endings each acting independently, could lead to a virtual anode effect.

Increasing current strength above threshold evoked a larger response, often at a shorter latency. This is shown for two of the most sensitive C mechanoreceptive units in Fig. 6 and two of the most sensitive C polymodal nociceptive units in Fig. 7. This effect was, however, often masked at suprathreshold currents by fatigue of the unit. A suprathreshold stimulus repeated at the same point on the receptive field evoked a smaller response or required a stronger stimulus. This fatigue was restricted to the point of stimulation; other hot spots for any particular unit, if more than one existed, had very similar thresholds to their initial TENS stim-

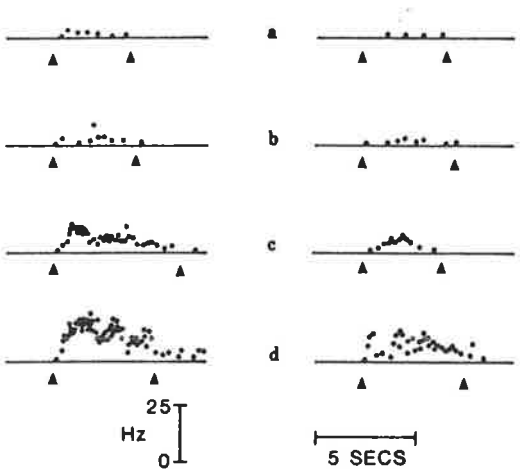


FIG. 6. Effect of increasing TENS current strength on the discharge of 2 C low-threshold mechanoreceptors, displayed side by side. Same display method as in Fig. 2. Current levels were *a*, 0.03 μA; *b*, 0.08 μA; *c*, 1.3 μA, and *d*, 5 μA. Note increased discharge and decreased latency to increased current strength.

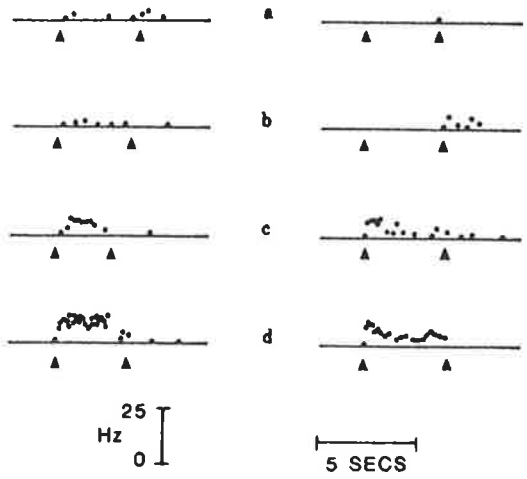


FIG. 7. Increasing TENS current on 2 polymodal C nociceptors. Same display method as in Fig. 2, same current strengths as in Fig. 6. Note increased discharge to increasing current strength and conversion of an 'off' response to the more normal 'on' response in the second unit.

ulation. Fatigue could persist for up to many minutes in some units, but no systematic studies on the time course of recovery were undertaken.

DISCUSSION

The animal experiments reported here confirm that our TENS device, at current levels corresponding to the threshold range for human subjects, selectively excites unmyelinated sensory C nerves in the rabbit skin. Our explanation for this selectivity is as follows. Because of cable properties of axons, the largest diameter, fastest conducting nerve fibers have the lowest threshold for extracellular electrical stimulation. Thus the recruitment of nerve fibers generally proceeds from largest to smallest. To reverse this order requires that the smallest-diameter nerve fibers are exposed to the greatest stimulus current density, i.e., are closest to the stimulating electrodes. This presupposes that they terminate more superficially in the skin.

The large-diameter sensory nerve fibers are thought to terminate mainly, if not exclusively, as anatomically distinct receptors, the most superficial in hairy skin being the Merkel cells, which lie in the basal layers of the epidermis (21). Other specialized endings lie much deeper. Thus, if small-diameter nerve fibers are going to have an anatomical advantage, they must terminate high within the epidermis. Cauna (7) reports penicilliate endings that appear to be derived from unmyelinated nerve fibers and have occassional axonal twigs extending into the epidermis as far as the stratum corneum. Immunofluorescence techniques have demonstrated the existence of substance P-containing nerve fibers within the epidermis (5, 8), with some fibers terminating just beneath the stratum corneum. Substance P is reportedly found only in unmyelinated C fibers (21). Thus there is compelling evidence to suggest that those nerve fibers that terminate most superficially within hairy skin are those with the smallest diameter axons.

TENS will preferentially engage the superficial fibers if needle surface electrodes are used to ensure that the electric current density within the skin falls rapidly with depth below the surface. Because of the insulating properties of the stratum corneum, the electric current will channel its way through this layer and only then start spreading. At least some of the small nerve fibers terminate just beneath the stratum corneum, giving them an advantage by their location.

Duration of the transcutaneous current pulse is also important when preferential stimulation of small nerve fibers is required. Ranck (16) has shown that durations should be much greater than 10^{-3} s. Then current thresholds are at the 'rheobase' level and largely independent of pulse duration. The length of time for which the probe contacted the skin for each tap by a subject was generally 1 s and often longer, placing small nerve fibers at less of a disadvantage.

The question as to which types of human nerve fibers are being stimulated by our TENS device has not been directly answered by these experiments. However, using the available data from the animal experiments and the work of others (1, 11, 23, 24, 26, 27), a convincing argument can be presented for polymodal C nociceptors, possibly with Aδ nociceptors, being stimulated. In the animal experiments only C fibers were excited by TENS, and as no low-threshold C fibers exist in man (11, 23, 24, 26, 27), only polymodal C nociceptors are left as a possible candidate. Aδ high-threshold afferents cannot be ruled out even though only one afferent in our

population of 17 rabbit Aδ receptors responded (and this may have been an aberrant fiber), as recent evidence has shown that in man Aδ and C nociceptors have similar properties (1, 27). This notion is further supported by nerve block experiments where it was shown that changes in fabric-evoked prickle sensation and in the sensation evoked by TENS occurred in parallel to changes in presumed small nerve fiber function (28) (and unpublished observations).

It was obvious in those experiments that prickle sensations persisted when all Aβ afferents were blocked and recovered together with pain and thermal sensations. The inherent difficulties in nerve block experiments (22), especially as the block itself is uncomfortable and leads to unusual sensations, make it difficult to determine whether these diffuse sensations were due solely to C fibers or what component should be ascribed to Aδ fibers. Reaction time experiments were not undertaken, as these are suitable only for clear easily defined sensations that do not require much central processing before a decision as to the presence of a stimulus is made.

An interesting sideline in our human data is that we have lowered the accepted threshold for sensations of electric current of ~1 mA to 0.08 μA. That the former is far too high has been shown in experiments by Grimnes (10). Under the conditions of his experiments most subjects reported that currents as low as 2 μA evoked the vibrotactile sensation of roughness. Our experiments confirm that subjects can sense currents much lower than previously thought, which as pointed out by Grimnes, is an important consideration in detecting current leaks that could become hazardous.

An approximate mathematical derivation is given in the APPENDIX of the relationship between threshold current and depth beneath the stratum corneum of the polymodal C nociceptors. The current thresholds obtained are consistent with at least some C fibers terminating high within the epidermis.

The data from our 162 subjects tested for both atopic status and electrical prickle sensitivity do not confirm the widely accepted opinion that atopic status confers a heightened sensitivity to skin irritation, at least as measured in our experiments. Variation of sensation and pain thresholds with time of day [and not with mood state of the subject (18)] could not explain this result as most subject testing was carried out between noon and 4:00 P.M., and the almost total overlap of the three populations (atopic, latent, and nonatopic) makes it unlikely that any major differences exist. This result is in agreement with our earlier study (28) in which no correlation of atopic status to fabric-evoked discomfort could be detected. Unfortunately there was no correlation between TENS threshold and fabric-evoked prickle threshold. Until evidence to the contrary is presented we conclude that sensitivity to skin irritation both by fabric and TENS is not directly related to atopic status as presently defined and that the TENS-evoked prickle threshold is not the same as fabric-evoked prickle threshold.

APPENDIX

Computation of TENS thresholds

The coordinate system used is shown in Fig. 8. Also shown is the relative disposition of the stimulating electrode and nerve axon. The disposition, though stylized, does represent the one with the lowest threshold among nerve axons terminating at a depth d beneath the stratum corneum. The electric current will be assumed to channel through the stratum corneum with negligible lateral spread. The diameter of the nerve axon will also be assumed negligibly small.

Since threshold excitation is being considered for very long current pulses, the capacitance of the nerve membrane can be neglected. Threshold depolarizations are reached by virtue of the stimulus current developing an ohmic voltage across the steady-state resistance of the membrane. A threshold depolarization of 20 mV will be assumed (13a). The standing polarization of the membrane does not affect the mathematical development and will be placed equal to zero.

Abbreviations

I	electric current
ρ	average resistivity of the epidermis beneath the stratum corneum
$V_o(Z)$	voltage along outside surface of nerve membrane
$V_i(Z)$	voltage along inside surface of nerve membrane
r_a	internal longitudinal resistance of axoplasm per unit length of axon
r_m	radial resistance of unit length of axon membrane

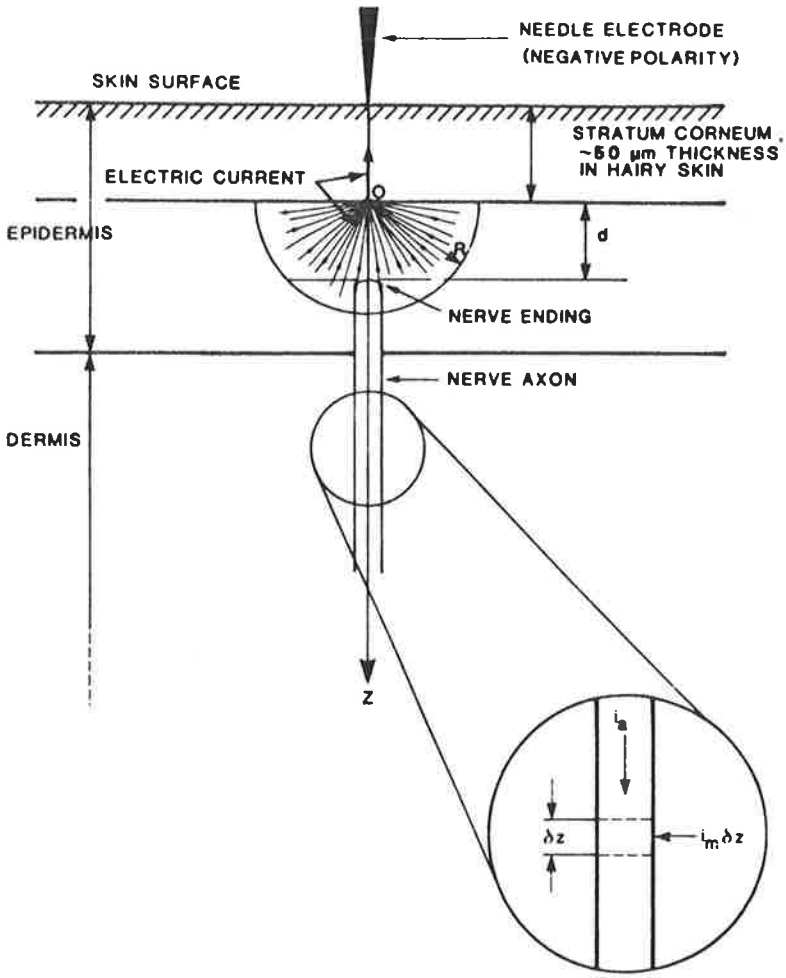


FIG. 8. Schematic diagram showing coordinate system and disposition of electrical stimulus and nerve axon. For further explanation see text.

i_m radial membrane current per unit length of axon; positive for inward current flow
i_a axoplasmic current flow; positive for current flow away from nerve ending
d depth of nerve ending beneath stratum corneum

For an isotropic spread of current beneath the stratum corneum, ohm's law gives

$$\delta V = -\frac{I\rho R}{2r^2} \qquad (1)$$

for the potential drop across a hemispherical shell of inner radius R and outer radius $R + \delta R$. *Equation 1* can be integrated to give

$$V = \frac{I\rho}{2R} \qquad (2)$$

where the zero of potential has been arbitrarily taken at infinity. *Equation 2* applies in particular along the outside surface of the nerve axon, so that

$$V_o(Z) = \frac{I\rho}{2Z} \qquad (3)$$

which will be written

$$V_o(Z) = V_o(d) \cdot \frac{d}{Z} \qquad (4)$$

This is the voltage variation along the outside surface of the axon membrane and it is now necessary to find the corresponding voltage along the inside.

The increase in axoplasmic current in going from Z to $Z + \delta Z$ is given by

$$\delta i_a = i_m \cdot \delta Z \tag{5}$$

which, by virtue of Ohm's law, becomes

$$\delta\left(-\frac{\delta V_i(Z)}{r_a \cdot \delta Z}\right) = i_m \cdot \delta Z \tag{6}$$

After some rearrangement, *Eq. 6* becomes

$$\frac{\delta^2 V_i(Z)}{\delta Z^2} = -r_a \cdot i_m \tag{7}$$

and Ohm's law can be used once again to give

$$\frac{\delta^2 V_i(Z)}{\delta Z^2} = -\frac{r_a}{r_m} \cdot [V_o(Z) - V_i(Z)] \tag{8}$$

It is convenient to write

$$\frac{r_a}{r_m} = \alpha^2 \tag{9}$$

where α is the reciprocal of the 'characteristic length' of the nerve axon (2). As a rule, the greater the length constant of a nerve axon, the more readily can it be stimulated. *Equations 4* and *9* enable *Eq. 8* to be written as

$$\frac{\delta^2 V_i(Z)}{\delta Z^2} - \alpha^2 V_i(Z) = -\alpha^2 V_o(d) \cdot \frac{d}{Z} \tag{10}$$

which must be solved to give the voltage along the inside surface of the axon membrane.

The substitutions

$$x = \alpha Z, \quad \text{and} \tag{11}$$

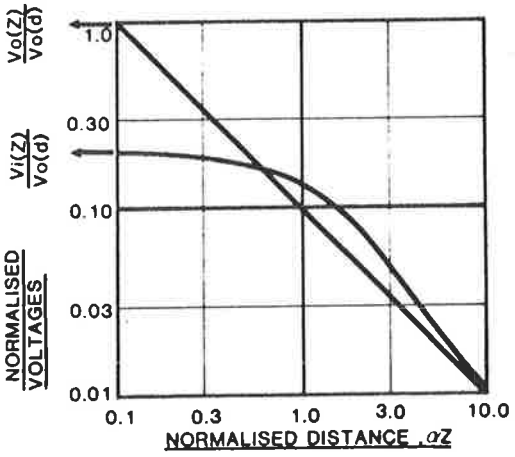


FIG. 9. Normalized voltages along inside and outside surfaces of the nerve membrane as a function of normalized distance beneath the stratum corneum ($\alpha = 5 \times 10^{-3}\ \mu m^{-1}$ for Z in micrometers). The curve has been computed for $\alpha d = 0.1$, i.e., the nerve ending is at the normalized depth of 0.1.

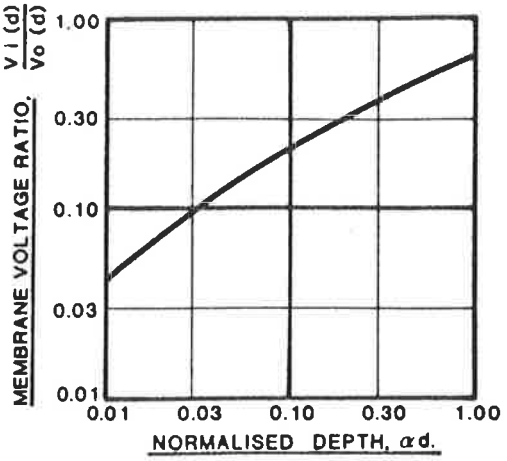


FIG. 10. Membrane voltage ratio at the nerve ending as a function of the normalized depth of the ending beneath the stratum corneum ($\alpha = 5 \times 10^{-3}\ \mu m^{-1}$ for d in micrometers).

$$y = \frac{V_i(Z)}{V_o(d)} \cdot \frac{1}{\alpha d} \tag{12}$$

enable *Eq. 10* to be written as

$$\frac{d^2y}{dx^2} - y = -\frac{1}{x} \tag{13}$$

Equation 13 will be integrated subject to the two boundary conditions

$$\frac{dy}{dx} = 0 \quad \text{for} \quad x = \alpha d$$

and

$$y \to 0 \quad \text{for} \quad x \to \infty$$

The first condition reflects the fact that the axoplasmic curvient must be zero at the nerve ending, the second that the voltage inside the axon membrane must tend to zero at large distances from the ending.

Integration of *Eq. 13* then gives

$$y = \tfrac{1}{2}e^{-x} \cdot [Ei(x) - Ei(\alpha d) + e^{2\alpha d} \cdot E_1(\alpha d)] + \tfrac{1}{2}e^{x}E_1(x) \tag{14}$$

where

$$Ei(x) = \int_{-\infty}^{x} \frac{e^{\gamma}}{\gamma}\, d\gamma \tag{15}$$

and

$$E_1(x) = \int_{x}^{\infty} \frac{e^{-\gamma}}{\gamma}\, d\gamma \tag{16}$$

are the exponential integral functions (27a). *Equation 14* together with *Eqs. 11* and *12* specify the voltage variation along the inside surface of

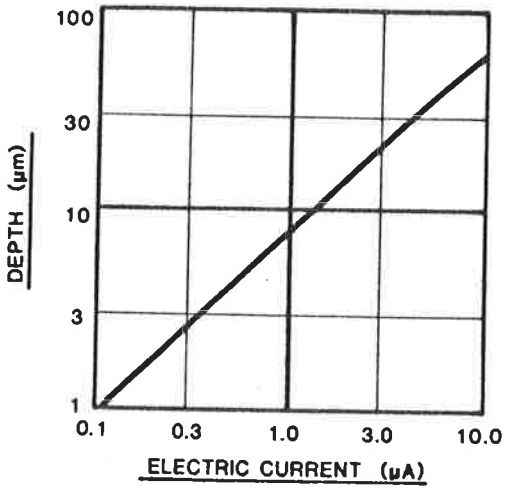


FIG. 11. Depth of nerve ending beneath the stratum corneum as a function of electric current at threshold for sensation.

the axon membrane. This variation and the voltage variation along the outside surface are shown in Fig. 9 for the case $\alpha d = 0.1$. For small-diameter nerve fibers, $\alpha \sim 5 \times 10^{-3}\ \mu m^{-1}$ (2) so that for this case $d = 20\ \mu m$. The voltage difference $V_o(Z) - V_i(Z)$ is a measure of the current flowing through the nerve membrane, and it can be seen that a reversal of the flow direction occurs at $\alpha Z \sim 0.6$. Thus, if the membrane is being depolarized by current in its distal part, then it is being hyperpolarized in its proximal regions.

Attention will now be directed to the nerve ending itself. For $x = \alpha d$, *Eq. 14* becomes

$$y = e^{\alpha d} E_1(\alpha d) \qquad (17)$$

or

$$\frac{V_i(d)}{V_o(d)} = \alpha d \cdot e^{\alpha d} \cdot E_1(\alpha d) \qquad (18)$$

Equation 18 is graphed in Fig. 10, and it can be seen that for nerve endings deep beneath the stratum corneum it is difficult to develop significant membrane depolarization potentials, since $V_i(d)$ is not much different to $V_o(d)$.

Equations 3, 4, and *18* can be combined to give the nerve ending depolarization in terms of the stimulating electric current, I. The result is

$$V_o(d) - V_i(d) = \frac{I\rho}{2\pi d}[1 - \alpha d e^{\alpha d} E_1(\alpha d)] \qquad (19)$$

At the threshold of action potential initiation, the following definitions will be used

$$V_o(d) - V_i(d) = V_T \qquad (20)$$

and

$$I = I_T \qquad (21)$$

Thus, at the excitation threshold, *Eq. 19* can be rearranged to give

$$I_T = V_T \cdot \frac{2\pi d}{\rho}[1 - \alpha d e^{\alpha d} E_1(\alpha d)]^{-1} \qquad (22)$$

Equation 22 is graphed in Fig. 11 using the following parameter values

$$V_T = 20\ mV$$

$$\rho = 110\ ohm\text{-}cm$$

$$\alpha = 5 \times 10^{-3}\ m^{-1}$$

The nerve ending depth has been made the dependent variable so the depth can be determined from knowledge of the electric current at threshold of sensation.

ACKNOWLEDGMENTS

We thank all our subjects for their cooperation and patience, Dr. P. Fergin for providing the clinical assessment, and A. Walker and J. Holloway for assistance during subject testing.

This work was supported by a grant from the Wool Research Trust Fund on the recommendation of the Australian Wool Corporation.

Address for reprint requests: P. Kenins, CSIRO Division of Textile Industry, Belmont, Victoria 3216, Australia.

Received 13 May 1987; accepted in final form 4 November 1987.

REFERENCES

1. ADRIAENSEN, H., GYBELS, J., HANDWERKER, H. O. AND VAN HEES, J. Response properties of thin myelinated (A-δ) fibers in human skin nerves. *J. Neurophysiol.* 49: 111–122, 1983.
2. BACH-Y-RITA, P. Tactile vision substitution: Past and future. *Int. J. Neurosci.* 19: 29–36, 1983.
3. BIRD, I. G. A precision regulator for DC proportional high voltage power supplies. *J. Phys. E Sci. Instrum.* 15: 1295–1296, 1982.
4. BISHOP, G. H. Responses to electrical stimulation of single sensory units of skin. *J. Neurophysiol.* 6: 361–382, 1963.
5. BJORKLUND, H., DALSGAARD, C-J., JONSSON, C-E., AND HERMANSSON, A. Sensory and autonomic innervation of non hairy and hairy human skin. *Cell Tissue Res.* 243: 51–57, 1986.
6. BURGESS, P. R. AND PERL, E. R. Cutaneous mechanoreceptors and nociceptors. In: *Handbook of Sen-*

sory Physiology. Somatosensory System, edited by A. Iggo. New York: Springer, 1973, p. 28–78.
7. CAUNA, N. Fine morphological changes in the penicillate nerve endings of human hairy skin during prolonged itching. *Anat. Rec.* 188: 1–12, 1977.
8. DALSGAARD, C-J., BJORKLUND, H., JONSSON, C-E., HERMANSSON, A. AND DAHL, D. Distribution of neurofilament-immunoreactive nerve fibres in human skin. *Histochemistry.* 81: 111–114, 1984.
9. GIBSON, R. H. Electrical stimulation of pain and touch, In: *The Skin Senses*, edited by D. R. Kenshallo. Springfield, IL: Thomas, 1968, p. 223–261.
10. GRIMNES, S. Electrovibration, cutaneous sensation of microampere current. *Acta Physiol. Scand.* 118: 19–25, 1983.
11. HALLIN, R. G. Human pain mechanisms studied with percutaneous microneurography, In: *Pain Measurement in Man: Neurophysiological Correlates of Pain*, edited by B. Bromm. New York: Elsevier, 1984, p. 39–53.
12. HANIFIN, J. M. AND RAJKA, G. Diagnostic features of atopic dermatitis. *Acta Dermatovenerol. Iugosl.* 92: 44–47, 1980.
13. HOKFELT, T., KELLERTH, J. O., NILSSON, G. AND PERNOW, B. Experimental immunohistochemical studies on the localization and distribution of substance P in cat primary sensory neurons. *Brain Res.* 100: 235–252, 1975.
13a. JACK, J. J. B., NOBLE, D. AND TSIEN, R. W. *Electrical Current Flow in Excitable Cells*, Oxford, UK: Clarendon, 1983.
14. LAITINEN, L. V. AND ERIKSSON, A. T. Electrical stimulation in the measurement of cutaneous sensibility. *Pain* 22: 139–150, 1985.
15. MASON, J. L. AND MACKAY, N. A. M. Pain sensations associated with electrocutaneous stimulation. *IEEE Trans. Biomed. Eng.* 23: 405–409, 1976.
16. RANCK, J. B. Extracellular stimulation. In: *Electrical Stimulation Research Techniques*, edited by M. M. Patterson and R. P. Kesner. New York: Academic, 1981, p. 1–36.
17. REILLY, J. P. AND LARKIN, W. D. Electrocutaneous stimulation with high voltage capacitive discharges. *IEEE Trans. Biomed. Eng.* 30: 631–641, 1983.
18. ROGERS, E. J. AND VILKIN, B. Diurnal variation in sensory and pain thresholds correlated with mood states. *J. Clin. Psychiatry* 39: 431–438, 1978.
19. SAUNDERS, F. A. Information transmission across the skin: High resolution tactile sensory aids for the deaf and the blind. *Int. J. Neurosci.* 19: 21–28, 1983.
20. SIGEL, H. Prick threshold stimulation with square-wave current: A new measure of skin sensibility. *Yale J. Biol. Med.* 26: 145–154, 1953.
21. SINCLAIR, D. *Mechanisms of Cutaneous Sensation*. Oxford, UK: Oxford Univ. Press, 1981, p. 363.
22. SWETT, J. E. AND BOURASSA, C. M. Electrical stimulation of peripheral nerve, In: *Electrical Stimulation Research Techniques*, edited by M. M. Patterson and R. P. Kesner. New York: Academic, 1981, p. 243–295.
23. TOREBJORK, H. E. Afferent C units responding to mechanical, thermal and chemical stimuli in human non glabrous skin. *Acta Physiol. Scand.* 92: 374–390, 1974.
24. TOREBJORK, H. E. Nociceptor activation and pain. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 308: 227–234, 1985.
25. TUCKETT, R. P. Itch evoked by electrical stimulation of the skin. *J. Invest. Dermatol.* 79: 368–373, 1982.
26. VALLBO, A. B., HAGBARTH, K-E., TOREBJORK, H. E., AND WALLIN, B. G. Somatosensory proprioceptive and sympathetic activity in human peripheral nerves. *Physiol. Rev.* 59: 919–957, 1978.
27. VAN HEES, J. AND GYBELS, J. C nociceptor activity in human nerve during painful and non painful skin stimulation. *J. Neurol. Neurosurg. Psychiatry.* 44: 600–607, 1981.
27a. WEAST, R. C. AND SELBY, S. M. (Eds.). *Handbook of Tables for Mathematics*. Cleveland, OH: CRC, 1967.
28. WESTERMAN, R. A., GARNSWORTHY, R. K., WALKER, A., KENINS, P. GULLY, R. L. AND FERGIN, P. Aspects of human cutaneous small nerve function: Sensations of prickle and itch. In: *29th IUPS Satellite Symp., Newcastle, Australia*, edited by L. A. Chahl, J. Szolcsanyi, and F. Lembeck. Budapest: Hungarian Academy of Science Press, 1985, p. 329–345.

JOURNAL OF NEUROPHYSIOLOGY
Vol. 59, No. 4, April 1988, *Printed in U.S.A.*

Identification of the Physical Stimulus and the Neural Basis of Fabric-Evoked Prickle

R. K. GARNSWORTHY, R. L. GULLY, P. KENINS, R. J. MAYFIELD, AND R. A. WESTERMAN

CSIRO Division of Textile Industry, Belmont, 3216, and Department of Physiology, Monash University, Clayton, Victoria 3168, Australia

SUMMARY AND CONCLUSIONS

1. The neurophysiological basis for the sensation of prickle evoked by contact of some fabrics with the skin is reported.

2. Single-unit sensory nerve recordings from the rabbit saphenous nerve were used to identify the receptors responsible for fabric-evoked prickle. These recordings showed that all low-threshold mechanoreceptors were activated by fabric, but they did not show differential response to prickly and nonprickly fabrics.

3. However, the response of some nociceptors, both Aδ and polymodal C, differed according to the prickliness of fabrics. Some of these receptors responded to fine von Frey hairs with buckling loads of at least 75 mgf. This suggested that the prickle stimuli on the fabric surface were protruding fiber ends that exerted loads of 75 mgf, or more, against the skin.

4. A Teflon replication technique was devised for estimating the density of these fiber ends. Estimates of the sensation magnitude of prickle from graded fabric sets by a panel of 55 subjects correlated (coefficient 0.91) with this measure of fiber end density.

5. We conclude from these results that fabric-evoked prickle is the result of low-grade activity in nociceptors and that the stimuli are protruding fiber ends exerting loads of ~75 mgf or more against the skin.

INTRODUCTION

When one considers the richness of sensations experienced by touching objects it is surprising how little we know of the neural basis for these sensations. Although a considerable amount of information is available as to the processing of simple stimuli, relatively little is known about complex sensations. For example, the elegant experiments of Mountcastle's group (16, 23), by combining physiological studies on monkeys and psychological studies on humans, have elucidated the coding mechanism of the sense of vibration. There are, however, more elusive sensations that are difficult to adequately describe and reliably produce. Unpleasant sensations such as prickle and itch are such a group, lacking a clear definition. It is not even known whether the two are separate sensations as evidenced by the term "prickling itch" found in the literature (9). Although itch has been defined as "an unpleasant sensation that provokes the desire to scratch" (6), so may tickle. As the latter can be evoked by extremely light touch it appears to be fundamentally different to itch, which is generally associated with nociceptors. Experiments using histamine (26, 29) and electrical stimulation (27) to evoke itch implicate activation of polymodal nociceptive C-fibers, though perhaps a subset of these receptors is involved (27).

The experiments reported here concern the sensation of fabric-evoked prickle, a problem of considerable interest to the garment industry. This type of prickle is often described as consisting of many fine pinpricks, and a minimum area of contact between fabric and skin is required for an unambiguous sensation. Subjects often report a desire to scratch. In our experiments this sensation was best evoked by placing a coarse fabric on the forearm and gently moving the fingers of the other hand over the back of the

cloth in a rocking motion with varying light pressure. Rapid movement results in a sensation of roughness and not prickle, whereas strong pressure either reduces the sensation of prickle or converts it to frank pain. It is not possible to evoke prickle on the areas of the arm that are most sensitive and have the richest innervation, i.e., the glabrous skin of the fingers and palm. Skin mechanical properties do appear to be a factor, as wetting the skin (either with water or by inducing sweating) markedly enhances the sensation in most subjects (30). Most coarse fabrics can evoke prickle in almost all subjects, though the sensitivity and magnitude of sensation varies widely (30). Thus it is unlikely that a chemical from the fabric is involved, nor is the atopic status of the individual related to this ability to perceive prickle (30).

By recording from all the different types of cutaneous mechanosensitive afferents in the saphenous nerve from the hairy skin of the rabbit's hind leg these experiments hoped to determine which types of receptors responded to the prickliness of a fabric rather than to other fabric properties. Once the receptors mediating prickle were identified, the main fabric property that activated those sensory receptors was sought. From this information a technique was devised to measure the physical stimulus for fabric prickle and this hypothesis tested on a large sample of fabrics using a test panel. A preliminary report has appeared (7).

METHODS

When these experiments were begun, the physical stimulus for fabric-evoked prickle was unknown. Fabrics of very similar structure and physical properties can vary greatly in the sensation of prickliness they evoke in subjects. From a graded series of fabrics, three were chosen for the animal experiments. These were similar except in the magnitude of the sensation of prickliness they evoked in six human subjects. These three fabrics, labeled A, B, and C, were reported by our subjects to evoke the following sensations: fabric A was nonprickly, fabric B moderately prickly, and fabric C very prickly. Prickliness of fabrics used in the animal experiments thus refers to the sensations evoked by the fabric in human subjects and does not imply that animals can experience prickle. However, as human and animal cutaneous sensory receptors are very similar (10, 24–29, 31), the physical stimulus for fabric-evoked prickle was determined in the animal experiments and the hypothesis tested in psychophysical experiments.

Animal experiments

All animal experiments were performed on rabbits. Adult animals of either sex were deeply anesthetized with urethan (0.5 g/ml) injected via the marginal ear vein. A tracheostomy was performed, the left hind leg was carefully shaved with animal clippers, and the saphenous nerve was prepared for stimulation and recording in the upper thigh. Standard dissection and recording procedures were used to record single cutaneous sensory receptors that were identified and classified as to type of mechanoreceptor by conduction velocity (measured to a supramaximal electrical stimulus applied to the nerve trunk 4 cm distal to the recording site) and mechanical stimuli applied to the receptive field, using established criteria [e.g., Burgess and Perl (3)].

For a recording to be accepted for analysis the receptor in question had to have the largest amplitude signal in the nerve bundle, with a minimum signal to noise ratio of 5 to 1. Signals were recorded on an FM tape recorder (Tandberg) and analyzed "off line." Analysis consisted of playing the recording through a 'Bak' window discrimator and using the TTL pulse so generated to trigger a 'Digital' VT240 computer with a 6809 microprocessor. This computer generated interval histograms using standard methods.

The three fabrics selected were part of the test fabrics used in a previous study (30). Initially a section of fabric 6 × 8 cm was applied to the skin of the rabbit and a roller (4.5 cm diameter, 2.5 cm wide and 50 g wt) rolled over the fabric across or along the leg at 0.5–2 cm/s. In later experiments, rate of movement was controlled by an electric motor attached to the handle of the roller. Effect of roller speed was not systematically investigated, however, it did not appear to influence sensation of prickle in humans or the pattern of discharge of sensory receptors in animals to prickly fabric as long as it was slow (<5 cm/s).

The receptive field of mechanoreceptors was explored with hand-held von Frey hairs made from nylon monofilament [0.3 mm diameter, 0.1 to 9 gram force (gf) buckling load] and fabric fibers (20–50 μm diam, 50–200 mgf buckling load).

Effect of moisture on the skin was examined by applying a thin film of distilled water onto the skin, excess water was removed by cotton swabs immediately before testing began. After 30 s water was reapplied before further testing.

Human experiments

Microneurography experiments were carried out using 'Haer' epoxylate-coated tungsten electrodes inserted into the radial nerve above the

elbow in healthy adult human volunteers. The experiments were approved by the Monash University Ethics Committee in accordance with the declaration of Helsinki. Standard recording and receptor identification procedures were used and all results recorded by a tape recorder for analysis as in the animal experiments described above. Nociceptors were identified as Aδ or C receptors by their conduction velocity as calculated from a supramaximal electrical stimulus applied transcutaneously through stick-on ECG electrodes over the radial nerve in the forearm. As the quality of microneurographic recordings was not as good as that obtained in the animal experiments, the criterion for acceptability was relaxed to a signal-to-noise ratio of 2 to 1. In some cases, however, nociceptor signals were accepted if their shape was sufficiently unique to reliably trigger the window discriminator, even if they were not the largest signals in the recording. The adequacy of this latter criterion was checked by manually counting discharges on a U-V record of selected responses and comparing them with the responses generated via the window discriminator.

Subjective estimates of prickliness were obtained from a panel of 55 subjects consisting of twenty 20–30 yr olds, twenty-four 30–45 yr olds and eleven 45–60 yr olds. There were 28 males and 27 females. Thirteen fabrics were presented, 7 in series A and 6 in series B. They did not include those used in the animal experiments.

Each sample was presented as a 12 × 18 cm rectangle with the fabric face covered with bleached cotton lining stitched to the edges. Subjects were thus unable to see the fabric tested and were instructed not to do so, nor to attend to any sensation other than prickle. The psychophysical methods of constant stimuli and magnitude estimation were used to determine threshold and sensation magnitude of prickle. Subjects were instructed to place the fabric on their forearm and were shown how to use gentle pressure with the other hand to evoke prickle. After instruction they were free to use whatever movement they felt was appropriate. A nonprickly and a very prickly fabric were initially presented to acquaint subjects with the sensation. They were asked to scale sensation magnitude from test fabrics relative to the two presented on a scale from 0 to 10. This procedure was chosen because the sensation of fabric-evoked prickle is diffuse and hard to define, so it was considered essential to provide a clear example that subjects could then use as a reference. The fabric chosen for the sensation of '10' was sufficiently above that expected during the experiment (7 being the average maximum reported) that no flattening at the top of the psychometric curve was expected. Each fabric was presented six times in random order for each series. After each trial, subjects were asked to give an estimate of the magnitude of the prickle. No feedback was given. Testing was done in a climatic room at 20 ± 1°C and 60 ± 5% relative humidity to standardize environmental factors. As the magnitude estimate scale was controlled from 0 to 10, the arithmetic mean was used to calculate the magnitude estimate for each fabric for all subjects (8).

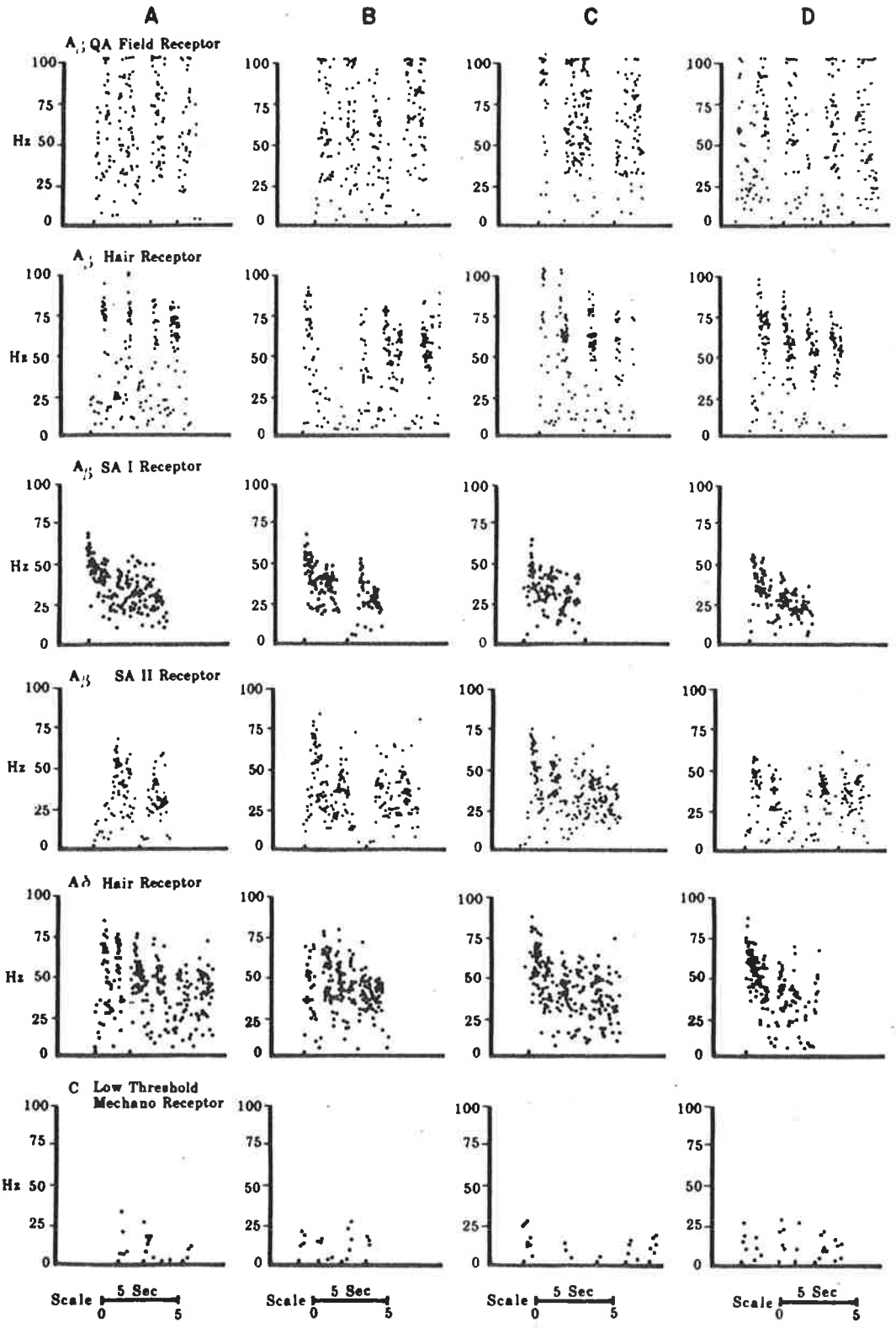
RESULTS

Animal experiments

The types of mechanoreceptors identified in rabbit hairy skin and tested for their ability to respond to the prickliness of fabric are shown in Table 1, with the number of single units studied and the number responding to the prickliness of fabric. All low-threshold receptors appeared to be equally activated by prickly as by nonprickly fabric, as shown in Fig. 1 where a typical example of each type of receptor and its response to the three fabrics is shown. There was no obvious change in discharge pattern or total amount of discharge evoked. However, a number of the Aδ nociceptors (2) and C polymodal nociceptors (31) showed a differential response to the three fabrics, as shown in Fig. 2 and 3. In all such cases, there was little or no response to nonprickly fabric but increased response to more prickly fabrics, with the Aδ nociceptors showing a much smaller response than the majority of the polymodal C nociceptors. The remaining Aδ nociceptors (2) and C polymodal nociceptors (6) did not respond to any fabric. Mechanical threshold of these

TABLE 1. *Receptors tested for fabric-evoked prickle*

Type	No. Tested	No. Differentially Sensitive to Prickly Fabric
Aβ		
Hair	15	0
Field	5	0
SA I	5	0
SA II	6	0
Aδ		
Hair	6	0
Nociceptor	4	2
C		
Low-threshold mechanoreceptor	12	0
Polymodal nociceptor	37	31


A
B
C
D
Aβ QA Field Receptor
Aβ Hair Receptor
Aβ SA I Receptor
Aβ SA II Receptor
Aδ Hair Receptor
C Low Threshold Mechano Receptor
Hz
100
75
50
25
0
Scale 5 Sec
0 5

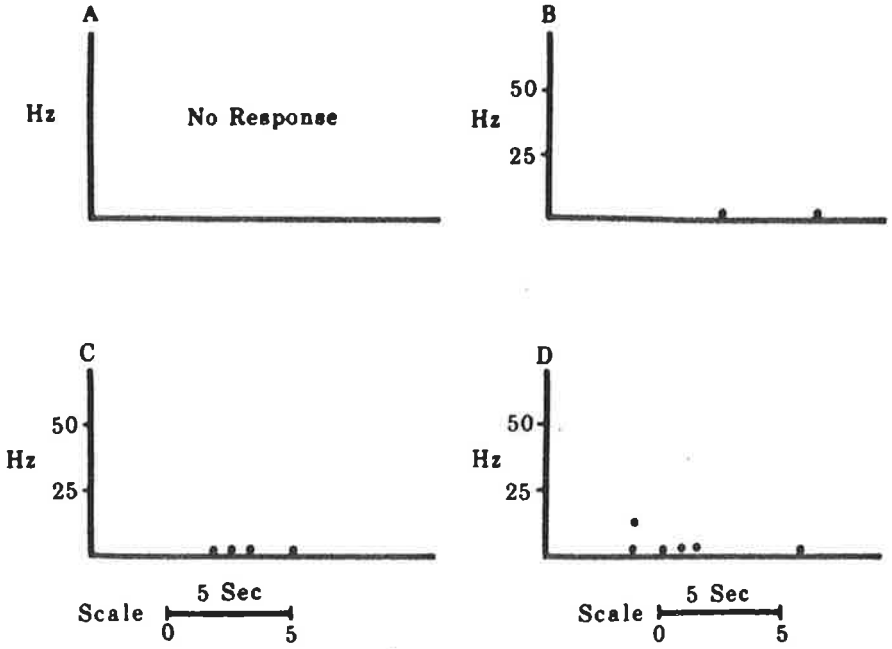


FIG. 2. Responses of an Aδ nociceptor recorded from the rabbit saphenous nerve to the same fabrics (*A*, *B*, and *C*) as in Fig. 1. Method of display as in Fig. 1 with *D* again being fabric *C* presented after moistening the skin. Note that although the response was small there was an increased discharge to the more prickly fabric and possibly a slight increase with moisture on the skin.

nociceptors as tested by standard nylon monofilament von Frey hairs was higher, as shown in Table 2.

The effect of applying water to the skin was tested on most of the above receptors as this is known to enhance prickle sensation in humans. No low-threshold mechanoreceptors showed any marked change in fabric evoked response, Fig. 1 (*D* compared with *C*) or developed a differential response to prickly fabric after water was applied to the skin. In contrast most prickle-sensitive nociceptors (17/26) showed an enhanced responsiveness to prickliness of fabric after application of water (as can be seen in Figs. 2 and 3). Those with an enhanced response had a lower average threshold (Table 2). The enhanced response consisted of increased and more regular discharge and this is consistent with the increased sensation of fabric-evoked prickle reported by subjects on wetting the skin. Nociceptors that failed to respond to fabric were still unresponsive after wetting the skin.

The total discharge evoked in 15 s (mean and standard deviation) by each of the three fabrics for the 26 prickly fabric-sensitive nociceptors studied in these experiments is plotted in Fig. 4. The number in brackets represents an estimate of the magnitude of prickle of each fabric, calculated as discussed later in this paper.

FIG. 1. Responses of single cutaneous mechanoreceptors recorded from the saphenous nerve of the rabbit to 3 different fabrics. Fabrics were placed on the skin and a 50-g roller moved over them. Fabrics (*A*, *B*, *C*) are presented in order of increasing prickliness with *C* (the most prickly) being also tested after the skin was moistened with a film of distilled water (*D*). Each *dot* represents the instantaneous frequency of an action potential relative to the preceding one. Roller movement back and forth across the fabric started after same delay in each recording but continued for a variable time. None of the different types of receptors tested (Aβ: hair, QA field, SA I, SA II; Aδ: hair; or C: low-threshold mechanoreceptive) showed a differential response to the prickliness of fabric, nor was the response to fabric *C* obviously altered by moisture on the skin.

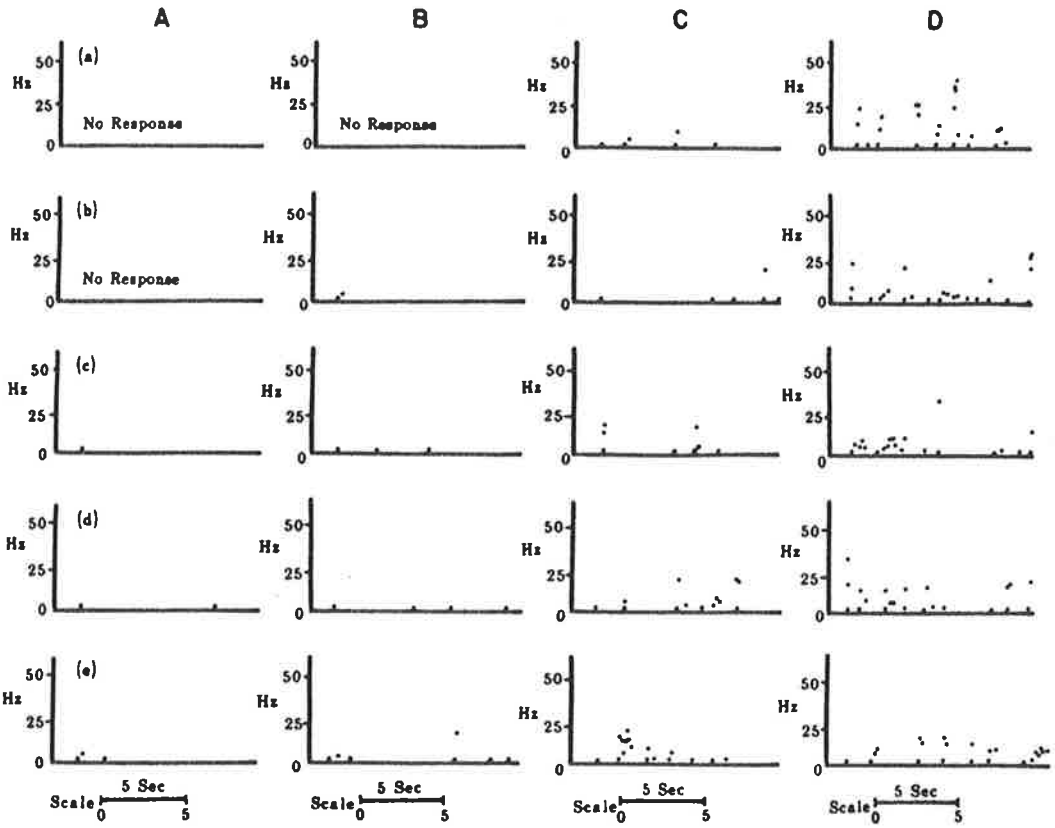


FIG. 3. Responses of 5 polymodal C nociceptors to the same fabrics (*A*, *B*, and *C*) as in Fig. 1 using the same display method, *D* again being fabric *C* after moistening the skin. The 5 were chosen to illustrate the variation of response encountered in the population studied: *a*, responds only to the most prickly (fabric *C*); *b*, to fabrics *B* and *C*; and *c*, *d*, and *e*, to all 3 fabrics. However, the response is progressively larger to more prickly fabrics and is enhanced after the skin was moistened.

The above results show that prickly fabrics are able to excite nociceptors. By definition nociceptors are high-threshold receptors, typical von Frey thresholds being 0.7–13.2 gf (28, 31). As no part of our fabric, e.g., fibers, penetrate the skin of man or animals (visual observations using a dissecting microscope), it is necessary to estimate the forces generated by fabrics on the skin under conditions that evoke prickle. Estimates of pressure applied by subjects over fabric when evoking the sensation of prickle yielded values of 2 to 10 gf/cm^2, so that pressures from the weave structure itself will be low. When the body of the fabric is held above the skin by fibers protruding from the fabric, reasonably large pressures can be generated at the fiber ends. These fiber ends thus act as von Frey hairs and the forces they generate will depend on the type of material, the diameter of the fiber, and the length of the fiber. A minimum length of ~0.2 mm must be assumed for these fibers, as below this length the deforma-

TABLE 2. *von Frey thresholds for the groups of nociceptors tested*

Nociceptor Type	No. Tested	Threshold, gf
Aδ fabric insensitive	2	4.9, 7.8
Aδ fabric sensitive	2	2.9, 2.9
C fabric insensitive	6	4.7 ± 0.9
C fabric sensitive	31	3.4 ± 0.8
C unchanged with wet skin	9	3.8 ± 0.9
C increased response with wet skin	17	3.2 ± 0.6

Values are means ± SD.

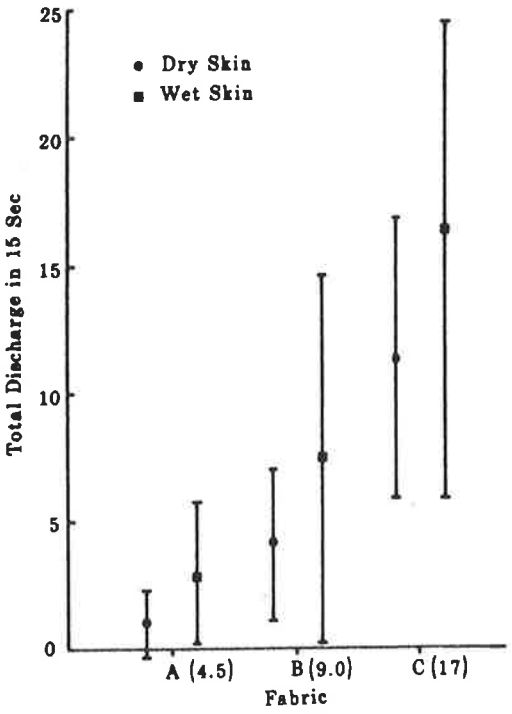


FIG. 4. Discharge of polymodal C nociceptors vs. prickliness of fabric. Total number of action potentials elicited by 15 s of stimulation are plotted on the ordinate (as the mean and standard deviation) for 26 nociceptors both before and after moistening the skin for each of the 3 fabrics. On the abscissa the number in brackets after each fabric represents our measure of the density (number per 10 cm^2) of high load-bearing fiber ends on the fabric surface. There is an increased response to more prickly fabrics, which also have a larger number of high load-bearing fiber ends. However, moisture on the skin, whilst evoking on average more discharge from the same fabric than on dry skin, also increases the variability as some receptors were not influenced by moisture.

tion of skin will lead to bottoming out of the fibers increasing the contact area as more fabric touches the skin and thus reducing the pressure at the fiber end. Measurements of buckling loads of coarse single fibers pulled from prickly fabrics and held at lengths similar to that protruding from fabric yielded average values in the range 70–100 mgf, though occasionally higher forces (up to 200 mgf) were recorded.

Von Frey hairs constructed from 40 μm diameter fibers with buckling loads of ~75 mgf were able to excite nociceptors that responded to prickly fabric, however, reproducibility of the response was poor. A sequence of responses from similar applications of such von Frey hairs applied within the 'hot spot' (as defined by a standard nylon monofilament von Frey hair) for four different polymodal nociceptive C receptors is shown in Fig. 5. Response varied from multiple discharges on some occasions, to a single action potential, or even none on others. Larger and more consistent responses were obtained after wetting the skin. This variability, which has been observed by others (28), made it difficult to define absolute threshold using single fabric fibers. However, most nociceptors responded a significant proportion of the time (20% or more) to fibers with buckling loads of 75 mgf or higher but failed to respond when buckling loads were reduced to 50 mgf. Nociceptors without a fabric response could be activated by fibers with greater buckling loads, however, it is doubtful whether these nociceptors could substantially contribute to fabric-evoked prickle, or that they constitute a distinct population of nociceptors.

Prickly fabric apparently evokes sensation by causing a low rate of discharge from nociceptors over a wide area of skin. The physical stimuli are fibers protruding from the surface that are able to generate loads of ~75 mgf or more at their point of contact with the skin. More detail on fabric and skin interaction is presented elsewhere (15).

Human experiments

Low-threshold mechanoreceptors, recorded in the radial nerve from hairy skin on the back of the hand and forearm, responded in essentially the same way to prickly as to nonprickly fabrics. Also, there was no obvious change in response after wetting the skin with distilled water. Typical examples of the 8 quickly adapting (QA) hair receptors, 9 QA field receptors, 6 SAI and 11 SAII receptors tested in these experiments are shown in Fig. 6.

Recording from small-diameter nerve fibers is extremely difficult and only three C and four Aδ nociceptor recordings were obtained. One C nociceptor, which was shown by its response to mechanical, thermal, and chemical stimulation to be polymodal (Fig. 7), and three Aδ nociceptors (Fig. 8), responded differentially to prickly fabric. The response of these nociceptors was enhanced by moistening the skin with distilled water. A

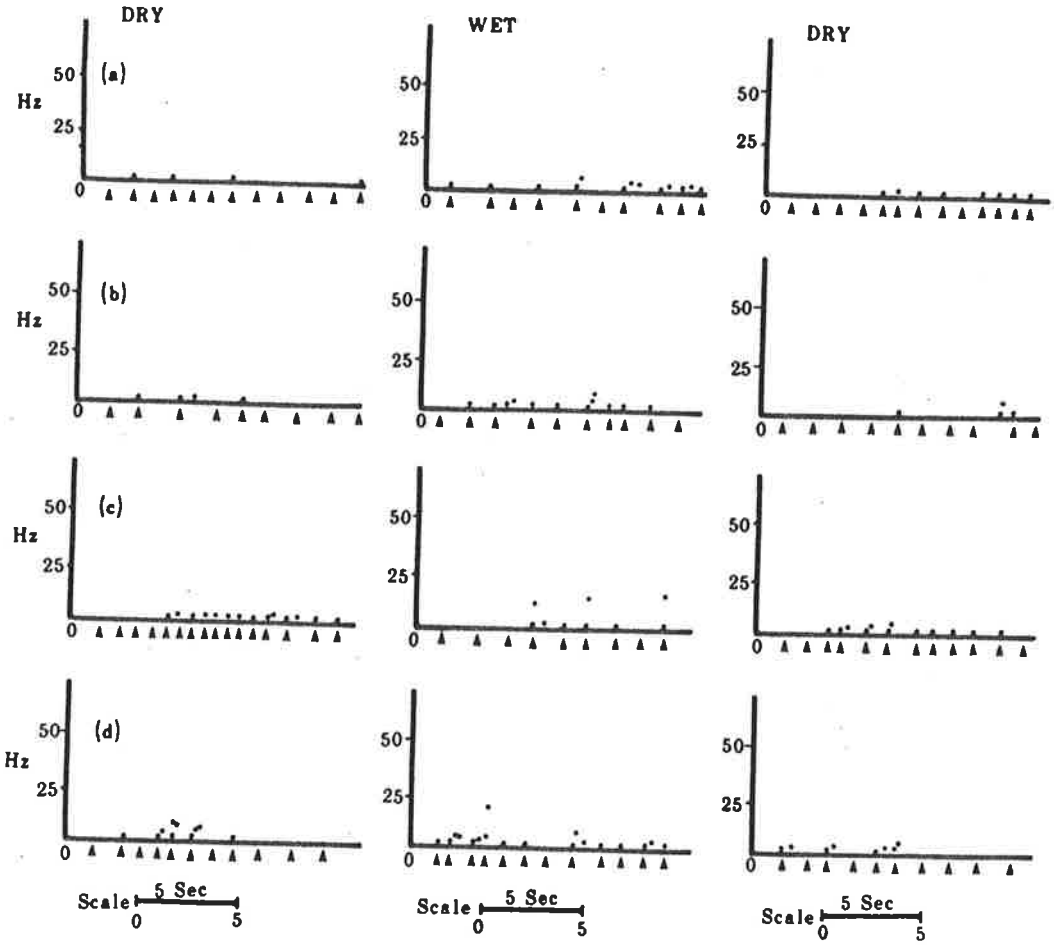


FIG. 5. Responses of 4 rabbit polymodal C nociceptors to a von Frey hair constructed from a single fiber from fabric *C*, buckling load (as measured on an electronic balance) 80 mgf, diameter 43 μm. Method of display as in Fig. 1, *arrows* mark moment of stimulation. Note variability of response and increased discharge when the skin was moistened (*middle*).

preliminary report of these findings has been given (7).

All the above results parallel those obtained in the animal experiments. However, the human Aδ nociceptor responses were comparable to the human polymodal C nociceptor response.

Once the basis for prickle had been identified from animal and human nerve recordings, a method was devised to estimate the density of high load-bearing fiber ends on a fabric surface. This method relied on creating a permanent impression of the protruding fabric fibers in stretched polytetrafluoroethylene (PTFE, Teflon) tape. Six samples of each fabric were mounted face down on 3-mm thick glass slides (60 × 40 mm) with double-sided adhesive tape. Teflon pipe thread tape (12 × 0.075 mm) was stretched over glass microscope slides and an impression of the fabric formed into the Teflon by bringing the former against the latter at a velocity of 0.5 cm/s to a final pressure of 4 gf/cm^2 and held at that pressure for 20 s. As teflon is pliable and nonelastic, a lasting impression of the high load-bearing fiber ends is thus prepared.

Due to variability of Teflon tape each slide had to be calibrated prior to use. One corner of each slide was tested for its response to

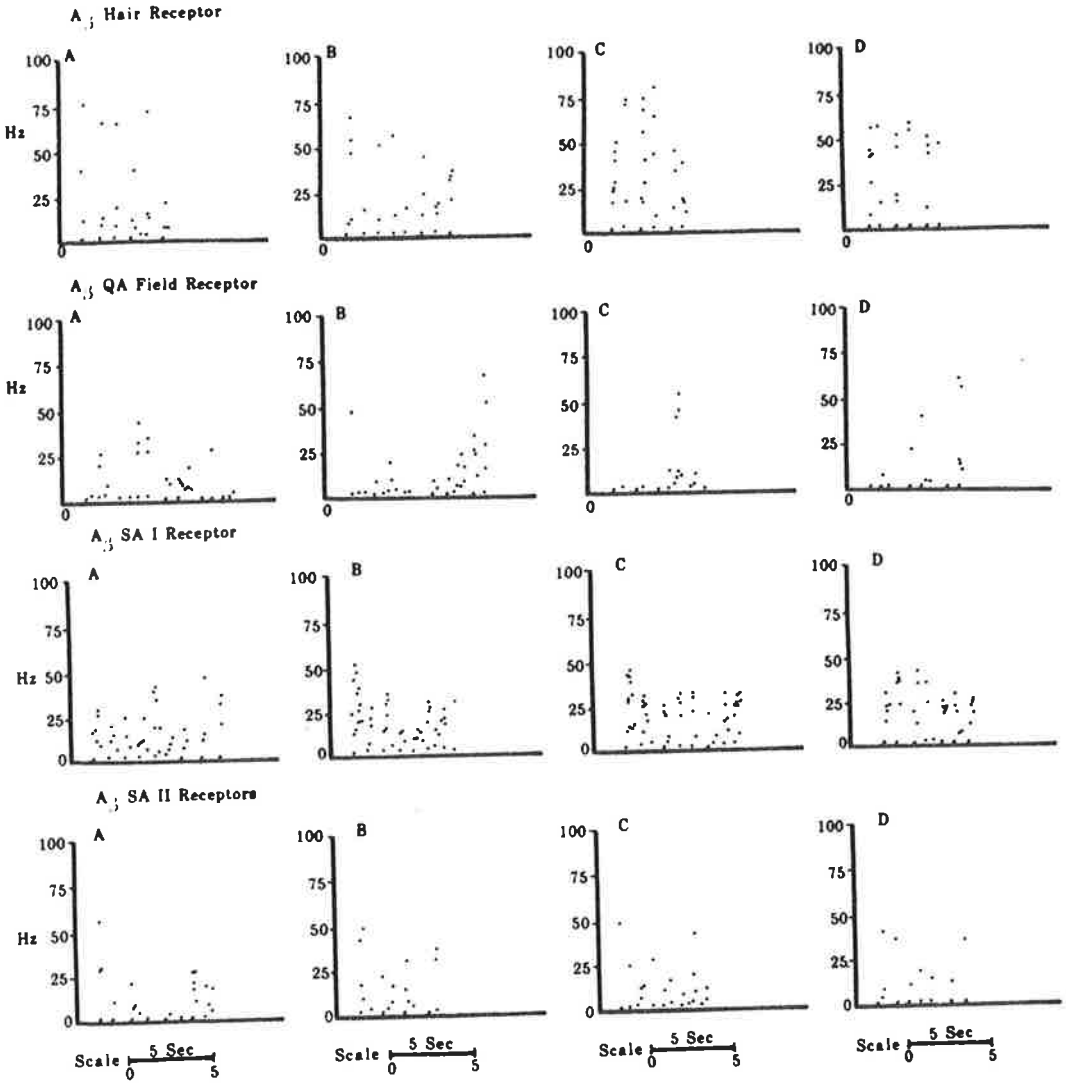


FIG. 6. Microneurographic recording from the radial nerve above the elbow in consenting human volunteers of single receptors from the hairy skin of the forearm and back of the hand. Display method as in Fig. 1, same 3 fabrics as for the animal experiments. A representative example of each type of Aβ receptor (hair, QA field, SA I, and SA II) is shown. As for the animal experiments no differential response to the prickliness of fabric could be discerned nor a change to the response to fabric *C* after moistening the skin (*D*).

four fibers with buckling loads of 75, 100, 130, and 175 mgf, respectively, as measured on an electronic balance. The impressions left by these fibers were evaluated by transmitted and incident light from a Wild stereo microscope using the following criteria for each depression: *1*. 75 mgf buckling load—no increase in transmitted light; *2*. 100 mgf—slight increase in transmitted light; *3*. 130 mgf—obvious increase in transmitted light; *4*. 175 mgf—strong increase as if it had penetrated the PTFE skin.

An 11.4 cm^2 area of Teflon impressed with the test fabric was examined within 10 min of imprint and the number of fibers falling into each of the following three categories was counted: <75 mgf, 75–175 mgf, and >175 mgf. As the criteria for these cate-

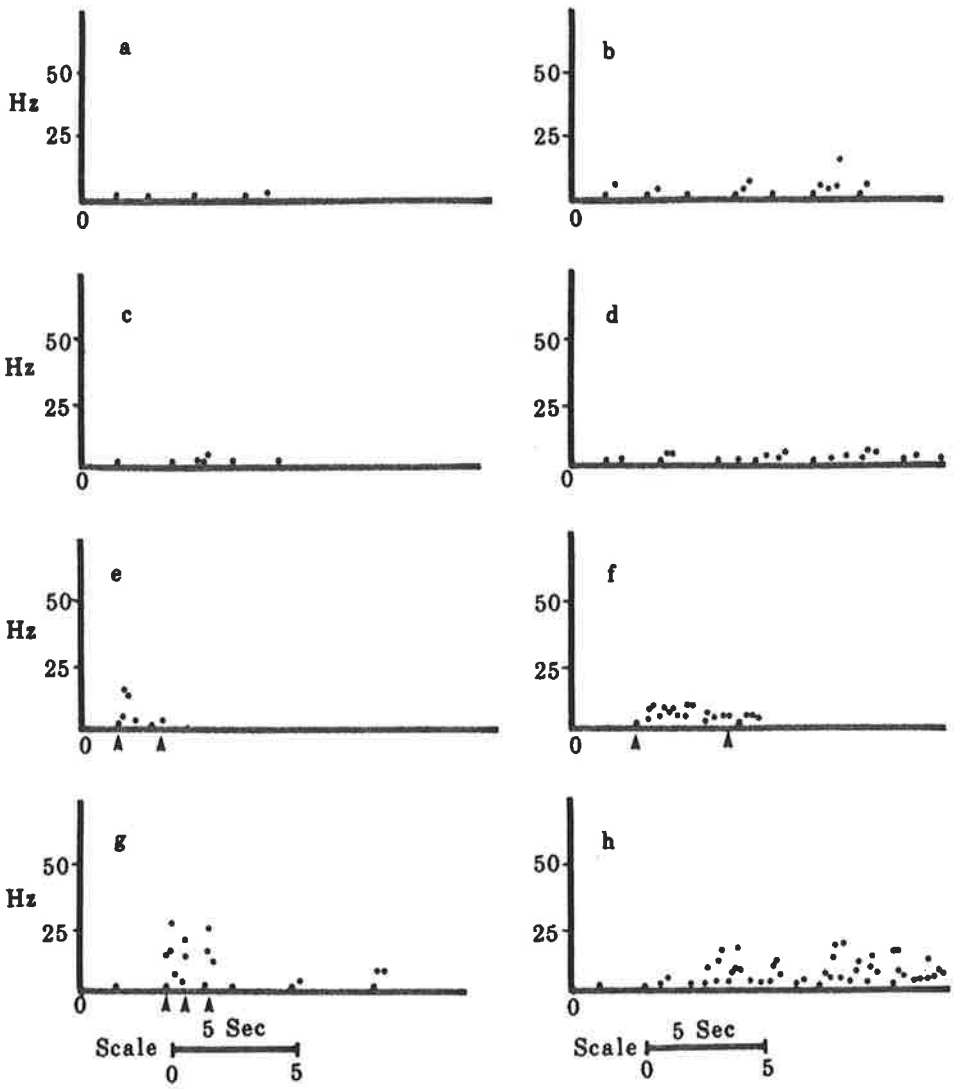


FIG. 7. Responses of a polymodal C nociceptor recorded microneurographically as in Fig. 6. Method of display as in Fig. 1. There was no response to fabrics *A* and *B* but *a* and *c* show the response to fabric *C* tested as previously. *b* and *d* show the response to the same fabric stimulus after moistening the skin, *e* shows the response to moderate pressure, and *f* shows the response to a 46°C probe lightly applied to the skin. *Arrows* in the preceding panels mark application of stimulus. *g*, Response to a drop of 1% capsaicin applied to the skin at the first *arrow*, subsequently pricked into the skin (with a fine needle) at second and third *arrow*. *h*, Response 60 s after *g*.

gories are somewhat subjective, further subdivision was not attempted. Counts were all done by one of the authors who was unaware of which particular fabric he was evaluating.

The 13 fabrics evaluated were divided into two sets, A and B, with 7 and 6 fabrics respectively, for subjective assessment of prickle sensation by 55 subjects. The values obtained for the prickle stimulus intensity (mean number of fiber ends exerting loads > 75, mgf/10 cm^2) and subjective sensation magnitude (arithmetic means for 55 subjects) are shown in Fig. 6. The data were fitted to Steven's psychophysical power law to give the correlation shown. By use of the formula $S_p = aI^n$ (where S_p = sensation magni-

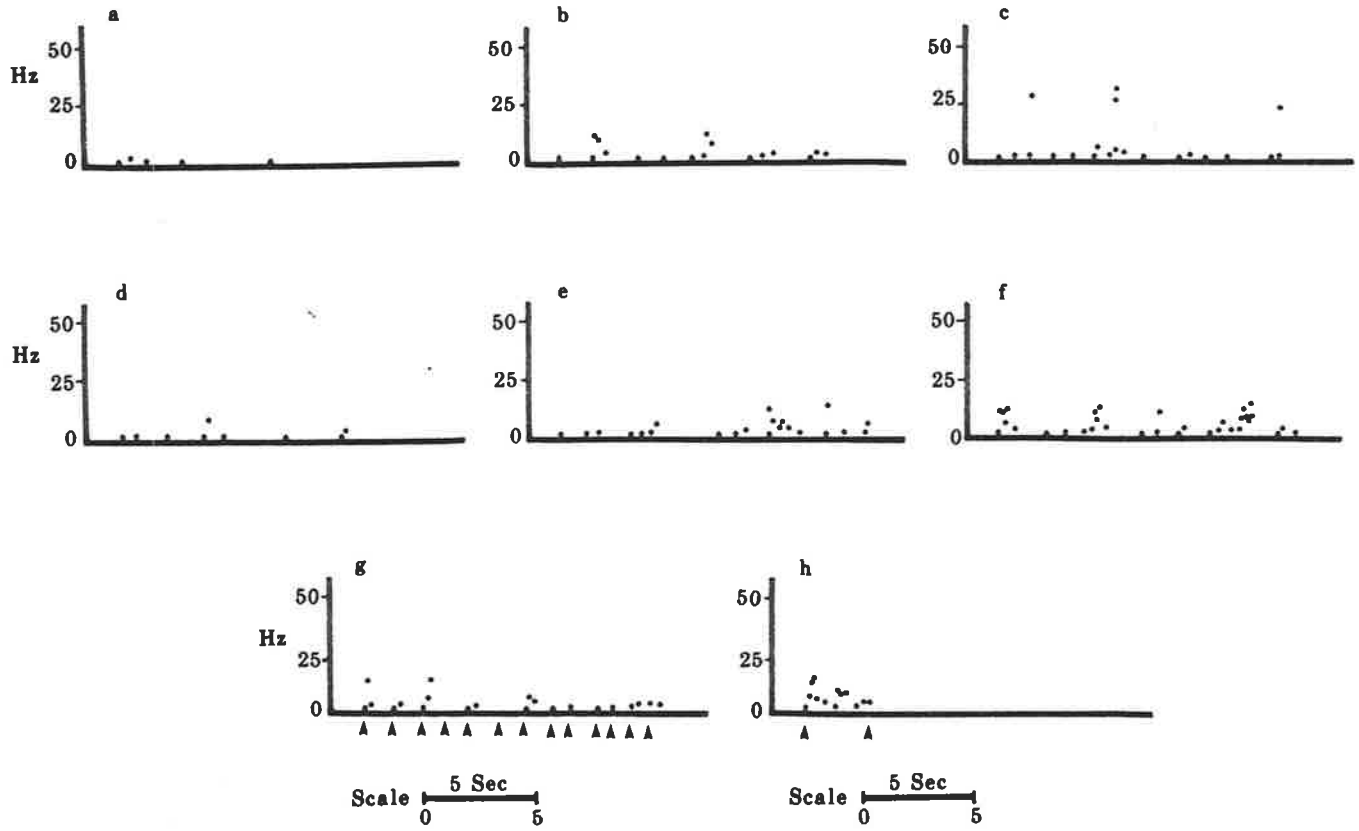


FIG. 8. Responses of two Aδ nociceptors (*a, b, c,* and *d, e, f, g, h*) recorded microneurographically as in Fig. 6, confirmed as Aδ by electrical stimuli applied to the distal nerve trunk. Method of display as in Fig. 1. Neither responded to fabric *A*. Response to fabric *B* (*a* and *d*) and fabric *C* (*b* and *e*) is similar to the animal and human polymodal C nociceptors (Figs. 3 and 8). Both also showed an enhanced response when the skin was moistened (*c* and *f* are the response to fabric *C* on moistened skin). Response of the second receptor to strong pressure (*h*) and the von Frey hair (*g*) constructed from a thread of fabric *C* (the same one used in Fig. 5). Moment of stimulation is marked by the *arrows*. These results further confirm the similarity of human Aδ and polymodal C nociceptors.

TABLE 3. *Effect of weighting fiber buckling loads on the correlation coefficient*

	Fabric Series A		Fabric Series B	
	Power exponent	Correlation coefficient	Power exponent	Correlation coefficient
$X+Y+Z$	0.97	0.82	0.71	0.68
$Y+Z$	0.73	0.91	0.51	0.99
$Y+2Z$	0.69	0.91	0.52	0.98
$Y+3Z$	0.66	0.91	0.52	0.95
$Y+5Z$	0.61	0.91	0.47	0.90

X, *Y*, and *Z* are the no. of fiber ends per 10 cm^2 of fabric with buckling loads of <75, 75–175, and >175 mgf, respectively.

tude, I_p = stimulus magnitude, and *a* and *n* are constants) fitted to the results (Table 3) give the equation

$$S_p = 0.54I^{0.66}$$

with a correlation coefficient of 0.91.

DISCUSSION

The aim of these experiments was to identify the neural basis of prickle. From the experiments reported here it is believed that fabric-evoked prickle is the result of low-level stimulation of Aδ mechano- and C polymodal nociceptors [the latter would be expected to contribute more because of their larger response as recorded in the animal experiments and their greater innervation density (31)] by fabric fibers protruding from the fabric and generating loads of ~75 mgf or more. These fibers do not penetrate the skin. Other fabric surface features are unlikely to contribute as they are unable to generate sufficient force over their area of skin contact with the pressures generated by clothing during normal wearing to excite nociceptors. Small loops could, however, become a problem in stiff nylon weaves as they are capable of exciting nociceptors (as are the edges of a flat surface) if sufficient force is applied (unpublished observations).

These results at present rely on the assumption that animal and human peripheral cutaneous receptors are closely similar. Microneurographic recordings reported by various workers (10, 24–29, 31) have confirmed that essentially the same cutaneous receptor types are present in man as in other mammals, and nociceptors are very similar in all mammals (3, 13, 20, 32). Some confirmation of this has been obtained by our microneurographic recordings from the radial nerve in consenting volunteer subjects. Again, only nociceptors showed a differential response to prickly fabric and Aδ nociceptors showed a similar response to polymodal C nociceptors. The similarity of C and Aδ nociceptors in man has been reported by others (1, 29). Thus, although insufficient data are available to establish the degree of similarity in these experimental conditions, Aδ nociceptors may provide a more important contribution to prickle sensation in man than might be the case in animals.

If we assume that a close similarity between animal and human receptors exists, our data at first sight appears to be at variance with van Hees and Gybels (29) who reported that low discharge rates in nociceptors (below 0.2 Hz) are not accompanied by any sensation. Although the discharge rates of nociceptors recorded in these experiments both in man and in rabbits generally were not much greater than 0.2 Hz, the innervation density of nociceptors is high (31), so that considerable summation is possible over the area of the fabrics used for testing. In fact a minimum area of 1–2 cm^2 is required even for a very prickly fabric, to obtain an unambiguous sensation of prickle (unpublished observations).

The correlation coefficient of 0.91 obtained for the relationship between sensation of prickle magnitude with the measure of the physical stimulus of prickle (number of protruding fabric hairs with buckling loads > 75 mgf) implies we have identified the major factor in fabric-evoked prickle. Similarly the power function for the sensation of 0.66 is within the range reported (8) for other sensa-

tions acting via skin receptors, e.g., hot 1.0, cold 1.6, pressure 0.8, and very close to that reported for discomfort (0.7) whilst differing radically from the 3.5 reported for electric shock, which is presumed to bypass the receptors and excite the nerve trunk directly. Attempts to increase the correlation coefficient by weighting fabric fibers as to their buckling loads were unsuccessful. Although it might be expected that fabric fibers exerting greater forces would evoke more discharge and thus contribute more to the sensation, the effect on the correlation coefficient was minimal as shown in Table 3, where X = <75, Y = 75–175, Z = >175 mgf buckling load. Individual counts for each category for the fabrics tested were X:20–90, Y:2–40, and Z:0–2. It is perhaps not surprising that fabric fibers with loads of <75 mgf (category X) do not contribute as these forces are too low to excite nociceptors. In fact, removal of category X, even though they are the most numerous, improves the correlation. Due to the broadness of categories Y and Z and the low number of counts of Z in commercially available fabrics, it was not possible to adequately assess their contribution by applying weighting factors. However, factors other than buckling load may also be important, perhaps spatial distribution of prickling fibers (clumping) or protection by less stiff hairs in hairy fabrics.

There was a marked variability between subjects in their sensitivity to prickle. A few of the possible reasons for this variability will now be discussed.

Skin mechanical properties are likely to influence prickle. Skin that is difficult to deform (hard, horny) would be more resistant to indentation. This effect is most probably the reason prickle is not felt in the thicker glabrous skin of the hand. Support for this proposition is also provided by the experiments on wetting the skin. Water softens skin (5, 17, 18) and enhances the sensation of prickle in subjects. The same prickly fabric or single fabric fiber will evoke a larger discharge from nociceptors if the skin is wet (Figs. 2, 3, 4, 8 and 9). The increased discharge of nociceptors to a standard prickly fabric after spreading a thin film of water on the skin makes it likely that the increased sensation of prickliness experienced by sweating subjects is due to hydration of the stratum corneum and not any chemicals released by the autonomic nervous system. Experiments searching for autonomic effects on nociceptors have failed to find evidence of any such effects (2, 22). Although sweating releases various chemicals, such as bradykinin, into the circulation, and the sensation of prickle is affected by the temperature of the skin (30), the effect of simply applying distilled water to the skin was so marked and prompt it was not possible to assess whether sweating, as distinct from the skin wetness it produces, has any small contributory effects. Skin hardness increases with age whilst prickle sensitivity decreases (unpublished observations) as would be predicted by these results.

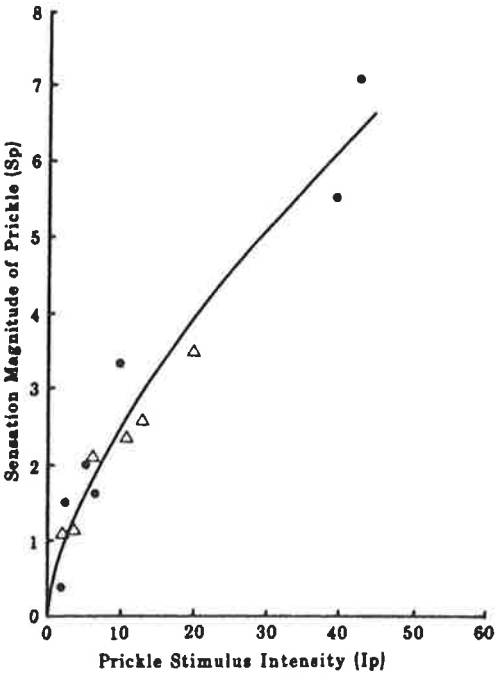


FIG. 9. Prickle sensation magnitude (Sp) versus prickle stimulus intensity (Ip). The psychophysical magnitude function for prickle measurements of both fabric series A (●) and B (△) (see Table 2) are plotted. Stevens' power law fitted to the data gives the curve shown for $Sp = 0.54\,Ip^{0.66}$, $r = 0.91$.

Innervation density, or effective density of nociceptors, is an obvious contribution to the variability of prickle sensation in subjects. Sensitive subjects may have more prickle-sensitive nociceptors, or nociceptors with a lower threshold (or nearer to the skin

surface and thus have an effectively lower threshold). Although sensitivity of subjects varies widely to many different types of stimuli, we know of no way to assess these factors in our subjects. Central, i.e., perceptual thresholds may also be a factor, perhaps the same sensory input will generate a larger affective component in sensitive subjects, or it may even vary with the mood state of the individual as reported for pain sensations (19), however, pain threshold is not affected by mood (21). We did not assess these factors in our experiments, e.g., by using a mood evaluation test, however, previous experiments (30) have shown that sensitive subjects tend to report more pricking points for a standard fabric, indicating that they are perceiving more stimulating points than less sensitive people. The effect of skin moisture, however, implies an involvement of skin hardness.

Previous experiments by our group (30) exclude atopy as a predisposing factor in prickle, despite the widely held belief by dermatologists in such a relationship. Mechanical rather than chemical factors appear to be most important in prickle, and skin flare responses to prickly fabric are probably due to mechanical activation of the axon reflex that polymodal C nociceptors are known to possess (4, 14). Cases of true allergy to fabric appear to be very rare (11, 12), most instances of rash being due to an immediate irritant response.

The results reported here have thus confirmed the utility of the combined physiological-psychological approach to the study of skin sensation introduced by Mountcastle and his colleagues. All the different types of sensory nerve fibers to the skin are accessible for recording, both in man and animals, and tactile sensations are possible only when these nerves are activated. If the physical basis for a sensation can be identified and varied in a controlled, graded manner it is possible to study even elusive sensations such as fabric-evoked prickle. Our experiments have established that fabric-evoked prickle is caused by short fiber ends that, although they do not penetrate the skin, generate sufficient force to evoke a low level of activity in nociceptors, both Aδ and polymodal C fibers. This previously unreported sensitivity to forces as low as 75 mgf, if applied over a sufficiently small area such as a fiber end (diameter 20–50 μm), underlies our discomfort to fabrics and defines this discomfort as a purely mechanical stimulus independent in the majority of cases of any chemical or the atopic status of the individual.

ACKNOWLEDGMENTS

We thank our subjects for participating in these experiments. B. Burns helped build some of the apparatus used and S. Mihajlovic wrote the computer programs to analyze the results.

This work was supported by a grant from the Wool Research Trust Fund on the recommendation of the Australian Wool Corporation.

Address for reprint requests: P. Kenins, CSIRO Division of Textile Industry, Belmont, Victoria 3216, Australia.

Received 13 May 1987; accepted in final form 4 November 1987.

REFERENCES

1. ADRIAENSEN, H., GYBELS, J., HANDWERKER, H. O. AND VAN HEES, J. Response properties of thin myelinated (Aδ) fibres in human skin nerves. *J. Neurophysiol.* 49: 111–122, 1983.
2. BARASI, S. AND LYNN, B. Effects of sympathetic stimulation on mechanoreceptive and nociceptive afferent units from the rabbit pinna. *Brain Res.* 378: 21–27, 1986.
3. BURGESS, P. R. AND PERL, E. R. Cutaneous mechanoreceptors and nociceptors. In: *Handbook of Sensory Physiology, Somatosensory System*, edited by A. Iggo. New York: Springer, 1973, p. 29–78.
4. CELANDER, O. AND FOLKOW, B. The nature of the distributions of afferent fibres provided with the axon reflex arrangement. *Acta. Physiol. Scand.* 29: 359–370, 1953.
5. DIKSTEIN, S. AND HARTZSHTARK, A. In vivo measurement of some elastic properties of human skin. In: *Bioengineering and the Skin*, edited by R. Marks and P. A. Payne. Lancaster, UK: MTP, 1981, p. 45–53.
6. Editorial. *Arch. Dermatol.* 99: p. 133, 1969.
7. GARNSWORTHY, R. K., MAYFIELD, R. J., GULLY, R. L., WESTERMAN, R. A. AND KENINS, P. Mechanisms in cutaneous sensations of prickle and itch evoked by fabrics. *Proc. 7th Int. Wool Text. Res. Conf.*, Tokyo, 1985, vol. III, p. 190–199.
8. GESCHEIDER, G. A. *Psychophysics Method and Theory.* New York: Wiley, 1976, p. 177.
9. GRAHAM, D. T., GOODELL, H. AND WOLFF, H. G. Neural mechanisms involved in itch, "itchy skin",

and tickle sensations. *J. Clin. Invest.* 30: 37–49, 1951.

10. HALLIN, R. G. Human pain mechanisms studied with percutaneous microneurography, In: *Pain Measurement in Man: Neurophysiological Corrlates of Pain*, edited by B. Bromm. New York: Elsevier, p. 39–53, 1984.
11. HATCH, K. L. *Chemicals and textiles, Part I, Dermatological Problems Related to Fibre Content and Dyes. Textile Res. J.* 54: 664–682, 1984.
12. HATCH, K. L. AND MAIBACH H. I. Textile fibre dermatitis. *Contact Dermatitis* 12: 1–11, 1985.
13. IGGO, A. Activation of cutaneous nociceptors and their actions on dorsal horn neurons, *Adv. Neurol.* 4: 1–9, 1974.
14. KENINS, P. Identification of the unmyelinated sensory nerves which evoke plasma extravasation in response to antidromic stimulation. *Neurosci. Lett.* 25: 137–140, 1981.
15. KENINS, P. The functional anatomy of the receptive fields of rabbit C polymodal nociceptors. *J. Neurophysiol.* 59: 1098–1115, 1988.
16. LA MOTTE, R. H. AND MOUNTCASTLE, V. B. Capacities of humans and monkeys to discriminate between vibratory stimuli of different frequency and amplitude: a correlation between neural events and psychophysical measurements. *J. Neurophysiol.* 38: 539–559, 1975.
17. MARKS, R. Techniques for the evaluation of emollients and keratolytics. *J. Soc. Cosmet. Chem.* 29: 433–440, 1978.
18. MARKS, R. Mechanical properties of the skin, In: *Biochemistry and Physiology of the Skin*, edited by L. A. Goldsmith. Oxford, UK: Oxford Univ. Press, 1983, vol. II, p. 1237–1254.
19. MELZACK, R. Measurement of the dimensions of pain experience, In: *Pain Measurement in Man: Neurophysiological Correlates of Pain*, edited by B. Bromm. New York: Elsevier, 1984, p. 327–348.
20. PERL, E. R. Pain and nociception. In: *Handbook of Physiology. The Nervous System. Sensory Processes*. Bethesda, MD: Am. Physiol. Soc. 1984, sect. I, vol. III, part 2, p. 915–975.
21. ROGERS, E. J. AND VILKIN, B. Diurnal variation in sensory and pain thresholds correlated with mood states. *J. Clin. Psychiatry* 39: 431–438, 1978.
22. SHEA, V. K. AND PERL, E. R. Failure of sympathetic stimulation to affect responsiveness of rabbit polymodal nociceptors. *J. Neurophysiol.* 54: 513–519, 1985.
23. TALBOT, W. H., DARIAN-SMITH, I., KORNHUBER, H. H. AND MOUNTCASTLE, V. B. The sense of flutter-vibration: Comparison of the human capacity with response patterns of mechanoreceptive afferents from the monkey hand. *J. Neurophysiol.* 31: 301–334, 1968.
24. TOREBJORK, H. E. Afferent C units responding to mechanical, thermal and chemical stimuli in human nonglabrous skin, *Acta. Physiol. Scand.* 92: 374–390, 1974.
25. TOREBJORK, H. E. Nociceptor activation and pain. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 308: 227–234, 1985.
26. TOREBJORK, H. E. AND HALLIN, R. G. Identification of afferent C units in intact human skin nerves. *Brain Res.* 67: 387–403, 1974.
27. TUCKETT, R. P. Itch evoked by electrical stimulation of the skin, *J. Invest. Dermatol.* 79: 368–373, 1982.
28. VALLBO, A. B., HAGBARTH, K-E., TOREBJORK, H. E. AND WALLIN, B. G. Somatosensory, proprioceptive and sympathetic activity in human peripheral nerves. *Physiol. Rev.* 59: 919–957, 1978.
29. VAN HEES, J. AND GYBELS, J. C nociceptor activity in human nerve during painful and nonpainful skin stimulation. *J. Neurol. Neurosurg. Psychiatry* 44: 600–607, 1981.
30. WESTERMAN, R. A., GARNSWORTHY, R. K., WALKER, A., KENINS, P., GULLY, R. AND FERGIN, P. Aspects of human cutaneous small nerve function: sensations of prickle and itch. In: *29th I.U.P.S. Satellite Symposium, Newcastle, Australia*, edited by L. A. Chahl, J. Szolcsanyi, and F. Lembeck. Budapest: Hungarian Academy of Sciences Press, 1985, p. 329–345.
31. WILLIS, W. D. The Pain System: The Neural Basis of Nociceptive Transmission in the Mammalian Nervous System, In: *Pain and Headache*, edited by P. L. Gildenberg. Basel: Karger, 1985, vol. 8, p. 7–77.
32. ZIMMERMAN, M. Neurophysiology of nociception. *Int. Rev. Physiol. Neurophysiol. II* 10: 179–221, 1976.

Properties of Transdermal Histamine Iontophoresis: Differential Effects of Season, Gender, and Body Region

Walter Magerl, Dipl.Psych., Rod A. Westerman, M.D., Ph.D., Bernd Möhner, and Hermann O. Handwerker, M.D.

Institut für Physiologie und Biokybernetik (WM, BM, HOH), University of Erlangen, Erlangen, F.R.G. and Department of Physiology (RAW), Monash University, Victoria, Australia

Histamine iontophoresis is demonstrated to be a reliable model for the study of inflammatory skin responses. It has the advantage of a non-invasive and uniform mode of application and is free of unwanted side effects. The wheal and flare responses to histamine are linearly related to dose over a wide range of stimulus strengths ($r = 0.88$). In summer, wheal responses were smaller, probably due to increased thickness of the epidermis. Female subjects generally expressed larger wheal responses than males, presumably due to differences in epidermal thickness and structure. There were significant regional differences in wheal, flare, and laser Doppler recorded flux responses. Ratings of itch sensations also showed clear, but less pronounced, differences of body regions. Significant regional differences of wheal and flare responses existed. Sensory discrimination of different stimulus levels was demonstrated with visual analogue scale ratings. *J Invest Dermatol 94:347–352, 1990*

Histamine has been used for decades as a means to induce itching and to study the early stages of cutaneous inflammatory responses. If applied to the superficial layers of the skin of primates it induces a triple response, i.e., local redness, replaced after a short time by a local edema (wheal), and a surrounding erythema (flare) [1]. So far, little attention has been paid to the mode of application. Pricking or injection via needles or vaccination pistols have commonly been used. There are some short notes in the literature suggesting that iontophoretic deposition of histamine might be superior to these methods by avoiding skin damage [2–6]. No thorough evaluation of this technique has been performed hitherto, although recently it was proposed that iontophoretic drug delivery may be the method of choice for a variety of ionized substances [7]. Even macromolecules, like insulin, were successfully delivered iontophoretically into the skin in diabetic animals and humans [8,9].

In this study we try to provide evidence that iontophoresis of histamine is a quantifiable method for dermatologic and pharmacologic studies in the skin with a high level of selectivity. A preliminary report [10] and some aspects of this technique, in particular those related to psychophysics, have been published recently [11,12].

Manuscript received March 15, 1989; accepted for publication September 16, 1989.

Most of the experiments were conducted at the Erlangen Department, but some at the II. Physiologisches Institut, University of Heidelberg, FRG.

This work was supported by the Deutsche Forschungsgemeinschaft, grant Ha 831/8. Dr. Westerman was an Alexander v. Humboldt research fellow.

Reprint requests to: Dr. Hermann O. Handwerker, Institut für Physiologie and Biokybernetik, University of Erlangen, Universitätsstrasse 17, D-8520 Erlangen, FRG.

Abbreviations:

ANOVA: analysis of variance
CGRP: calcitonin gene-related peptide
LED: light emitting diode
SAS: statistical analysis system
SP: substance P
VAS: visual analogue scale

MATERIALS AND METHODS

Histamine dihydrochloride (SIGMA) was prepared as a 1% aqueous solution. This solution was supplemented with 2.5% methylcellulose and left for 24 h to form a hydrogel matrix. For the purpose of application we used a small pencil-like perspex rod. A hemispheric chamber 5 mm in diameter and with a volume of 50 μl was drilled into one end of this rod. The base of the chamber was formed by a small plate of chlorided silver, which served as an anode (see Fig 1). The histamine gel solution was introduced into this cavity. The methylcellulose gel held the histamine in the cavity space. This "chemode" allowed a precise histamine application to any spot of the skin surface. Histamine ions were driven from the chamber to the skin by electrical pulses from an isolated constant current source (World Precision Instruments WPI 305 B). A 3 × 3 cm foam rubber coated silver electrode nearby moistened with tyrode served as a reference (Fig 1). The position of the reference electrode is not critical. Intensities of iontophoretic stimuli could be varied by changing either current strength or time of application and were calculated, following Coulomb's law, as the product of current and time: $q = I \times t$, where q is the charge given in mC, I is the current given in mA, and t is the time given in seconds.

Wheal and flare responses were marked with water-resistent pens 10 min after histamine application and copied to translucent paper for planimetry. The wheal and flare areas were measured and the diameters of circles having the same area were calculated. Experiments were performed to control non-specific, e.g., hydrogel and/or current-related, responses. These included the application of hydrogel, either with or without histamine and no current applied, and also the application of current via the hydrogel only or the histamine-hydrogel-store using iontophoresis with reversed polarity, i.e., with the histamine-containing "chemode" used as a cathode. Concomitant sensations of itching were estimated by means of a visual analogue scale (VAS) at 10-sec intervals cued by signal tones. The VAS was a 31-element LED scale, the left end of which was defined as "threshold" and the right end as "maximal itching." The light-emitting diode (LED) scale was controlled by the subject via a linear potentiometer lever to indicate the itch intensity. A mark at the 30% level of the scale was defined as "itching strong enough

0022-202X/90/$03.50

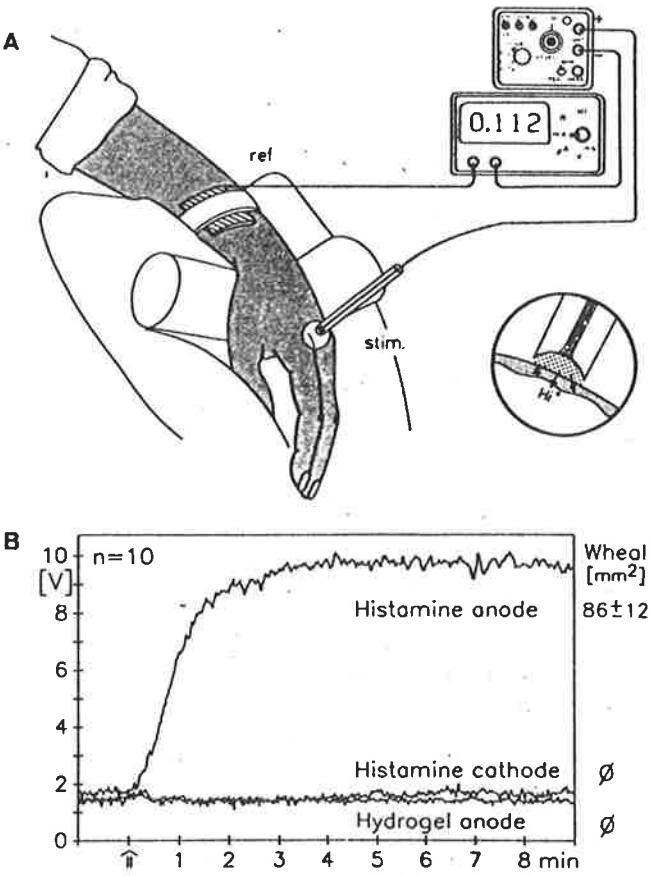


Figure 1. *A*. Principle of the experimental set-up. The area of interest (here, e.g., the dorsum of the hand) is rested in a comfortable position. The anode (stim.) containing histamine in a gel is placed on the spot to be stimulated and a larger reference cathode (ref.) is fixed nearby (here, e.g., at the wrist). Both are wired to an output-controlled constant current unit (upper right corner). Histamine ions (Hi^+) are driven to the skin by repulsion of charges from the anode. The inset shows the principle of ion transition to the skin in more detail. *B*: Comparison of anodal histamine iontophoresis to different control conditions, i.e., hydrogel anode and histamine cathode, respectively (n = 10) in wheal and flux response. Note that a significant wheal and flux response occurs exclusively under the histamine anode.

to be scratched" (intervention point), an analogy with the drug request point of pain research [13]. This scale had performed well in previous studies on the effect of nonsteroidal anti-inflammatory drugs [14,15] on experimental pain.

Four different experiments have been conducted with this experimental design. The experimental subjects were healthy and in particular were not suffering from dermatologic diseases. Volunteers were recruited among the students of the universities of Heidelberg and Erlangen and among the staff of the respective Departments of Physiology. All subjects were of caucasian origin. Before each experiment, an instruction on the experimental setting, the use of the rating scale, and the aims of the study were given. A first stimulus was delivered as a cue. This was not used for the data evaluation.

Experiment 1 was performed during winter time (December to February) in 48 subjects (27 male, 21 female; age range, 19–52 years; median age, 26 years) receiving stimuli of different strength in randomized order.

Experiment 2 was conducted in a different group of 23 subjects (11 male, 12 female; age range, 15–31 years, median age, 23 years) during summer time (June to August). The aim of the first two experiments was to study the relationship between stimulus strength and the size of wheal and flare reactions, the effects of the gender, and of the season. The test area was the skin of the ventral forearms.

Experiment 3 was designed to assess the responses of different body regions to histamine iontophoresis. Eight body regions were studied in 20 subjects (10 male, 10 female; age range, 24–34 years; median age, 26 years): forehead, ventral thorax, upper arm (ventral side), lower arm (ventral side), hand (dorsal side), thigh (ventral side), lower leg (lateral side), and foot (dorsal side). These eight sites were tested with identical current pulses of 10 mC (10 mA, 10 sec). The experiments were conducted during the summer time (May to July). In addition to the parameters mentioned above, the flare response was also measured by two laser Doppler flowmeters (Periflux PF2, perimed, Stockholm, Sweden). Both flowmeters were placed in standard holders positioned at 90° angles to each other and at 45° angles to the longitudinal axis of the respective body region. The skin site at which the blood flow was measured by this method (flux) was fixed at 8 mm distance to the spot of iontophoresis. Flux values are presented as output voltages of the Laser Doppler flowmeter set to gain 10, 1.5 sec time constant, and an upper frequency limit of 12 kHz.

Experiment 4 was conducted in six subjects (four male, two female). Its aim was to test the acuity of sensory discrimination of different levels of itch. Two levels of stimulation (0.25 and 1.35 mC) were applied to three different spots in the innervation zones of the superficial radial nerves of both hands (i.e., 2 × 3 stimuli/subject) in random order. Current strength was changed and the application time was always 10 sec. After each stimulus, itching was assessed for 8 min on the VAS as described above. To avoid evaporative cooling (which may suppress itching, according to literature [16,17]), remaining traces of gel were wiped off in these experiments immediately after stimulation.

Statistical analysis was performed on a main frame computer system by means of "Statistical Analysis Systems" (SAS Institute Inc., Cary, North Carolina, USA). Procedures included analysis of regression and analysis of variance. For group comparisons, whole sample t test and chi-square test were used. Duncan's multiple range test was employed in cases of multi-stage comparisons. Levels of $p < 0.05$ were considered to be significant.

RESULTS

The responses to iontophoretic stimulation were strictly related to anodal stimulation through a histamine-loaded hydrogel store for currents up to 1 mA. All other types of current application, i.e., anodal or cathodal through plain hydrogel and even cathodal stimulation through a histamine-loaded hydrogel, induced neither wheal nor flare responses. The latter was carefully controlled by laser Doppler flowmetry (Fig 1*B*).

The diameters of the wheal and flare areas in ventral forearm skin were linearly correlated with the logarithm of charge over the range of 0.05 to 30 mC in normal subjects (winter sample, n = 48). The respective correlation coefficients were r = 0.88 in both wheal and flare diameter (Fig 2*A*). Threshold values were calculated by regression analysis and were slightly lower for flare (0.04 mC) than for wheal responses (0.05 mC). Very similar results were obtained when the areas of wheal and flare were correlated instead of the diameters. Flares and wheals were correlated to r = 0.89. However, in a later study (experiment 3) in which the body region was varied, instead of the stimulus strength a lower correlation coefficient of r = 0.54 was found. Thus, much of the covariance of wheal and flare sizes in experiment 1 may have been caused by the variance of stimuli. When this factor of variance was partialed out, there was still a significant partial correlation of wheal and flare responses of r = 0.60, which is in good agreement with the result of experiment 3.

Measurements of wheal responses were repeated in summer (experiment 2) in 23 subjects, to estimate seasonal and gender effects. In general, wheals were smaller in summer at all stimulus levels resulting in a parallel shift of the regression line. In summer, charges had approximately to be doubled to achieve wheals equivalent to those in the winter group (Fig 2*B*). Wheal responses of the summer group were further analyzed for the effect of gender. There was a highly significant difference between the value for male and female

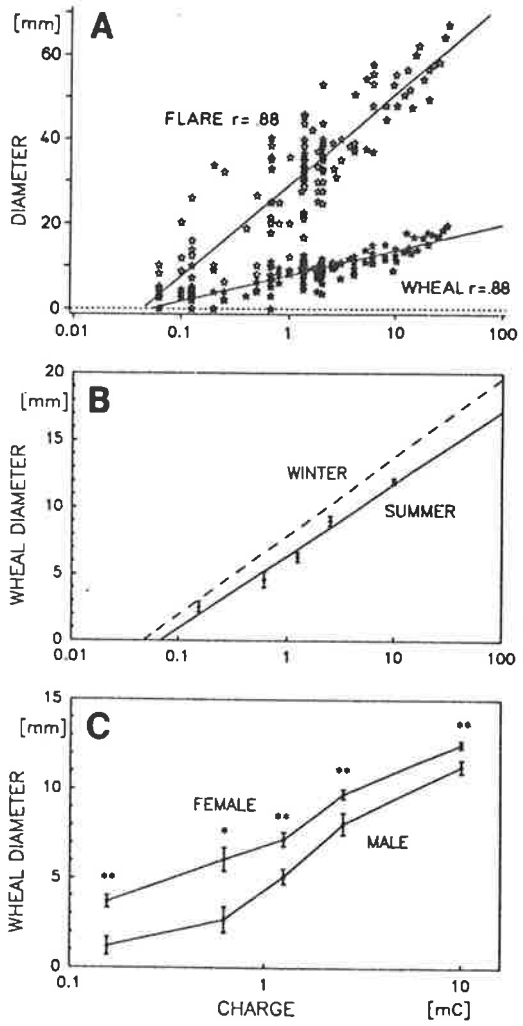


Figure 2. Wheal and flare responses to histamine iontophoresis under various conditions. *A*: Linear relationship of wheal *(solid stars)* and flare responses *(open stars)* to logarithm of charge. Correlation coefficient was 0.88 in both cases (experiment 1, n = 48). *B*: Comparison of the wheal responses of the subjects tested in winter (n = 48) to those of subjects measured during summer (experiment 2, n = 23). The *broken line* is equivalent to the regression line of the winter group shown in *A*. The summer group was treated with a comparable range of stimuli. Since the data was obtained with the method of constant stimuli (0.156, 0.625, 1.25, 2.5, and 10 mC) in the summer group, the data is presented both as mean ± SEM of the respective levels of iontophoresis *and* with the respective regression line *(solid line)*. The latter shows unequivocally the parallel shift to lower responses in the summer group. The correlation coefficients were r = 0.88 in both, summer and winter sample. *C*: Gender-related differences in wheal responses, as measured on the ventral forearm. The summer group of *B*, further subdivided according to gender, shows significant differences at all stimulation intensities. Female subjects (n = 12) in general gave bigger responses than male subjects (n = 11). Data as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$.

($p < 0.0001$, F test). Wheals were significantly bigger in the female subgroup at all stimulus levels (Fig 2*C*). Correlation coefficients (charges vs wheal sizes) were calculated separately for both gender subgroups and were 0.90 and 0.93 for the male and female subgroup, respectively.

Sizes of wheal and flare responses varied with the skin region tested. This was studied in 20 subjects (experiment 3). Wheal areas, flare areas, flux, and mean itch ratings during eight minutes after stimulus application were obtained from eight body regions. With each of these four data sets a two-way analysis of variance (ANOVA) was computed to assess the effects of the factors "gender" and "body region." Each of the four ANOVA revealed a highly significant effect of "body region" (all $p < 0.0001$) for the parameters wheal sizes, flare sizes, flux responses, and itch ratings (see Fig 3). In all three objective measures (wheal size, flare size, and flux response) there was a clear proximo-distal gradient of responses in both upper and lower limbs. This was not the case in itch reports.

In addition, a significant "gender" effect was found for the wheals ($p < 0.05$). The latter is due to larger wheals of the female subjects in most body regions, in agreement with what was found in experiment 2. Statistically significant differences were only obtained for the hand. In all other body regions, except the foot, there was also a tendency for smaller responses in male subjects, namely in the forehead, upper arm, and upper and lower thigh they had responses that were, by one half to more than one standard deviation, smaller than in females. However, these differences were not statistically significant, due to the small number of subjects (10 males vs 10 females). To demonstrate the differences between the body regions more clearly, the four data sets "wheal size," "flare size," "flux responses," and "mean itch ratings" were standardized by transforming them into z values (i.e., expressing them as differences from the grand mean in units of the standard deviation). These standardized data are shown in Fig 4.

Variations of wheals and flares at different body sites were similar: forehead, foot, and hand showed small reactions, the shoulder large reactions. However, as mentioned above, flare and wheal sizes correlated but moderately (r = 0.54). Mean itch ratings did not vary as conspicuously as the skin reactions. The lowest ratings were obtained at the forehead.

The question of whether the subjects were able to differentiate two different, unknown levels of histamine iontophoresis (0.25 mC and 1.35 mC) applied to the dorsa of their hands was tackled in experiment 4. As in the other experiments, itching developed with a latency of about half a minute after current delivery, climbed to a maximum after about 1 or 2 min, and usually wore off within 8 to 15 min (Fig 5*A*).

Two different, though interrelated, parameters were extracted from the record of ratings, maximum and the average of the 12 ratings of the third and fourth minute. Both had an equally good discriminative power ($p < 0.01$, t test for whole samples) (Fig 5*B*). This was in agreement with the data obtained from a larger group of subjects in experiment 3 (see above) where a correlation coefficient of r = 0.96 was obtained between the maximal itch rating in each trial and the average rating during 8 min (144 pairs of measurements from 20 subjects and 8 body regions).

Intraindividually, there was almost no overlap of the responses to the two different charges applied in experiment 4. Two-way analysis of variance with the independent variables "stimulus charge" and "subjects" revealed that "stimulus charge" was the more significant variable (F test, $p \ll 0.0001$), though there was a minor contribution by the second factor "subjects" ($p < 0.001$, F test), indicating that the perceived itching depended mainly on the stimulus charge, but stable intra-individual differences also occurred. The overall performance of the model is shown in the percentage of variance explained by these two factors with an R^2 of 69.4 and 74.4% for maximum and average rating, respectively.

DISCUSSION

Iontophoretic histamine delivery has been used in a small number of studies in the past [2–6,18,19]. Where possible, stimulus parameters were reconstructed from these studies: currents ranged from 0.5–2 mA, equivalent to current densities of 0.15–12.5 mA/cm². Application times varied widely in different studies, total charge delivery was 5–450 mC, equivalent to charge densities of approximately 2–150 mC/cm². We have extended this range mainly to lower currents of 0.01–3 mA (0.05–15 mA/cm²), by delivering charges of 0.05–30 mC (0.25–150 mC/cm²), because higher current densities induce increasingly painful burning sensations during the stimulus application. From various combinations of time and current it became quite clear that total charge transferred intradermally was the parameter correlating best with skin reactions, provided that the time of delivery was kept short enough to avoid

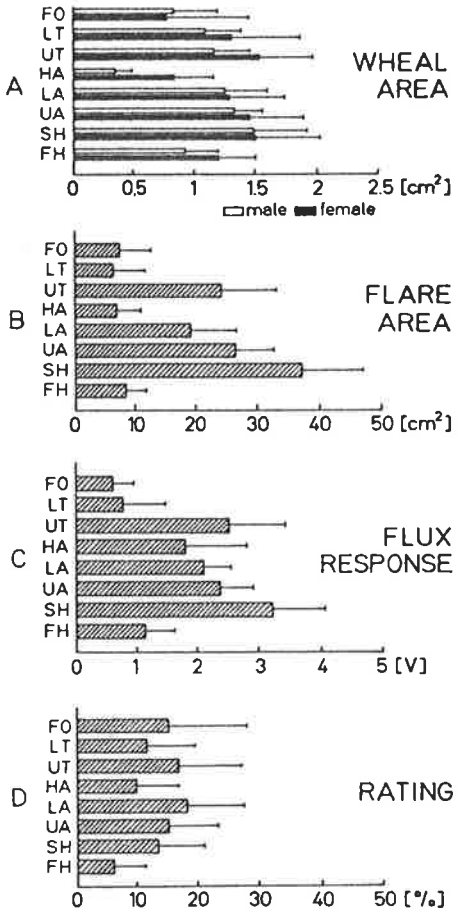


Figure 3. Regional differences in responses to histamine iontophoresis. The diagrams represent the wheal *(A)* and flare areas *(B)*, flux responses *(C)*, and mean ratings during 8 min after stimulus application *(D)*. Eight body regions were tested with identical stimuli of 10 mC in 20 subjects (10 male, 10 female). The data shown represent mean plus 1 SD. An influence of gender was found only in the wheal data *(A)*. Though they were significantly different only in the hand, other body regions, namely, the upper and lower thigh, upper arm, and forehead, showed clearly smaller responses in male subjects, though these did not reach statistical significance, presumably due to the small number of subjects (10 males vs 10 females). On either limb wheal, flare, and flux responses were smaller the more distal the point of measurement was. Wheal and flare data in cm^2, flux values in output voltage of the Laser Doppler flowmeter, and mean itch ratings in % of the rating scale. FO, foot; LT, lower thigh; UT, upper thigh; HA, hand; LA, lower arm; UA, upper arm; SH, shoulder; FH, forehead.

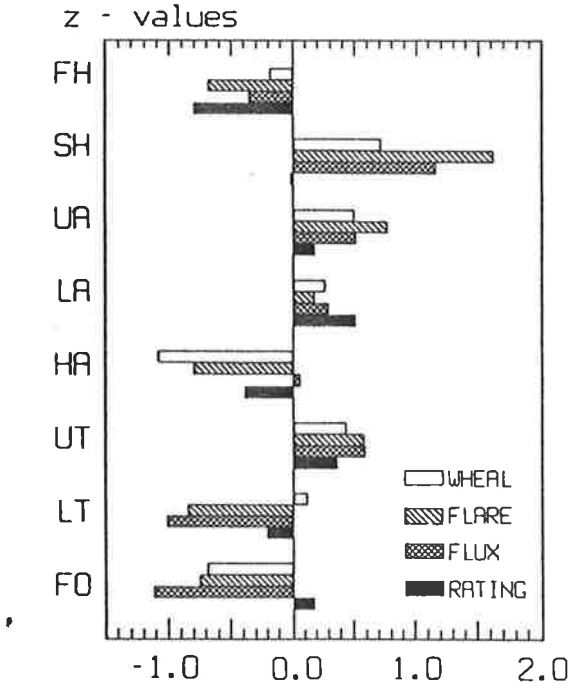


Figure 4. Regional differences in wheal, flare, flux, and rating data. The data base is identical with that used for Fig 3. All measurements were expressed in z values, i.e., as deviations from the population mean of the respective parameter over all body regions in units of the standard deviation ($([x_i - \bar{x}]/s)$, where x_i is the mean value obtained in a particular body region, $\bar{x}$ the mean over all body regions, s the standard deviation of the whole sample. The columns represent mean values obtained from 20 subjects. Note the strong responses in the shoulder (greater than 1 SD shift) and the weak responses, e.g., in forehead, hand, and foot.

clearance of histamine from the tissue within stimulation time. In the range of stimuli used, wheal responses were elicited only under the histamine anode. All other conditions never resulted in wheals. One has to be careful, however, to use excessive current densities (> 5 mA/cm^2 equivalent to > 1 mA in these experiments), because these can possibly result in direct electrical lesioning of the skin, when applied for a longer period of time. Since current pulses were usually kept short (5–20 sec), no such effects have been encountered with currents below 1.5 mA (i.e., 7.5 mA/cm^2). Higher current densities sometimes elicited long-standing local discolorations, which may probably be due to a slight superficial burning. This is even more likely in denervated skin. Although the subjects will easily tolerate this, it should and could be avoided by using a lower level of current for a longer stimulation time, which will give an equivalent transfer of charged molecules.

It is quite obvious from our results that this type of histamine iontophoresis is a technique of high sensitivity and selectivity to study skin responses in humans. Wheal and flare responses were highly correlated with histamine doses in a range of almost three orders of magnitude. Follicular wheals were observed with charges as low as 0.05 mC. This association with hair follicles is in agreement with the premise that transdermal delivery uses skin appendages as the major penetration pathways [7]. Control experiments disclosed that responses to iontophoresis of histamine were strictly due to the anodal mode of application. Neither passive diffusion nor hydrogel and/or direct-current effects were any source of influence on the responses.

Several factors were found which may influence the histamine responsiveness. There was a striking difference in wheal responses in winter compared to summer season, probably due to epithelial thickening of the skin under the influence of higher levels of ultraviolet radiation in summer. It has been demonstrated that ultraviolet light of the A band induces thickening of the horny layer of the skin [20]. Differences were also found between the reactions of male and female subjects. Since permeability of the stratum corneum is the main obstacle influencing responses [21,22], differences in this layer between male and female skin may be responsible for these findings. Bronaugh and co-workers [23] have reported that percutaneous absorption is enhanced in the female rat skin in vitro compared to the thicker skin of male rats. Sex-related differences could be prevented by early castration, which suggests that androgen levels are an important factor [22]. Different skin properties may be responsible also for the significant regional differences seen. Holbrook and Odland [24] have shown a wide variation of thickness of the stratum corneum. Differences in corneocyte size and volume were considered to be partly responsible for regional variances of skin permeability [25–27]. Considerable differences in permeability still exist between areas, which have both equal size and volume of corneo-

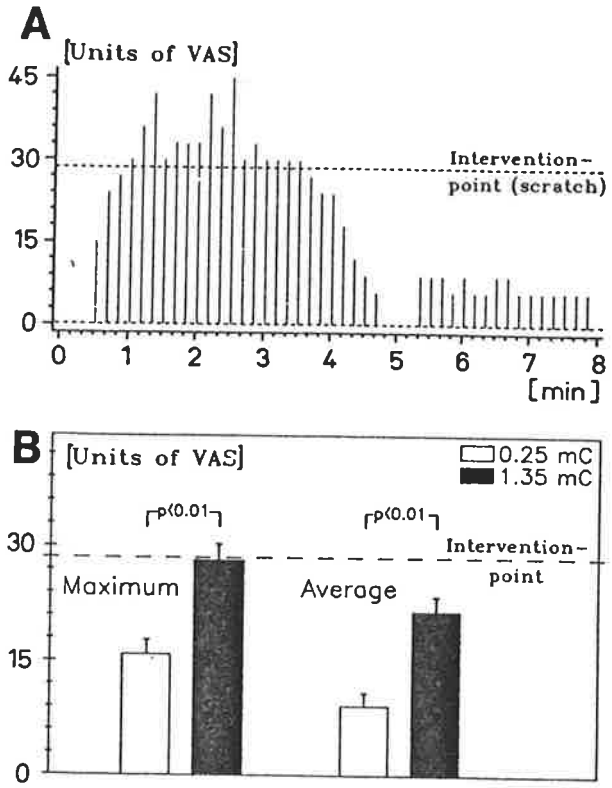


Figure 5. Estimation of the magnitude of itch perception following histamine iontophoresis. *A*: A specimen of an itch rating on a visual analogue scale following a 10-sec iontophoretic stimulus starting at $t = 0$ (stimulus = 1.35 mC). *Ordinate:* percent of the full rating scale. The *broken line* represents the level of itching, which would be responded to by scratching under non-experimental conditions (intervention point). *B*: Discrimination of two levels of iontophoresis. The subjects discriminate equally in both maximum and average of ratings to different stimuli (0.25 vs 1.35 mC) (mean ± SEM, $n = 18$, $p < 0.01$, whole sample t test).

cytes. Because the intercellular rather than transcellular routes of penetration are important, the physico-chemical properties of the intercellular cement have to be taken into account. The lipid composition of the intercellular space, e.g., the role of flat ceramide liposomes, has thus been invoked to explain the influence of species, gender, and body region [28–30].

We have shown previously [11] that in skin sites pretreated with capsaicin the neurogenic flare component of the triple response was strikingly impaired, while wheal responses, which are dependent upon plasma extravasation, seemed to be unaffected. The same areas, which were almost unresponsive to chemical stimulation, also displayed a 95% reduction of neurogenic vasodilatation to electrical stimulation of the skin in humans [31]. In both models, vasodilatation due to a release of neuropeptides from the receptive endings of unmyelinated C-fibers is impaired as a sequel of the depletion of these peptides, e.g., substance P (SP) and calcitonin gene-related peptide (CGRP) [32].

It is likely that neuropeptide release from primary afferent C-fibers due to noxious stimulation takes part in the process of local tissue repair. Destruction of unmyelinated primary afferents in rats by neonatal treatment with capsaicin leads to a considerably greater vulnerability of the skin to damage compared to controls [33]. In human skin, C-fiber deafferentiation often leads to trophic changes [34,35]. Afferent C-fibers may thus play a crucial role in the regeneration of the epidermis. On the other hand, neuropeptide release may play also a trophic role in healthy skin. It has been reported that SP is a potent mitogen stimulating DNA synthesis in arterial smooth muscle cells, in human skin fibroblasts [36], and in a mouse epidermal cell line (Pam 212 cells) in vitro [37], thereby being a likely candidate for stimulation of epidermal proliferation. This could also play some role in the seasonal differences we have demonstrated, since ultraviolet light is able to induce discharges of low frequency in cutaneous polymodal nociceptors [38].

Iontophoresis of histamine and/or other agents which activate C-nociceptors may be a suitable model to test the integrity of the sensory and neurovascular functions of the skin, in other words, to check the functional state of the "nocifensor system" [39–41]. A number of substances, including serotonin, dopamine, and a cholinergic derivative, carbachol, have been successfully used for iontophoresis and are all able to elicit noxious sensations [Grämer, Magerl, and Handwerker; in preparation]. A first study using this model of histamine iontophoresis in patients suffering from atopic dermatitis has now been performed showing clear changes in atopic skin compared to the skin of control subjects [42].

Regarding our method of histamine iontophoresis from the technical point of view, it is of interest whether the type of storage itself will affect the rate of transport. Using Stokes' equation to estimate molecule transport in gel matrices, it can be shown that in low concentration gels the electric mobility of ions is equivalent to their mobility in a sucrose solution [43]. This is certainly true for the hydrogel used (2.5% methylcellulose, viscosity coefficient: 0.5 Pa s at 20°C). Mobility of molecules is also dependent on the molecular weight, but approaches almost free mobility for molecular weights below 10,000 [44] (molecular weight of histamine is about 111). It has been reported recently that in an in vitro model, transport from a gel matrix through a model membrane is membrane-controlled for electrically assisted transport (i.e., iontophoresis), and that electrical mobility is indeed unity for small molecules [45]. One single gel store for application contained approximately 5×10^{-6} mol of histamine cations, from which, following Coulomb's law, only 1.04×10^{-8} mol $\times$ mC^{-1} were removed by current delivery. The maximal stimulus used (30 mC) thus utilized only 6% of the total store. It can be assumed, thereby, that transport rates were linearly related to the calculated charge values. The depth and area penetrated by iontophoresis of a similar compound (potassium ferroferricyanide) in an in vitro agar gel model has been found to correlate well with the in vivo dilator effects of the same iontophoretically applied doses of acetylcholine in forearm skin [46,47]. Of course, the conjecture that the amount of histamine (or other ionized substances) gaining access to the neurovascular region of the skin is linearly related to the stimulus charge does not imply that the current is carried exclusively by the respective ions. Depending on the acidity of the solution, proton transport may be partly responsible for current delivery. Electrolytes on the skin surface or within the penetration pathways can also contribute. It has been shown in the cornea, which is a comparable model, that empirical values of iontophoresis were about two thirds of the theoretically calculated amount [48].

In addition to those mentioned above, a variety of substances, including acetylcholine, metacholine, sodium nitrite, and sodium nitroprusside, were iontophoretically applied to test exclusively the responsiveness of the endothelium and/or smooth muscle [46]. The latter can be combined with the iontophoresis of substances, such as histamine, which trigger the axon reflex cascade via excitation of C-afferents. This approach could be a valuable means of assessing the relative importance of the neuron, endothelium, or smooth muscle component under various pathologic conditions, e.g., diabetic or alcoholic neuropathy, which can affect both the neuronal and vascular responsiveness.

We wish to thank Dr. Pamela Waddell for valuable suggestions, Mr. K. Burian for the preparation of the figures, and Ms. D. Bechtle for editing the manuscript.

REFERENCES

1. Lewis T: The blood vessels of the human skin and their responses. Shaw & Sons, London, 1927
2. Aaron TH, Abramsom HA: Inhibition of histamine whealing in human skin by pyribenzamine-hydrochloride using iontophoretic technique. Proc Soc Exp Biol Med 65:272–273, 1947
3. Perry DJ, Falk MS, Pillsbury DM: A comparison of the effect of antihistamine drugs in human subjects by means of histamine iontophoresis. J Invest Dermatol 2:461–468, 1948
4. Hearin DL, Mori PP: A study of some anti-histamine compounds by histamine iontophoresis. J Invest Dermatol 14:391–394, 1950
5. Shelley WB, Melton FM: Relative effect of local anesthetics on experimental histamine pruritus in man. J Invest Dermatol 15:299–300, 1950
6. Shelley WB, McConahy JC, Hesbacher EN: Effectiveness of antihistaminic compounds introduced into normal human skin by iontophoresis. J Invest Dermatol 15:343–344, 1950
7. Tyle P: Iontophoretic devices for drug delivery. Pharmaceut Res 3:318–326, 1986
8. Kari B: Control of blood glucose levels in alloxan-diabetic rabbits by iontophoresis of insulin. Diabetes 35:217–221, 1986
9. Stephen RL, Petelenz TJ, Jacobson SC: Potential novel methods for insulin administration. I. Iontophoresis. Biomed Biochim Acta 43:6890–6893, 1984
10. Westerman RA, Magerl W, Handwerker HO, Szolcsányi J: Itch sensation evoked by percutaneous microiontophoresis of histamine on human hairy skin. Proc Aust Physiol Pharmacol Soc 14:79P, 1985
11. Handwerker HO, Magerl W, Klemm F, Lang E, Westerman RA: Quantitative evaluation of itch sensation. In: Schmidt RF, Schaible HJ, Vahle-Hinz C (eds.). Fine afferent nerve fibers and pain. Weinheim, Verlag Chemie (VCH), 1987, pp 461–473
12. Magerl W, Handwerker HO: A reliable model of experimental itching by iontophoresis of histamine. In: Dubner R, Gebhart GF, Bond MR (eds.). Pain Research and Clinical Management, Vol.3. Proceedings of the Vth World Congress on Pain. Amsterdam, Elsevier, 1988, pp 536–540
13. Wolff BB: Behavioural measurement of human pain. In: Sternbach RA (ed.). The psychology of pain. New York, Raven Press, 1978, pp 129–168
14. Anton F, Handwerker HO, Kreh A, Reeh PW, Walter E, Weber E: Influence of acetylsalicylic and salicylic plasma levels on psychophysical measures of long-standing natural pain stimuli. In: Fields HL, Dubner R, Cervero F (eds.). Advances in Pain Research and Therapy, Vol.9. New York, Raven Press, 1985, pp 781–790
15. Forster C, Anton F, Reeh PW, Weber E, Handwerker HO: Measurement of the analgesic effects of aspirin with a new experimental algesimetric procedure. Pain 32:215–222, 1988
16. Shelley WB, Arthur RP: The neurohistology and neurophysiology of the itch sensation in man. Arch Dermatol 76:296–323, 1957
17. Fruhstorfer H, Hermanns M, Latzke L: The effects of thermal stimulation on clinical and experimental itch. Pain 24:259–269, 1986
18. Winquist RA, Stamm SJ: Arterialized capillary sampling using histamine iontophoresis. J Pediat 76:455–458, 1970
19. Hunt CE: Capillary blood sampling in infants. Pediatrics 51:501–506, 1973
20. Frosch PJ, Duncan S, Kligman AM: Cutaneous biometrics. I. The response of human skin to dimethyl sulphoxide. Br J Dermatol 102:263–274, 1980
21. Guy RH, Hadgraft J: Mathematical models in percutaneous absorption. In: Maibach HI, Lowe NJ (eds.). Models in dermatology, Vol.2. Basel, Karger, 1985, pp 170–177
22. Bronaugh RL, Maibach HI: In vitro models for human percutaneous absorption. In: Maibach HI, Lowe NJ (eds.). Models in dermatology, Vol.2. Basel, Karger, 1985, pp 178–188
23. Bronaugh RL, Stewart RF, Congdon ER: Differences in permeability of rat skin related to sex and body site. J Soc Cosmet Chem 34:127–135, 1983
24. Holbrook KA, Odland GF: Regional differences in the thickness (cell layers) of the human stratum corneum: an ultrastructural analysis. J Invest Dermatol 62:415–422, 1974
25. Plewig G, Marples BM: Regional differences of cell size in the human stratum corneum. Part I. J Invest Dermatol 54:13–18, 1970
26. Plewig G: Regional differences of cell size in the human stratum corneum. Part II: Effect of sex and age. J Invest Dermatol 54:19–23, 1970
27. Rougier A, Lotte C, Corcuff P, Maibach HI: Relationship between skin permeability and corneocyte size according to anatomic site, age and sex in man. J Soc Cosmet Chem 39:15–26, 1988
28. Elias PM, Cooper ER, Korc A, Brown BE: Percutaneous transport in relation to stratum corneum structure and lipid composition. J Invest Dermatol 76:297–301, 1981
29. Elias PM: Structure and function of the stratum corneum permeability barrier. Drug Develop Res 13:97–106, 1988
30. Landmann L: The epidermal permeability barrier. Anat Embryol 178:1–13, 1988
31. Magerl W, Szolcsányi J, Westerman RA, Handwerker HO: Laser Doppler measurements of skin vasodilatation elicited by percutaneous electrical stimulation of nociceptors in humans. Neurosci Lett 82:349–354, 1987
32. Buck SH, Burks TF: The neuropharmacology of capsaicin: review of some recent observations. Pharmacol Rev 38:180–226, 1986
33. Maggi CA, Borsini F, Santicioli P, Gepetti P, Abelli L, Evangelista S, Manzini S, Theodorsson-Norheim E, Somma S, Amenta F, Bacciarelli C, Meli A: Cutaneous lesions in capsaicin-pretreated rats. A trophic role of capsaicin-sensitive afferents? Naunyn Schmiedeberg Arch Pharmacol 336:538–545, 1987
34. Breslauer F: Die Pathogenese der trophischen Gewebsschäden nach der Nervenverletzung. Dtsch Z Chir 150:50–81, 1919
35. Lewis T, Marvin HM: Observations relating to vasodilatation arising from antidromic impulses to herpes zoster and throphic effects. Heart 14:27–47, 1927
36. Nilsson J, Euler AM, Dalsgaard CJ: Stimulation of connective tissue cell growth by substance P and substance K. Nature 315:61–63, 1985
37. Tanaka T, Danno K, Ikai K, Imamura S: Effects of substance P and substance K on the growth of cultured keratinocytes. J Invest Dermatol 90:399–401, 1988
38. Szolcsányi J: Selective responsiveness of polymodal nociceptors of the rabbit ear to capsaicin, bradykinin and ultraviolet irradiation. J Physiol 388:9–23, 1987
39. Lewis T: The nocifensor system of nerves and its reactions. Brit Med J 194:431–435, 491–494, 1937
40. Lembeck F: Sir Thomas Lewis's nocifensor system, histamine and substance P-containing primary afferent nerves. Trends Neurosci 6:106–108, 1983
41. Lembeck F: The 1988 Ulf von Euler lecture. Substance P: from extract to excitement. Acta Physiol Scand 133:435–454, 1988
42. Heyer G, Hornstein O, Handwerker HO: Skin reactions and itch sensation induced by epicutaneous histamine application in atopic dermatitis and controls. J Invest Dermatol 93:492–496, 1989
43. Rodbard D, Crambach A: Unified theory for gel electrophoresis and gel filtration. Proc Nat Acad Sci USA 65:970–977, 1970
44. Weber K, Osborn M: The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J Biol Chem 244:4406–4412, 1969
45. Bannon YB, Corish J, Corrigan OI: Iontophoretic transport of model compounds from a gel matrix across a cellophane membrane. Drug Develop Ind Pharm 13:2617–2630, 1987
46. Westerman RA, Widdop RE, Hannaford J, Low A, Roberts RGD, Kent P, Sideris K, Yip T, Hales JRS, Stephens FRN: Laser Doppler velocimetry in the measurement of neurovascular function. Aust Phys Eng Sci Med 11:53–66, 1988
47. Westerman RA, Sideris K, Widdop RE, Kent P, Roberts RGD, Yip T: Spread of iontophoretically injected ions in a tissue model. Aust Phys Eng Sci Med 12 (in press)
48. Hughes L, Martin DM: A fresh look at iontophoresis. Arch Ophthalmol 102:1825–1829, 1984

In: Clin. Exper. Neurol. 23:81-89
(1987) M. Eadie (ed) Adis Press,
Sydney.

Electrically Evoked Skin Vasodilatation: A Quantitative Test of Nociceptor Function in Man

R.A. Westerman, A. Low,* A. Pratt,* J.S. Hutchinson.* J. Szolcsanyi,† W. Magerl,‡ H.O. Handwerker‡ and W.M. Kozak***

Traditional tests of nociceptor function in humans, such as measurement of thresholds for pain sensation evoked by noxious heat,[1] electrical stimulation,[2] or mechanical pressure,[3] provide useful but limited information about the function of nociceptors in the areas of skin tested. However, a quantitative test is needed for the study of cutaneous nociceptor activity in such diseases as diabetic or other polyneuropathy both to detect abnormalities and to enable more accurate monitoring of the course of the disease.

This paper summarizes the technique we have developed for recording local cutaneous vasodilatation in response to noxious percutaneous electrical stimulation. Microvascular dilator responses to electrical stimulation of skin were described in Lewis' classic studies[4,5] but, to our knowledge, have not been applied in neurological disease or for the quantitative study of neurogenic vasodilatation.

Materials and Methods

The local nociceptor-induced vasodilatation (that is, the axon reflex part of Lewis' triple response) was recorded from the hairy skin of the anterior forearm or the dorsum of the foot in most subjects (consenting volunteers) but was also recorded from other hairy and glabrous skin sites on the upper and lower extremities. A Periflux Pf 1 laser doppler flowmeter (Perimed, Sweden) was used to

*Department of Physiology, Monash University, Clayton, Victoria, †Department of Pharmacology, Medical University, Pecs, Hungary, ‡II Physiological Institute, University of Heidelberg, Heidelberg, F.R.G. and **Biomedical Engineering Program, Carnegie-Mellon University, Pittsburgh, U.S.A.

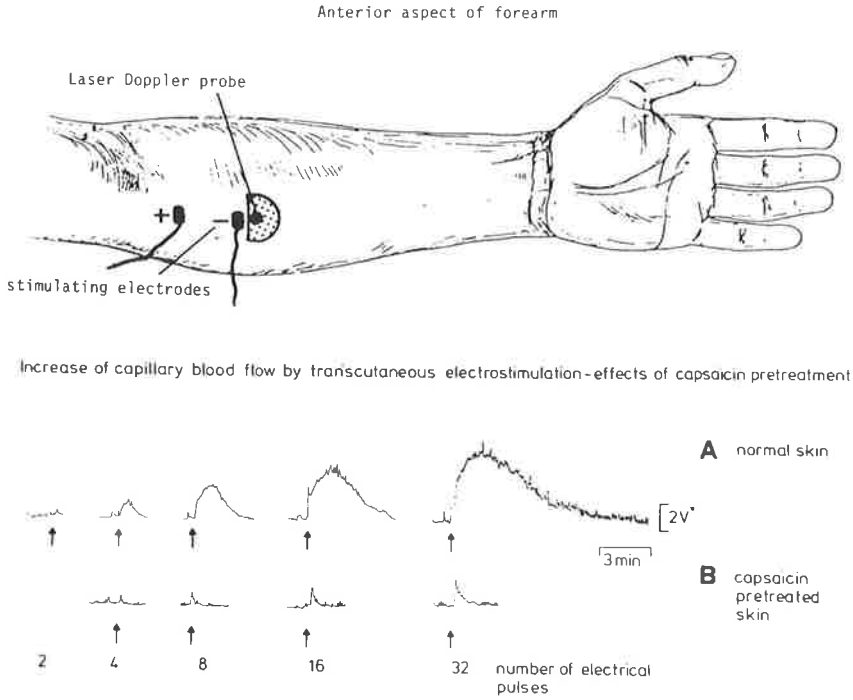


Figure 1. Upper diagram shows typical arrangement of stimulating electrodes and laser probe on forearm: panels A and B show increase of microvascular blood flux by transcutaneous electrical stimulation of normal skin and capsaicin-pretreated skin, respectively. The number of stimulus pulses required to evoke each dilator response are shown below. The time and laser signal voltage calibrations bars apply to both A and B.

record the changes in skin blood flux. The laser doppler probe holder was fixed to the skin by a double-sided adhesive disc. An indifferent anodal electrode was applied 5 cm proximal to the test site, where the cathode was a 30 mm^2 gold-plated disc electrode taped onto the skin 1 cm from the laser probe (Figure 1). Although a range of stimulus parameters were explored in the preliminary tests (Westerman RA, Szolcsanyi J, Magerl WM, Handwerker HO, unpublished observations) it was found that the minimum stimulation giving reproducible responses in normal skin was a series of pulses each of 150 volts, 0.5–1.0 ms in duration, at a frequency of 2 Hz. The total number of pulses delivered in each brief train ranged from 1 to 32. All healthy subjects reported that this stimulus produced pain at the cathode, and several did not consent to receive the train of 32 stimuli.

An area of forearm skin was then desensitized with capsaicin in 6 volunteers. A 1% ethanol solution of capsaicin[6] was applied 3 or 4 times daily for 3 or 4 days until its application no longer evoked redness and pain. The capsaicin-treated skin area was tested on the following day.

The area of each vasodilator response was measured as the voltage–time

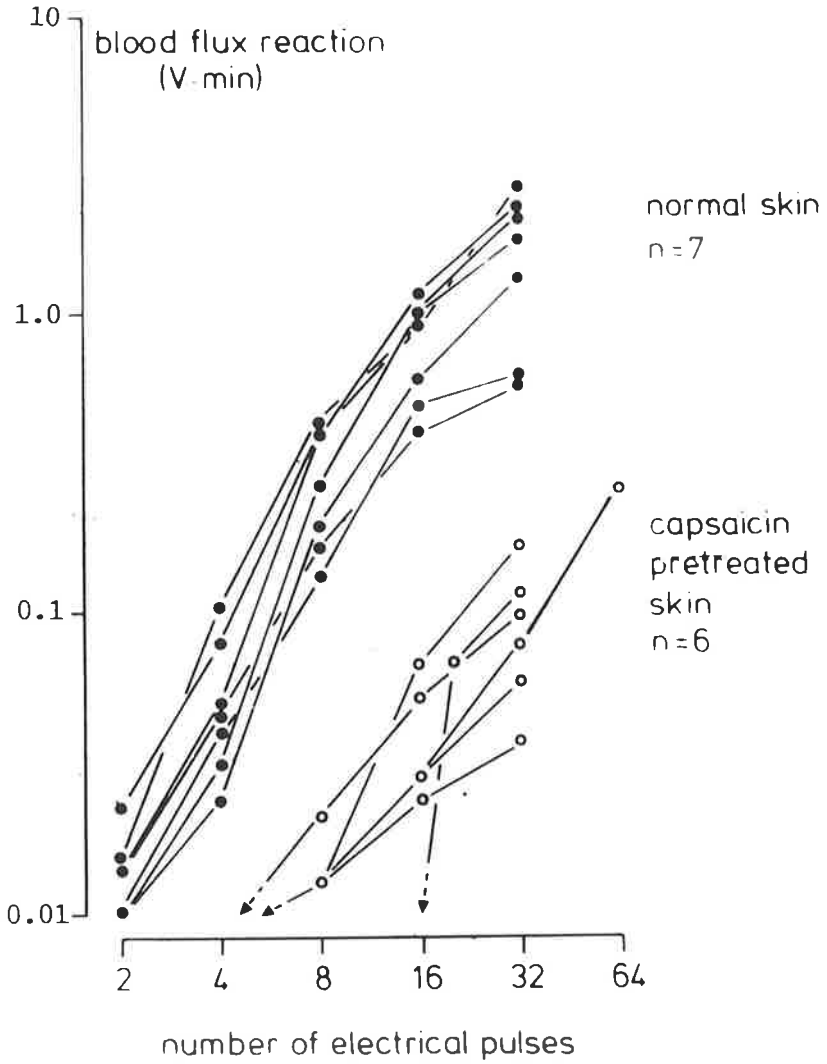


Figure 2. The increase of microvascular blood flow after transcutaneous electrical stimulation with 0.5 ms pulses at 2 Hz, and total numbers of stimuli 2, 4, 8, 16, 32, and 64 (in one case). The data shown are for normal and capsaicin-treated skin of the same subjects. The transient vasodilatations (like those shown in Figure 1) are plotted logarithmically as areas (volt min) on the ordinate and the number of pulses on the abscissa, to give a typical dose-response plot.

integral using a Zeiss MOP image analysis computer with a magnetic tablet and stylus attached. The logarithms of responses and stimuli were then plotted (Figures 2 and 4). The means, standard errors of the means, and 95% confidence limits were calculated for all normal subjects and are shown in Figure 4.

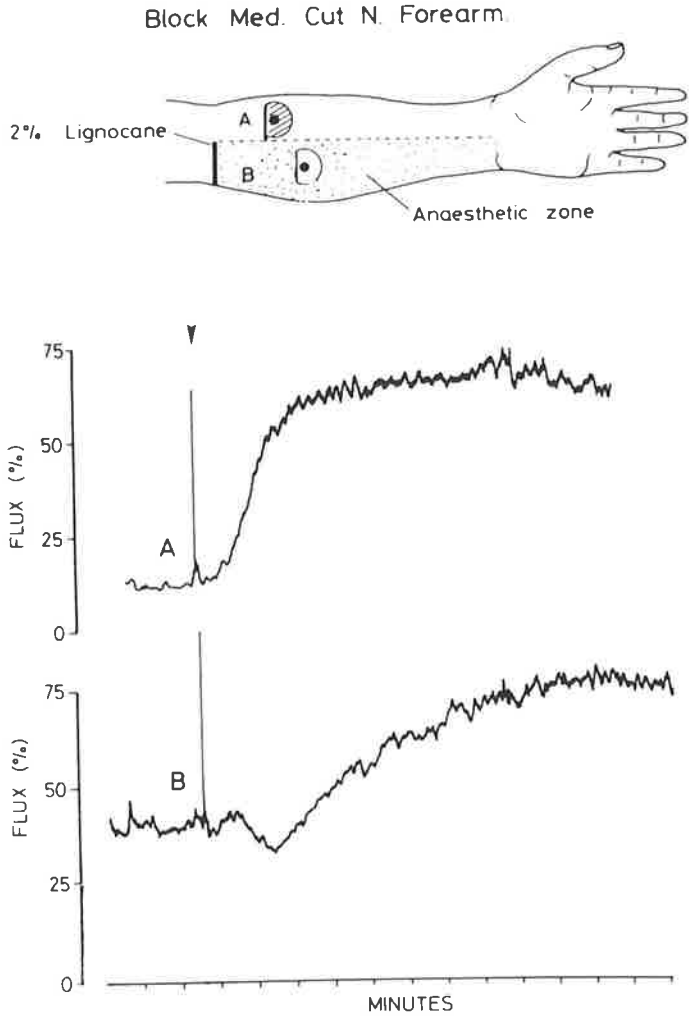


Figure 3. Flux changes are shown for normal and locally anaesthetized skin after increased stimulation compared to Figures 1 and 2 (total 64 pulses). The arrow indicates the stimulus artefact. The medial cutaneous nerve was blocked 6 minutes before record B. Note that the resting skin blood flux (37%) after local anaesthetic is elevated compared to that in unanaesthetized skin A (12%).

Results

In 27 experiments a reproducible increase of skin blood flux was recorded by the laser probe in response to transcutaneous electrical stimulation with the parameters described. These stimuli were perceived as painful and must be

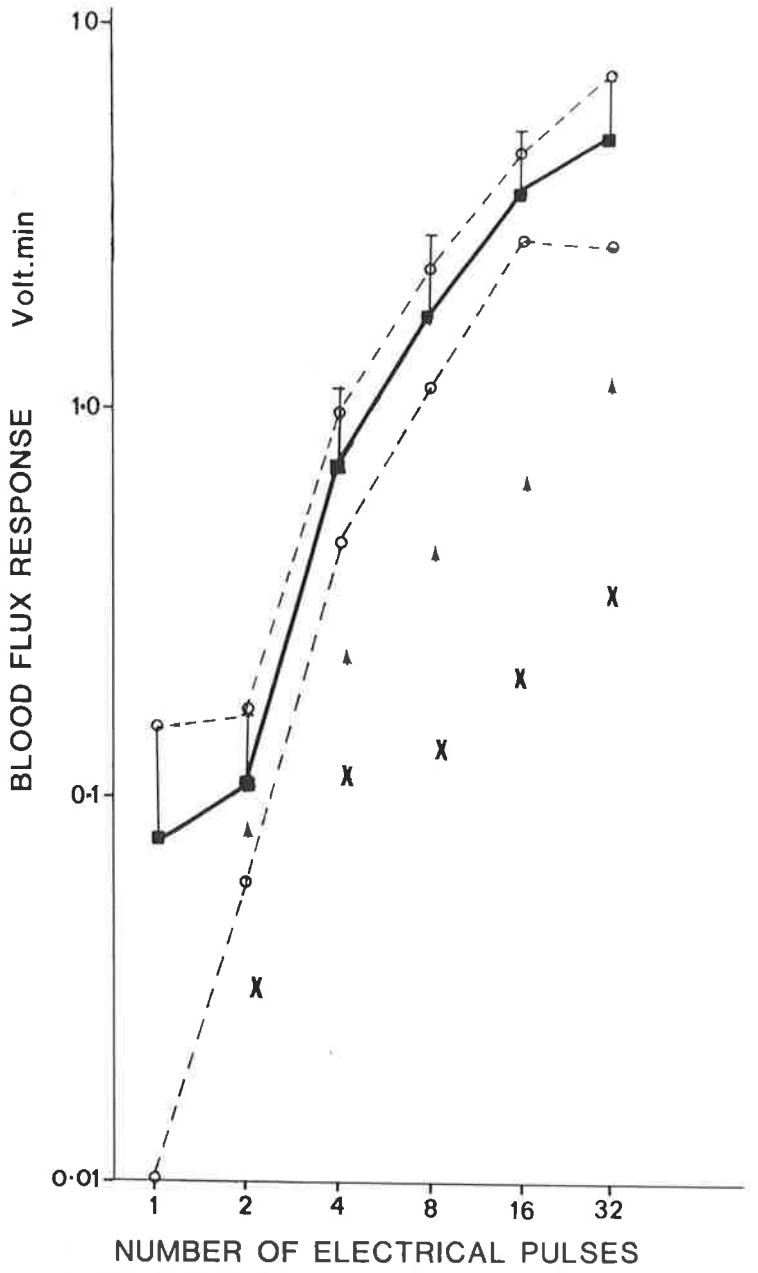


Figure 4. A logarithmic plot of responses to the electrical stimulus pulse series for 15 healthy volunteers similar to those shown in Figure 2. Means (squares) and standard errors (vertical bars) are shown, as well as the 95% confidence limits for the population data (broken line). Also depicted are results for electrical percutaneous stimulation of one subject with polyneuropathy (X) and one with sympathetic neuropathy (▲) associated with posterior root damage.

presumed to activate nociceptors and other C-fibres. The geometry of the recording probe and stimulating electrodes is shown in Figure 1 together with a series of vasodilator responses to the stimulus trains of 2, 4, 8, 16 and 32 pulses before and after capsaicin treatment. The amplitude of the microvascular blood flux increase and its duration are seen to depend on the 'dose' of electrical stimulation expressed as the total number of electrical pulses. In the normal skin at higher stimulus doses the vasodilatation was often preceded by a transient vasoconstriction, and this can just be seen in the response to 32 pulses at the arrow. The capsaicin-desensitized skin gave very markedly reduced responses to the same series of electrical stimulus pulses, applied in the same manner. The perceived pain from the electrical stimulation of capsaicin-treated skin was much reduced, and in two of the subjects the noxious heat threshold was found to be elevated by 2.3 °C and 3.1 °C, respectively. The other capsaicin-treated subjects were not tested in this manner, but elevated noxious heat thresholds have been reported in previous studies using capsaicin.[6]

The polygraph skin blood flux records were measured for the total area of each blood flux response, and the results are depicted graphically in Figure 2. The responses before and after capsaicin treatment indicate that, although vasodilator response and pain perception are both markedly reduced, some vasodilatation remains, evoked by larger total numbers of electrical stimulus pulses.

Figure 3 shows 2 responses to the same total stimulus (64 pulses) in the same subject, in whom approximately half of the anterior surface of the forearm was anaesthetized by 2% lignocaine block of the medial cutaneous nerve of the forearm. The large and prolonged vasodilator response seen in A was evoked by 64 pulses at 2 Hz applied near the laser probe at site A on the lateral (unanaesthetized) side of the forearm. In B, the resting skin blood flux is seen to be already elevated to 37% 6 minutes after the nerve block, compared with the resting flux of about 12% before the stimulus train was applied to the non-anaesthetized skin of the lateral side. Note that in B the stimuli did not evoke a perception of pain, but a brief vasoconstriction during the first 2 minutes was followed by considerable vasodilatation with increase in microvascular flux to reach an almost identical figure to that produced at the unanaesthetized site, A.

In Figure 4 the mean flare responses, standard errors of the means and the 95% confidence limits of the normal subjects' responses to the standard noxious electrical stimulation are shown. The reduced blood flux responses from a patient with dorsal root damage neuropathy (▲) and another with chronic polyneuropathy (×) show the degree to which this nociceptor-mediated response is reduced in both patients.

Discussion

The inflammatory response to necrosis or deep infection in the feet of patients with long standing diabetes mellitus is often accompanied by only slight superficial redness of the skin. This suggests a possible impairment of the neurogenic vasodilatation, that is, the axon reflex portion of the inflammatory triple response.[4,7]

This suggestion is supported by the findings of Hutchison *et al.*[8] that the histamine flare response, as measured by the rise in skin temperature in response to intradermally administered histamine, is reduced in diabetics.

Our technique was developed as a non-invasive quantitative test of how nociceptor function contributes to the axon reflex flare evoked by percutaneous electrical stimulation. The responses resemble closely in form and duration the 'local' vasodilatation obtained by Blumberg and Wallin[9] from intraneural microstimulation in microneurography. The responses are not reflexes requiring CNS participation since they persist after local anaesthetic block of the medial cutaneous nerve of the forearm (Figure 3). The same painful electrical stimuli applied at skin sites remote from the laser probe failed to evoke vasodilatation, as did non-painful levels of stimulation (less than 60–80 V) near the laser probe. The presence of an early vasoconstrictor response, seen as transient falls in skin blood flux with higher stimulation parameters, suggests that sympathetic vasoconstrictor fibres are excited by these levels of electrical stimulation which evoke an axon reflex flare (as in Lewis' triple response). Capsaicin pretreatment is known to reduce the substance P levels in epidermal primary afferents and their small cell bodies in the dorsal root ganglia.[10] Capsaicin treatment might therefore be predicted to shift the electrical stimulation dose-response plot in a similar manner to neuropathic states and result in impaired nociceptor function. A comparison of the data in Figures 2 and 4 supports this view.

After capsaicin pretreatment (Figure 2) the presence of some residual vasodilatation evoked by a larger total number of pulses suggests either that capsaicin desensitization of the nociceptor was incomplete or that other mechanisms may be involved in the vasodilation.

Other small nerve fibres in the skin which can be activated at the stimulus levels used include sudomotor (sympathetic postganglionic cholinergic fibres) and/or active sympathetic vasodilator fibres, whose transmitter is not known.[11] Of these, the cholinergic sudomotor fibres are not likely to participate in the response because atropine does not affect the local flare response[9] or our percutaneous vasodilatation (Westerman RA, Szolcsanyi J, Magerl WM, Handwerker HO, unpublished observations). We cannot exclude possible involvement of active vasodilator fibres.[11]

The sensitivity of the skin flare response, albeit transient, to very few stimulus pulses tempts us to consider a possible physiological role for this mechanism in local microvascular blood flow regulation. Further evidence of the sensitivity of the response of cutaneous nociceptors is found in the skin redness evoked by 'prickly' fabrics.[12] These sensations of 'prickle', and often small amplitude local vasodilatation, can be evoked by very small currents (10^{-7} A) applied so as to stimulate only the most superficial epidermal nerve fibres (Kenins P, Garnsworthy R, Gully R, Westerman R, Walker A, unpublished observations) which are shown by immunofluorescence to include substance P containing unmyelinated primary afferent fibres. The stimulus parameters and protocol shown in the present paper to be effective in evoking a neurogenic vasodilatation are proposed as a quantitative test of cutaneous nociceptor function contributing to axon reflex flare. This proposal is now being tested in diabetic patients with neuropathy.

Summary

Direct stimulation of intact forearm skin affects adjacent microvascular blood flux. Pulses of current, known to activate C-fibres effectively, were applied over a period of 1–16 seconds at 2 Hz using transcutaneous stimulation. An increase of up to 50% was observed in skin microvascular blood flux. Increased blood flux correlated well with increasingly painful sensations. Some subjects responded to one or two pulses at 2 Hz, 0.5 ms in duration and 150 volts. A response onset latency of 4–15 s, lasting up to 5 minutes, was recorded. At higher frequencies (4–8 Hz) and more pulses (16–32) vasoconstriction was frequently observed before the usual flux increase. After administration of local anaesthesia (2% ligocaine) the resting skin blood flux increased, but electrical stimulation still produced vasodilatation. The local cutaneous flare response to electrical stimulation was abolished or greatly reduced by capsaicin pretreatment. Excitation of small intracutanous forearm nerve C-fibres produces increased microvascular blood flux which is dependent on local release of vasodilator substances. Thus the neurogenic flare (axon reflex) may have a physiological role in regulating skin blood flow, and nociceptor function may be measured by applying the aforementioned transcutaneous electrical stimulation.

Note Added in Proof: A reduction in the neurogenic vasodilator response to electrical stimulation has now been observed in 25 diabetics with clinical symptoms of early sensory neuropathy.

Acknowledgements

We thank Mr C. Hogan for technical assistance, Mr V. Jones (Hadland Photonic, Mt Waverley) for lending us the Periflux laser doppler blood flux meter, and all the volunteer subjects who participated. The financial support of the Australian Research Grants Scheme and Monash Special Research Grants (to R.A.W.) and of the Lions International Diabetes Institute (to W.M.K.) is also gratefully acknowledged.

References

1. Beihl R, Treede RD, Bromm B. Pain ratings and short radiant heat pulses. In: Bromm B, ed. Pain measurement in man. Amsterdam: Elsevier, 1984; 397–408.
2. Notermans SLH. Measurement of the pain threshold determined by electrical stimulation and its clinical application I. Methods and factors possibly influencing the pain threshold. Neurology 1966; 16: 1071–1086.
3. Anton F, Kreh A, Reeh PW, Handwerker HO. Algesimetry using natural stimuli of long duration. In: Bromm B, ed. Pain measurement in man. Amsterdam: Elsevier, 1984; 455–462.
4. Lewis T. The blood vessels of the human skin and their responses. London: Shaw & Sons, 1927.
5. Lewis T. Pain. New York: MacMillan, 1942.
6. Jansco N, Jansco-Gabor A, Szolcsanyi J. Direct evidence for neurogenic inflammation and its prevention by denervation and by pretreatment with capsaicin. Br J Pharmacol Chemother 1967; 31: 138–151.

7. Lewis T, Grant RT. Vascular reactions of the skin to injury – II. The liberation of a histamine like substance in injured skin; the underlying cause of factitious urticaria and wheals produced by burning; and observations upon the nervous control of certain skin reactions. Heart 1924; 11: 209–265.
8. Hutchison KJ, Johnson BW, Williams HTG, Brown GD. The histamine flare response in diabetes mellitus. Surg Gynaecol Obstet 1974; 139 (4): 566–568.
9. Blumberg H, Wallin BG. Evidence of neurally mediated vasodilatation in the hairy skin of the human foot. J Physiol 1987; 382;105–121.
10. Gibbins IL, Furness JB, Costa M, MacIntyre I, Hillyard CJ, Girgis S. Co-localization of calcitonin gene-related peptide-like immunoreactivity with substance P in cutaneous, vascular and visceral sensory neurons of guinea pigs. Neurosci Lett 1985; 57: 125–130.
11. Westerman RA, Blumberg H, Hollerback M, Jäger M, Kiss J. Direct microneurographic and pharmacological evidence for the existence of sympathetic neurons mediating active vasodilatation in human skin. Proc Aust Physiol Pharmacol Soc 1986; 17(1): 23P.
12. Garnsworthy R, Mayfield R, Gully R, Westerman R, Kenins P. Mechanisms in cutaneous sensations of prickle and itch evoked by fabrics. In: Proceedings of the 7th International Wool Textile Research Conference, Tokyo 1985; III: 190–199.

Neuroscience Letters, 82 (1987) 349–354
Elsevier Scientific Publishers Ireland Ltd.

8

NSL 04957

Laser Doppler measurements of skin vasodilation elicited by percutaneous electrical stimulation of nociceptors in humans

Walter Magerl[1,*], Janos Szolcsányi[2], Rod A. Westerman[3] and Hermann O. Handwerker[1,*]

[1]II. Physiologisches Institut der Universität Heidelberg, Heidelberg (FRG), [2]University Medical School of Pécs, Department of Pharmacology, Pécs (Hungary), and [3]Department of Physiology, Monash University, Clayton, Vic. (Australia)

(Received 29 Juni 1987; Revised version received and accepted 30 July 1987)

Key words: Human skin; Cutaneous nociceptor; Electrical stimulation; Neurogenic inflammation; Laser Doppler flowmetry; Vasodilatation; Axon reflex

This study was designed to assess the quantitative features of the skin protecting mechanism of axon reflexes by monitoring stimulus related changes in skin blood flow using laser Doppler flowmetry. Skin vasodilatation induced by trains of brief transcutaneous electrical pulses (0.5 ms, 150 V) was strictly related in magnitude to the number of pulses (trains of 1–32). This vascular response was reduced by 95% when the skin had been pretreated by painting it for two days with a 1% alcoholic solution of capsaicin. The sensitivity of vascular responses to activation of afferent C-fibers indicates a role of this mechanism in the protection of the skin against noxious events.

It has been known since the last century that the stimulation of a certain class of nerve fibers with cell bodies in the dorsal root ganglia exerts an influence on blood flow in the skin [1, 10, 14]. This effect was termed 'antidromic vasodilatation' since it was experimentally demonstrated by antidromic excitation of afferent units [5, 8]. The same neurons were also found to be responsive to natural stimulation of their receptive endings in the skin and to give rise to characteristic patches of vasodilatation surrounding the stimulation sites. The effect was conjectured to work as an axon reflex [3, 11] spreading locally via the branches of the unmyelinated afferent neurons, This socalled 'nocifensor system' [12] was supposed to have a physiological role in protecting the vital functions of the skin against injury [2, 11]. The impairment or loss of this type of peripheral nerve function, e.g. in traumatic nerve injury or in neuropathy, is thus supposed to lead to trophic changes often observed in denervated skin.

Correspondence: H.O. Handwerker. *Present adress: Institut für Physiologie und Biokybernetik, Universität Erlangen, Universitätsstrasse 17, D-8520 Erlangen, F.R.G.

In spite of the long history of the research on the 'nocifensor system', precise clinical routines for testing the functional state of this system are not yet established. The present study presents a non-invasive method for testing the neurogenic vasodilatation in human subjects which may also be useful for clinical investigations. Part of the present data have been published previously in short form [19].

Electrical stimulation of the volar surface of the forearm was performed in 7 consenting male subjects (29–54 years old), including the authors, with a silver plate contact electrode (cathode) 5 mm in diameter moistened with tyrode, using series of brief electrical stimuli appropriate to excite the axons of cutaneous C-nociceptors. A larger (5 × 7 cm) silver plate near the wrist served as reference. Constant voltage stimuli were delivered capacity-coupled via an isolation unit from a Grass S 8 electrical sti-

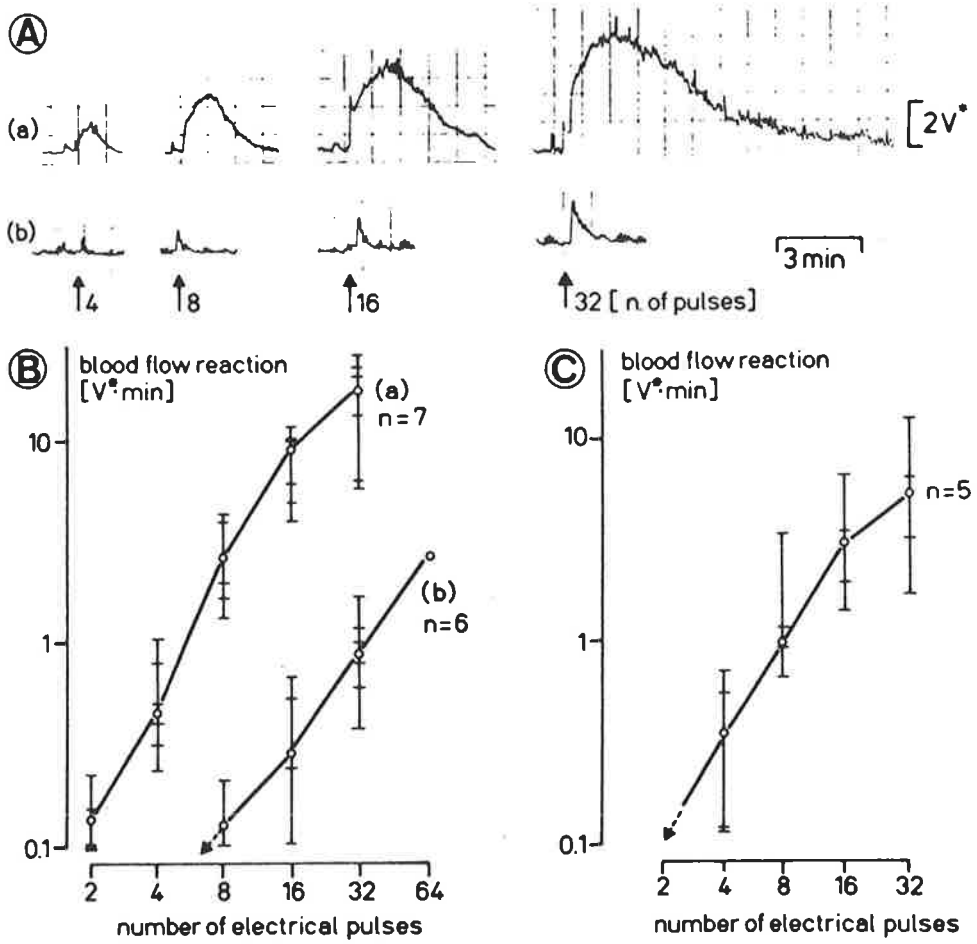


Fig. 1. Axon reflex vasodilatation in the vicinity of skin sites stimulated by trains of electrical pulses. A: dilatory responses of one subject to percutaneous electrical stimulation of the ventral forearm with an ascending series of trains consisting of respective numbers of pulses as indicated below (arrows) in normal skin (a) and capsaicin-pretreated skin (b). B: dose–response relationship of electrical stimulation and reflex vasodilatation in untreated skin (a) and capsaicin-pretreated skin (b). The 6 subjects participating in the capsaicin experiment served also as controls. C: dose–response relationship following intracutaneous bipolar stimulation (60 V) through intradermal needles.

mulator. Pulses of 0.5 ms duration and 60–150 V were delivered at frequencies of 1–8 Hz. Vasodilatation responses in the superficial skin layers were monitored 10 mm apart from the stimulated skin area by laser Doppler flowmetry (Periflux PF1, Perimed KB, Stockholm, Sweden). The light of a 2 mW He–Ne laser is frequency shifted in the skin by the motion of particles, mainly red blood cells. The degree of Doppler shift is differentially processed and leads to voltage readings (V*) which are linearly related to the number and velocity of erythrocytes in a given volume of tissue. Since absorption and reflection of the laser light is affected by differences in properties of the skin, laser Doppler flowmetry allows recording of relative but not absolute blood flow changes [17, 18]. Subjective ratings of pain were assessed by means of a visual analogue scale.

Stimuli of 60–90 V were strong enough to activate the muscles underlying the stimulated area. Tapping-like sensations were felt at the stimulation site, but using test stimuli of 8 pulses (2 Hz, 0.5 ms) neither pain was sensed, nor vasodilatation occurred. The threshold values for the occurrence of both pain and flare responses were at the same level of stimulation, usually at 110–130 V. Therefore 150 V pulses were used in the experimental sessions.

At that strength of stimulation trains of 2–4 pulses elicited vasodilatation responses clearly discernible from spontaneous fluctuations in blood flow as well as from artefacts due to muscle contractions. Longer trains of stimuli resulted in longer lasting responses of several minutes (up to 12 min) (see Fig. 1A).

Ascending stimulation series with trains of 1, 2, 4, 8, 16, 32 and sometimes 64 pulses (2 Hz, 0.5 ms) were employed to study the relationship of the number of pulses and the magnitude of the vasodilatation response. Subsequent stimulations were performed only when blood flow levels had returned to baseline for at least 2 min. Vasodilatation responses were quantified as area under the curve and refer to the integral of flowmeter output voltages over time [V*·min]. A strong correlation with the size of stimulus trains was found (see Fig. 1B).

Stimuli of the strength required for eliciting vasodilatation responses were always painful. Pain increased with longer stimulus trains. The stimuli presumably excited the terminal branches of C-nociceptors of the stimulated skin area, but not unmyelinated fibers of more deeply located nerve trunks since radiating pain sensations were never reported. Accordingly the vasodilatation was restricted to the area surrounding the stimulation site. Blood flow in skin areas 5 cm apart was not affected.

Interindividual variability of the dilatation responses was more pronounced (8.59 ± 7.04 V*·min, mean $\pm$ S.D., range: 2.64–21.41 V*·min, $n=6$) than intraindividual variability checked in one subject at different skin sites (4.22 ± 2.93, V*·min, mean $\pm$ S.D., range: 2.13–11.38 V*·min, $n=8$) under a standard condition (20 pulses, 2 Hz, 0.5 ms).

Variation of the frequency within a pulse train between 1 and 8 Hz seemed to have no effect on the response magnitude, when equal numbers of pulses per train were applied.

The latency of the vasodilatation was checked by means of a 1-s pulse train of 8 Hz in 3 subjects where no muscle artefacts were encountered. Latencies of 4.9 ± 0.6

s (mean ± S.E.M.), $n = 11$) were found. This is in good agreement with latencies reported in vintage studies on antidromic vasodilatation using different methods [1]. The shapes of the responses resembled those registered in antidromic vasodilatation in dog [1] and in cat [10].

Comparable results were obtained with bipolar intracutaneous stimulation ($n = 45$) using 30-gauge intradermal needle electrodes inserted in parallel at a distance of 15 mm in 5 subjects. In these experiments the skin blood flow in the region between the needles was measured (Fig. 1C). Forty to 60 V proved to be sufficient to elicit an increase in blood flow with intradermal stimulation which was preceded by sensations of a sharply stinging pain at the needle sites.

Injecting the stimulated site with 0.5 ml of a 1% lidocain solution resulted in a complete abolition of the response to a standard stimulus (20 pulses, 2 Hz, 0.5 ms), while responses at sites injected with saline (5.92 ± 1.56 V*·min, mean ± S.E.M., $n = 5$) were not significantly different from a control stimulus (5.99 ± 1.68 V*·min, mean ± S.E.M., $n = 5$) applied 20–40 min prior to injection. This test conclusively demonstrated the neurogenic origin of the vasodilatation.

Capsaicin pretreatment was used to test the involvement of peptidergic afferent C-fibers in the vasodilator response. Six skin patches (2 × 3 cm) at the volar side of the forearms of 3 subjects were painted with a solution of 1% capsaicin in 80% ethanol up to 10 times in 2–3 h intervals. The painted areas had been pretreated with dimethylsulfoxide (DMSO) for 1 min. Capsaicin paintings caused marked erythemas

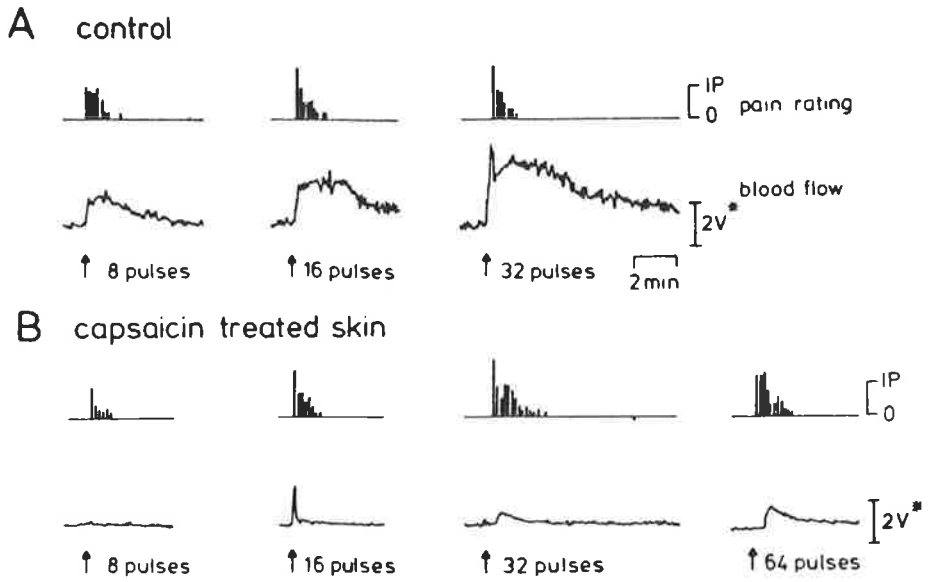


Fig. 2. Pain and blood flow responses to percutaneous electrical stimulation (150 V, 2 Hz, 0.5 ms) in normal and capsaicin-pretreated skin (original recordings). Blood flow recordings are distorted by artefacts due to muscle twitching during stimulation in several instances. IP, intervention point at one third of the visual analogue scale defined as subject's withdrawal from the stimulus under non-experimental circumstances.

and burning sensations that became weaker with repetitive paintings. Testing was not started prior than 2 days after completion of the capsaicin paintings to allow the local inflammation and hyperalgesia to subside. Capsaicin treatment resulted in an increase in pain threshold to radiant heat by approximately 2°C and in a complete abolition of itching to iontophoretic application of histamine [7].

Flare reactions to the electrical stimuli used in this study were no longer observed. More sensitive monitoring by laser Doppler flowmetry indicated that the vascular reaction was reduced to 5% of control values. This resulted in a parallel shift of the dose–response curves to the right. Threshold responses required at least trains of 8, often 16 or more pulses (Fig. 1B) 3–6 days after capsaicin desensitization. It has been described previously that flare responses following other types of stimuli are also suppressed after capsaicin [4, 6, 7, 9, 18].

While vasodilatation was markedly reduced in capsaicin-pretreated skin sites, this did not apply to the pain induced by the stimulation (Fig. 2). This may be due to the fact that electrical stimulation unlike natural stimulation does not involve transduction via receptive membranes, but has direct access to the conductile part of the axon [13] which most likely is not impaired by topical treatment of the skin with capsaicin. The secreto-sensory endings of polymodal C-nociceptors are thus the main targets of this procedure [15]. In addition the pain induced by transcutaneous electrical stimulation may be partly mediated by nociceptive nerve elements insensitive to capsaicin, e.g. by mechanoceptive A-δ-units [16]. The latter most probably do not contribute to the antidromic vasodilatation.

The technique described may be useful as an objective method to monitor altered properties of afferent cutaneous C-fibers and to detect changes in the nocifensive system of the skin under various conditions of neuropathic lesions characterized by a diminished number of fibers or in an altered neuropeptide content of unmyelinated afferents. The capabilities of the method are illustrated by the model of topical capsaicin administration which presents an experimentally induced deterioration of the nociceptor functions. The differences shown in the magnitude of axon reflexes between normal and capsaicin-pretreated skin are drastic in such a manner that changes by far minor than those demonstrated will surely be detected by the method of laser Doppler flowmetry.

This work was supported by the DFG, Grant Ha 831/8. R.A.W. held a v. Humboldt fellowship. J.S. was a guest professor at the University of Heidelberg while performing this study. We wish to thank these institutions.

1 Bayliss, W.M., On the origin from the spinal cord of the vasodilator fibres of the hind-limb and on the nature of these fibres, J. Physiol. London, 26 (1901) 173–209.
2 Breslauer, F., Die Pathogenese der trophischen Gewebsschäden nach der Nervenverletzung, Deut. Z. Chir., 150 (1919) 50–81.
3 Bruce, A.N., Vasodilator axon reflexes, Q. J. Exp. Physiol., 6 (1913) 339–354.
4 Carpenter, S.E. and Lynn, B., Vascular and sensory responses of human skin to mild injury after topical treatment with capsaicin, Br. J. Pharmacol., 73 (1981) 755–758.
5 Celander, O. and Folkow, B., The nature and the distribution of afferent fibres provided with the axon reflex arrangement, Acta Physiol. Scand., 29 (1953) 359–370.

6 Foreman, J.C., Jordan, C.C., Oehme, P. and Renner, H., Structure-activity relationships for substance P-related peptides that cause wheal and flare reactions in human skin, J. Physiol. (London), 335 (1983) 449–465.
7 Handwerker, H.O., Magerl, W., Klemm, F., Lang, E. and Westerman, R.A., Quantitative evaluation of itch sensation. In R.F. Schmidt, H.J. Schaible and C. Vahle-Hinz (Eds.), Fine Afferent Nerve Fibres and Pain, VCH, Weinheim, 1987, pp. 461–473.
8 Hinsey, J.C. and Gasser, H.S., The component of the dorsal root mediating vasodilatation and the Sherrington contracture, Am. J. Physiol., 92 (1930) 679–689.
9 Jancsó, N., The role of nerve terminals in the mechanisms of inflammatory reactions, Bull. Millard Fillmore Hosp. Buffalo N.Y., 53 (1960) 54–77.
10 Langley, J.N., Antidromic action. Part I, J. Physiol. (London), 57 (1923) 428–446.
11 Lewis, T., The blood vessels of the human skin and their responses, Shaw & Sons, London, 1927.
12 Lewis, T., The nocifensor system of nerves and its reactions, Br. Med. J., 194 (1937) 431–435, 491–494.
13 Rollman, G.B., Behavioural assessment of peripheral nerve function, Neurology 25 (1975) 339–342.
14 Stricker, S., Untersuchungen über die Gefässwurzeln des Ischiadicus, Sitzungsber. Kaiserl. Akad. Wiss. Wien, 3 (1876) 173–185.
15 Szolcsányi, J., Capsaicin-sensitive chemoceptive neural system with dual sensory efferent function. In L.A. Chahl, J. Szolcsányi and F. Lembeck (Eds.), Antidromic Vasodilatation and Neurogenic Inflammation, Akadémiai Kiadó, Budapest, 1984, pp. 27–55.
16 Szolcsányi, J., Anton, F., Reeh, P.W. and Handwerker, H.O., Selective excitation by capsaicin of mechano-heat sensitive nociceptors in rat skin, Brain Res., submitted.
17 Tenland, T., On laser Doppler flowmetry, Linköping Studies in Science and Technology Dissertations No. 83, Linköping University Medical Dessertations No. 136, Linköping, 1982.
18 Toth-Kasa, I., Jancsó, G., Bognar, A., Husz, S. and Obal, F., Capsaicin prevents histamine-induced itching, Int. J. Clin. Pharmacol. Res., 6 (1986) 163–169.
19 Westerman, R.A., Magerl, W., Szolcsányi, J., Handwerker, H.O., Electrically evoked cutaneous axon reflex flare in man, Proc. of the IUPS, Vol. 16, 1986, P 318.01.

Neuroscience Letters, 81 (1987) 177–182
Elsevier Scientific Publishers Ireland Ltd.

NSL 04862

Non-invasive tests of neurovascular function: reduced responses in diabetes mellitus

Roderick A. Westerman[1], Robert E. Widdop[2], Craig Hogan[1] and Paul Zimmet[3]

Departments of [1]Physiology and [2]Pharmacology, Monash University, Clayton, Vic. (Australia) and [3]The Lions International Diabetic Institute, Royal Southern Memorial Hospital, Caulfield, Vic. (Australia)

(Received 4 May 1987; Revised version received 15 June 1987; Accepted 18 June 1987)

Key words: Skin vasodilatation; Axon reflex; Laser Doppler flowmetry; Iontophoresis; Diabetes mellitus; Neuropathy; Acetylcholine; Sodium nitrite

Painful transcutaneous electrical nerve stimulation (TENS) of the foot dorsum evoked axon reflex vasodilatation, measured by laser Doppler flowmetry. In addition, acetylcholine (ACh) and sodium nitrite ($NaNO_2$) were iontophoresed to cause vasodilatation by endothelium dependent and independent mechanisms, respectively. Compared with healthy volunteers, diabetic patients with clinically diagnosed neuropathy showed reduced electrical axon reflex flare and ACh responses, but not $NaNO_2$ responses. Such reduced cutaneous nocifensor functions may contribute to some symptoms and complications of diabetes mellitus.

Diabetes mellitus often leads to disturbances of small nerve fibres and blood vessels in the skin. As a consequence, pain, sweating disorders, frequent skin injury and infections are common in diabetics. Sensory nerve damage and reduced skin blood flow can even lead to ulceration and loss of digits [1]. Current methods of nerve testing only assess damage to large nerves (myelinated A-fibres) [3]. However, smaller nerves (C-fibres) may also be affected early in the disease [1, 4] and a more sensitive, specific and portable method of assessing their function would be a major development for evaluating diabetic neuropathy in both clinical and research settings [4]. To this end, we have developed a non-invasive technique for measuring small nerve fibre function by monitoring skin blood flow during noxious transcutaneous electrical nerve stimulation (TENS) [16, 18].

This study was conducted on 19 healthy subjects (age range: 20–63 years) and 30 diabetic patients, who were all currently receiving insulin therapy (age range: 21–88 years). According to previous clinical histories, patients were catagorized as those with or without previous large fibre neuropathy. All subjects gave their informed con-

Correspondence: R.A. Westerman, Department of Physiology, Monash University, Clayton, Vic. 3168, Australia.

sent to the studies which were approved by the Monash University and Royal Southern Memorial Hospital Ethics Committees. All subjects were seated in a room with an ambient temperature of 21–24°C, with the leg slightly extended so that the foot could be supported comfortably approximately 20 cm above the ground. Skin blood flow was measured with a helium–neon laser Doppler flowmeter (Periflux Model Model PF1d, Perimed, Sweden) [11]. Vasodilatation was evoked by either transcutaneous electrical nerve stimulation (TENS) or the iontophoretic application of the endothelium-dependent vasodilator, acetylcholine (ACh), and the endothelium-independent vasodilator, sodium nitrite ($NaNO_2$) [5]. TENS was applied immediately adjacent to the laser probe, as has been described for the stimulation of the forearm [16, 18] (see Fig. 1A for stimulation parameters). A battery-powered constant current stimulator (WPI 502R) was used to provide a direct (galvanic) current for drug iontophoresis [17]. ACh and $NaNO_2$ were dissolved in an inert aqueous gel (2% methyl cellulose) and applied to an Ag–AgCl plug in a hemispherical 50 μl chamber. This was part of a cylindrical perspex applicator which served as the active electrode and fitted into the laser probe holder. Therefore, by using small currents of brief duration, it is possible to provide a discrete transfer of a polar vasoactive drug into the epidermis in the immediate skin area under the probe (dose is expressed as the total charge in milliCoulombs). The resulting change in blood flow is measured by the laser Doppler probe after its re-insertion into the holder. Since this study was chiefly designed to examine the C-nociceptor nerve function clinically, time constraints allowed testing of only one concentration of each test drug in each patient. From preliminary experiments and dose–response curves, the following stimuli were chosen, using 1% solutions of ACh chloride and $NaNO_2$: anodal current of 0.2 mA for 10 s (ACh) and cathodal current of 0.2 mA for 20 s ($NaNO_2$). ACh was normally applied to the laser site previously tested by adjacent TENS, while a new site on the foot dorsum was used to apply $NaNO_2$. At these low current levels, the inert gel used to dissolve the drugs caused negligible effect on skin blood flow. Vasodilator responses were quantified by measuring the area of each response, calculated as the voltage·time integral and plotting these as described previously [16]. However, if the vasodilator responses to ACh and $NaNO_2$ were still elevated at 4 min, this time was chosen as the period for calculation of the V·time integral.

The results of TENS performed in both control and diabetic patients are shown in Fig. 1A. TENS was perceived as painful in control subjects, while diabetic patients with neuropathy barely perceived the stimuli and could not always confirm the number of pulses received. In such patients the stimuli rarely evoked any discomfort. In all control subjects, TENS produced vasodilatation, the size of which was dependent on the number of pulses applied. This can be seen from the linear stimulus–response curve, obtained with 1–16 noxious electrical pulses (Fig. 1A). All vasodilator responses returned to baseline levels within 2–4 min. By contrast, there was a progressive rightward shift of the pulse-related axon reflex vasodilator response curve in diabetic subjects without and with clinical neuropathy, respectively (Fig. 1A). For the diabetic patients with clinical neuropathy this corresponds to approximately a one log unit reduction in responses, and is statistically significant for each stimulus

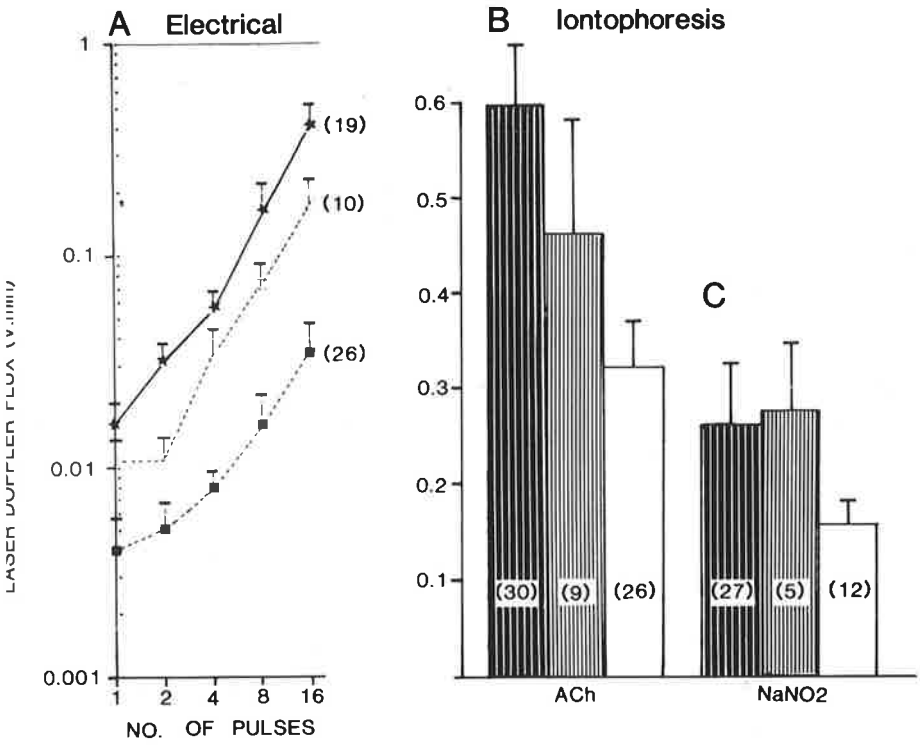


Fig. 1. Increases in microvascular blood flow (defined as laser Doppler flux) on the foot dorsum of control subjects and diabetic patients in response to TENS, and iontophoretic application of ACh and $NaNO_2$ (see text for abbreviations). A: effect of TENS (150 V, 0.75 ms, at 2 Hz). Data plotted on double log axes with the number of electrical pulses delivered to foot dorsum on the abscissa, and the corresponding changes in blood flow, calculated as a V·min integral from the laser Doppler analog record, plotted on the ordinate. Each point on the graph is the mean, and vertical bar the S.E.M., of responses from control subjects (stars, $n=19$), diabetic patients without neuropathy (points, $n=10$) and with neuropathy (closed squares, $n=26$). Each point in the latter group was significantly different from the corresponding control response ($P<0.05$, t-test). B: effect of ACh (1%, 0.2 mA, 10 s). C: effect of $NaNO_2$ (1% 0.2 mA, 20s). Each column is the mean, and the vertical bar the S.E.M., of responses for control subjects (dark hatch), diabetic patients without neuropathy (light hatch) and with neuropathy (open column); n for each group is given inside each column. Only the reduced ACh response obtained from diabetic patients with neuropathy was significantly different from the control response ($P<0.05$, t-test).

point ($P<0.05$, t-test). The results from the iontophoresis of ACh and $NaNO_2$ are summarized in Fig. 1B, C. ACh (2 mC) caused immediate vasodilatation in all control subjects, although only 8 out of 30 responses were complete within the 4-min calculation period. The effect of ACh in the diabetic patients paralleled the effects of TENS, in that ACh caused less vasodilatation in both diabetic groups (Fig. 1B). However, this was only significant in patients with diabetic neuropathy ($P<0.05$, t-test). These differences are probably underestimated somewhat because a greater percentage of responses in both diabetic groups had returned to resting levels within 4 min (4 out of 9 responses from diabetics without, and 8 out of 26 from diabetics with

neuropathy, respectively). The iontophoresis of $NaNO_2$ also caused vasodilatation in control subjects, although compared with ACh, these responses were smaller and more variable in that there was often a latency of approximately 1 min before vasodilatation commenced. Thereafter this usually remained elevated in both control and diabetic patients during the observation period (4 min). In only the diabetic patients with neuropathy, there was a trend towards reduced $NaNO_2$-induced vasodilatation (Fig. 1C), however, this was not significantly different from the control group ($P > 0.05$, t-test).

The present study has shown that diabetic patients with diagnosed large fibre neuropathy also exhibit other neurovascular changes that can be readily detected with the aforementioned techniques. Axon reflex vasodilatation evoked by TENS persists after local proximal anaesthesia but is markedly attenuated by pretreatment of the skin with capsaicin [16], a neurotoxin known to deplete primary afferent fibres of substance P [7]. The axon reflex vasodilatation is totally absent in skin 15 days after total denervation [19]. Thus, this neurogenic flare is thought to involve the local antidromic stimulation of C-fibres which causes the release of substance P (and possibly other mediators) [9, 18]. Similar results in man have also recently been obtained using painful intraneural stimulation of the superficial peroneal nerve at the ankle [2]. As in the forearm [16], TENS evoked a transient pulse-related flare in the foot dorsum, which occurred only if the stimulus was painful and applied in the immediately adjacent area to the laser probe. However, in both groups of diabetics, there were shifts to the right in the stimulus–response curve. This was more marked in the diabetic patients with clinical neuropathy and therefore suggests an additional dysfunction of primary afferent fibres. This is consistent with an earlier report of reduced histamine-induced axon reflex flare in diabetic patients, measured indirectly by the rise in skin temperature following intradermally injected histamine [6]. In a recent preliminary communication [12], axon reflex vasodilatation was also evoked by the electrophoresis of ACh. This was achieved by applying 10% ACh with a current of 1 mA for 5 min (i.e. 300 mC) to an outer ring, and then measuring the resultant spread of the flare to the centre of the ring, using a laser Doppler flowmeter. Moreover, in this abstract it was claimed that these axon reflex responses were reduced in patients with diabetic neuropathy (although no data were presented) [12].

In this and other [6, 12] studies, the reduced axon reflex vasodilatation in patients with diabetic neuropathy suggests loss of afferent C-fibre function. However, since this neurogenic flare depends ultimately on the functional integrity of the microvasculature in series with the primary afferent nerve fibres, it is possible that damage to either the vascular endothelium or the vascular smooth muscle itself could contribute to, or even be largely responsible for, the observed effects. Therefore, ACh or $NaNO_2$ were iontophoresed directly across the skin barrier into the area measured by the laser probe in order to examine vascular endothelial and smooth muscle function, respectively. This technique differs to that previously described (see ref. 12), in that lower concentrations of vasodilator drugs were iontophoresed using smaller currents of much briefer duration and the skin immediately iontophoresed with ACh was examined directly, not a distant site. These parameters and geometry were insuf-

icient to evoke axon reflex vasodilatation, i.e. spreading flare, which is consistent with the relative insensitivity of normal isolated polymodal nociceptors to applied ACh [8]. Moreover, the local vasodilator responses to ACh and $NaNO_2$ were also largely unaffected by pretreatment of the skin with capsaicin [13] and even persisted in skin denervated 15 days previously [19]. ACh is thought to cause vasodilatation mainly via the release of endothelium-derived relaxing factor (EDRF) from the vascular endothelium (with a lesser contribution from axon reflex mechanisms), while nitrovasodilators act directly on the blood vessels [5]. Thus, the reduced iontophoretic vasodilator responses to ACh, but not $NaNO_2$, in patients with diabetic neuropathy suggest that endothelial function is impaired although the microvascular smooth muscle appears to be responding normally.

Another possible contribution to the reduced TENS- and ACh-induced vasodilator responses exhibited by diabetic patients with neuropathy may occur because of an increased skin electrical impedance associated with reduced sudomotor activity [10]. However, in the present study, this is unlikely to be a major factor because the TENS used markedly suprathreshold stimulation for C-fibre activation, while a constant current stimulator was used for drug iontophoresis. Moreover, the fact that both ACh and $NaNO_2$ were not altered uniformly would argue against an increased skin electrical impedance as the only factor involved to limit drug penetration (although transport numbers are not identical).

Substance P binding sites have recently been demonstrated on endothelial cells [14], and this would explain the strictly endothelium-dependent vasodilator nature of substance P [5]. Therefore, it is possible that impaired endothelial function, demonstrated using iontophoretic ACh, may have contributed to the reduced electrically evoked axon reflex in patients with and without diabetic neuropathy. This could occur if part of the vasodilatation evoked by TENS is due to neurogenically released substance P causing the release of EDRF. However, the ability of substance P to cause histamine release from mast cells [9] would be unaffected, and so this component of the axon reflex would presumably be intact. Thus, impaired endothelial function (either EDRF synthesis or release) could exacerbate any primary afferent dysfunction. In this context, recent evidence suggests that vascular changes may be causally important in the development of diabetic neuropathy [15]. Therefore these present results would implicate the vascular endothelium as a primary target of this metabolic disorder and suggest that reduced skin nocifensor functions may contribute to some of the symptoms and complications of diabetes mellitus. Further studies are in progress using other cholinergic and nitrovasodilators in order to verify and extend these findings.

Supported by the Apex-Australian Diabetes Foundation.

1 Asbury, A.K. and Brown, M.J., Clinical and pathological studies of diabetic neuropathies. In Y. Goto, A. Horiuchi and K. Kogure (Eds.), Diabetic Neuropathy, Excerpta Medica, Elsevier, Amsterdam, 1982, pp. 50–57.

2 Blumberg, H. and Wallin, B.G., Direct evidence of neurally mediated vasodilatation in hairy skin of human foot, J. Physiol. (London), 382 (1987) 105–121.

3 Dorfman, L.J., The distribution of conduction velocities (DVC) in peripheral nerves: a review, Muscle Nerve, 7 (1984) 2–11.
4 Dyck, P.J., Karnes, J.L., Daube, J., O'Brien, P. and Service, F.J., Clinical and neuropathological criteria for the diagnosis and staging of diabetic polyneuropathy, Brain, 108 (1985) 861–880.
5 Furchgott, R.F., The role of endothelium in the responses of vascular smooth muscle to drugs, Annu. Rev. Pharmacol. Toxicol., 24 (1984) 175–197.
6 Hutchison, K.J., Johnson, B.W., Williams, H.T. and Brown, G.D., The histamine flare response in diabetes mellitus, Surg. Gynecol. Obstet., 139 (1974) 566–568.
7 Jansco, N., Jansco-Gabor, A. and Szolcsanyi, J., Direct evidence for neurogenic inflammation and its prevention by denervation and by pretreatment with capsaicin, Br. J. Pharmacol., 31 (1967) 138–151.
8 Koschorke, G.M., Westerman, R.A. and Zimmermann, M., Histamine excites non-myelinated fibres in experimental neuroma of sural nerve in cat, Neurosci. Lett., Suppl. 22 (1985) S32.
9 Lembeck, F., Sir Thomas Lewis's nocifensor system, histamine and Substance P-containing primary afferent nerves, Trends Neurosci., 6 (1983) 106–108.
10 Low, P.A., Zimmerman, B.R. and Dyck, P.J., Comparison of distal sympathetic with vagal function in diabetic neuropathy, Muscle Nerve, 9 (1986) 592–596.
11 Nilsson, G.E., Tenland, T. and Oberg, P.A., Evaluation of a laser Doppler flowmeter for measurement of tissue blood flow, IEEE Trans. Biomed. Eng., 27 (1980) 597–604.
12 Parkhouse, N. and Le Quesne, P.M., Laser Doppler measurement of acetylcholine-induced axon reflex flare to assess nociceptive C-fibre function, J. Physiol. (London), 381 (1986) 3P.
13 Roberts, R.G.D., Westerman, R.A., Widdop, R.E., Kotzmann, R.R. and Zimmet, P., Effects of capsaicin on cutaneous axon reflex vasodilatation in man, Proc. Aust. Physiol. Pharmacol. Soc., 18 (1987) 95P.
14 Stephenson, J.A., Burcher, E. and Summers, R.J., Autoradiographic demonstration of endothelium-dependent 125I-Bolton-Hunter Substance P binding to dog carotid artery, Eur. J. Pharmacol., 124 (1986) 377–378.
15 Thomas, P.K., Vascular factors in the causation of diabetic neuropathy, Trends Neurosci. 10 (1987) 6–8.
16 Westerman, R.A., Low, A., Pratt, A., Hutchinson, J.S., Szolcsanyi, J., Magerl, F.W., Handwerker, H.O. and Kozak, W.M., Electrically evoked skin vasodilatation: A quantitative test of nociceptor function in man, Clin. Exp. Neurol., 23 (1987) 81–89.
17 Westerman, R.A., Magerl, F.W., Handwerker, H.O. and Szolcsanyi, J., Itch sensation evoked by percutaneous microiontophoresis of histamine on human hairy skin, Proc. Aust. Physiol. Pharmacol. Soc., 16 (1985) 79P.
18 Westerman, R.A., Magerl, F.W., Szolcsanyi, J., Handwerker, H.O., Low, A., Pratt, A., Widdop, R.E. and Kozak, W.M., Vasodilator axon reflexes. In P.M. Vanhoutte (Ed.), Mechanisms of vasodilatation IV, Raven New York, in press.
19 Westerman, R.A., Widdop, R.E., Hogan, C., Hannaford, J., and Roberts, R., unpublished observations.

Australasian Physical & Engineering
Sciences in Medicine (1988) Vol. 11 No 2

LASER DOPPLER VELOCIMETRY IN THE MEASUREMENT OF NEUROVASCULAR FUNCTION*

R.A. Westerman[1], R.E. Widdop[2], J. Hannaford[2], A. Low[1], R.G.D. Roberts[1], P. Kent[1], K. Sideris[1], T. Yip[1], J.R.S. Hales[3] and F.R.N. Stephens[3].

Departments of [1]Physiology and [2]Pharmacology, Monash University, Clayton. 3168. Victoria.
[3]C.S.I.R.O. Division of Animal Production, Blacktown. N.S.W.

Summary

Since the first use of laser Doppler velocimetry (LDV) 16 years ago to measure tissue blood flow there has been no general acceptance of its clinical usefulness. The previous difficulty of measuring cutaneous blood flow made the introduction of the laser Doppler flowmeter (LDF) 8 years ago seem ideal because of its simplicity to use, and its provision of a continuous non-invasive quantitative measurement of changes in local microcirculatory activity. However, the present paper discusses some limitations of the technique and caveats about its use before emphasizing its greatest area of usefulness for the sensitive measurement of relative changes in blood flow under dynamic conditions.

In particular, the sources and extent of variability in neurovascular responses to transcutaneous electrical and iontophoretically applied stimuli are defined in order for these to be minimized. Examples of clinical and experimental studies, with particular reference to diabetes mellitus, illustrate the use of LDF for dynamic changes in skin and muscle microvascular blood flow.

Introduction

Many neurological disorders such as diabetic, neoplastic and other neuropathies, lead to disturbances of thin nerve fibres which have not been easily measured in the past. Methods for measuring tissue blood flow in absolute terms have been long sought, but most techniques used to date have the disadvantage of being invasive. Recently semiquantitative techniques of laser Doppler velocimetry (LDV) and laser Doppler flowmetry (LDF) first used 16 years ago have proved useful in continuously monitoring the blood flux changes in skin microvessels (1). The laser Doppler flowmeter (LDF) uses a low power laser (usually helium neon 2-3mW) to generate a non-injurious beam of infrared light which passes through an optical fibre to illuminate a region of tissue. Photons entering the tissue are scattered by moving red blood cells and undergo a frequency shift according to the Doppler principle. Some of this light is then collected and delivered via other optical fibres to a photodetector whose electrical output is processed to yield a continuous reading which in many conditions is proportional to the local blood flow. The laser probe senses average blood flow in a region of tissue approximately a hemisphere, with a radius of one millimetre. The measurement, referred to as blood flux, is continuous and has high spatial and temporal resolution (100 ms) so dynamics associated with local skin blood flow regulation or cardiac output change can be followed.

The previous difficulty of measuring cutaneous blood flow made the laser Doppler flowmeter (LDF) seem ideal because of its simplicity to use, and its provision of a continuous non-invasive quantitative measurement of local microcirculation, but as yet there has been no general acceptance of its clinical usefulness (2). However, the limitations of the technique and some caveats about its use should be made at the outset before emphasizing its greatest usefulness for the measurement of relative changes in blood flow under dynamic conditions.

The physical principles of LDV are scientifically attractive and have led to an array of different LDF devices with some appreciable differences in their implementation of the principles. These include in particular, the wavelength of the laser light used (3, 4), the number, dimensions and geometry of the fibre optics conveying the reflected signal, the signal processing (5) and filtering of the reflected laser signal, and the mathematical algorithms used to derive a voltage output proportional to tissue blood flow. Examples of such instruments are those described by Tenland (3) and Haumschild (4). These principles, parameters and their implementation have been recently reviewed in detail by Kilpatrick et al. (6) and by Svensson (7).

Address for correspondence: Associate Professor R.A. Westerman, Department of Physiology, Monash University, Clayton. 3168. Vic.

** A brief account of this paper was presented at the Conference EPSM 86, Sydney, August, 1986 under the title "Non-invasive monitoring of skin blood flow"*

Manuscript received 8 May 1988, revised 3 June 1988.

Standardization of LDF instruments

Because it utilises Doppler shifts in the range of helium neon laser light wavelength 632.8 nM or infra red 760-800 nM, LDV is sensitive to all movements on a microscopic scale. The devices which employ two fibre optics for pickup of the reflected signal and differential recording (3, 8) in our experience have proven more sensitive and less subject to movement artifact. Filtering and improvements in fibre optic cables (6, 9) have further reduced this problem. Until now, most LDF devices have claimed validation of their particular configuration and signal processing by comparison with other established methods of blood flow measurements. These include: 133 xenon clearance (7, 10, 12), optical plethysmography (13, 14), electromagnetic flowmetry (9), microspheres (15, 16, 17) and in vitro fluid models (3, 4).

Good correlations have been demonstrated with microsphere flowmetry (15, 16) and 133 xenon clearance (10), both of which are accepted and accurate methods. Figure 1 depicts such an experiment using LDF and microsphere measurements. In the merino sheep, measurement of flank skin blood flow, simultaneously using microspheres and LDF during the continuous infusion of epidermal growth factor intravenously, was performed over an 8 hour period in a warm environment. In spite of the useful correlation it is clear that the LDF is not measuring absolute blood flow (11). In addition, examining intestinal mucosa blood flow by LDF in the dog, significant correlations with electromagnetic flowmetry were obtained by Shepherd and Riedel (18, 19). A comparison between plethysmography, LDF and heat thermal clearance gave results strongly suggesting the different methods measure skin blood flow changes at

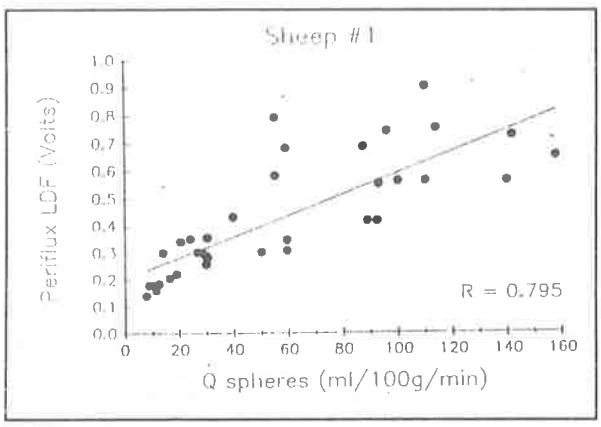


Fig. 1: Comparison of two different measures of changes in skin blood flow on a sheep's flank during 8 hours of continuous intravenous infusion of epidermal growth factor. The sheep was comfortably lying in a metabolic cage in a warm environment and both Periflux Pf1d Laser Doppler flowmeter and radioactive microsphere measurements of skin blood flow were made simultaneously at approximately 3/4 - 1 hour intervals. The linear regression of the data points over the range of flow from 10-160 ml.100g^{-1}.min^{-1} showed the relationship between LDF flux and Q spheres has a correlation coefficient R=0.795. This includes data points at 8 hours with flows around 150 ml.100g^{-1}.min^{-1} when active vasodilatation was likely to be more established.

different depths in vascular beds in the skin (20) and the observations of Johansson et al. in a gastrointestinal application (8) support this. However, in order to make the most useful inter-experimental comparisons some reproducible standard of blood flow is required against which varying types of LDF can be calibrated - and unfortunately this is still lacking.

Limitations of the LDV technique

Apart from the general problems of movement sensitivity and lack of a reproducible standard of blood flow already discussed, the following additional sources of variability are recognized:

(i) dependence of readings on blood vessel walls and non cellular elements such as lipid droplets.

(ii) biological variables such as skin thickness, pigmentation and capillary density may affect LDF readings.

(iii) haematological changes, e.g. haematocrit in the absence of flow change may affect LDF reading.

(iv) uncertainty about the depth of 2 mW laser penetration and therefore the volume of tissue whose microvasculature is monitored (20).

(v) uncertainty about whether LDV can distinguish blood flow through capillary beds from that through arteriovenous shunts.

(vi) response amplitude (% increase in laser Doppler flux) depends upon resting level of flux (see Figure 8).

Experimental and clinical uses of LDF

Use of LDF has been extensive and includes monitoring of skin blood flow in a wide variety of situations including sickle cell disease (21), autonomic and diabetic neuropathy (22, 23), fetal scalp blood flow in labour (24), perfusion of skin flaps (25, 26), monitoring of burns and hypothermia and shock (9). Other relatively static situations in which LDF has been used are in microprobe measurements of subcutaneous and intramuscular flow (27, 17), and in endoscopic measurement of gastrointestinal blood flow (8).

A much more powerful use of LDF lies in the sensitivity of the technique to quantify relative changes in blood flow in response to various graded stimuli. In this way the reactivity (i.e. responsiveness and sensitivity) of a microvascular bed may be tested. Examples of this application of LDV are found in the paper of Karanfilian et al. (28). The simple but elegant techniques of inducing reactive hyperaemia of limb skin in response to a standard ischaemic stimulus demonstrate unequivocally the value of quantitating dynamic vasodilator responses when trying to measure subtle functional disturbances of microvasculature resulting from chronic peripheral arterial disease. It also illustrates the philosophy of using LDV most effectively to measure changes without at-

tempting to extract or extrapolate absolute flow data from the measured response. However, the response amplitude measured as a % increase in LDF flux may depend upon the resting level of flux (29).

Neurovascular responses, axon reflexes and neurogenic inflammation

The rest of this paper will examine various aspects of the neurogenic inflammatory defense mechanisms against injury or infection including axon reflex vasodilatation in skin and muscle in health and disease and the useful clinical and experimental applications of LDV in these systems to monitor the dynamic changes in microvascular blood flux resulting from applying standard graded stimuli of various types.

Nocifensor mechanisms in the skin

There are many defensive (nocifensor) reactions of blood vessels or cells occurring in skin, muscle and pia arachnoid evoked by noxious stimuli and mediated by nerves or by chemical agents. These humoral, cellular and vascular mechanisms are illustrated diagramatically in Figure 2. The final response measured by LDF is microvascular dilatation probably involving only epidermal microvessels particularly postcapillary venules. It is important to note that the mechanism responsible involves the serial interaction of three elements: (a) polymodal nociceptors and primary afferent fibres, (b) microvascular endothelium, and (c) micro- vascular smooth muscle. Thus dysfunction of any one or more of these components will reduce the dilator response elicited and it is necessary to test each component separately.

Methods

Traditional tests of nociceptor function in humans, such as measurement of thresholds for pain sensation evoked by noxious heat, electrical stimulation, or mechanical pressure, provide useful but limited information about the function of nociceptors in the areas of skin tested (30). However, a quantitative test for the study of cuta-

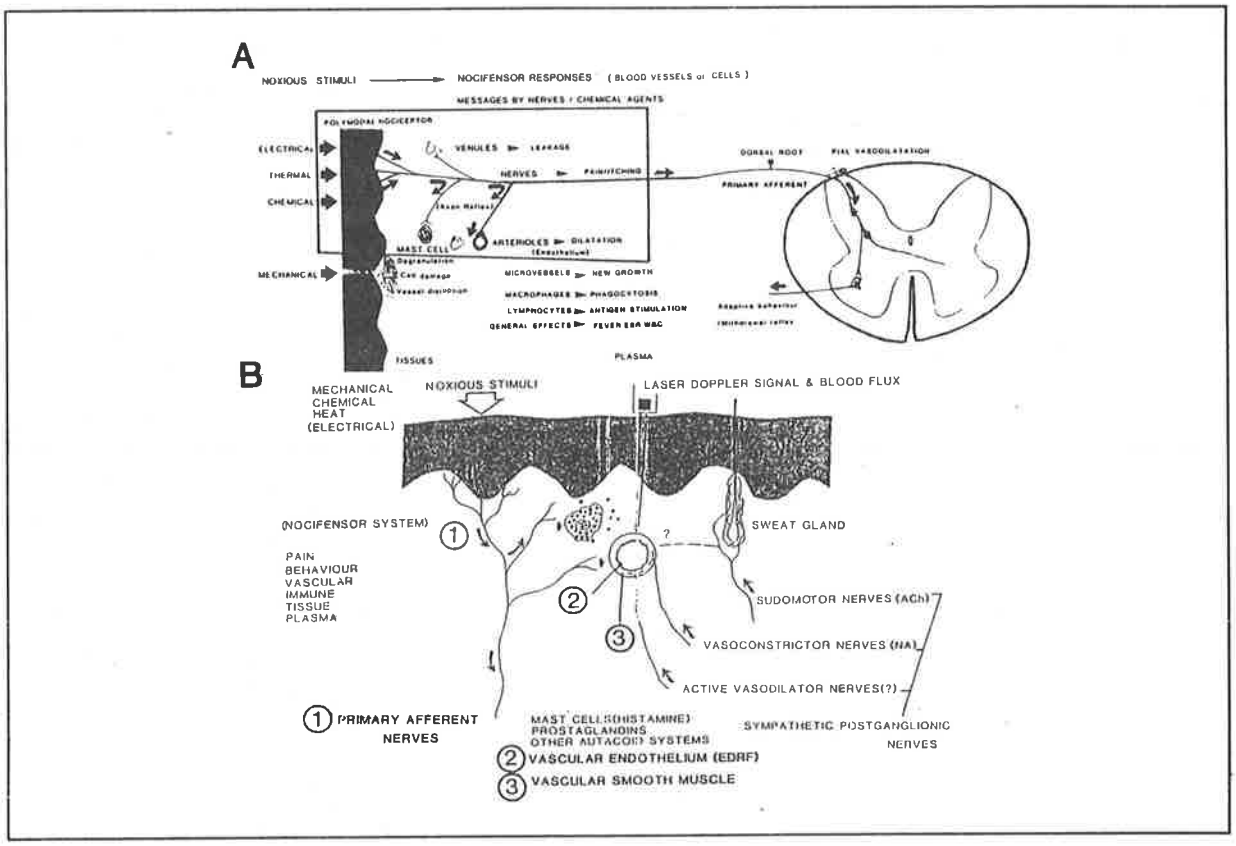


Fig. 2: A:. Shows many nocifensor (defensive) or damage-control reactions which occur in mammalian skin in response to noxious stimuli of any type. Such nocifensor responses involve blood vessels or cells and are mediated by neurogenic peptides or other autacoid chemicals. These include: release of peptides (e.g. substance P, calcitonin gene-related peptide, neurokinin A) from axon reflex branches of C-nociceptor primary afferents; degranulation of mast cells with release of histamine, 5 hydroxytryptamine etc; induction of prostaglandin and other autacoid synthesis, tachykinin release; microvascular dilatation, plasma extravasation and leakage of circulating antibodies, lymphocytes and macrophages; increased phagocytosis and antibody production; general effects such as fever, leucocytosis and increased erythrocyte sedimentation rate; adaptive behaviour, such as feeling pain, initiating withdrawal and protective flexor reflexes and volitional movements.

B: Enlarged diagram of region indicated by rectangle in A. The neurovascular interactions of (1) polymodal C-nociceptor/primary afferent nerve, (2) microvascular endothelium, (3) vascular smooth muscle occur serially and are tested separately by non-invasive LDF measure of microvascular dilator responses. (See text).

neous nociceptor activity in such diseases as diabetic (or other) polyneuropathy has been described (23, 31, 32, 33) and is summarized below. This utilizes the technique for recording local cutaneous vasodilatation in response to noxious percutaneous electrical stimulation. This so-called electrical axon reflex is measured by LDF. In addition, the effects of peptides released from sensory nerves and of tachykinins, prostaglandins, histamine and other vasoactive autacoids acting on skin microvessels can be assessed by quantitative iontophoretic application of such substances and LDF measurement of resulting blood flow changes. Detailed descriptions of both axon reflex (A) and drug iontophoretic tests (B) are given in the following sections.

A. Electrically evoked axon reflex

The local nociceptor-induced vasodilatation (that is, the axon reflex part of Lewis' triple response) is recorded from the hairy skin of the anterior forearm or the dorsum of the foot. A Periflux Pf1d laser Doppler flowmeter (Perimed, Sweden) is used to record the changes in skin blood flux. The laser Doppler probe holder is fixed to the skin by a double- sided adhesive disc. An indifferent anodal electrode is applied 5 cm proximal to the test site, where the cathode is a 30 mm2 gold-plated disc electrode taped onto the skin approximately 5 mm from the laser probe. A graded increase in the stimulus voltage permits the noxious electrical stimulus theshold to be determined and recorded. The minimum stimulation giving reproducible responses in normal skin is a series of pulses each of 150 volts, 0.75 ms in duration, at a frequency of 2Hz delivered by a fully isolated Grass S88 stimulator with constant voltage output. The total number of pulses delivered in each brief train ranged from 1 to 32. (All healthy nondiabetic subjects report that this stimulus produced pain at the cathode, and usually do not receive the train of 32 stimuli, only 16 pulses.) The same sized responses are also obtained using a constant-current isolated stimulator (HSE Neurostim 50) with the other parameters frequency and pulse duration the same. This indicates that provided the stimulus is supramaximal for C-nociceptors, constant voltage and constant current stimulation are equally effective. The geometry of the recording probe and stimulating electrodes is shown in Figure 3A together with a series of vasodilator responses to the stimulus trains of 2, 4, 8 and 16 pulses in a normal subject (Figure 3B). The area of each vasodilator response was measured as the voltage-time integral (see below). The means, standard errors of the means, and 95% confidence limits are calculated for all normal and diabetic subjects and the logarithms of responses and stimuli are plotted. The amplitude of the microvascular blood flux increase and its duration are seen to depend on the 'dose' of electrical stimulation expressed as the total number of electrical pulses.

B. Iontophoresis of vasoactive compounds

The subject is seated in a room with an ambient temperature between 20-23°C. When the forearm is being tested,

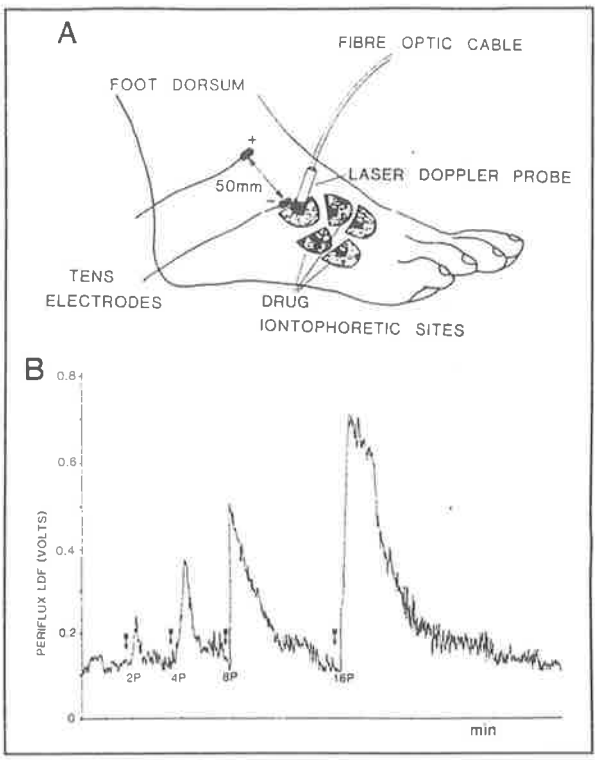


Fig. 3: A: Arrangement of LDF probe holders and TENS electrodes on the human foot dorsum for evoking electrical axon reflex and iontophoretic vasodilator responses.
Note that when electrically stimulating the laser probe sits in the probe holder adjacent to the electrodes, and that the electrodes are 50 mm apart, cathode adjacent to probe 5 mm away.

B: Examples of graded axon reflex dilatations evoked by 2, 4, 8 and 16 noxious TENS pulses (150 V, 0.75 ms duration, 2 Hz frequency) in a consenting normal volunteer. These responses depend on functioning nociceptor/primary afferent, microvascular endothelium and smooth muscle.

the arm is comfortably positioned on the arm of the chair with its anterior surface facing upwards. For testing the foot dorsum, the leg is extended and supported approximately 30 cm above the ground. Relative changes in skin blood flow is measured with a LDF. The LDF measures basal blood flow by sitting in a plastic probe holder that is fixed to the skin by a double-sided adhesive disc.

A battery powered constant current stimulator (WPI 502R) is used to provide a direct (galvanic) current for drug iontophoresis. The drugs are dissolved in an inert aqueous gel (2-3% methylcellulose) to produce a 1% aqueous solution, and are applied to a Ag-AgCl or stainless steel plug in a hemispherical 50µl chamber. This is part of a cylindrical perspex applicator which serves as the active electrode and fits into the laser probe holders adhered to the skin (see Figure 3A). An indifferent electrode of cotton gauze wet with distilled water is attached to the ankle or wrist of the subject. After removing the laser probe from the measuring site, the drug is applied into the epidermis in the immediate skin area under the probe, with small currents of short duration (dose is expressed as the total charge in millicoulombs, mC). To transfer the drugs into the skin, the

polarity of the active electrode has to be the same charge as the drug. Following iontophoresis, the resulting change in blood flow is detected by replacing the LDF probe into its holder on the surface of the skin. The major determinants of the iontophoretic procedure are represented in Figure 4A.

Dilator responses to acetylcholine (ACh), an endothelium-dependent agonist, and to sodium nitroprusside (NaNP), an endothelium-independent nitrodilator are obtained using iontophoresis on the forearm and the foot dorsum of subjects. The doses are altered by varying the time of exposure to the current, which was kept constant at 0.2 mA. Thus, a charge of 2 mC applied to the skin occurs with 0.2 mA current for 10s, while 4 mC equals 20s of 0.2 mA current. For acetylcholine chloride, anodal currents are used to transfer the cation (ACh^+) during iontophoresis, and for sodium nitroprusside cathodal currents are employed to pass the anion. Each application of drug is given to a different site, unless no response is recorded initially. At these low current levels, the inert gel used to dissolve the drugs causes negligible effect on skin blood flow and are usually imperceptible to the subject. Doses of between 0.25 mC and 8 mC of ACh, and 1-8 mC of NaNP have been used and an example of the latter is illustrated in Figure 4B. If higher currents than 0.2 mA are employed there is a possibility of local electrically or thermally induced axon reflex vasodilatation depending on current density.

Assessment of Recordings

Vasodilator responses obtained from electrical and iontophoretic procedures are quantified by measuring the area under the curve of each response, as recorded on an I.C.I. DP600 Pen Recorder. The area, in mm^2 is measured using image analysis programmes of an IBM PC-AT compatible computer with a magnetic tablet and stylus attached. For TENS, the area measured is the peak above the resting base-line, until the response returns to the resting flux level. However, after drug applications, the response does not always return to base-line particularly for larger doses, so the area under the curve is calculated for the first four minutes of the response. The value finally obtained is expressed as a V.min integral, and this reflects response onset-latency and amplitude.

Sources of variability

The major sources of variability are summarised below.

A. General
   (i) Equipment:
   - Variable voltage output for standard calibration signal
   - laser gain settings, time constant, filters
   - laser probe at variable distance from skin surface
   - analogue chart recorder settings
   - measurements of area under curve with digitising tablet.

   (ii) Operator error:
   - Different operators performing and/or analysing neurovascular tests

   (iii) Subject:
   - age
   - sex
   - time of testing
   - anxiety (vasoconstrictor tone)
   - current medication
   - atopic/non-atopic status
   - ingestion of vasoactive compounds, e.g. caffeine
   - resting level of baseline flux
   - site to site variability

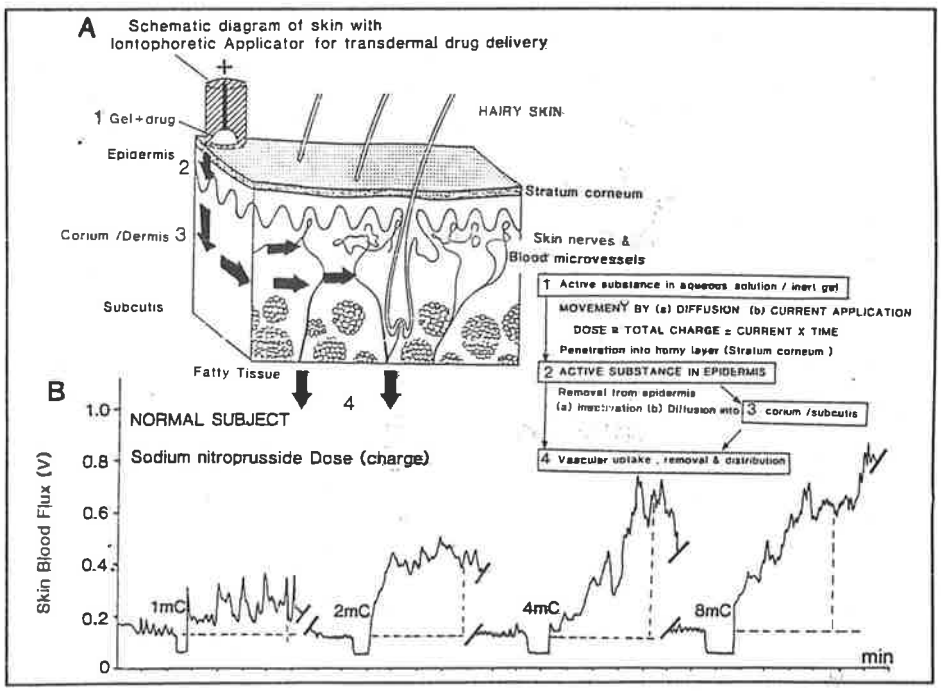


Fig. 4: A: Diagram of iontophoretic application of vasoactive compound to skin surface, including factors determining the penetration, accumulation and clearance of drug in epidermis, corium and subcutis.

B: Examples of graded dilator responses evoked by sodium nitroprusside iontophoresis. Various charges were applied (1, 2, 4, 8 milli Coulombs) by altering the time of iontophoresis (5-40 sec) while the current was kept constant (0.2 mA). The periods of zero flux indicate varying times of iontophoresis when the LDF probe was momentarily removed for drug application.

(iv) Site of measurement, e.g. forearm vs. foot dorsum:
- stratum corneum thickness
- sweat pore distribution and density
- primary afferent innervation and density
- microvascular bed distribution and density
- permeability differences
- ambient temperature

B. Tens
- stimulus current not of C-fibre strength
- variable placement of cathodal electrode adjacent to laser probe.

C. Iontophoresis
- variable drug concentrations
- inadequate mixing of drug in vehicle (e.g. inert gel)
- choice of charge used to transfer drug depends on both current strength and duration of iontophoresis
- incorrect polarity for drug to be transferred
- large current strengths (>0.2-0.4 mA) may evoke axon reflex
- skin clearance of drugs by mechanisms shown in Figure 4.

Results

A. Variability studies

Many of the general sources of variability listed in the Methods section (A(i), (ii)) are obvious and the appropriate steps are taken to standardise there. Others such as the measurements of different sites within and between subjects (i.e. Methods A(iii), (iv)) can only be determined by systematic, controlled testing. Our laboratory has been concerned mainly with neurovascular responses obtained from forearm and foot dorsum skin regions and we have defined intra- and inter-subject variability for TENS (see Figures 5A, B) and ACh- (see Figures 6A, B) evoked responses obtained on the forearm. A comparison of forearm responses with those obtained on the foot dorsum is also illustrated in Figure 7.

Notwithstanding site to site variation, body temperature is also an important variable since this will affect resting skin temperature and therefore resting skin blood flow (29). Figure 8 shows that an increase in resting skin blood flux will depress the neurovascular dilator responses to ACh (4 mC) and will thus also reduce neurovascular responses evoked by TENS.

B. Clinical Studies

Having established the limits of variation of these neurovascular tests, the next most obvious step was to test whether there were any small nerve fibre deficits in disease states which are known to cause large fibre neuropathies. These neurovascular tests were performed in patients with diabetes mellitus and examples are illustrated in Figure 9.

These and other results (23, 32, 33) clearly demonstrate markedly reduced TENS responses in diabetic patients with clinically diagnosed large fibre neuropathy. Such impaired nocifensor mechanisms may contribute to some of the symptoms and complications of diabetes such as pain, frequent skin injuries and infections. However, since the axon reflex responses depend ultimately on the integrity of the microvasculature in series with the primary afferent nerve fibres, it is important to test at this microvascular level by drug iontophoresis for a contributing vascular deficit. It is now well known that different drugs may cause vasodilatation indirectly via the release of a substance (EDRF) from the endothelium or directly by stimulating the vascular smooth muscle (34). We have iontophoresed ACh as an endothelium-dependent vasodilator and sodium nitrite and sodium

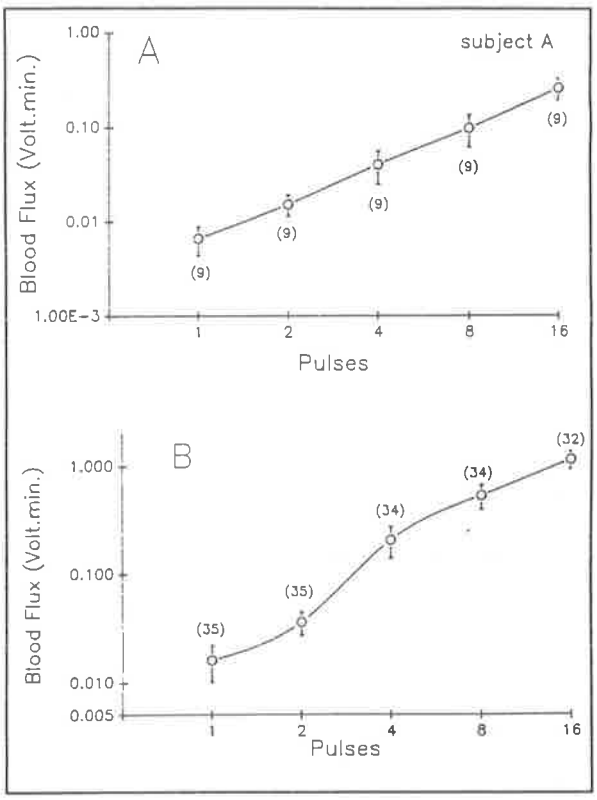
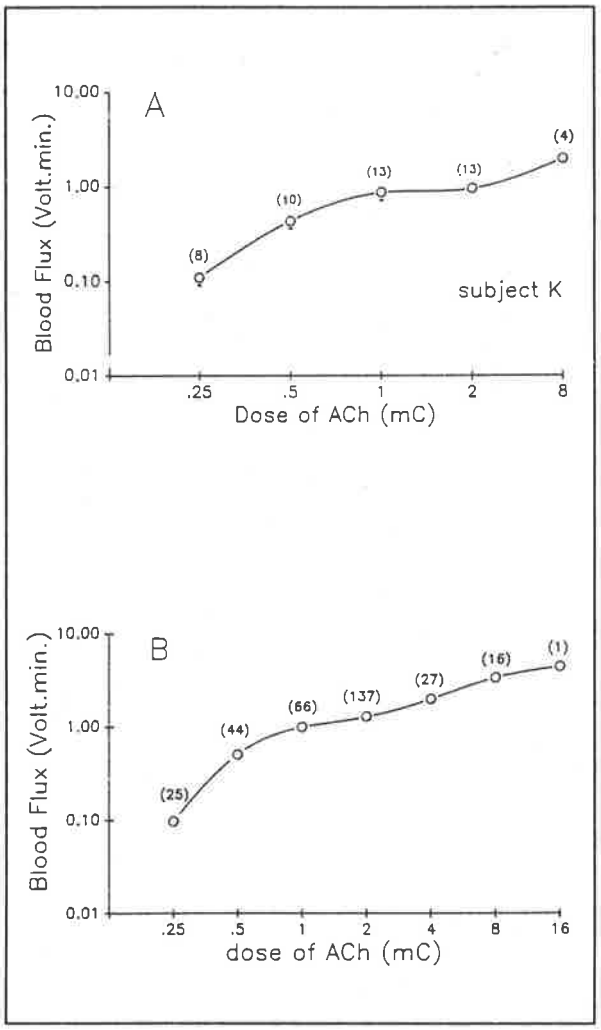


Fig. 5: Intrasubject variability to TENS:
A: Shows the variability of LDF blood flux responses to graded noxious TENS (1-16 pulses; 150 V, 0.75 ms, 2 Hz) applied to the same general site i.e. forearm of the same subject on different days. Abscissa- flux change as Volt.min integral (log scale). Ordinate - number of stimulus pulses (log scale). Each point is the mean with standard error as vertical bars. Number of observations in brackets.

Intersubject variability to TENS:
B: Variability of LDF blood flux responses to graded noxious TENS (1-16 pulses; 150V 0.75 ms 2Hz) applied to the same general site, i.e. forearm of different subjects. Abscissa-flux change as Volt.min integral (log scale). Ordinate - number of stimulus pulses (log scale). Each point is the mean with standard error as vertical bars. Number of observations in brackets.

Note: For all the variability studies in Figure 5, 6 and 10, the same laser Doppler flowmeter (Periflux Pf1d) was used to record all responses, but several operators recorded and analysed the data.



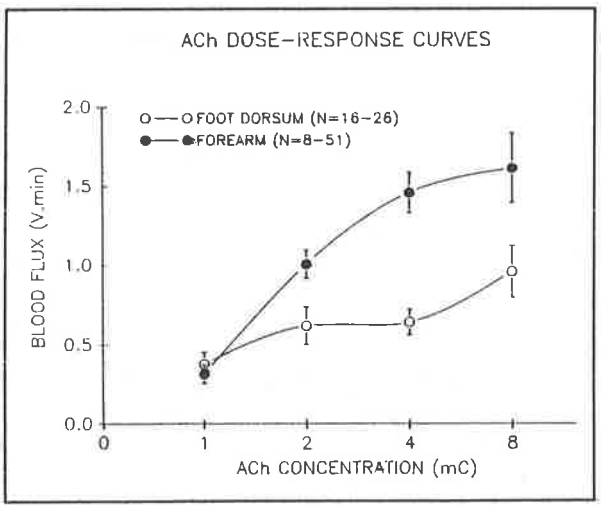


Fig. 7: (above) Shows LDF blood flux responses to graded doses of acetylcholine chloride (ACh), 1-8 milli Coulomb, applied iontophoretically to either the forearm (closed symbols) or foot dorsum (open symbols) of different subjects. Abscissa-flux change as a Volt.min integral. Ordinate - dose of ACh as mC iontophoretic charge (log scale). Each point is the mean with standard error as vertical bars, number of observations in brackets.

Fig. 6: (left)

Intrasubject variability to ACh:

A: Shows the variability of LDF blood flux responses to graded doses of acetylcholine chloride (ACh), 0.25-4 milli Coulomb, applied iontophoretically to the forearm of the same subject on different days. Abscissa-flux change as Volt.min integral (log scale). Ordinate - dose of ACh as mC iontophoretic charge (log scale). Each point is the mean with standard error as vertical bars, number of observations in brackets.

Intersubject variability to ACh:

B: Shows the variability of LDF blood flux responses to graded doses of acetylcholine (ACh), 0.25-16 milli Coulomb, applied iontophoretically to forearm of different subjects. Abscissa-flux changes as Volt.min integral (log scale). Ordinate - dose of ACh as mC iontophoretic charge (log scale). Each point is the mean with standard error as vertical bars, number of observations in brackets.

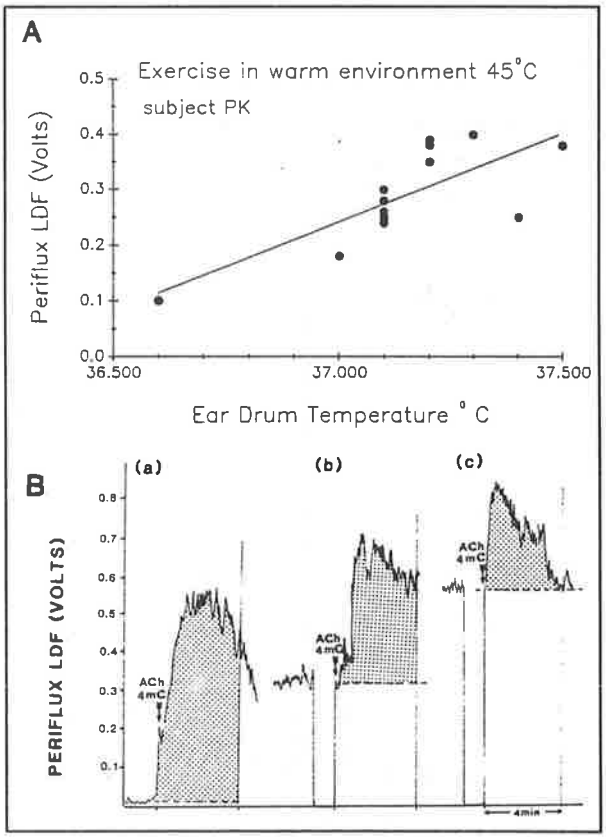


Fig. 8: (above)

A: Shows LDF resting flux increasing with core temperature as the healthy subject exercised in a warm environment 45°C, 30% relative humidity.

B: Shows flux changes in response to iontophoretic ACh 4mC applications superimposed on different resting levels of LDF, corresponding to approximate core temperatures of 36.5°C (a), 37.0°C (b) and 37.5°C (c).

Note: the response amplitude is reduced at higher resting LDF skin flux levels.

nitroprusside as smooth muscle vasodilators (endothelium-independent) in control and diabetic patients (23, 32, 33). From these studies we have determined that diabetic patients with neuropathy exhibit impaired vascular endothelial, but not smooth muscle, function which could partly contribute to the depressed TENS responses (for detail discussion, see refs 23 and 32).

The question of variability between operator and LDF instruments is an important part of establishing the reliability and reproducibility of a test. Evidence about the contribution of this to overall variability is shown in Figure 10A, B which compares 1986 control and diabetic data with corresponding 1987 data for TENS and Figure 10C, D which similarly compares responses to iontophoretic application of ACh.

C. Animal studies

The use of animal models of diabetes mellitus including alloxan, streptozotocin and genetic types of diabetes in the rat are well documented (33). Recently, the equivalent of TENS has been used in anaesthetized rats by electrically stimulating intradermal needle electrodes placed overlying the saphenous neurovascular bundle (33). Skin blood flow was measured distal to the electrodes in an adjacent shaved region innervated by the saphenous nerve (Figure 11). In agreement with studies in man, electrical stimulation evoked pulse-dependent vasodilatation (Figure 11). Similar experiments have also been performed in the STZ rat diabetic model. This study showed that, in comparison to age-matched control animals, rats tested 60 days after the administration of STZ exhibited markedly depressed electrical axon reflex responses (33; Figure 12A). However, the application of the non-invasive neurovascular tests described in the present paper to such animal models lies in the ability to follow progress of the disease from onset to the development of complications (Figure 12A) and to measure the effects of various treatments such as the newer aldose reductase inhibitor compounds ready for clinical trial in human diabetes mellitus. The effect of an aldose reductase inhibitor, Sorbinil, in reversing the decline in neurovascular function and partly restoring axon reflex dilator responses is shown in Figure 12B.

Discussion

To date, methods of nerve testing have assessed damage mainly to larger myelinated A-fibre nerves (30, 35). The results presented in this paper indicate that the monitoring of skin blood flow changes using LDF during TENS provides an additional sensitive and portable method of

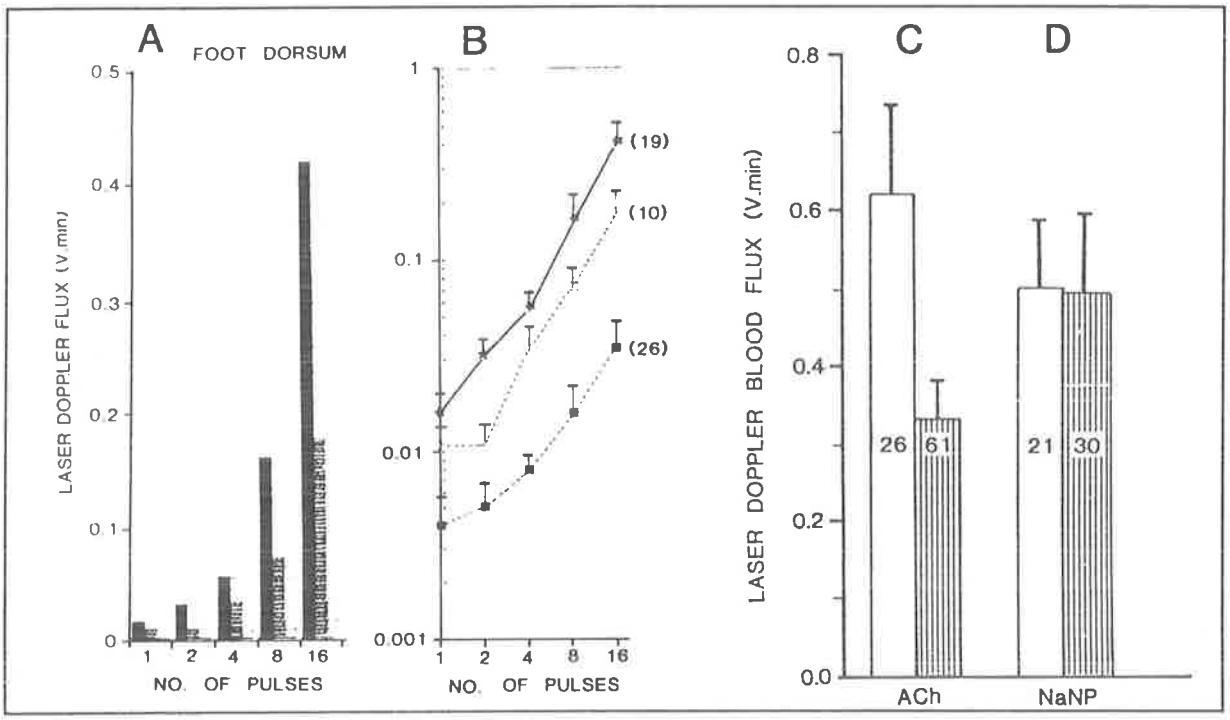


Fig. 9: Comparison of skin microvascular LDF blood flux changes in normal and diabetic foot dorsum skin evoked by (A, B) noxious TENS and iontophoresis of (C) acetylcholine (endothelial-dependent) and (D) sodium nitroprusside (endothelium-independent).

A: Shows linear plot of flux change, as a Volt.min integral, evoked by 1-16 TENS pulses in normal subjects (solid histograms), diabetic patients without neuropathy (dark hatched histograms) and with neuropathy (light hatched histograms).

B: Shows same data as depicted in A but plotted on double log axes. Normal subjects: solid line/stars., diabetic patients without neuropathy: dotted line/open squares., and with neuropathy: dotted line/closed squares.

C, D: The effect of iontophoretic application of (C) acetylcholine chloride (ACh), 2 mC, and (D) sodium nitroprusside (NaNP), 4 mC, in control subjects (open histograms) and diabetic patients with neuropathy (hatched histograms).

Note: Only diabetic patients with neuropathy showed significantly reduced responses from corresponding control responses for both TENS and ACh ($p<0.05$, t-test). Each point is the mean with standard error as vertical bars. The numbers indicate the n for each group.

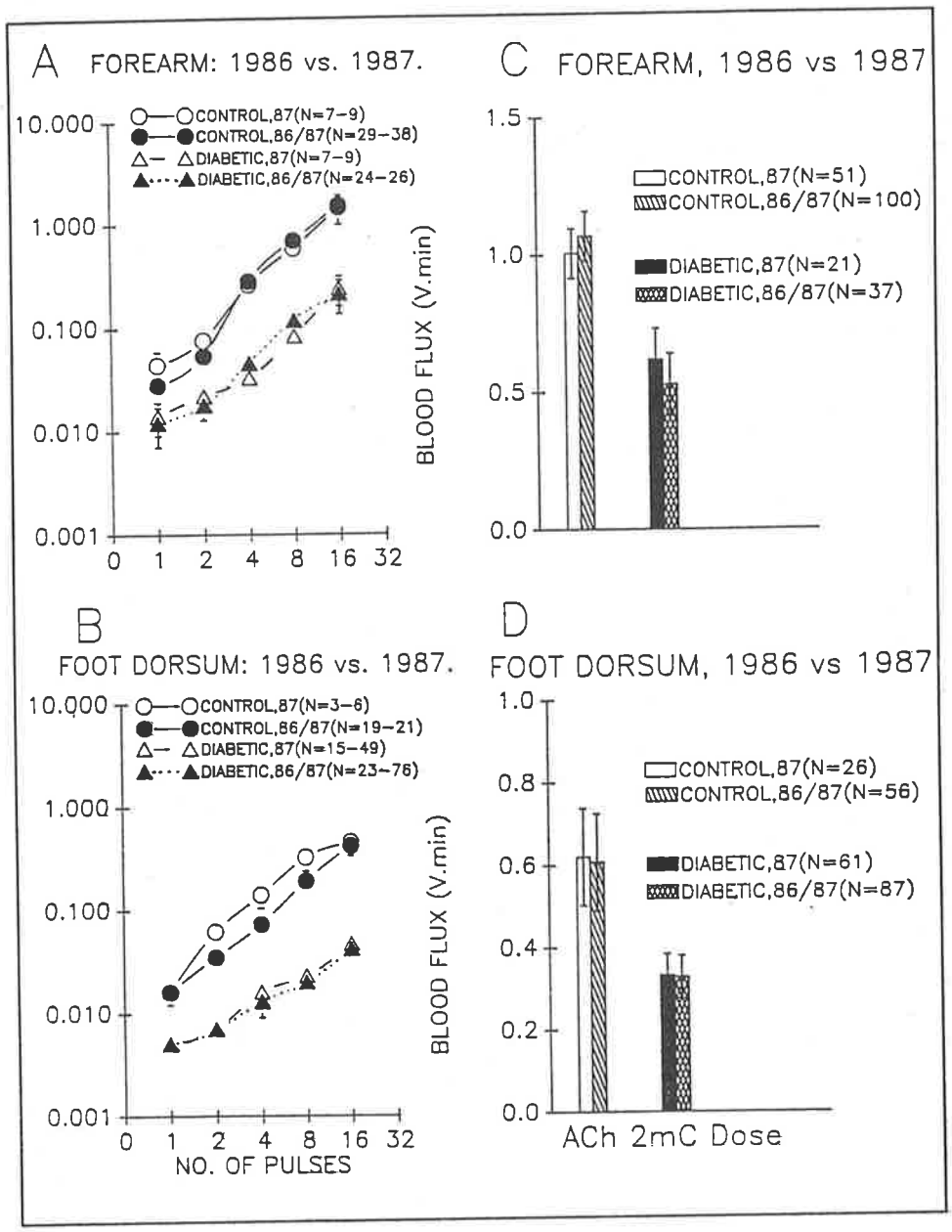


Fig. 10:

Compares the cumulative data gathered over two years (1986/1987) with that of the preceding year (1986) for TENS responses obtained on the (A) forearm and (B) foot dorsum and similarly for a standard ACh dose on the (C) forearm and (D) foot dorsum. A different operator was responsible for the recording/analysis of these data in each year.

These figures show the highly reproducible nature of the TENS and iontophoretic stimuli tested in both control subjects and diabetic patients with neuropathy (see text).

evaluating smaller C-fibre nerve function. Indeed the suggestions that these nerves may be affected early in diabetes mellitus (36, 37) has been confirmed in the present study. Moreover, the combination of drug iontophoresis together with TENS represents a unique non-invasive 'neurovascular testing kit' which utilises the strongest feature of LDF, viz its dynamic sensitivity. The importance of testing each individual component separately is highlighted in the present study since the reduced TENS responses in diabetic patients could not be attributed to impaired vascular smooth muscle vasodilatation. This was determined by the iontophoresis of ACh and nitrovasodilators (23, 32, 33), all of which exert negligible effect via the stimulation of the primary afferent nerves (38, 39).

Therefore, the following protocol is recommended to test neurovascular function for clinical purposes (refer to Figure 2):

1. Noxious transcutaneous electrical nerve stimulation (TENS) evoked axon reflex dilatation of skin microvessels by neurogenic peptide release and so tests the function of components 1, 2 and 3 simultaneously (see Figure 2, 3).

2. Iontophoretic application of muscarinic agonist acetylcholine chloride elicits endothelium-dependent dilatation of epidermal vessels in the skin iontophoresed. This tests the function of 2, 3. (See Figure 2, 4).

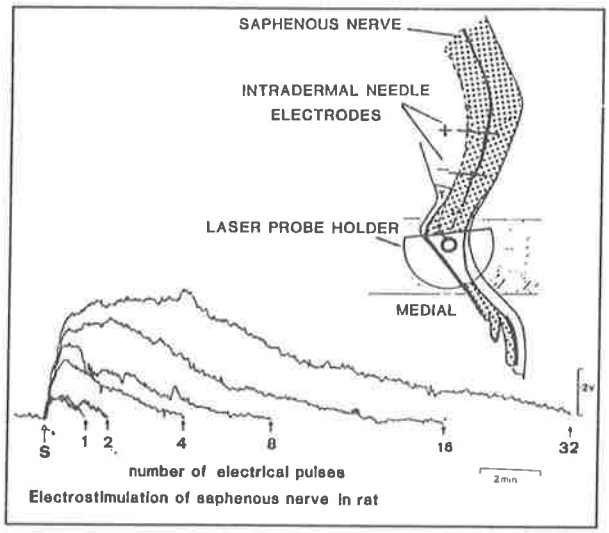


Fig. 11:
Axon reflex responses in rat hairy skin evoked by noxious electrical stimulation (100V, 1 ms 2 Hz) applied by intradermal needle electrode. The rat was anaesthetised to minimize movement and prevent discomfort during the noxious TENS. Flux responses to trains of 1-32 electrical pulses (superimposed) are shown by arrows. Inset shows diagramatically the location of LDF probe and stimulating electrodes.

3. Iontophoretic application of nitrodilators such as sodium nitroprusside evokes direct endothelium-independent dilator responses testing the microvascular smooth muscle reactivity, i.e. function 3 (see Figure 2, 4).

It should be stressed that the process of iontophoresis involves passing polar drugs across the skin using small, direct currents (40). As such, the exact drug concentration, location and distribution in the skin is unknown since a known charge (current x duration) is applied to the testing skin site, in which mechanical and electrical properties of skin will also vary (41, 42, 43). Nevertheless, as long as standard stimuli are chosen, relative dynamic changes in skin blood flow in normal and disease states can be determined and with small doses (up to 8mC) the drug appears confined to epidermis (44). Quantifying dynamic changes holds true also for TENS responses, since it is our experience that, while resting flux baselines may vary slightly from site to site, the absolute differences in mean resting flux between normal subjects and diabetic patients with neuropathy is negligible compared to the dramatic dynamic changes (i.e. flux differences) that occur (23, 32, 33).

Some of these neurovascular tests have also proven extremely useful in animal models in which more invasive techniques may be used (45). In the present study, the STZ rat model of diabetes parallels the clinical situation with depressed TENS-evoked vasodilatation. While drug iontophoresis is more difficult in the hairy skin of animals (42), the ability to perform repeated serial testing in animals adds a further degree of versatility to these tests. The appreciable restoration of axon reflex mechanisms following treatment with aldose

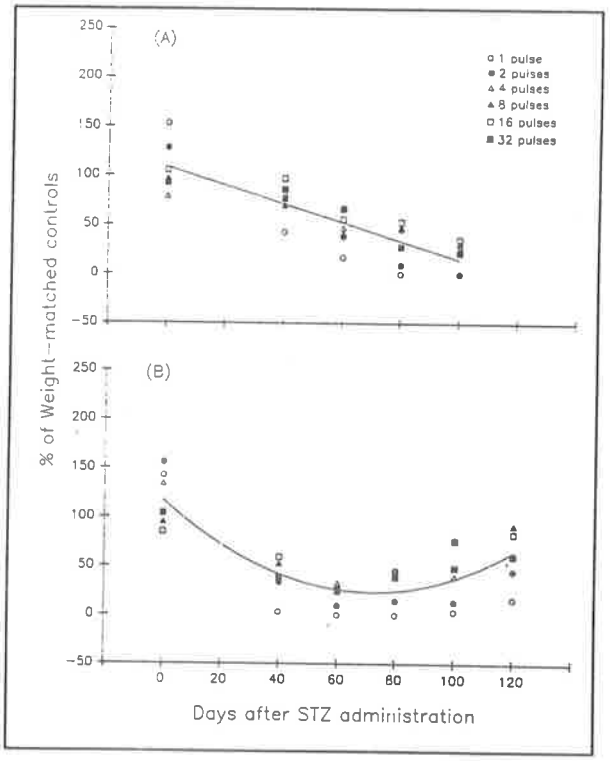


Fig. 12:

A: LDF readings of electrical axon reflex responses in paw skin of streptozotocin-diabetic rats compared with those of weight- matched controls (expressed as %) evoked by noxious electrical TENS (100V, 1.0 ms, 2Hz; 1, 2, 4, 8, 16, 32 pulses). Untreated STZ diabetic rats showed a progressive reduction of their axon reflexes with time. Each point is a mean of n=6 rats. This decline in responses is indicated by the unsubstantiated linear regression. At 100 days the responses to all stimuli are significantly reduced compared with the pre-diabetic control responses using an unpaired t-test (p<0.05 - 0.02).

B: LDF readings of electrical axon reflex response in diabetic rats treated with the aldose reductase inhibitor, Sorbinil, compared with those of weight-matched controls (expressed as %) evoked by noxious electrical TENS. Each point is a mean of n=7 rats. Note the sorbinil treated rats show a partial recovery and restoration of the reduced axon reflex responses. At 60 days responses are sigmificantly reduced compared to day 0 (p<0.05 - 0.001) while responses at 120 days are not significantly different from those at day 0.

reductase inhibitors again illustrates the usefulness of these techniques (Figure 12). Furthermore, it is easy to see the application of these techniques in the clinical setting for the early detection of, for example, diabetic neuropathy and for measuring the progress of the disease as well as the efficacy of subsequent therapy to be monitored objectively.

New directions and uses

(a) Transdermal delivery systems

Interest and research in these techniques has recently increased greatly (46, 47, 48) because of the useful bypass it provides for a substance to enter directly into systemic circulation without initial exposure to portal circulation and liver metabolism as occurs in the oral/enteric absorption route. Notwithstanding this, the only routine iontophoretic drug use in medicine is the pilo-

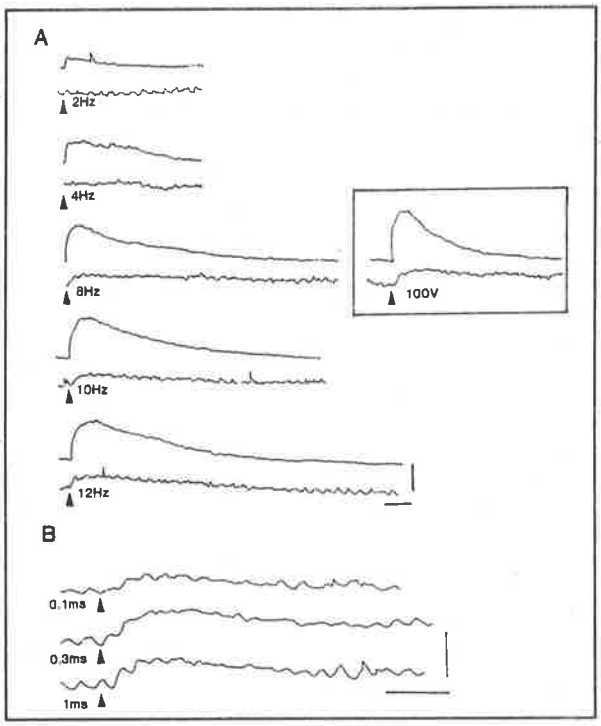


Fig. 13:

A. LDF flux changes simultaneously recorded from the hindpaw skin (upper trace in each panel) and surface of hindlimb calf muscle (lower trace in each panel) in the anaesthetised rat in response to noxious electrical stimulation applied to the distal cut end of the isolated L5 dorsal root (arrows). This antidromic electrical stimulation (40V. 0.5ms, 10s.) elicits an axon reflex type of blood flow change in the gastrocnemius muscle which increases with pulse frequency/number as shown and also with amplitude (see inset. response to 100V. 0.5ms, 10s. 8Hz).

B. Responses obtained from muscle to stimulation as above with pulses of increasing duration (50V. 20s. 10Hz). A "hunting" fluctuation in resting LDF flux in muscle was often seen.

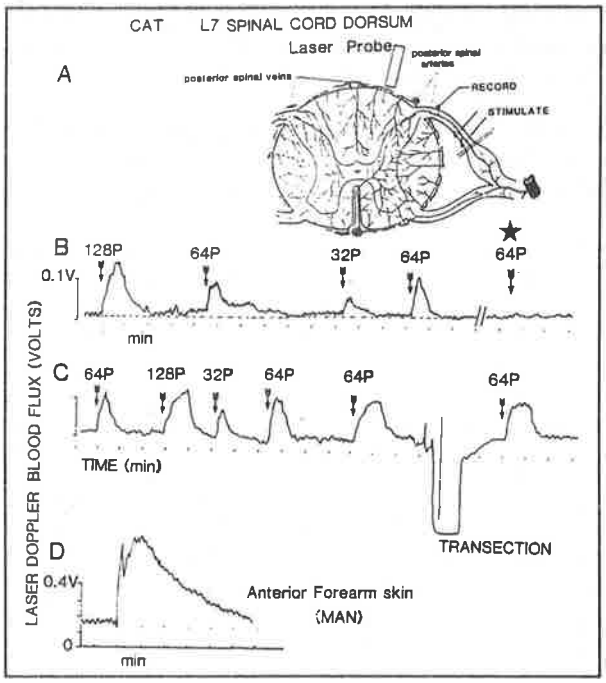


Fig. 14: Flux increases in pia-arachnoid vessels on L7 segment of cord dorsum of anaesthetised cat in response to noxious electrical stimulation of the central cut end of dorsal root transected and placed on platinum stimulating electrodes.

A: Cat spinal cord in TS showing major blood supply from posterior spinal artery and vein, stimulating electrodes, laser probe location and electrode recording ingoing afferent action potential are shown.

B, C: LDF blood flux responses, as volts, with the number of stimulus pulses (40V, 0.75 ms, 2Hz) shown at arrow for each stimulus train applied. Time in minutes. NOTE: (1) For all stimuli there was a C-deflection present in the afferent volley except at the asterisk where 64 pulses at only A-fibre stimulus strength (5 volts, 0.1 ms, 2 Hz) were applied - and no flux change was observed. (2) Transection of the spinal cord at segmental level T12/L1 had no effect on the flux increase evoked by train of 64 noxious pulses.

D: For comparison, axon reflex flux change on anterior forearm skin of an experimenter in response to 16 noxious TENS pulses (150V, 0.75 ms, 2 Hz).

carpine test for cystic fibrosis (44). Our variability data and the obvious precision in dose-response relationships suggests that this technique only requires more quantitative definition (40) to become much more useful for diagnostic and perhaps therapeutic systems.

(b) Axon reflexes in muscle

The incidence of chronic pain states involving skin, muscle and myofascial tissue, as well as disturbances of the interaction between sympathetic and sensory nerves and the common occurrence of painful neurovascular disorders is now known (49, 50, 51). This greatly widens the scope of the present neurovascular function test system for basic research into the mechanisms underlying these and other disturbances (52) and for clinical monitoring of treatment in these conditions (52). Chronic pain states involving muscle (e.g. Repetitive Strain Injury) provides a fertile area for such research. Figure 13 demonstrates that by antidromically stimulating the peripheral cut end of dorsal roots in the anaesthetised rat, muscle LDF blood flux changes occur closely resembling axon reflexes evoked simultaneously in skin by the same stimulus. The question of what afferent fibres (probably group 3 and 4) provoke such responses remains to be elucidated. However, from the available evidence (53, 54, 55) it seems likely that the smallest afferent nerves including nociceptors (i.e. the group 3 and 4 fibres) in muscle are activated by chemical changes in their environment such as occur in fatigue, severe overuse and associated microtrauma. It could be functionally significant if they contribute to axon reflex vasodilatation and prolonged hyperaemia in such fatigued or overused muscle. This is clearly an important area for further research and the development of single fibre-optic needle probes will facilitate this (9).

(c) Neural transmission of pain

An important role for the substance P-containing (SP) peptidergic primary afferent fibres in the transmission and relay of pain sensation is established by the elegant microprobe measurements of Duggan et al. (56, 57) who quantified SP release in substantia gelatinosa of cat

dorsal horn during noxious stimulation. A remarkable and no less significant release of SP occurs at the same time in the pia-arachnoid overlying active dorsal root conveying such nociceptor afferent input (54). Figure 14 illustrates the dilator function of such afferent activity in which brief LDF flux increases occur in the pia ipsilateral to the noxious sensory stimulation. These are roughly dose-dependent on the total number of stimulus pulses, but do not occur in roots rostral or caudal to that stimulated, and are unaffected by spinal cord transection 6-7 segments above the test-root to interrupt descending pathways. The functional role of such SP release and pia-arachnoid vasodilatation is not clear, but it may reduce or modify presynaptic inhibition during noxious sensory stimulation.

It is clear from all the examples provided that the technique of LDV can provide reliable reproducible responses with a variability no greater than many other clinically useful tests already established in medicine. If the search for a true standard flow model succeeds, much wider use and application of the LDV technique must soon follow.

Acknowledgements

Support for the research described above has been generously provided from the following sources, and is acknowledged with gratitude: Australian Research Grants Scheme, Monash Special Research Grants, Clive and Vera Ramaciotti Foundation, William Buckland Trust, Rebecca L. Cooper Research Foundation, National Health and Medical Research Council, Apex-Australian Diabetes Foundation, Boehringer Ingelheim. Clinical studies on diabetic patients were performed at the Lions International Diabetes Institute, Royal Southern Memorial Hospital, and the assistance of the Institute physicians and support staff is greatly appreciated.

References

1 Riva C, Ross B, Benedek GB. *Laser Doppler measurements of blood flow in capillary tubes and retinal arteries.* Invest. Opthalmol 1972; 11: 936-44

2 Lancaster JF, Lucarotti M, Leaper DJ. *Laser Doppler velocimetry.* J. Roy. Soc. Medicine 1988; 80: 729-30

3 Nilsson GE, Tenland T, Oberg PA. *Evaluation of laser Doppler flowmeter for measurement of tissue blood flow.* IEEE Trans Bio-med Eng. 1980; 27:597-604.

4 Haumschild, Daniel J. *Microvascular blood flow measurement by laser Doppler flowmetry.* TSI Application Note, (BPM 403, St. Paul, TSI Inc. 1986; 1-7

5 Ahn H, Johansson K, Lundgren O, Nilsson GE. *In vivo evaluation of signal processors for laser Doppler tissue flowmeters.* Med. & Biol. Eng. & Comput. 1987; 25: 207-211

6 Kilpatrick D, Kajiya F, Ogasawara Y. *Fibre optic laser Doppler measurement of intravascular velocity.* Aust. Phys. Eng. Sci. 1988; 11(1): 5-14.

7 Svensson H, Pettersson H, and Svedman P. *Laser Doppler flowmetry and laser photometry for monitoring free flaps.* Scand. J. Plast. Reconst. Surg. 1985; 19:245-249.

8 Johansson K, Ahn H, Lindhagen J. *Tissue penetration and measuring depth of laser Doppler flowmetry in the gastrointestinal application.* Scand. J. Gastroenterol. 1987; 22(9): 1081-1088.

9 Salerud GE, Oberg PA. *Single-fibre laser Doppler flowmetry.* Med. & Biol. Eng. & Comput. 1987; 25: 329-334

10 Stern MD, Lappe DL, Bowen PD, et al. *Continuous measurement of tissue blood flow by laser Doppler spectroscopy.* Am J. Physiol 1977; 232(4): H441-8

11 Holloway GA, Watkins DW. *Laser Doppler measurement of cutaneous blood flow.* J. Investigat. Dermatol. 1977; 69: 306-309

12 Engelhart M, Kristensen JK. *Evaluation of cutaneous blood flow responses by 133 Xenon washout and a laser-Doppler flowmeter.* J. Investigat. Dermatol 1983; 80: 12-15

13 Wunderlich RW, Folger RL, Giddon DB, Ware BR. *Laser Doppler blood flow meter and optical plethysmography.* Review of Scientific Instruments 1980; 51: 1258-62

14 Wester RC, Maibach HI, Guy RH, Novak E. *Minoxidil stimulates cutaneous blood flow in human balding scalps: pharmacodynamics measured by laser Doppler velocimetry and photopulse plethysmography.* J. Investig. Dermatol. 1984; 82: 515-517

15 Kiel JW, Riedel GR, Di Resta GR, Shepherd AP. *Gastric mucosal blood flow measured by laser Doppler velocimetry.* Am. J. Physiol. 1985; 249: G539-G545.

16 Kvietys PR, Shepherd AP, Granger DN. *Laser Doppler, H2 clearance and microsphere estimates of mucosal blood flow.* Am. J. Physiol. 1985; 249 : G221-G227.

17 Holmstrom A, Lewis DH. *Regional blood flow in skeletal muscle after high energy trauma.* Acta Chir. Scand. 1983; 149: 453-458

18 Shepherd AP, Riedel GL. *Continuous Measurement of Intestinal Mucosal Blood Flow by Laser Doppler Flowmetry.* Am. J. Physiol. 1982; 242 : G668-G672.

19 Shepherd AP, Riedel GL. *Laser Doppler velocimetric studies of intestinal blood flow during selective vasodilation.* Microvasc. Res. 1983; 25: 223-63

20 Saumet JL, Dittmar A, Leftheriotis G. *Non-invasive measurement of skin blood flow: comparison between plethysmography, laser-Doppler flowmeter and heat thermal clearance method.* Int. J. Microcirc: Clin Exp. 1986; 5: 73-83

21 Rodgers GP, Schecter AN, Noguchi CT. *Periodic microcirculatory flow in patients with sickle cell disease.* N. Engl. J. Med. 1984; 311: 1534-8

22 Low PA, Neumann C, Dyck PJ, Fealey RD, Tuck RR. *Evaluation of skin vasomotor reflexes by using laser Doppler velocimetry.* Mayo Clin. Proc. 1983; 58: 583-92

23 Westerman RA, Widdop RE, Hogan C, Zimmet P. *Non-invasive tests of neurovascular function: reduced responses in diabetes mellitus.* Neurosci. Lett. 1987; 81: 177-182

24 Smits TM, Aarnoudse JG. *Variability of scalp blood flow during labour. Continuous transcutaneous measurement by the laser Doppler technique.* Br. J. Obstet. Gynaecol. 1984; 91: 524-31

25 Moneta MR, Benichoux R. *Monitoring of the perfusion of a free skin flap by the skin temperatures and laser Doppler velocimetry. Comparative study in the rat.* Eur. Surg. Res. 1987; 19/S187: 64

26 Micheels J, Alsbjorn B, Sorensen B. *Laser Doppler flowmetry. A new non-invasive measurement of microcirculation in intensive care?* Resuscitation 1984; 12: 31-9

27 Gush RJ, King Ta, Jayson MIV. *In vivo measurement of subcutaneous and intramuscular tissue blood flow by a laser Doppler microprobe.* Int. J. Microcirc. Clin Exp. 1984; 3: 433

28 Karanfilian RG, Lynch TG, Lee BC, Hobson RW. *The assessment of skin blood flow in peripheral vascular disease by laser Doppler velocimetry.* The American Surgeon 1984; 50: 641-4

29 Ninet J, Fronek A. *Laser Doppler flux monitored cutaneous responses to local heating and cooling.* VASA 1985; Band 14(1): 38-43

30 *Jamal GA, Hansen S, Weir AL, Ballantyne JP. The neurophysiologic investigation of small fibre neuropathies.* Muscle & Nerve 1987; 10: 537-545

31 Westerman RA, Low A, Pratt A, Hutchinson JS, Szolcsanyi J, Magerl W, Handwerker HO,Kozak WM. *Electrically evoked skin vasodilation: a quantitative test of nociceptor function in man.* In: Clin. Exper. Neurol. 1987; 23: 81-89

32 Westerman RA, Widdop RE, Hannaford J, Hogan C, Roberts RGD, Zimmet P. *Non-invasive tests of neurovascular function.* Clin. Exper. Neurol. 1988; 24: 129-137

33 Westerman RA, Widdop RE, Low A, Hannaford J, Kozak WM, Zimmet P. *Non- invasive tests of neurovascular function: reduced axon reflex responses in diabetes mellitus of man and streptozotocin-induced diabetes of the rat.* Diabetes Res. & Clin. Practice 1988; in press

34 Furchgott R. *Role of endothelium in responses of vascular smooth muscle.* Circulation Research 1983; 53: 557-573

35 Dorfman LJ. *The distribution of conduction velocities (DCV) in peripheral nerves: a review.* Muscle & Nerve 1984; 7: 2-11

36 Asbury AK, Brown MJ. *Clinical and pathological studies of diabetic neuropathies.* In Y. Goto, A. Horiuchi and K. Kogure (eds) Diabetic Neuropathy, Excerpta Medica, Elsevier, Amsterdam 1982; pp.50-57

37 Dyck PJ, Karnes JL, Daube J, O'Brien P, Service FJ. *Clinical and neuropathological criteria for the diagnosis and staging of diabetic polyneuropathy.* Brain 1985; 108: 861-880

38 Roberts RGD, Westerman RA, Widdop RE, Kotzmann RR, Zimmet P. *Effects of capsaicin on cutaneous axon reflex vasodilatation in man.* Proc. Aust. Physiol. Pharmacol. Soc. 1987; 18(1): 95P

39 Lindblad LE, Ekenvall L, Ancker K, Rohman H, Oberg PA. *Laser Dopppler flowmeter assessment of iontophoretically applied norepinephrine on human finger skin circulation.* J. Invest. Dermatol. 1986; 87 : 634-636.

40 Trubatch J, van Harreveld A. *Spread of iontophoretically injected ions in a tissue.* J. theor. Biol. 1972; 36: 355-366

41 Elias PM, Cooper ER, Korc A, Brown BE. *Percutaneous transport in relation to stratum corneum structure and lipid composition.* J. Invest. Dermatol . 1981; 76: 297-301

42 Wahlberg JE. *Skin clearance of iontophoretically administered chromium (51Cr) and sodium (22Na) ions in the guinea pig.* Acta Dermatovener (Stockholm) 1970; 50: 255-262

43 Edelberg R. *Relation of electrical properties of skin to structure and physiologic state.* J. Investig. Dermatol. 1977; 69: 324-327

44 Sato K, Feibleman C, Dobson RL. *The electrolyte composition of pharmacologically and thermally stimulated sweat: a comparative study.* J. Invest. Dermatol. 1970; 55(6): 433-438

45 Low A, Westerman RA, Kozak WM. *Effect of insulin on reduced axon reflex vasodilatation in Streptozotocin diabetic rats.* Proc. Aust. Physiol. Pharmacol. Soc. 1987; 18(1): 97P.

46 Heilmann K. *Therapeutic systems: rate-controlled drug delivery: concept and development.* 2nd Edition, Stuttgart, Georg Thieme Verlag 1984

47 Kydonieus AF, Berner B. (eds) *Transdermal delivery of drugs.* Boca Raton Fla: CRC Press 1987; 3 volumes

48 Gale R. *Transdermal delivery systems: technological aspects. In: Transdermal Nitroglycerin in Angina.* Ciba-Geigy Symposium, Adis Press, Sydney 1987

49 Ochoa J. *The newly recognised painful ABC syndrome: thermographic aspects.* Thermology 1986; 2(2): 65-107

50 Nakamura M, Ferreira SH. *A peripheral sympathetic component in inflammatory hyperalgesia.*

Europ. J. Pharmacol. 1987; 135: 145-153

51 Roberts WJ, Foglesong ME. *A neuronal basis for sympathetically maintained pain.* Thermology 1986; 2(2): 2-6

52 Westerman RA, Roberts RGD, Westerman DA, Kotzmann RR, Widdop RE, Carter B. *Action of topical capsaicin on affected dermatomes in postherpetic neuralgia.* Clin. Exper. Neurol 1988; 25: In press.

53 Mense S, Stahnke M. *Responses in muscle afferent fibres of slow conduction velocity to contractions and ischaemia in the cat.* J. Physiol. 1983; 342: 383-397

54 Mense S, Meyer H. *Bradykinin-induced modulation of the response behaviour of different types of feline group III and IV muscle receptors.* J. Physiol. 1988; 398: 49-63

55 Woolf CJ, Wall PD. *Relative effectiveness of C primary afferent fibers of different origins in evoking a prolonged facilitation of the flexor reflex in the rat.* J. Neuroscience 1986; 6: 1433-1442

56 Duggan AW, Hendry IA. *Laminar localisation of the sites of release of immunoreactive substance P in the dorsal horn with antibody-coated microelectrodes.* Neurosci. Lett. 1986; 68: 134-140

57 Duggan AW, Morton CR, Zhao ZQ, Hendry IA. *Noxious heating of the skin releases immunoreactive substance P in the substantia gelatinosa of the cat:* a study with antibody microprobes. Brain Res. 1987; 403: 345-349

11

Vasodilatation: Vascular Smooth Muscle, Peptides, Atuonomic Nerves, and Endothelium, edited by Paul M. Vanhoutte, Raven Press, Ltd., New York, 1988.

VASODILATOR AXON REFLEXES

*R. A. Westerman, †F.W. Magerl, ††J. Szolcsanyi, †H. O. Handwerker, *A. Low, *A. Pratt, **R. Widdop, and *W. Kozak

Department of *Physiology and **Pharmacology, Monash University, Clayton 3168 Victoria, Australia; †II Physiological Institute, University of Heidelberg, D-6900 Heidelberg, Federal Republic of Germany; and ††Department of Pharmacology, Medical University, Pecs, Hungary

Bayliss (1) was the first researcher to observe antidromic responses in the skin upon stimulation of nerve fibers in the dorsal root. This response requires an intact peripheral sensory innervation and is most easily evoked when the stimulation excites the smaller myelinated and unmyelinated nerve fibers (9). Various agents applied to the skin, particularly if noxious or injurious, induce a local flare reaction. This is due to dilatation of the smaller blood vessels, long believed to be initiated by the release of vasodilator substance(s) from smaller sensory nerve fibers of the skin. Lewis (15) proposed that such a response would be protective, by promoting movement of nutrients and metabolites in regions of tissue damage. He described the spreading of hyperalgesia around skin injury and from antidromic stimulation of cutaneous nerves as well as the flare and triple response to injury (16,17) in terms of nocifensor functions.

CURRENT VIEWS

Some more recent evidence supports Lewis's view of a neurogenic system in skin controlling elements of defense, such as inflammation (13). Some peptidergic sensory neurons in the skin with small-diameter C-fibers (unmyelinated) contain either somatostatin (which can be released or depleted by capsaicin) or substance P, whereas others contain cholecystokinin or bombesin and are unaffected by capsaicin. The presence of vasoactive intestinal peptide (VIP) and fluoride-resistant acid phosphate indicates the existence of additional subgroups of primary afferent fibers. The remarkable degree of localization of calcitonin gene-related peptide (CGRP), together with substance P in cutaneous vascular and visceral sensory neurons (5), suggests that yet another potent vasodilator, CGRP, may be involved in neurogenic inflammation of the skin. The original view of Bayliss (1), Lewis (15), and others was obtained with rather crude stimulating and recording techniques and held that antidromic vasodilatation was confined to the skin and the conjunctiva. However, Hilton and Marshall (8) demonstrated vasodilatation in skeletal muscle of the cat following antidromic stimulation of the dorsal root; similar vasodilatation has been observed in hind limb muscles of the rat (R. A. Westerman et al., unpublished observations). This deep vasodilatation requires prolonged stimulation and lacks the exquisite sensitivity of the cutaneous phenomena where a single hair of the cowhage nettle (Fig. 1A) or a single electrical pulse at C-fiber strength (Fig. 1C and E) elicits a small relatively brief dilator response. The participation of histamine, substance P, and other tachykinins in this response, as well as the necessary integrity of the primary afferent neuron, is accepted (12,14,19,20,26). The neurotoxin capsaicin has proved to be a useful tool for examining the role of substance P in these events (7,11). Given the numerous endogenous transmitters and autocoids in skin, to define the diversity of their cellular actions and interactions on adjacent nerve endings and microvessels resembles a puzzle of daunting complexity.

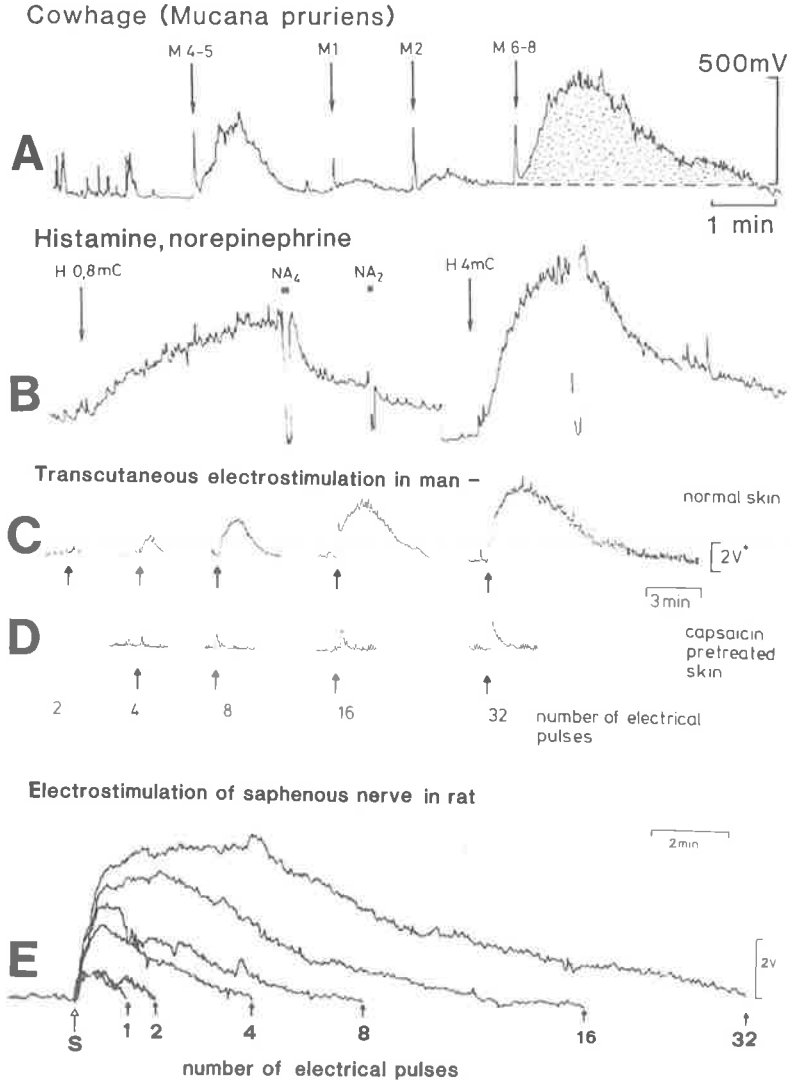


FIG. 1. A: Responses of skin blood flow to insertion (arrows) of cowhage spicules. B: Laser Droppler recording of skin blood flow after iontophoretic application of histamine (H) and norepinephrine (NA). C: Normal control responses of human forearm to transcutaneous electrical stimulation. D: Capsaicin pretreatment of skin markedly reduces blood flow responses to electrical stimulation. E: Hairy skin of rat paw shows graded antidromic vasodilatation evoked by electrical stimulation of the saphenous nerve. The standard stimulus protocol of 1,2,4,8,16, and 32 pulses, (150 V, 0.5 msec, 2+Hz) is applied (arrows) in parts C,D, and E. Calibrations for laser blood flow signal (volts) and time (min) are shown for parts A-E.

QUESTIONS ON MECHANISMS

Although axon reflexes are of physiological significance in the nocifensive sense, they probably do not contribute to the regulation of microvascular blood flow (13). However, substance P is a potent releaser of histamine from

peritoneal and cutaneous mast cells (see Fig. 2 as well as refs. 3 and 10). Histamine has been implicated in active vasodilatation (6), and there is direct evidence that sympathetic postganglionic axons in cutaneous nerves are responsible for active vasodilatation in response to whole-body warming (22). Thus the question of other physiological roles of axon reflexes in the control of the cutaneous microcirculation remains unresolved. Furthermore, a range of endogenous mechanisms exist whereby vasoconstriction can be inhibited. Important among these are the adenine nucleotides, prostaglandin-like substances, and histamine (21). Finally, the vascular endothelium itself is intimately involved with the responses of vascular smooth muscle (4).

MEASUREMENT STRATEGIES

To this end, advantage was taken of newer technology to reexamine neurogenic vasodilatation. The strategy was to sensitively monitor changes in microvascular blood flow using a Periflux PF1d laser Doppler device and measure the response as a voltage time integral, i.e., the area under the blood flow response curve (23). This enables the change in flow to be measured, although the resting flow cannot be interpreted directly as absolute skin blood flow. The polymodal C-nociceptors, which mediate the flare of the axon reflex, are excited by a variety of adequate stimuli, including noxious, thermal, mechanical, chemical, or electrical stimuli. Several different methods of exciting vasodilator axon reflexes were examined, for example, by application of cowhage endopeptidase, percutaneous iontophoresis of vasodilator drugs, and electrical stimulation of skin nerves by various techniques. For example, graded flux increases (vasodilatations) were seen in response to insertion of 1,2,4 to 5 or 6 to 8 spicules of cowhage (Mucana pruriens) near the laser probe (Fig. 1A). The vasoactive ingredient in cowhage is an endopeptidase (mucanain) which produces strong itch sensations and a chemically induced flare when inserted into the skin. This is comparable with the reaction to iontophoretically applied histamine given as two doses, 0.8 and 4.0 mC (millicoulombs) (Fig. 1B; see also ref. 24). The axon reflex response is dependent on nociceptor stimulation but not on participation

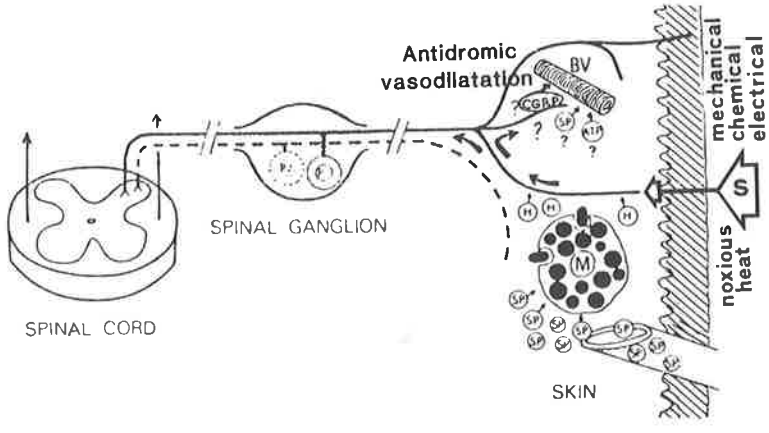


FIG. 2. Illustration of the mechanisms that interact to produce antidromic vasodilatation and itch or pain sensations. (Modified from ref. 10.) Noxious stimuli (heat, mechanical, electrical, or chemical) applied to skin result in degranulation of mast cells and excitation of nociceptor primary afferent endings. Two results follow: Primary afferent nerve impulses entering the central nervous system evoke sensations of prickle, itch, burning, or pain; and histamine (H), substance P (SP), and various tachykinins and autocoids (not shown) are believed to act on either nerve endings, small blood vessels, or mast cells. Most are potent dilators of skin microvessels. Antidromic nerve activity and mast cell products cause vasodilatation and spreading hyperalgesia.

of the central nervous system (2). Proximal nerve block with a local anesthetic drug does not affect the skin response to percutaneous nerve stimulation except to abolish accompanying itch or pain sensations mediated by nociceptor input to the central nervous system (23). If electrical stimuli at a strength adequate to excite C-nociceptors are delivered to the distal cut end of the saphenous nerve of the rat (Fig. 1E), brief vasodilatations are elicited. These are associated with the release of substance P, as demonstrated by blister-base superfusion studies (26). The brief flare responses elicited by electrical stimulation are gradable, with the intensity of stimulation expressed as the total number of pulses applied. The antidromic response in blood flow to electrical stimulation is similar in the glabrous or hairy skin of the rat paw, human forearm, hand, or foot dorsum (Fig. 1C,D, and E; Fig. 3). These responses are evoked by percutaneous electrodes on intact skin or by intracutaneous needles; all closely resemble those elicited by direct intraneural microstimulation (2) and presumably utilize the same antidromic mechanisms.

The results of capsaicin pretreatment of the skin on the human forearm and foot dorsum (2,23) and of hairy and glabrous skin of the rat paw (R. A. Westerman et al., unpublished observations) are similar: a marked reduction in blood flow response to comparable stimulation is observed (Fig. 1D and E; Fig. 3). This is consistent with the known desensitization and depletion of substance P in primary afferents by capsaicin treatment (13).

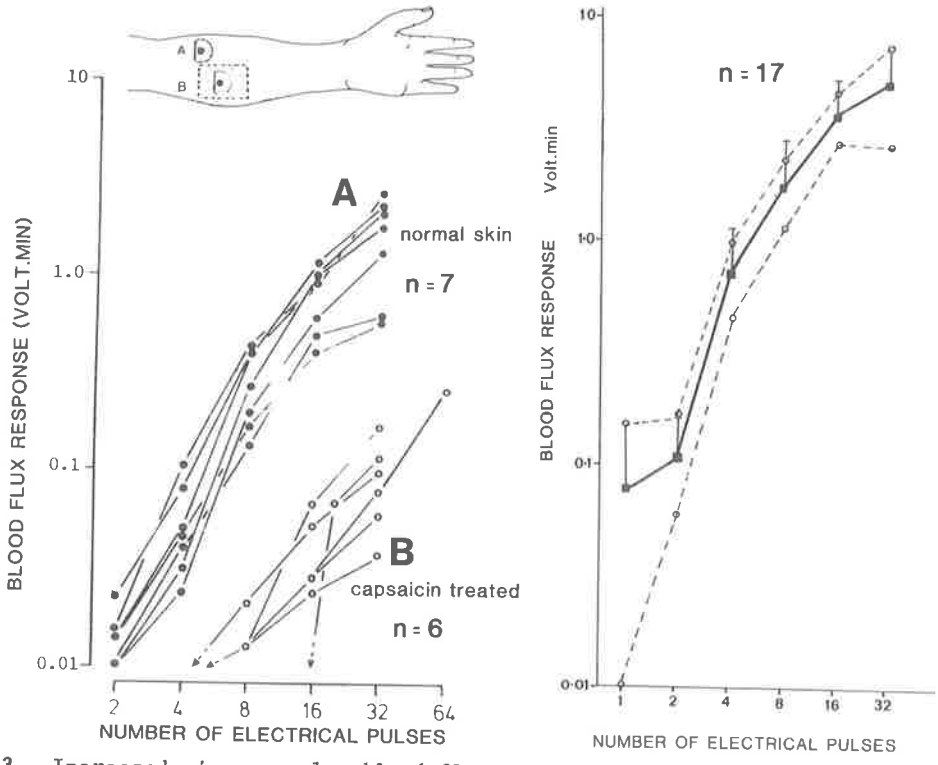


FIG. 3. Increased microvascular blood flow responses to percutaneous electrical stimulation of human forearm with stimulus parameters used in Fig. 1C,D, and E. Responses of normal skin (●) and capsaicin-treated skin (○) in the same subjects are shown. The transient flow increases (like those in Fig. 1C and D) are plotted logarithmically as areas (volt • min) on the ordinate and as the number of electrical pulses on the abscissa. In addition, mean responses with standard errors (vertical bars) and 95% confidence limits (dashed lines) from 17 normal volunteers are plotted logarithmically against number of pulses to give a typical dose-response graph.

CLINICAL SIGNIFICANCE

The signals obtained with photoplethysmography or laser Doppler techniques are not an absolute measure of microvascular blood flow. Nevertheless, local vasodilator axon reflexes evoke brisk increases in skin blood flow which can be sensitively detected by these techniques and can allow the change from the resting flow to be measured. The measurement and logarithmic plotting of flux response • time integral permits comparison of flux reaction to a standard stimulus protocol given to normal subjects and patients with suspected C-fiber neuropathy. This is a more quantitative approach to measuring neurogenic flare and may provide a useful noninvasive clinical test of nociceptive function. Further, among the various diseases commonly associated with neuropathy of thin nerve fibers, diabetes is notable for also disturbing microvascular function. Unfortunately, current electrophysiological methods (nerve conduction studies, electromyography) only permit the assessment of damage to large myelinated nerve fibers. Therefore, tests of the function of C-fibers and of the microvascular reactivity in diabetes would be particularly useful; one possibility is the iontophoretic technique (see, e.g., Fig. 1B) for in vivo pharmacological study of the microvascular responses to a range of cholinergic, histaminergic, and alpha- and beta-adrenergic agonists and blockers (18,25). These substances are applied locally to the intact skin zone being monitored by the laser Doppler flow meter, and, provided that the currents are small (less than 0.4 mA), there is no effect from the iontophoresis itself. It is hoped that this micropharmacological approach, together with the sensitive flow monitoring provided by the laser Doppler, will prove to be a valuable technique in exploring the mechanisms underlying the vasodilatation caused by the axon reflexes. In this context we have recently demonstrated reduced axon reflexes in diabetics (25).

REFERENCES

1. Bayliss, W. M. (1901): On the origin from the spinal cord of the vasodilator fibres at the hind limb and on the nature of these fibres. J. Physiol., 26: 73-209.
2. Blumberg, H., and Wallin, B. G. (1987): Direct evidence of neurally mediated vasodilatations in hairy skin of the human foot. J. Physiol., 382:105-121.
3. Erjavec, F., Lembeck, F., Florjanc-Irman, T., Skofitsch, G., Donnerer, J., Saria, A., and Holzer, P. (1981): Release of histamine by substance P. Naunyn Schmiedebergs Arch. Pharmacol., 317:67-70.
4. Furchgott, R. (1983): Role of endothelium in responses of vascular smooth muscle. Circ. Res., 53:557-573.
5. Gibbins, I. L., Furness, J. B., Costa, M., MacIntyre, I., Hillyard, C. J., and Girgis, S. (1985): Colocalization of calcitonin gene related peptide-like immunoreactivity with substance P in cutaneous, vascular and visceral sensory neurons of guinea pigs. Neurosci. Lett., 57:125-130.
6. Heitz, D. C., and Brody, M. J. (1975): Possible mechanisms of histamine release during active vasodilatation. Am. J. Physiol., 228:1351-1359.
7. Helme, R. D., and McKernan, S. (1985): Neurogenic responses following topical application of capsaicin in man. Ann. Neurol., 18:505-509.
8. Hilton, S. M., and Marshall, J. M. (1960): Dorsal root vasodilatation in cat skeletal muscle. J. Physiol., 229:277-288.
9. Hinsey, J. C., and Gasser, H. S. (1930): The component of the dorsal root mediating vasodilatation and the Sherrington contracture. Am. J. Physiol., 92:679-689.
10. Hökfelt, I., Johnson, O., Kellerth, J. O., Ljungdahl, A., Nilsson, G., and Pernow, B. (1977): Immunohistochemical distribution of substance P. In: Substance P, edited by U.S. von Euler and B. Pernow, pp. 117-145. Raven Press, New York.
11. Jansco, N., Jansco-Gabor, A., and Szolcsanyi, J. (1967): Direct evidence for neurogenic inflammation and its prevention by denervation and by pretreatment with capsaicin. Br. J. Pharmacol. Chemother., 31:138-151.

12. Kenins, P., Hurley, J. V., and Bell, C. (1984): The role of substance P in the axon reflex in the rat. Br. J. Dermatol., 111:551-559.
13. Lembeck, F. (1983): Sir Thomas Lewis' nocifensor system, histamine and substance P containing primary afferent nerves. Trends Neurol. Sci., 6:106-108.
14. Lembeck, F., and Gamse, R. (1982): Substance P in peripheral sensory processes. In: Substances P in the Nervous System: CIBA Foundation Symposium, Vol. 91, edited by R. Porter and M. O'Conner, pp. 33-54. Pitman, London.
15. Lewis, T. (1927): The Blood Vessels of the Human Skin and Their Responses. Shaw and Sons Ltd., London.
16. Lewis, T. (1937): The nocifensor system of nerves and its reactions. Lecture I. Br. Med. J., 1:431-434.
17. Lewis, T. (1937): The nocifensor system of nerves and its reactions. Lecture II. Br. Med. J., 1:491-494.
18. Lindblad, L. E., and Ekenvall, L. (1986): Alpha adrenoceptors in vessels of human finger skin. Acta Physiol. Scand., 128:219-222.
19. Lundberg, J. M., Saria, A. Rosell, S., and Folkers, K. (1984): A substance P antagonist inhibits heat induced oedema in the rat skin. Acta Physiol. Scand., 120:145-146.
20. Nagy, J. I. (1982): Capsaicin's action on the nervous system. Trends Neurol. Sci., 5:362-365.
21. Vanhoutte, P. M., and Leusen, I. (editors) (1981): Vasodilatation, pp. 1-536. Raven Press, New York.
22. Westerman, R. A., Blumberg, H., Hollerback, M., Jäger, M., and Kiss, J. (1986): Direct microneurographic and pharmacological evidence for the existence of sympathetic neurons mediating active vasodilatation in human skin. Proc. Aust. Physiol. Pharmacol. Soc., 17(1):23P.
23. Westerman, R. A., Low, A., Pratt, A., Hutchinson, J. S., Szolcsanyi, J., Magerl, F. W., Handwerker, H. O., and Kozak, W. (1987): Electrically evoked skin vasodilatation: A quantitative test of nociceptor function in man. In: Clinical and Experimental Neurology, Vol 3, edited by M. Eadie, pp. 81-89. Adis Press, Sydney.
24. Westerman, R. A., Magerl, F. W., Handwerker, H. O., and Szolcsanyi, J. (1985): Itch sensation evoked by percutaneous microiontophoresis of histamine on human hairy skin. Proc. Aust. Physiol. Pharmacol. Soc., 16(1):79P.
25. Westerman, R. A., Widdop, R. E., Hogan, C., Low, A., and Zimmet, P. (1987): Reduced axon reflex responses in human diabetes mellitus: Non-invasive tests of neurovascular function. Proc. Aust. Physiol. Pharmacol. Soc., 18(1):96P.
26. White, D. M., and Helme, R. D. (1985): Release of substance P from peripheral nerve terminals following electrical stimulation of sciatic nerve. Brain Res., 336:27-31.

2.

12

In: Clinical and Experimental Neurology ,

24: 129-137 (1987).

Non-invasive Tests of Neurovascular Function

R. A. Westerman, R.E. Widdop,† J. Hannaford,† C. Hogan,* R. Roberts* and P. Zimmet‡*

The neurogenic nocifensor system of skin, originally described by Lewis,[1] depends upon activity in primary afferent nerves, especially the release of peptides, particularly substance P, from the C-polymodal nociceptors.[2] The protective mechanisms evoked by nociceptor activity are summarised in Figure 1.

Recently, we have developed and reported a non-invasive technique for measuring small nerve fibre function by monitoring skin blood flow during noxious transcutaneous electrical nerve stimulation (TENS).[3,4] If the polymodal C-nociceptor is functioning, such noxious stimulation results in cutaneous axon reflex vasodilatation which can be measured with a laser Doppler flowmeter.

This paper describes impairments to some of these mechanisms in subjects with diabetes mellitus. Diabetes mellitus often leads to disturbances of small nerve fibres and blood vessels in the skin. Consequently, pain, sweating disorders, frequent skin injury and infections are common in diabetics.[5,6] Sensory nerve damage and reduced skin blood flow can even lead to skin ulceration and loss of digits.[5] Current methods of nerve testing assess damage only to large myelinated A-nerve fibres.[7] However, smaller nerves (C-fibres) may also be affected early in the disease[5,8] and a more sensitive, specific and portable method of assessing their function would be a major development for evaluating diabetic neuropathy in clinical, epidemiological and research settings.[8]

Departments of *Physiology and †Pharmacology, Monash University, Clayton, Vic. ‡Lions International Diabetic Institute, Royal Southern Memorial Hospital, Caulfield, Vic.

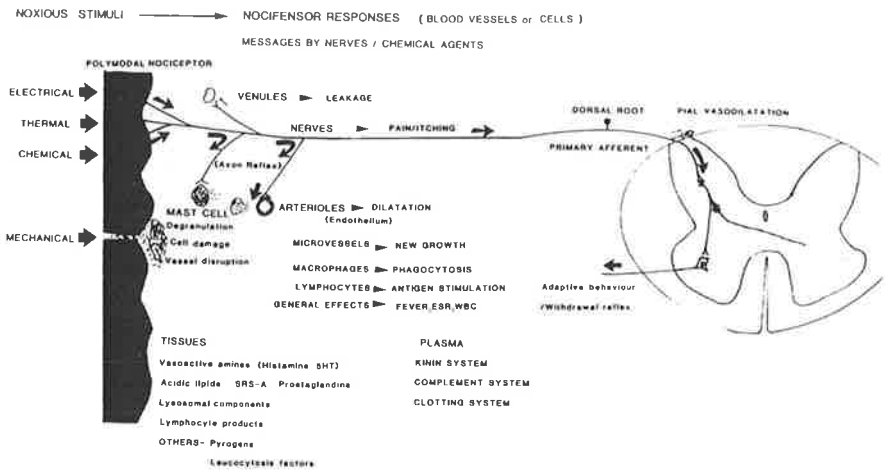


Figure 1. Summary of the mechanisms which are involved in the nocifensor responses evoked by noxious stimulation of skin. Adequate stimulation (mechanical, chemical, thermal or electrical) results in responses of blood vessels or cells, mediated by nerves or chemical agents.

Several results follow: primary afferent nerve impulses arising from polymodal nociceptors enter the CNS to evoke sensations of prickle, itch, burning or pain. Reflex withdrawal or voluntary adaptive behaviour ensues. Histamine, serotonin, substance P and other autacoids act on nerve endings, small blood vessels or mast cells. Arteriolar vasodilatation and venular leakage result, as well as the activation of various plasma and tissue systems, which are listed. Antidromic (axon reflex) nerve activity and mast cell products produce vascular flare and spreading hyperalgesia. The microvascular endothelium is involved in vasodilatation.

Materials and Methods

The results presented here extend our earlier observations in healthy subjects and diabetic patients, all of whom were currently receiving insulin therapy.[9] According to previous clinical histories, patients were categorised as being with or without previous large fibre neuropathy.[5] All subjects gave informed consent to the studies which were approved by the Monash University and Royal Southern Memorial Hospital Ethics Committees. They were seated in a room with an ambient temperature of 21–24 °C, with the leg slightly extended so that the foot could be supported comfortably approximately 20 cm above the ground. Skin blood flow was measured with a helium–neon laser Doppler flowmeter (Periflux Model PF1d, Perimed, Sweden). A plastic holder was attached to the dorsum of the foot by a double-sided adhesive disc and the laser probe was placed in the holder to measure microvascular blood flow. This is recorded as an analog output voltage signal, which is directly proportional to the product of the number of erythrocytes within the epidermal measuring volume and their mean velocity.[10]

Skin vasodilatation was evoked by either TENS or the techniques of percutaneous iontophoresis of vasodilator agents.[11] For TENS, the cathode was a 30 mm^2 gold-plated disc electrode taped onto the skin 6 mm from the laser probe while a similar

indifferent anodal electrode was placed 5 cm proximal to the test site. Preliminary tests showed that the minimum stimulation giving reproducible responses in normal foot dorsum skin was a series of pulses of 150 V, 0.75 ms in duration, at 2 Hz. The total number of pulses delivered in each brief train ranged from 1 to 16 and, in healthy subjects, this produced pain or discomfort at the cathode site, as described for the stimulation of the forearm.[3,4] A battery-powered constant current stimulator (WPI 502R) was used to provide a direct (galvanic) current for drug iontophoresis. Acetylcholine and sodium nitrite ($NaNO_2$) were used as endothelium-dependent and endothelium-independent vasodilators, respectively.[12] The acetylcholine and $NaNO_2$ were dissolved at 1% concentrations (w/v) in an inert aqueous gel (2% methyl cellulose) and applied to an Ag–AgCl plug in a hemispherical 50 μL chamber. This was part of a cylindrical perspex applicator which served as the active electrode and fitted into the laser probe holder. Therefore, it was possible to provide a discrete transfer of a polar vasoactive drug into the epidermis in the immediate skin area under the probe by using small currents of brief duration (expressed as the total charge in milliCoulombs). The resulting change in blood flow is measured by the laser Doppler probe after its re-insertion into the holder. Since this protocol was chiefly designed to examine the C-nociceptor nerve function clinically, time constraints allowed testing of only one concentration of each test drug in each patient. From preliminary experiments the following stimuli were chosen, using 1% solutions of acetylcholine chloride and $NaNO_2$: anodal current of 0.2 mA for 10 s (acetylcholine) and cathodal current of 0.2 mA for 20 s ($NaNO_2$). Acetylcholine was normally applied to the laser site previously tested by adjacent TENS, while a new site on the dorsum of the foot was used to apply $NaNO_2$. At these low current levels, the inert gel used to dissolve the drugs had a negligible effect on skin blood flow.

Vasodilator responses were quantified by measuring the area of each response, calculated as the voltage·time integral using a Zeiss MOP image analysis computer with a magnetic tablet and stylus attached. For the TENS data, the logarithms of responses and stimuli were then plotted as previously described.[3] The means and standard errors of the means were calculated for the standard iontophoretic stimuli. However, if the vasodilator responses to acetylcholine and $NaNO_2$ were still elevated at 4 min, this time was chosen as the period for calculation of the voltage·time integral.

In addition, the TENS and iontophoretic stimuli were tested in a 39 year old man who had accidently completely severed his left ulnar and median nerve (radial nerve intact) 15 days previously.

Results

In a previous study, we examined cutaneous neurovascular changes in 19 healthy subjects and 30 diabetic patients.[9] We have now extended these findings to 29 control and 40 diabetic patients, and essentially the same results have been obtained (see Figure 2). TENS was perceived as painful in all control subjects and this produced vasodilatation, the size of which was dependent on the number of

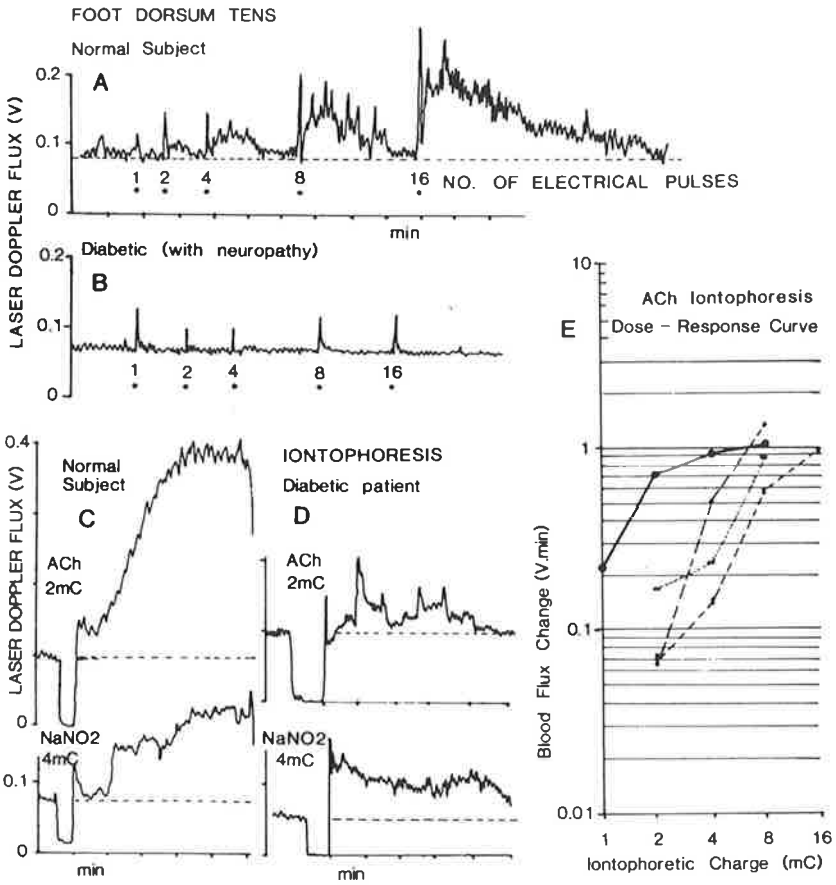


Figure 2. Recordings of changes in skin microvascular blood flow in the foot dorsum of 2 control subjects and 2 diabetic patients with neuropathy, in response to transcutaneous electrical nerve stimulation (TENS) and the iontophoresis of acetylcholine (ACh) and sodium nitrite ($NaNO_2$). Increases in blood flow were measured by a laser Doppler flowmeter and are given as increases in voltage (V).

(A) Effect of TENS (1–16 pulses, 150 V, 0.75 ms, at 2 Hz) and (C) acetylcholine (1%, 0.2 mA, 10 s) and $NaNO_2$ (1%, 0.2 mA, 20 s) in 2 control subjects.

(B) Effect of TENS and (D) acetylcholine and $NaNO_2$ in 2 diabetic patients with neuropathy.

(E) Mean dose–response curve to acetylcholine in 6 control subjects (full line) and individual dose-response curves to acetylcholine in 3 diabetic patients with neuropathy. Data plotted on double log axes, with the dose of acetylcholine (expressed as the charge in milliCoulombs (mC)) against the corresponding increase in skin blood flow calculated as a V min integral.

pulses applied. This can be seen as a progressively increasing response when the number of noxious electrical pulses was increased (Figure 2A). All vasodilator responses returned to baseline levels within 2–4 minutes. In contrast, diabetic patients with clinical neuropathy could barely perceive the stimuli and could not

always confirm the number of pulses received. To date, we have performed TENS in 31 patients with clinical neuropathy. In this group, the pulse-related axon reflex vasodilator responses evoked by TENS were markedly depressed (Figure 2B). In addition, in the subacutely denervated subject, TENS caused negligible effects in the denervated skin (Figure 3).

To test the functional integrity of the microvasculature, acetylcholine and $NaNO_2$ were also iontophoresed across the epidermis of the foot dorsum. In 29 control subjects tested, acetylcholine caused immediate vasodilatation with only approximately 25% of the responses completed in the 4 minute calculation period (n = 45 responses). $NaNO_2$ also caused vasodilatation although, compared with acetylcholine, these responses were smaller and more variable in that there was sometimes a delay of 1–2 minutes before vasodilatation commenced, but thereafter remained elevated (Figure 2C). In contrast, in diabetic patients with neuropathy, acetylcholine caused markedly less vasodilatation (n = 36 responses) than controls, while $NaNO_2$-induced vasodilatation was less affected (n = 18 responses) (Figure 2D). In addition, in 6 normal subjects and 3 diabetic patients with neuropathy, dilator responses to graded iontophoretic doses (in milliCoulombs) of acetylcholine were recorded. In comparison with the mean control data, the dose–response curve to acetylcholine in each of the diabetic patients was shifted to the right (Figure 2E).

In one additional subject with ulnar and median nerve lesion (see Figure 3), acetylcholine (2 mC) as well as histamine dihydrochloride (2 mC) and sodium nitroprusside (8 mC) were iontophoresed across both the intact and denervated skin regions (see Figure 3). Vasodilator responses to all 3 drugs were obtained at both sites, although these were smaller in the denervated region. Since skin electrical resistance was also increased by approximately 10000 ohms, acetylcholine and histamine (each 100 µL of 1% w/v) were injected intradermally to both the intact and denervated skin. It was found that acetylcholine and histamine both caused a local wheal and flush at and immediately surrounding the injected sites in both the intact and denervated skin. However the axon reflex spreading flare caused by histamine in the intact skin was absent in the denervated region. Acetylcholine caused a less marked axon reflex flare compared with histamine, and again this was seen only in the intact skin.

Discussion

The present study has illustrated the value of a series of non-invasive neurovascular function tests in the clinical setting. These results confirm and extend our earlier study,[9] indicating that diabetic patients with diagnosed large fibre neuropathy also exhibit other neurovascular changes that can readily be detected with the aforementioned techniques. Axon reflex vasodilatation evoked by TENS persists after local proximal anaesthesia but is markedly attenuated by pretreatment of the skin with capsaicin, a neurotoxin known to deplete primary afferent fibres of substance P.[13] This neurogenic flare is thought to involve the local antidromic stimulation of C-fibres which cause the release of substance P (and

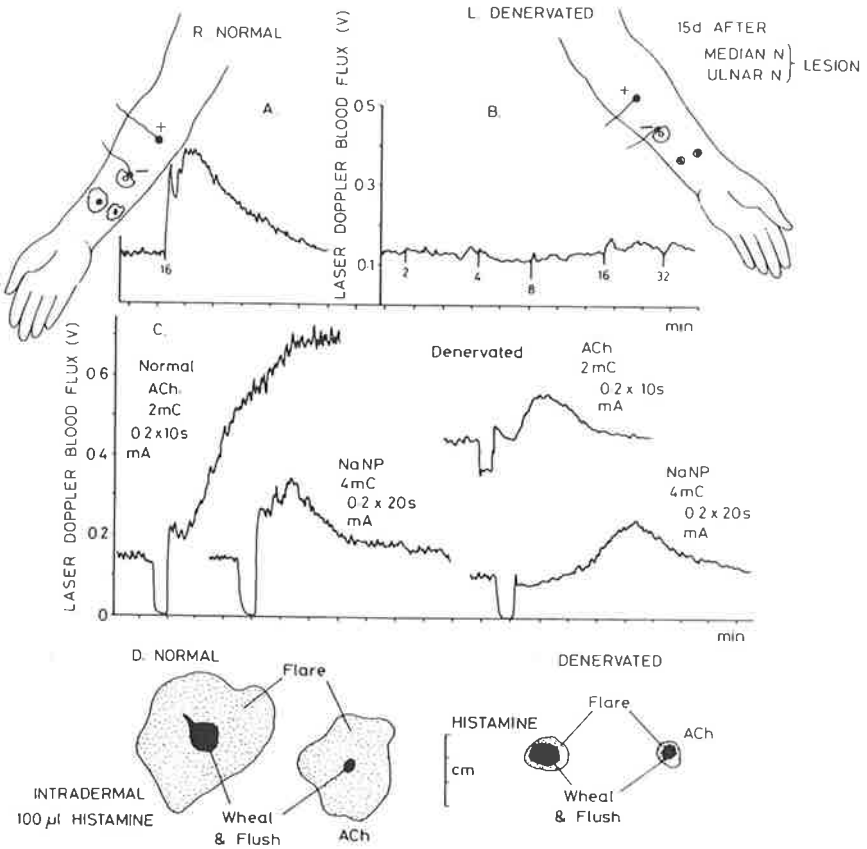


Figure 3. (A) Normally innervated skin response to 16 pulses (150 V, 0.75 ms, 2 Hz).

(B) Illustrates the absence of any vasodilator responses of hairy skin of the left forearm to noxious TENS (2–32 pulses, 150 V, 0.75 ms, 2 Hz) applied 15 days after accidental complete ulnar and median nerve transection by a chain saw.

(c) In denervated skin, vasodilator responses to acetylcholine and sodium nitroprusside are evident but reduced, compared with responses of normally innervated skin to the same iontophoretic charge.

(D) Intradermal injections of acetylcholine and histamine (100 µL 1% w/v) were made into corresponding innervated and denervated areas of right and left forearm, respectively. Tracings of wheal (dark) and flare (stippled) are shown. Axon reflex flare (which is neurogenic) is abolished for both substances in denervated skin, but the wheal and flush immediately surrounding the acetylcholine and histamine site are both normal, suggesting that local direct effects on microvasculature persist after denervation.

possibly other mediators).[2,3] Confirmation that this is a neurogenic event was obtained by the absence of electrical axon reflex vasodilatation in skin 15 days after total denervation, which is consistent with the original observations of Lewis.[1] Similar axon reflex flare responses in man have also recently been obtained using

painful intraneural stimulation of the superficial peroneal nerve at the ankle.[14] As in the forearm,[3] TENS evoked a transient pulse-related flare in the dorsum of the foot, which occurred only if the stimulus was painful and applied in the area immediately adjacent to the laser probe. However, in both groups of diabetic patients (with and without neuropathy), there were shifts to the right in the stimulus–response curve. This was more marked in the diabetic patients with clinical neuropathy and therefore suggests an additional dysfunction of primary afferent fibres.[9] This is consistent with an earlier report of reduced histamine-induced axon reflex flare in diabetic patients.[6] However, in that previous study, vasodilatation was assessed indirectly by the rise in skin temperature following intradermally injected histamine.

In this and other[6,9,15] studies, the reduced axon reflex vasodilatation in patients with diabetic neuropathy suggests loss of afferent C-fibre function. However, since the neurogenic flare depends ultimately on the functional integrity of the microvasculature in series with the primary afferent fibres, it is possible that functional impairment or damage to either the vascular endothelium or the vascular smooth muscle itself could contribute to or even be largely responsible for the observed effects. Therefore acetylcholine or $NaNO_2$ was iontophoresed directly across the skin barrier into the area measured by the laser probe in order to examine vascular endothelial and smooth muscle function, respectively. This technique differs from other iontophoretic techniques[15] in that low concentrations of vasodilator drugs were iontophoresed using small currents of much briefer duration. Further the skin immediately iontophoresed by acetylcholine was examined and *not* a distant site. These parameters and geometry were insufficient to evoke axon reflex vasodilatation (i.e. spreading of flare), which is consistent with the relative insensitivity of normal isolated polymodal nociceptors to applied acetylcholine.[16] Moreover, the local vasodilator responses to acetylcholine and to $NaNO_2$ were also largely unaffected by pretreatment of the skin with capsaicin.[17] Acetylcholine is thought to cause vasodilatation mainly via the release of endothelium-derived relaxing factor (EDRF) from the vascular endothelium (with a lesser contribution from axon reflex mechanisms), while nitrovasodilators act directly on the blood vessels.[12] Thus, the reduced iontophoretic vasodilator responses to acetylcholine, but not to $NaNO_2$, in patients with diabetic neuropathy suggest that endothelial function is impaired although the microvascular smooth muscle appears to be responding normally.

Another possible contribution to the reduced TENS- and acetylcholine-induced vasodilator responses exhibited by diabetic patients with neuropathy may be an increased skin electrical impedance associated with reduced sudomotor activity.[18] This was highlighted by the iontophoretic results in the denervated skin (Figure 3). This region of skin was extremely dry and the skin electrical impedance was markedly elevated. Also, as part of the nursing care, the skin was treated daily with barrier cream. This may have limited drug transfer across the skin and may have contributed to the reduced acetylcholine-induced dilator response. However the microvasculature appeared to be functioning normally, and independently of nerves, since intradermal injections of acetylcholine and histamine still caused similar local vasodilatation in normal and denervated skin (in spite of the absence

of an axon reflex flare in the latter). Furthermore, in the present study, impedance changes were unlikely to be a major factor because the TENS used markedly suprathreshold stimulation for C-fibre activation, while a constant current stimulator was used for drug iontophoresis. Moreover, the fact that responses to both acetylcholine and $NaNO_2$ were not altered uniformly would argue against an increased skin electrical impedance limiting drug penetration as the only factor involved (although the transport numbers of the two compounds are not identical).

It is possible that impaired endothelial function, demonstrated using iontophoretic acetylcholine, may have in fact contributed to the reduced electrically evoked axon reflex in both groups of diabetic patients with and without diabetic neuropathy. This could occur if part of the vasodilatation evoked by TENS is due to neurogenically released substance P causing the release of EDRF. Substance P binding sites have recently been demonstrated on endothelial cells,[19] and this would explain the strictly endothelium-dependent vasodilator nature of substance P.[12] Thus, impaired endothelial function (either EDRF synthesis or release) could augment any primary afferent dysfunction. In this context, recent evidence suggests that vascular changes may be causally important in the development of diabetic neuropathy.[20] The present results would implicate the vascular endothelium as a primary target for this metabolic disorder and suggest that reduced skin nocifensor functions may contribute to some of the symptoms and complications of diabetes mellitus. Thus, this study has emphasised the importance of testing different components of the axon reflex response separately, using the non-invasive methods described.

Summary

This paper describes the value of non-invasive neurovascular function tests in the clinical setting. Painful transcutaneous electrical nerve stimulation (TENS) of the dorsum of the foot evoked axon reflex vasodilatation, measured by laser Doppler flowmetry. In addition, acetylcholine and sodium nitrite ($NaNO_2$) were iontophoresed to cause vasodilatation by endothelium-dependent and -independent mechanisms, respectively. Compared with healthy volunteers, diabetic patients with clinically diagnosed neuropathy showed reduced electrical axon reflex flare responses. These responses in one additional subject were absent in a region of denervated skin. Acetylcholine responses, but not $NaNO_2$ responses, were also depressed in patients with diabetic neuropathy. Such reduced cutaneous nocifensor functions may contribute to some symptoms and complications of diabetes mellitus.

Acknowledgements

This study was supported in part by Apex-Australian Diabetes Foundation and Monash Special Research grants.

References

1. Lewis T. The blood vessels of the human skin and their responses. London: Shaw & Sons, 1927.
2. Lembeck F. Sir Thomas Lewis's nocifensor system, histamine and substance-P-containing primary afferent nerves. Trends Neurol Sci 1983; 6: 106–108.
3. Westerman RA, Low A, Pratt A, *et al*. Electrically evoked skin vasodilatation: a quantitative test of nociceptor function in man. Clin Exp Neurol 1987; 23: 81–89.
4. Westerman RA, Magerl FW, Szolcsanyi J, *et al*. Vasodilator axon reflexes. In: Vanhoutte PM, ed. Mechanisms of vasodilatation IV. New York: Raven Press, 1987 (in press).
5. Asbury AK, Brown MJ. Clinical and pathological studies of diabetic neuropathies. In: Goto Y, Horiuchi A. Kogure K, eds. Diabetic neuropathy. Amsterdam: Excerpta Medica, 1982; 50–57.
6. Hutchison KJ, Johnson BW, Williams HT, Brown GD. The histamine flare response in diabetes mellitus. Surg Gynecol Obstet 1974; 139: 566–568.
7. Dorfman LJ. The distribution of conduction velocities in peripheral nerves: a review. Muscle Nerve 1984: 7: 2–11.
8. Dyck PJ, Karnes JL, Daube J, O'Brien P, Service FJ. Clinical and neuropathological criteria for the diagnosis and staging of diabetic polyneuropathy. Brain 1985; 108: 861–880.
9. Westerman RA, Widdop RE, Hogan C, Zimmet P. Non-invasive tests of neurovascular function: reduced responses in diabetes mellitus. Neurosci Lett 1987; (in press).
10. Nilsson GE, Tenland T, Oberg PA. Evaluation of a laser Doppler flowmeter for measurement of tissue blood flow. IEEE Trans Biomed Eng 1980; 27: 597–604.
11. Westerman RA, Magerl FW, Handwerker HO, Szolcsanyi J. Itch sensation evoked by percutaneous microiontophoresis of histamine on human hairy skin. Proc Aust Physiol Pharmacol Soc 1985; 16: 79P.
12. Furchgott RF. The role of endothelium in the responses of vascular smooth muscle to drugs. Annu Rev Pharmacol Toxicol 1984; 24: 175–197.
13. Jansco N, Jansco-Gabor A, Szolcsanyi J. Direct evidence for neurogenic inflammation and its prevention by denervation and pretreatment with capsaicin. Br J Pharmacol 1967; 31: 138–151.
14. Blumberg H, Wallin BG. Direct evidence of neurally mediated vasodilatation in hairy skin of the human foot. J Physiol 1987; 382: 105–121.
15. Parkhouse N, Le Quesne PM. Laser Doppler measurement of acetylcholine-induced axon reflex flare to assess nociceptive c-fibre function. J Physiol 1987; 384: 3P.
16. Koschorke GM, Westerman RA, Zimmermann M. Histamine excites non-myelinated fibres in experimental neuroma of sural nerve in cat. Neurosci Lett 1985; 22 (suppl.): S32.
17. Roberts RGD, Westerman RA, Widdop RE, Kotzmann RR, Zimmet P. Effects of capsaicin on cutaneous axon reflex vasodilatation in man. Proc Aust Physiol Pharmacol Soc 1987; 18: 95P.
18. Low PA, Zimmerman BR, Dyck PJ. Comparison of distal sympathetic with vagal function in diabetic neuropathy. Muscle Nerve 1986; 9: 592–596.
19. Stephenson JA, Burcher E, Summers RJ. Autoradiographic demonstration of endothelium-dependent 125-I-Bolton-Hunter substance P binding to dog carotid artery. Eur J Pharmacol 1986; 124: 377–378.
20. Thomas PK. Vascular factors in the causation of diabetic neuropathy. Trends Neurol Sci 1987; 10: 6–8.

:betes Research and Clinical Practice, 5 (1988) 49–54
evier

.C 00203

Non-invasive tests of neurovascular function: reduced axon reflex responses in diabetes mellitus of man and streptozotocin-induced diabetes of the rat*

R. Westerman[1], R. Widdop[2], A. Low[1], J. Hannaford[2], W. Kozak[1] and P. Zimmet[3]

epartments of [1] Physiology and [2] Pharmacology, Monash University, Clayton, Vic. 3168, Australia, and [3] Lions International Diabetes Institute, RSMH, Caulfield, Vic. 3162, Australia

(Received 1 December 1987, accepted 4 January 1988)

y words: Skin vasodilatation; Axon reflex; Drug iontophoresis; Endothelium; (Diabetes mellitus)

mmary

ne prevalence of neuropathy and microangiopathy complicating diabetes mellitus led to our development non-invasive tests of neurovascular function. On the foot dorsum of consenting normal subjects, diabetic atients, normal and streptozotocin-induced (STZ) diabetic rats, transcutaneous electrical nerve stimulation TENS) with 1, 2, 4, 8, 16 pulses at 150 V, 0.75 ms, at 2 Hz, evokes transient cutaneous axon reflex vasolatation measured by a laser Doppler (Periflux Pfld). This tests the integrity of both the polymodal nociptor/primary afferent nerves and microvessels in the skin. TENS-evoked axon reflexes are reduced in abetics (particularly with neuropathy) and progressively in rats 40–100 days after STZ administration. This ould be reversed in rats by a single injection of 3 units of soluble insulin at 100 days post STZ. The icrovascular endothelium and smooth muscle are tested in man by measuring vasodilatation induced by ntophoretic application of 2 mC acetylcholine (ACh) and 4 mC sodium nitroprusside (NaNP), respectively. iabetics show reduced ACh-evoked endothelium-dependent vasodilator responses, but the direct smooth uscle (endothelium-independent) responses evoked by NaNP are not reduced. Such functional neurovasular disturbances probably underlie many complications of diabetes mellitus, and the potential for these to e reversible with appropriate therapy can now be examined with such neurovascular tests.

Introduction

Diabetes mellitus may lead to a number of neurovascular complications among which peripheral neuropathy and microangiopathy are quite prevalent. In the skin, disturbances of small nerve fibres

A preliminary account of these results was presented at the International Diabetes Federation Western Pacific Region Congress, 1987.

Address for correspondence: Prof. R.A. Westerman, Department of Physiology, Monash University, Clayton, Vic. 3168, ustralia.

and blood vessels may be manifest as pain, ulceration, reduced skin blood flow and loss of digits [1]. Such loss of function has been attributed to abnormalities in the unmyelinated (C) fibres [2]. Conventional methods of nerve testing only assess the function of large myelinated nerve fibres [3], although the afferent C fibres are affected in diabetes [1,4].

The neurogenic nocifensor system, originally described by Lewis [5], is an integral part of this primary afferent system. The flare component of Lewis' triple response is reduced in diabetes mellitus [6,7]. We have examined the effect of diabetes on this axon reflex mechanism by measuring changes in skin blood flow in the rat and man by laser Doppler flowmetry [8].

Materials and methods

Animals and patients

Female Wistar rats (150–190 g) were made diabetic by a single intravenous injection of streptozotocin (70 mg/kg, Upjohn Co.), while age-matched control rats received a vehicle (citrate buffer) injection. In the clinical studies, healthy subjects and diabetic patients with clinical neuropathy, who were receiving insulin, were studied [9,10]. All experiments were approved by the Monash University and Royal Southern Memorial Hospital Ethics Committees.

Electrical stimulation

In man, axon reflex vasodilatation was evoked by transcutaneous electrical nerve stimulation (TENS) applied to the foot dorsum. The resultant flare was measured adjacent (6–8 mm) to the cathode stimulating electrode by a Periflux Pfld laser Doppler flowmeter [9,11]. Analogous experiments were performed in rats, maintained under halothane anaesthesia, in which needle stimulating electrodes were placed intradermally overlying the saphenous neurovascular bundle. Skin blood flow was measured in the adjacent shaved region of the saphenous nerve distribution, distal to the electrodes.

In all experiments, electrical stimulation consisted of a series of 1–32 pulses delivered at 2 Hz (rats: 100 V, 1 ms; man: 150 V, 0.75 ms). The resultant neurogenic vasodilatation was quantified for both intensity and duration by measuring the area under the curve (voltage · time integral).

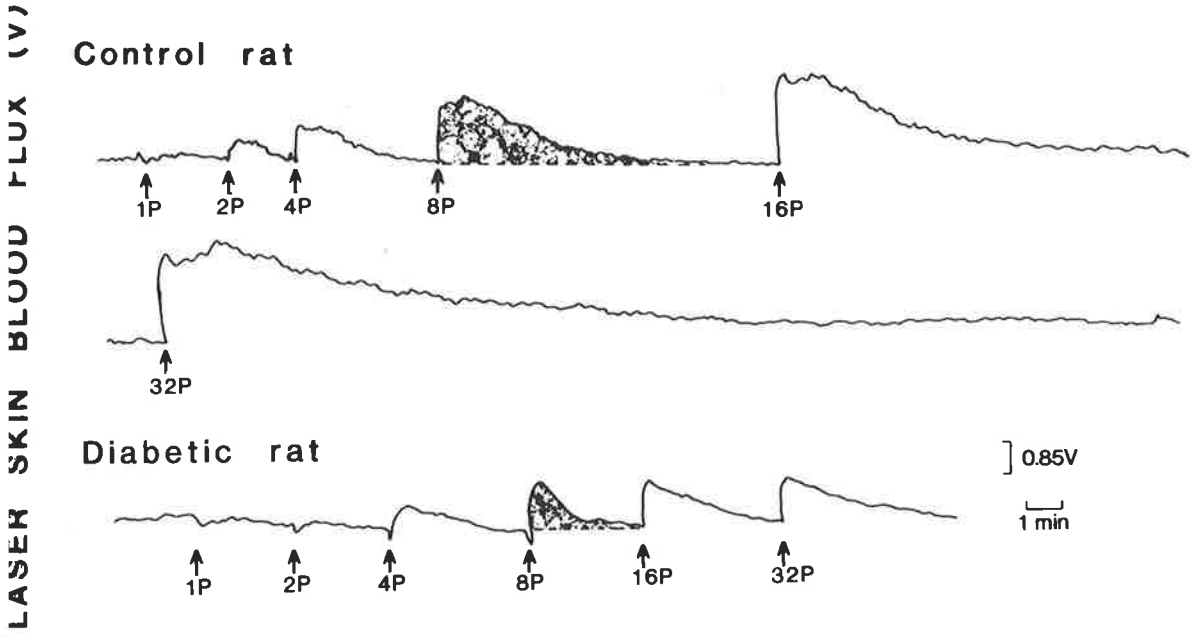
Drug application

The technique of iontophoresis [12] was also used to pass vasodilator drugs across the skin of the foot dorsum. Acetylcholine (ACh) or sodium nitroprusside (NaNP), dissolved as 1% aqueous solutions in 2% methyl cellulose gel, was applied in a 50-μl electrode chamber to the skin. A 0.2 mA galvanic (direct) current was then passed across the skin using a battery-powered constant-current stimulator (WPI 502R) [9,10]. For ACh, an anodal current was applied for 10 s while a cathodal current of 20 s was used for NaNP.

Results

Animals

In both control and diabetic rats, electrical stimulation was tested in the same animals at 20-day intervals, up to 100 days post streptozotocin (STZ) treatment [13]. In control rats, electrical stimulation produced vasodilatation, the magnitude of which was dependent on the number of electrical pulses received (see Fig. 1). In these animals there were no significant differences in the axon reflex responses tested over the initial 60-day test period, although responses tested at 80 and 100 days were significantly reduced. In contrast, there was a progressive reduction in the diabetic responses, beginning at 40 days post STZ. Fig. 1 shows the stimulus–response curve of a diabetic rat tested 60 days after STZ. It is evident that, when compared to the pre-diabetic level, there is a marked reduction in the axon reflex responses. This finding was consistently observed in rats tested 60–100 days after STZ. The effect of insulin on the axon reflex responses in rats 100 days after STZ was also examined. At this time insulin (3 units) was injected subcutaneously 20–30 min before testing. Insulin did not alter resting skin blood flow, however, it returned the stimulus–response curve to the pre-diabetic position [13].



g. 1. Recordings of changes in skin microvascular blood flow in response to electrical stimulation in an age-matched control rat (upper o panels) and a diabetic rat (lowermost panel) tested 60 days after the administration of vehicle and streptozotocin (70 mg/kg i.v.), spectively. The electrical stimulation consisted of a series of 1–32 pulses (100 V, 1 ms, at 2 Hz) applied to intradermal needles in the in overlying the saphenous nerve. Increases in blood flux were measured immediately adjacent to the cathodal site by a Periflux Pfld ser Doppler flowmeter, and are given as increases in voltage (V). The magnitude and duration of the vasodilatation were determined by the area under the curve (e.g., 8P, stippled area) which is measured as a voltage · min integral.

atients

ENS, applied to the foot dorsum of control and iabetic patients, was perceived as painful only in ontrol subjects. As in rats, this produced a stimlus-related vasodilatation which returned to aseline levels within 2–4 min. However, in diabetic atients with neuropathy, the responses to electrical imulation were significantly depressed when comared to control subjects, while diabetic patients ithout neuropathy exhibited responses which lay etween those of control and neuropathic subjects ee Fig. 2).

When iontophoresed across the foot dorsum, oth ACh (2 mC) and NaNP (4 mC) caused vasoilatation in control subjects. This was immediate or ACh while there was often a delay of approxiately 1 min using NaNP. Due to time constraints, nly one concentration of each drug was tested in ese patients, and the voltage · time integral was alculated for a 4-min period if the response had not returned to resting levels. In comparison with control subjects, diabetic patients with neuropathy exhibited depressed responses to ACh, although those to NaNP appeared relatively normal (see Fig. 3).

Discussion

Over the past 2 years, a familiar pattern of responses has emerged from these neurovascular tests [9,10]. Diabetic patients with large fibre neuropathy also exhibit abnormalities of the primary afferent C fibres, which confirms earlier studies [6,7]. This assumption is based on the marked reduction of TENS responses by pretreatment of skin with capsaicin [11], a substance known to deplete substance P from primary afferent fibres [14]. Furthermore, responses to TENS were abolished in a region of skin which had been denervated accidentally [10].

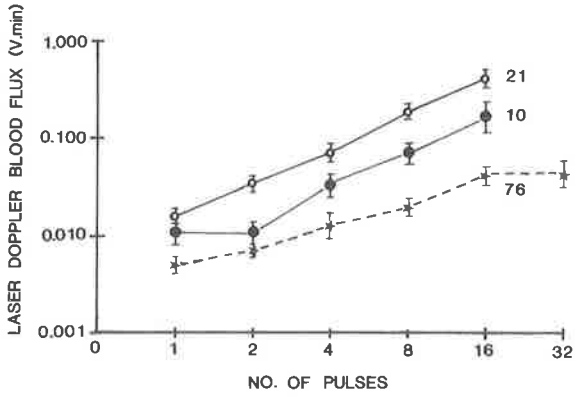


Fig. 2. Increases in microvascular blood flow (defined as laser Doppler blood flux) on the foot dorsum of control subjects and diabetic patients in response to noxious transcutaneous electrical nerve stimulation (TENS). The TENS consisted of a series of 1–32 pulses (150 V, 0.75 ms, at 2 Hz). Data plotted on double log axes with the number of electrical pulses delivered to foot dorsum on the abscissa, and the corresponding changes in blood flow, calculated as a $V \cdot$ min integral from the laser Doppler analog record, plotted on the ordinate. Each point on the graph is the mean, and vertical bar the SEM, of responses from control subjects (open circles, $n = 21$), diabetic patients without neuropathy (closed circles, $n = 10$) and with neuropathy (stars, $n = 76$ for 1–16P, $n = 23$ for 32P). Each point in the latter group was significantly different from the corresponding control response ($P < 0.05$, t-test).

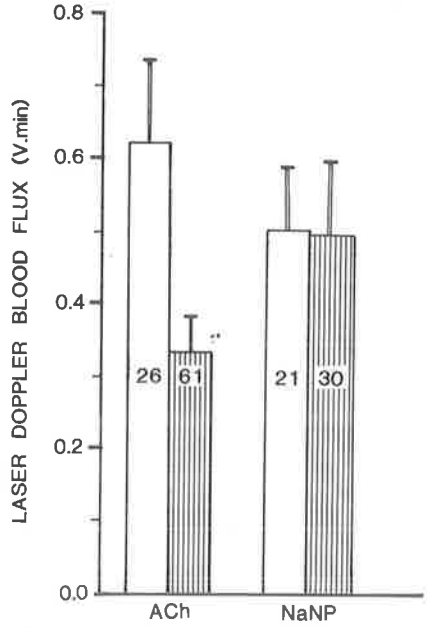


Fig. 3. ACh: The effect of iontophoretic application of acetylcholine (1%, 0.2 mA, 10 s). NaNP: The effect of iontophoretic application of sodium nitroprusside (1%, 0.2 mA, 20 s). Each column is the mean and the vertical bar the SEM of responses obtained on the foot dorsum for control subjects (open columns) and diabetic patients with neuropathy (hatched columns). The n for each group is given in each column. Only the reduced ACh response obtained from diabetic patients with neuropathy was significantly different from the control response ($P < 0.05$, t-test).

Thermal discrimination thresholds, reflecting small nerve fibre function, have also recently been reported to be elevated in diabetic patients with painful neuropathy [15], and so this is consistent with the present study.

The results of the rat study parallel those in man, in that the vasodilatation produced was dependent on the number of electrical pulses applied. Moreover, these responses were reduced in the STZ-diabetic rat which suggests that this animal model is a good approximation of the clinical situation. It was also found that insulin could normalise the axon reflex responses in the diabetic rats. This would suggest that there was a reversible functional deficit, rather than a structural one. One sensory transmitter peptide, substance P, is known to be dependent on axoplasmic transport for its deposition in the terminal branches of the primary afferent nerve fibre [16]. Defective axoplasmic transport occurs in diabetics [17] and this impairment can be reversed by insulin [18]. Therefore it is possible that diabetes has affected the axoplasmic transport of substance P and its subsequent involvement in axon reflex vasodilatation.

In the present studies, the reduced TENS responses in patients with diabetic neuropathy suggest impaired C fibre function. However, the neurogenic flare depends ultimately on the functional integrity of the cutaneous microvasculature in series with the primary afferent fibres. Thus damage to either the vascular endothelium or the smooth muscle itself could contribute to the observed effects. Considerable interest has recently focussed on the vascular endothelium-derived relaxing factor which is responsible for the vasodilatation observed to a variety of substances including substance P and ACh, but not to the nitrovasodilators [19]. The

ıal dilator effects of ACh and NaNP remain in n pretreated with capsaicin, while those of TENS ɔ depressed [20]. This suggests that their vasodi- or actions occur largely independently of the C res, that is, at the level of the microvasculature. us, the results of the present study suggest that ɔ vascular smooth muscle is able to dilate to sim- r extents in both control and diabetic subjects. ıis is consistent with other studies in which so- ım nitrite was used [9,10]. Therefore, the reduced ıtophoretic vasodilator responses to ACh impli- te the vascular endothelium as a primary target · this metabolic disease. These results are con- tent with reduced endothelium-dependent relax- on caused by ACh and histamine in aortic rings tained from the STZ-diabetic rat [21] and the ontaneously diabetic BB Wistar rat [22]. A recent ıew also describes several other dynamic pro- sses which are impaired in endothelial cells of dia- tic patients [23]. Thus, it is possible that impaired dothelial function per se may have also contrib- ɔd to the reduced TENS responses, especially ıce substance P is an integral component of the on reflex mechanism, and it also causes vasodi- ation by an endothelium-dependent mechanism. In conclusion, this study has illustrated the value a series of non-invasive tests of neurovascular nction in the clinical setting. A number of se- ential events which result in cutaneous axon re- x vasodilatation can be examined independently. ır results suggest that the combination of im- ired C fibre and endothelial function with re- ıced neurogenic inflammation is likely to contrib- ɔ to some of the symptoms of diabetes mellitus. ırthermore, these neurovascular tests allow for ɔ early detection of diabetic neuropathy, and for llowing progress of the disease and the efficacy of bsequent therapy with, for example, aldose re- ıctase inhibitors.

ɔknowledgements

ıis study was supported in part by Apex-Austra- n Diabetes Federation, a Monash Special Re- arch grant, the Rebecca L. Cooper Research ɔundation and the Diabetes Support Association.

References

1 Asbury, A.K. and Brown, M.J. (1982) Clinical and pathological studies of diabetic neuropathies. In: Y. Goto et al. (Eds.), Diabetic Neuropathy. Excerpta Medica, Amsterdam, pp. 50–57.
2 Asbury, A.K. and Gilliatt, R.W. (1984) The clinical approach to neuropathy. In: A.K. Asbury and R.W. Gilliatt (Eds.), Peripheral Nerve Disorders. Butterworth, London, pp. 1–20.
3 Dorfman, L.J. (1984) The distribution of conduction velocities (DVC) in peripheral nerves: a review. Muscle Nerve 7, 2–11.
4 Dyck, P.J., Karnes, J.L., Daube, J., O'Brien, P. and Service, F.J. (1985) Clinical and neuropathological criteria for the diagnosis and staging of diabetic polyneuropathy. Brain 108, 861–880.
5 Lewis, T. (1927) The Blood Vessels of the Human Skin and their Responses. Shaws and Sons Ltd., London.
6 Hutchison, K.J., Johnson, B.W., Williams, H.T.G. and Brown, G.D. (1974) The histamine flare in diabetes mellitus. Surg. Gynec. Obstet. 139, 566–568.
7 Hutchison, K.J., Overton, T.R., Biltek, K.B., Nixon, R. and Williams, H.T.G. (1983) Skin blood flow during histamine flare using the clearance of epicutaneously applied Xenon-133 in diabetic and non-diabetic subjects. Angiology 34, 223–230.
8 Nilsson, G.E., Tenland, T. and Oberg, P.A. (1980) Evaluation of a laser Doppler flowmeter for measurement of tissue blood flow. IEEE Trans. Biomed. Eng. 27, 597–604.
9 Westerman, R.A., Widdop, R.E., Hogan, C. and Zimmet, P. (1987) Non-invasive tests of neurovascular function: reduced responses in diabetes mellitus. Neurosci. Lett. 81, 177–182.
10 Westerman, R.A., Widdop, R.E., Hannaford, J., Hogan, C., Roberts, R. and Zimmet, P. (1987) Non-invasive tests of neurovascular function. Clin. Exp. Neurol. 24, 129–137.
11 Westerman, R.A., Low, A., Pratt, A. et al. (1987) Electrically evoked skin vasodilatation: a quantitative test of nociceptor function in man. Clin. Exp. Neurol. 23, 81–89.
12 Sloan, J.B. and Soltani, K. (1986) Iontophoresis in dermatology: a review. J. Am. Acad. Dermatol. 15, 671–684.
13 Low, A., Westerman, R.A., Kozak, W.M. and Zimmet, P. (1987) Effect of insulin on reduced axon reflex vasodilatation in streptozotocin diabetic rats. Proc. Aust. Soc. Physiol. Pharmacol. 18, 97P.
14 Jancso, N., Jancso-Gabor, A. and Szolcsanyi, J. (1967) Direct evidence for neurogenic inflammation and its prevention by denervation and by pretreatment with capsaicin. Br. J. Pharmacol. 31, 138–151.
15 Heimans, J.J., Bertelsmann, F.W. and Van Rooy, J.C.G.M. (1986) Large and small nerve fiber function in painful diabetic neuropathy. J. Neurol. Sci.. 74, 1–9.
16 Pernow, B. (1983) Substance P. Pharmacol. Rev. 32, 85–141.
17 Tomlinson, D.R. and Mayer, J.H. (1984) Defects of axonal transport in diabetes mellitus – a possible contribution to

the aetiology of diabetic neuropathy. J. Auton. Pharmacol. 4, 59–72.

18 Sidenius, P. and Jakobsen, J. (1982) Reversibility and preventability of the decrease in slow axonal transport velocity in experimental diabetes. Diabetes 31, 689–693.

19 Furchgott, R.F. (1984) The role of endothelium in the responses of vascular smooth muscle to drugs. Annu. Rev. Pharmacol. Toxicol. 24, 175–197.

20 Roberts, R.G.D., Westerman, R.A., Widdop, R.E., Kotzmann, R.R. and Zimmet, P. (1987) Effects of capsaicin on cutaneous axon reflex vasodilatation in man. Proc. Aust. Physiol. Pharmacol. Soc. 18, 95P.

21 Oyama, Y., Kawasaki, H., Hattori, Y. and Kanno, M. (198(Attenuation of endothelium-dependent relaxation in aort from diabetic rats. Eur. J. Pharmacol. 131, 75–78.

22 Meraji, S., Jayakody, L., Senaratne, M.P.J., Thomsor A.B.R. and Kappagoda, T. (1987) Endothelium-depender relaxation in aorta of BB rat. Diabetes 36, 978–981.

23 Porta, M., La Selva, M., Molinatti, P. and Molinatti, G.M (1987) Endothelial cell function in diabetic microangiopathy Diabetologia 30, 601–609.

Life Sciences, Vol. 45, pp. 49-57
Printed in the U.S.A.

Pergamon Press

NEUROGENIC VASODILATION IN THE RAT HAIRY SKIN MEASURED USING A LASER DOPPLER FLOWMETER

Amy Low and Roderick A Westerman

Department of Physiology, Monash University,
Clayton, Victoria 3168, Australia

(Received in final form April 25, 1989)

Summary

Intradermal stimulation of the saphenous nerve of anesthetized rats at C-fiber strength elicited stimulus -dependent vasodilator responses in saphenous-innervated hairy skin. A laser Doppler flowmeter (Periflux, Pf1d) was used to measure these neurovascular responses non-invasively. Capsaicin pretreatment significantly attenuated the vasodilator response. Furthermore, intraperitoneal administration of atropine or mepyramine significantly reduced the skin vascular responses. The responses of halothane-anesthetized rats were significantly attenuated when compared with rats anesthetized with pentobarbitone. Results of this study suggest the presence of endothelium-dependent vasodilator peptidergic, cholinergic and histaminergic contributions to electrically-evoked axon reflex vasodilation in the rat hairy skin.

Antidromic conduction in the primary afferent nerves which results in peripheral arteriolar vasodilation following stimulation of the dorsal root ganglion was observed many years ago (1-4) and led to the postulate of a specific cutaneous neurogenic vasodilator system. Antidromic vasodilation was shown to be mediated by small unmyelinated, peptide-containing nerve fibers which were sensitive to capsaicin (5-10). Release of peptides such as substance P (SP) from the terminal endings of these primary afferent fibers causes dilation of the adjacent microvessels in the skin either directly (11,12) or via mast cell degranulation (13-15) which releases vasoactive compounds such as histamine. Although the initial local dilator response is rapid in onset (16), the spreading dilation of this axon reflex phenomenon (1,2) is a slow event which may last for many minutes.

In the late 60's evidence was obtained for the presence of cholinergic and histaminergic vasodilator systems in the skin of the dog limb (17-19). Furthermore, the presence of a third vasodilator system with an unidentified substance responsible for prolonged and sustained dilation in the skin of the dog paw was noted (20,17,21,18). The behavior of this latter system was not accounted for by either the cholinergic or histaminergic vasodilator systems, but does resemble more recently described peptide-mediated vasodilation in the skin (12,22-24).

0024-3205/89 $3.00 + .00

Most of the data supporting the existence of active cutaneous vasodilation, distinct from that mediated by peptide-containing primary afferent fibers, came from studies on the skin of the dog limb (17-19) and human skin during hyperthermia (25). Participation of these active neurogenic vasodilator systems in the rat skin is less defined or not recognised (12,26). In humans, mechanisms underlying neurogenic inflammation and other dilator responses have been studied by Blumberg and Wallin (27) using intradermal electrical stimulation.

Peripheral vasodilation can be measured as the area of the flare, a rise in skin temperature or pressure, Evans Blue dye-leakage due to plasma extravasation, a change in volume (plethysmography), and radioactive or thermal blood flow clearance. The recent technique of measuring changes in microcirculation over a relatively small volume (a few cubic millimeters) under the skin using a laser Doppler flowmeter (Periflux, Pf1d) provides a simple and non-invasive means for studying electrically-evoked microvascular changes in the skin resulting from axon reflex vasodilation (16). Furthermore, since sensitivity of blood vessels may vary according to their size, this technique is an ideal method for accurately quantifying responses of the microvasculature in the skin.

This study, therefore aims to re-examine the components of cutaneous neurogenic vasodilation and to evaluate the efficiency of the laser Doppler flowmeter in measuring peripheral vasodilation in the hairy skin of the rat. Such an animal model is of importance in the many conditions, such as diabetes mellitus, in which neurovascular function is impaired and cutaneous nocifensor reactions are thereby reduced.

Methods

Female Wistar rats (160-250 g, 8-12 wk) were anesthetised with either halothane (1.5% with a mixture of Nitrous Oxide and Oxygen in a ratio of 3:1) or pentobarbitone (40 mg/kg, i.p.). Depth of anesthesia was checked using foot pinch test; anesthesia was considered adequate when the flexor withdrawal reflex was absent. The rat was placed supine on a heating pad set at 38°C to maintain constant body temperature. The skin area innervated by the saphenous nerve (28,29) on one leg was thoroughly but gently shaved with an electric shaver. A commercial depilatory compound was initially used to remove remnants of hair after shaving, but because it caused rashes, its use was abandoned and results from these rats were excluded from analysis.

For monitoring microvascular changes in skin from noxious electrical stimulation, a laser Doppler flowmeter (Periflux, Pf1d) which allows for direct real time measurement of cutaneous microvascular changes was used. Laser beams, carried by the optical fibers, are emitted and some of these are scattterd by moving red blood cells (RBCs) and undergo a frequency shift (Doppler effect). Those scattered in static structures remained unshifted in frequency. The relative portion of light which has undergone Doppler shift was shown to be linearly related to the volume of moving RBCs for a given volume of tissue of interest while the mean Doppler frequency is linearly related to the average RBC velocity (30, 31). The photodetector output signal,

comprising information on the volume of RBCs and their velocity for the given volume, is then converted to a continuous electrical signal. This quantity represents a derivative of the first moment of the unnormalized power spectrum of the photodetector output signal, and is referred to as blood flux. In a piece of tissue whose blood vessels are dilated, for example, both the volume of RBC (hence, the number) and/or their velocity would be reflected as an increase in blood flux.

The cut-off frequency and time constant used were 12 kHz and 1.5 s, respectively. A stable baseline recording (5-10 min) was obtained before the stimulation protocol commenced. The leg was placed on a foam pad so that the probe of the flowmeter could just sit flush on the skin without exerting any pressure on the skin. The probe holder was attached to the skin and the pad by a double-sided adhesive (3M, No.2181) at the distal end of the skin area innervated by the saphenous nerve, approximately 5-10 mm above the ankle. A pair of intradermal stimulating needles were placed 1 cm apart along the saphenous neurovascular bundle proximal to the probe with the cathode needle distal to the anode.

Rectangular 1 ms electrical pulses from a Grass Model S8 stimulator and Model S1 Isolation Unit were applied at 2 Hz to the saphenous nerve underlying the skin in trains of 4, 8, 16 and 32 pulses. The voltage was initially varied to determine the threshold for C-fiber activation as described by Kenins (32). In preliminary experiments, compound action potentials were recorded from the saphenous nerve exposed and isolated by dissection from its neurovascular bundle. The nerve was placed on a pair of platinum recording electrodes, covered with paraffin oil. The electrodes were connected to a Tektronix 122 preamplifer. Following this, a supramaximal voltage for C-fiber was used in all experiments. A train of electrical pulses was delivered after the preceeding vasodilation had subsided to the baseline. The response, defined as the area under the curve, was measured as an integral of voltage and time interval using the Zeiss MOP (Carl Zeiss, FRG) image analysis device.

Capsaicin desensitization and drug pretreatment

1% capsaicin (Sigma) in 10% ethanol, 10% Tween 80 and 80% 0.9% physiological saline (33) was administered subcutaneously to 6 female Long Evans rats (180-200 g, 70-80 days) over 3 successive days with a total dosage of 200 mg/kg (60, 60, 80 mg/kg/day). As capsaicin injections could be painful, rats were premedicated each day with a mixture of Ketamine (analgesic) and Xylazine (tranquilizer) in a ratio of 5:1 mg/ml, and this was administered intramuscularly (0.13 ml/kg) prior to capsaicin injection. The final dose was administered 24 h before testing.

Mepyramine maleate (10 mg/kg, i.p., n=7, Sigma) or atropine sulphate (1 mg/kg, i.p., n=7, Sigma) were given 15 mins before testing. These dosages were chosen because they produce maximal effect in the rat in vivo (R.E. Widdop, personal communication); these are also similar to those used in other studies (12,26).

Statistics

Data are expressed as the means ± SEM. The significance of differences was calculated by the Student t-test for paired or unpaired data as appropriate. The acceptable level of

significance is $p<0.05$ (two-tailed).

Results

Intradermal nerve stimulation with amplitude of 100 and 50 V, 2 Hz frequency and 1 ms duration for 4 s yielded similar vasodilator responses in the skin (n=3, Fig.1A). As amplitude was decreased to 20 V, a parallel reduction in the response was observed. Further reduction did not elicit any obvious dilator response. The appearance of the vasodilator response corresponded to the presence of C-fiber action potentials observed on the oscilloscope. Recruitment of C-fibers was found to be maximal between 50-65 V for intracutaneous needle electrodes, with an initial recruitment commencing at about 25 V. For direct antidromic nerve stimulation, it was 15 V and 6 V, respectively.

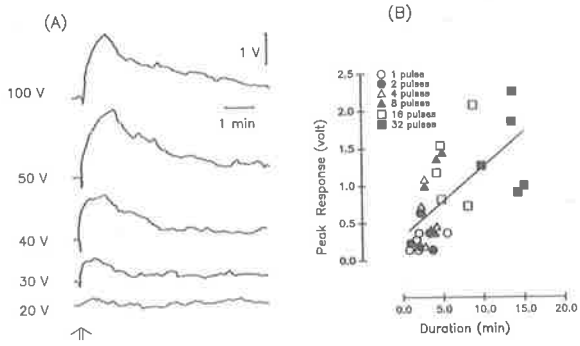


FIG. 1

A. Cutaneous vasodilator responses to noxious intracutaneous electrical stimulation with varying amplitudes (20-100 V, 2 Hz frequency and 1 ms pulse duration for 4 s). Supramaximal recruitment of C-fibers was observed at 50-65 V, after which no change in the size of the vasodilator response was observed (cf. 50 and 100 V stimulations). B. Scatter diagram showing the relationship between peak and duration of the vasodilator response to trancutaneous nerve stimulation. Data was obtained from 5 animals. The least-squares regression is represented by the line with $r=0.64$ (n=30, $p<0.01$, two-tailed).

Antidromic stimulation (40 V, 2 Hz, 1 ms for 4 s) of the intact saphenous nerve directly with platinum bipolar electrodes yielded identical-sized response when compared with intracutaneous stimulation (n=4). There was no difference in the size of responses from antidromically stimulating the distal end of acutely transected saphenous nerve (n=3) although an increase in baseline was observed. Contralateral nerve stimulation did not evoke any vascular responses (n=3). Similarly, ipsilateral stimulation of a different nerve on the leg did not produce a response (n=3). The peak and duration of the vasodilator responses (the time for the response to return to within 10% of the baseline before stimulation) to electrical stimulation with various numbers of stimulus pulses (1,2,4,8,16,32) were both significantly correlated with $r=0.64$ (n=30, $p<0.01$, Fig.1B).

Vasodilator responses to all the standard stimuli were

significantly reduced following capsaicin pretreatment (Fig.2A). The skin vasodilator responses were also significantly reduced following pretreatment with mepyramine (histamine H1 receptor antagonist) and atropine (acetylcholine muscarinic receptor antagonist) (Fig.2B).

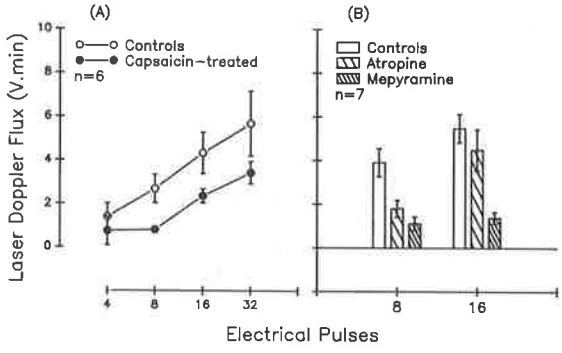


FIG. 2
Effect of (A) capsaicin and (B) atropine and mepyramine on skin microvascular responses to electrical stimulation. Capsaicin desensitization with a total dose of 200 mg/kg, s.c. administered over 3 days (60,60,80 mg/kg) significantly attenuated the dilator responses ($p<0.01$, paired t-test, two-tailed). Atropine (1 mg/kg, i.p., 15 min before) reduced the responses significantly ($p<0.03$, paired t-test, two-tailed). Mepyramine (10 mg/kg, i.p., 15 min before) also resulted in significant reduction ($p<6e-6$, paired t-test, two-tailed).

Effect of anesthetics: halothane compared with pentobarbitone

The vasodilator responses to electrical stimulation in halothane-anesthetized rats were significantly smaller than those responses obtained from the pentobarbitone-anesthetized animals for the same stimulus pulse-trains (Fig.3). No significant difference in the resting baseline was noted with the use of either halothane or pentobarbitone.

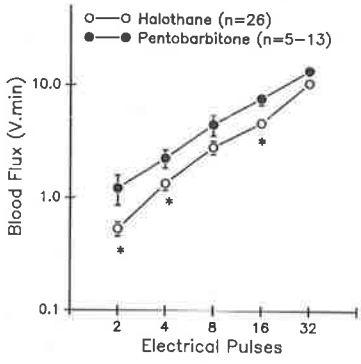


FIG. 3
Effect of halothane compared with pentobarbitone on electrically -evoked vasodilator responses. Significant attenuation of vaso -dilator responses was obtained from halothane-anesthetized rats when compared with those obtained from pentobarbitone-anesthetized (*$p<0.05$ - unpaired t-test, two-tailed).

Discussion

Measurement of electrically-evoked axon reflex vasodilation using the laser Doppler flowmeter

The spreading vasodilation of the triple response is an axon reflex phenomenon (4). This represents an end result of a cascade of events originating from stimulation of primary afferent fibers,

in particular, the nociceptor afferent nerve fibers (4,34). While its neurogenic component is a rapid event, the lateral spread and increasing intensity of the dilation is a relatively slow and time-dependent phenomenon involving mast cell degranulation, release of histamine and activation of adjacent nociceptor endings. The laser Doppler flowmeter accurately measures the vasodilator response as an integral of a change in output voltage with respect to time. This allows for sensitive measurement of even the smallest flux changes during the axon reflex and particularly useful in a rat model since a flare in the rat skin is invisible.

Axon reflex vasodilation in the rat hairy skin

This study has demonstrated that vasodilation can be produced by stimulation of a cutaneous nerve supplying its zone of innervation, provided that the stimulus parameters used were adequate to elicit C-fiber action potentials (35). Recruitment of C-fibers was found to be maximal between 50-65 V with 2 Hz, 1 ms pulses for 4 s, for intradermal needle electrodes with an initial recruitment commencing at about 25 V. The size of the response was found to be dependent on the number of C-fibers activated. For direct nerve stimulation, the initial recruitment commenced at 6 V with 2 Hz, 1 ms pulses for 4 s and the maximal recruitment occurred at 15 V.

Comparison between intradermal and direct antidromic nerve stimulation, with parameters supramaximal for activating C-fibers in both situations, showed similar dilator responses. This effectively eliminates possible contributions to the size of the vasodilator response from other sources. Furthermore, vasodilation persisted following acute decentralization eliminating central nervous system reflex contributions. Moreover, ipsilateral (stimulation of different nerve on the same leg) and contralateral nerve stimulation did not cause any dilation. These observations confirm the previous proposition that the vasodilator response is a local peripheral event (4,5,27).

In this study, reduced stimulus-dependent vasodilator responses were obtained following systemic capsaicin pretreatment. This is consistent with previous reports (5,33,36, also see 37). Lembeck and Donnerer (9) showed that SP content in the dorsal roots, saphenous nerves and hind paw skin of adult rats declined to 60-70% of control 4 days after capsaicin administration. In single nerve fiber studies, Kenins (32) reported that only the polymodal fibers in the rat skin were responsive to capsaicin and not other types of C-fibers (low threshold mechanoreceptors and thermoreceptors - warm, cold and 'freezing'). Hence, the significantly reduced vasodilator response seen following capsaicin pretreatment is consistent with the depletion of SP from the polymodal nociceptors in the skin. However, the action of capsaicin on co-localized neuropeptides in primary afferent fibers and dorsal root ganglia, such as somatostatin (SS) and calcitonin gene-related peptide (CGRP) cannot be excluded since there is evidence to suggest that capsaicin does not only deplete SP but also SS (8), Neurokinin A and CGRP (38).

While ample evidence exits to support the mediation of the axon reflex vasodilation by SP, the participation of other vasoactive peptides, which have been recently identified in the SP-containing

primary afferent fibers (eg. SS and CGRP) (39,40) cannot be excluded as possible mediators of the dilation (38). Interactions of co-localized neuropeptides in primary afferent fibers in neurogenic inflammation have been suggested (41). Furthermore, there is increasing evidence for the presence of purinergic mechanisms in the skin mediated by small-diameter afferent fibers (42).

It has been shown that SP not only directly acts on the microvasculature of the skin causing dilation of the blood vessels (11,12), it is also capable of histamine release from adjacent mast cells in rat and human skin (13-15). In this study, reduced responses following administration of mepyramine, a histamine H1 antagonist were observed confirming previous reports on the contribution of histamine from mast cells to the flare (4,43,44).

In contrast to the findings of Gamse and Saria (26), administration of atropine, a acetylcholine muscarinic antagonist in this study caused reduction in the vasodilator response suggesting some contribution of cholinergic mechanism to cutaneous vasodilation in the rat hind leg (12) as demonstrated in the trigeminal territory (45,46). Because the noxious electrical stimulation is at a strength supramaximal to excite C-fibers, it is highly possible that sympathetic postganglionic fibers of all types are activated by the stimuli used and these include sudomotor, cholinergic fibers as well as noradrenergic vasoconstrictor fibers. Evidence that the latter are excited is seen in the initial vasocontriction - the rapid fall in flux following stimulation lasting 20-30 s preceding each vasodilator flux increase as seen in Fig.1A.

Effect of Anesthetics

Mediators of the neurogenic flare such as SP, histamine and acetylcholine have been shown to elicit relaxations in isolated blood vessels only with intact endothelial cells (see 47). Recently, it has been reported that halothane reversibly interfered only with endothelium-dependent relaxations in vitro although the precise mechanism of this action of halothane is unknown (48, Low & Neild, unpublished observations). Significant attenuation of the vasodilator responses observed in halothane -anesthetized animals suggests for the first time in an in vivo situation that these cutaneous vasodilator responses are in part endothelium-mediated mechanisms. While it was reported that halothane caused dilatation in dogs, no dilator effect of halothane was observed in this study since the resting baseline was the same for both anesthetics (between and within rats). This observation eliminates the possible effect of the anesthetics on the vascular tone in the skin of the rat.

This study has demonstrated the usefulness of a rat model using noxious intracutaneous electrical nerve stimulation to elicit neurovascular responses. Laser Doppler flowmetry is sensitive in measuring electrically-evoked axon reflex dilator responses non-invasively. Results of this study indicated the presence of endothelium-mediated peptidergic, cholinergic and histaminergic mechanisms in the axon reflex vasodilation in the rat hairy skin.

Acknowledgement

We are most grateful to Dr Tim Neild and Professor Morley Sutter for helpful criticisms of the manuscript. Support of Rebecca L. Cooper Foundation, Monash Special Research Grants and Australian Research Grant Scheme are also gratefully acknowledged.

References

1. W.M. BAYLISS, J. Physiol. (Lond.) 26 173-209 (1901).
2. W.M. BAYLISS, J. Physiol. (Lond.) 28 276-299 (1902).
3. J.N. LANGLEY, J. Physiol. (Lond.) 57 428-446 (1923).
4. T. LEWIS, The Blood Vessels of the Human Skin and their Responses, Shaw, London (1927).
5. N. JANSCO, A. JANSCO-GABOR and J. SZOLCSANYI, Br. J. Pharmac. Chemother. 31 138-151 (1967).
6. R. GAMSE, P. HOLZER and F. LEMBECK, Br. J. Pharmac. 68 207-213 (1980).
7. J.E. BERNSTEIN, R.M. SWIFT, K. SOLTANI and A.L. LORINCZ, J. Invest. Derm. 76 394-395 (1981).
8. R. GAMSE, D. LACKNER, G. GAMSE and S.E. LEEMAN, Naunyn-Schmiedeberg's Arch. Pharmac. 316 38-41 (1981).
9. F. LEMBECK and J. DONNERER, Naunyn-Schmiedeberg's Arch. Pharmac. 316 240-243 (1981).
10. R.A. WESTERMAN, A. LOW, A. PRATT, J.S. HUTCHINSON, J. SZOLCSANYI, W. MAGERL, H.O. HANDWERKER, and W.M. KOZAK, In Clinical and Experimental Neurology 23 (Eadie, M., ed.) p81-89, Adis Press, Sydney (1987).
11. D. HALLBERG and B. PERNOW, Acta. Physiol. Scand. 93 277-285 (1975).
12. F. LEMBECK and P. HOLZER, Naunyn-Schmiedeberg's Arch. Pharmac. 310 175-183 (1979).
13. F. ERJAVEC, F. LEMBECK, T. FLORJANE-IRMAN, G. SKOFITSCH, J. DONNERER, A. SARIA and P. HOLZER, Naunyn-Schmiedeberg's Arch. Pharmac. 317 67-70 (1981).
14. C.M.S. FEWTRELL, J.C. FOREMAN, C.C. JORDAN, P. OEHME, H. RENNER and J.M. STEWART, J. Physiol. 330 393-411 (1982).
15. J.C. FOREMAN, C.C. JORDAN and W. PIOTROWSKI, Br. J. Pharmac. 77 531-539 (1982).
16. R.A. WESTERMAN, R. WIDDOP, A. LOW, J. HANNAFORD, W. KOZAK and P. ZIMMET, Diabet. Res. Clin. Prac. 5 49-54 (1988).
17. B.G. ZIMMERMAN, J. Pharmac. Exp. Ther. 152 81-87 (1966).
18. M.J. BRODY and R.A. SHAFFER, Am. J. Physiol. 218 470-474 (1970).
19. T.F. ROLEWICS and B.G. ZIMMERMAN, Am. J. Physiol. 223 939-943 (1972).
20. B.G. ZIMMERMAN, Am. J. Physiol. 214 62-66 (1968).
21. L. BECK, A.A. POLLARD, S.O. KAYAALP and L.M. WEINER, Fedn. Proc. 25 1596-1610 (1966).
22. S. ROSELL, L. OLGART, B. GAZELIUS, P. PANOPOULOS, K. FOLKERS and J. HORIG, Acta. Physiol. Scand. 111 381-382 (1981).
23. J.L. JORIZZO, A.A. COUTTS, R.A.J. EADY and M.W. GREAVES, Eur. J. Pharmac. 87 67-76 (1983).
24. P. DEVILLIER, D. REGOLI, A. ASSERAF, B.DESCOURS, J. MARSAC and M. RENOUX, Pharmac. 32 340-347 (1986).
25. L.B. ROWELL, In Vasodilatation (Vanhoutte, P.M. and Leusen, I., eds.) p1-17, Raven Press, New York (1981).
26. R. GAMSE and A. SARIA, J. Auton. Nerv. Syst. 19 105-111 (1987).

27. H. BLUMBERG and B.G. WALLIN, J. Physiol. 382 105-121 (1987).
28. J.E. SWETT and C.J. WOOLF, J. Comp. Neurol. 231 66-77 (1985).
29. N. JANSCO, In Pharmacology of Pain (Lim, R.K.S., Armstrong, D., and Pardo, E.G., eds.) p33-55, Pergamon Press, Oxford (1968).
30. G.E. NILSSON, T. TENLAND and P.A. OBERG, IEEE Trans. Biomed. Eng. BME-27(10) 597-604 (1980).
31. R.A. WESTERMAN, R.E. WIDDOP, J. HANNAFORD, A. LOW, R.G.D. ROBERTS, P. KENT, K. SIDERIS, T. YIP, J.R.S. HALES and F.R.N. STEPHENS, Aust. Phys. Eng. Sci. Med. 11(2) 53-66 (1988).
32. P. KENINS, Neurosci. Lett. 29 83-88 (1982).
33. J. SZOLCSANYI, In Tachykinin Antagonists (Hakanson, R. and Sundler, F., eds.) p45-53, Elsevier Science Publishers, B.V. (1985).
34. F. LEMBECK, Br. J. Derm. 109(Suppl.25) 1-9, (1983).
35. J.C. Hinsey and H.S. GASSER, Am. J. Physiol. 99 679-689 (1930).
36. J. SZOLCSANYI, Agents Actions 23(1/2) 4-11 (1988).
37. J.I. NAGY, Trends Neurosci. 5(10) 362-365 (1982).
38. F.J. DIEZ GUERRA, M. ZADI, P. BREVIS, I. MacINTYRE and P.C. EMSON, Neurosci 25 839-846 (1988).
39. I.J. GIBBINS, J.B.FURNESS, M. COSTA, I. MacINTYRE, C.J. HILLYARD and S. GIRGIS, Neurosci. Lett. 57 125-130 (1985).
40. I.J. GIBBINS, D. WATTCHOW and B. COVENTRY, Brain Res. 414 143-148 (1987).
41. R. GAMSE, M. POSCH, A. SARIA and G. JANSCO, Acta. Physiol. Hung. 69(3-4) 343-354 (1987).
42. R.E.W. FYFEE and E.R. PERL, Proc. Natl. Acad. Sci. USA 81 6890-6893 (1984).
43. J.A. KIERNAN, Q. J. Exp. Physiol. 60 123-130 (1975).
44. D.A.A. OWEN, E. POY, D.F. WOODWARD and D. DANIEL, Br. J. Pharmac. 69 615-623 (1980).
45. R. COUTURE and A.C. CUELLO, J. Physiol. 346 273-285 (1984).
46. R. COUTURE, A.C. CUELLO and J.L. HENRY, Br. J. Pharmac. 84 637-643 (1985).
47. R.F. FURCHGOTT, Cir. Res. 53 557-573 (1983).
48. S.M. MULDOON, J.L. HART, K.A. BOWEN and W. FREAS, Anesthesiology 68 31-37 (1988).

Frontiers in Diabetes
Editor: F. Belfiore, Catania

Reprint
Publishers: S. Karger, Basel
Printed in Switzerland

Molinatti GM, Bar RS, Belfiore F, Porta M (eds): Endothelial Cell Function in Diabetic Microangiopathy: Problems in Methodology and Clinical Aspects. Front Diabetes. Basel, Karger, 1990, vol 9, pp 127–138

Non-Invasive Tests of Neurovascular Function in Human and Experimental Diabetes Mellitus

R.A Westerman[a], *A.M. Low*[a], *R.E. Widdop*[b], *T.O. Neild*[a], *C.A. Delaney*[a]

[a]Departments of Physiology and [b]Pharmacology, Monash University, Clayton, Victoria, Australia

Recently, increased interest in the interactions between endothelium and other vascular and neural structures [11, 28] led to the development of non-invasive tests of neurovascular function in human and experimental diabetes mellitus [24, 30, 34]. These have demonstrated reduced neurogenic inflammatory responses in diabetes mellitus [25, 33], particularly in patients with clinical evidence of neuropathy. Such impairment of neurovascular responses in diabetics with small fibre neuropathy accords well with previous observations of reduced histamine flare response in diabetics [13].

It has been known since the last century that stimulation of particular nerve fibers with their cell bodies in the dorsal root ganglia influences blood flow in the skin [3, 26]. Responses of the same neurons to natural stimulation of their receptor endings in the skin gave rise to vasodilation surrounding the stimulation sites. This effect was termed axon reflex or antidromic vasodilatation [3, 17].

Nocifensor System

The functional significance of neurogenic (axon reflex) inflammatory responses as a 'nocifensor' or damage-control system was first described by Sir Thomas Lewis [18], and has been recently re-elaborated [15, 16]. This system involves the interactive participation of both small nerves and blood vessels. The defensive actions triggered by a noxious stimulus exciting the nociceptive afferent pathway include: mediation of pain sensation, with resulting adaptive behavioural responses; release of neuropeptides from nociceptive afferent branches; vasodilation and plasma extravasation; macrophage induction and increased phagocytosis; stimulation of lymphocytes and antibody production; effects on plasma kinins, complement and

clotting systems; general systemic effects, including fever, leucocytosis and increased erythrocyte sedimentation rate [32]. These effects depend upon the release and action of many chemical mediators including neuropeptides substance P (SP), calcitonin gene-related peptide (CGRP), neurokinin A (NKA), neuropeptide Y (NPY), mast cell degranulation products – vasoactive amines, histamine, 5-hydroxytryptamine (5HT); acidic lipids, prostaglandins (PG-) and other autacoids; slow reacting substance of anaphylaxis; lysosomal products; lymphocyte products; leucocytosis factors; pyrogens. The importance of the protective pain sensation as well as other multiple efferent functions of the nociceptive afferent terminals [27], for example in the prevention of pressure damage to feet in diabetics [4, 23], emphasizes the importance of developing sensitive routine measures of small nerve fibre function.

Neurovascular Function Test Strategy

Because the extent of erythema (flare) produced by this nocifensor system depends on normal cutaneous vascular reactivity as well as the nociceptive afferent C-fibres, a dysfunction of any one of the serial components of the microvascular dilator cascade (fig. 1, 2) could result in reduced axon reflex dilator response [31, 33]. These include the nociceptive afferent C-fibres (I), the vascular endothelium (II), and smooth muscle (III) (fig. 1, 2).

In the present study noxious transcutaneous electrical nerve stimulation (TENS) is used at a current strength sufficient to excite nociceptive C-fibres and evoke a sensation of discomfort and an axon reflex [30, 33, 34]. The latter tests all the components I, II, and III (fig. 1, 2), although sensations of discomfort depend only on the nociceptive C-fibres. For this reason it is also necessary to measure the direct local red reaction [17] which is non-neurogenic and this is sensitively measured using laser Doppler velocimetry [32]. The red reaction can be produced in a variety of ways, is confined to the area stimulated, and will reveal any intrinsic vascular dysfunction such as may be associated with microangiopathy or occlusive vasculopathy [25]. Mechanical pressure is used by Le Quesne [24, 25], but the endothelium-dependent vasodilation without nociceptor participation can be evoked by very small doses (1, 2, 4 mC) of acetyl-beta-methacholine (MeCh), pilocarpine (Pil) or acetylcholine (ACh) iontophoretically applied directly onto the skin under the laser Doppler probe [30, 33, 34]. This local dilator response evoked by muscarinic agonists does not depend upon functioning nociceptive C-fibres because it is present in locally anaesthetised or denervated skin [31], and is reduced when microvascular endothelium function is impaired [19]. It tests the function of

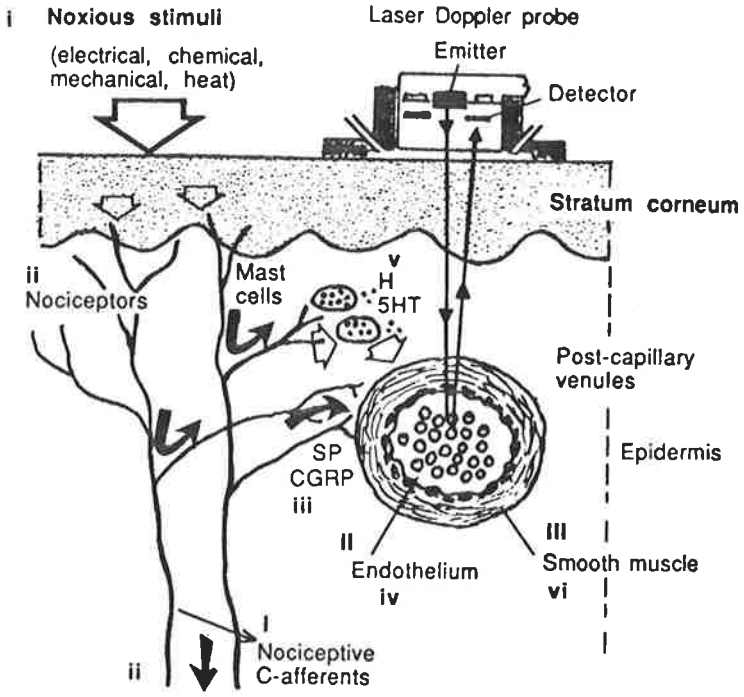


Fig. 1. Simplified schematic diagram of the cascade mechanism involved in axon reflex vasodilatation. In sequence, i = a noxious stimulus activates primary nociceptive afferent fibres (I); ii = pain sensation occurs via orthodromic afferent propagation to CNS; iii = release of neuropeptides e.g. SP, CGRP, from terminal endings via antidromic conduction (axon reflex); iv = endothelium-dependent dilatation of skin microvessels by SP etc.; v = degranulation of mast cells by SP and histamine also causes vasodilatation of skin microvessels; vi = some other mechanisms including ACh release from sympathetic sudomotor endings may be involved in cutaneous vasodilatation (see text). The vasodilatation resulting from iii, iv, v, vi is partly dependent on release of EDRF from luminal cells of microvessels, and can be measured by laser Doppler velocimetry [32].

both vascular endothelium and smooth muscle, i.e. components II, III of the dilator cascade, and is mediated by the release of endothelium-derived relaxing factor (EDRF). However, it should be pointed out that muscarinic agonists are also capable of reducing noradrenaline release from sympathetic nerves responsible for constrictor tone and this could contribute to microvascular relaxation. The final test is of component III – that is, vascular smooth muscle reactivity. This involves iontophoretic application of a directly acting nitrodilator such as sodium nitrite ($NaNo_2$) [31, 33] or sodium nitroprusside (NaNP) [32, 34]. This strategy of testing the serial elements of the axon reflex flare, whose interactions are summarized in figure 2, has particular applicability in diabetic neuropathy because of the previous difficulty in measuring any neural function except the conduction

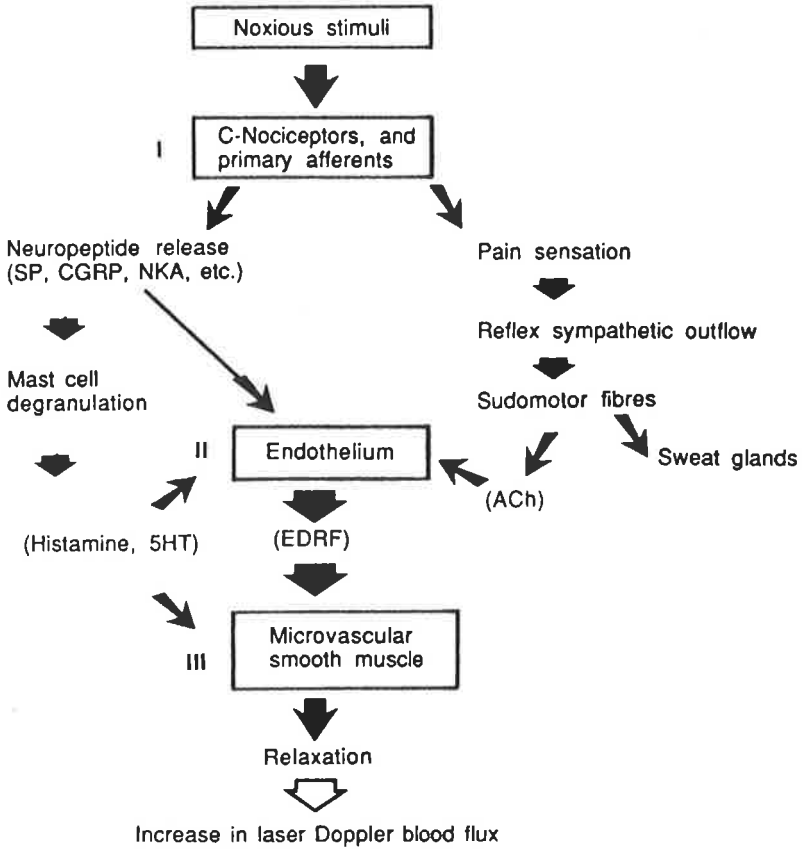


Fig. 2. Cutaneous axon reflex microvascular dilator cascade. Diagram summarizes the interactions described in the text leading to relaxation of microvessels and increased laser Doppler flux.

velocity of the larger myelinated sensory and motor nerve fibres [8] and the prevalence of small fibre disturbances in diabetes [2, 12].

Results

Examples of human cutaneous electrical axon reflex dilator responses are shown in figure 3. When the current strength is below C-nociceptor threshold, as in figure 3a, no stimulus-dependent axon reflex is seen, only an increased oscillation of the flux record, occurring a few minutes after the stimulus. When the current pulse trains are noxious (i.e. painful), stimulus-dependent flux increases are seen irrespective of the type of laser Doppler device used (fig. 3b, c). Compared with helium neon laser light (633 nm), the infrared laser light (827 nm) of the diode Doppler penetrates more

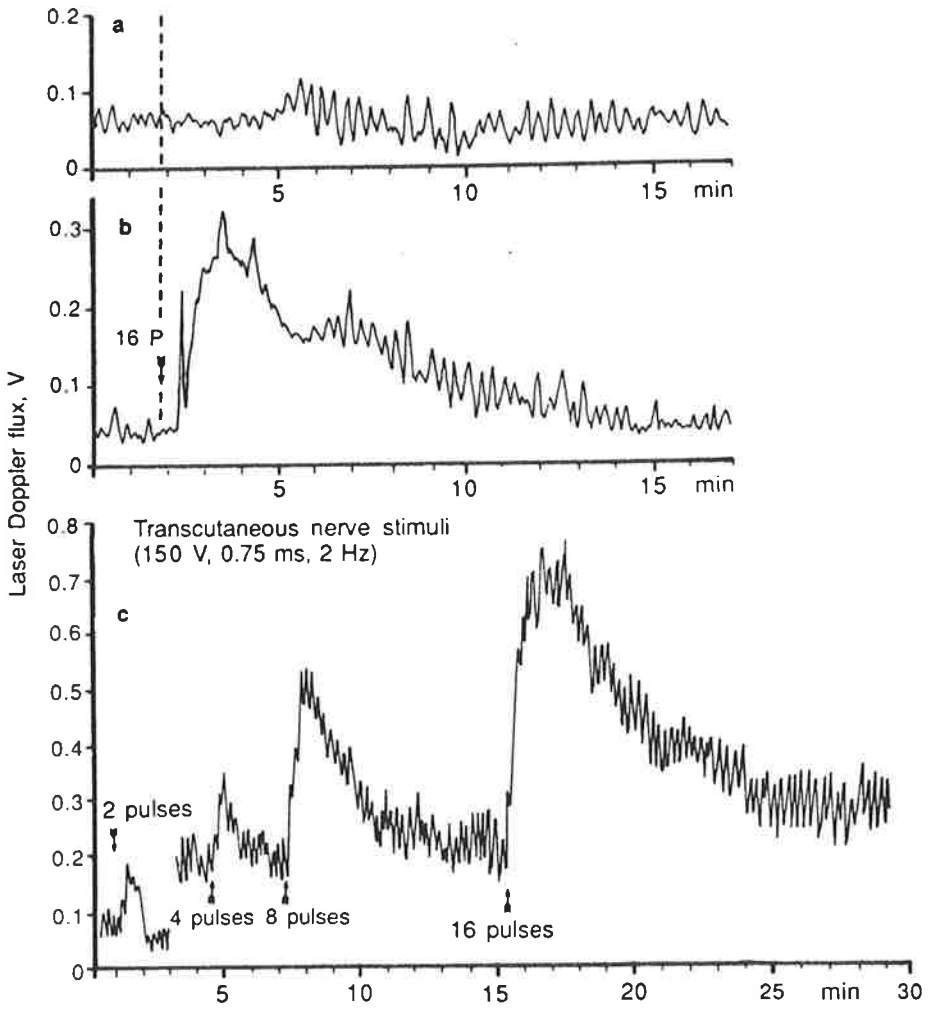


Fig. 3. a, b Helium-neon (633 nm) laser Doppler flowmeter (Periflux Pfld) measures cutaneous blood flux changes (volts) with time (min). *a* At broken line, 16 transcutaneous electrical nerve stimuli (TENS) at a non-noxious current strength (40 V, 0.75 ms, 2 Hz) are applied adjacent to the laser Doppler probe on the forearm of a non-diabetic volunteer. *b* At arrow, 16 noxious TENS pulses (150 V, 0.75 ms, 2 Hz; for constant current stimulation, the same supramaximal level of TENS was achieved by 45 mA) applied at the same skin site. *c* Infrared diode (827 nm) laser Doppler velocimeter (Diodopp) measures the cutaneous blood flux changes evoked by graded trains of 2, 4, 8, 16 noxious TENS pulses. Stimulus-dependent blood flux changes (transient vasodilator responses) are evoked.

deeply into the skin and therefore reflected light from deeper vascular beds is measured, prolonging the flux change in response to the same stimulus (compare 16P in fig. 3b and c). Such axon reflex responses are reduced significantly in diabetics [33, 34].

Other functional tests of polymodal small fibres include warm thermal acuity [10] and the usefulness of an improved automated method for the measurement of thermal thresholds in normal subjects [14] and diabetic patients [7] has been established using the Medelec TTT device. Such data may be compared with the axon reflex tests of nociceptive afferent function in order to help define a subgroup of diabetic patients who primarily display dysfunction of the microvascular endothelium (fig. 4 provides an example of such concordance studies).

Warm stimuli from a Peltier thermode are applied using a two alternative forced-choice psychophysical paradigm with 4 or 6 reversals. The results for 55 non-diabetic volunteers (fig. 4a) and 91 diabetic patients (fig. 4b) tested on the foot dorsum are shown in figure 4. Of the 91 diabetics, 60 had a warm threshold higher than the upper 99% confidence limit for non-diabetic subjects and of these, 18 had warm thresholds exceeding 10 °C (the upper limit of the Medelec TTT).

When comparisons of warm perception threshold against electrical axon reflex dilator responses in the same diabetic subjects are made (fig. 4c), three groups of responses are obvious: those at the top left of the graph (29/67) who exhibit clearly reduced electrical axon reflex (EAR) dilator responses together with elevated warm perception threshold (WPT); those at the lower right quadrant (11/67) with both normal WPT and EAR; and finally a group in the lower left quadrant (27/67). This latter anomalous group exhibit putatively abnormally reduced EAR but apparently normal WPT which may indicate functional deficit beyond component I.

Therefore to discriminate further between endothelial or smooth muscle dysfunction, skin blood flux changes in response to iontophoretic application of endothelium-dependent chemical stimuli (ACh, MeCh) or endothelium-independent stimuli of the direct nitrodilator type ($NaNo_2$, NaNP) can be measured. Typical records are seen in ref. [31, 32]. Significantly reduced dilator responses to iontophoresed ACh and MeCh are found in diabetic patients compared with non-diabetic patients. No significant reduction of responses to $NaNO_2$ or NaNP was found in diabetic patients.

Because the multiple sensory-motor functions of cutaneous nociceptive afferents are also demonstrable in the rat, we have developed both in vivo [20, 34] and in vitro [19] methods of measuring neurovascular function in the streptozotocin-diabetic rat model and these in vitro techniques are directly applicable for studying microvascular reactivity of excised human arteries.

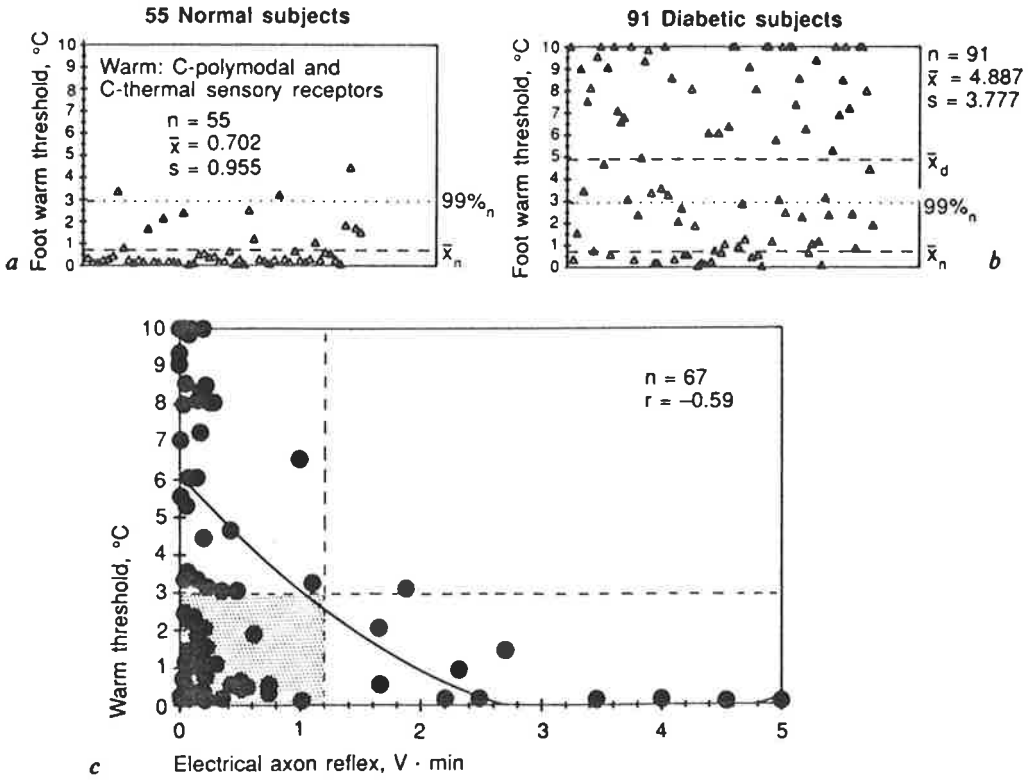


Fig. 4. Scattergram plots of warm perception thresholds recorded on foot dorsum in normal volunteers *(a)* and diabetic patients *(b, c)* using a Medelec TTT automated device described by Jamal et al. [14]. *a, b* Means and SD are shown for warm thresholds in normal (0.70 ± 0.96 °C) and diabetic (4.89 ± 3.78 °C) patients having similar age distribution. *c* Compares warm perception threshold (Y-axis) as a test primarily of C-fibre function and electrical axon reflex dilator responses (X-axis) as a test of total neurovascular function in the same diabetic patients. Broken lines show 99% confidence limits for warm thresholds (horizontal line) and for electrical axon reflex (vertical line). Warm threshold values below 3.0 °C are probably normal, and axon reflex responses smaller than 1.2 V·min are probably abnormally reduced (stippled zone). Responses in *c* fall into three main groupings (see text). Although not shown in here, a similar distribution of elevated WPT was found on the anterior forearm of diabetic patients and is an even more impressive functional deficit at this site because clinical large fibre neuropathy is infrequent here [7].

Some of the contributions to the electrically evoked axon reflex dilator responses in anaesthetized rat paw skin are shown in figure 5 [20]. Significant reductions in the vasodilator responses to stimulus pulse trains of 8 and 16 pulses (2 Hz, 100 V, 1 ms) were observed in the animals after pretreatment with systemic capsaicin in a dose sufficient to desensitize C-nociceptors (fig. 5a). Similar reductions were observed 15 min after pretreatment with an H_1 histamine antagonist, mepyramine maleate (fig. 5b) and with a muscarinic cholinergic antagonist, atrophine sulphate (fig. 5c).

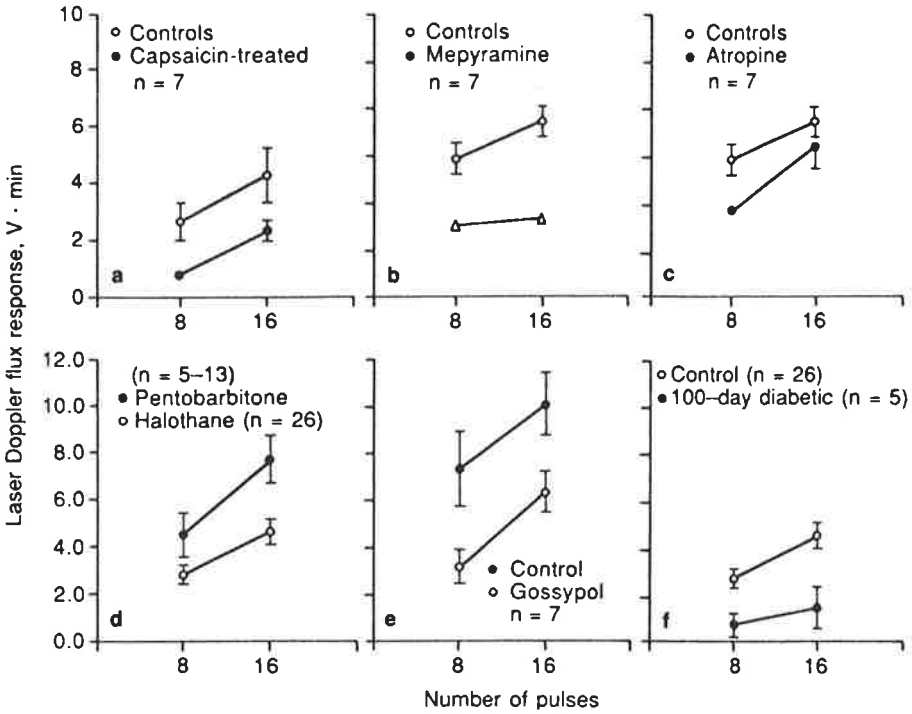


Fig. 5. a–f Recording with Periflux Pfld laser Doppler (633 nm) flowmeter of axon reflex vasodilatation from rat paw skin in areas supplied by the saphenous nerve, in response to transcutaneous noxious electrical stimulation (TENS) with 8 and 16 pulses (100 V, 1 ms, 2 Hz) applied to intradermal needle electrodes. Blood flux changes are expressed as Volt·min (integral of area under blood flux response: time record). Effect of *(a)* capsaicin, *(b)* mepyramine, *(c)* atropine on cutaneous axon reflex responses to noxious TENS. [For methods see ref. 20, 34.] *(d)* Effect of halothane surgical anaesthesia compared with pentobarbitone 40 mg/kg on electrically evoked axon reflex responses showed significant attenuation of vasodilator responses ($p < 0.05$, unpaired t-test, two-tailed). *(e)* Effect of gossypol infusion, 80 mg/kg retrogradely into the femoral artery for 30 min prior to retesting, showed significantly reduced electrical axon reflex dilator responses ($p < 0.05$, unpaired t-test, two-tailed). *(f)* The effect of untreated experimental diabetes mellitus, 100 days after streptozotocin diabetes was induced (70 mg/kg i.v.), significantly reduced electrically evoked axon reflex dilator responses ($p < 0.05$, unpaired t-test, two-tailed).

Because halothane inhibits endothelium-dependent vascular relaxation in vitro [22], vasodilator responses in halothane-anaesthetized rats were tested and found to be significantly smaller than those responses obtained from the pentobarbitone-anaesthetized rats for the same stimulus pulse-trains (fig. 5d). Gossypol, both in vivo and in vitro, is reported to inhibit endothelium-dependent relaxation [1, 9] and the effect of gossypol treatment in vivo (80 mg/kg given over 30 min at 0.1 ml/min) is shown in figure 5e to produce significant reduction in the size of the electrically evoked axon reflex dilator responses. Finally, untreated experimental diabetes

mellitus (streptozotocin-induced, 100 days) also significantly reduces the electrical axon reflex dilator responses (fig. 5f).

Gossypol and halothane have also been used in our rat in vitro arterial preparation to verify whether they are selective inhibitors of endothelium-dependent relaxations. Using a Mulvany-Halpern myograph for measurement of circumferential force in the central tail artery segment, dose-response relationships for the relaxant effects of cumulative doses of carbachol (Carb) and sodium nitroprusside (NaNP) were established before and after gossypol (30 μM in DMSO, 30 min, followed by 30 min washout). It was found that gossypol significantly attenuated the relaxant effects of Carb, but not NaNP. The effect of gossypol was not reversible and histological examination of silver nitrate stained preparations revealed extensive damage to the endothelium [19]. Exposure of the artery to halothane, in 95% O_2, 5% CO_2, markedly reduced relaxation to Carb, but had no effect on NaNP, and this effect of halothane was reversible [19].

Discussion

Activity of nociceptor nerves in both human and rat skin can cause vasodilation which is mediated initially by peptide substances such as substance P, CGRP, etc., released by primary sensory nerves and is known as 'axon reflex vasodilatation'. Relative changes in blood flux may be accurately monitored non-invasively using sensitive laser Doppler devices [32].

It has been suggested that the loss of nociceptive C-fibre function is of central importance to the development of neuropathic foot lesions in diabetes [4, 25, 34]. Clearly, impairment of nociceptive afferent function leads to reduced axon reflex flare evoked by chemical stimulation with acetylcholine [24, 25], noxious transcutaneous electrical stimulation [30, 33, 34], and noxious heat. However, ACh, histamine (Hi) and SP have all been shown to initiate endothelium-dependent vasodilatation mediated by the release of a relaxing factor [21, 28].

In our in vivo rat preparation we have presented direct evidence that electrically evoked axon reflex vasodilatation is endothelium-dependent (fig. 5). These results are confirmed by the in vitro results of Low et al. [19], also presented here. Therefore alternative explanations of reduced axon reflex flare in diabetes mellitus must include primary endothelial dysfunction in the microvasculature perhaps due to the presence of mascrovascular disease such as atherosclerosis [6] or a metabolic disturbance of endothelial secretory functions. This latter view is supported by the rapid reversal of abnormal levels of von Willebrand factor in diabetic patients after only 30

days of treatment with Statil (ICI 128436), an aldose reductase inhibitor [5]. Thus in testing neurovascular function in diabetic patients, demonstration of impairment of the spreading neurogenic axon reflex flare, whether electrically evoked [30, 33, 34] or chemically induced by prolonged circumferential ACh iotophoresis [24, 25], should be followed by specific tests of the direct endothelial function. This can be achieved by brief iontophoretic application of ACh, and MeCh, dilator responses to which are then measured at the site of application [33, 34], rather than at a distant site [24, 25].

Normal vasodilator responses to these substances are good evidence of the release by endothelium of its relaxing factor (EDRF), and the ability of the vascular smooth muscle to respond to this [11, 28]. In addition, the microvascular smooth muscle function itself may be tested by measuring the laser Doppler flux responses to iontophoretically applied directly acting nitrodilator compounds, $NaNO_2$ or NaNP [33, 34].

Now that other sensitive non-invasive tests of C-fibre function are available [7, 14], we recommend that if a detailed measure of neurovascular function is required (e.g. for initial assessment of a newly diagnosed diabetic or to monitor a patient's progress with new therapy), the warm perception threshold should be measured at two sites, volar forearm near the wrist, and on the foot dorsum.

Finally, the advantage of examining concordance between two different measures of polymodal C-fibre function can be seen from the comparison shown in figure 4c. This revealed two expected groups of patients: those with normal nociceptive function to both tests, EAR and WPT, seen at the bottom right quadrant of figure 4c and those with apparently impaired nociceptive afferent function to both tests. The latter appear in the upper left quadrant of figure 4c and show both elevated WPT and reduced EAR.

Lastly, a third group of neurovascular responses was defined in which patients appeared to have WPT within the normal range, but EAR was reduced (fig. 4c, stippled zone). This group of patients contains some who display a primary vascular endothelial dysfunction [29] while having functional C-nociceptive afferents. These patients are defined after applying the additional tests of endothelium-dependent dilatation (e.g. ACh, MeCh) and of vascular smooth muscle function (e.g. NaNP). These patients also tend to perceive the noxious TENS pulses as painful, unlike many diabetics. An alternative explanation of a near normal WPT and functional nociceptive afferent pathway in the presence of reduced EAR may lie in the dual afferent system (C-thermal receptors and polymodal C-nociceptors) which convey sensations of warmth in man [14]. The assessment of small and large fibre function in long term diabetic patients with and without symptoms of painful neuropathy is now becoming an important part of their detailed clinical assessment [35]. It is hoped that an adequate case has

now been made for the non-invasive measurement of microvascular endothelial function, and of neurovascular interactions in diabetes [34].

Conclusion

We must now acknowledge that endothelial cells are obligatory participants in the blood flux modulation and microvascular relaxation which accompany neurogenic inflammation. Measurement of axon reflex dilator responses using sensitive laser Doppler devices enables relative changes in blood flux to be accurately monitored non-invasively. The serial testing of nociceptive C-afferent fibre function, by measuring axon reflex dilator responses as well as endothelium-dependent and direct smooth muscle dilator responses to iontophoresed chemicals, tests all three major components which mediate microvascular dilatation in the skin. By testing the thermal threshold perception as well, a more comprehensive assessment of nociceptive afferent function is obtained. Applied serially, such non-invasive measures of neurovascular function enable the progress of disease or toxic states to be monitored, and the results of current or new therapies (e.g. aldose reductase inhibitors) in diabetic neuropathy on each component of the microvascular dilator cascade to be more accurately evaluated.

References

1 Alheid, U.; Dudel, C.; Forstermann, U.: Br. J. Pharmacol. *92:* 237–240 (1987).
2 Asbury, A.K.; Brown, M.J.: in Goto, Horiuchi, Kogure, Diabetic neuropathy, pp. 50–57 (Excerpta Medica Elsevier, Amsterdam 1982).
3 Bayliss, W.M.: J. Physiol., London *26:* 173–209 (1901).
4 Boulton, A.J.M.: Med. Clins. N. Am. *72:* 1513–1530 (1988).
5 Braund, W.J.; Moore, J.C.; Williams, S.C.; Walter, S.; Brown, G.; Barrow, B.; Turner, R.C.: Proc. Aust. Diabet. Soc., abstr. 22, August (1987).
6 Colwell, J.A.; Winocour, P.D.; Lopes-Virella, M.; Halushka, P.V.: Am. J. Med. *75:* 67–80 (1983).
7 Delaney, C.A.; Westerman, R.A.; Widdop, R.E.; Hannaford, J.; Carter, B.E.; Roberts, R.G.D.: Proc. Aust. Physiol. Pharmacol. Soc. *19:* 192P (1988).
8 Dorfman, L.J.: Muscle Nerve *7:* 2–11 (1984).
9 Dudel, C.; Forstermann, U.: Eur. J. Pharmacol. *145:* 217–221 (1988).
10 Fruhstorfer, H.; Lindblom, U.; Schmidt, W.G.: J. Neurol. Neurosurg. Psychiat. *39:* 1071–1075 (1976).
11 Furchgott, R.: Circulation Res. *53:* 557–573 (1983).
12 Horowitz, M.; Harding, P.; Maddox, A.; Akkermans, L.; Wishart, J.; Chatterton, B.; Shearman, D.: Diabetes Res. clin. Pract. *5:* suppl. 1, p. S585 (1988).
13 Hutchinson, K.J.; Johnson, B.W.; Williams, H.T.; Brown, G.D.: Surgery Gynec. Obstet. *139:* 566–568 (1974).

14 Jamal, A.; Hansen, S.; Weir, A.I.; Ballantyne, J.P.: J. Neurol. Neurosurg. Psychiat. *48:* 354–360 (1985).
15 Jancso, N.: Bull. Millard Fillimore Hosp. Buffalo *53:* 54–77 (1960).
16 Lembeck, F.: Trends Neurosci. *6:* 106–108 (1983).
17 Lewis, T.: The blood vessels of the human skin and their responses (Shaw & Sons, London 1927).
18 Lewis, T.: Br. med. J. *194:* 431–435, 491–494 (1937).
19 Low, A.M.; Neild, T.O.; Westerman, R.A.: Proc. Aust. Physiol. Pharmacol. Soc. *20(1):* 15 (1989).
20 Low, A.M.; Westerman, R.A.: Life Sci. *45(1):* 49–57 (1989).
21 Luscher, T.F.; Cooke, J.P.; Houston, D.S.; Neves, J.; Vanhoutte, P.M.: Mayo Clin. Proc. *62:* 601–606 (1987).
22 Muldoon, S.M.; Hart, J.L.; Bowen, K.A.; Freas, W.: Anesthesiology *68:* 31–33 (1988).
23 Parkhouse, N.; Le Quesne, P.M.; Scurr, J.: Br. J. Surg. *74:* 534 (1987).
24 Parkhouse, N.; Le Quesne, P.M.: J. Neurol. Neurosurg. Psychiat. *51:* 28–34 (1988).
25 Parkhouse, N.; Le Quesne, P.M.: New Engl. J. Med. *318:* 1306–1309 (1988).
26 Stricker, S.: Kaiserl. Akad. Wiss, Wien *3:* 173–185 (1876).
27 Szolcsanyi, J.; Westerman, R.A.; Magerl, F.W.; Pinter, E.: Regulat. Peptides *22:* 180 (1988).
28 Vanhoutte, P.M.; Rubanyi, G.M.; Miller, V.M.; Houston, D.S.: A. Rev. Physiol. *48:* 307–320 (1986).
29 Westerman, R.A.; Delaney, C.A.; Roberts, R.G.D.; Pratt, A.; Kent, P.; Low, A.: Neurosci, Lett., suppl. 34, p. S170 (1989).
30 Westerman, R.A.; Low, A.M.; Pratt, A.; Hutchinson, J.S.; Szolcsanyi, J.S.; Magerl, F.W.; Handwerker, H.O.; Kozak, W.M.: Clin. exp. Neurol. *23:* 81–89 (1987).
31 Westerman, R.A.; Widdop, R.E.; Hannaford, J.; Hogan, C.; Roberts, R.G.D.; Zimmet, P.: Clin. exp. Neurol. *24:* 129–137 (1988).
32 Westerman, R.A.; Widdop, R.E.; Hannaford, J.; Low, A.M.; Roberts, R.G.D.; Kent, P.; Sideris, K.; Yip, T.; Hales, J.R.S.; Stephens, F.R.N.: Aust. Phys. Eng. Sci. Med. *11:* 53–66 (1988).
33 Westerman, R.A.; Widdop, R.E.; Hogan, C.; Zimmet, P.: Neurosci. Lett. *81:* 177–182 (1987).
34 Westerman, R.A.; Widdop, R.E.; Low, A.M.; Hannaford, J.; Kozak, W.M.; Zimmet, P.: Diabetes Res. clin. Pract. *5:* 49–54 (1988).
35 Ziegler, D.; Mayer, P.; Wiefels, K.; Gries, F.A.: Pain *34:* 1–10 (1988).

Prof. R.A. Westerman, Department of Physiology, Monash University, Clayton, Vic. 3168 (Australia)

neuroReport

1

NeuroReport 2, 421 – 424 (1991)

Chronic nicotine exposure enhances cutaneous axon reflexes in the rat

Jennifer A. Grunfeld[CA], Garry J. Tiedemann and Roderick A. Westerman

Department of Physiology, Monash University, Clayton, VIC 3168, Australia

THE effect of chronic nicotine exposure on the cutaneous neurogenic flare response was investigated non-invasively in rats. Axon reflexes were evoked by transdermal iontophoresis of acetylcholine, and resultant changes in skin microvascular blood flux were measured by laser Doppler flowmetry. Rats given five injections of nicotinesulphate (1 mg kg^{-1} i.p.) daily for 14 days were compared to saline-injected littermates. Axon reflexes were enhanced by 143% after 7 days exposure to nicotine, and by 336% after 14 days exposure. Controls did not display these increases. Axon reflexes measured 7 days after nicotine cessation were similar to pre-nicotine levels. These results may suggest that nicotinic cholinoceptors on skin nociceptors and primary afferents upregulate in response to chronic nicotine exposure, and return to normal following nicotine cessation.

Key words: Nicotine, Nociceptors, Vasodilation, Receptors, nicotinic, Receptors, muscarinic, Receptors, cholinergic, Upregulation, Iontophoresis, Laser Doppler flowmetry, Vasodilator agents

[CA]Corresponding Author

Introduction

Cutaneous neurovascular responses to acetylcholine (ACh) are of two types, nicotinic and muscarinic. Stimulation of nicotinic cholinoceptors found on cutaneous nociceptive sensory nerves[1,2] produces sensations of prickle, itch and burning pain,[3,4,5] and a flare which spreads via the axon reflex. This is a component of Lewis' inflammatory triple response.[6] Local vasodilator responses, mediated by endothelial muscarinic cholinoceptors,[7] depend on functional microvascular endothelium.[8,9,10] These responses are abolished by atropine,[8] and are limited to the area stimulated.[10]

Douglas and Ritchie[2] demonstrated that the excitatory action of ACh on cutaneous unmyelinated fibres is abolished by hexamethonium but not by atropine. Parkhouse and Le Quesne[1] showed that pilocarpine produces direct vasodilation identical to that evoked by ACh, but no flare. These observations imply that initiation of the axon reflex depends on stimulation of nicotinic cholinoceptors.

It is commonly accepted that chronic exposure to an agonist causes receptors to exhibit diminished responsiveness to stimulation.[11] Indeed, this phenomenon, agonist-induced downregulation, has been observed in some classes of peripheral nicotine cholinoceptor.[12,13] Paradoxically, chronic exposure to nicotinic agonists causes upregulation of some central nicotinic receptors, first demonstrated in rat brain by Schwartz and Kellar,[14] and subsequently in mouse brain by Marks, Burch and Collins.[15] Post-mortem studies by Benwell and Balfour[16] detected upregulation in the brains of chronic smokers. Studies supporting these original findings were reviewed recently by Wonnacott,[17] who suggested that agonist-induced upregulation is unique to central nicotinic receptors. However, Hahn[18] showed sensitisation of cutaneous sensory nerves mediating the axon reflex in chronic smokers, and interpreted this as indirect evidence of nicotine-induced upregulation of nicotinic cholinoceptors on cutaneous sensory nerves.

The aim of this study was to isolate the effects of chronic nicotine exposure on specific neurovascular responses in skin. It investigated (a) nicotine-induced changes in ACh-evoked cutaneous axon reflexes in rats; and (b) whether these effects are reversed by cessation of nicotine exposure.

Materials and Methods

Animals used: Female Wistar rats (180–220 g at 7–9 weeks) obtained from Monash University Central Animal House were housed in the Physiology Department Animal House (12 h light, 12 h dark) with food and water *ad libitum*.

Treatment schedule: Ten rats were given intraperitoneal i.p. injections of nicotine sulphate (1 mg kg^{-1}) in 0.2 ml saline, five times daily for 14 days. Eight littermates received parallel injections of 0.2 ml saline as controls.

Testing schedule: Rats were tested at day 0, then randomly assigned to the nicotine-exposed or the control group. Testing was repeated at days 7 and 14 of treatment, and at seven days post-treatment (day 21). The growth rates of rats were determined from daily body weight measurements.

Testing Procedure: Although not painful, the testing procedure was performed under general anaesthesia (pentobarbitone sodium, 40 mg kg^{-1} i.p.) to eliminate voluntary movements, which produce potentially complicating changes in cutaneous blood flow.

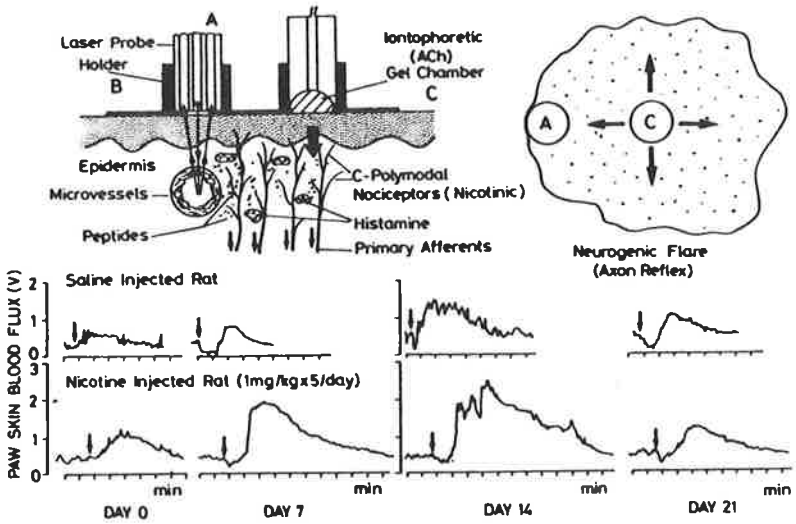


FIG. 1. Diagrammatic section (above left) of laser Doppler probe (A), probe holder (B), 8 mm (centres) from iontophoretic gel chamber (C) on the rat thigh skin. Polymodal C-nociceptors, stimulated by ACh iontophoresis, release peptides which excite adjacent nociceptors initiating the inflammatory cascade, and the spreading neurogenic flare (above right). Examples of skin blood flux changes during axon reflex are shown for a control rat and a nicotine-injected rat.

Anaesthesia was considered adequate when a foot pinch did not elicit the flexor withdrawal reflex.

The anaesthetised rat was laid supine, with hindpaws extended, on a 37° C heated pad to maintain constant body temperature. The probe/electrode chamber of Parkhouse and Le Quesne,[1] who elicited cutaneous axon reflexes in humans by ACh iontophoresis, was redesigned for use on rats. Our chamber has two compartments 8 mm apart; one receives an iontophoretic electrode, and the other holds a laser probe (Fig. 1). This separation of iontophoretic and recording sites ensures that responses measured are neurogenic flares.[1] The chamber was placed on the shaved medial thigh without impeding blood flow, and pinned into a foam block to ensure a fluid-tight seal.

The testing procedure involved transdermal iontophoresis of a vasoactive substance on the medial thigh, while resultant changes in skin microvascular blood flux were measured by laser Doppler flowmetry, to quantify the axon reflex vasodilator response. The procedure was performed six to eight hours after the previous injection, to discount short-term nicotine interactions.

Iontophoresis: Transdermal iontophoresis delivers a precise quantity of polar substance. Drugs iontophoresed were: acetylcholine chloride (ACh); hexamethonium bromide, a nicotinic antagonist; and atropine sulphate, a muscarinic antagonist.

Drugs, dissolved in distilled water, were blended with 4% methylcellulose gel, producing 1% aqueous drug–gel solutions. A hemispherical perspex chamber containing 80 μl of drug–gel solution on a stainless steel electrode was inserted into the remote orifice of the chamber.

A battery-powered constant current stimulator (WPI 305-R) passed a direct current through the gel; an indifferent electrode placed on the rat's forepaw completed the circuit. The iontophoretic electrode was cationic, the same polarity as the drugs applied. A constant current of 0.2 mA was passed for 20, 40 or 80 s; the product of current and time gives a charge of 4, 8 or 16 mC, which determines the amount of drug transferred.[19]

Studies of receptor antagonism were performed in control rats using consecutive iontophoreses of 16 mC ACh, 16 mC hexamethonium or atropine, and 16 mC ACh, at 20 min intervals.

Blood flux measurement: The Periflux PF1d Laser Doppler Flowmeter (Perimed, Sweden) provided continuous real-time measurements of microvascular changes elicited by ACh iontophoresis.[1,8,10,19] A stable baseline recording was obtained for 5–10 min before stimulation commenced.[8] Vasodilator responses to ACh were monitored until blood flux returned to its resting level.

Microvascular dilator responses were quantified by measuring the area under the voltage-time curve recorded on an ICI DP600 chart recorder. This area was integrated using the Sigmascan image analysis package on an IBM AT compatible PC with a magnetic tablet and stylus attached. Blood flux response (V min) is the integral of flux (V) and time (min).

Statistical analysis: Data are expressed as the mean ± s.e.m. Bartlett and Cochran tests for homogeneity of variance required square root transformations of weekly data, and log transformations of cholinergic antagonist data, before analysis of variance (ANOVA) could be legitimately used. Having thus achieved homogeneity of variance, the data were analysed using ANOVA followed by Student Newman Keuls multi-range tests. Results where $p < 0.05$ are termed significant in this paper.

Results

Effect of chronic nicotine exposure on axon reflex: Examples of vasodilator responses to 16 mC ACh iontophoresis before, during and after chronic nicotine exposure are shown compared with control responses in Fig. 1. Onset latency is constant; however, responses obtained during nicotine exposure show increased amplitude and duration.

Axon reflex vasodilator responses evoked by graded iontophoretic doses of ACh (4 mC, 8 mC, and 16 mC) in control and nicotine-exposed rats are shown in Fig. 2. Nicotine-exposed rats exhibited significantly enhanced axon reflexes at all doses on days 7 and 14. Seven days after nicotine cessation, responses to 4, 8, and 16 mC ACh iontophoresis were comparable to those at day 0. Responses in control rats were unaccountably elevated by 0.346 V min at day 0 compared to the remainder of the testing period.

The extent of axon reflex enhancement in nicotine-exposed rats is clearly shown in Fig. 3. Responses to 16 mC ACh increased from 1.42 ± 0.36 V min at day 0 to 3.46 ± 0.61 V min (143% increase) after seven days of nicotine administration. After a further seven days of nicotine treatment, responses had reached 6.19 ± 0.82 V min, a total increase of 336%. Responses at day 21 were 1.48 ± 0.32 V min, not significantly different from those at day 0.

Cholinergic antagonist studies: Hexamethonium iontophoresis (16 mC) reduced the size of the axon reflex vasodilator response to ACh from 2.03 ± 0.60 V min to 0.21 ± 0.07 V min, a 90% decrease. Iontophoresis of 16 mC atropine did not significantly alter the response (control 2.03 ± 0.60 V min; atropine 2.26 ± 0.70 V min) (Fig. 4).

Effect of nicotine on growth: The control group's growth rate was comparable to that of age-matched rats in the animal house. During the 14 day injection period, growth rates of nicotine-exposed rats were at first slightly retarded, then parallel to but on average 10 g behind the controls. Following cessation of injections, the nicotine-exposed group showed supranormal growth rates, overtaking the controls after 9 days, and were on average 10 g heavier after 21 days. Growth retardation during nicotine exposure, and post-cessation weight gain, were expected, and have been previously reported.[20]

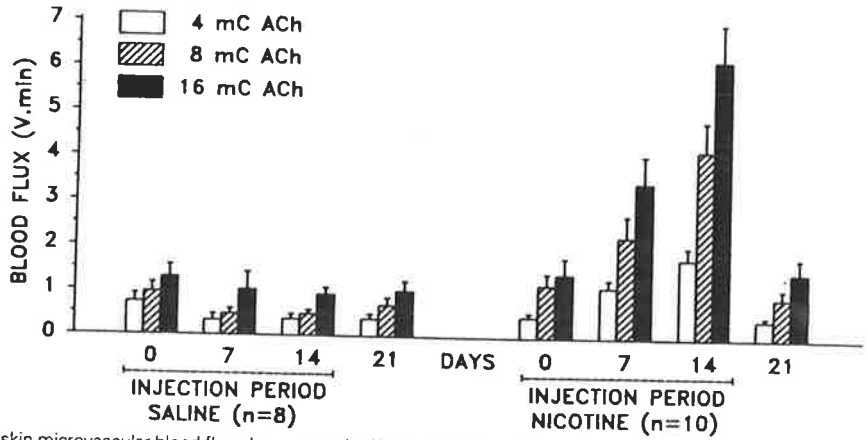


FIG. 2. Comparison of skin microvascular blood flux changes evoked by 4, 8 and 16 mC graded iontophoretic doses of ACh in rats injected i.p. with either saline or nicotine for 14 days, and then allowed to recover for 7 days. Dose-responses are significantly enhanced at days 7 and 14 ($p < 0.05$).

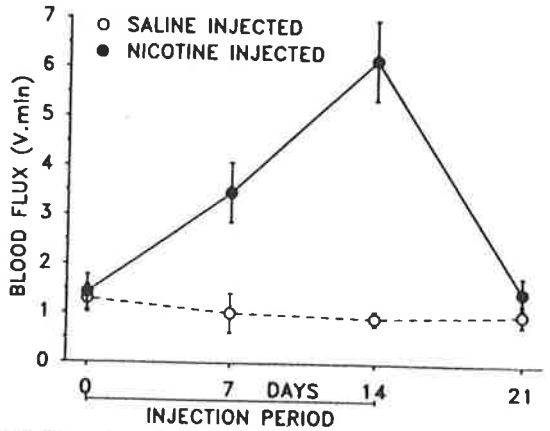


FIG. 3. Comparison of skin microvascular blood flux changes evoked by 16 mC graded iontophoresis in rats injected i.p. with either saline or nicotine for 14 days, and then allowed to recover for 7 days. Responses of nictoine-exposed rats are significantly enhanced at days 7 and 14 ($p < 0.05$).

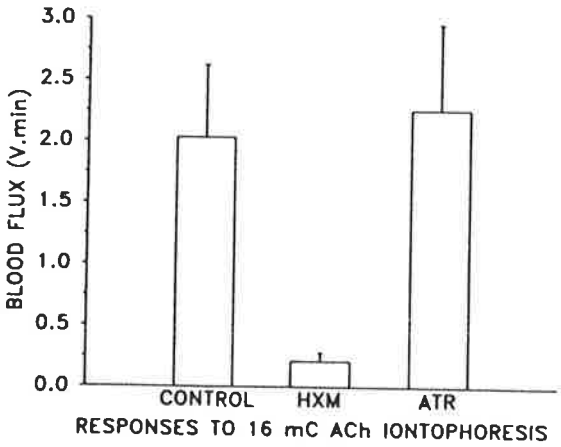


FIG. 4. Effects of hexamethonium (HXM) and atropine (ATR) on skin microvascular blood flux changes evoked by 16 mC ACh iontophoresis ($n = 8$). Responses are significantly attenuated by HXM ($p < 0.05$).

Discussion

Our results provide direct evidence, supporting previous findings,[1,2,4,5,18] that it is the nicotinic cholinoceptors on primary sensory nerves in rat skin which mediate the axon reflex flare. This phenomenon is distinct from the muscarinic endothelium-dependent vasodilation also produced by ACh,[9] which persists in denervated skin.[8]

The primary finding of this study is that both 7 and 14 days exposure to nicotine in rats causes a significant increase in the size of the neurogenic axon reflex flare evoked by ACh iontophoresed 8 mm from the laser probe. This is reversed by cessation of exposure. Our results confirm, in rats, Hahn's association of nicotine with axon reflex enhancement in chronic smokers,[18] while eliminating the confounding effects of the many other substances in cigarette smoke.

Alterations in the kinetics or sequencing of neuropeptide mediators might explain this enhancement of axon reflex flare; however, such effects of nicotine have never been reported.

Alternatively, the present findings, revealing selective sensitisation of cutaneous nicotinic receptors on primary sensory nerves during chronic nicotine exposure, with resulting enhancement of ACh-evoked axon reflex, can be interpreted in line with previous findings describing upregulations of nicotinic receptors in rat brains during chronic nicotine exposure.[13,14]

Similarly, the rapid attenuation of sensitised axon reflexes in the nicotine exposed group, when tested 7 days after the last nicotine administration, may be interpreted as a return to normal nicotinic receptor levels. This phenomenon has been described in central nicotinic receptors following cessation of nicotine exposure,[21,22] but has not previously been reported in nicotinic cholinoceptors on peripheral nerves.

If the nicotine-induced upregulation is the mechanism of axon reflex enhancement, it would refute Wonnacott's suggestion that central nicotinic receptors are unique in exhibiting agonist-induced upregulation.[17]

Although *in-vitro* analyses of certain peripheral nicotinic cholinoceptors have shown agonist-induced downregulation,[12,13] these must be verified by *in-vivo* studies, which are essential when investigating the neurovascular system. When subject to normal regulatory control, these receptors also may exhibit upregulation in response to chronic agonist exposure.

The paradox of upregulation of central nicotinic cholinoceptors in response to chronic nicotine exposure has been explained in pharmacodynamic terms. During continuous administration, nicotine may act as a functional antagonist, causing prolonged desensitisation,[23] whch results in compensatory upregulation of central nicotinic cholinoceptors.[14,15,21] If repeated administration of nicotine causes protracted functional blockade of nicotinic cholinoceptors on cutaneous sensory nerves, this may signal the cell to upregulate the receptor. However, the predominant nictotinic receptor subtypes expressed in brain differ from those found in peripheral tissues.[17,24]

Rats are widely used to study diabetic neuropathies and vascular changes.[8] We have proven the rat useful as a model of nicotine-induced neurovascular changes, for investigations inappropriate in human subjects. The simple, non-invasive nature of our testing procedure will enable extension of research into nicotine's effects on cutaneous receptor function in humans.

Conclusion

Our findings establish that nicotinic cholinoceptors on cutaneous nociceptive nerves, but not muscarinic receptors on vascular endothelium,[9] are sensitised by chronic exposure to nicotine for 7 and 14 days in rats. This suggests that chronic nicotine exposure may induce upregulation of nicotinic cholinoceptors on nociceptive skin nerves in the rat. Further, this effect is reversed by cessation of nicotine. Although this remains to be confirmed by ligand binding[23] and by more specific receptor antagonists (eg. tetrodotoxin or dihydro-beta-erythroidin[21]), both the daily dose of nicotine (5 mg kg^{-1}) and the time course of its effect on cutaneous sensory nerves correspond to those described for the upregulation of nicotinic receptors in rat brain.[21,22]

Given that stimulation of cutaneous nociceptor endings produces impulses which travel centrally to produce the sensation of pain, and to axon collaterals to initiate neurogenic vasodilation, it now remains to be determined if chronic smokers experience enhanced pain perception as a result of chronic nicotine exposure.

References

1. Parkhouse N, Le Quesne PN. *J Neurol Neurosurg Psychiatry* **51**, 28–34 (1988).
2. Douglas WW, Ritchie JM. *J Physiol (Lond)* **150**, 501–514 (1960).
3. CIBA Foundation Study Group No. 1. Wolstenholme GEW, O'Connor M, eds. *Pain and itch, nervous mechanisms*. London: Churchill, 1959; 3–59, 84–97.
4. Keele CA, Armstrong D. *Substances producing pain and itch*. London: Arnold, 1964
5. Holton P, Perry WLM. *J Physiol (Lond)* **114**, 240–251 (1951).
6. Lewis T. *BMJ* **194**, 431–435, 491–494 (1937).
7. Augus JA, Cocks TM. *Pharmacol Ther* **41**, 303–351 (1984).
8. Low AM, Westerman RA. *Life Sci* **45**, 49–57 (1989).
9. Furchgott RF. *Ann Rev Pharmacol Toxicol* **24**, 175–197 (1984).
10. Westerman RA, Magerl FW, Szolcsányi J *et al.* Vasodilator Axon Reflexes. In: Vanhoutte PM ed. *Vasodilation: vascular smooth muscle, peptides, autonomic nerves, and endothelium*. New York: Raven Press, 1988; 107–112.
11. Creese I, Sibley DR. *Ann Rev Pharmacol Toxicol* **21**, 357–391 (1981).
12. Robinson D, McGee R. *Mol Pharmacol* **27**, 409–417 (1985).
13. Berg DK, Boyd T, Halvorsen SW, *et al. Trends Neurosci* **12**, 16–21 (1989).
14. Schwartz RD, Kellar KJ. *Science* **220**, 214–216 (1983).
15. Marks MJ, Burch JB, Collins AC. *J Pharmacol Exp Ther* **226**, 817–825 (1983).
16. Benwell MEM, Balfour DJK, Anderson JM. *J Neurochem* **50**, 1243–1247 (1988).
17. Wonnacott S. *Trends Pharmacol Sci* **11**, 216–219 (1990).
18. Hahn A. *The effect of smoking on small nerve and blood vessel function in man*. B.Sc. Hons. Thesis: Monarch University, 1990.
19. Westerman RA, Widdop RE, Hannaford J, *et al. Aust Phys Eng Sci Med* **11**, 53–66 (1988).
20. Levin ED, Morgan MM, Galvez C, *et al. Physiol Behav* **39**, 441–444 (1987).
21. Schwartz RD, Kellar KJ. *J Neurochem* **45**, 427–433 (1985).
22. Marks MJ, Stitzel JA, Collins AC. *J Pharmacol Exp Ther* **235**, 619–628 (1985).
23. Weber M, David-Pfeuty T, Changeux J-P. *Proc Natl Acad Sci USA* **72**, 3443–3447 (1975).
24. Lindstrom J, Schoepfer R, Conroy WG, *et al. CIBA Found Symp* **152**, 23–42 (1990)

ACKNOWLEDGEMENTS. We sincerely thank Mr David Caddy for his excellent statistical advice, and Professor R. Hales, Dr T. Nield and Dr D. Walker for helpful discussions during the preparation of this report. This study was supported in part by the National Heart Foundation, the Australian Research Council, and the National Health and Medical Research Council.

Received 21 May1991; accepted 16 June 1991

neuroReport

NeuroReport 4, 467–470 (1993)

Denervation impairs cutaneous microvascular function and blister healing in the rat hindlimb

R. W. Carr, C. A. Delaney, R. A. Westerman[CA] and R. G. D. Roberts

Department of Physiology Monash University, Clayton, Victoria 3168, Australia

SKIN sensory nerve nocifensor functions were investigated non-invasively in rats by measuring neurogenic inflammation and blister healing-rate after unilateral hindlimb denervation. Axon reflexes were evoked by transdermal iontophoresis of acetylcholine (ACh) or noxious electrical stimulation (TNS). Sodium nitroprusside (SNP) evoked direct dilator responses. Resultant changes in skin microvascular blood flux were measured by laser Doppler flowmetry. Compared with their sham-operated control limbs, denervation reduced inflammatory responses (ACh or TNS) by more than 85% and SNP responses by 28% ($p < 0.05$). Healing of dry-ice blisters raised on the hindpaw 14d post-denervation was significantly slower to complete healing (42d) than controls (26d) and initial inflammation was attenuated, confirming that innervation is important for inflammation and blister-healing.

Key words: Denervation; Neuropeptides; Blister healing; Neurogenic inflammation; Axon reflexes; Laser Doppler flowmetry; Microvascular reactivity; Iontophoresis; Acetylcholine; Sodium nitroprusside

[CA] Corresponding Author

Introduction

Since Lewis[1] first assigned nocifensor functions to cutaneous primary afferent nerves, this role,[2] and that of their neuropeptides has been extensively studied.[3,4] Because of recent reports of possible trophic functions of neuropeptides,[5] in wound healing,[6,7] we aimed to demonstrate the importance of sensory nerves in neurovascular responses and cutaneous wound healing processes. Our working hypothesis was that a reduction in sensory nerve functions would impair neurogenic inflammation and wound healing.

When a noxious stimulus either electrical, chemical, thermal or mechanical impinges on the skin, polymodal nociceptors respond with a discharge both orthodromically to the spinal cord and antidromically along collateral branches generating the so called 'axon reflex'. Axon reflexes result in neuropeptide release from all arborised nerve terminals and produces subsequent vasodilatation through action directly on vascular endothelium or smooth muscle or via the liberation of vasoactive substances from mast cells.[8]

The project strategy in the rat was to denervate one hindlimb surgically and to measure (a) electrically and chemically evoked axon reflexes, as indicators of neurogenic inflammation; (b) microvascular dilator responses to direct iontophoretic application of endothelial excitant ACh and smooth muscle relaxant SNP; and (c) blister area[8] daily, to derive blister healing rate in glabrous skin of both denervated and sham control hindpaws.

Materials and Methods

Animals used: Female Wistar rats (185–254 g), aged 8–12 weeks were used. Rats were housed at 19–23°C, on a 12 h light/dark cycle, and caged in groups of four except for recovery periods of 5–10 days following surgery, when they were individually caged to minimize autotomy. Rats were fed commercial rat chow and watered *ad libitum*.

Testing schedule: On day 0, skin flare in response to noxious electrical skin stimulation[9] and iontophoresis of acetylcholine (ACh) and nitroprusside (SNP) was measured by laser Doppler velocimetry.[10] Unilateral hindpaw sensory denervation was immediately performed and fourteen days later neurovascular function was reassessed as above. Sodium pentobarbitone (40 mg kg^{-1} i.p.) was used for general anaesthesia with supplementary doses (10 mg kg^{-1}) as required. During all surgical and testing procedures, body temperature was maintained at 37°C.

Denervation: In one hindlimb cutting sciatic and saphenous nerves resulted in denervation of that hind footpad. In a sham operation on the contralateral limb, nerves were isolated but not cut, each animal thus served as its own control.

Neurovascular Testing: *(a) Noxious Transcutaneous Electrical Stimulation* was performed on the plantar aspect of the hind foot pad with a pair of 27G needle electrodes placed intradermally, 1 cm apart on the hairline of the heel, with cathode distal to anode for stimulation of the innervation zone of posterior tibial and saphenous nerves.[8] A series of 2, 4, 16 rectangular electrical pulses of noxious current strength (150 V, 2 Hz, 1 ms duration) were delivered to the test site, while skin blood flow was recorded immediately distal to the cathode.[9] *(b) Iontophoresis of vasoactive compounds* has been described in detail previously.[10] Doses of iontophoretically applied drugs used, expressed in terms

of the total charge transferred in milliCoulombs (mC), were ACh 4 mC (direct), ACh 16 mC (indirect) and 8 mC SNP (direct).

Blood flux responses to vasodilator stimuli were recorded using the Periflux Pf1d laser Doppler flowmeter in the arrangement described previously.[10] Axon reflex responses to electrical stimulation and to indirect ACh iontophoresis were integrated between stimulus offset and the point at which flux returned to baseline. Responses to iontophoresis of ACh and SNP directly under the laser probe were integrated over the first 4 min of the response, as these responses often do not return to baseline for many minutes. In all cases, the response was defined as the integral of the voltage-time trace analysed and measured using an LC digitizing tablet.

Wound healing: Induction of blisters: Uniform, controlled blisters were created bilaterally 14 days after hindlimb denervation by application of a standardized 7 mm diameter pellet of solid carbon dioxide (dry ice) for a period of 30 s at a constant pressure of 0.25 N. Blisters were induced in a concave part of the foot pad at the base of the toes to minimize post-wound trauma and lameness. Measurements of the physical dimensions of the wound margin (delineated as the neurogenic flare border, or the border of the scab) were taken daily.

Statistics: Data are expressed as means and standard error of the mean (s.e.m.). Statistical analysis was performed using analysis of variance ANOVA with *post-hoc* analysis and Student's *t*-test for both paired and unpaired data. Statistical significance was taken as $p < 0.05$ for ANOVA or *t*-test. Pearson's R-value was used as an indicative significance for linear correlation where appropriate.

Results

Neurovascular testing: Due to the incidence of autotomy full results for only 12 out of 15 animals are available. Vasodilator responses elicited via the axon reflex were significantly reduced by more than 85% in the denervated compared with sham operated limb 14 days post-surgery for both chemical and transcutaneous noxious electrical stimuli (TNS).

Responses to noxious TNS: Electrical axon reflex (EAR): Skin flare responses to noxious electrical stimulation (16P, Fig. 1B, C) were reduced significantly by 96% in the denervated limb 14 days post surgery compared with sham operated limbs ($p < 0.01$). In addition responses to 16P were also significantly reduced in the sham operated side 14 days post surgery compared with the untreated response on day 0 ($p < 0.05$).

Chemically evoked neurovascular responses: Inset in Figure 2A is an example of a chemically evoked neurovascular response. *Chemical axon reflex*: The ACh-evoked indirect (axon reflex) response in the denervated limb was significantly reduced by 85% at 14 days post surgery compared with sham operated control limb responses at that time (paired *t*-test, $p < 0.001$). There was no significant difference in the size of the chemical axon reflex responses on days 0 and 14 in the sham operated limb (paired *t*-test, $p > 0.05$) (Fig. 2A).

Smooth muscle and endothelium-dependent responses: The response to the direct nitrodilator SNP (8 mC) was significantly reduced in the denervated limb on day 14 ($p < 0.05$) compared with the sham treated limb, while the response to the endothelium dependent ACh (4 mC) directly applied was not significantly changed by denervation ($p > 0.05$) (Fig. 2B, C). There was a trend for both direct nitrodilator and ACh responses in sham treated legs to be reduced on day 14 compared with day 0 but this was not significant ($p > 0.05$, Fig. 2B, C).

Effects of denervation on blister healing: Healing-rate profiles for denervated and sham-operated rats (i.e. different legs of the same animal) are shown in Figure 3. Figure 3A shows the overall profiles for denervated

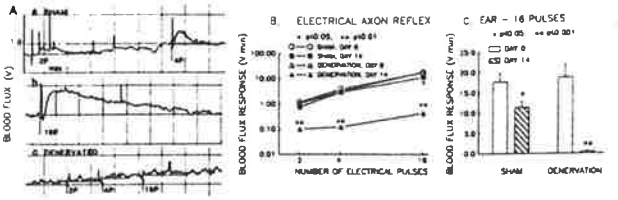


FIG. 1. *Denervation effects on electrically-evoked neurogenic inflammation.* Axon reflex blood flux responses to indirect (remote) stimuli in both sham operated and denervated limbs at day 0 before surgery and 14 days post-operatively. Each animal is its own control (group $n = 12$). **A**: shows typical dilator responses for 2, 4, 16 pulses (150 V, 2 Hz,1 ms) of noxious electrical stimulation. Abscissa is skin blood flux (Volts), ordinate time (min). **B**: depicts axon reflex dilator responses to 2, 4 and 16 noxious electrical pulses (150 V, 2 Hz, 1 ms duration). The Y-axis is the volt.min integral on a logarithmic scale against number of pulses on the horizontal (linear). * indicates mean denervated 14d response significantly different from untreated day 0 value, $p < 0.05$.** indicates significance of $p < 0.01$. **C**: shows the significant reduction in electrically-evoked axon reflex responses in denervated ($p < 0.001$) and sham ($p < 0.05$) limbs, compared with the pre-operative control values for the same animals.

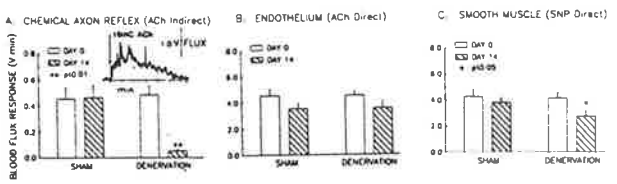


FIG. 2. *Denervation effects on chemically-evoked neurogenic inflammation, and microvascular function.* **A, B, C**: Histograms showing direct dilator responses in both sham-operated and denervated limbs at day 0 (before surgery) and 14 days post-operatively. Each animal is its own control (group $n = 12$). Blood flux response is volt.min ± s.e.m. **A**: shows chemical axon reflex dilator responses to 16 mC of iontophoretic acetylcholine remote from the recording probe. Compared with the responses on sham-operated limb, the **denervated limb showed significantly reduced chemical axon reflexes ($p < 0.01$). **B**: shows the microvascular dilator response to direct endothelial excitant ACh (4 mC) directly under the laser probe which is not significantly reduced in denervated skin compared with either controls on day 0 or sham-operated limbs on day 14, $p > 0.05$. **C**: shows the responses to direct nitrodilator SNP (8 mC), *indicates statistically significant decrease compared with sham treated day 14 value, $p < 0.05$.

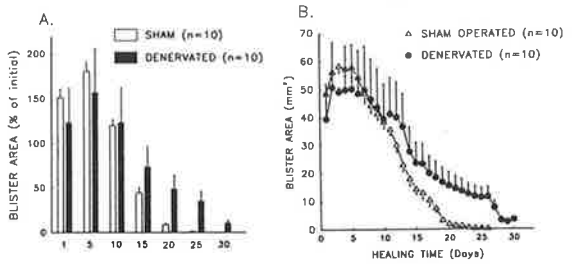


FIG. 3. *Blister healing rate after denervation.* Comparison of healing time in sham-operated (open symbols, broken line) and denervated (filled symbols, solid black line) limbs of the same animals. **A:** Histograms of area relative to initial blister size are shown against time and **B:** shows raw healing profiles of area retraction. Significant reduction in healing profile shape and overall duration ($p < 0.05$) is evident in denervated compared with sham-operated limbs.

and sham-operated limbs, with a statistically significant difference between the two ($F(1,18) = 5.78$, $p < 0.05$), the initial blister inflammatory response being attenuated in the denervated paw. In addition, the overall healing times of the two limbs were significantly different ($p < 0.0001$, paired t-test), the denervated limb showing a mean blister-healing time of 42d compared with 26d for blisters on the sham-operated limb. Figure 3B shows the high degree of data variability in wound area measurement of the denervated limbs. Photographs of typical denervated and sham-operated limb blisters are shown in Figure 4.

Discussion

Wound healing involves a complex, integrated series of events with many neural, humoral, cellular and vascular elements. The role of sensory nerves in the initial inflammatory response to injury is well documented.[5,8] Lewis[1] reported that cutting skin nerve fibres in humans abolished the 'normal' triple response (skin flare, flush, wheal) to all modalities of noxious stimulation. This observation has since been consolidated with studies of surgical and chemical lesioning (chronic capsaicin) demonstrating both the functional and morphological demise of primary afferent 'nocifensor' nerves.[3] Consistent with the notion that local inflammation, evident as a spreading red flare, is a nerve mediated phenomenon, the present study found significantly attenuated dilator responses 6 mm from the site of noxious electrical and chemical stimulation. Since White and Helme[11] found a 65% reduction in substance P-like immunoreactivity 10 days after both surgical and chemical lesions, it is likely that the presence of even a small response to both stimulus modalities 14 days after surgical denervation in the present study, may be attributed to a residual level of peptide in the nerve terminal. The effect of movement artifact of tissue elements, particularly following TNS cannot however be discounted. The present observation of a significantly reduced dilator response to 16 noxious electrical pulses may reflect some alteration in neural or vascular elements, or both.

Denervation altered sensitivity: This phenomenon which commonly follows surgical interruption of innervation, shifts the dose-response curve to the left. Fleming[12] described this as a state in which the amount of substance required to produce a given biological response is less than normal. In the case of vascular smooth muscle, the increased sensitivity is non-specific and is not accompanied by any changes in receptor density or affinity.[13] Whilst denervation super-sensitivity has been demonstrated for a number of constrictor agents[12] including adrenaline, the response to dilators is less well documented due to the distinct loss of vessel tone following denervation. No evidence of neurovascular dilator denervation supersensitivity was observed in the current study. In fact a tendency toward generally reduced vasodilator responsiveness was observed. As no changes in resting blood flux occurred, this tendency is unlikely to be artefactual. Reduced dilator sensitivity after denervation suggests a dose-response shift to the right and may indicate a change in the level of soluble guanylate cyclase, the cytoplasmic enzyme through which nitroprusside mediates its effect.[14] Measurement of a full dose-response relation for SNP on each animal would be desirable to determine any shift and to properly eliminate any ceiling effect of the dilation. Further, the reduction in response to the nitrodilator SNP seen here in denervated rat skin, also has been demonstrated in humans with a genetic defect in which primary sensory denervation is the prominent feature.[15] The non-significant reduction in response size observed both for the endothelium-dependent action of acetylcholine and for the vessel smooth muscle itself (activated by direct nitrodilator) in sham operated limbs, possibly indicates some effect of surgery either directly or indirectly. This observation is consistent with that of Helme and Andrews[3] in which they also found a reduced cellular inflammatory response (leucocyte and protein exudate) in sham operated legs. The 'sham' response phenomena may be mediated by stress-related hormones or reflexes and seems worthy of further study.

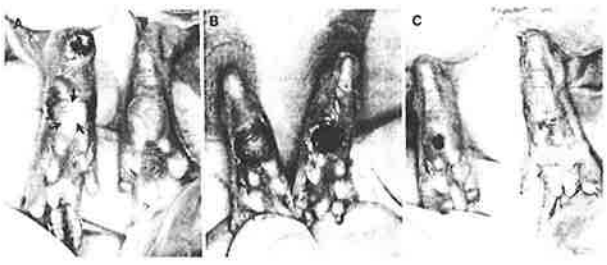


FIG. 4. Photographs of healing blisters on sham-operated and denervated rat paws. **A:** The plantar aspect of paws 4 days after induction of cryothermal blister. The foot on the left was denervated 14 days prior to blister formation (arrows) and shows less plasma extravasation or oedema fluid in the blister, and the presence of spontaneous lesions and dystrophic skin compared with the sham-operated right limb. **B, C:** Non-operated control rat paws showing appearance of normal blister healing at B: 7 days and C: 16 days after blister induction.

Denervation—pathophysiology: The surgical denervation in the present study is highly non-selective and non-specific with all nerve types lesioned. The role of sympathetic post-ganglionic neurones is well established in microvascular regulation. Recently Hottenstein[16] *et al* demonstrated that neonatal capsaicin treatment in rats (which chemically lesions capsaicin-sensitive nerves) resulted in a lower persistent vasoconstriction in the gut, suggesting that peptidergic afferent nerves also may regulate resting vascular tone with active dilatation. Furthermore, interactions between C-fibre afferents and sympathetic post-ganglionic responses have been demonstrated.[17] Both classes of peptidergic and post-ganglionic sympathetic fibres were sectioned in the current study resulting in loss of vascular innervation and calibre regulation. Activity of vascular smooth muscle may be necessary to retain normal function, integrity and responsiveness, and thus quiescence of the vessel may in itself contribute to the reduced vascular responses presently observed. The presence of spontaneous cutaneous lesions in capsaicin densensitized animals,[16] suggested a trophic role of capsaicin sensitive afferents. In all present cases of surgical denervation, spontaneous lesions were apparent on the plantar aspect of the foot pad and the skin was dystrophic and lustreless. Observations in the present study support the incidence of spontaneous lesions (Fig. 4) attributable to nerve section but do not isolate primary afferent (capsaicin sensitive) sensory nerves as the specific influence due to the non-selective denervation technique.

Mechanisms of neuropeptide action in inflammation and repair: The nervous system contributes to inflammation and inflammatory disease,[18] but the precise mechanisms and sequence are unknown. Neurogenic inflammation and subsequent anti-inflammatory actions of neuropeptides have been demonstrated convincingly by antidromic stimulation of dorsal roots.[19] A further action of neuropeptides in activating the immune system and modulating lymphocytes has been demonstrated[20] and substance P, either exogenous or endogenous, activates regional lymph nodes.[21] Calcitonin gene-related peptide (CGRP) has improved wound healing and musculocutaneous critical flap survival in the rat[22] while capsaicin-evoked sensory denervation decreased the survival of such experimental critical flaps.[23] One possible mechanism mediating these effects is increased blood flow,[23] as neuropeptides are potent vasodilators.[6] Another possibility stems from the demonstrated stimulation of connective tissue cell growth.[24] An effect of neuropeptides on angiogenesis during the earlier phase of blister healing[25] is possible but not proven, and is rendered less likely by the present observations suggesting that the later stage of blister healing (after day 15) is most affected by denervation. Finally, it is known that the presence of nerve injury may create postural asymmetries between the hindlimbs during stance and walking and may alter self-licking behaviour. A contribution of such asymmetries to different blister-healing rates cannot be excluded.

Conclusion

Overall, following denervation the reduced axon reflex and wound healing rate probably reflects reduced peptide availability with a consequent decline in neurogenically triggered humoral and cellular responses to injury, and delayed healing. Reduced microvascular responsiveness to dilators such as SNP is a new finding which would also be expected to retard healing of cutaneous wounds, perhaps due to denervation-evoked desensitization of vascular smooth muscle.

References

1. Lewis T. *Br Med J* **194**, 431–434; 491–494 (1937).
2. Lembeck F. *Trends Neurol Sci* **6**, 106–108 (1983).
3. Helme RD and Andrews PV. *J Neurosci Res* **13**, 453–459 (1985).
4. Szolcvsanyi J. *Agents Act* **23**, 4–11 (1988).
5. Maggi CA, Borsini F, Santicioli P *et al. Naunyn-Schmiedeberg's Arch Pharmacol* **336**, 538–545 (1987).
6. Kjartansson J and Dalsgaard C-J. *Eur J Pharmac* **142**, 355–358 (1987).
7. Skerret P. *J Science* **249**, 625–626 (1990).
8. Helme RD, White DM and Andrews PV. *Exp Brain Res* **59**, 382–387 (1985).
9. Low AM and Westerman RA. *Life Sci* **45**, 49–57 (1989).
10. Grunfeld JA, Tiedemann GJ and Westerman RA. *NeuroReport* **2**, 421–424 (1991).
11. White DM and Helme RD. *Brain Res* **336**, 27–31 (1985).
12. Fleming WW. *Rev Neurosci* **2**, 43–90 (1976).
13. Nasseri A, Barakeh JF, Abel PW *et al. J Pharmacol Exp Ther* **234**, 350–357 (1985).
14. Angus JA, Cocks TM. *Med J Aust* **146**, 250–253 (1987).
15. Westerman RA, Block A, Nunn A *et al. Clin Exper Neurol* **29**. In press (1993).
16. Hottenstein OD, Pawlik WW, Remak G *et al. Naunyn-Schmiedeberg's Arch Pharmacol* **343**, 179–184 (1991).
17. Shyu BC, Olausson B and Andersson SA. *Acta Physiol Scand* **137**, 85–91 (1989).
18. Basbaum AI and Levine JD. *Can J Physiol Pharmacol* **69**, 647–651 (1991).
19. Pinter E and Szolcsanyi J. *Agents Act* **25**, 240–242 (1988).
20. Payan DG, Levine JD and Goetzl EJ. *J Immunol* **132**, 1601–1604 (1984).
21. Helme RD, Eglezos G, Dandie GW *et al. J Immunol* **139**, 3470–3478 (1987).
22. Kjartansson J, Dalsgaard C-J and Jonsson C-E. *Plast Reconstr Surg* **79**, 218–221 (1987).
23. Kjartansson J, Lundeberg T, Samuelson UE *et al. Acta Physiol Scand* **134**, 89–94 (1988).
24. Nilsson J, von Euler AM and Dalsgaard C-J. *Nature* **315**, 61–63 (1985).
25. Kishimoto S. *J Invest Dermatol* **83**, 219–223 (1984).

ACKNOWLEDGEMENTS: This study was supported by the Australian Research Council, the Shepherd Foundation Research Trust and the Rebecca L. Cooper Research Foundation.

Received 18 January 1993; accepted 17 February 1993

NeuroReport 4, 635–638 (1993)

Maternal nicotine exposure enhances cutaneous axon reflexes in the neonatal rat

Jennifer A. Grunfeld, Garry J. Tiedemann and Roderick A. Westerman[CA]

Department of Physiology, Monash University, Clayton, Victoria 3168, Australia

[CA] Corresponding Author

CHRONIC nicotine exposure enhances axon reflexes in adult rats. Since smoking mothers expose their infants to nicotine, this study investigated whether late gestational and or lactational maternal nicotine exposure affects neonatal axon reflexes. Osmotic pumps, implanted subcutaneously in adult female rats, delivered either nicotine (5 mg kg^{-1} day^{-1}) or saline. Axon reflex responses of infant progeny, evoked by iontophoresis of 2 mC acetylcholine, were measured by laser Doppler flowmetry. Nicotine-exposed infants showed significantly enhanced axon reflexes during both gestational and lactational nicotine exposure, which recovered after exposure ceased. Controls did not exhibit these changes. Maternal nicotine exposure reversibly sensitized nicotinic cholinoceptors on infant cutaneous sensory nerves, but not muscarinic cholinoceptors on vascular endothelium. This may result from upregulation of cutaneous nicotinic cholinoceptors.

Key words: Nicotine; Osmotic pump; Nociceptors; Iontophoresis; Vasodilator agents; Laser Doppler flowmetry; Axon reflex; Receptors, nicotinic; Upregulation; Infants

Introduction

Infants of smoking mothers are exposed to nicotine prenatally, as nicotine freely crosses the placenta,[1] and postnatally, as it is found in breast milk.[2] Recent studies[3] have demonstrated that chronic nicotine exposure causes enhanced cutaneous axon reflexes in adult rats, with rapid attenuation following nicotine cessation. This was interpreted in line with descriptions of nicotinic cholinoceptor upregulation in rat brain during chronic nicotine exposure.[4] Slotkin *et al*[5] subsequently demonstrated that foetal exposure to nicotine produced effects similar to those in mature brain, that is, agonist-induced nicotinic cholinoceptor upregulation.

The aim of this study was to investigate in neonatal rats: (a) the effects of maternal nicotine exposure during late gestation and/or lactation on acetylcholine (ACh) evoked cutaneous axon reflexes; and (b) whether these effects are reversed by cessation of maternal nicotine exposure. Cholinergic antagonist studies were performed to confirm that cutaneous axon reflex responses measured were mediated by nicotinic cholinoceptors. A preliminary report of the findings has appeared.[6]

Materials and Methods

Animals used: Pregnant Wistar rats (7–9 weeks old) obtained from Monash University Central Animal House were housed in the Department of Physiology Animal House (12 h light, 12 h dark) with food and water *ad libitum*.

Treatment schedule: Three cohorts, each comprising eight pregnant rats, were implanted with Alzet mini-osmotic pumps, model 2001 (1.0 μl h^{-1}, 7 d). Pumps were implanted subcutaneously (s.c.) between the scapulae, under neurolept analgesia (fentanyl 50 μg kg^{-1}, droperidol 2.5 mg kg^{-1} i.m.). In each cohort, six rats received pumps delivering nicotine sulphate (5 mg kg^{-1} day^{-1}) in saline, and two rats received saline pumps as controls. The three cohorts were treated as follows: Cohort A (Late Gestational Exposure): Rats in Cohort A received 7-day pumps at 14 days of gestation, so that saline/nicotine exposure ceased on the day of parturition; Cohort B (Late Gestational and Lactational Exposure): Rats in Cohort C received 7-day pumps at 14 days of gestation, and at parturition, providing saline/nicotine exposure during both the last week of pregnancy and the first week of lactation; Cohort C (Lactational Exposure): Rats in Cohort B received 7-day pumps at parturition, providing saline/nicotine exposure during the first week of lactation.

Testing schedule: Infant progeny of each cohort were tested at day 0 (birth), day 7 and day 14 (cohorts B and C only).

Testing procedure: Testing was performed under neurolept analgesia (fentanyl 40 μg kg^{-1}, droperidol 2 mg kg^{-1} s.c.) to eliminate voluntary movements which produce potentially complicating changes in cutaneous blood flow. Rats were positioned on a 37°C heat pad, presenting the lateral thigh. A probe-electrode chamber applied to this site permitted iontophoretic stimulation and blood flux measurement. Microvascular blood flux responses elicited by transdermal iontophoresis of 2 mC acetylcholine chloride (ACh) were measured by laser Doppler flowmetry (Periflux Pfld), to quantify the axon reflex response. The arrangement of the iontophoretic chamber and

laser Doppler probe, together with details of the techniques of transdermal drug delivery and measurement of resulting blood flux, are based on methods previously described.[3] Cholinergic antagonist studies were performed on infant rats from Cohort B both during and after saline/nicotine exposure, using hexamethonium bromide (HXM), a nicotinic antagonist and atropine sulphate (ATR), a muscarinic antagonist. A control response was elicited by 4 mC ACh. The test site was then pretreated with 4 mC HXM, and retested 20 min later with 4 mC ACh. This procedure was repeated at another site, using 4 mC ATR instead of HXM.

Statistical analysis: Data are expressed as the mean ± s.e.m. Bartlett and Cochran tests for homogeneity of variance were used; these required square root transformations of weekly data, and log transforms of both types of cholinergic antagonist data, to enable the legitimate application of analysis of variance (ANOVA). After thus achieving homogeneity of variance, the data were analysed using ANOVA followed by Student Newman Keuls multi-range tests. In this paper, results where $p < 0.05$ are termed significant.

Results

Examples of cutaneous axon reflex responses to 2 mC ACh iontophoresis from infant progeny of nicotine-exposed mothers in Cohort C, are compared to control responses in Figure 1. Onset latency was constant, however, during maternal nicotine exposure, response amplitude and duration were increased. Seven days after cessation of nicotine exposure, responses were similar to pre-exposure levels. Infants of control mothers did not exhibit these changes. The effects of late gestational and/or lactational nicotine exposure on cutaneous axon reflexes (Cohorts A,B,C) are compared to saline controls in Figure 2. Blood flux response (V.min) is the integral of flux (V) and time (min).

Cohort A (Late Gestational Exposure): Late gestational maternal nicotine exposure produced significantly enhanced axon reflex responses in infant rats, when measured on the day of parturition ($p < 0.05$). At day 0, nicotine-exposed infants in cohort A exhibited mean responses of 1.154 ± 0.047 V.min ($n = 40$), which were 150% greater than control values (0.462 ± 0.051 V.min, $n = 16$). Seven days after nicotine cessation (day 7), responses from nicotine-exposed infants (0.477 ± 0.021 V.min) were similar to control responses (0.507 ± 0.066 V.min). Control responses did not differ significantly during the testing period.

Cohort B (Late Gestational and Lactational Exposure): Following late gestational maternal nicotine exposure, axon reflex responses in infant rats were significantly enhanced on the day of parturition ($p < 0.05$). Maternal nicotine exposure, continued during early lactation, produced significant further enhancement ($p < 0.05$). Responses at day 0 (1.052 ± 0.027 V.min) were 144% greater than control values (0.432 ± 0.053 V.min). At day 7, responses (1.727 ± 0.058 V.min) were 268% greater than control values (0.469 ± 0.034 V.min). By contrast, at day 14, responses (0.470 ± 0.023 V.min) were similar to control values (0.482 ± 0.051 V.min). Control responses did not differ significantly during the testing period.

Cohort C (Lactational Exposure): Maternal nicotine exposure during the first week of lactation produced significantly enhanced infant axon reflexes at day 7 ($p < 0.05$). Day 0 responses (0.494 ± 0.031 V.min) were similar to control values (0.466 ± 0.033 V.min). Responses at day 7 (0.968 ± 0.060 V.min) were 106% greater than control values (0.469 ± 0.034 V.min). Day 14 responses (0.489 ± 0.031 V.min) were similar to control values (0.472 ± 0.031 V.min). Control responses did not differ significantly during the testing period. Each rat pup is sustained *in utero* by its own unique placenta and amniotic sac. Therefore, data were analysed in terms of numbers of rat pups, rather than

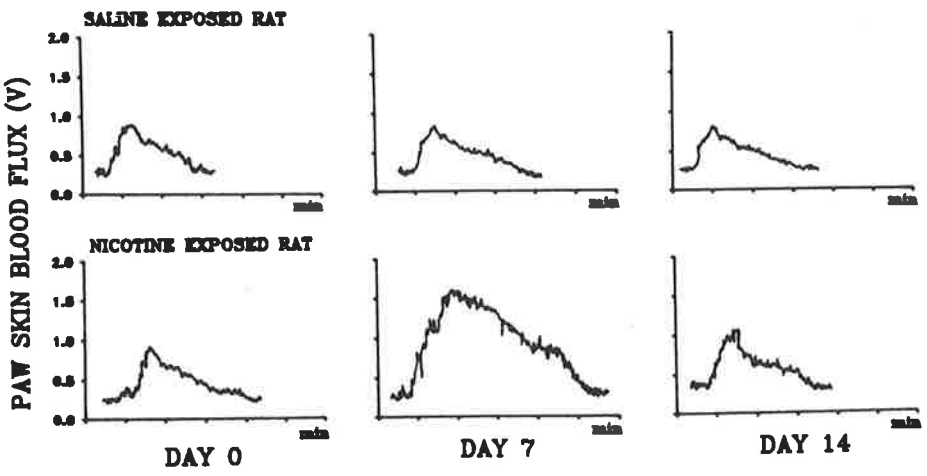


FIG. 1. Examples of cutaneous axon reflex responses to 2 mC ACh iontophoresis from progeny of nicotine-exposed mothers in Cohort C are shown in comparison to control responses: at day 0, before exposure; at day 7, after 7 days of lactational exposure; at day 14, 7 days after cessation of exposure.

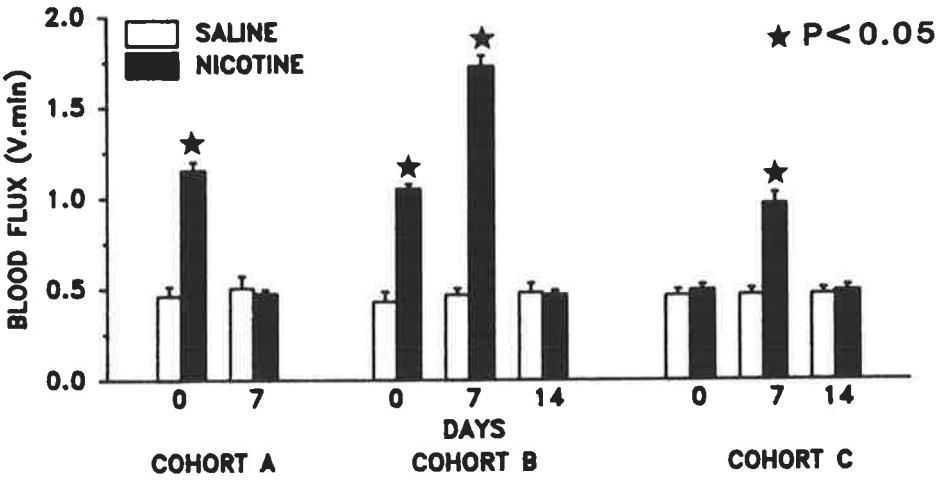


FIG. 2. Comparison of skin microvascular blood flux responses evoked by 2 mC ACh in infant progeny of nicotine-exposed mothers and saline controls. Cohort A (Late Gestational Exposure): infants exposed to saline (n = 16) or nicotine (n = 40) during the last 7 days of gestation, then allowed to recover for 7 days. Cohort B (Late Gestational and Lactational Exposure): infants exposed to saline (n = 16) or nicotine (n = 50) during the last 7 days of gestation and the first 7 days of lactation, then allowed to recover for 7 days. Cohort C (Lactational Exposure): infants exposed to saline (n =14) or nicotine (n = 39) during the first 7 days of lactation, then allowed to recover for 7 days.

Table 1. Litter cohort data

Cohort	Treatment	Day 0	Day 7	Day 14
Cohort A (N = 8)	Saline	0.456 ± 0.052	0.489 ± 0.064	—
	Nicotine	1.151 ± 0.078	0.486 ± 0.051	—
Cohort B (N = 8)	Saline	0.432 ± 0.028	0.453 ± 0.015	0.473 ± 0.064
	Nicotine	1.032 ± 0.038	1.721 ± 0.051	0.470 ± 0.013
Cohort C (N = 8)	Saline	0.457 ± 0.049	0.468 ± 0.050	0.460 ± 0.049
	Nicotine	0.496 ± 0.040	1.013 ± 0.079	0.505 ± 0.037

Comparison of skin microvascular blood flux responses evoked by 2 mC ACh in infant progeny of nicotine-exposed mothers and saline controls. In this table, data have been analysed in terms of number of litters (N). Statistical analysis produced results comparable to those achieved when rat pup data were analysed independently ($p < 0.05$).

numbers of litters. However, some researchers consider that data such as these should be treated in terms of litter 'N' rather than progeny 'n'. If the present data are re-analysed in this manner, the differences between the treatment groups and their controls is still significant ($p < 0.05$). This analysis is presented in Table 1. Similar analyses of cholinergic antagonist data produced results consistent with those presented below.

Cholinergic Antagonist Studies: Results of cholinergic antagonist studies performed on both saline- and nicotine-exposed infants from cohort B (Table 2) are summarized as histograms in Figure 3. Pretreatment of the test site with 4 mC HXM significantly reduced the size of the axon reflex response to 4 mC ACh by 87% to 90% at days 0, 7 and 14 ($p < 0.05$). Pretreatment with ATR did not significantly alter the size of axon reflexes at day 0, 7, or 14. These results take into account both nicotine-exposed and control infants.

Discussion

The principal finding of the present study is that maternal nicotine exposure, both during the last week of gestation and the first week of lactation, results in significantly enhanced cutaneous axon reflexes in neonatal rats. This phenomenon is reversed 7 days following cessation of nicotine exposure. The occurrence of nicotine-induced changes in rat pup axon reflexes may be explained by previously reported mechanisms such as transplacental[1] and lactational[2] transfer of nicotine from mother to infant. The fact that control pups from saline-exposed mothers did not exhibit these changes discounts the possibility that procedural effects, such as handling, or repeated anaesthesia, could be responsible for the observed changes.

The results of cholinergic antagonist studies demonstrate that axon reflex responses in infant rats are abolished by HXM but not by ATR. This establishes that the functional kinetics of cutaneous nicotinic cholinoceptors in nicotine-exposed neonatal rats are similar to those observed in control and adult rats.[3] Furthermore, the present results support previous findings[3,7] that nicotinic cholinoceptors on primary sensory nerves in rat skin mediate the axon reflex. This vasodilator response is distinct from that elicited by ACh at muscarinic

Table 2. Cholinergic antagonist data

Drug used	Saline			Nicotine		
	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
Control	0.933 ± 0.083	1.079 ± 0.068	0.964 ± 0.102	2.266 ± 0.060	3.971 ± 0.106	0.987 ± 0.078
After HXM	0.124 ± 0.018	0.108 ± 0.015	0.101 ± 0.019	0.240 ± 0.010	0.410 ± 0.034	0.117 ± 0.023
After ATR	0.928 ± 0.084	1.104 ± 0.053	0.981 ± 0.084	2.253 ± 0.078	3.812 ± 0.105	0.941 ± 0.048

Mean blood flux responses are expressed as V.min ± s.e.m. In both nicotine-exposed infants (n = 50) and saline controls (n = 16) from Cohort B, responses are seen to be significantly attenuated by pretreatment with HXM, but not by ATR ($p < 0.05$).

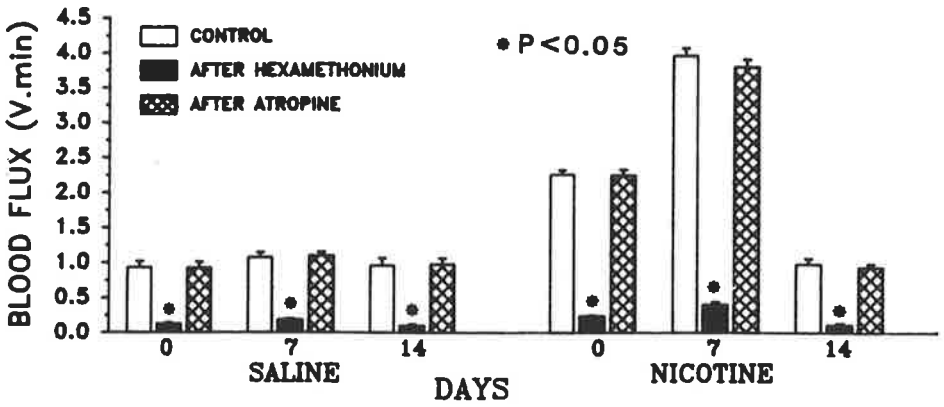


FIG. 3. Cholinergic antagonist studies: effects of 4 mC hexamethonium (HXM) and 4 mC atropine (ATR) iontophoresis on skin microvascular blood flux changes evoked by iontophoretic application of 4 mC ACh, in nicotine-exposed infants (n = 50) and saline controls (n = 16) from Cohort B.

cholinoceptors on microvascular endothelium,[8] which persists even after denervation.[9] These data also suggest that the muscarinic cholinoceptors at sympathetic sudomotor terminals on sweat glands[10] are not involved in the enhanced dilator responses. Alterations in the kinetics or sequencing of neuropeptide mediators might explain the observed enhancement of the axon reflex flare, however such effects of nicotine have not been reported. Although paradoxical,[11] the most suitable interpretation of the present findings is in line with descriptions of nicotinic cholinoceptor upregulation in infant rat brain.[5] This phenomenon is also seen in the brains of adult rats[12] and humans[13] when exposed to nicotine.

Some explanations which have been offered for the upregulation of central nicotinic cholinoceptors following chronic nicotine administration are: (1) Nicotine may interfere with release or metabolism of ACh, reducing available synaptic ACh, with resultant upregulation of nicotinic cholinoceptors;[4,14] (2) During continuous administration, nicotine may act as a functional antagonist, causing prolonged desensitization,[15] which results in compensatory upregulation of central nicotinic cholinoceptors;[4,12,14] (3) Another possible mechanism could involve occupation of cholinoceptor by agonist, with resulting phosphorylation and upregulation[16] of the nicotinic cholinoceptors. Although the predominant nicotinic receptor subtypes expressed in brain may differ from those found in peripheral tissue,[11] these mechanisms may also be involved peripherally.

Conclusion

These findings establish that, during maternal nicotine exposure, infant rats receive nicotine via placental and lactational transfer, causing a significant, reversible sensitization of nicotinic cholinoceptors on cutaneous sensory nerves. The time course of this nicotine-induced sensitization, and that of the post-cessation attenuation, is similar to that seen in adult rats.[3] Cutaneous muscarinic cholinoceptors are not affected. Our interpretation of these data in light of Slotkin's evidence for nicotinic receptor upregulation in infant rat brain during chronic maternal nicotine exposure[5] suggests that a similar phenomenon may occur peripherally. This remains to be confirmed by ligand binding studies.

These findings have implications for the infants of smoking mothers, who have been shown to be at greater risk of apnoeic attacks, Sudden Infant Death Syndrome,[17] low birth weight and failure to thrive,[18] and who may incur other risks related to nicotinic cholinoceptor upregulation such as reduced intellectual capacity[19] or altered pain perception.[20]

References

1. Luck W, Nau H, Hansen R *et al. Dev Pharmacol Ther* **8**, 384–395 (1985).
2. Luck W and Nau H. *Brit J Clin Pharm* **18**, 9–15 (1984).
3. Grunfeld JA, Tiedemann GJ and Westerman RA. *NeuroReport* **2**, 421–424 (1991).
4. Schwartz RD and Kellar KJ. *Science* **220**, 214–216 (1983).
5. Slotkin TA, Orband-Miller L and Queen KL. *J Pharmacol Exp Ther* **242**, 232–237 (1987).
6. Grunfeld JA, Tiedemann GJ and Westerman RA. *Proc Aust Physiol Pharmacol Soc* **22**(2): 152P (1991).
7. Douglas WW and Ritchie JM. *J Physiol* **150**, 501–514 (1960).
8. Furchgott RF and Zawadzki JV. *Nature (Lond)* **288**, 373–376 (1980).
9. Low AM and Westerman RA. *Life Sci* **45**(1), 49–57 (1989).
10. Shields SA, MacDowell KA, Fairchild SB *et al. Psychophysiol* **24**(1), 312–320 (1987).
11. Wonnacott S. *TIPS* **11**, 216–219 (1990).
12. Schwartz RD and Kellar KJ. *J Neurochem* **45**, 427–433 (1985).
13. Benwell MEM, Balfour DJK and Anderson JM. *J Neurochem* **50**(4), 1243–1247 (1988).
14. Marks MJ, Burch JB and Collins AC. *J Pharmacol Exp Ther* **226**, 817–825 (1983).
15. Weber M, David-Pfeuty T and Changeux J-P. *Proc Natl Acad Sci USA* **72**, 3443–3447 (1975).
16. Huganir RL, Delcour AH, Greengard P *et al. Nature* **321**, 74–77 (1986).
17. Luck W et Nau H. *J Pediatr* **107**, 816–820 (1985).
18. Schwartz-Bickenbach D, Schulte-Hobein B *et al. Toxicol Lett* **35**, 73–81 (1987).
19. Skarping G, Willers S and Dalene M. *J Chromatog* **454**, 293–301 (1988).
20. Carr RW, Delaney CA *et al. Proc Aust Neurosci Soc* **4**, 176 (1993).

ACKNOWLEDGEMENTS: This study was partly supported by grants from the Australian Research Council and the National Health and Medical Research Council to R.A.W. which are gratefully acknowledged. We sincerely thank Mr David Caddy, Dr Tim Neild and Dr David Walker for helpful discussions during preparation of this report.

Received 25 January 1993; resubmitted 23 March 1993; accepted 29 March 1993

In: Clin Exper Neurol 30: 39-61, (1993)
Edited by M Eadie, C Lander, M Pender

THE ROLE OF SKIN NOCICEPTIVE AFFERENT NERVES IN BLISTER HEALING

R.A. Westerman*, R.W. Carr*, C.A. Delaney*, M.J. Morris† and R.G.D. Roberts*

Department of Physiology*, Monash University, Clayton, Victoria and Department of Biological Sciences†, Deakin University, (Geelong Campus) Waurn Ponds, Victoria

SUMMARY

Because sensory neuropeptides improve survival of critical skin and muscle flaps in rats, skin nociceptive sensory nerve function in blister healing was examined. Sensory nerve ablation by unilateral hindlimb denervation or cutaneous axon reflex enhancement by 14 days systemic nicotine treatment (5 mg $kg^{-1}day^{-1}$) decreased and increased, respectively, peripheral motor functions of nociceptive (peptidergic) skin nerves. Effects on nociception were measured by a radiant heat tail-flick test. Axon reflex flares were evoked by transdermal iontophoresis of acetylcholine or noxious electrical stimulation under pentobarbitone 40 mg kg^{-1} anaesthesia. Resultant changes in cutaneous microvascular blood flux were measured non-invasively by laser Doppler flowmetry. In nicotine-treated rats compared with placebo-treated controls, acetylcholine-evoked axon reflex flare was enhanced by 240% ($p<0.01$) without enhancement of electrically evoked flare. Thus, nicotine-sensitized nociceptors show stimulus specificity in their enhancement of neurogenic flare responses. No significant changes were seen in other endothelial-dependent or smooth muscle-dependent microvascular dilator responses. Nicotine-treated rats had prolonged tail-flick withdrawal latencies to noxious radiant heat stimuli compared with placebo-treated controls ($p<0.05$), suggesting an anti-nociceptive or analgesic effect of nicotine-treatment. Neurogenic effects on wound healing rate were assessed by measuring the dimensions of standardized blisters twice daily. The blisters were raised on hindpaw glabrous skin using a constant weight and diameter of compressed dry ice pellet applied for 30 secs at constant force. Dry-ice blisters raised on the hindpaw 14 days post-denervation were significantly slower to heal completely (42 days) than controls (30 days: $P<0.05$) and the surrounding inflammation was reduced. By contrast, nicotine-treated rats showed more rapid blister healing (25 days) than controls (30 days), seen only in the later phase after day 15. Finally, resting substance P release from blisters, after direct cutaneous nerve stimulation, appears to be enhanced in nicotine-treated rats. Thus nociceptive innervation appears critical for inflammation and rapid healing of blisters in rat skin. The

data signal a possible important role for neuropeptides in these processes and question the function of nicotinic receptors on sensory nerves.

Lewis[1] and more recently Lembeck[2] described neurogenic inflammation and its possible 'nocifensor' or protective actions. When a noxious stimulus impinges on the skin, polymodal nociceptors respond with a discharge both orthodromically to the spinal cord and also antidromically along collateral branches (the 'axon reflex') generating a local flush[1,2,3]. The axon reflex results in release of neuropeptides eg, substance P (SP) and calcitonin gene related peptide (CGRP), from arborised nerve terminals[2,3,4]. Subsequent vasodilatation occurs through their direct actions on vascular endothelium[5] or smooth muscle or their actions via the liberation of vasoactive substances from mast cells[6] (Fig 1). The neuropeptides, tachykinins and eicosanoids involved in this cascade trigger the following spectrum of defensive and healing processes: macrophages become phagocytic; lymphocytes in regional nodes are activated; microvessels dilate and plasma extravasation occurs; the kinin and complement systems are activated. Fever, leucocytosis and altered erythrocyte sedimentation rate may follow if tissue damage results in accumulation of lysosomal products, pyrogens and leucocytosis factors. Important roles of nociceptive primary afferent skin nerves in the axon reflex flare[1], microvascular reactivity[6] and wound healing[7] have been reported recently in a study[7] which used unilateral denervation as the primary strategy to in effect ablate the skin sensory nerves. In that study, denervation adversely affected all measured nocifensor functions of skin sensory nerves.

Based on these data, the present study tested the hypothesis that increases or decreases in sensory nerve responsiveness will alter the neuropeptide release and therefore the rate of wound healing. Its strategy employed the effect of systemic nicotine treatment, which enhances axon reflexes[8,9], to putatively increase sensory nerve responsiveness. Thus, it was proposed that chronic nicotine exposure should (i) increase neurogenic inflammation, (ii) alter nociception, (iii) enhance neuropeptide release and (iv) alter the wound healing rate. The present paper reports the testing of these proposals.

METHODS

Animals used

The rat husbandry, anaesthesia, neurovascular testing by noxious electrical transcutaneous nerve stimulation (TNS), iontophoretic application of acetylcholine (ACh) and sodium nitroprusside (SNP), and the use of laser Doppler velocimetry to measure the microvascular blood flux responses to noxious stimulation of paw skin have been described in detail in preceding papers[7,8,9]. Rats received electrical and iontophoretic stimulation on days 0, 14, and 28.

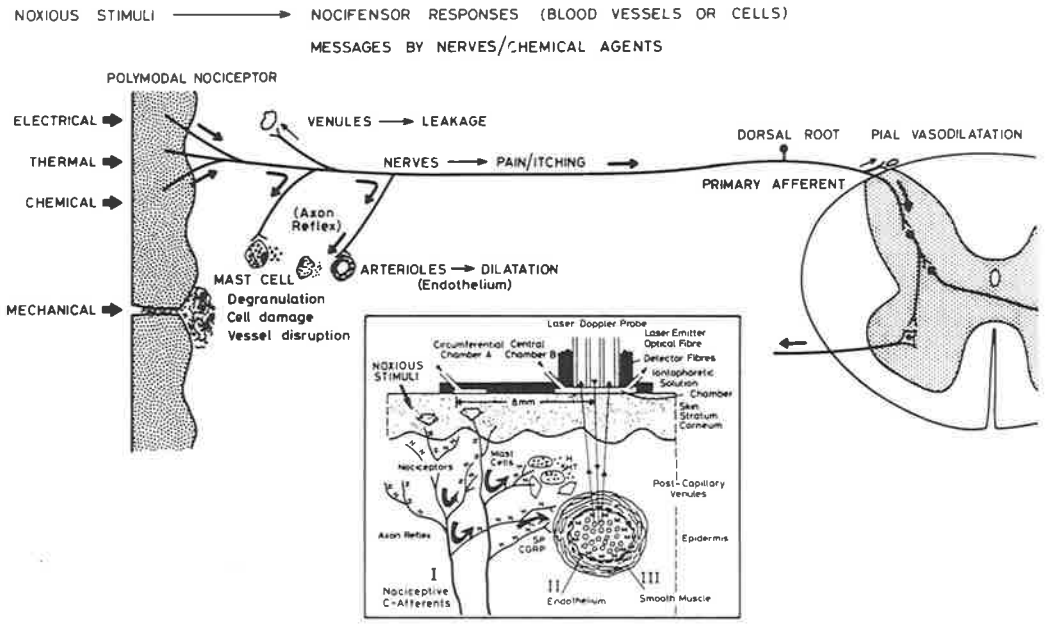


Fig 1 Neurovascular interactions in skin. When a noxious stimulus, either electrical, chemical, thermal or mechanical impinges on the skin, polymodal nociceptors respond with a discharge both orthodromically to the spinal cord and antidromically along collateral branches generating the so-called 'axon reflex'. Nocifensor responses to noxious stimulation listed on diagram are triggered by neuropeptide release and are categorised as cellular, humoral, vascular, immunological & systemic. **INSET:** Indirect stimulation via noxious electrical pulses at the arrow, or by iontophoresis of ACh at A, evokes an axon reflex.This results in neuropeptide release from arborised sensory C-fibre terminals and subsequent vasodilatation through an action directly on vascular endothelium or smooth muscle or via the liberation of vasoactive substances from mast cells. Laser Doppler velocimetry (LDV) was used to assess cutaneous vasodilatation in response to specific quantified stimuli as an indicator of neural and vascular function. The responses to direct vascular stimuli applied by iontophoresis via chamber B were also recorded in this way.

Nicotine administration

The naturally occuring isomer (-)-nicotine (hydrogen tartrate salt) dissolved in sterile 0.9% saline at a dose of 5 mg $kg^{-1}day^{-1}$ was administered systemically by an Alzet mini-osmotic pump[9]. This device, implanted subcutaneously between the scapulae, delivered the solution over a period of 14 days at a fixed rate of 0.5 ± 0.1 µl/hr. A perspex pellet, identical in size to the Alzet-2002 pump, served as a placebo implant[17].

Tail-flick *(Noxious Thermal Latency Test)*

Each animal was tested with the tail flick test on days 0,14 and 28 (Fig 2). The rat was placed in a darkened immobilising perspex chamber with its tail protruding. A K-type thermocouple provided feedback for temperature control; a halogen heat lamp (54°C) focussed onto the tail elicited a reflex tail-withdrawal (flick). The tail-flick, sensed optically by an infra-red beam chopping circuit and a stop watch, triggered synchronously with the heat lamp onset, was stopped automatically by a beam chopper pulse. Rats were placed in the perspex chamber for 20 mins acclimatisation, the noxious thermal stimulus was applied and the reflex tail-flick latency recorded. Tail temperature was recorded 10 cm from the tail tip using a digital thermometer. Stimulation at a distance 6 cm from the tail tip was performed twice on each rat at days 0, 14 and 28 (Fig 3A). In a small number of rats, a 3rd stimulus was applied randomly either 2 cm rostral or caudal to the initial stimulus site, i.e. 8 or 4 cm from the tail tip (Fig 3A). Based on these data, the order and site of stimulus application (2 x 6 cm, 1 x either 8 cm or 4 cm) was randomised.

Electrical nerve stimulation

Noxious transcutaneous pulses were applied as previously described[7,10,11]. The hind legs of rats anaesthetised with pentobarbitone (40 mg/kg, i.p) were shaved. Intradermal needle electrodes were placed distal to the cathode, 1 cm apart, on the hair line of the heel, for stimulation of the area innervated by the posterior tibial and saphenous nerves. A heatpad maintained body temperature at 37°C. A series of rectangular electrical pulses (150V, 2 Hz, 1 ms duration) were delivered to the site while blood flow was concurrently recorded by laser Doppler immediately distal to the cathode[7]. In each animal, the electrical stimulation consisted of short trains of 2, 4, 16 pulses from a Grass S8 stimulator and SIU5A isolation unit[7].

Neurovascular testing

Dual probe-holder/iontophoretic electrode chambers[7,8,9] attached by double-sided adhesive discs on the medial aspect of the hindlimb (secured to a contoured foam pad) minimised fluid leakage without impeding blood flow. The 2 circular chambers (6 mm diameter) were separated by 6 mm, allowing remote stimulation of test skin areas and thus only nerve-mediated alterations in blood flow at the recording site[7,8,9]. This indirect test was performed using ACh on all rats in an attempt to reproduce enhanced nerve function mediated by nicotine, previously described[8,9].

Iontophoresis of vasoactive compounds

This was carried out as described previously[7,8,9,10,11] and was performed after electrical stimulation[7]. Acetylcholine chloride (ACh) and sodium nitroprusside (SNP) as

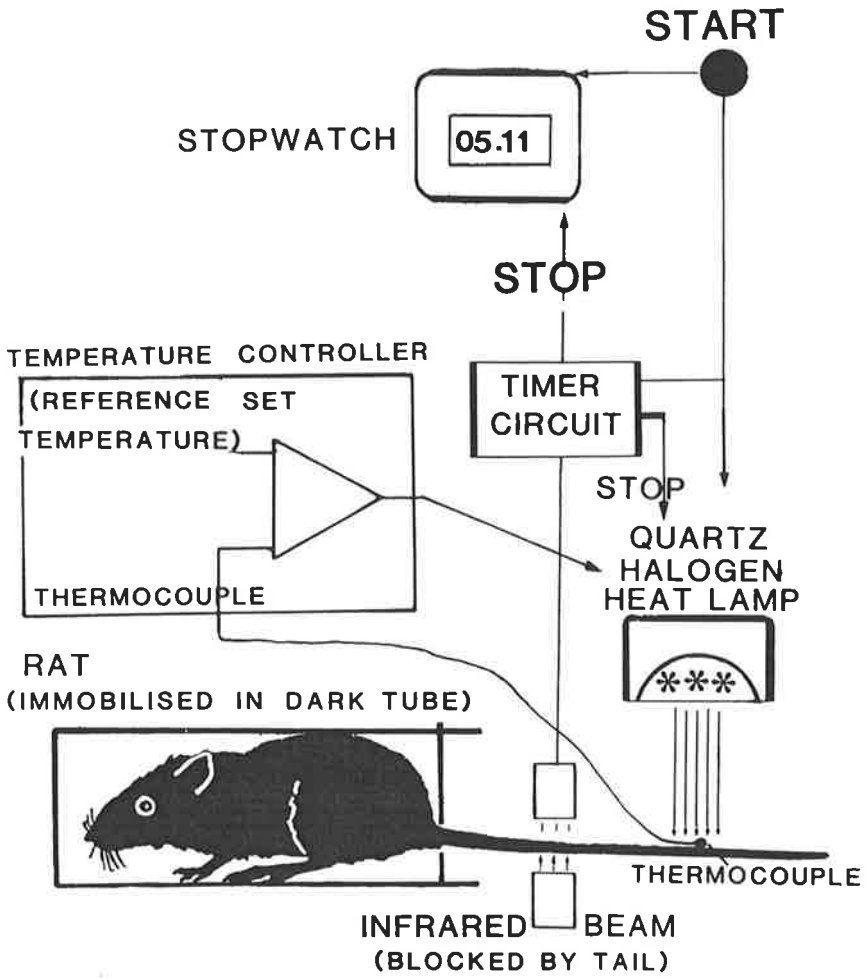


Fig 2 Tail-flick apparatus. A focused noxious radiant heat lamp was temperature regulated via a feedback thermistor and delivered a noxious heat pulse synchronously with the starting of a timer. Tail withdrawal was detected via an infra-red beam chopping circuit that stopped the timer and established a tail flick latency indicative of nociceptive sensitivity.

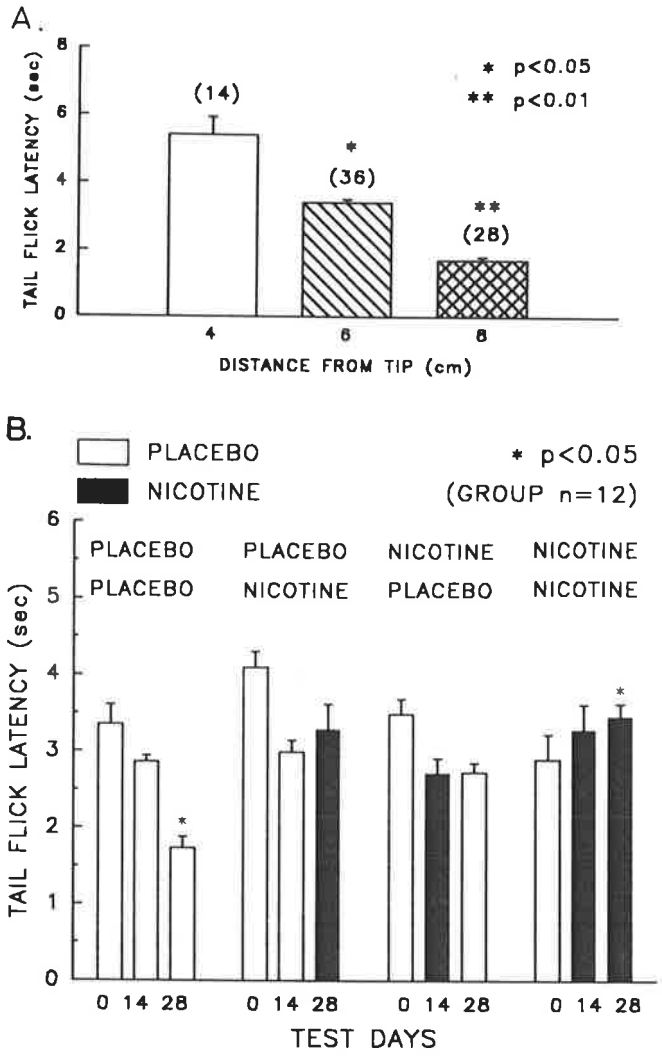


Fig 3 Tail flick withdrawal latency. A. Histograms showing tail-flick latencies (s) to 54°C noxious radiant heat stimuli at different locations (4,6,8 cm from the tip) along the rat's tail. All responses shown were obtained on day 0, before nicotine or placebo implants. "*" indicates a significant reduction in tail-flick latency compared with those obtained 4 cm from the tail tip ($p<0.05$): "**" indicates a significance of $p<0.01$. B. Time (s) to withdraw from a noxious stimulus of 54°C applied 6cm from tail tip on days 0, 14, and 28 shown in each group of placebo (28d); placebo (14d) - nicotine (14d); nicotine (14d) - placebo (14d) and nicotine (28d) animals "*" indicates a significant reduction or increase in latency compared with the untreated day 0 value, $p<0.05$.

1% w/v solutions in inert 4% methyl cellulose gel in distilled water were each applied in a hemispherical 50 µl electrode chamber to the skin: a constant current stimulator provided a 0.2 mA galvanic (direct) current through the gel, with an indifferent electrode on the rat's forepaw[7,8,9]. Doses of ACh (direct) 4mC (0.2 mA X 20s), ACh (indirect) 16 mC (0.2mA X 80s), SNP (direct) 8mC (0.2 mA x 40) which produce sub-maximal responses were selected during pilot observations[7,8,10,11].

Blood flux responses

Laser Doppler velocimetry (LDV) with the Periflux PF1d (Perimed, Sweden) as previously described[10,11] was used non-invasively to assess cutaneous vasodilatation in response to specific quantified electrical or chemical vasodilator stimuli as an indicator of neural and vascular function. Typical dilator responses are shown in Fig 4 for iontophoretic stimuli applied either remotely from a circumferential chamber, or directly onto the skin (being monitored by LDV). Axon reflex dilator responses to noxious electrical stimulation have been shown in previous reports[7,10,11].

Wound healing: induction of blisters

Uniform, controlled blisters were created 14 days after implanting a nicotine or placebo pump. A standardised 7mm diameter pellet of solid carbon dioxide (dry ice) was applied for a period of 30 secs at a constant pressure of 0.25N. Blisters were induced in a concave part of the foot pad at the base of the toes to minimise post-wound trauma and lameness[7].

Assessment of blister-healing

Daily measurement of physical dimensions of the wound margin, delineated as the neurogenic flare border or the border of the scab, whichever was the larger, assessed the wound retraction/healing. Vernier calipers and a 6x lens with an overlay grid were used to determine the longitudinal and cross-sectional dimension and the total area, respectively[7].

Neuropeptide release study

To test the effect of nicotine treatment on neuropeptide release from skin sensory nerves, substance P (SP) release was measured during periods before, during, and after direct electrical stimulation of the sciatic nerve[4]. Using methods previously described[12], the SP was assayed in the fluid collected from paw suction blisters[4,13] raised after 14 days of systemic nicotine or placebo treatment. Twelve rats in each of the 2 groups (nicotine treated and control) were screened with chemical neurovascular tests and implanted with pumps. Fourteen days after implantation, animals were retested using the chemical neurovascular test battery.

Suction blister creation

Induction of bilateral suction blisters was performed with the animal under anaesthesia in a similar manner to that first described by Kiistala[13] in 1968. The device[4] consisted of a large brass plate with two 8mm diameter ports manifolded to a single outlet and attached to a venturi water pump for suction. The solid brass plate was heated with

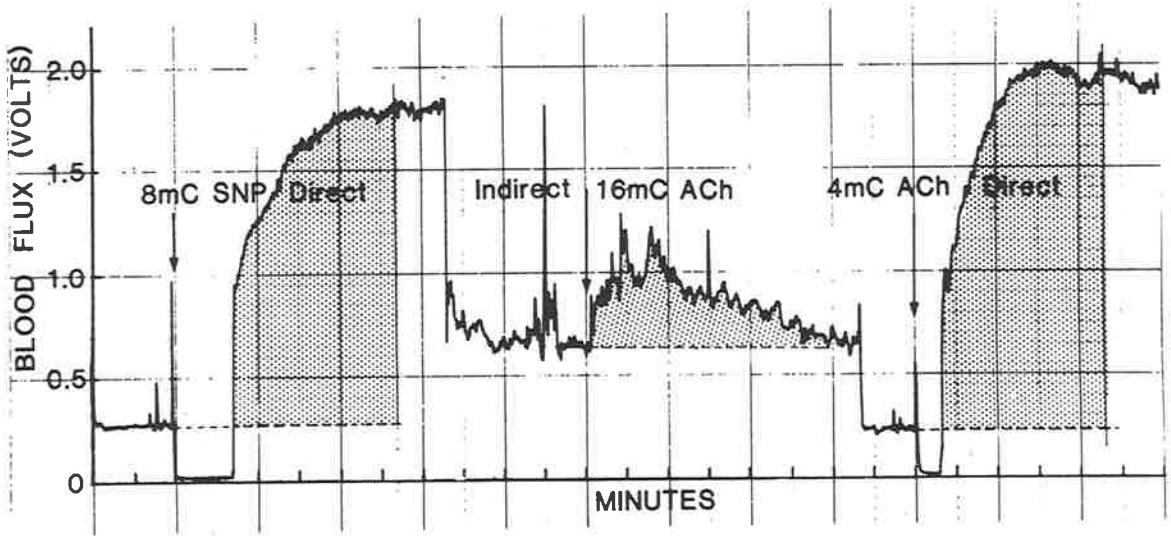


Fig 4 Blood flux responses. Recording of cutaneous blood flow by Periflux PF1d laser Doppler flowmeter (Perimed, Sweden), provided a continuous, real-time, non-invasive measure of evoked microvascular flux changes. Microvascular dilator responses to stimulation were quantified by determining the area beneath the voltage-time response recorded on an ICI DP600 chart recorder. Responses to indirect ACh iontophoresis[2,3] (centre) were integrated between the stimulus offset and the point at which the flux returned to baseline. Responses to iontophoresis of SNP (left) and ACh (right) directly under the laser probe were integrated over the first 4 mins of response.

a 10 ohm, 25W resistor to 42°C and maintained via a K-type thermocouple feedback. With the animal laid supine on a heated pad (37°C), the plantar aspects of the hind feet were placed over the suction ports and secured with an elastic strap and an airtight seal was assured with the use of high vacuum silicone grease (Ajax Chemicals). Application of a 30 kPa negative pressure (-220T) for a period of 30 mins (or to effect) resulted in an epidermal-dermal separation, and thus a blister. The epidermal layer was removed surgically. The foot was placed in a fluid-tight perspex clamp with the blister base exposed and the perspex cover forming a small fluid well.

Blister fluid collection

Standard Krebs-Ringer buffer (138 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM NaH_2PO_4, 11 mM $NaHCO_3$ 1 mM $C_{22}H_{38}O_7$, 11 mM $C_6H_{12}O_6$), 0.3% aprotinin (a protease inhibitor) and 0.25% bovine serum albumin were aliquoted as 300 µl samples into the blister well. Fluid was aspirated from the blister well after 15 mins using a Terumo µ-100 insulin syringe with a 29G needle (<1µl dead space), and collected into acetic acid (final concentration 0.1 M). Samples were centrifuged at 12,000 rpm for 2-3 minutes to remove any contaminants (especially blood cells or platelets) and 220 µl of supernatant was aspirated for assay. Samples were collected serially in triplicate for the resting, stimulation and recovery periods.

Nerve stimulation

The sciatic nerve was isolated surgically and transected in the upper thigh. To evoke peptide release the nerve was stimulated using two platinum electrodes (cathode distal to anode), at 50 V, 8 Hz, 1 ms duration[4,16]. Periods of 15 mins were used, after which blister fluid was removed, frozen and stored for assay.

Neuropeptide assay – substance P

Radioimmunoassay of samples was performed by a method similar to that previously detailed by Morilak *et al*[12] in which samples were collected into polyethylene tubes on ice, centrifuged, aspirated vortexed then frozen and allowed to stand at -80°C. Samples were thawed, reconstituted in 100 ml of buffer and lyophilised. The substance P (SP) content was determined by radioimmunoassay using an antiserum raised against SP in the rabbit, and iodinated SP labelled with the Bolton and Hunter reagent. The SP detection limit was 0.3 – 0.4 pcg per sample.

Statistics

Data were expressed as means and standard errors of the mean (sem). Statistical analysis was performed using analysis of variance (ANOVA) with post-hoc analysis, for both paired and unpaired data. Statistical significance was taken as $p<0.05$ for ANOVA or t-tests. Pearson's r value was used as an indicative measure of significance for linear correlations where appropriate.

Table 2 Results from random testing of 6 rats for nicotine exposure. Urinary cotinine levels (μgl^{-1}) tabulated for 2 groups of rats (n=3) with placebo exposure and 7 days nicotine exposure.

TREATMENT GROUP	PLACEBO (14 days)	NICOTINE (14 days)
cotinine (μg/l)	83.33 ± 34.75	4140 ± 1099

Mean body weights of rats (n=12)

The mean body weight of each of the 4 groups of animals viz. placebo-placebo; placebo-nicotine; nicotine-placebo and nicotine-nicotine measured at days 0, 14 and 28 is shown in Table 3. No statistically significant differences ($p>0.05$) were found when tested by multiple ANOVA.

Table 3 Mean body weights of rats (n=12) in each of the four groups viz. placebo-placebo; placebo-nicotine; nicotine-placebo and nicotine-nicotine measured at days 0, 14 and 28. No statistically significant differences ($p>0.05$) were found when tested by multiple ANOVA.

TABLE OF BODY WEIGHT (grams)	PLACEBO (28d)	PLACEBO (14d) NICOTINE (14d)	NICOTINE (14d) PLACEBO (14d)	NICOTINE (28d)
DAY 0	213.83 ± 3.35	213.33 ± 5.01	211.27 ± 3.63	215.92 ± 3.01
DAY 14	225.42 ± 3.78	225.00 ± 4.24	219.83 ± 3.51	227.75 ± 2.60
DAY 28	231.36 ± 3.91	223.91 ± 3.23	226.00 ± 3.07	229.42 ± 2.09

Tail-flick test

Results from the 4 nicotine treatment regimens in rats on days 0, 14 and 28 are shown in Fig 3B.

Placebo-implanted animals showed a significant reduction in tail-flick latency over the experimental period but the reduction was significant only by the 3rd testing (post hoc analysis $p<0.05$). In contrast, animals which received nicotine throughout the 28 day period showed a progressive statistically significant increase in withdrawal latency ($p<0.05$). The 2 combinations of placebo and nicotine showed no consistent trend.

Neurovascular testing

Fig 4 shows typical responses to neurovascular tests. Fig 5 shows the mean blood flux response to a chemically-evoked axon reflex (AR) measured at days

0, 14 and 28 with the 4 nicotine treatment regimens. In each case placebo implants had no effect on the AR response to ACh. However, a statistically significant increase ($p<0.05$) in the size of the AR response was observed after 14 days of nicotine treatment. Further, this nicotine-induced AR enhancement reverted to normal following cessation of nicotine therapy.

By contrast, none of the nicotine treatment schedules produced a significant change in the AR responses to electrical stimulation ($p>0.05$, Fig 6) or to directly applied microvascular dilators SNP or ACh ($p >0.05$, see Figs 7A,7B).

Stimulus specificity was tested, but no correlation was found between thermal and chemical nociceptive function tests at day 0 ($r=-0.187$, $n=32$, $p>0.05$) or following 14 days of nicotine treatment ($r=0.139$, $n=33$, $p>0.05$).

Wound healing

Fig 8B shows the healing rate profiles of nicotine pre-treated rats (circles) and placebo treated or control rats (triangles). No significant difference was found for orthogonal comparisons of profile shape between groups ($F_{(1,22)} = 2.13$, $p > 0.05$). However, a divergence of the profiles is apparent at approximately day 16 after blister induction, highlighted by the enlarged inset (B), indicating a trend towards decreased healing time from this point onwards. On repeated measures analysis this is significantly different ($p<0.05$).

Neuropeptide release

Substance P (SP) release (pg/200µl) was assayed in blister fluid during rest, stimulation and recovery, samples being collected over 15 minute periods (Fig 9). Control rats showed a stimulus dependent increase in SP levels but this was not statistically significant. In rats treated with nicotine no such stimulus dependent increase was observed - instead there was a tendency for the absolute level of SP to be elevated in basal terms but the change fell short of statistical significance ($p=0.061$). By contrast, Table 4 shows that the increase in SP release with direct nerve stimulation correlated significantly with an indicator of nociceptor function, viz, the size of the chemical axon reflex response in control animals ($r=0.945$, $n=6$, $p<0.01$). However, there was no significant correlation for stimulated SP release levels and tests of vascular endothelial reactivity and smooth muscular function.

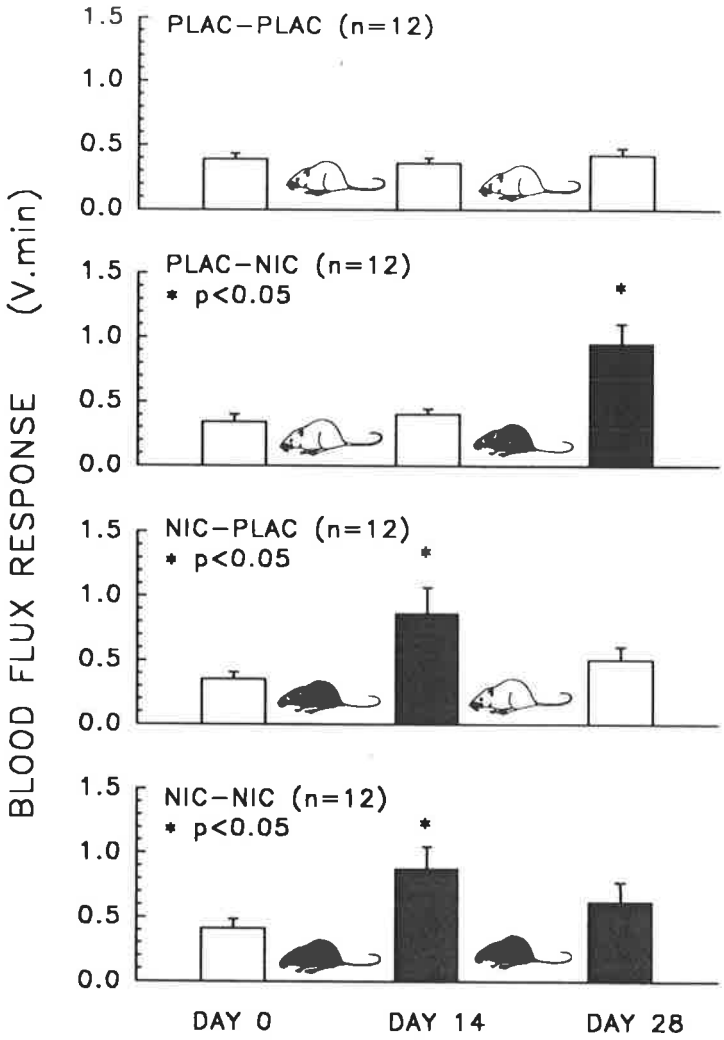


Fig 5 Nicotine effects on chemical axon reflex. Nicotine Treatment Schedule. In a 2 way cross-over arrangement, rats were on a 28 day schedule and received either a nicotine or placebo implant for the first 14 days followed by a reimplant of either placebo or nicotine for the remainder. The histograms show the effect of nicotine on the chemically evoked axon reflex response, measured at days 0, 14 and 28, in the 4 treatment regimens. Open bars represent responses following 14 days of placebo implant and shaded bars represent those following 14 day nicotine treatment. Black filled rats indicate nicotine treatment during the corresponding 14 day period, while unfilled rats indicate placebo. "*" indicates a significant increase in size of the axon reflex compared to day 0 ($p<0.05$).

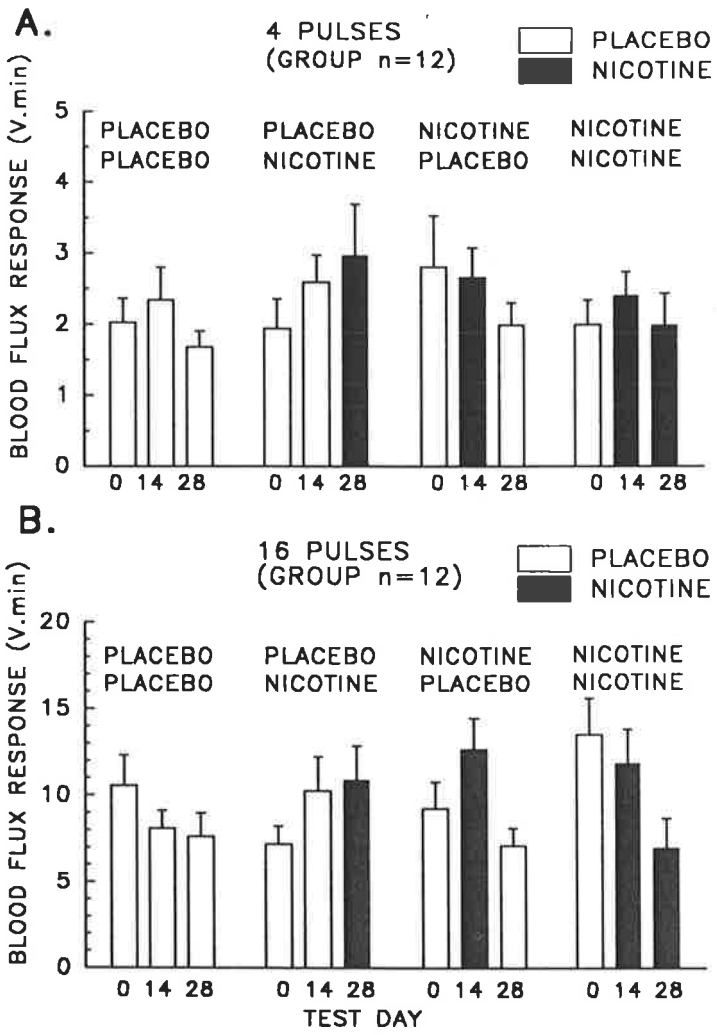


Fig 6 Nicotine effects on electrical axon reflex. Blood flux dilator responses are shown to noxious electrical stimulation (150V, 2Hz, 1msec) of (A) 4 pulses and (B) 16 pulses in the 4 nicotine treatment groups, placebo (28d); placebo (14d) – nicotine (14d); nicotine (14d) – placebo (14d) and nicotine (28d), on each test day, 0, 14 and 28. Stimuli were administered by a cathode remote from the LDV probe, the anode being 2cm further away (see methods). Open histograms show dilator responses after placebo treatment; filled histograms depict responses following 14 days of systemic nicotine. There was no significant difference between responses in either group on any test day ($p>0.05$).

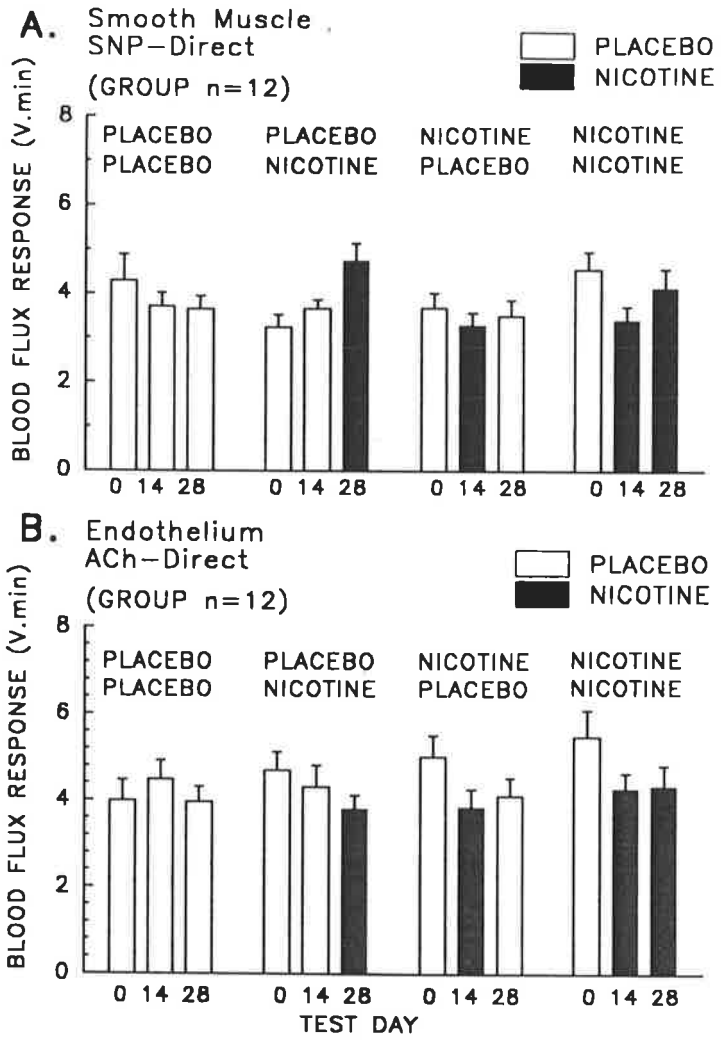


Fig 7 Nicotine microvascular effects. Histograms showing blood flux dilator responses (volt.min ± sem) in each nicotine treatment group, placebo (28d); placebo (14d) - nicotine (14d); nicotine (14d) - placebo (14d) and nicotine (28d) on each test day 0,14 and 28. Open histograms show responses in untreated animals or following placebo treatment. Solid histograms show responses after 14 days of preceeding nicotine treatment. The graphs show responses elicited with (A) the endothelium independent nitro-dilator, SNP (8mC) and (B) the endothelium-dependent dilator, ACh (4mC).

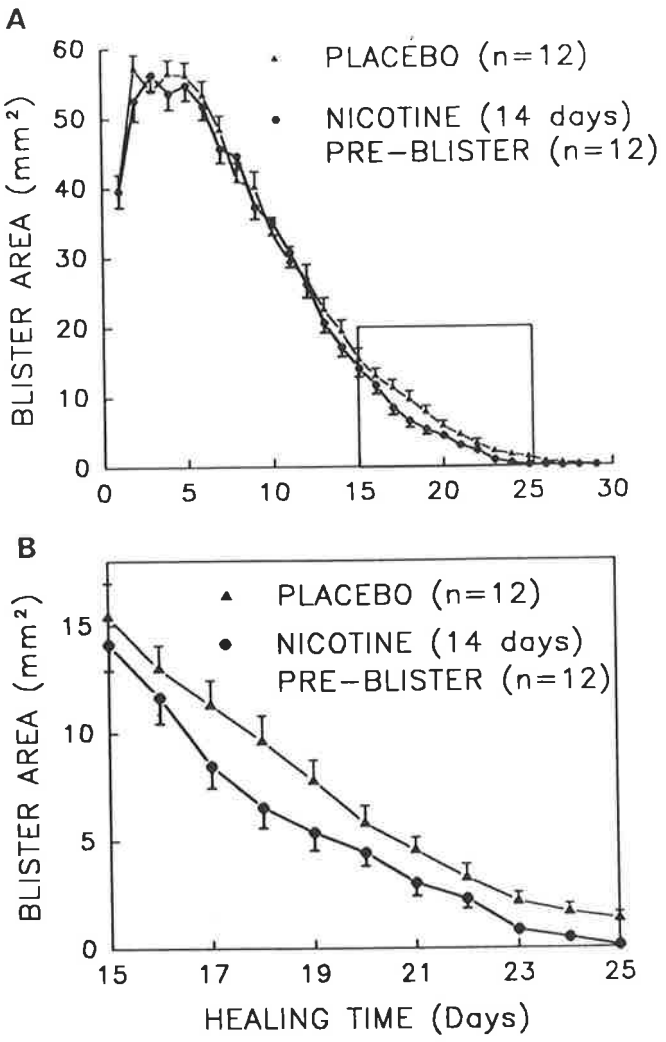


Fig 8 Blister healing rate after nicotine treatment. **A:** Healing rate profiles of nicotine pre-treated rats (circle) and placebo treated or control rats (triangle). No significant difference was found for orthogonal comparisons of profile shape between groups ($F_{(1,22)} = 2.13$, $p > 0.05$). However, a divergence of the profiles is apparent at approximately day 16 after wounding, highlighted by the enlarged inset **(B)**, indicating a trend towards decreased healing time from this point onwards. On repeated measures analysis this portion is significantly different ($p<0.05$).

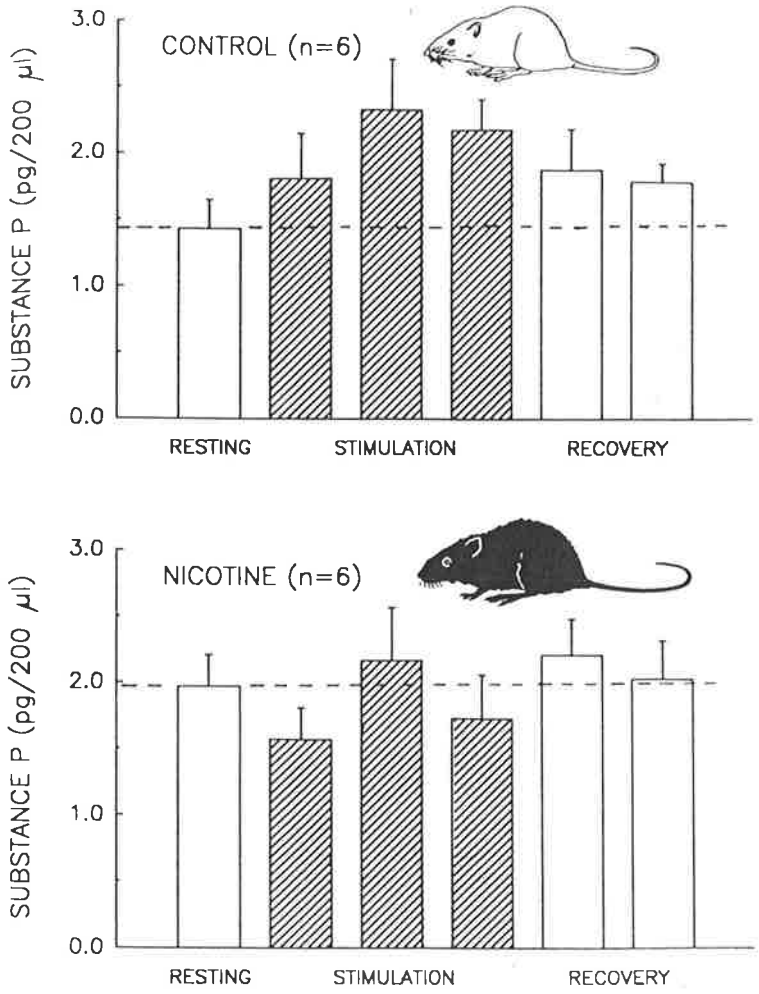


Fig 9 Substance P release. Levels of SP (pcg/200µl) assayed in blister fluid during rest, stimulation and recovery. Samples were collected over 15 min periods. Dotted lines show the initial (resting) level of SP release (n=6). Control rats showed a stimulus dependent increase in SP levels which was not statistically significant. In rats treated with nicotine no such stimulus dependent increase was observed. Instead there was a tendency for the absolute level of SP to be elevated in basal terms but there was not significant (p=0.061). The SP levels are near the lowest detection limits of the assay and the data should be considered indicative only.

Table 4 Relationship between substance P (SP) release in response to direct electrical stimulation of the sciatic nerve, and axon reflex blood flux responses to indirectly applied ACh. The correlation between the size of the chemical axon reflex and the net increase in SP release upon stimulation (ie. amount released upon stimulation minus amount released during rest periods) is statistically significant (r=0.945, n=6, p<0.01)

Net SP release (pg/200 µl)	Chemical Axon Reflex (V.min.)
0.185	0.319
0.245	0.252
0.620	0.293
0.635	0.329
0.780	0.396
1.810	1.116

DISCUSSION

The overall project strategy was to alter sensory nerve function in 2 ways: viz. (i) denervation – to impair axon reflexes, neurogenic inflammation, nociception, wound healing rate (reported by Carr *et al*[7]) and (ii) nicotine treatment – to enhance axon reflexes[8,9], neurogenic inflammation, nociception, neuropeptide release and wound healing rate.

Denervation has adverse effects on all nocifensor functions of skin sensory nerves, which clearly are important for inflammation and speed the late phase of blister-healing[7,17]. The present study provides evidence that systemic nicotine-treatment enhances neurogenic axon reflex flare if evoked by ACh (Fig 5), but not the axon reflex evoked by noxious TNS (Fig 6) – i.e., the effect is stimulus specific. This may be interpreted in terms of the present understanding of cutaneous neurovascular mechanisms[2,3,6,7,8] (Fig 1). All noxious stimuli impinging on the skin evoke axon reflexes resulting in neuropeptide release[3,6,7] from nerve terminals, leading to vasodilatation evident as skin reddening[7,8]. Dilator responses for noxious electrical stimulation[7] were not enhanced (Fig 6), which suggests that nicotinic acetylcholine receptors (nAChr) are not involved in producing the electrically-evoked axon reflex flare. Such stimulus specificity (Table 5) also points to different transduction mechanisms being involved in initiating the electrical and chemical axon reflex flare. Maintained electrical depolarization induces a large electrical gradient across nerve terminals producing a receptor

potential and thus a response[14]. Chemical stimuli such as ACh bind to specific receptors to open cationic channels[14], whilst thermal and mechanical stimulus transduction involve different mechanisms[14]. Furthermore, there are no microvascular effects of short nicotine exposure (Fig 7) which is explicable by the presence of nAChr on primary sensory nerves[7], but only muscarinic AChr on microvasculature[5].

Table 5 Stimulus specificity: Correlation between thermal and chemical (ACh) nociceptive function. Tail flick latency (sec) against integrated area of chemical axon reflex response (V.min.). First row shows results for rats on day 0 before nicotine or placebo implant (r=0.187, n=32, p>0.05) and second row shows results for the same animals after 14 days nicotine treatment (r-0.139, n=33, p>0.05). Linear regressions and p values indicate lack of statistical significance for both correlations.

Day	Correlation coefficient (r)	Number of animals	Significance (p)
0	–0.187	32	>0.05
14	0.139	33	>0.05

It is believed that synthesis of neural nAChr is regulated by phosphorylation[15], and is dependent on such factors as activity levels, and desensitisation by chronic exposure to agonist or antagonists[16]. Thus the enhanced flare response is best interpreted in the light of nAChr upregulation[7,8,16]. In Figure 3B the effect of prolonged tail-flick latency seen with N-treatment is significant and indicates some form of anti-nociception. Regional variation in sensory acuity or innervation density is excluded by testing at the same level in all rats[17], and the test is not dependent on basal skin temperature[17]. There are published data suggesting that such an action may be mediated centrally, peripherally, or both[18,19,20].

The nAChr blocker hexamethonium, which does not cross the blood brain barrier, has no effect on the anti-nociceptive effect of nicotine[19,20,21,22]. This suggests a central site of N-anti-nociceptive action. The mechanism underlying N-analgesia may involve participation of endogenous opioid peptides, since naloxone (opiate antagonist) blocked the anti-nociceptive effect of systemic nicotine in rats[22]. Supporting this, are the observations of Eiden *et al*[23] demonstrating release of metenkephalin from adrenal chromaffin cells after N-treatment. Another possible mechanism involves nicotine interaction with substance P (SP) , which has anti-nociceptive properties[24]. SP does not act on opioid receptors[22], but produces naloxone-reversible anti-nociception in rats after both intracerebral and systemic administration[20,22]. SP may induce release of metenkephalin from nociceptive brain regions[25], but SP-induced anti-nociception does

not block N-induced anti-nociception[22,24]. In spite of these findings[20-25],it is still possible that nicotine may partly mediate its effects at the periphery. Noxious stimuli induce peptide release from skin nerves[25], as does nicotine. Interactions between the nicotine-evoked actions on eicosanoids[26], peptides and tachykinins modulate nociceptor sensitivity[27]. Nevertheless, in spite of N-enhanced neurogenic inflammation[8,9], N-induced changes in the amount of peptide released in response to measured stimuli have not previously been reported. Therefore the trend shown in Fig 9 needs to be confirmed.

Denervation has been shown to prolong blister healing time in recent reports by Carr *et al*[7,17]. The effect is noted as a reduction of the acute inflammation surrounding the blister during the first 7 days after induction. The healing profiles diverge dramatically after 10 days, with a significant prolongation in healing time from 26 days (sham) to 42 days for the denervated hindlimb. This conforms with the finding of decreased survival of experimental critical flaps after sensory denervation with capsaicin[28]. The converse also applies, in that calcitonin gene related peptide (CGRP) increases survival of such flaps[29]. In the case of nicotine treatment, the only group of N-treated animals which showed a significant decrease in the blister-healing rate was the 14 day pre-blister nicotine treatment group. The effect was significant during the latter part of the healing profile, after day 15. In addition, healing was shortened by 5 days. Although SP release correlated significantly with an index of sensory nerve function, the axon reflex flare (Table 4), it is difficult to imagine that the mechanism underlying this effect is simply enhanced neuropeptide release at the time of blister induction. This would be expected to be manifest as a divergence of the healing profile early, analogous with the early reduction in inflammation acompanying denervation[7]. Since the divergence in healing profiles occurs late, it is presumed that some other mechanisms may be involved. These may involve increased local blood flow[30], alterations in the local turnover of eicosanoids[31], or modulation of growth factors[32]. Furthermore, there may be dose-dependent and/or time-dependent processes such as changes in SP or neuropeptide kinetics[33], which could mediate the nicotine effect. If higher doses are effective, the topical application of nicotine may be a better method of achieving appropriate levels locally[34]. The present result is tantalising, and in conflict with the observed adverse effects of smoking on skin flap healing[35,36], although Hahn[37] has shown that smoking also increases axon reflexes, presumably via nicotine. If these effects are nicotine-dependent, how are the nAChr on sensory nerves involved in the process? Clearly these questions should be explored further.

Primary skin sensory nerves which mediate neurogenic inflammation (axon reflex flare) are sensitised by chronic exposure to nicotine. Axon reflex responses

to ACh applied by iontophoresis are enhanced, but not those evoked by noxious transcutaneous electrical nerve stimulation. This indicates stimulus specificity of the polymodal nociceptors which may respond to both stimulus modalities by different excitation mechanisms. Also it suggests that nicotinic ACh receptors are not involved in the electrical axon reflex response. Nicotine treatment is anti-nociceptive for noxious thermal stimuli, and this may reflect either a central or a peripheral desensitisation effect. Nicotine treatment appears to produce a small alteration in the substance-P release during resting, nerve-stimulated and recovery collection periods. Nictotine pretreatment also accelerated the late phase of blister healing by 5 days.

REFERENCES

1. Lewis T. The nocifensor system of nerves and its reactions. I. British Medical Journal 1937; 1: 431-434; II. ibid. 1937; 1:491–494.
2. Lembeck F. Sir Thomas Lewis's nocifensor system, histamine and substance P-containing primary afferent nerves. Trends in Neurological Science. 1983; 6:106–108.
3. Foreman JC, Jordan CC. Neurogenic inflammation. Trends in Pharmacological Science 1984; 5:116–119.
4. White DM, Helme RD. Release of substance P from peripheral nerve terminals following electrical stimulation of the sciatic nerve. Brain Research 1985; 336:27–31.
5. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature (Lond.) 1980; 288:373–376.
6. Devillier P, Regoli D, Asseraf A *et al.* Histamine release and local responses of rat and human skin to substance P and other mammalian tachykinins. British Journal of Pharmacology 1986; 32:340–347.
7. Carr RW, Delaney CA, Westerman RA, Roberts RGD Denervation impairs cutaneous microvascular function and blister healing in the rat hindlimb. NeuroReport 1993; 4/5:467–470.
8. Grunfeld JA, Tiedemann GJ, Westerman RA. Chronic nicotine exposure enhances cutaneous axon reflexes in the rat. NeuroReport 1991; 2:421–424.
9. Grunfeld JA, Tiedemann GJ, Westerman RA. Maternal nicotine exposure enhances cutaneous axon reflexes in the neonatal rat. NeuroReport 1993; 4:635–638.
10. Low A, Westerman RA. Neurogenic vasodilatation in the rat hairy skin measured using a laser Doppler. Life Sciences 1989; 45:49–57.
11. Westerman RA, Low AM, Widdop RE, Neild TO, Delaney CA. Non-invasive tests of neurovascular function in human and experimental diabetes mellitus. In: GM Molinatti, RS Barr, S Belfiore & M Porta (Eds). Frontiers in Diabetes. Karger, Basel 1990; 9:127–138.
12. Morilak DA, Morris M, Chalmers J. Release of substance P in the tractus solitarius measured by in vivo microdialysis: response to stimulation of the aortic depressor nerve in rabbits. Neuroscience Letters 1988; 94:131–137.
13. Kiistala U. Suction blister device for separation of viable epidermis from dermis. Journal of Investigative Dermatology 1968; 50:129–137.
14. Iggo A. Sensory receptors in the skin of mammals and their sensory functions. Reviews in Neurology 1985; 141:599–613.
15. Huganir RL, Delcour AH, Greengard P *et al.* Phosphorylation of the nicotinic acetylcholine receptor regulates its rate of desensitization. Nature 1986; 321:774–776.
16. Wonnacott S. The paradox of nicotinic acetylcholine receptor upregulation by nicotine. Trends in Pharmacological Science 1990; 11:216–219.

17. Carr RW. Sensory nerve function in cutaneous wound healing. BSc Hons Thesis, 1992, Monash University.
18. Bonnycastle DD, Cook L, Ipsen J. The action of some analgesic drugs in intact and chronic spinal rats. Acta Pharmacologica et Toxicologica 1953; 9:332–336.
19. Sahley TL, Berntson GG. Antinociceptive effects of central and systemic administration of nicotine in the rat. Psychopharmacology 1979; 65:279–283.
20. Tripathi HL, Martin BR, Aceto MD. Nicotine-induced antinociception in rats and mice: correlation with nicotine brain levels. Journal of Pharmacology and Experimental Therapeutics 1982; 221:91–96.
21. Mattila MJ, Ahtee L, Saarnivaara L. The analgesic and sedative effects of nicotine in white mice, rabbits and golden hamsters. Annals of Medicine and Experimental Biology Fenn 1986, 46:78–84.
22. Molinero MT, Del Rio J. Substance P, nicotinic acetylcholine receptors and antinociception in the rat. Neuropharmacology 1987; 26:1715–1720.
23. Eiden LE, Giraud P, Dave JR *et al.* Nicotinic receptor stimulation activates enkephalin release and biosynthesis in adrenal chromaffin cells. Nature 1984; 312:661–663.
24. Frederickson RCA, Burgis V, Harrell EC *et al.* Dual actions of substance P on nociception: possible role of endogenous opioids. Science 1978; 199:1359–1362.
25. Naranjo JR, Sanchez-Franco F, Garzon J *et al.* Analgesic activity of substance P in rats: apparent mediation by metenkephalin release. Life Sciences 1982; 30:441–446.
26. Sonnenfeld T, Wennmalm A. Inhibition by nicotine of the formation of prostacyclin-like activity in rabbit and human vascular tissue. British Journal of Pharmacology 1980; 71:609–613.
27. Khalil Z, Helme RD. Sequence of events in substance P mediated plasma extravasation in rabbit skin. Brain Research 1990; 527:292–298.
28. Kjartansson J., Dalsgaard C-J., Jonsson C-E. Decreased survival of experimental critical flaps in rats after sensory denervation with capsaicin. Plastic and Reconstructive Surgery 1987; 79, 218–221.
29. Kjartansson J, Dalsgaard C-J. Calcitonin gene-related peptide increases survival of a musculocutaneous critical flap in the rat. European Journal of Pharmacology 1987; 142:355–358.
30. Kjartansson J, Lundberg T, Samuelson U E, Dalsgaard C-J, Heden P. Calcitonin gene-related peptide (CGRP) and transcutaneous electrical nerve stimulation (TENS) increase cutaneous blood flow in a musculocutaneous flap in the rat. Acta Physiologia Scandinavica 1988; 134: 89–94.
31. Nadler JL, Velasco JS, Horton R. Cigarette smoking inhibits prostacyclin formation. Lancet, 1983;1248–1250.
32. Nilson G, von Euler AM, Dalsgaard C-J. Stimulation of connective tissue cell growth by substance P and substance K. Nature, 1985; 315:61–63.
33. Nadel JA. Regulation of neurogenic inflammation by neutral endopeptidase. American Review of Respiratory Diseases 1992; 145 548–552.
34. Watcher MA, Wheeland RG. The role of topical agents in the healing of full-thickness wounds. Dermatological Surgery and Oncology 1989; 15:1189–1195.
35. Craig S, Rees TD. The effects of smoking on experimental skin flaps in hamsters. Plastic and Reconstructive Surgery 1985; 75:842–846.
36. Sherwin MA, Gastwirth CM. Detrimental effects of cigarette smoking on lower extremity wound healing. Journal of Foot Surgery 1990; 29:84–87.
37. Hahn A. The effect of smoking on small nerve and blood vessel function in man: a comparison with diabetes mellitus. Hons BSc. Thesis, Dept. of Physiology, Monash University, Clayton. 1990.

In: Clin. Exper. Neurol., 25: 71-84 (1988).

Effects of Topical Capsaicin on Normal Skin and Affected Dermatomes in Herpes Zoster

R.A. Westerman, R.G.D. Roberts,* R.R. Kotzmann,† D.A. Westerman,‡ C. Delaney,* R.E. Widdop** and B.E. Carter**

Clinical disorders of sensation are routinely assessed by bedside examination tests which lack sensitivity and quantitation.[1] In particular, disorders of thin nerve fibre function such as those that occur in post-herpetic neuralgia, diabetes and other neuropathies[2] have been difficult to diagnose and measure in the absence of clinical or other evidence of dysfunction.[3] Now non-invasive tests which measure nociceptor and primary afferent function are available.[4–6]

The present study used infrared emission thermography and thermal threshold testing to estimate the degree of desensitisation in the primary afferents innervating a skin zone or dermatome induced by repeated topical application of capsaicin.[7,8] This solenaceous plant derivative, *trans*-8-methyl-*N*-vanillyl-6-nonenamide, is widely used as a skin rubefacient and also as a hot spice in foods. It is a neurotoxin, probably specific for unmyelinated peptidergic sensory fibres.[7,9,10] Its effects are reversible in human skin[8,11] and it does not appear to affect adversely autonomic functions or blood vessels[8] (cf ref. 12). Capsaicin elevates noxious heat thresholds, and desensitises the C-polymodal nociceptors, reducing the axon reflex dilator responses to noxious electrical stimulation.[5,7] Therefore, it was proposed as a treatment for chronic post-herpetic neuralgic pain[13] although there was no measure of cutaneous nociceptor desensitisation nor of the most efficacious concentration of the active ingredient in this study. The present report describes the results of non-invasive measures of nociceptor function before and after capsaicin desensitisation in normal subjects and in the affected dermatome(s) of patients with herpes zoster.

* Department of Physiology, Monash University, Clayton, Vic. † Department of Radiology, Royal Southern Memorial Hospital, Caulfield, Vic. ‡ Royal Melbourne Hospital, Parkville, Vic. ** Department of Clinical Pharmacology, Austin Hospital, Heidelberg, Vic.

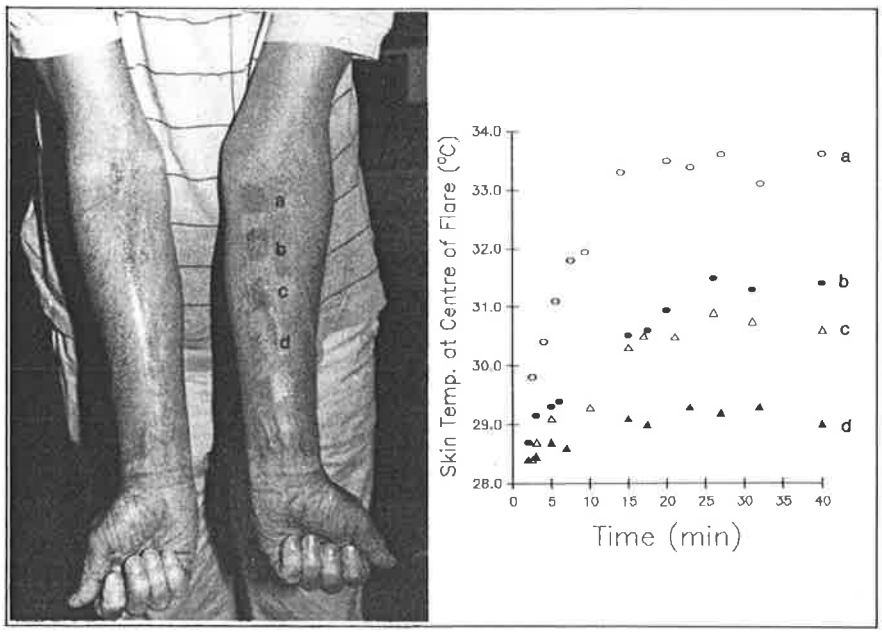


Figure 1. (A) Photograph taken 40 minutes after application of graded concentrations of capsaicin cream (a, 0.4%; b, 0.2%; c, 0.1%, d, 0.05%) to the left arm of a healthy volunteer. Graded inflammatory skin flares are seen at a, b, c, d. As a control, corresponding sites on the right forearm were treated only with ointment base and show no flares.
(B) Intermittent temperature readings at left forearm sites a, b, c, d taken with a handheld emission thermograph (Biotherm C-600M) show graded warming during 40 minutes following the application of capsaicin cream (0.4%, 0.2%, 0.1%, 0.05% respectively). The maximum warming occurred at 25–30 minutes after capsaicin application and was 4.5 °C.

Methods

Fifteen patients with post-herpetic neuralgia, ranging in age from 56 to 91 years (mean 75 years), and 69 normal subjects were used in this study. In 9 normal subjects, capsaicin cream (0.01%–0.4%) was applied acutely to the anterior skin of the forearm while for the patients it was applied to the post-herpetic dermatomes for periods ranging from 4 to 16 weeks (0.01% or 0.05% capsaicin cream applied 3 or 4 times daily to the herpes-affected skin zone). The capsaicin was diluted and dissolved in sorbolene cream base that was inert when tested separately (Figure 1). Thermal thresholds were measured for both warm and cold stimuli using the Medelec TTT microprocessor-controlled Peltier device. This has a thermode with a skin contact surface measuring 80 × 30 mm. The device uses a forced-choice psychophysical paradigm with 2, 4, 6, or 8 reversals selectable. The method of use and results in some clinical conditions are described by Jamal *et al.*[1,3,4] This device was used to measure warm and cold thresholds in the herpes-affected dermatomes and in the corresponding contralateral unaffected dermatome (Table 1).

Table 1. Details of subjects and responses to capsaicin

Patient ID	Age (years)	Affected dermatome	TTT readings °C						% capsaicin used	Thermal threshold difference °C (desensitisation)			
			Normal side		Before capsaicin PHN side		After capsaicin PHN side			Before capsaicin		After capsaicin	
			Warm	Cold	Warm	Cold	Warm	Cold		H	C	H	C
2000	79	L Trigem I	0.05	0.05	0.33	0.05	7.45	0.05	0.05	+0.28	0	+7.12	0
2001	79	R Trigem I, II			1.05		8.15	2.75	0.05	—	0	+7.10	0
2002	61	R Th 5, 6 (6, 7)	0.45	0.25	1.05	0.55	0.60	0.55	0.01	+0.6	+0.3		
							2.10	1.35	0.05			+1.05	−0.40
2003	91	L Th 2, 3	—	—	0.55	0.55	1.15	0.20	0.05	—	—	+0.60	−0.35
					0.35		0.15	0.60	0.01				
2004	86	R Th 7, 8	—	—	0.25	0.40	1.05	0.25	0.05	+0.4	−0.15	+0.80	−0.15
					0.05	0.10	0.55	0.25	0.17				
2005	71	R Th 3	0.25	0.10	4.05	0.50	>10	0.10	0.05	+3.8	+0.4	+6.05	−0.40
2006	77	R Th 5, 6	0.30	0.05	0.65	0.25	6.5		0.05	−0.3	+0.2	+5.85	−0.20
2007	80	L Th 2, 3	1.05	0.45	4.15	3.35	6.05	6.05	0.05	+3.1	+2.9	+1.90	+2.0
2009	73	L Th 3			2.40	2.0	>10	2.0	0.05	—	—	+7.60	0
2010	76	L Trigem. I	—	—	0.55	0.20	0.65	0.10	0.01	—	—	+0.10	−0.10
2012	80	R Th 5	0.25	0.20	1.35	0.95	1.55	1.65	0.01				−0.05
					0.25	0.20	5.05	0.15	0.05	—	—	+4.80	
2016	56	L C6 (Sup Rad)	0.15	0.10	0.45	0.35	2.10	0.65	0.05	+0.30	+0.25	+2.05	+0.3
2008	76	R Trigem I, II	—	—	4.35	0.65	7.5	0.30	0.01	—	—	+3.00	−0.30
2016	65	R Th 5	0.25	0.15	1.00	1.55	—	—		+0.75	+1.4		
2011	78	L Th 5, 6	—	—						+8.55	+8.5		
	n = 15								n = 8	8		13	13
	$\bar{x}$ = 75.2								S = ±1.19	0.7		3.69	0.08
	S = ± 9.06								x^2 = 1.42	0.99		2.8	0.62
	x^2 = 82.2								2.06	0.98		7.86	0.38

Abbreviations: PHN = post-herpetic neuralgia, H = hot, C = cold, R = right, L = left, Th = thoracic, Trigem = trigeminal.

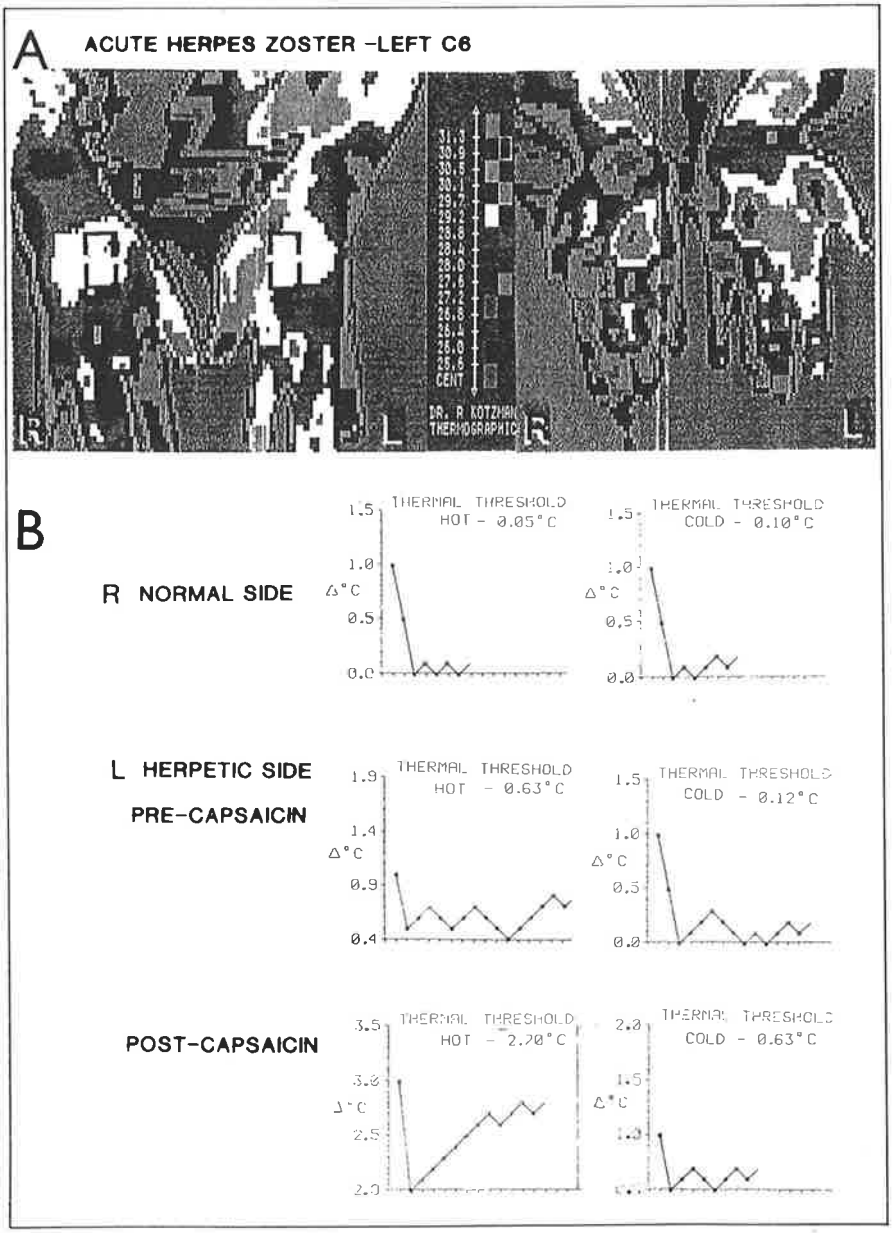


Figure 2. (A) Infrared emission thermographs of both hands in a patient with herpes zoster in the skin area supplied by the left superficial radial nerve, within the C6 dermatome. The left hand exhibits warming of 1.5–2.0 °C compared to the right in the skin zone supplied by the herpetic sensory nerve. (B) The warm and cold detection thresholds after 4 weeks of desensitisation with 0.05% capsaicin cream applied topically 3 or 4 times daily.

Infrared emission thermograms of affected and normal skin areas were taken with an Agema 782 system and with a handheld Biotherm C-600M (Figures 1 and 2).

The assessment of pain intensity before and after capsaicin treatment was made by each patient using a linear visual analog (VAS) scale recorded with pencil and paper, described in the McGill Short Form Pain Questionnaire.[14] The length (in mm) of the patient's mark estimating pain intensity was calculated as a fraction of the 100 mm VAS line length, and the difference between the pre- and post-capsaicin VAS rating was calculated as a percentage pain relief (Table 2).

Table 2. Summary of changes produced by capsaicin treatment

Patient ID	VAS rating of pain intensity		Pain relief (%)	TTT warm threshold elevation °C
	Before capsaicin	After capsaicin		
2000 (Fig. 3)	0.76 (0.05%)	0.12	−84	+7.12
2001	0.65 (0.05%)	0.35	−46	+7.10
2002	0.78 (0.01%)	0.43	−45	+0.30
	(0.05%)	0.9	−88	+1.05
2003	0.58 (0.05%)	0.13	−78	+0.60
	(0.01%)	0.43	−26	
2004	0.84 (0.05%)	0.19	−77	+0.80
2005				+6.05
2006	0.65 (0.05%)	0.9	−86	+5.85
2007	0.54 (0.05%)	0.11	−80	+1.90
2009	0.64 (0.05%)	0.28	−56	+7.60
2010	0.77 (0.01%)	0.49	−36	+0.10
2012	0.65 (0.01%)	0.41	−40	
	(0.05%)	0.8	−88	+4.80
2016 (Fig. 2)	0.32 (0.05%)	0.4	−87	+2.07
2008*	0.79 (0.01%)	0.75	−5	+3.0
2014†	0.72 (0.05%)	0.92	'Much worse'	—withdrew
2011‡	0.71	0.71	0	TTT >10 before

Pain relief with: 0.05% $n = 10$, $x = 77\%$, $S = 14.5\%$, $x^2 = 209.3$
: 0.01% $n = 5$, $x = 30\%$, $S = 15.8\%$, $x^2 = 250.3$

*This patient had a history of shingles, but had probable cluster headaches and was referred to a neurologist. †This patient was unable to persist with capsaicin for more than 3 days, and withdrew because of intolerable burning discomfort. ‡This patient had a dorsal rhizotomy 10 years before testing but was still denervated in the affected dermatomes with TTT >10 °C to both warm and cold stimuli.

Results

Figure 1 shows that the acute inflammatory responses to graded doses (0.4%, 0.2%, 0.1%, 0.05%) of topical capsaicin ointment applied to normal forearm skin

reached their maximum by 25–30 minutes, the extent of warming being dose-dependent and consistent with the capsaicin-evoked temperature elevation already described.[8] During the acute vesicular phase of herpes zoster, skin temperature in the affected dermatome may be elevated by 1–4 °C. An example of one patient's thermogram is shown in Figure 2A. Presumably this largely reflects the degree of neurogenic inflammation evoked by virus-containing primary afferent nerve fibres being similar to the immediate effects of topical capsaicin in elevating skin temperature (Figure 1). Thermal thresholds to warm and cold stimuli were measured at the wrist for normal subjects (Figure 3) and on the herpes-affected dermatome and also on the contralateral unaffected dermatome of patients (Figures 2B and 4; Table 1). The distribution and low variability of warm and cold thresholds measured at one site, viz. for normal forearm/wrist skin, shown here agree closely with published data.[1,3,4]

Capsaicin was then used to desensitise the painful herpetic skin zone by topical application of 0.05% or 0.01% cream 3 or 4 times daily for 4 weeks, after which thermal thresholds for warm and cold detection were again measured. This comparison permitted estimation of the extent of capsaicin-induced desensitisation of nociceptors in treated skin, as shown in Table 2.

Figures 2B and 4 show examples of the warm and cold threshold Medelec printouts, and Figure 4 gives the thermal threshold results obtained in one female patient aged 78 years who had suffered from post-herpetic neuralgia in the left ophthalmic division of the trigeminal nerve for 4 years. The initial pre-capsaicin warm threshold for the post-herpetic dermatome was 0.33 °C, only slightly higher than the corresponding normal skin. After 22 days of treatment with 0.05% capsaicin cream the warm threshold showed an elevation of 7.12 °C. By contrast, there was no difference between cold detection thresholds of normal and herpetic skin even after topical capsaicin treatment. This patient (No. 2000; Table 1, 2) achieved a high degree of pain relief (84%) from the capsaicin. The warm threshold elevation (2.07 °C) but not pain relief (87%) was less for the patient in Figure 2B after 0.05% capsaicin treatment of the affected left C6 hand skin (No. 2016; Table 1, 2).

Thermal threshold data for all 15 patients are presented in Table 1. The dermatomes affected included the trigeminal in 4 patients, the cervical in 1 and the thoracic in the remaining 10 patients. For technical reasons not all patients in this series had pre-capsaicin thermal threshold measurements. The difference between both thermal detection thresholds on corresponding normal and herpes-affected skin indicates the extent of desensitisation caused by herpetic damage. Before capsaicin treatment, often warm detection and sometimes cold detection thresholds were elevated in the herpetic dermatome by comparison with the corresponding normal skin. The mean elevation of warm threshold in untreated herpes-affected skin was 1.19 °C and for cold detection was 0.70 °C. However in some instances the affected dermatome was hypersensitive to warm stimuli and exhibited a lower threshold (Table 1). The average desensitisation resulting from 4 weeks of topical capsaicin treatment was 3.69 °C in the case of the warm detection threshold and 0.08 °C for the cold detection threshold.

Varying elevation of warm detection thresholds and the corresponding patient estimate of pain relief calculated from the VAS rating scale before and after capsaicin was seen in capsaicin-desensitised skin zones compared with their corres-

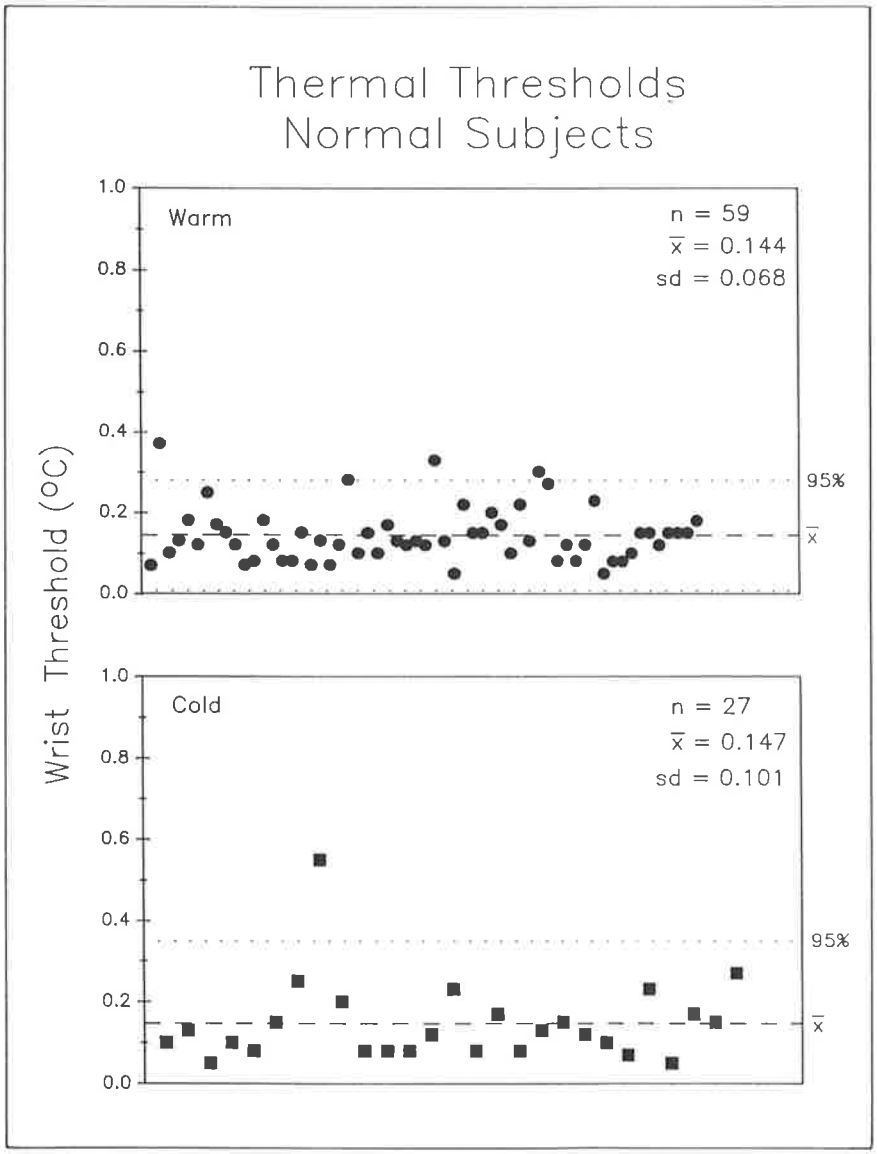


Figure 3. Thermal detection thresholds to warm stimuli measured by a Peltier device (Medelec TTT).
(A) (Upper) The warm detection thresholds (mean 0.144 ± 0.07 °C) measured on the anterior aspect of the forearm above the wrist for $n = 87$ observations on 59 normal subjects. The mean and 99% confidence limits are indicated as solid and broken lines respectively.
(B) (Lower) Cold detection thresholds (mean 0.147 ± 0.10 °C) measured on the anterior aspect of the forearm above the wrist for $n = 36$ observations on 27 normal subjects. The mean and 99% confidence limits are shown.

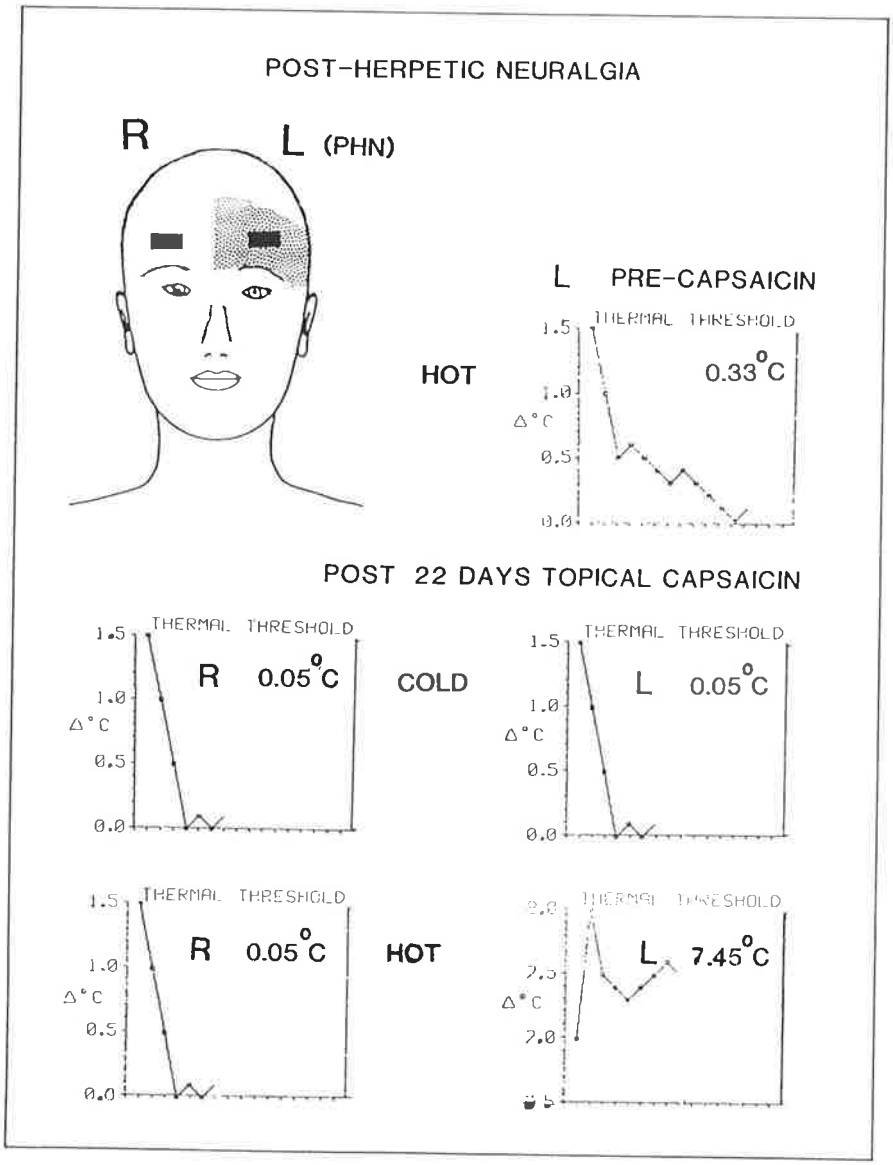


Figure 4. Thermal thresholds of a 78-year-old woman with post-herpetic neuralgia of 4 years duration. (A) (Upper, left) The herpetic dermatome (L, trigeminal ophthalmic division) (stippled) and the sites of application of Peltier thermal probe (solid). (B) (Upper, right) The warm threshold tested on herpetic skin was 0.33 °C pre-capsaicin. (C) (Centre) After 22 days of treatment thrice daily with 0.05% capsaicin cream the warm threshold on herpetic skin was markedly elevated by 7.45 °C compared to normal side 0.05 °C. (D) (Lower) Cold thresholds on capsaicin-treated herpetic and normal skin were identical and normal.

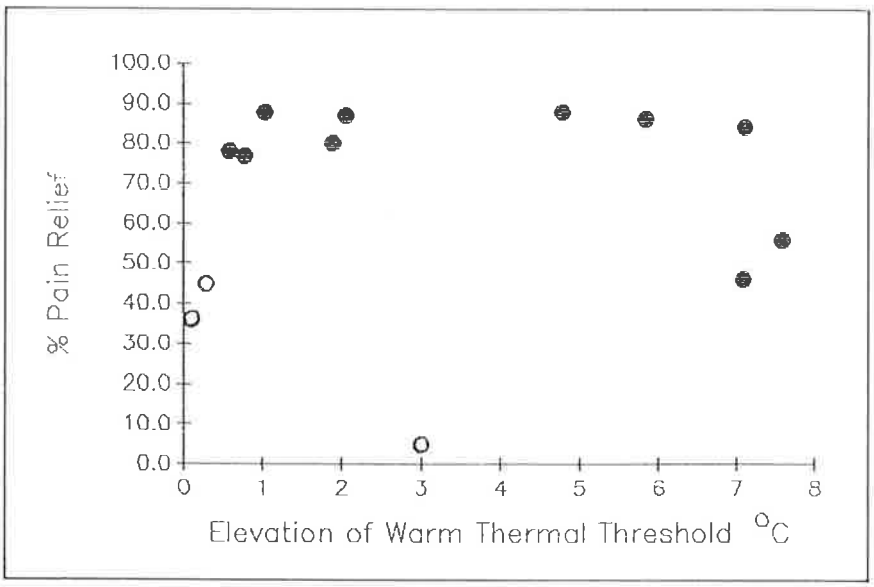


Figure 5. Abscissa: The elevation in °C of the warm detection thresholds for 12 patients measured by Medelec TTT on herpes-affected skin after 4 weeks of topical capsaicin treatment. Filled circles indicate 0.05% and open circles 0.01% topical capsaicin cream treatment.
Ordinate: Pain relief calculated from patient estimates of pain using a visual analog scale rating.[14] Pain estimates were made as a mark on 2 linear 100 mm scale ranging from NO PAIN to WORST POSSIBLE PAIN and were calculated as a fraction for each subject before (x) and after (y) capsaicin treatment. The pain relief (z) was then calculated by expressing the pain after capsaicin treatment as a percentage of the pain before capsaicin, according to the formula $z = (x - y)/x$ (see Table 2). No significant correlation existed between the extent of pain relief and the warm threshold elevation ($r = 0.069$).

ponding normal skin (Figure 5; Table 2). The mean elevation of the TTT warm threshold in capsaicin-treated skin was 3.69 °C ($n = 13$), but no consistent alteration in cold threshold was observed (Table 1). On average, the degree of pain relief achieved by 0.01% capsaicin was lower, only 30%, compared with a mean 77% pain relief from 0.05% capsaicin.

Discussion

Repeated chronic capsaicin treatment of normal skin has been shown to produce desensitisation of peptide-containing primary afferent nerves[7,15,16] which is reversible[8,11] and does not adversely affect microvascular or autonomic function[8] (cf ref. 12). This suggested a possible topical therapy for post-herpetic neuralgia[13] which appeared to be effective in relieving pain in about 75% of the 14 cases treated. However, although temperature sensitivity has been reported to be re-

duced and pain and thermal thresholds to be elevated in neuropathy,[16] no estimate of capsaicin-evoked desensitisation was made in Bernstein's study[13] except for subjective pain ratings before and after treatment—and this study did not use a standard scale such as the McGill visual analog scale (VAS) for pain rating.[14] The present study relies on the fact that normal detection thresholds for warm sensations require intact C-thermal and polymodal sensory fibres: impairment of this primary afferent transmission by capsaicin treatment elevates warm thermal detection thresholds and provides a measure of the extent of desensitisation of treated skin.[16,17] Therefore it was not unexpected to find no appreciable effect of capsaicin treatment on the cold threshold which depends upon A-delta cold afferents (Figure 3). This finding can be correlated with the subjective estimates (i.e. VAS pain ratings) before and after treatment. The results reported in the present preliminary study raise a number of questions, as follows.

What is the Optimum Concentration of Topical Capsaicin to Achieve Maximum Pain Relief in Treating Post-herpetic Neuralgia?

In the present study, the concentrations of capsaicin in aqueous cream base used for desensitisation treatment of post-herpetic neuralgia dermatomes were 0.05% or 0.01% applied 3 to 4 times daily for 4 weeks. The lower concentration has the advantage of a milder acute response to each application of topical capsaicin and reduced risks of severe discomfort after inadvertent contamination of conjunctiva or mucosa. However, in the limited preliminary trial reported here, the degree of pain relief was less with the lower 0.01% capsaicin than with the 0.05% preparation (Table 2).

The apparent efficacy of low concentrations of topical capsaicin 0.05% and 0.01% in the present study, and 0.025% used by Bernstein *et al.*[13] suggests a possible selective uptake mechanism for capsaicin and makes it desirable to test the minimum effective concentration able to achieve satisfactory desensitisation. Nevertheless, 0.05% appeared more effective than 0.01% in our group of patients, based on the average pain relief of 77% and 30%, respectively. A prospective double-blind controlled trial is now being planned to test this important question.

What is the Optimum Duration for which Topical Capsaicin Desensitisation should be Continued?

In this study of 15 patients, 4 were treated continuously for between 3 and 4 months. The continued application of topical capsaicin 3–4 times daily maintained a high degree of pain relief in these 4 patients. The remaining patients received capsaicin cream over shorter periods, and their pain relief is shown in Table 2. In 3 of these patients a reduction of capsaicin strength from 0.05% to 0.01% resulted in a return of pain sufficient to require the use of analgesics. Therefore in future studies all patients will be treated continuously for 6 months before we attempt to

discontinue treatment. There is as yet no answer to this question, but because capsaicin desensitisation is reversible[8, 11, 16] the topical application should continue for some time.

What is the Correlation Between the Extent of the Cutaneous Nociceptor Desensitisation Measured by Warm Threshold Elevation, and Pain Relief Obtained by Capsaicin Treatment?

Even with the limited numbers of subjects in this preliminary study, many different dermatomes were affected by herpes zoster. It is well recognised that different skin regions have different stratum corneum thicknesses and therefore differing penetrability to the topical capsaicin.[18] Furthermore, different innervation density and different sensory thresholds for warm, cold and two-point discriminations are well recognised.[19] For these reasons, until a larger number of patients is tested for each of the affected dermatomes, any correlation of pain relief with desensitisation can only be very approximate (see Figure 5; Table 2). The surprising feature of the present study is the apparently poor relationship between degree of pain relief and warm threshold elevation afforded by topical capsaicin treatment shown in Figure 5 and Tables 1 and 2. This could be explained in part by the fact that warm detection is not solely a function of C-polymodal nociceptors, but if it is primarily desensitisation of these nociceptors which is to be measured one should examine noxious heat pain thresholds.[17] (This is not practicable with the Medelec TTT device.) This is also begs the questions as to which sensory fibres are affected by capsaicin treatment,[9, 10] and as to what degree of herpetic damage to sensory nerves has occurred in affected skin before capsaicin treatment is applied. The usefulness of thermal threshold testing will probably lie in the prediction of which patients are most likely to respond well to capsaicin treatment, and therefore to the selection of those more likely to benefit from other forms of treatment.

Are There Any Adverse Effects or Complications from Chronic Repeated Capsaicin Treatment?

In one of the patients in the present study the application of capsaicin caused the reappearance of small vesicles at the margin of the capsaicin appplication. This may be interpreted as reactivation of the herpes zoster which occurred 16 months after the original attack of shingles. The frequency of this occurrence is not known.

Are All Types of Post-herpetic Neuralgia Pain Suitable for Topical Capsaicin Desensitisation Treatment?

One of the patients studied (No. 2011) had been treated by dorsal rhizotomy 3 years earlier, but had been afforded no pain relief by this procedure. The pain was still perceived as being present in the truncal dermatomes T3, 4 although the skin

was totally desensitised to warm and cold stimuli and both thresholds were elevated by more than 10 °C. In this case, post-herpetic neuralgic pain appeared to involve elements of causalgia associated with sensory nerve destruction.[20] The attempted desensitisation of peripheral nociceptors in such a denervated dermatome should not be expected to afford any appreciable amelioration of pain—nor did it in this patient. Theoretically this may result from the viral neuropathy itself in the absence of any neurosurgical destruction. In such cases one might expect to find greater thermal desensitisation of the herpetic dermatome before capsaicin treatment, and consequently less pain relief. This is an important matter, but remains to be tested.

The present results indicate some complexities in what is reportedly a simple and frequently effective treatment for the pain of cutaneous nociceptor origin described as post-herpetic neuralgia.[13] The need for further objective measurement of small nerve function in this condition is emphasised by the findings. It is hoped that such studies will predict those patients most likely to respond to topical capsaicin treatment, and identify subgroups of patients with different mechanisms of pain pathogenesis.

Summary

Hyperalgesia and allodynia, lasting for months or even years, occurs in the form of post-herpetic neuralgia in approximately 70% of adults previously infected with the varicella herpes zoster virus.

The present study aimed at testing the analgesic desensitising actions and reversibility of repeated application of topical capsaicin on disordered polymodal nociceptors and peptidergic sensory fibres mediating warm and pain sensation. Cutaneous nociceptor desensitisation was measured using the Glasgow automated thermal threshold test (Medelec TTT). For normal subjects ($n = 69$) the mean forearm warm threshold was 0.15 ± 0.07 °C and the cold threshold was 0.14 ± 0.10 °C. A variable degree of partial desensitisation of herpes-affected skin was found in 15 patients with post-herpetic neuralgia before capsaicin treatment where the mean threshold elevation for warm detection was 1.19 °C and 0.7 °C for cold detection, compared with the corresponding normal skin.

In preliminary studies of 15 patients with post-herpetic neuralgia, good pain relief averaging 30% or 77% occurred in the affected dermatome(s) after 3 to 4 weeks of 0.01% or 0.05% capsaicin cream respectively, applied 3–4 times daily. The warm thresholds, after chronic capsaicin treatment, increased between 0.1 and 7.60 °C, the average elevation being 3.69 °C. By contrast cold thresholds after capsaicin altered inconsistently and by only an average of 0.08 °C. The results suggest that elevation of the warm threshold may indicate the desensitisation achieved by capsaicin treatment of skin polymodal nociceptors. Cold detection, being dependent upon A-delta cold fibre function, is unaffected by capsaicin treatment.

There was a poor correlation between pain relief and elevation of warm detection in response to capsaicin treatment. Generally, it was found that those patients with less initial desensitisation to warm detection as a consequence of post-herpetic neuralgia experienced better pain relief after capsaicin was applied.

The method used permits determination of the minimum effective desensitis-

ing dose of capsaicin, enables patient compliance and progress to be monitored and should allow the prediction of patients likely to achieve the best response to treatment.

Acknowledgements

The authors sincerely thank all subjects, both normal volunteers and patients, who participated in this study. This research was partly supported by Australian Research Grants Scheme, the Clive and Vera Ramaciotti Foundation, and by Boehringer Ingelheim.

References

1. Jamal GA, Hansen S, Weir AI, Ballantyne JP. An improved automated method for the measurement of thermal thresholds. 1. Normal subjects. J Neurol Neurosurg Psychiatry 1985; 48: 354–360.
2. McLeod JG, Pollard JD. Neuropathies in systemic diseases: hidden and overt. In: Asbury AK, Gilliatt RW, eds. Peripheral nerve disorders. London: Butterworths, 1984; 92–125.
3. Jamal GA, Weir AI, Hansen S, Ballantyne JP. An improved automated method for the measurement of thermal thresholds. 2. Patients with peripheral neuropathy. J Neurol Neurosurg Psychiatry 1985; 48: 361–366.
4. Jamal GA, Hansen S, Weir AI and Ballantyne JP. The neurophysiologic investigation of small fiber neuropathies. Muscle Nerve 1987; 10: 537–545.
5. Westerman RA, Low A, Pratt A, *et al.* Electrically evoked skin vasodilatation: a quantitative test of nociceptor function in man. Clin Exp Neurol 1987; 23: 81–89.
6. Westerman RA, Widdop RE, Hogan C, Zimmet P. Non-invasive tests of neurovascular function: reduced responses in diabetes mellitus. Neurosci Lett 1987; 81: 177–182.
7. Magerl FW, Szolcsanyi J, Westerman RA, Handwerker HO. Laser Doppler measurements of skin vasodilatation elicited by percutaneous electrical stimulation of nociceptors in humans. Neurosci Lett 1987; 82: 349–354.
8. Roberts RGD, Westerman RA, Widdop RE, Kotzmann RR, Payne R. Effects of capsaicin on cutaneous vasodilator responses in humans. J Invest Dermatol 1988; submitted for publication.
9. Jessell TM, Iversen LL, Cuello AC. Capsaicin-induced depletion of substance P from primary sensory neurones. Brain Res 1978; 152: 183–188.
10. Anand P, Bloom SR, McGregor GP. Topical capsaicin pretreatment inhibits axon reflex vasodilatation caused by somatostatin and vasoactive intestinal polypeptide in human skin. Br J Pharmacol 1983; 78: 665–669.
11. Lundblad L, Lundberg JM, Anggard A, Zetterstrom O. Capsaicin-sensitive nerves and the cutaneous allergy reaction in man. Allergy 1987; 42: 20–25.
12. Duckles SP. Effects of capsaicin on vascular smooth muscle. Naunyn Schmiedeberg's Arch Pharmacol 1986; 333: 59–64.
13. Bernstein JE, Bickers DR, Dahl MV, Roshal JY. Treatment of chronic postherpetic neuralgia with topical capsaicin. J Am Acad Dermatol 1987; 17: 93–96.
14. Melzack R. The short-form McGill pain questionnaire. Pain 1987; 30: 191–197.
15. Jancso N, Jancso-Gabor A, Szolcsanyi J. Direct evidence for neurogenic inflammation and its prevention by denervation and by pretreatment with capsaicin. Br J Pharmacol 1967; 31: 138–151.
16. Szolcsanyi J. Selective responsiveness of polymodal nociceptors of the rabbit ear to capsaicin bradykinin and ultraviolet irradiation. J Physiol 1987; 388: 9–23.
17. Fruhstorfer H, Goldberg JM, Lindblom U, Schmidt WG. Temperature sensitivity and pain thresholds in patients with peripheral neuropathy. In: Zotterman Y, ed. Sensory functions of the skin in primates. Oxford: Pergamon Press, 1976; 507–517.

18. Elias PM, Cooper ER, Korc A, Brown BL. Percutaneous transport in relation to stratum corneum structure and lipid composition. J Invest Dermatol 1981; 76: 297–301.
19. Weddell G. The anatomy of cutaneous sensibility. Br Med Bull 1945; 3: 167–172.
20 Cousins MJ. Introduction to acute and chronic pain: implications for neural blockade. In: Cousins MJ, Bridenbaugh PO, eds. Neural blockade in clinical anaesthesia and management of pain. 2nd Ed. Philadelphia: J.B. Lippincott, 1988; 739–752.

R.G.D. Roberts and R.A. Westerman
Department of Physiology, Monash University, Melbourne

Capsaicin:
Hot Stuff for Pain Relief

the main active, hot ingredient in Hungarian
lli and curry powders. This pungent chemical
lly obtained in its pure form more than 100
/ oily extraction from various members of the
family (nightshade plants) and is responsible
rkable heating properties of these condiments.
oid, trans-8-methyl-N-vanillyl-6-nonenamide,
lily synthesised. Although its pharmacological
Molnar 1965) and physiological effects (Buck
6; Holzer 1988) have been investigated exten-
he last 50 years, its therapeutic role has only
n considered (Bernstein 1987; Jansco et al.
eck 1988).

gical

ological actions
in have been
in both human
l experiments
ently indicated
werful, specific
which exerts its
marily on a
f the unmyeli-
bres. In peri-
rves C-fibres
tsynaptic sym-
bres and slow
, high threshold
fferents which
arious somatic
. Within this
geneous group
tide-containing
olymodal noci-

ceptors, capable of responding to various natural noxious stimuli (mechanical, chemical and thermal) and largely responsible for mediating prickle, itch and acute and chronic pain sensation. It is primarily these fibres which are susceptible to capsaicin.

Polymodal nociceptors normally respond to stimuli by releasing neuropeptides at their peripheral and central nerve endings. This comes about because the natural stimulus causing the initial release from the peripheral ending, e.g. in the skin, muscle, gut, etc., simultaneously activates the process whereby an action potential is established, sending a stimulus to the dorsal root ganglion cell and then through the central connection of the primary afferent fibre to the dorsal horn of the spinal cord. At least one of the neuropeptides released from the central connections of these fibres is believed to act as the synaptic transmitter for this pain pathway (Lembeck 1988). In the periphery, stimulation of a single C-fibre nerve ending activates collateral preterminal branches of that nerve ending producing what is known as an 'axon reflex'. In this way the effect of the stimulus spreads, e.g. in the surface of the skin for a few millimetres. This usually produces a flare resulting from the vasoactive effect of neuropeptides released from the C-fibre nerve endings, hence a neurally

Salient Points

- Topical capsaicin produces significant pain relief in both the acute phase of a shingles attack and in chronic postherpetic neuralgia.
- Low concentrations of capsaicin (Capsig 0.025%) are more effective in the acute phase.
- Higher concentrations of capsaicin (Finalgon diluted to 0.05%) are more effective in chronic postherpetic neuralgia.
- Pain relief tends to be less in more caudally affected dermatomes.
- Higher initial thermal threshold suggests effective pain relief is less likely.
- Visual analogue pain rating scales provide good estimates of pain relief.
- Long lasting pain relief is not provided by surgical treatment.

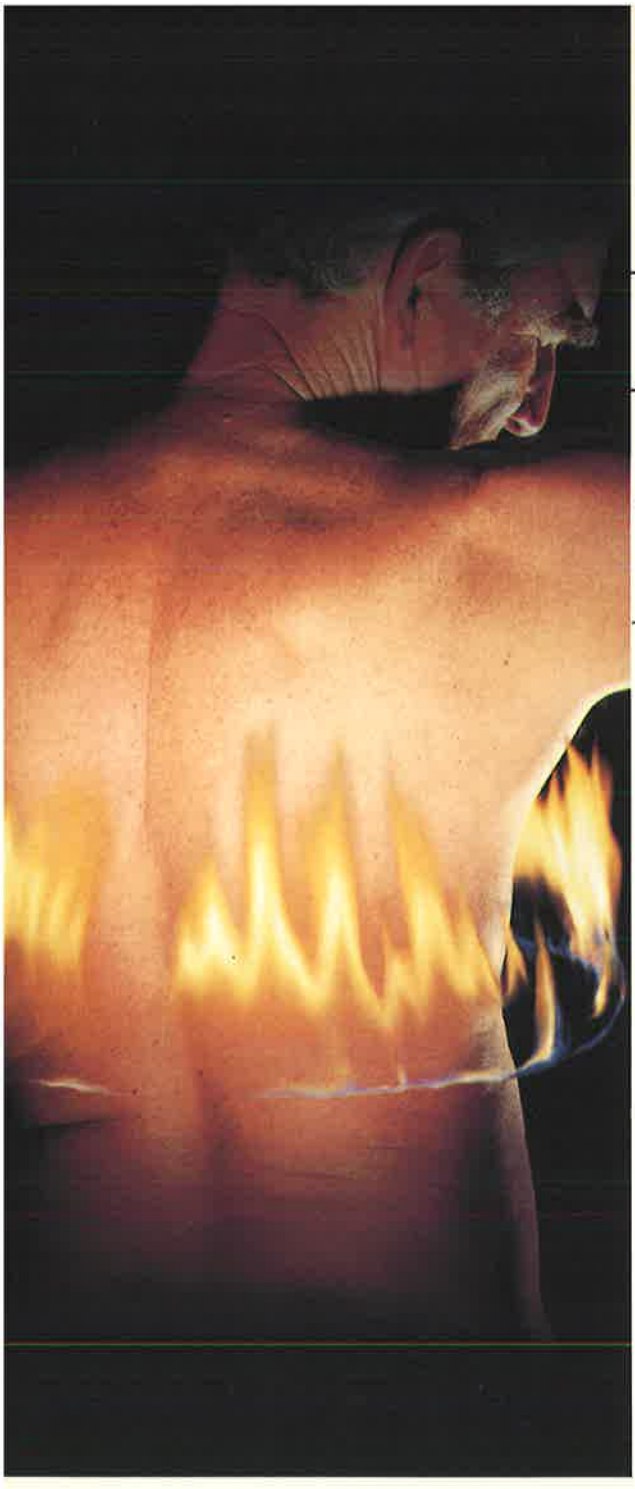
CAPSAICIN IS A SPECIFIC NERVE TOXIN AFFECTING PRIMARILY A SUBGROUP OF UNMYELINATED C-FIBRES

vasodilatation-neurogenic inflammation
3).
m vasodilatation, other protective functions
peptides released by noxious stimuli include
n of plasma protein and blood cells; mast cell
on; histamine and tachykinin release;
phagocytosis by macrophages; chemotaxis
hils; proliferation of lymphocytes and
synthesis of acidic lipids and prostaglandins;
the kinin, complement and clotting systems;
of fever by pyrogen production; and antigen
and growth of new microvessels (Buck &
Chahl 1988; Holzer 1988; Lembeck 1988).
ation of capsaicin, e.g. to the skin, in suffici-
concentration appears to activate these
ociceptor fibres causing them to release such
dilator neuropeptides as substance P and
ene-related peptide inducing dilation of the
dermal vessels, hence the rubefacient effect
. Simultaneously, the sensation of pain is
ne central connections. The neurotoxic action
becomes apparent on repeated applications
pheral (vasodilatation) and central (burning
ffects are progressively lessened, inducing a
nsitisation to noxious stimuli (Buck & Burks
l 1988; Holzer 1988). How does this
desensitisation occur?
animals administration of successively
oses of capsaicin on consecutive days induces
om primary afferent C-fibres and tissues
y these fibres of substance P and calcitonin
peptide, in addition to several other
es including somatostatin, cholecystokinin,
testinal polypeptide, corticotrophin-releasing
in and neurokinin A. Radioimmunoassay of
nerve has shown reduction in substance P
n whereas in structures not innervated by
rents (ventral horn and midbrain structures)
remain unchanged.
istochemical identification of neuropeptide-
bres, which is a more sensitive technique than
oassay to detect these changes, indicates that
nic sympathetic C-fibres,which also contain
les (vasoactive intestinal polypeptide,
e Y, galanin), are unaffected by capsaicin
Following this regimen of capsaicin
t, animals develop a long lasting desensitisa-
ul stimuli without any effect on their response
sory stimuli. These effects have been demon-
ingle fibre recordings of identified receptor
ensory myelinated and unmyelinated fibres
in of rats receiving repeated topical appli-
% capsaicin (Kenins 1982). The evidence
at there is a specific subgroup of primary
afferent nerve fibres which is susceptible to the toxic effects of capsaicin. This subgroup includes unmyelinated neuropeptide-containing polymodal nociceptors with perhaps the involvement of a small group of the thinnest myelinated fibres which also subserve nociception. While acute application of capsaicin causing an initial release of neuropeptide from susceptible nerve endings is usually associated with hyperaesthesia (where warm stimuli feel uncomfortably hot), repeated exposure progressively depletes these nerves of neuropeptide resulting in desensitisation. Since neuropeptides are synthesised in the nerve cell bodies of the dorsal root ganglion and transported to the nerve terminals by axonal transport, progressive depletion at the terminals may be due to the inability of the capsaicin-treated nerve to resupply the nerve terminals with neuropeptide. Local application of capsaicin to rat sciatic nerve blocks fast axonal transport of substance P and somatostatin (Gamse et al. 1982; Jansco et al. 1980), whereas capsaicin-treated neonatal rats exhibit reduced substance P synthesis in the dorsal root ganglion (Harmar et al. 1981). These effects, combined or singly, would account for the neuropeptide-depleting effect of capsaicin, given the neuropeptide release it evokes.

Therapeutic Uses of Capsaicin

Postherpetic Neuralgia

Based on the known pharmacology of capsaicin, the search for therapeutic uses led immediately to the painful neurocutaneous eruption associated with the *Herpes zoster varicella* virus. Although seen in the very young, this is commonly an infection of the elderly (Burgoon



Break the circle of postherpetic neuralgia

Now there is Capsig, a new topical analgesic cream specifically for postherpetic neuralgia. By depleting substance P, Capsig interrupts pain transmission between the skin periphery and the CNS.

In clinical trials, over 70% of patients who completed 28 days' therapy experienced substantial or complete pain relief.[1,2]

New Capsig Cream

(capsaicin 0.025%)

rst against postherpetic neuralgia

Pharmaceuticals Pty. Ltd., 1408 Centre Road, Clayton, Vic. 3168. Ph. (03) 542 9777.

SIGMA SKIN CARE

petic neuralgia and topical capsaicin, Watson, C.P.N. et al, Toronto, Canada, Pain , 33 Elsevier Science Publ. pp. 333-340. 2. Treatment of chronic postherpetic neuralgia with topical capsaicin, Bernstein, J.E. et al, J. Am. Acad. Derm.,

IN PRELIMINARY TRIALS, THE RATE OF USEFUL PAIN RELIEF IN POSTHERPETIC NEURALGIA WAS ABOUT 65%

m 196 patients, aged 19-93 (average = 70) years*

	Acute herpetic pain – duration < 10 weeks			Postherpetic neuralgia – duration > 10 weeks		
ength	0.025%	0.05%	All patients	0.025%	0.05%	All patients
	60	46	57	26	49	33

topical capsaicin 0.025% and 0.05% applied 3 times daily for 1 month in the treatment of patients suffering from acute herpetic pain) weeks) and postherpetic neuralgia (duration > 10 weeks). Per cent pain relief was calculated from the difference between visual analogue at the 2 visits, 1 month apart.

4.2% of postherpetic neuralgia patients are an 20 years, while 47.5% are over 70 years 986). Because many pharmacological, anaes- urgical therapies have been proposed for the t of postherpetic neuralgia, a multimodal s been advocated which aims to alleviate acute ise risks of developing refractory pain in those zoster and improve any impediments which ress in pain management (Portenoy 1986). advocated therapies is consistently effective cts.

pes zoster is followed by marked destruction s nerves (Hasegawa 1971) with the axon reflex on of neurogenic origin found to be markedly ansco et al. 1983), consistent with a loss of ction. The continuing pain following herpes tion is therefore most likely comparable to a pain of partial deafferentation.

of its relatively specific effects on le-containing unmyelinated sensory nerve eir polymodal receptors, capsaicin may have ic effect in chronic pain states involving ociceptive pathways. Bernstein et al. (1986) he first preliminary report on the effects of saicin in alleviating pain in 14 patients with neuralgia. A similar larger study by Watson in 33 patients confirmed Bernstein's findings in relief in about 65% of patients. Westerman attempted to quantify the degree of desensi- ieved after topical application of capsaicin in f 0.05% nonylvanillamide 3 times daily to matomes of postherpetic neuralgia patients ng thermal perception threshold increase over atment period. Warm and cold sensations are unmyelinated (C-) and thinly myelinated (A- ry fibres, respectively; the former contain the les substance P and calcitonin gene-related bbins et al. 1987) and so should be desensi- reater extent by repeated topical capsaicin. ely, the degree of pain relief assessed by a visual rating scale did not correlate significantly with the degree of desensitisation achieved by topical capsaicin therapy. However, the use of a microprocessor-controlled thermal threshold measuring device Medelec TTT (Westerman et al. 1988) did help in the optimum selection of patients with postherpetic neuralgia most likely to respond to topical capsaicin and provided pointers for future research. Patients with more elevated thermal thresholds (initial desensitisation) before capsaicin treatment, who presumably had more extensive viral damage to nociceptor sensory pathways, achieved less pain relief with topical capsaicin. There was a non-significant trend for greater pain relief in patients who demonstrated greater thermal desensitisation after 1 month's capsaicin treatment. However, the overall rate of useful pain relief (30% or better) is only about 65% in the 3 preliminary trials published to date (Bernstein et al. 1986; Watson et al. 1988; Westerman et al. 1988) [table 1].

Many questions concerning the use of capsaicin in postherpetic neuralgia remain unanswered. Although capsaicin concentrations of 0.025% (Bernstein et al. 1986; Watson et al. 1986) and 0.05%[1] (Westerman et al. 1988) were almost equally effective, the optimum concentration to achieve maximum pain relief in treating postherpetic neuralgia is not yet known; neither is the optimum duration of treatment for which topical capsaicin should be continued. Adverse effects only of an uncomfortable nature have been reported, including local burning and irritation severe enough to cause cessation of treatment in 2 patients; reactivation of the vesicular herpetic eruption usually at the border of treated dermatome and untreated skin, occurring infrequently (2 patients); and inadvertent but painful introduction of capsaicin into conjunctiva, mouth or other mucocutaneous junctions (avoided by using disposable gloves to apply capsaicin cream).

1 Capsaicin is available in Australia as 'Capsig' cream (0.025%) Sigma Pharmaceuticals and as 'Finalgon' cream and ointment (Boehringer Ingelheim) 0.17% and 0.4% nonylvanillylamide, respectively. 'Finalgon' also contains another rubefacient, butoxyethyl nicotinate.

POSSIBLE OTHER TOPICAL USES INCLUDE ARTHRITIS, RHINITIS AND PSORIASIS VULGARIS

challenging patients are 1 in 3 who fail to useful pain relief from topical capsaicin ırgical or radiotherapeutic dorsal rhizotomy temporary pain relief but the postherpetic turns intractably and, not surprisingly, is sponsive to topical capsaicin treatment. l placebo controlled trials using 0.025% and ical capsaicin on postherpetic neuralgia are presently in progress are most timely. xtensive knowledge of the pharmacology of ıd its effects on peptidergic afferents, estions about its pain relieving effect on post-ralgia remain unanswered.

erapeutic Uses

(1988) describes preliminary observations ther possible therapeutic uses of capsaicin y to target tissue with the aim of desensitising ısitive afferents, including treatments for lpaert et al. 1983), rhinitis (Wolf et al. 1987) soriasis vulgaris (Bernstein et al. 1986). In ore recently, capsaicin has been shown to be he treatment of painful diabetic neuropathy ipapa 1989) and the alleviation of chronic following amputation (Rayner et al. 1989). ive specificity of capsaicin's action on fibres suggests that it may prove to have herapeutic uses and be more than just 'hot

es

al. Effects of topically applied capsaicin on moderate and oriasis vulgaris. Journal of the American Academy of logy 15: 504, 1986

TF. The neuropharmacology of capsaicin: review of some servations. Pharmacological Reviews 38(3): 179, 1986

al. The natural history of herpes zoster. Journal of the ı Medical Association 164 (3): 265, 1956

tidromic vasodilatation and neurogenic inflammation. ology and Therapeutics 37: 275, 1988

t al. Effects of capsaicin on inflammation and on the P content of nervous tissue of rats with adjuvant arthritis. nces 32: 1827, 1983

Capsaicin applied to peripheral nerve inhibits axoplasmic of substance P and somatostatin. Brain Research 239: 447,

l. Two immunohistochemically identified populations of ı gene-related peptide (CGRP)-immunoreactive axons in kin. Brain Research 414: 143, 1987

al. Cycloheximide-sensitive synthesis of substance P by dorsal root ganglia. Nature 284: 267, 1980

Hasegawa T. Further electron microscopic observations of herpes zoster virus. Archives of Dermatology 103: 45, 1971

Holzer P. Local effector functions of capsaicin-sensitive sensory nerve endings: involvement of tachykinins, calcitonin gene-related peptide and other neuropeptides. Neuroscience 24(3): 739, 1988

Jansco G, et al. Direct evidence for an axonal site of action of capsaicin. Naunyn-Schmiedebergs Archives of Pharmacology 313: 91, 1980

Jansco G, et al. Impairment of axon reflex vasodilatation after herpes zoster. Clinical and Experimental Dermatology 8: 27, 1983

Kenins P. Responses of single nerve fibres to capsaicin applied to the skin. Neuroscience Letters 29: 83, 1982

Lembeck F. Substance P: from extract to excitement. Acta Physiologica Scandinavica 133: 435, 1988

Molnar J. Die pharmakologischen wirkungen des capsaicins, des scharf schmeckenden wirkstoffes im paprika. Arzneimittel-Forschung 15(7): 718, 1965

Portenoy R. Postherpetic neuralgia: a workable treatment plan. Geriatrics 41 (11): 34, 1986

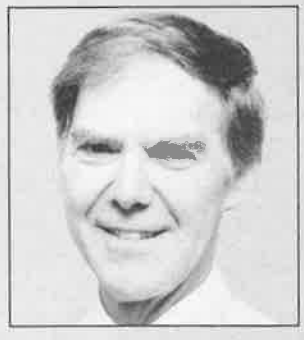
Rayner HC, et al. Relief of local stump pain by capsaicin cream. Lancet 2: 1276, 1989

Ross DR, Varipapa RJ. Treatment of painful diabetic neuropathy with topical capsaicin. New England Journal of Medicine 321 (7); 474, 1989

Watson CPN, et al. Post-herpetic neuralgia and topical capsaicin. Pain 33: 333, 1988

Westerman RA, et al. Effects of topical capsaicin on normal skin and affected dermatomes in herpes zoster. Clinical and Experimental Neurology 25: 71, 1988

Wolf G, et al. Anderungen des nasalen volumstromes nach lokalen Application des Neuropeptides Substanz-P und von Capsaicin. Laryngology Rhinology and Otology 66: 412, 1987



R. G. D. Roberts **R. A. Westerman**

Mr R. G. D Roberts is Lecturer in Medical Biophysics at Swinburne Institute of Technology and is presently completing a PhD in the Department of Physiology, Monash University.

Professor R. A. Westerman is Associate Professor in Physiology, Monash University and Consultant Neurophysiologist at the International Diabetes Institute, Caulfield General Medical Centre.

Author's address: Professor R.A. Westerman, Department of Physiology, Monash University, Clayton, Victoria 3168 (Australia).

Agents Actions 37 (1992)

0065-4299/92/020053-07 $ 1.50 + 0.20/0

22

Effects of capsaicin on cutaneous vasodilator responses in humans

R. G. D. Roberts, R. A. Westerman, R. E. Widdop[1], R. R. Kotzmann[2+] and R. Payne[3]

Department of Physiology, Monash University, Clayton, Victoria, Australia
[1] Department of Clinical Pharmacology, Austin Hospital, Heidelberg, Victoria, Australia
[2] Department of Radiology, Caulfield General Medical Centre, Caulfield, Victoria, Australia and
[3] Department of Physiology, University of Aberdeen, Scotland, U.K.

Abstract

The neurovascular responses to noxious electrically evoked axon reflex (EAR) stimulation and iontophoretic application of endothelium-dependent [acetylcholine (ACh)] and independent [sodium nitrite ($NaNO_2$)] vasodilator substances were examined in human forearm skin using laser Doppler flowmetry, before and after repeated topical applications of the neurotoxin capsaicin (chronic capsaicin pre-treatment). Following 3 or 4 days of capsaicin pre-treatment the normal vasodilator response to acute application of capsaicin was significantly reduced as was the EAR response. There were, however, no significant changes in the vasodilator responses to either ACh or $NaNO_2$, suggesting that the neurogenic axon reflex is in series with the endothelial and microvascular smooth muscle mechanisms of vasodilatation. Recovery of normal EAR occurred within 2–4 weeks of cessation of capsaicin pre-treatment.

Introduction

Elements of the cutaneous neurogenic inflammatory response can be separated into axon reflex vasodilation induced by noxious stimuli (e.g. mechanical, chemical, thermal, or electrical) applied to the skin, releasing vasodilator substances from nociceptor nerve endings [1–4], and more direct effects which can be induced by iontophoresis of vasodilator agents that are endothelial-dependent or -independent in their action [5, 6]. The primary afferent neurotoxin capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) has been used to desensitise nociceptor C-fibre afferents from human skin [7, 8], in order to investigate further their participation in the mechanism of the neurogenic inflammatory response. In the present study, we examine: (i) the time course and nature of neurovascular responses to acute application of varying concentrations of capsaicin, (ii) the correlation between cutaneous blood flux change and temperature and (iii) the recovery of neurovascular responses after several days of chronic capsaicin desensitisation, using non-invasive techniques. Some of these results have been presented in preliminary form [9, 10].

[+] Deceased 3 Jan. 1991.

Methods and materials

Subjects

Eighteen normal healthy Caucasian volunteers, 5 women and 13 men, aged between 19 and 56 years,

were the subjects for the experiments described. No medication had been taken by any of the subjects prior to the test. Informed consent according to the Helsinki II declaration was obtained. The protocol was approved by the Monash University Human Ethics Committee.

Methods

All experiments were carried out in a quiet room at a thermo-neutral temperature of 22–24 °C in which direct air flow was prevented from falling on and affecting the subjects who were comfortably clothed and seated.

In the first series of experiments, changes in skin blood flow (defined as laser Doppler blood flux) were recorded in 9 subjects using a Periflux Pf1d laser Doppler flowmeter (Perimed, Sweden), according to the technique described previously [4, 6]. In brief, normal cutaneous dilator responses to our standard noxious electrically evoked axon reflex stimulation of 150 V, 2 Hz, 0.75 ms duration (EAR) were obtained from the volar surface of both the left (L) and right (R) forearm of each subject. The intensity of the resultant flare was monitored adjacent (6–8 mm) to the cathode stimulating electrode by the laser Doppler. In addition, the iontophoresis of 2 mC of 1% acetylcholine chloride (ACh) and 4 mC of 1% sodium nitrite ($NaNO_2$) in 3% methyl cellulose gel was performed [6]. This was achieved by inserting an applicator containing the drug into the laser probe holder. A direct current was then applied to the skin area immediately under the probe, and the resultant change in blood flow was measured by the laser Doppler after its reinsertion into the holder. Vasodilator responses evoked by either EAR or drug iontophoresis were recorded on an ICI 2 channel pen recorder and quantified by measuring the area of each response as a V·min integral. This encompassed the total EAR response [4, 6], while 4 min was chosen as the cutoff point for drug responses [6]. Immediately following this assessment of each subject's response to the standard electrical and iontophoretic tests, capsaicin 1% in ethanol was applied to a rectangular area of the volar surface of the L forearm, approximately 30 cm^2, and the blood flow monitored for the next 15–30 min (acute capsaicin treatment). The R forearm served as the contralateral control at the times of testing. Sometimes, immediately preceding the capsaicin application and for the next 15–30 min, a standard electrical stimulus of either 2 or 4 pulses (150 V, 2 Hz, 0.75 ms) was given at regular intervals of 3–4 min. During these procedures the surface temperature of treated skin was monitored simultaneously with blood flux, using either a calibrated Biotherm C-600 infrared emission thermometer or Agema 782/disco thermograph system. These instruments were directed to focus on a small area of treated skin, immediately adjacent to the probe holder, i.e. the laser Doppler measurement site. Skin temperature recorded by the Biotherm unit was read directly from the digital display every 2.5 min or was available as an analogue output on the 2 channel pen recorder. The Agema system was calibrated according to the manufacturer's specifications against a black body and a known 30 °C reference temperature source so that the colour scale could be directly related to surface temperature. Skin temperature was, thus, determined from the analysis of coloured thermograms printed at 2.5 min intervals from the continuous video of infrared emission. The average temperature of the skin viewing area was determined by counting the number of pixels at each temperature (colour) in the area and averaging their combined sum.

A second series of experiments was carried out to examine the characteristics of this acute capsaicin response in more detail. In 7 subjects, standard electrical stimuli of 1, 2 or 4 pulses (150 V, 2 Hz, 0.75 ms) were applied to a previously untreated forearm skin area at regular intervals immediately before and for several minutes following the application of different concentrations of a capsaicin cream preparation (0.001–0.005% capsaicin in an inert sorbolene cream base). Resultant skin blood flux changes were monitored as above with the laser Doppler.

The third experimental investigation involved the assessment of responses to chronic effects of capsaicin. In 6 subjects, reapplication of 1% capsaicin in ethanol to the initially treated L forearm area was continued at approximately 4 h intervals for the next 3 or 4 days (chronic capsaicin pre-treatment) until its application no longer evoked redness or pain [4]. On the day following this desensitisation, the initial test procedures of EAR and drug iontophoresis were repeated. Recovery of function was assessed by repeating these procedures in all subjects at 1 and 2 weeks, and in 2 of these subjects also at 4 weeks, after the conclusion of the chronic capsaicin pre-treatment.

The Mann–Whitney single tailed test was used to

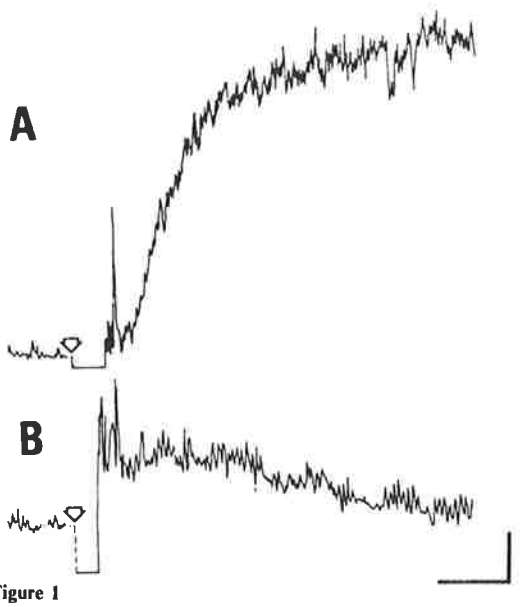


Figure 1
Laser Doppler skin blood flux recorded before and after topical application of 1% capsaicin in ethanol to the left mid-forearm. (A) Naive skin, initial application of capsaicin – broad inverse arrow. (B) Similar acute capsaicin response in the same subject as in (A) after 4 days chronic capsaicin pre-treatment. Time bar = 3 min, flux bar = 0.1 V.

compare groups, before and after the various procedures. A p value of 0.05 was selected as the minimal level of statistical significance.

Results

Typical blood flux responses (expressed in volts) measured after acute topical application of 1% capsaicin in ethanol to normal and chronically capsaicin pre-treated skin of one subject are shown in Fig. 1A and B, respectively. Initial exposure to 1% capsaicin in naive subjects caused a marked vasodilation equivalent to an increase in flux of 0.66 ± 0.22 V (mean $\pm$ SEM, $n = 9$) measured as the maximum (plateau) vasodilatory response, usually achieved after about 20 min exposure to capsaicin. When this procedure was repeated after chronic capsaicin pre-treatment, the peak plateau vasodilatation was significantly reduced by 65% ($p < 0.02$). Parallel changes in skin surface temperature and blood flux as shown in Fig. 2. were observed in all cases where both were measured. After a brief fall in skin temperature due to evaporative cooling of the solvent ethanol, an average increase in skin temperature of 1.87 ± 1.11 °C (mean $\pm$ SEM, $n = 9$), which was significantly different from pre-application resting values ($p < 0.05$), occurred on initial capsaicin

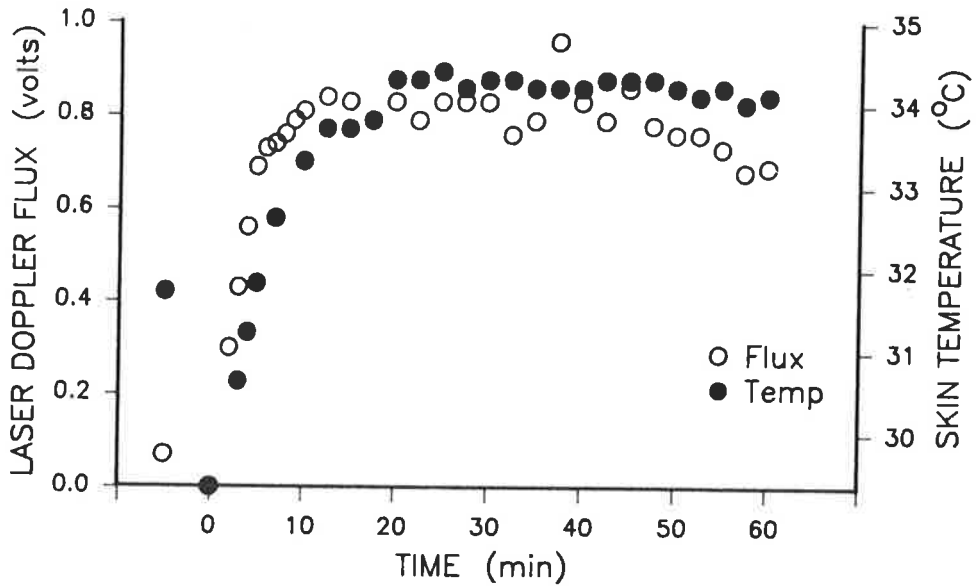


Figure 2
Simultaneous changes in laser Doppler blood flux and skin surface temperature in a single subject, measured by infrared thermography, following acute topical application of capsaicin (1% in ethanol) to normal naive forearm skin. Mean pre-treatment levels indicated by pre-zero data points.

exposure. In contrast, however, the maximal increase in skin temperature following acute application of capsaicin to skin previously desensitised by chronic capsaicin pre-treatment was only 0.64 ± 0.59 °C (mean $\pm$ SEM, $n=6$), which was not significantly different from the resting skin temperature immediately prior to capsaicin application ($p=0.33$).

In the separate experiments, in which flux responses to a standard short train of electrical pulses were measured, before and after the acute application of capsaicin cream of varying concentrations, variable effects were observed. Responses from one subject are shown in Fig. 3, where a capsaicin concentration of 0.001% was not effective in eliciting any change in either resting skin blood flux or the response to EAR, but an increase in concentration to 0.005% produced increases in both. Essentially, at a critical threshold of concentration there occurred a slowly rising resting flux on which was superimposed an increased response to a standard electrical stimulus (Fig. 3C). At concentrations above the threshold, the resting flux rose rapidly, as in the response to 1% capsaicin in alcohol (Fig. 1A), making it difficult to determine precisely the size of the superimposed EAR response during this phase. Results from 9 subjects tested are presented in Fig. 4. On an average a non-significant increase in EAR occurred even at 0.001%, becoming significant at a capsaicin concentration of 0.0025% in an inert sorbolene base ($p<0.05$).

Results from the chronic capsaicin pre-treatment series of experiments are shown in Figs. 5 and 6. In Fig. 5, skin blood flux increases to electrical stimuli applied to the forearm skin area either untreated with capsaicin (control) or chronically capsaicin pre-treated are graphed as V·min integral responses to 1, 2, 4, 8, 16 and 32 pulses. Mean responses in 6 subjects from skin untreated with capsaicin (control) are compared with those from their capsaicin-treated skin, at varying intervals after the completion of the period of chronic capsaicin pre-treatment. In agreement with earlier work [4, 6], responses to electrical stimulation above 2 pulses were significantly reduced after capsaicin pre-treatment ($p<0.05$), but the present experiments indicate that subjects show a progressive recovery within 2–4 weeks to values of 70–80% of, and not significantly different from, control. The equivalent responses to drug iontophoresis on

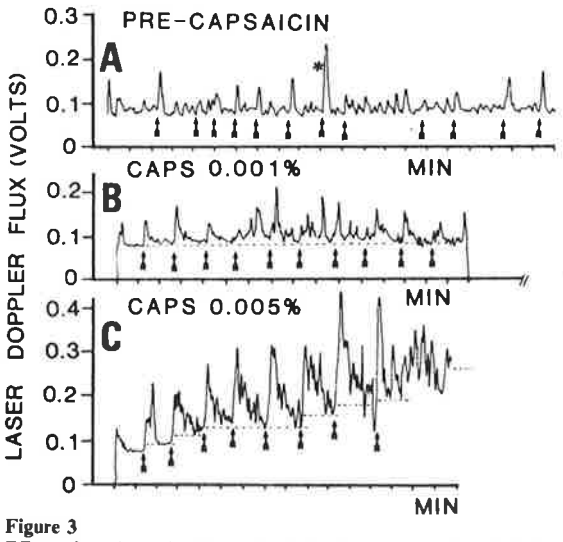


Figure 3
Effect of noxious electrical stimuli in the presence of capsaicin cream. (A) Laser Doppler skin flux responses in normal skin to repeated noxious electrical stimuli of 2 pulses at arrows (150 V, 2 Hz, 0.75 ms) showing typical variability – including response to 4 pulses at asterisk – immediately prior to (B), where similar responses followed the application of 0.001% capsaicin cream producing only increased "resting" activity. In (C), when the dose was increased to 0.005% capsaicin, both an increased response to electrical stimulation and a slow rise in the baseline occurred.

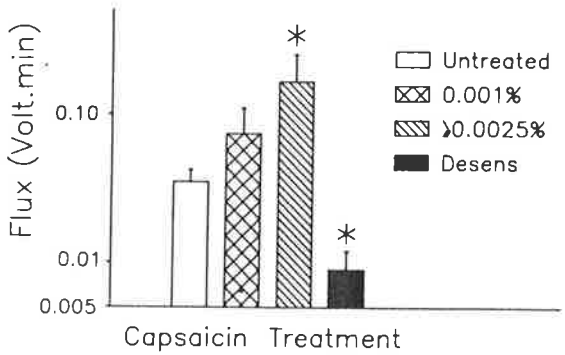


Figure 4
Effect of variable concentration of capsaicin. Open histogram shows EAR flux changes to 2 pulses applied to normal untreated forearm skin. This is compared with similar responses in the period of up to 20 min following acute application of capsaicin – 0.001% and 0.0025%–0.005% (pooled data) – in an inert sorbolene cream base applied on separate occasions at the same site ($n=9$, mean $\pm$SEM). The EAR is significantly increased in skin acutely treated with capsaicin at the higher concentrations. Responses from chronically pre-treated (desensitised) skin are also shown for comparison. Asterisks indicate responses significantly different from normal ($p<0.05$).

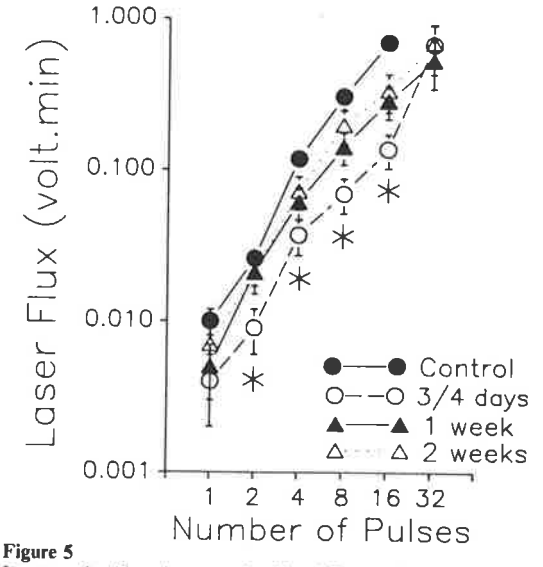


Figure 5
Increases in skin microvascular blood flow, defined as a V·min integral of laser Doppler flux, from forearms of 6 subjects before and following chronic capsaicin pre-treatment, in response to noxious electrical stimuli (150 V, 2 Hz, 0.75 ms) of a graded number of pulses (1–32). Each point on the graph is the mean, and vertical bar the SEM of responses of: normal untreated skin – a pooled average from left (L) pre-capsaicin-treated forearms and right (R) control forearms at the times of testing – (filled circles), skin chronically pre-treated with capsaicin for 3–4 days (open circles), and capsaicin-treated skin one (filled triangles) and two (open triangles) weeks after the cessation of chronic capsaicin pre-treatment. An asterisk indicates a value significantly less than the corresponding control value ($p < 0.05$).

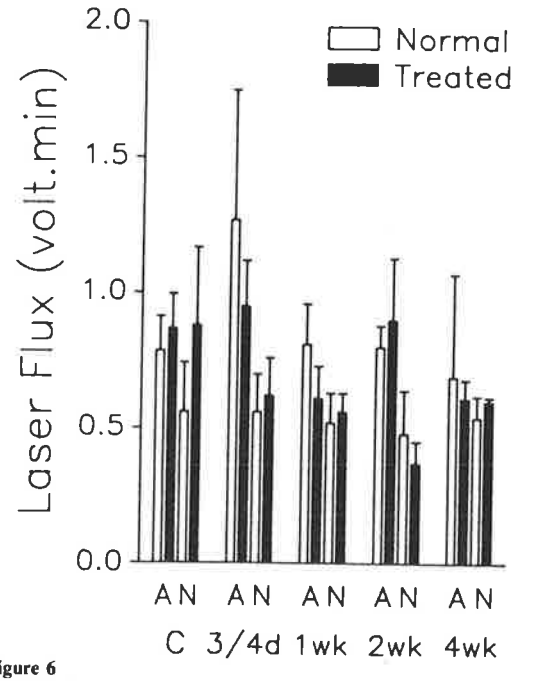


Figure 6
Increases in skin microvascular blood flow, defined as a V·min integral of laser Doppler flux, before and following chronic capsaicin pre-treatment, in response to iontophoresis of 2 mC ACh (A) and 4 mC $NaNO_2$ (N) on control (open bar) and capsaicin-treated (filled bar) forearms of 6 subjects. Each column is the mean, and vertical bar the SEM of responses before chronic capsaicin pre-treatment (C), immediately after 3–4 days of chronic capsaicin pre-treatment (3/4d) and 1, 2 and in two subjects only, 4 weeks after its cessation. No significant differences were observed among any of the ACh responses or any of the $NaNO_2$ responses ($p > 0.05$).

chronic capsaicin pre-treated skin are shown in Fig. 6. The vasodilator responses to ACh, an endothelium-dependent vasodilator, and $NaNO_2$, an endothelial-independent vasodilator [5], are displayed in histogram form. While there was some variation in responses between testing periods, there were no significant differences in vasodilator responses to either ACh or $NaNO_2$ between capsaicin-treated and control forearms at any time following chronic capsaicin pre-treatment ($p > 0.05$).

Discussion

The acute application of capsaicin caused marked vasodilatation as a result of its action to release and deplete potent vasodilator neuropeptides such as substance P (SP) and calcitonin gene-related peptide (CGRP) from susceptible C-fibre primary afferents [11–13]. Increases in skin temperature were temporally associated with these blood flux responses but reflected both intra- and inter-subject variations in factors such as skin thickness and skin test site [14]. The vasodilator responses, both to reapplication of capsaicin and to electrical stimulation, of chronically desensitised skin were markedly depressed compared with their respective controls, in accordance with a reduced supply of vasoactive neuropeptides being available for release from the pre-terminal nerve endings after continuous capsaicin exposure. The fact that some desensitisation was still apparent in the EAR at 2–4 weeks post capsaicin pre-treatment [15] may indicate that the time for recovery is dependent on the restoration of mechanisms such as axonal transport and neuropeptide resynthesis [16, 17]. This present serial measurement of the recovery of cutaneous axon

reflex dilator function is of clinical significance if repeated topical capsaicin application is to be used therapeutically, such as in the treatment of postherpetic neuralgia [18, 19]. Moreover, this ready reversal of capsaicin desensitisation provides support for the continued acceptance of this technique for use in human subjects to investigate cutaneous neurovascular function [16, 20, 21].

Further support for the specificity of action of capsaicin derives from the observations of unaltered sympathetic nerve activity in skin treated with capsaicin. When capsaicin-provoked vasodilator response reached maximum on acute topical application and the skin blood vessels were fully dilated, only unopposed vasoconstriction was observed in response to electrical stimulation. Similarly, there was no difference in resting blood flux between normal and capsaicin-pre-treated (desensitised) skin, indicating that sympathetic vasoconstrictor tone was unaltered by capsaicin. Clearly, these observations suggest that sympathetic vasoconstrictor mechanisms are intact in capsaicin-treated skin, inferring that these sympathetic nerves are not adversely affected by topical capsaicin [16]. An effect of capsaicin on sympathetic vasodilator fibres cannot, however, be discounted by these results [21].

The experiments investigating responses to different concentrations of capsaicin in a cream base applied acutely to capsaicin-naive skin demonstrated a wide intra- and inter-subject variability in vasodilatory response. The results suggest that the acute action of capsaicin on susceptible C-fibres may be concentration-dependent, with an initial effect occurring only when the dose exceeds a certain threshold and the response at higher concentrations being determined by the sensitivities of the various mechanisms by which the vasoactive neuropeptides are released from their sites of storage [3, 11, 16]. This is supported by the observations of capsaicin-induced cutaneous hyperalgesia [6, 14, 15] and subjective reports by the present volunteers describing enhanced discomfort from the same EAR immediately after capsaicin application, presumably as a consequence of increased nociceptive afferent discharge related to increased amounts of neuropeptide being released for a given stimulus.

It is well-known that ACh exerts its vasodilator effect via the endothelium while nitro-vasodilators act directly on vascular smooth muscle [5]. However, it is conceivable that the small current (0.2 mA) used to effect iontophoresis, or the drugs themselves, could stimulate neuropeptide release from primary afferent fibres [22–24]. Previous reports [4, 6] and the present study show that neurogenically mediated vasodilatation by axon reflex mechanisms is markedly reduced in capsaicin-desensitised skin. Accordingly, the activity of the other elements of the vasodilator mechanism – the endothelium and vascular smooth muscle – in capsaicin-desensitised skin were also tested in this study. Since responses to iontophoresed ACh and $NaNO_2$ were unchanged throughout the experimental period (Fig. 6), the indication is that neither the endothelial-dependent mechanisms nor the direct arteriolar smooth muscle relaxing mechanisms of vasodilatation were affected by capsaicin. These findings further suggest that neither of these types of vascular responses requires a primary afferent sensory input when activated by iontophoretic application of appropriate compounds. Similarly, the local vasodilator responses to ACh and $NaNO_2$ have been shown to persist in skin accidentally denervated 15 days previously [25].

Thus, it is proposed that the neurogenic peptidergic component of the Lewis triple response [2] is in series with the endothelial and microvascular smooth muscle mechanisms, and that each of these components of the dilator response may be tested separately and non-invasively by the methods described above. Moreover, capsaicin appears to affect only the peptidergic primary afferent component of the cutaneous vasodilator mechanisms. The advantages of these kinds of tests have recently been demonstrated where dysfunction of primary afferent and/or endothelial mechanisms was observed in diabetic patients with neuropathy [6, 25]. These neurovascular tests, therefore, allow for the early detection of conditions such as diabetic neuropathy, and for the appropriate therapy to be commenced and re-evaluated at regular intervals.

Acknowledgements

This work was supported by the Australian Research Grants Scheme, Apex-Australia Diabetes Foundation and the Rebecca L. Cooper Foundation.

We wish to thank the subjects for their cooperation, B. Carter and P. Kent for technical assistance and J. Hannaford, A. Low and J. Loke for assistance with the figures.

Received 16 May 1991; accepted by H. O. Handwerker
4 February 1992

References

[1] N. Jansco, A. Jansco-Gabor and J. Szolcsanyi, *Direct evidence for neurogenic inflammation and its prevention by*

denervation and by pretreatment with capsaicin. Br. J. Pharmacol. Chemother. *31,* 138–151 (1967).
[2] F. Lembeck, *Sir Thomas Lewis's nocifensor system, histamine and Substance-P containing primary afferent nerves.* Trends Neurosci. *6,* 106–108 (1983).
[3] J. Wallengren and R. Hakanson, *Effects of substance P, neurokinin A and calcitonin gene-related peptide in human skin and their involvement in sensory nerve-mediated responses.* Eur. J. Pharmacol. *143,* 267–273 (1987).
[4] W. Magerl, J. Szolcsanyi, R. A. Westerman and H. O. Handwerker, *Laser Doppler measurements of skin vasodilatation elicited by percutaneous electrical stimulation of nociceptors in humans.* Neurosci. Lett. *82,* 349–354 (1987).
[5] J. A. Angus and T. M. Cocks, *Vasodilatation and the discovery of endothelium-derived relaxing factor.* Med. J. Aust. *146,* 250–253 (1987).
[6] R. A. Westerman, R. E. Widdop, C. Hogan and P. Zimmet, *Non-invasive tests of neurovascular function: Reduced responses in diabetes mellitus.* Neurosci. Lett. *81,* 177–182 (1987).
[7] P. Anand, S. R. Bloom and G. P. McGregor, *Topical capsaicin pretreatment inhibits axon reflex vasodilatation caused by somatostatin and vasoactive intestinal polypeptide in human skin.* Br. J. Pharmacol. *78,* 665–669 (1983).
[8] J. E. Bernstein, R. M. Swift, K. Soltani and A. L. Lorincz, *Inhibition of axon reflex vasodilatation by topically applied capsaicin.* J. Invest. Dermatol. *76,* 394–395 (1981).
[9] R. G. D. Roberts, R. A. Westerman, R. E. Widdop, R. R. Kotzmann and B. Carter, *Effects of capsaicin on cutaneous axon reflex dilatation in man.* Proc. Aust. Physiol. Pharmacol. Soc. *18,* 95P (1987).
[10] R. G. D. Roberts, R. A. Westerman and P. Kent, *Acute neurovascular responses to TENS and capsaicin.* Proc. Aust. Physiol. Pharmacol. Soc. *19,* 184P (1988).
[11] T. M. Jessell, L. L. Iversen and A. C. Cuello, *Capsaicin-induced depletion of substance P from primary sensory neurones.* Brain Res. *152,* 183–188 (1978).
[12] I. L. Gibbins, D. Wattchow and B. Coventry, *Two immunohistochemically identified populations of calcitonin gene-related peptide (CGRP)-immunoreactive axons in human skin.* Brain Res. *414,* 143–148 (1987).
[13] A. Franco-Cereceda, *Calcitonin gene-related peptide and tachykinins in relation to local sensory control of cardiac contractility and coronary vascular tone.* Acta Physiol. Scand. *133* (Suppl. 569), 1–63 (1988).
[14] W. J. Culp, J. Ochoa, M. Cline and R. Dotson, *Heat and mechanical hyperalgesia induced by capsaicin.* Brain *112,* 1317–1331 (1989).
[15] D. A. Simone and J. Ochoa, *Early and late effects of prolonged topical capsaicin on cutaneous sensibility and neurogenic vasodilatation in humans.* Pain *47,* 285–294 (1991).
[16] C. A. Maggi, *Capsaicin and primary afferent neurons: From basic science to human therapy?* J. Auton. Nerv. Syst. *33,* 1–14 (1991).
[17] D. R. Tomlinson and J. H. Mayer, *Defects of axonal transport in diabetes mellitus – a possible contribution to the aetiology of diabetic neuropathy.* J. Auton. Pharmacol. *4,* 59–72 (1984).
[18] J. E. Bernstein, D. R. Bickers, M. V. Dahl and J. Y. Roshal, *Treatment of chronic postherpetic neuralgia with topical capsaicin.* J. Am. Acad. Dermatol. *17,* 93–96 (1987).
[19] R. A. Westerman, R. G. D. Roberts, R. R. Kotzmann, D. A. Westerman, C. Delaney, R. E. Widdop and B. Carter, *Effects of topical capsaicin on normal skin and affected dermatomes in herpes zoster.* Clin. Exp. Neurol. *25,* 71–84 (1989).
[20] B. Lynn, *Capsaicin: Actions on nociceptive C-fibres and therapeutic potential.* Pain *41,* 61–69 (1990).
[21] Y. Kurozawa, Y. Nasu and T. Nose, *Response of capsaicin pretreated skin blood vessels to exercise.* Acta Physiol. Scand. *141,* 181–184 (1991).
[22] L. A. Chahl, *Antidromic vasodilatation and neurogenic inflammation.* Pharmacol. Ther. *37,* 275–300 (1988).
[23] W. W. Douglas and J. M. Ritchie, *The excitatory action of acetylcholine on cutaneous non-myelinated fibres.* J. Physiol. (Lond.) *150,* 501–514 (1960).
[24] N. Parkhouse and P. M. Le Quesne, *Quantitative objective assessment of peripheral nociceptive C fibre function.* J. Neurol. Neurosurg. Psychiat. *51,* 28–34 (1988).
[25] R. A. Westerman, R. E. Widdop, J. Hannaford, C. Hogan, R. Roberts and P. Zimmet, *Non-invasive tests of neurovascular function.* Clin. Exp. Neurol. *24,* 129–137 (1988).

1 to day 6. He is now 4 months old and has remained free of the clinical features of chickenpox or shingles.

Varicella in a woman from 4 days before to 2 days after delivery is dangerous because the baby will lack maternal antibodies. The rash starts 5–10 days after birth and the case fatality rate is high.[1] Passive immunisation with VZIG or ZIG attenuates without preventing the illness. We suggest combination of this treatment with acyclovir since viral replication starts as early as primary inoculation. Little, if any, toxicity is observed. "Contact isolation" should be adopted for mother and infant. This approach may be the safest, until a safe and effective active immunisation, which could then be coupled to passive immunisation, is generally available.

JOSEPH HADDAD
UMBERTO SIMEONI
JEAN MESSER
DANIEL WILLARD

Neonatal Service,
Hôpital de Hautepierre,
67098 Strasbourg, France

1. Enders G. Varicella-zoster virus infection in pregnancy. *Progr Med Virol* 1984; 29: 166–96.

LOWERED SKIN BLOOD FLOW AND ERYTHROCYTE SPHERING IN COLLAPSED FUN-RUNNERS

SIR,—Elite athletes are usually sufficiently well trained and informed to avoid heat-induced illnesses. However, enthusiastic amateurs in endurance events may be so highly motivated as to exercise to the point of collapse which, if not properly treated, can be fatal.[1]

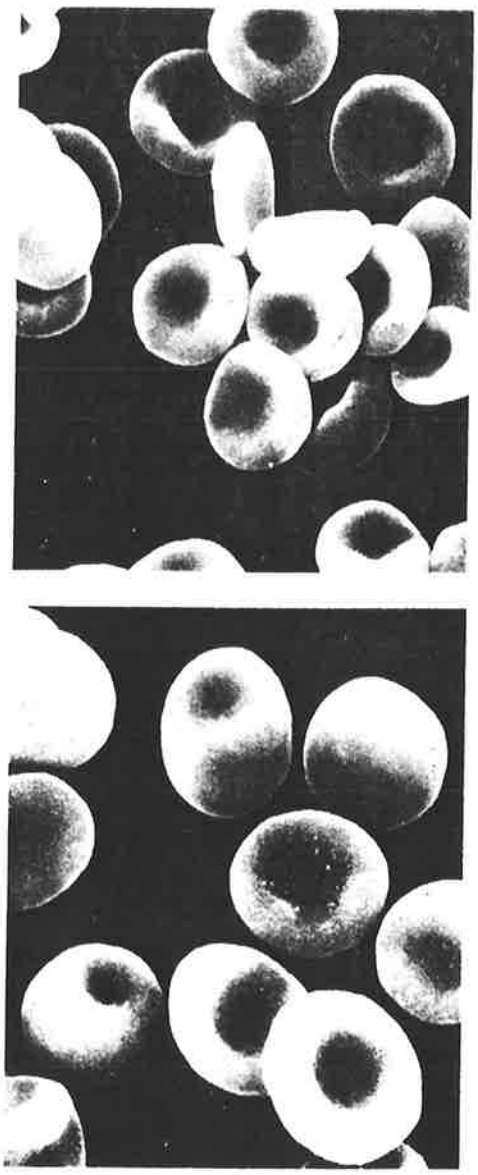
Research in animals and clinical experience[2] strongly implicate circulatory phenomena in heatstroke. One of us has recently proposed[3] that the high skin blood flow necessary to lose body heat during heat stress falls in advanced stages of heat stress. The consequences of very high core temperatures (above about 41·5°C) may be fatal; death seems to be due to diffuse cellular and neurological damage, probably resulting from microthrombi and coagulative necrosis from disseminated intravascular coagulation.[2] Erythrocyte sphering would make this situation worse and has been observed in hyperthermic sheep (unpublished). We describe here a study in collapsed athletes of both skin blood flow and erythrocyte shape.

The Sun City-to-Surf 14 km fun-run was held in Sydney on Aug 4, 1985, between 1000 and 1130 hours in full sunshine, with an average wind speed of 3 m/s, and with ambient dry and wet bulb temperatures of about 12·5°C and 10·0°C, respectively (relative humidity 70%), and a corrected effective temperature of 13·5°C. Runners who collapsed and presented for medical attention and volunteers who completed the race without collapsing were examined 5–15 min after they stopped running. They lay on their sides in rooms at 14–18°C ambient air temperature, which should have been a cool environment for them. Observations were restricted to young adult males.

As an indicator of skin blood flow we measured the tissue perfusion index (TPI) using a Stephens Tissue Perfusion Monitor (Perfusion Diagnostics Australia, Northbridge, Sydney). The method is non-invasive. Time-integrated photoelectric plethysmography is used to sense rhythmic changes in erythrocyte flux in the superficial microvasculature. Measurements were taken on the mid-outer surface of the forearm. Rectal temperature was measured at a depth of 10 cm, using a resistance thermometer calibrated against laboratory thermocouples. A fingerprick blood sample was taken for assessment of erythrocyte shape by scanning electron microscopy.

Of 36 collapsed runners 31 were males aged 19–36 years. 9 had rectal temperatures of 42·0–42·5°C, compared with 38·3–40·3°C in the 20 male volunteers. TPI measured in 7 patients with rectal temperatures of 41·4–42·5°C was 54 ± 14 units (mean ± SE) compared with 117 ± 21 units in the 20 healthy volunteers. The high variability of skin blood flow was probably due to the widely varying physiological states of patients and volunteers. Our data suggest that skin blood flow in patients was about half that in volunteers. In the most severely affected patient core temperature fell from 42·5°C to 37·8°C over 1·6 h while TPI doubled from 30 to 60 units before falling to resting levels. Such a reduction in skin blood flow would greatly restrict the loss of body heat and lead to a further increase in body temperature. Possibly, the increase in skin blood flow in response to thermoregulatory demands during physical exertion becomes sufficiently great to reduce central venous pressure, eliciting reflex cutaneous vasoconstriction via low-pressure baroreceptors.[4]

The most severely affected patient not only exhibited the lowest skin blood flow but also had the highest rectal temperature (42·5°C) and the most conspicuous change in erythrocyte morphology. He was mentally obtunded when admitted and within a few minutes became combative and confused; his skin was faintly moist and cyanosed. In this patient, about 10% of erythrocytes had become spherical (figure). The prevalence of spherical erythrocytes was not estimated in every other patient or volunteer, but such cells were conspicuous in all patients and were found in only one volunteer



Sphering of erythrocytes, conspicuous in all collapsed runners (lower) and not detectable in healthy runners (upper).

Field of view was rotated until dimples on spheres were identified and possible confusion with a direct face view of a disc was thereby avoided.

(rectal temperature 40·0°C, 0·5% spherical erythrocytes). Such cells would increase blood viscosity, which is detrimental to tissue perfusion, and would probably be unable to assume the "parachute" shape usually associated with capillary perfusion. Heatstroke death is thought to be caused by microthrombi and coagulative necrosis; erythrocyte sphering may be an important step in this process.

We thank the many athletes who volunteered to act as controls; H. I. Clements Pty Ltd, Sydney, for the loan of the IVAC thermometers; and Miss S. Westerman for technical assistance.

Commonwealth Scientific and Industrial Research Organisation (CSIRO), Prospect (Sydney), New South Wales, Australia — J. R. S. HALES

Mater Misericordiae Hospital, North Sydney — F. R. N. STEPHENS

CSIRO, Prospect — A. A. FAWCETT

Department of Physiology, Monash University, Clayton, Melbourne, Victoria — R. A. WESTERMAN

CSIRO, Prospect — J. D. VAUGHAN

Department of Medicine, Westmead Medical Centre, Sydney — D. A. B. RICHARDS

Menzies Foundation, North Sydney — C. R. B. RICHARDS

1. To LB. Fatal heat stroke in a "fun run". *Med J Aust* 1980; ii: 104–05.
2. Khogali M, Hales JRS, eds. Heat stroke and temperature regulation. Sydney: Academic Press, 1983.
3. Hales JRS. A case supporting the proposal that cardiac filling pressure is the limiting factor in adjusting to heat stress. *Yale J Biol Med* (in press).

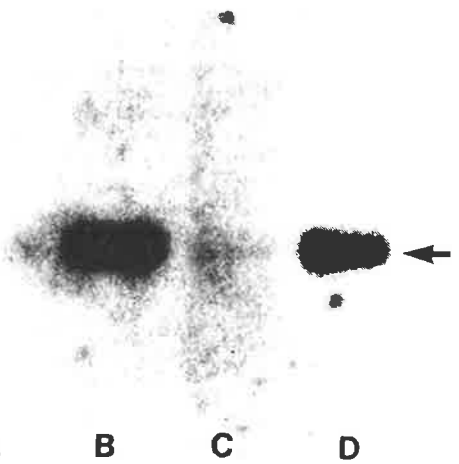
N-*myc* ONCOGENE AMPLIFICATION IN RHABDOMYOSARCOMA AT RELEASE

SIR,—Amplification of the N-*myc* oncogene is a clinically important prognostic variable in neuroblastoma where it strongly correlates with advanced disease and rapid tumour progression.[1,2] N-*myc* amplification has also been reported in other neuroectodermal tumours, including retinoblastoma[3] and astrocytoma.[4] This apparent restriction to tumours of neuroectodermal origin has led to the suggestion[5] that N-*myc* amplification may be a useful marker in the differential diagnosis of those anaplastic small round cell tumours (neuroblastoma, rhabdomyosarcoma, Ewing's sarcoma, and lymphoblastic leukaemia/lymphoma) that are difficult to classify by conventional or immunohistological techniques. This report describes N-*myc* amplification in an embryonal rhabdomyosarcoma.

A 15-month-old boy was admitted, in October, 1982, to the Hospital for Sick Children, London, with a 6-week history of an enlarging palatal mass. This was an embryonal rhabdomyosarcoma, clinical stage III. Radiotherapy and chemotherapy produced a temporary remission but the tumour recurred locally and with pulmonary metastases after 18 months. The original tumour and tissue taken from the palatal recurrence at post mortem exhibited characteristic cytoplasmic cross-striations and expressed desmin[6] on immunohistological staining, features that support the diagnosis of rhabdomyosarcoma and rule out neuroblastoma.

N-*myc* amplification was demonstrated by Southern blotting (figure). DNA was extracted from snap-frozen tumour tissue and digested with *Eco*R1. Restriction fragments were separated by electrophoresis and hybridised with a ^{32}P-labelled N-*myc* probe. Autoradiographs were exposed for 48 hours on preflashed Kodak XAR-5 film with an intensifier screen. The N-*myc* gene copy number was estimated relative to placental DNA single copy intensity and molecular weights were calculated from lambda *Hind*III restriction fragment standards.

To ensure that the amplification seen in the recurrent tumour was not due to a loading error in the total amount of DNA applied to the gel, the filter was washed and rehybridised with the DNA probe L2·30. This probe recognises a 2·2 kb *Eco*R1 fragment present in the human genome as a single copy. There were bands of equal intensity in both the original and the relapse samples (not shown), thus eliminating the possibility of loading error.



Southern blot demonstrating N-*myc* amplification in recurrent rhabdomyosarcoma.

(A) 10 µg human placental DNA; 2 kb band of single copy intensity faintly visible.
(B) 1 µg DNA from neuroblastoma cell line "Kelly" which has 100-fold amplification of N-*myc* gene;[7] intense 2 kb band represents 10 copies of N-*myc* per haploid genome.
(C) 10 µg DNA from first biopsy specimen; faint 2 kb band of single copy intensity.
(D) 10 µg DNA from recurrent tumour; intense 2 kb band representing 10-fold amplification.
Arrow = 2·0 kb.

The increase in N-*myc* gene copy number during the disease in this patient supports the hypothesis[1] that N-*myc* amplification is concerned primarily with progression of the malignant phenotype rather than with the initiation of neoplasia. The possibility that the amplification was related to the cytotoxic therapy cannot be definitely excluded but we have not seen such amplification in several other rhabdomyosarcoma patients treated with the same drugs.

This report demonstrates N-*myc* amplification in a tumour of mesodermal origin and suggests the need for caution in using such data for differential diagnosis. Nevertheless, the striking correlation between amplification of N-*myc* and rapid tumour progression will ensure that it remains, in the context of neuroblastoma at least, a clinically important prognostic variable.

We thank Dr P. Rabbits, Dr N. Spurr, and Dr F. Gilbert for donating reagents. Clinical and pathological materials were kindly supplied by Dr J. Pritchard and Dr J. Pincott. This work was supported by the Imperial Cancer Research Fund and the Child Health Research Appeal Trust.

ICRF Oncology Laboratory, Institute of Child Health, London WC1N 1EH — J. A. GARSON, J. CLAYTON, P. MCINTYRE, J. T. KEMSHEAD

1. Seeger RC, Brodeur GM, Sather H, et al. Association of multiple copies of the N-*myc* oncogene with rapid progression of neuroblastomas. *N Engl J Med* 1985; **313**: 1111–16.
2. Brodeur GM, Seeger RC, Schwab M, Varmus HE, Bishop JM. Amplification of N-*myc* in untreated human neuroblastomas correlates with advanced disease stage. *Science* 1984; **224**: 1121–24.
3. Lee WH, Murphee AL, Benedict WF. Expression and amplification of the N-*myc* gene in primary retinoblastoma. *Nature* 1984; **309**: 458–60.
4. Garson JA, McIntyre PG, Kemshead JT. N-*myc* amplification in malignant astrocytoma. *Lancet* 1985; ii: 718–19.
5. Schwab M, Ellison J, Busch M, et al. Enhanced expression of the human gene N-myc consequent to amplification of DNA may contribute to malignant progression of neuroblastoma. *Proc Natl Acad Sci USA* 1984; **81**: 4940–44.
6. Altmannsberger M, Weber K, Droste R, Osborne M. Desmin is a specific marker for rhabdomyosarcomas of human and rat origin. *Am J Pathol* 1985; **118**: 85–95.
7. Schwab M, Alitalo K, Klempnauer KH, et al. Amplified DNA with limited homology to *myc* cellular oncogene is shared by human neuroblastoma cell lines and neuroblastoma tumour. *Nature* 1983; **305**: 245–48.

Clinical and Experimental Pharmacology and Physiology (1989) **16**, 403–415

OBSERVATIONS ON A NEW NON-INVASIVE MONITOR OF SKIN BLOOD FLOW

J. R. S. Hales, F. R. N. Stephens, A. A. Fawcett, K. Daniel, J. Sheahan, R. A. Westerman and S. B. James

Commonwealth Scientific and Industrial Research Organisation, Ian Clunies Ross Animal Research Laboratory, Prospect (Sydney), New South Wales, Australia

(Received 10 August 1988; revision received 13 January 1989)

SUMMARY

1. A 'tissue perfusion monitor' (TPM) to non-invasively provide an index of skin blood flow (SkBF) has been developed; it employs photoelectric plethysmographic principles to measure changes in the nett flux of red blood cells in superficial microvasculature.

2. The 'tissue perfusion index' (TPI) varies in proportion to SkBF, provided local haemoglobin concentration does not change significantly. TPI of humans and experimental animals has been shown to indicate reliably, well established phenomena such as decreased SkBF in response to mechanical restriction, cold or Valsalva's manoeuvre, or increased SkBF in response to heat, acetylcholine, sodium nitrite or local nerve blockade.

3. SkBF in sheep was varied between 1 and 156 mL/100g per min as measured with radioactive microspheres. Simultaneous measurements were made using the TPM and four laser-Doppler instruments. The TPI yielded a correlation coefficient of 0.938, and when data were expressed as percentage change, the regression line did not differ significantly from the line of identity and the root-mean-square-error was 6.2%. Data for the laser-Doppler indices of SkBF were, respectively, 0.549–0.786, highly significant deviations in slopes, and 13.6–16.7%.

4. Thus, the TPI is a reliable index of changes in SkBF. Compared with some other available instruments, the TPM is more precise; it is also less sensitive to movement artefact, can be completely portable by battery operation, probes can be multiplexed to a single meter and it is likely to be much less expensive than current lasers.

5. Applications include, for example, experimental investigations of SkBF in man and animals, clinical uses such as evaluation of the efficacy of regional nerve blockade or of circulatory restitution after reconstructive surgery, and clinical tests of neurovascular function.

Key words: blood flow measurement, laser-Doppler, microspheres, plethysmography, reconstructive surgery, skin.

Correspondence: Dr J. R. S. Hales, CSIRO, P.O. Box 239, Blacktown, NSW 2148, Australia.

INTRODUCTION

Changes in gross cardiovascular parameters such as heart rate and blood pressure may be so small as to be clinically or even experimentally undetectable, and therefore it can be critically important to assess activity at the effector site of the cardiovascular system, that is, the highly sensitive microcirculation.

The very early physicians relied on subjective indices of changes in skin blood flow (SkBF), namely, visualization of changes in skin colour or feeling for a temperature change. Since the 1930s, photoelectric plethysmography has been employed with limited success (Hertzman 1937a,b,1938), and since at least the late 1940s, more quantitative studies have involved highly sophisticated techniques necessitating considerable skill, such as venous occlusion plethysmography, local heat dissipation, or washout of radioactive inert gases (Ludbrook 1981; Lassen *et al.* 1983). Approximately 16 years ago a completely non-invasive technique employing Doppler shifts in laser light to detect the velocity of movement of red blood cells in superficial microvasculature was first reported (Riva *et al.* 1972), and approximately 7 years ago the first commercial instrument based on this principle became available (the Periflux by Perimed KB, Sweden). Since that time at least three laser-Doppler-based 'blood flow monitors' have been manufactured (the LD 5000 by Medpacific Corp., the LaserFlo by TSI Inc. and a prototype made in Japan). In all cases the wave frequency of emitted light is shifted, according to the Doppler principle, by erythrocytes moving in any direction; positive and negative effects result in the parameter sensed being the nett speed of erythrocytes which is broadly proportional to blood flow provided local haematocrit remains insignificantly changed. We have developed an instrument based on photoelectric plethysmography and report here our observations on its validity in providing an index of changes in SkBF.

METHODS

Subjects

Observations on humans were made on 30–45 year old healthy males with the approval of the Monash University Standing Committee on Ethics in Human Experimentation. Animal experiments were performed on five Merino sheep, two trained to lie quietly in ventral recumbency, while conscious and minimally restrained by cords over the shoulders and hips, and three trained to stand quietly held by a yoke in a climatic room; relevant protocols were approved by the Animal Care and Experimentation Ethics Committee, CSIRO, Prospect.

Tissue perfusion monitor

The tissue perfusion monitor (TPM) is based on photoelectric plethysmographic principles, that is, the reflection of light by a tissue varies with the quantity of red blood cells present. Classically, photoelectric plethysmography involves measurement of the total transmission through or reflection from a tissue, of light from a nearby, localized source (Hertzman 1937a,b). The TPM is unique in that only the pulsatile component of reflected infrared light is processed over and above a non-pulsatile level of reflection characteristic of the non-perfused tissue. That is, a d.c. component is ignored while an a.c. signal is processed as the pulse rate multiplied by the height of the curve generated by the flux of red cells into and out of the microvasculature; this quantity is defined as the 'tissue perfusion index' (TPI). Two light-emitting diodes (LED) provide the light, and the sensor is a phototransistor, all mounted in a probe module which can be encapsulated in a range of probes such as for placement on the skin surface (Fig. 1) or against

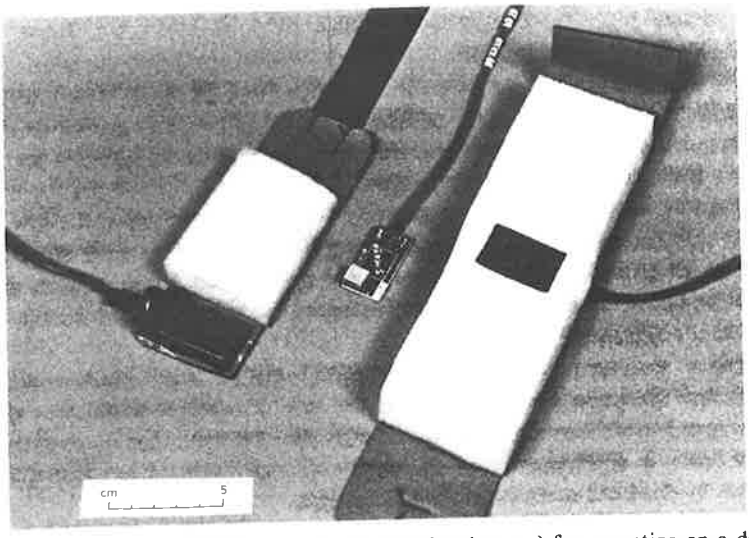


Fig. 1. The two commonly used TPM probes (with associated wraps) for mounting on a digit (left) or a relatively flat surface such as the forearm (right). A common module of electrical components (centre) is clipped into each.

intestinal mucosa. The volume of tissue observed may be widely varied for particular applications and in the current study approximated an elliptical area 10×3 mm to an undefined depth.

Figure 2 illustrates the basic principles of a commercial prototype of the TPM. Thus, the excitated probe (1) feeds to an operational amplifier (2). The 'raw' signal can at this point be recorded for analysis of the characteristics of the pulsatile microvascular perfusion curve. The output of the amplifier passes in two directions, to a band pass filter (3) which has been adapted to remove unwanted artefact from the microvascular 'perfusion' input signal. A Schmitt trigger circuit (4) is fed to a monostable multivibrator circuit (5) which regularizes the trigger signals. The occurrence of regularized trigger signals is averaged in the integrator (6) and fed into a voltmeter (7) calibrated to read beats per minute (heart rate) which is also recordable. The circuit section '5–6' also connects (via a flashing LED heart rate indicator) with a multiplier circuit (10) which electronically multiplies the output of the monostable multivibrator (5) with the pulse amplitude averager (9) to supply an integrator (12) but being modified for TPI scale calibration as desired, by

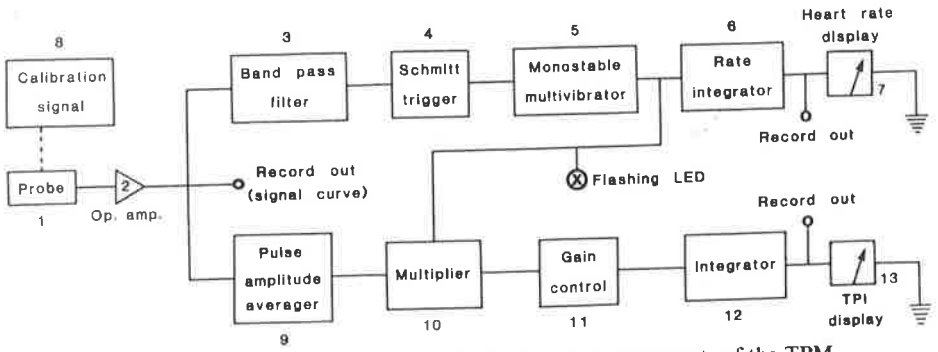


Fig. 2. Schematic representation of the fundamental components of the TPM.

the TPI gain control (11). The integrator (12) drives the TPI display which can be digital or analogue and also has a recorder outlet.

The probe modules are factory tuned by adjusting the LED excitation current to yield a 'standard' (suitable, for example, for the normal human digit) or 'high' probe sensitivity which has to date been set for convenience at three times standard sensitivity (suitable, for example, for normal human limb or torso, or for sheep skin). The entire equipment (probe and meter) is standardized against an *in vitro* reference signal (No. 8 in Fig. 2) by adjusting the TPI gain control. This reference consists of an oscillating voltage supplied to the probe LED while the probe is mounted on an empirically selected, reflective red surface; the meter reading so generated (usually set at mid-scale for convenience) approximates the TPI of healthy human digital pulp skin in a thermally comfortable environment.

Each probe is mounted in a 12 mm thick sponge rubber wrap which is compressed to approximately half thickness on application to the skin. Too much application pressure will mechanically hinder local blood flow, whereas too little pressure may lead to artefacts due to, for example, variations in configuration of the probe–skin interface, direct illumination of the phototransistor by the LED or entry of extraneous light.

In the quantitative comparisons with microsphere measurements, the millivolt outputs of the TPM and Periflux models were continuously recorded on a physiological recorder (Devices M19) and a strip chart recorder (ICI, DP600), respectively. Neotrace 200 ZEF, Grass (Model 79D) or Elmeasco (Model 485) recorders were used for the 'trend' recordings which are illustrated.

Laser-Doppler instruments used

Three commercially available instruments and one prototype rely on laser-Doppler velocimetry to obtain an index of SkBF. The former three, that is, the Periflux (Model Pf 1d and 2B, Perimed, Sweden) and the LaserFlo (Model BPM 403, TSI Inc., USA) have become widely used, and we were fortunate in having a Japanese-made prototype available to us. These were all used according to the manufacturers' instructions. The probes of the Periflux models and Japanese prototype employed the identical plastic Periflux holders attached to the skin by double-sided adhesive rings. The probe of the LaserFlo was held against the skin by a reproducible pressure achieved by elastic straps left in place for the duration of the entire experimental period.

Radioactive microsphere measurements

The sheep had chronic catheters in a femoral artery for 'reference organ' blood sampling, and in the left ventricle for dose injection. The microspheres used were nominally 15 μm in diameter, labelled with ^{141}Ce, ^{51}Cr, ^{113}Sn, ^{103}Ru, ^{95}Nb and ^{46}Sc (New England Nuclear Co., USA); approximately 0.3×10^6 per kg bodyweight was injected per dose and the reference sample was withdrawn at approximately 20 mL/min. On completion of the period of observations, the sheep was killed by an overdose of barbiturate anaesthetic, full depth skin samples were dissected from under each probe site, and the radioactivity assayed and blood flows calculated. All of these procedures have been previously described in detail (Hales 1974; King & Hales 1982).

Experimental protocols

Demonstrations of established blood flow responses (i) To demonstrate that TPI decreases when SkBF is known to decrease, after the establishment of a 'control' level of TPI for a finger or

forearm, three treatments were separately applied, namely, application of a tourniquet to the upper arm, application of iced water to the contralateral arm, and performance of Valsalva's manoeuvre. (ii) To demonstrate that TPI increases when SkBF is known to increase, sodium nitrite and acetylcholine were separately applied iontophoretically to the human forearm, and in three conscious sheep, TPI and microsphere measurements were made in a thermoneutral and again in a hot environment.

Validation by comparison with the established microsphere technique TPI and the outputs of the three laser-Doppler instruments were monitored from 12 sites on the midsides of each of two restrained conscious sheep. Throughout the period of observations, on four sites, four TPM probes remained unmoved; on a different four sites, the plastic mounts remained unmoved and the Periflux and Japanese Prototype probes were rotated between sites to obtain three readings for each position, spanning the time of microsphere dose injection; on the third group of four sites, the elastic straps remained in place and the LaserFlo probe was rotated between sites to obtain readings at times comparable to the other laser-Doppler instruments. Epidermal growth factor, which is known to be an exceptionally potent stimulus to increase skin capillary blood flow (Carter *et al.* 1988), was infused (5.4 μg/kg per h, i.v.) for 3.5 h. The six doses of microspheres were injected when progressive increases in SkBF were indicated by one or more of the monitors. These data were treated in two ways. First, the regression equation and correlation coefficient were calculated for TPI versus blood flow rate. Second, to enable comparisons of all instruments with the microsphere-measured blood flow rate, all data were expressed in a common unit, namely, percentage of maximum level. Standard statistical analyses were applied (Snedecor & Cochran 1980), that is, the correlation coefficient, significance of the difference between each correlation coefficient and that for the TPM, regression equation, and significance of difference in slope of the regression line from unity (by standard *t*-test). Further, to provide a direct estimate of the mean difference between the blood flow measurements obtained by each instrument and what was taken to be the true values for blood flow (obtained using microspheres), the

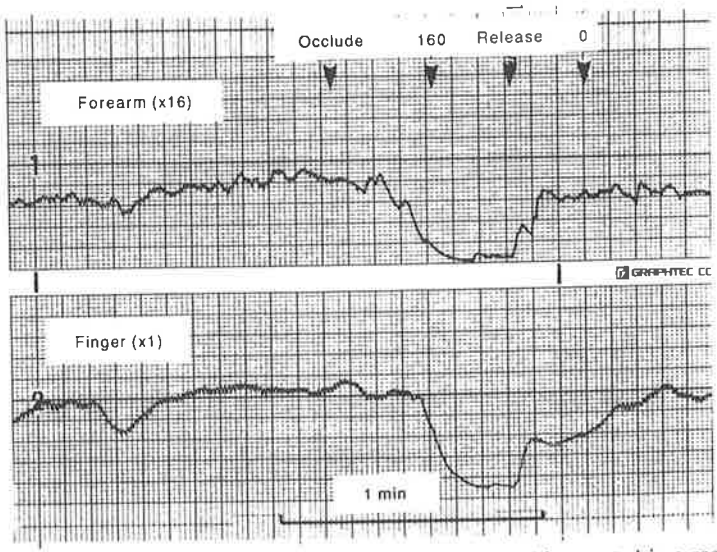


Fig. 3. TPI of the forearm and an index finger of a healthy human subject seated in a room with an air temperature of approximately 25°C. The marked falls in TPI were induced using a sphygmomanometer inflated to the pressures indicated (mmHg). Values in parentheses denote amplification relative to 'normal' finger perfusion. Time constant = 500 ms, full scale = 1 V.

'root-mean-square-error' was calculated, that is, the square root of the mean value for the squar of the difference between the instrument and microsphere measurement of blood flow.

RESULTS

Demonstrations of established blood flow responses

The expected decreases in SkBF were clearly demonstrated in human subjects in response to a tourniquet (Fig. 3), to contralateral limb cooling (Fig. 4) and to Valsalva's manoeuvre (Fig. 4).

The expected increases in SkBF were clearly demonstrated using locally applied sodium nitrite or acetylcholine in humans (Fig. 5), and by exposure of sheep to a hot environment. In the latter, simultaneous measurements were made with microspheres, and there was an increase in TPI of magnitude comparable with that of the microsphere values in seven of eight measurements (Table 1).

Comparisons of the TPM and three laser-Doppler instruments with microsphere measurements

In Fig. 6, TPI readings are plotted against absolute values for blood flow ranging from 1 to 156 mL/100 g per min. The regression equation is $y=0.961x+3.313$ and correlation coefficient = 0.939.

Figures 7–10 present, respectively, the data expressed as per cent of maximum response, for the TPM, Periflux, LaserFlo and Japanese prototype. The statistical analyses for these

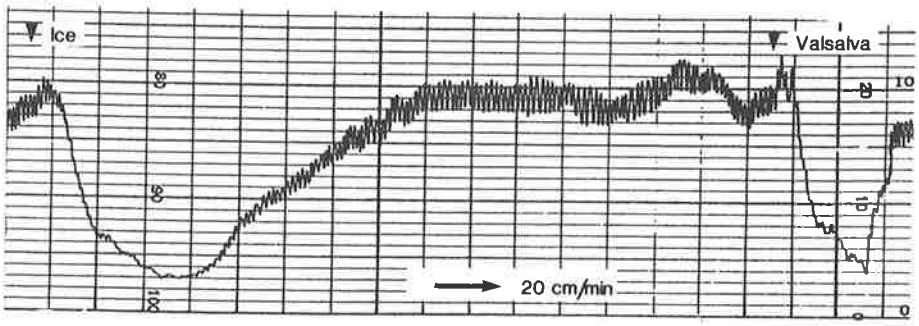


Fig. 4. Original recording of the TPI of an index finger of a healthy human subject seated in a room with an air temperature of approximately 23°C. Note the fall in TPI within a few seconds of applying ice to the contralateral arm or of commencing Valsalva's manoeuvre. Instantaneous read-out.

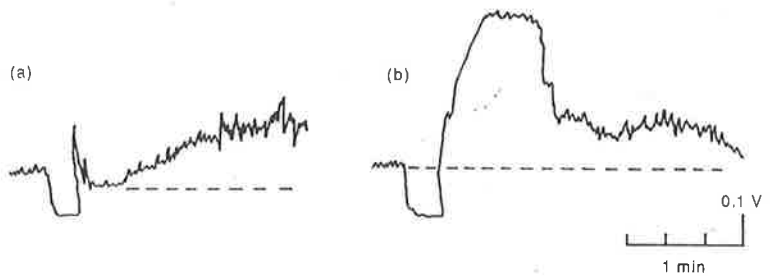


Fig. 5. Changes in skin blood flow measured as TPI when (a) sodium nitrite (dose = 4 mC) and (b) acetylcholine (dose = 2 mC) are applied iontophoretically to the human forearm.

Table 1. Skin blood flow rate (mL/100 g per min) and TPI (mV) of three conscious sheep standing in a thermoneutral (TN) environment and then after ambient temperature had been raised to 44°C (Heat)

Animal	Blood flow		TPI	
	TN	Heat	TN	Heat
1	1.2	4.2	13	25
	5.6	6.1	20	20
	4.4	11.4	18	33
2	15.7	16.8	40	35
	5.6	17.0	7	59
	5.9	24.7	20	71
3	8.6	9.9	17	19
	3.3	13.7	10	30
Mean	6.3	13.0	18	37
s.e.m.	1.5	2.3	4	7

comparisons with microspheres, are given in Table 2. The regression line relating TPI to microspheres was the only one not significantly different from the line of identity, and TPM showed a root-mean-square-error of less than half that of the best of the other three instruments. All instruments yielded statistically significant correlation coefficients, however, that for the TPM versus microspheres was highly significantly different from that for each other instrument versus microspheres.

DISCUSSION

A non-invasive, low skill method for the assessment of SkBF, particularly for clinical use but also for some experimental applications, has been a longstanding need. Also, the most common method used to obtain quantitative measurements of human SkBF, namely, venous occlusion plethysmography, may be used for only (i) discrete rather than continuous measurements, (ii) relatively large tissue masses, and (iii) is applicable only to limbs under conditions in which

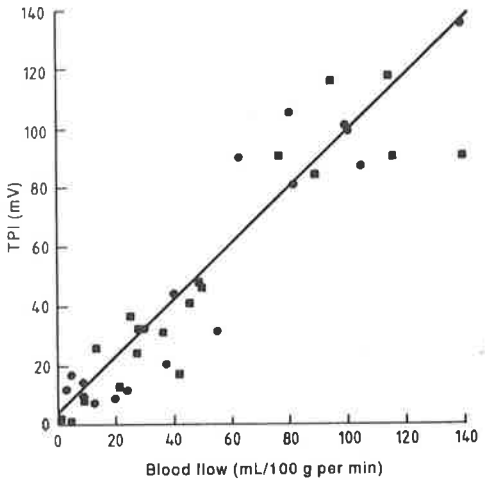


Fig. 6. Skin TPI of four sites with four probes in each of two conscious sheep (■, ●). SkBF was quantitatively measured using microspheres while epidermal growth factor was infused (i.v.) to elicit the demonstrated increases in SkBF.

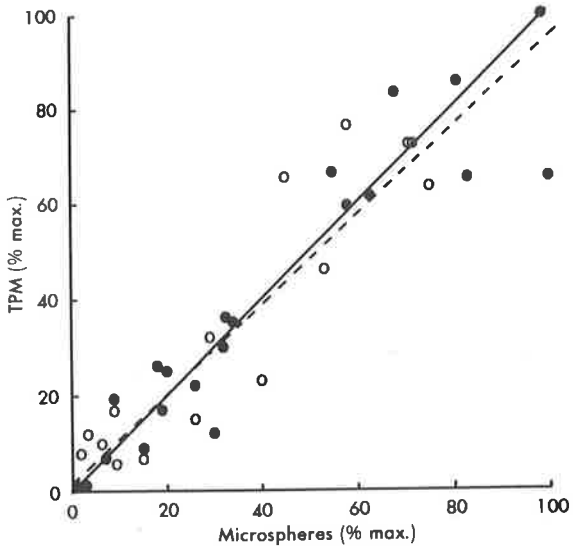


Fig. 7. The same data as in Fig. 6 plotted using common units for the two methods of measuring tissue perfusion (TPM and microspheres), namely, the percentage of their maximum values. Solid line represents the line of identity and the dotted line represents the regression line. The two symbols denote data from two sheep.

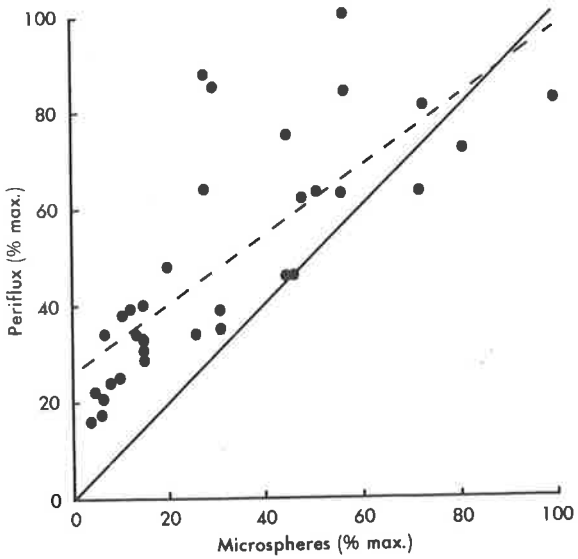


Fig. 8. Skin perfusion in a sheep as a percentage of the maximum levels indicated by the Periflux laser-Doppler compared with microspheres. The solid line represents the line of identity and the dotted line represents the regression line.

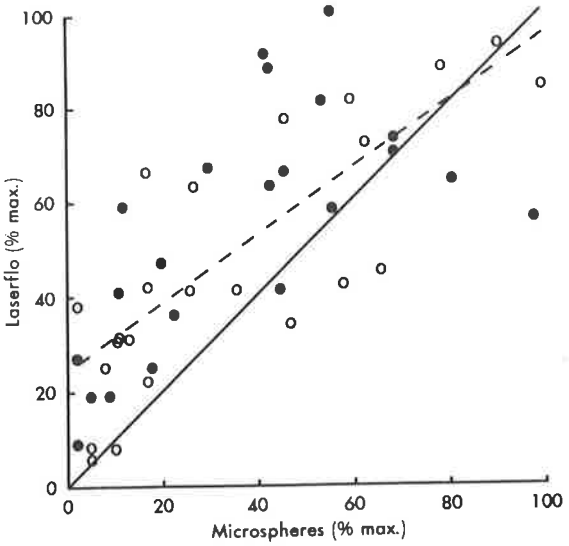


Fig. 9. Skin perfusion in two sheep (●, ○) as a percentage of the maximum levels indicated by the LaserFlo compared with microspheres. The solid line represents the line of identity and the dotted line represents the regression line.

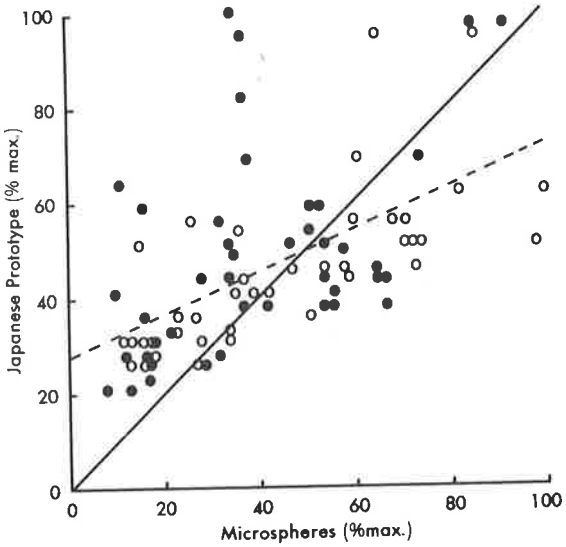


Fig. 10. Skin perfusion in two sheep (●, ○) as a percentage of the maximum levels indicated by the Japanese prototype compared with microspheres. The solid line represents the line of identity and the dotted line represents the regression line.

Table 2. Simultaneous comparisons between the percentage change in skin blood flow as indicated by five instrum..nts and radioactive microsphere measurements. Observations in two conscious, restrained sheep with blood flows of 1–156 mL/100 g per min stimulated by i.v. epidermal growth factor

	TPM	Perifluxes	LaserFlo	'Japan Prot.'
Regression equation	$y=0.925x+1.875$	$0.710x+26.0$	$0.702x+24.3$	$0.441x+27.6$
Correlation coefficient (r)	0.938	0.786	0.742	0.549
P for r difference*	—	<0.01	<0.001	<0.001
P for slope deviation†	>0.05	<0.05	<0.01	<0.001
Root-mean-square-error	6.2	15.7	16.7	13.6
Number of observations	38	31	44	82

*Significance of difference between r for TPM and each other instrument.
†Significance of deviation of slope of regression line from line of identity.

muscle blood flow remains unchanged (Detry *et al.* 1972; Ludbrook 1981). Photoelectric plethysmography is applicable to the skin of all body regions (Hertzman 1937a,b; Hertzman *et al* 1947; Hertzman & Randall 1948) but attempts at its use in a quantified fashion have met with limited success (Hertzman *et al.* 1946; Bini *et al.* 1980). The relatively recent introduction o several instruments based on laser-Doppler principles purportedly largely solved the abov problems (Westerman *et al.* 1988a). However, the available instruments might not only b regarded as quite expensive, but in each case the fibre-optic lead to the probe is sensitive t movement, and publications to date indicate that their overall precision as an indicator of SkBF i questionable.

In advocating the application of this new instrument, there are two fundamental questions t be answered. First, does the TPM respond in a predictable manner when 'textbook' responses i SkBF are evoked? This has been clearly demonstrated in two ways. (i) The marked decreases i TPI with limb occlusion or as a reflex response to either a remote cold stimulus or t compromised venous return induced by Valsalva's manoeuvre. It is worth noting that with th first of these procedures, that is, mechanical stoppage of the complete blood supply, TPI fell to level which differed insignificantly from zero; this contrasts with studies of laser-Dopple instruments under comparable conditions, which have invariably yielded a significantly positiv and quite variable perfusion value at zero SkBF (Engelhart & Kristensen 1983; Johnson *et a* 1984; Saumet *et al.* 1986). Presumably, this is due to the fact that laser-Doppler instrumen measure blood volume flow only as a derived function of the speed of movement of th erythrocytes and no distinction is made between erythrocytes and other moving structures with the field, for example, collagenous materials making up tissue elements. This means that, f example, laser-Doppler estimates of heat-induced increases in SkBF would be unreliable, as a accurate knowledge of instrument output at zero SkBF is necessary. The TPM does not suffer th shortcoming, and in fact, the following supports this point. (ii) The increases in TPI on exposu of sheep to a warm environment accurately reflected the quantitative changes in SkBF measur by microspheres in seven of eight comparisons (Table 1). The good correlation between the tw methods is even more surprising when it is recognized that SkBF is such a labile parameter a each microsphere measurement takes place only over a period of time of no more than 'a f seconds'; TPI values corresponding to these times were read from the continuous record, where in many comparable applications of the TPM, TPI would normally be averaged over a mu longer period to allow for normal fluctuations in SkBF. Also, the drug-induced increases in T

correspond with the well established enhancement of SkBF by nitrodilators such as sodium nitrite, sodium nitroprusside or nitroglycerine acting directly on the microvascular smooth muscle (Bowman & Rand 1980) or endothelium-dependent-dilators such as acetylcholine and acetyl-β-methacholine (Furchgott 1984).

The second question to be answered is whether skin TPI is a precise indicator of SkBF. As radioactive microsphere measurements of tissue blood flow rely on simple physical principles, the technique has become widely accepted as a standard against which other methods may be compared (Hales 1974; Warren & Ledingham 1974; Heymann *et al.* 1977); this is particularly so with tissues such as skin for which the anatomy precludes the timed collection of venous outflow or, for example, electromagnetic monitoring of arterial supply. The TPM and four laser-Doppler instruments were therefore compared with microsphere measurements of SkBF.

The provision of a reliable and precise measure of changes in SkBF by the TPM is shown by the high value for the correlation coefficient of the relationship between TPI and microsphere values for SkBF, the significant difference between this and the other correlation coefficients, and the non-significant difference between the regression line and the line of identity. These data are for different probes on different body locations and on different animals. The Periflux also yielded an acceptable correlation, and this has been reported previously for skin when compared with venous-occlusion plethysmography (Johnson *et al.* 1984; Saumet *et al.* 1986). However, as with the other laser-Doppler instruments that we tested on skin, other investigations have yielded much poorer correlations for other tissues, such as renal cortex (Stern *et al.* 1979) and intestinal mucosa (Kvietys *et al.* 1985), when compared with microsphere or hydrogen clearance measurements. Shepherd *et al.* (1987) have reported acceptable correlations between LaserFlo measurements of hepatic or gastric mucosal blood flow, and total blood flow measured by collection of venous outflow or electromagnetically, respectively. However, as with the skin studies by Johnson *et al.* (1984) and Saumet *et al.* (1986) employing the Periflux, the LaserFlo showed highly significant variations in slope of the regressions obtained for different sites or for different occasions on what was supposed to be the same site. Our poor correlation between the LaserFlo and microspheres agrees with current comparisons of the LaserFlo with forearm plethysmography (G. L. Brengelmann & M. Savage, pers. comm.). The favoured explanation offered in the above publications was that the small volume of tissue sensed by the laser-Doppler probe was not representative of the very much larger tissue volume included in the measurement by the other method. It appears therefore, that our failure to experience this shortcoming with the TPM may be at least partly attributable to the much larger tissue volume viewed by the TPM probe.

Smolander and Kolari (1985) did not obtain a linear relationship between the Periflux and venous occlusion plethysmography and suggested that besides the importance of the detected tissue volume, significant influences on the Periflux could come from variable characteristics of blood flow through cutaneous venules and accumulation of sweat. In addition to these factors, the widely different fundamental principles of operation of the TPM and laser-Doppler instruments should render the former more likely than the latter to be able to provide a measure of SkBF. Thus, the laser-Dopplers are said to be measuring velocity of particle movement whereas they in fact are measuring speed (Haumschild 1986). Their algorithms are applied on the assumption that speed is equal to velocity, which of course is true sometimes but is not necessarily so. Thus, 'usable' correlations between Doppler shifts in light wave frequency and blood volume flow would be anticipated but close correlations should not be expected. As our photoelectric plethysmography depends upon variations in light reflection with variations in skin colour due to pulsatile changes in erythrocyte content, a relatively superior correlation with blood volume flow could be expected.

The LaserFlo and Japanese prototype utilize algorithms in microprocessor-based signal analyzers to provide an actual 'flow' readout. However, this is of limited value if the signal is based on speed rather than velocity and the flow being computed is in such a minute volume of tissue as to be unrepresentative of the 'greater' organ in which the experimenter or clinician is interested. The TPM could be regarded as equally quantitative because the signal derived from its *in vitro* calibration is equal to the TPI of digits of healthy humans in a thermally comfortable environment; but this has been empirically determined and therefore, at least for the time being, we would prefer to advocate the use of the TPM only to measure changes in blood flow. Nevertheless, whether the skin being studied is 'relatively vasoconstricted' or 'relatively vasodilated' is clear by comparison with the human digital perfusion defined above. This is a distinct advantage of the TPM over the laser-based instrument which correlated best with microspheres, that is, the Periflux, the latter therefore being useful only for measurements relative to the starting reference level.

It is also worth noting that the fibre-optic lead, which is an essential part of the laser-Doppler system, is very sensitive to movement; thus, its application is limited to co-operative, non-moving subjects or parts of subjects, a shortcoming not experienced with the TPM which employs a highly flexible electrically conductive lead of essentially unlimited length. Two further advantages of the TPM compared with currently available instruments are that it can be battery powered, making it highly portable; and several probes can be very simply multiplexed to the one meter, greatly reducing the cost of recording from several body sites (so long as simultaneous measurements are not essential).

The following applications of the TPM are envisaged. (i) In experimental studies such as to check the common assumption that under many conditions, changes in forearm volume provide a measure of SkBF of the forearm (Tripathi & Nadel 1986), and often that this in turn is representative of the remainder of the body excluding acral regions. The TPM has been used in a study of the pathophysiology of heat/exercise exhaustion in fun-runners (Hales *et al.* 1986). (ii) As a clinical tool, such as to assess the efficacy of regional nerve blocks (J. R. S. Hales, F. R. N. Stephens, W. B. Conolly, J. Stamell, unpubl. data), or monitoring the progress of tissue revascularization and viability following burns and other forms of trauma or after reconstructive surgery; it should be useful in clinical tests of skin small nerve function and microvascular reactivity in conditions such as diabetes where neuropathological signs may be measured in otherwise symptomless patients (Westerman *et al.* 1988b).

ACKNOWLEDGEMENTS

The commercial prototype instruments were manufactured by Cumbrae Electronics P/L Lasers (Sydney) and Scientific Industrial Automation (Canberra). We are grateful to Optek Technology Inc. (Taipei) for supply of the optoelectrical components.

REFERENCES

Bini, G., Hagbarth, K.-E., Hynninen, P. & Wallin, B. G. (1980) Thermoregulatory and rhythm-generating mechanisms governing the sudomotor and vasoconstrictor outflow in human cutaneous nerves. *Journal of Physiology*, **306**, 537–552.

Bowman, W. C. & Rand, M. J. (1980) *Textbook of Pharmacology*, 2nd edn, Blackwell, Oxford.

Carter, N. B., Fawcett, A. A., Hales, J. R. S., Moore, G. P. & Panaretto, B. A. (1988) Circulatory effects of a depilatory dose of epidermal growth factor in sheep. *Journal of Physiology*, **403**, 27–39.

Detry, J-M. R., Brengelmann, G. L., Rowell, L. B. & Wyss, C. (1972) Skin and muscle components of forearm blood flow in directly heated resting man. *Journal of Applied Physiology*, **32**, 506–511.

Engelhart, M. & Kristensen, J. K. (1983) Evaluation of cutaneous blood flow responses by ^{133}Xenon washout and laser-Doppler flowmeter. *Journal of Investigative Dermatology*, **80**, 12–15.

Furchgott, R. F. (1984) The role of endothelium in the responses of vascular smooth muscle to drugs. *Annual Reviews of Pharmacology and Toxicology*, **24**, 175–197.

Hales, J. R. S. (1974) Radioactive microsphere techniques for studies of the circulation. *Clinical and Experimental Pharmacology and Physiology* Suppl. 1, 31–46.

Hales, J. R. S., Stephens, F. R. N., Fawcett, A. A., Westerman, R. A., Vaughan, J. D., Richards, D. A. B. & Richards, C. R. B. (1986) Lowered skin blood flow and erythrocyte sphering in collapsed fun-runners. *Lancet*, **1**, 1494–1495.

Haumschild, D. J. (1986) Microvascular blood flow measurement by laser Doppler flowmetry. *TSI Application Note.*

Hertzman, A. B. (1937a) Photoelectric plethysmography of the nasal septum in man. *Proceedings of the Society for Experimental Biology and Medicine*, 37, 290–292.

Hertzman, A. B. (1937b) Photoelectric plethysmography of the fingers and toes in man. *Proceedings of the Society for Experimental Biology and Medicine*, 37, 529–534.

Hertzman, A. B. (1938) The blood supply of various skin areas as estimated by the photoelectric plethysmograph. *American Journal of Physiology*, **124**, 328–340.

Hertzman, A. B. & Randall, W. C. (1948) Regional differences in the basal and maximal rates of blood flow in the skin. *Journal of Applied Physiology*, **1**, 234-241.

Hertzman, A. B., Randall, W. C. & Jochim, K. E. (1946) The estimation of the cutaneous blood flow with the photoelectric plethysmograph. *American Journal of Physiology*, **145**, 716–726.

Hertzman, A. B., Randall, W. C. & Jochim, K. E. (1947) Relations between cutaneous blood flow and blood content in the finger pad, forearm and forehead. *American Journal of Physiology*, **150**, 122–132.

Heymann, M. A., Payne, B. D., Hoffman, J. I. E. & Rudolph, A. M. (1977) Blood flow measurements with radionuclide-labelled particles. *Progress in Cardiovascular Disease*, **20**, 55–79.

Johnson, J. M., Taylor, W. F., Shepherd, A. P. & Park, M. K. (1984) Laser-Doppler measurement of skin flow: comparison with plethysmography. *Journal of Applied Physiology*, **56**, 798–803.

King, R. B. & Hales, J. R. S. (1982) An automated solid state detector and computer based analysis system for multiple blood flow measurements using radioactive microspheres. *Proceedings of the Australian Physiological and Pharmacological Society*, **13**, 29P.

Kvietys, P. R., Shepherd, A. P. & Granger, D. N. (1985) Laser-Doppler, H_2 clearance, and microsphere estimates of mucosal blood flow. *American Journal of Physiology*, **249**, G221–227.

Lassen, N. A., Henriksen, O. & Sejrsen, P. (1983) Indicator methods for measurement of organ and tissue blood flow. In: *Handbook of Physiology*, Sec. 2, Vol. III, Part 1, pp. 21–63. American Physiological Society, Bethesda.

Ludrook, J. (1981) Limb volume plethysmography as an index of small vessel perfusion. In: *Progress in Microcirculation Research* (Ed. D. Garlick), pp. 388–396. University of New South Wales, Committee in Postgraduate Medical Education, Sydney.

Riva, C., Ross, B. & Benedek, G. B. (1972) Laser Doppler measurements of blood flow in capillary tubes and retinal arteries. *Investigative Ophthalmology*, **11**, 936–944.

Saumet, J. L., Dittmar, A. & Leftheriotis, G. (1986) Non-invasive measurement of skin blood flow: Comparison between plethysmography, laser-Doppler flowmeter and heat thermal clearance method. *International Journal of Microcirculation: Clinical & Experimental*, **5**, 73–83.

Shepherd, A. P., Riedel, G. L., Kiel, J. W., Haumschild, D. J. & Maxwell, L. C. (1987) Evaluation of an infrared laser-Doppler blood flowmeter. *American Journal of Physiology*, **252**, G832–839.

Smolander, J. & Kolari, P. (1985) Laser-Doppler and plethysmographic skin blood flow during exercise and during acute heat stress in the sauna. *European Journal of Applied Physiology*, **54**, 371–377.

Snedecor, G. W. & Cochran, W. G. (1980) *Statistical Methods* (7th edn). The Iowa State University Press, Ames, USA.

Stern, M. D., Bowen, P. D., Parma, R., Osgood, R. W., Bowman, R. L. & Stein, J. H. (1979) Measurement of renal cortical and medullary blood flow by laser-Doppler spectroscopy in the rat. *American Journal of Physiology*, **236**, F80–87.

Tripathi, A. & Nadel, E. R. (1986) Forearm skin and muscle vasoconstriction during lower body negative pressure. *Journal of Applied Physiology*, **60**, 1535–1541.

Warren, D. J. & Ledingham, J. G. G. (1974) Measurement of cardiac output distribution using microspheres. Some practical and theoretical considerations. *Cardiovascular Research*, **8**, 570–581.

Westerman, R. A., Widdop, R. E., Hannaford, J. *et al.* (1988a) Laser Doppler velocimetry in the measurement of neurovascular function. *Australasian Physical and Engineering Sciences in Medicine*, **11**, 53–66.

Westerman, R. A., Widdop, R. E., Low, A., Hannaford, J., Kozak, W. & Zimmet, P. (1988b) Non-invasive tests of neurovascular function: reduced axon reflex responses in diabetes mellitus of man and streptozotocin-induced diabetes of the rat. *Diabetes Research and Clinical Practice*, **5**, 49–54.

Int J Microcirc: Clin Exp **12**: 99–104, 1993.

Technical Note/Short Communication

Evidence for skin microvascular compartmentalization by laser-Doppler and photoplethysmographic techniques

J.R.S. HALES, R.G.D. ROBERTS[1], R.A. WESTERMAN[1], F.R.N. STEPHENS and A.A. FAWCETT[2].

School of Physiology and Pharmacology, University of New South Wales, Sydney, [1]Department of Physiology, Monash University, Melbourne, and [2]CSIRO, Division of Animal Production, Sydney, Australia.

Received 13 August 1992; accepted 25 September 1992

Key words: laser-Doppler, photoplethysmographic techniques, AVA (arteriovenous anastomoses)

Abstract. Indices of perfusion were obtained using commercial laser-Doppler instruments with differing wavelengths on metatarsal skin of sheep (which is abundant with AVAs) and compared with quantitative measurements of AVA and capillary blood flow using microspheres; an infra-red photoplethysmograph was also compared. The data provide physiological evidence that laser-Doppler instruments of longer wavelength sense to a greater depth, and our photoplethysmograph behaves similarly to a "near-infra-red" laser. Thus, we suggest that near-infra-red light, either laser-Doppler or photoplethysmograph, should be used to measure a parameter most representative of total local blood flow, and light of a very much shorter wavelength, probably green light, would allow more confidence in estimates of true capillary perfusion. Because the commonly used visible red light can, but does not necessarily detect deep AVA perfusion, the influence depending upon the magnitude of the AVA perfusion, measurements by an instrument based on such red light is difficult to define.

Introduction

Blood flow in skin serves several purposes, viz., regulation of blood pressure and body temperature, metabolite exchange and assistance with the protective barrier provided by skin. Therefore there are widely varying requirements for perfusion of the nutrient pathways (capillaries) or of the non-nutrient, high volume, low resistance pathways provided by arteriovenous anastomoses (AVAs). Of course, the possible extent of physiological and anatomical variations between capillaries and AVAs also varies markedly with body region and animal species. However, with the exception of some animal studies in which microsphere techniques could be applied [5], it is rare to find attempts to define which of the cutaneous microvascular compartments is being measured.

quantitatively measure capillary blood flow under each individual probe and the total femoral flow through AVAs as previously described [4]. Phentolamine bolus doses of 1–10 mg were injected to elicit marked increases in flow through AVAs with decreases or only small increases in flow through capillaries.

Results and discussion

In metatarsal skin of sheep [9] as with some human regions such as plantar skin [2], the superficial plexus of capillaries lies generally within approximately 1 mm of the skin surface, whereas AVAs are much deeper, generally at 2–3 mm, with some of the venules and arterioles in between. Thus, if the characteristics of equipment designed to monitor microvascular perfusion can be manipulated to penetrate to different depths it should be possible to distinguish between the different microvascular compartments – still clearly bearing in mind that the presence of AVAs and the relative depths of compartments varies enormously between species and body regions. Two basic characteristics of optically-based instruments determine their depth of penetration into tissues, viz., wavelength [1] and emitter-sensor separation [8].

Gush and King [3], while pointing out that the shorter wavelength green light (543.5 nm) is absorbed much more by blood than is red light

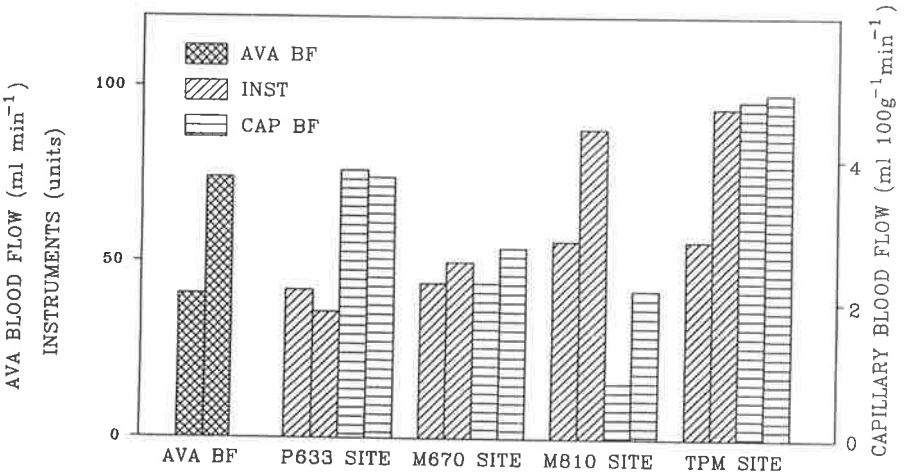


Fig. 1. One complete set of simultaneous measurements of total limb blood flow passing through AVAs, capillary blood flow at each of the four different probe sites and instrument responses. First bar in each pair is the control and second is the response to 1 mg of phentolamine.

halving of capillary flow, whereas the TPM increased 1.4-fold. That is, 'large' but not 'moderate' increases in AVA perfusion completely obliterated the P633 response to changes in capillary perfusion, whereas the TPM was always dominated by the AVA flow but was still influenced by the capillary flow.

With respect of the importance of emitter-sensor separation, our data are inconclusive. Thus, while we do not doubt theoretical considerations [8] supported by some experimental observations [7, 8], on the one hand our M670 and P633 showed indistinguishable responses despite their very different fibre separations (Fig. 1), and on the other hand our TPM clearly detected much deeper blood flow than did our P633 (Fig. 2); but the TPM had both wider emitter-sensor separation and higher wavelength. Notwithstanding this, comparisons with the TPM must be made cautiously because of its entirely different principle of operation; it is included in this study because of its potential value in monitoring total local skin blood flow, and not because of its wavelength and emitter-sensor characteristics.

In conclusion, the present pilot observations provide physiological evidence that laser-Doppler instruments of longer wavelength sense to a greater depth, and our TPM behaves similarly to the M810. Since the red light can, but does not necessarily detect deep AVA perfusion, the influence depending upon the magnitude of the AVA perfusion, measurements by an instrument based on red light are difficult to define. Thus we suggest that measurements could be defined more confidently if a much lower wavelength, probably green light, was used to measure superficial capillary perfusion and an infra-red system, either laser-Doppler or TPM was used to measure a parameter more representative of total local blood flow. The latter would have the further advantage of being less susceptible to regional anatomical variations in the distance of even the capillaries below the skin surface due, say, to variations in thickness of the cornified epithelium.

Investigators should specify, particularly while instrument design is evolving so rapidly, (a) instrument and probe characteristics, (b) species and (c) microvascular anatomy of the body region.

Acknowledgement

This work was supported by an Australian Research Council grant to J.R.S.H. and F.R.N.S.

In: Clin. Exper. Neurol., 26: 51-63, (1989)

Concordance Between Different Measures of Small Sensory and Autonomic Fibre Neuropathy in Diabetes Mellitus

R.A. Westerman, C. Delaney,* A. Ivamy-Phillips,* M. Horowitz† and A. Roberts.‡*

Diabetes mellitus commonly leads to neuropathic complications which include disturbances of the smallest nerve fibres. Symptoms may be those of sensory or autonomic dysfunction such as dysaesthesia, paraesthesia, pain or disturbance of sweating.[1] Until recently, conventional clinical neurophysiological tests such as nerve conduction velocity (NCV) have measured the function of only the larger myelinated nerve fibres.[2] Now, the neurophysiological assessment of small sensory nerve fibre neuropathies has been greatly advanced by the development of an improved automated method for measurement of thermal thresholds[3–5] and by other non-invasive tests of neurovascular function which measure electrically evoked axon reflex and endothelial-dependent vasodilator responses.[6–9]

Thermal threshold detection for warm stimuli depends upon functional C-thermal and C-polymodal nociceptors and their primary afferent fibres. The axon reflex tests of cutaneous microvascular dilatation depend upon the integrity of 3 serial components – C-polymodal peptidergic primary afferents, microvascular endothelium and smooth muscle. Autonomic neuropathy is also a well recognised complication of diabetes[10] and may result in abnormal gastrointestinal motility as well as impotence, faecal or urinary incontinence, postural hypotension or sweating disorders.[1,11] Delayed oesophageal and gastric emptying measured by scintigraphy has been demonstrated both in insulin-dependent and non-insulin dependent diabetics even without symptoms.[11,12]

There are little data regarding the prevalence of abnormal oesophageal or gastric emptying in diabetics,[11] partly because of the previous lack of sensitive and

* Department of Physiology, Monash University, Clayton, Vic.
† Department of Medicine, University of Adelaide, SA.
‡ Department of Endocrinology, Royal Adelaide Hospital, SA.

simple tests, and the fact that asymptomatic patients may have functional abnormalities of small sensory or autonomic nerves.[9–12] Furthermore, sparse information exists about simultaneous prevalence of small cutaneous sensory neuropathy. Because both warm threshold detection and electrical axon reflex share a common functional component in the C-polymodal nociceptor/unmyelinated afferent pathway, we have sought a correlation between both these test methods applied to the skin of the forearm and the dorsum of the foot in each of 25 diabetic patients in whom gastric and oesophageal emptying has been recently measured. This provided an opportunity to examine the impact of diabetes mellitus on both cutaneous sensory and gastro-oesophageal vagal unmyelinated nerve fibres. A short communication about this has already been presented.[13]

Methods

Twenty-five patients were selected initially by random numbers from the diabetic outpatient register of the Endocrine Department, Royal Adelaide Hospital. Both Type I and II diabetics were included in the study.

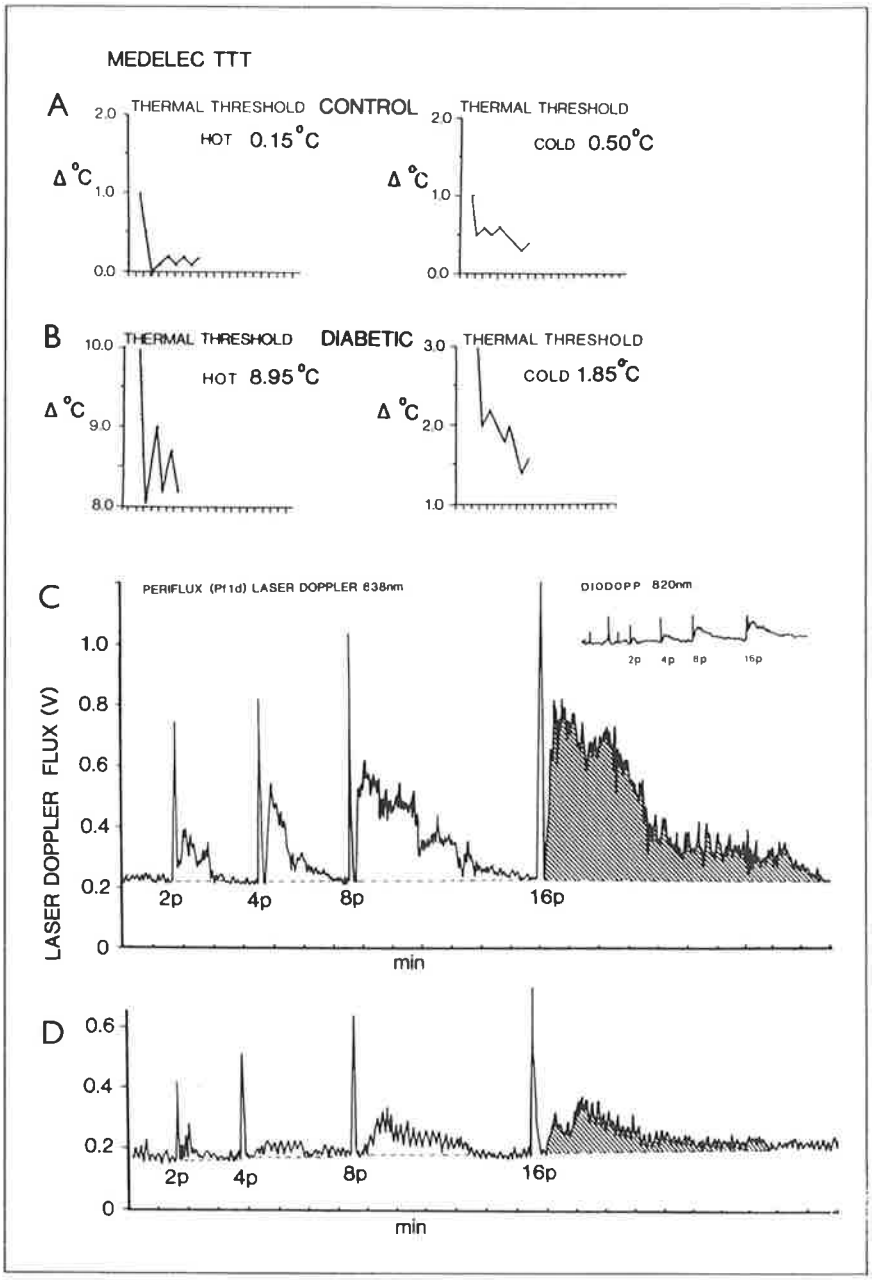
In the Medelec TTT automated thermal threshold test[3] thermal stimuli, warm or cold, are delivered to the skin surface through a metal Peltier thermode. The magnitude and duration of the current applied regulate the thermal stimulus and a constant rate of change of temperature (1°C per second) is provided for each stimulus. Thermal stimuli are provided during one of 2 time windows shown to the subject by illuminated LEDs and the subject must indicate using a switch in which time period a stimulus occurred. The subject's success rate is analysed by the microprocessor using the 'Up-Down Transform Rule' to define the temperature which the subject can reliably detect. Sample records appear in a recent paper concerning sensory neuropathy in herpes zoster,[14] as well as in Figure 1.

The electrical axon reflex vasodilator responses of forearm or foot dorsum skin[6] are evoked by noxious transcutaneous electrical stimulation at a pulse strength adequate to excite C-polymodal nociceptors (150V, 0.75ms, 45–50mA). Trains of such pulses (1, 2, 4, 8, 16) at 2Hz are used to provoke stimulus-dependent cutaneous axon reflex vasodilation which is measured by a helium neon (638nm) laser Doppler velocimeter (Periflux Pf1d). This technique, which has demonstrated reduced axon reflex responses in diabetic patients[7,8] and rats,[8] has been recently reviewed.[9] Because reduction of the electrically evoked axon reflex may result from microvascular endothelial dysfunction as well as from primary afferent neuropathy, iontophoretic tests of endothelium and microvascular smooth muscle functions are applied.[7–9] Acetylcholine chloride, pilocarpine or methacholine is the endothelium-dependent stimulus, and sodium nitroprusside is the endothelium-independent nitrovasodilator now used.

Figure 1. Examples of records – thermal thresholds and electrical axon reflexes. *A,B:* show examples of the triple T printout for warm and cold detection thresholds for a normal subject (A) and a diabetic patient (B), recorded in both instances from the skin of foot dorsum. *C,D:* show sample records of the laser Doppler flux change corresponding to cutaneous axon reflex vasodilator responses evoked by 2,4,8,16 noxious TENS pulses in a normal subject (C) and diabetic patient (D). All records were taken from the skin of the foot dorsum in the same normal subject, and the same diabetic patient as in A,B.

The cross-hatching shows, as an example, the total flux response to 16 noxious TENS pulses and such areas are measured as Volt.min integrals by a digitising tablet in order to derive the data presented in Figures 5,6,7.

The main records shown in C,D were obtained using a Periflux Pf1d laser Doppler velocimeter with a 638 nm helium neon light source. Inset in C shows the responses from the control subject simultaneously recorded using an infra red diode laser Doppler (DIODOPP, Applied Laser Technology) having a wavelength of 820 nm. Responses from both machines are similar.


MEDELEC TTT
A
THERMAL THRESHOLD CONTROL
HOT 0.15°C
Δ °C
2.0
1.0
0.0
THERMAL THRESHOLD
COLD 0.50°C
B
THERMAL THRESHOLD DIABETIC
HOT 8.95°C
10.0
9.0
8.0
THERMAL THRESHOLD
COLD 1.85°C
3.0
2.0
1.0
C
PERIFLUX (PF1d) LASER DOPPLER 638nm
DIODOPP 820nm
2p 4p 8p 16p
LASER DOPPLER FLUX (V)
1.0
0.8
0.6
0.4
0.2
0
min
D
0.6
0.4
0.2
0
2p 4p 8p 16p
min

The size of the laser Doppler flux response recorded against time is measured as the voltage: time integral using a digitising tablet and area measuring programme.

Measurement of gastric emptying was performed by a double isotope test which measures both solid and liquid gastric emptying simultaneously and has been described previously.[11,12] The scintillation camera was behind the seated subjects who fasted from 1900 (for solids) or 2400 (for liquids) from the previous day. The subject ate the solid meal over a 5-minute period and then immediately drank the dextrose solution, the study continuing for 2 hours after completion of the whole meal.

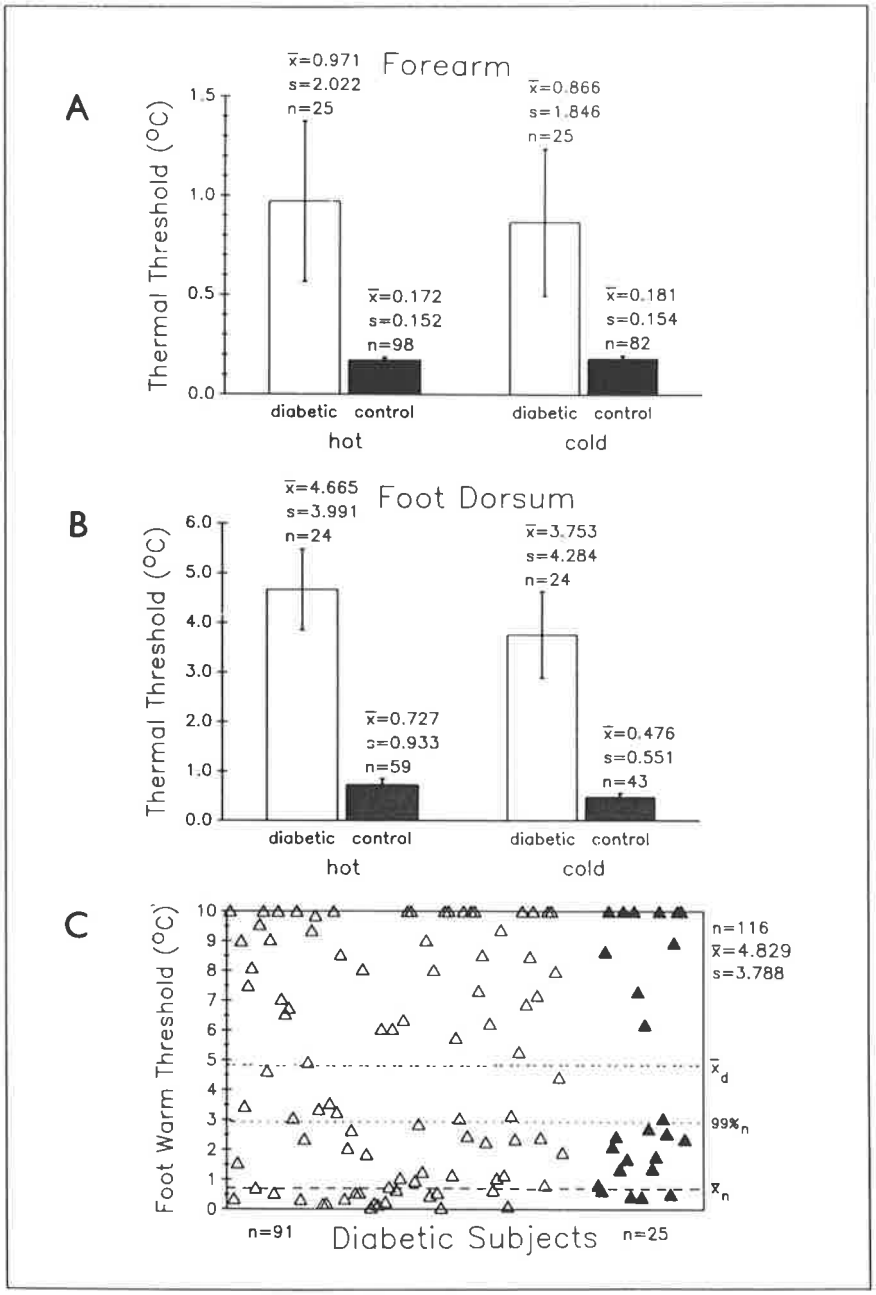
Data were corrected for patient movement, radionuclide decay, Compton scatter and gamma-ray attenuation as previously described.[15] From the histograms of solid and liquid emptying (expressed as a percentage of the total meal remaining within the stomach vs. time) several parameters were derived for later analysis. For the solid portion of the meal these parameters were the lag period before food left the stomach and the percentage remaining at 100 minutes after meal completion (T_{100}%). For the liquid component the percentage remaining at 10 minutes after meal completion (T_{10}) and the time for 50% emptying (T_{50}) were obtained. The percentage of liquid remaining at 10 minutes was used as an index of the early phase of gastric emptying.

Results

Examples of the automated thermal threshold test record and the electrically evoked axon reflex are shown in Figure 1A and B, respectively, for foot dorsum of one normal subject and one diabetic patient (see also Figures 2, 4 reference 14; Figure 3 reference 9). The thermal thresholds for both warm and cold are elevated and the electrical axon reflex dilator responses reduced in the diabetic compared with the normal subject. In Figure 2, thermal thresholds to warm and cold stimuli are shown as histograms for the forearm and foot dorsum sites, both on normal subjects and diabetic patients. It is noteworthy that although none of the diabetic patients tested in this study had any symptoms relating to the forearms, mean thermal thresholds (n = 25) for both warm and cold stimuli applied to the forearm were elevated (W = 0.97°C ± 2.02°C and C = 0.87°C ± 1.8°C) compared with the mean control values of W = 0.17°C ± 0.15°C and C = 0.18°C ± 0.15°C (n = 53), respectively. In the case of the foot dorsum, 14 of 25 diabetic patients tested had symptoms suggesting mild to moderate distal neuropathy. Thermal thresholds for warm detection in these patients were elevated more (mean = 4.67°C ± 3.99°C) than for cold detection (mean = 3.75°C + 4.38°C). The distribution of warm thresholds for the 25 patients in the concordance study is not discernably different from that of 91 other unselected diabetic patients from the Lions International Diabetes Institute in Melbourne (Figure 2C).

There is a positive correlation between thermal thresholds for both warm and cold vs. recent HbA_1C (as an index of glycaemic control) illustrated in Figure 3; 9

Figure 2. Distributions of thermal thresholds. *A:* Thermal threshold data of 25 diabetic patients compared with 82 to 98 normal subjects to hot and cold stimuli applied to the forearm. Means ± S.D. are shown above each histogram. *B:* Thermal thresholds to hot and cold stimuli applied to the foot dorsum of 24 diabetic patients compared with 43 to 59 normal subjects. Means ± S.D. are shown above each histogram. *C:* Scattergram plot of warm perception thresholds recorded on the foot dorsum in 91 diabetic patients randomly selected from the Lions International Diabetes Institute (open triangles) and the 25 diabetic patients in the Royal Adelaide Hospital concordance study (filled triangles).


A
Forearm
x̄=0.971
s=2.022
n=25
x̄=0.866
s=1.846
n=25
x̄=0.172
s=0.152
n=98
x̄=0.181
s=0.154
n=82
Thermal Threshold (°C)
1.5
1.0
0.5
0.0
diabetic control
hot
diabetic control
cold
B
Foot Dorsum
x̄=4.665
s=3.991
n=24
x̄=3.753
s=4.284
n=24
x̄=0.727
s=0.933
n=59
x̄=0.476
s=0.551
n=43
Thermal Threshold (°C)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
diabetic control
hot
diabetic control
cold
C
Foot Warm Threshold (°C)
10
9
8
7
6
5
4
3
2
1
0
n=116
x̄=4.829
s=3.788
x̄d
99%n
x̄n
n=91
Diabetic Subjects
n=25

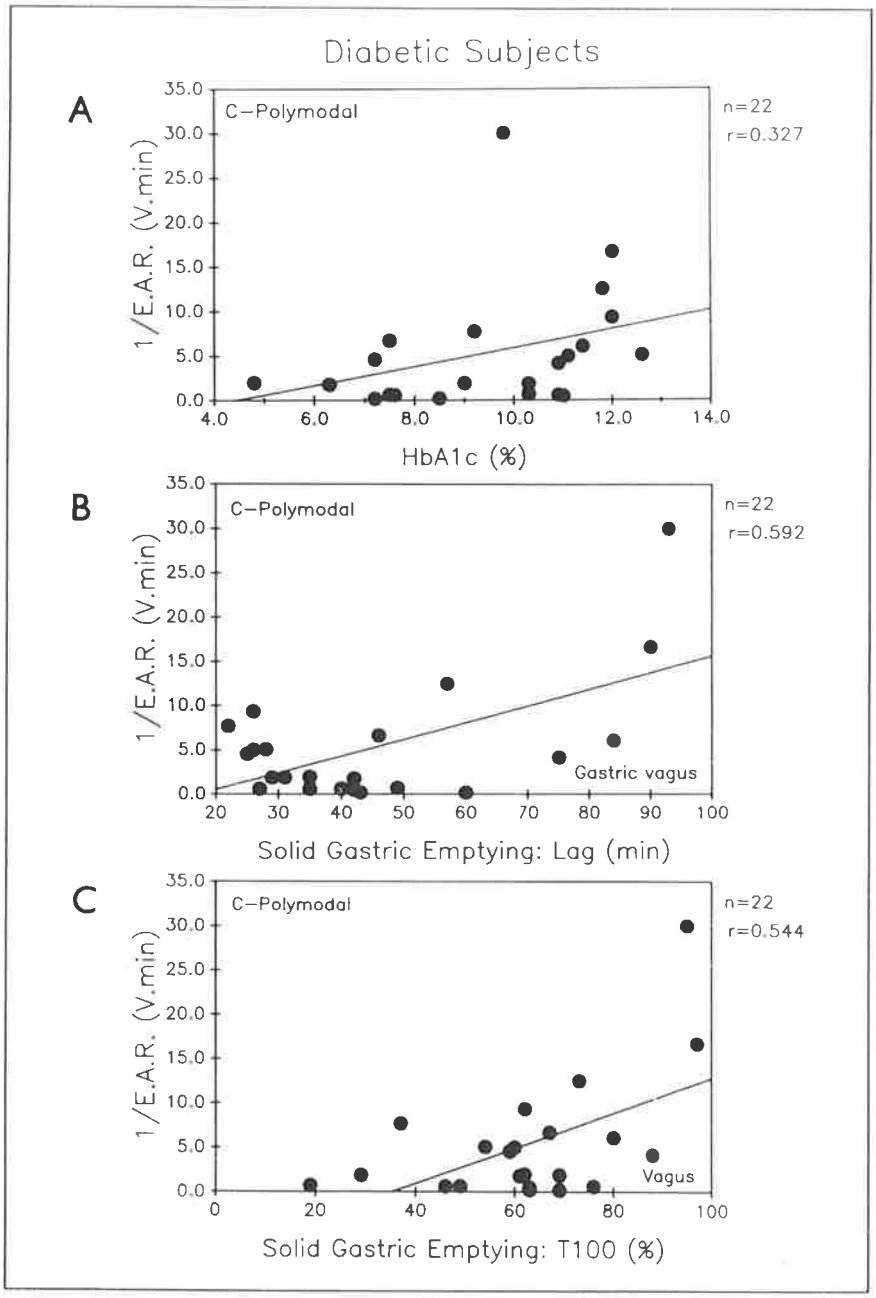


Figure 5. For each graph A,B,C the ordinate indicates the inverse plot of electrical axon reflex flux response to 8 + 16 noxious TENS pulses (expressed as $V.min^{-1}$) against *A:* HbA_1C (%); *B:* Solid gastric emptying: Lag (min); *C:* Solid gastric emptying: T_{100}%.

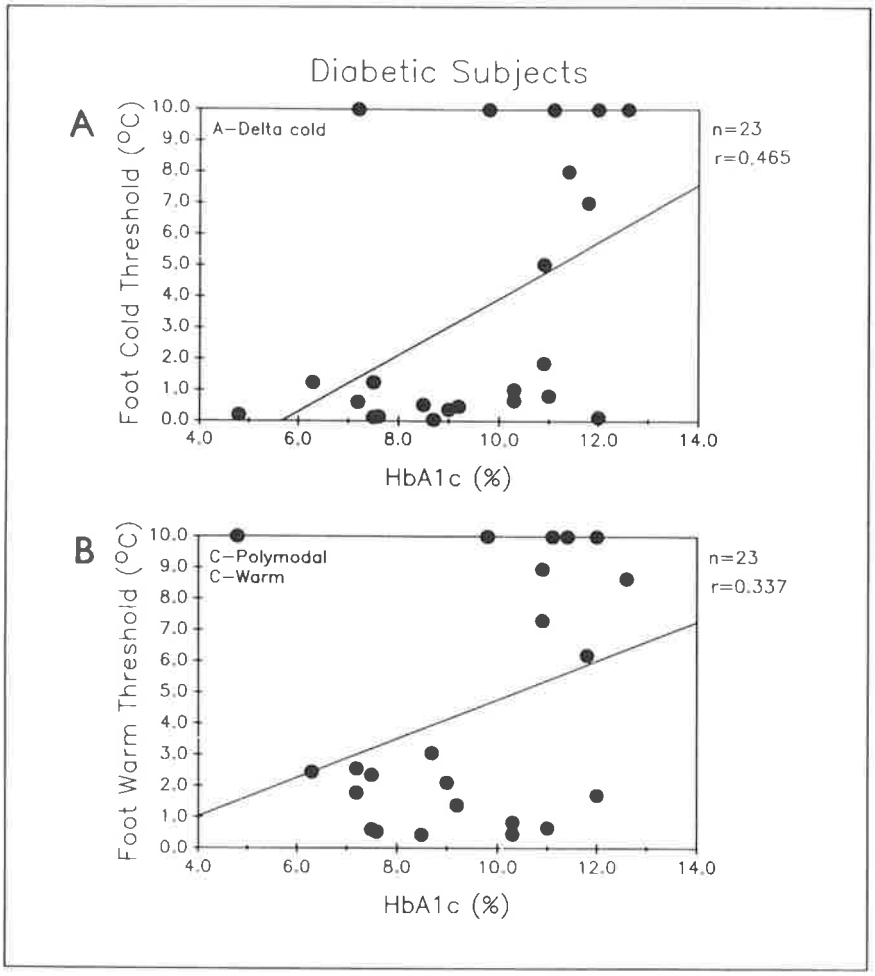


Figure 3. Thermal thresholds recorded from the foot dorsum in 23 RAH diabetic patients for *A:* cold; *B:* warm plotted against $HbA_1C\%$. Linear regressions and r values are shown.

of 23 patients show thermal thresholds exceeding the 99% confidence limits for normal controls, and most of these (8 of 23) had HbA_1C levels exceeding the acceptable maximum of 8 per cent.

The concordance between thermal thresholds for warm and cold stimuli applied to the foot dorsum and gastric emptying $T_{100}\%$ is shown in Figure 4. Nine of 24 diabetic patients showed cold threshold and 10 of 24 had warm thresholds well above the 95% confidence limits for normal control subjects.

The noxious TENS-evoked axon reflex responses and the acetylcholine-evoked dilator responses in diabetic patients have been recently described[8,9] and their reduction is illustrated again in Figure 5. Because impairment of electrical axon reflex leads to reduced flux responses, the *inverse* of electrical axon reflex is

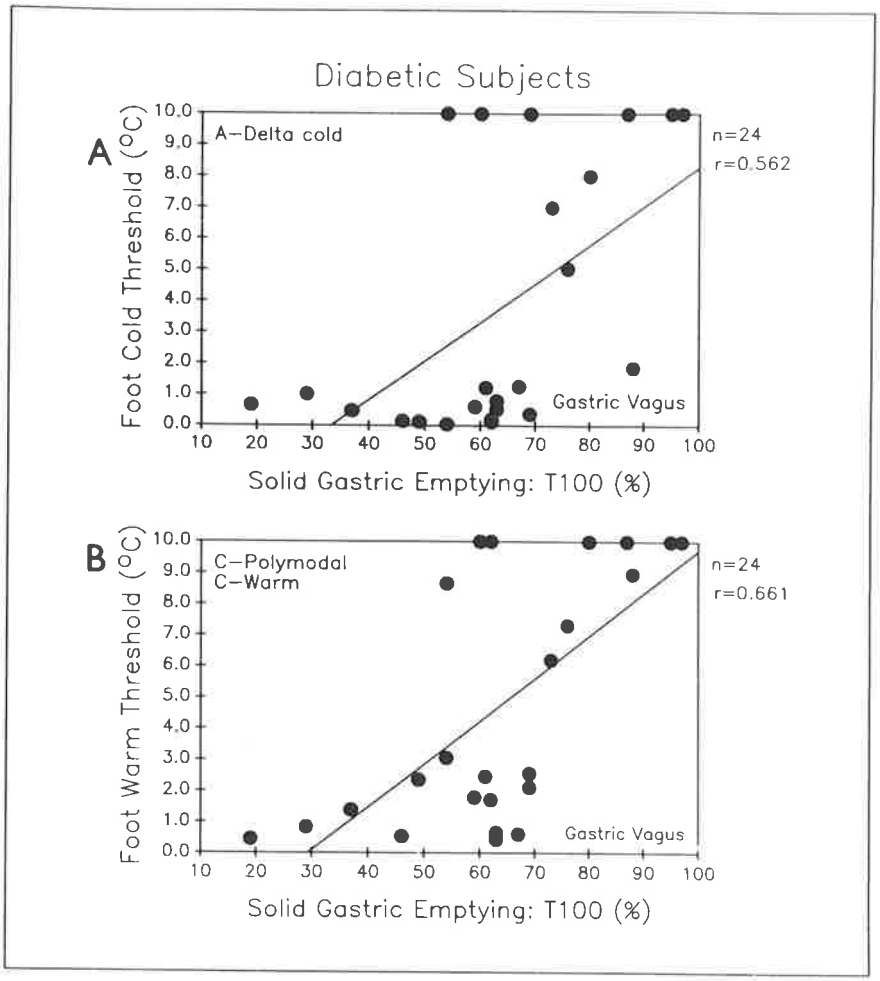


Figure 4. Thermal thresholds recorded from the foot dorsum in 24 RAH diabetic patients for *A:* cold; *B:* warm, plotted against solid gastric emptying: T_{100}%. Linear regressions and r values are shown.

plotted in each ordinate of Figure 5A,B,C to depict the positive correlation in the same direction as other graphs, where increased thermal thresholds indicate impairment. This figure shows that the extent to which TENS-evoked axon reflex responses are reduced is greater in the presence of poor glycaemic control as shown by elevated HbA_1C levels. Many of these patients had symptoms or clinical signs of neuropathy. In addition, reduction in size of cutaneous electrical axon reflex dilator responses correlates positively with delayed gastric emptying – both lag and T_{100}%.

Figure 6 shows the correlation between warm thresholds (WTT) and electrical axon reflex responses (EAR) recorded from the same diabetic patients. The correlation coefficient is −0.43 and indicates that there is a negative relationship

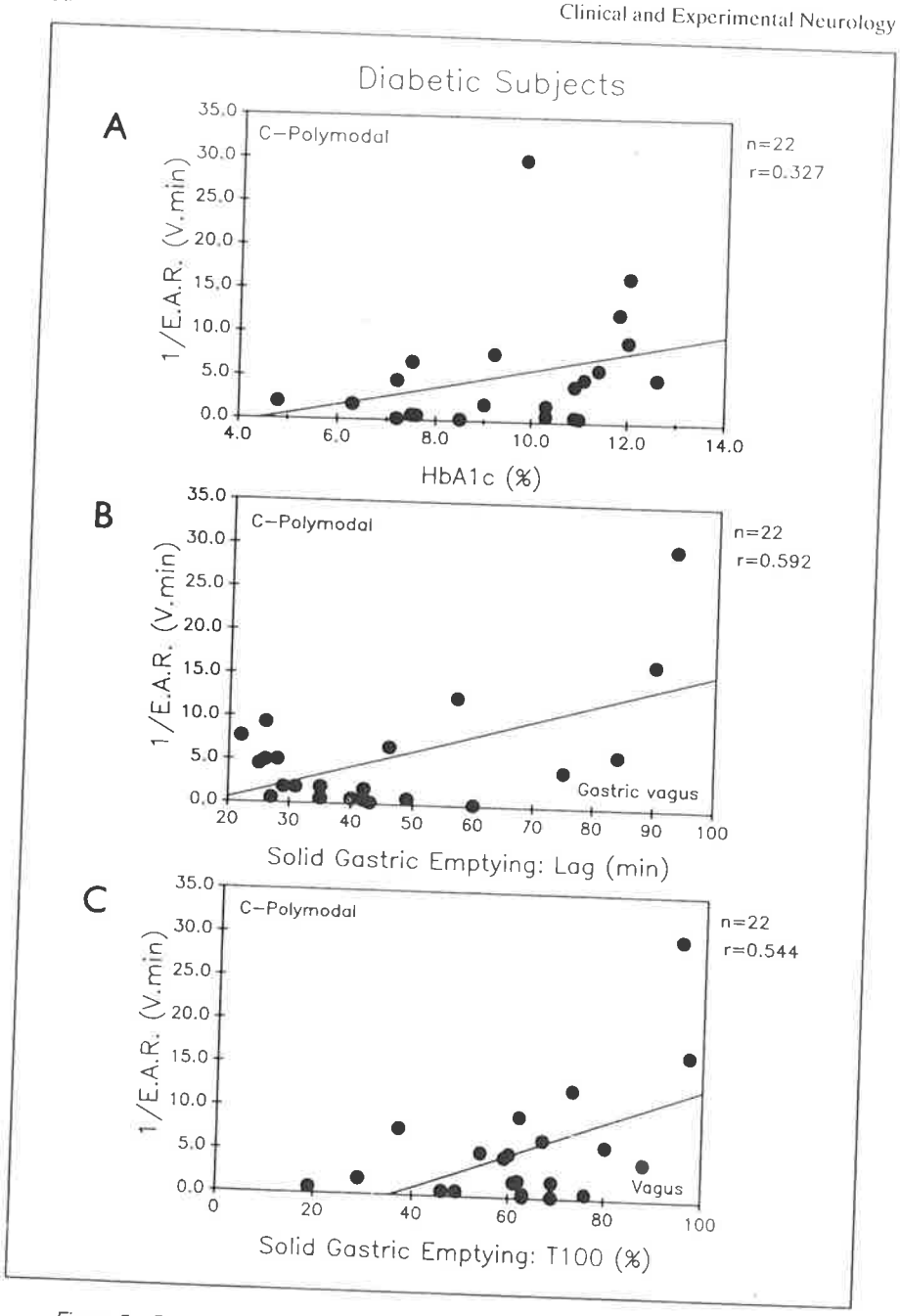


Figure 5. For each graph A,B,C the ordinate indicates the inverse plot of electrical axon reflex flux response to 8 + 16 noxious TENS pulses (expressed as $V.min^{-1}$) against *A:* HbA_1C (%); *B:* Solid gastric emptying: Lag (min); *C:* Solid gastric emptying: T_{100}%.

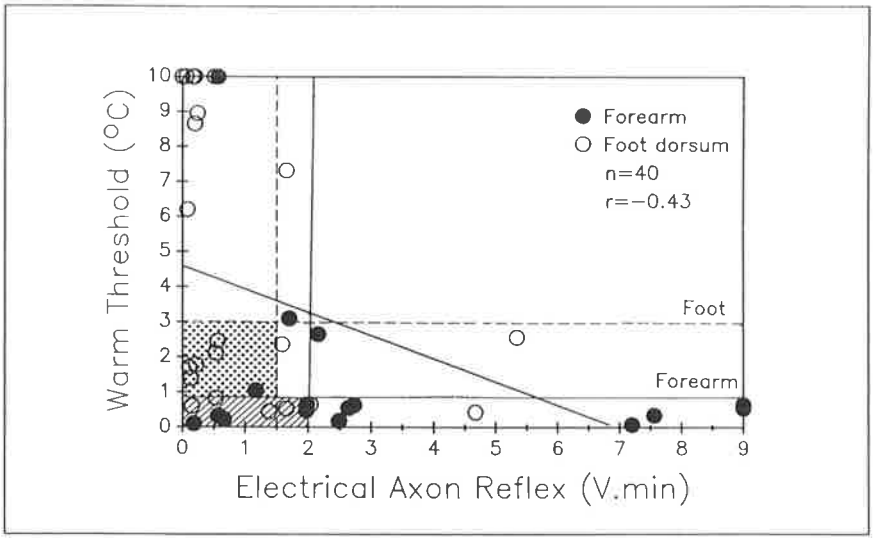


Figure 6. Compares warm perception threshold (Y axis) as a test primarily of C-fibre function and electrical axon reflex dilator responses (X axis) as a test of total neurovascular function in the same diabetic patients. Broken lines show 99% confidence limits for warm thresholds (horizontal line) and for electrical axon reflex (vertical line). Warm threshold values below 3.0°C are probably normal, and axon reflex responses smaller than 1.5V.min are probably abnormally reduced (stippled zone) for foot dorsum, while WPT below 0.9°C and EAR greater than 2V.min are probably normal for forearm. Responses fall into three main groupings (see text). By comparison of forearm responses (open circles) and foot dorsum responses (filled circles) it is seen that a similar distribution of elevated WPT was found on the anterior forearm of diabetic patients and is an even more impressive functional deficit at this site because clinical large fibre neuropathy is infrequent here.

between elevation of warm threshold and magnitude of the electrical axon reflex response. There is a particular subgroup of 9 patients (15 responses) whose combined WTT/EAR data lie near the origin and identify those patients with reduced EAR responses whose WTT are approximately normal. It is these patients which are responsible for the downward skewing of the linear regression line away from the higher warm threshold values at the lowest EAR values.

Finally, of interest is the correlation of HbA_1C and gastric emptying (i.e. vagal nerve fibre function) tests with endothelial-dependent responses measured by iontophoretic application of acetylcholine: this tests microvascular reactivity.

These correlations are shown in Figure 7, and reveal a positive correlation between reduced gastric motility and reduced ACh-evoked dilator responses. Delayed solid gastric emptying is shown by prolonged lag and T_{100}% values.

Discussion

The finest cutaneous nerve fibres subserve both sensory and autonomic function, but the axon reflex, iontophoretic dilator and thermal threshold tests

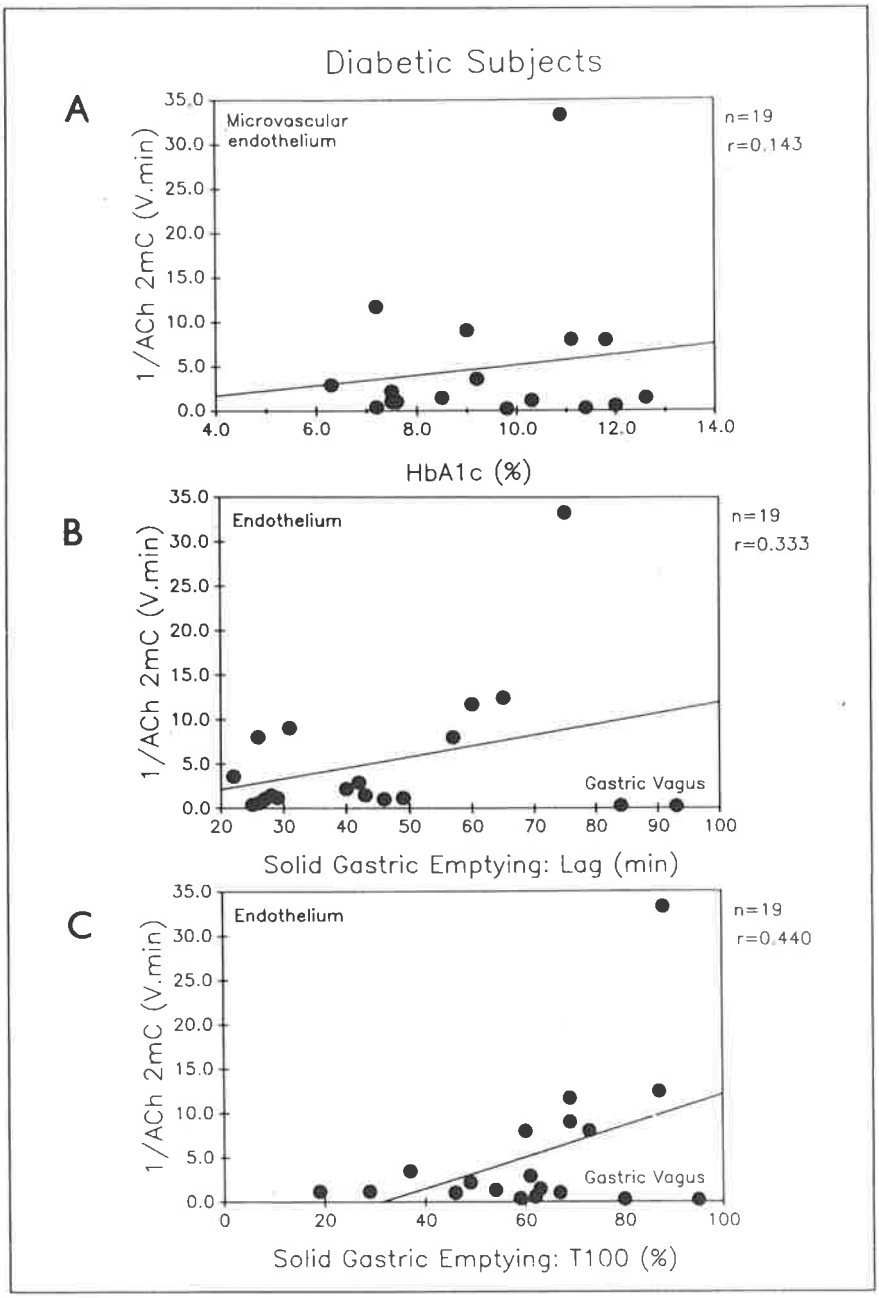


Figure 7. For each graph A,B,C the ordinate indicates the inverse plot of laser Doppler flux responses to iontophoretic application of 2 mC acetylcholine (expressed as V.min-1) against *A:* HbA_1C (%); *B:* Solid gastric emptying: lag(min); *C:* Solid gastric emptying: T_{100}%.

described in the present paper measure only the former. The A delta cold fibres are thinly myelinated, but appear to be implicated in the small fibre neuropathy in the diabetic patients presently studied. However, from the Figure 2 data it is evident that the elevation of warm thresholds on the foot dorsum is more marked than that for cold detection – and both show much more impairment on the foot dorsum than the forearm, indicating that the foot is the predilection site for small fibre neuropathy. The positive correlation with reduced gastric motility and prolonged emptying times indicated that the presence of gastric vagal neuropathy is likely to be accompanied by small cutaneous sensory fibre dysfunction. The electrical axon reflex is a test of serial functions of C-polymodal nociceptors and their peptidergic primary afferent fibres, as well as the adjacent epidermal microvascular endothelium, and the smooth muscle.

From Figures 6 and 7 there is a substantial group (9 of 25) of patients whose thermal thresholds are normal (indicating functional C-thermal, C-polymodal and A delta nerve fibres), but whose EAR is still impaired. Their positive correlation with reduced ACh endothelium-dependent responses suggests that this group suffers predominantly microvascular endothelial dysfunction rather than neuropathy, and their expected prognosis with treatment may be very different from that of those patients with predominantly small fibre neuropathy. All 3 tests of small nerve fibre function show a strong concordance between elevated HbA_1C and functional impairment – either elevated thermal thresholds, reduced electrically evoked axon reflex flare and delayed gastric emptying. There are indications of reversibility of the haematological indices dependent upon endothelial function after aldose reductase inhibitor (ARI) treatment in man[16] and of neuropathy and EAR reduction in the rat.[17] Therefore it is probable that, in the future, when safer and effective aldose reductase inhibitors are more widely available, the reversibility of this thin fibre neuropathy and endothelial dysfunction may become more apparent.

Of the 25 patients tested in this study, 6 could be classed as within normal limits in relation to all 3 variables, but 8 of 25 displayed objective evidence of C-fibre neuropathy – thermal perceptual impairment (C and A delta sensory fibres), reduced neurogenic inflammatory flare (peptidergic nociceptive afferents) and delayed gastro-oesophageal emptying (vagal afferent/efferent fibres).

Most importantly, the correlates between 3 different methods of measuring small nerve fibre function should emphasise the value of these techniques in the quantitative assessment of some of the more obscure neuropathies and microvasculopathies as well as those occurring in diabetes mellitus. These methods should also improve our epidemiological knowledge of small fibre neuropathy and enable progress with better glycaemic control or new treatments such as aldose reductase inhibitors to be monitored more accurately.

Summary

This study presents concordance data from 3 different tests of small nerve-fibre function on the same diabetic patients and also examines the effects of hyperglycaemia. Thus the TTT–Glasgow automated thermal threshold test, EAR–

Electrically evoked axon reflex flare, and GOE–Gastric emptying of a mixed sold/liquid meal and oesophageal emptying of a solid bolus were all measured on 25 diabetic patients. The TTT, EAR and GOE all gave values ranging from within the normal reference range for non-diabetics to markedly dysfunctional readings. Mean warm perception (WPT) on the foot dorsum averaged 0.73°C ± 0.93 for normal controls, but was 4.67°C ± 3.99 in the 25 diabetics. Cold perception thresholds (CPT) were 0.48°C ± 0.55 for normal subjects and 3.75°C ± 4.28 for diabetics. In the same normal subjects the mean EAR flare laser flux responses (for 8 and 16 noxious TENS pulses) was 2.8 V.min, while for diabetics the mean was 0.2 V. min. Solid and liquid gastric 50% emptying times and oesophageal emptying for non-diabetics were within normal range (mean 78 min and 18 min, 18 sec respectively) but for the 25 diabetics emptying times ranged from normal to very prolonged (mean 114 min and 30 min, 68 sec respectively). A plot of 3 measured variables (TTT, EAR and GOE) showed a high degree of correspondence between the gastro-oesophageal emptying delays and the presence of reduced electrical axon reflex and elevated thermal thresholds. Of 25 patients, 6 could be classed as within normal limits on all 3 variables, but 8 of 25 displayed objective evidence of C-fibre neuropathy – thermal perceptual impairment (C- and A-delta sensory fibres), reduced neurogenic inflammatory flare (peptidergic nociceptive afferents) and delayed gastro-oesophageal emptying (vagal afferent/efferent fibres).

Acknowledgements

The authors are pleased to acknowledge that this research has been partly supported by the NH & MRC, ARGS, and the Clive and Vera Ramaciotti Foundation. The assistance of the staff of the Lions International Diabetes Institute and the Endocrinology ward and unit, Royal Adelaide Hospital, is very much appreciated. The concordance study at the RAH was specifically supported by ICI Australia and their generous assistance is gratefully acknowledged.

References

1. Asbury AK, Brown MJ. Clinical and pathological neuropathies. *In*: Goto Y, Horinchi A, Kogure K (Eds.) Diabetic neuropathy. Amsterdam: Excerpta Medica, 1982: 50–57.
2. Dorfman LJ. The distribution of conduction velocities in peripheral nerves: a review. Muscle and Nerve 1984; 7: 2–11
3. Jamal GA, Hansen S, Weir AI, Ballantyne JP. An improved automated method for measurement of thermal thresholds. 1. Normal subjects. J Neurol Neurosurg Psychiat 1985; 48: 354–360.
4. Jamal GA, Hansen S, Weir AI, Ballantyne P. An improved automated method for the measurement of thermal thresholds. 2. Patients with peripheral neuropathy. J Neurol Neurosurg Psychiat 1985; 48: 361–366.
5. Jamal GA, Hansen S, Weir A, Ballantyne JP. The neurophysiologic investigation of small fibre neuropathies. Muscle and Nerve 1987; 10: 537–545.
6. Westerman RA, Low A, Pratt A, *et al.* Electrically evoked skin vasodilatation: a quantitative test of nociceptor function in man. Clin Exp Neurol 1987; 23: 81–89.
7. Westerman RA, Widdop RE, Hogan C, Zimmet P. Non-invasive tests of neurovascular function: reduced responses in diabetes mellitus. Neurosci Lett 1987; 81: 77–82.
8. Westerman RA, Widdop RE, Low A, Hannaford J, Kozak W, Zimmet P. Non-invasive

tests of neurovascular function: reduced axon reflex responses in diabetes mellitus of man and streptozotocin-induced diabetes of rat. Diabetes Research and Clinical Practice 1988; 5: 49–54.
9. Westerman RA, Widdop RE, Low A, Neild TO, Delaney CA. Non-invasive tests of neurovascular function in human and experimental diabetes mellitus. In: G Molinatti, R Bar, S Belfiore, M Porta (Eds) Frontiers in Diabetes: Endothelial cell function in diabetic microangiopathy: problems in methodology and clinical aspects. Basel: Karger, (1989) In press.
10. Ewing DJ, Clarke BF. Diabetic autonomic neuropathy – present insights and future prospects. Diabetes Care 1986; 6: 648–665.
11. Horowitz M, Harding PE, Maddox A, *et al.* Gastric and oesophageal emptying in insulin-dependent diabetes mellitus. J Gastroenterol Hepatol 1986; 1: 97–113.
12. Horowitz M, Harding PE, Maddox AF, *et al.* Gastric and oesophageal emptying in patients with Type II (non-insulin-dependent) diabetes mellitus. Diabetologia 1989; 32: 151–159.
13. Westerman RA, Delaney CA, Horowitz M, Pratt A, Kent P, Roberts A. Concordance between different measures of small nerve-fibre function in diabetes mellitus. Aust NZ J Med 1989; 19 (Suppl); 633.
14. Westerman RA, Roberts RGD, Kotzmann RR, *et al.* Effects of topical capsaicin on normal skin and affected dermatomes in herpes zoster. Clin Exper Neurol 1989; 25: 71–84.
15. Collins PJ, Horowitz M, Cook DJ, Harding PE, Shearman DJC. Gastric emptying in normal subjects – A reproducible technique using a single scintillation camera and computer system. Gut 1983; 24: 1117–1125.
16. Braund WJ, Moore JC, Williams SC, *et al.* Effect of aldose reductase inhibition on endothelial and microvascular abnormalities in diabetes mellitus. Proc Aust Diabetes Soc Abstract 22: August 1987.
17. Low AM, Westerman RA. Neurogenic vasodilatation in the rat hairy skin measured using a laser Doppler flowmeter. Life Sci 1989; 45: 49–57.

In: Clin Exper Neurol 29: 149-160 , (1992)
Edited by M Eadie, C Lander, M Pender

CONFOUNDING FACTORS IN NON-INVASIVE TESTS OF NEUROVASCULAR FUNCTION IN DIABETES MELLITUS

R.A. Westerman*, L.E. Lindblad†, D. Wajnblum*, R.G.D Roberts* and C.A. Delaney*

Department of Physiology*, Monash University, Clayton, Melbourne and Department of Clinical Physiology†, Södersjukhuset, Stockholm

SUMMARY

Disturbances of neurovascular function in the extremities may occur in patients with diabetes mellitus, exposure to toxic substances and chronic exposure to vibrating hand tools, as well as in Raynaud's phenomena. In these conditions symptoms of paraesthesia, finger numbness and blanching occur, so nerve conduction studies, vibration and temperature threshold measurements and neurovascular function tests are used for objective assessment of neurological dysfunction. The aim of the present study was to examine some factors which may confound quantitative neuro-vascular function measurements if used to assess neuropathy in diabetics. All subjects were consenting volunteers without exposure to known neurotoxic chemicals. The 5 groups were (a) healthy non-diabetic subjects not exposed to vibration (n=10, mean age 52.3 yrs) (b) 2 insulin dependent and 8 non-insulin dependent diabetic subjects with a mean of 6 years treatment (n=10, mean age 55.7 yrs) (c) maintenance employees exposed to high frequency pneumatic hand tools (n=10, mean age 52.2 yrs) (d) subjects who were not diabetic or exposed to vibrating tools, but were being treated with the ACE-inhibitor enalapril 20 mg daily for hypertension (n=5, mean age 54 yrs) (e) subjects who had smoked more than 10 cigarettes daily for at least 15 yrs (n=10, mean age 51 yrs). Neurovascular tests included axon reflex responses measured by laser Doppler velocimeter evoked on the dorsum of the finger by iontophoresis of acetylcholine 16mC in a circumferential chamber: cutaneous microvascular dilator responses to endothelial stimulation by iontophoretic application of the muscarinic agonist pilocarpine 16 mC and to direct nitrodilator sodium nitroprusside 16 mC. The skin temperature of the digits was held between 33^0 and 34^0C during testing and dilator responses were measured as flux change by on-line computer analysis using 'Perisoft'. There was a significant reduction ($P<0.05$) in the neurovascular responses of both diabetics and vibration - exposed subjects to acetylcholine and, in the case of vibration-exposed subjects, to pilocarpine, but nitroprusside responses were not

significantly different. Our findings of reductions in neurovascular responses in diabetics and in subjects exposed to higher frequency vibration is consistent with recent epidemiological findings. Furthermore, subjects treated with an ACE-inhibitor (enalapril) showed significant reduction in acetylcholine-evoked axon reflex responses, while the test group of smokers showed a significant reduction in their dilator response to pilocarpine. These data suggest that the neurovascular test battery used was sufficiently sensitive for the interpretation of its results to be confounded by environmental factors such as exposure to vibration, smoking or some concurrent medication, unless a meticulous history is taken.

Neuropathy commonly occurs in diabetes mellitus[1,2] and involves disturbances of sensory, motor, and small nerve fibre function including autonomic function [1,2,3,4]. Diagnostic tests which are recommended to determine the nature and extent of neuropathy include nerve conduction/EMG studies, quantitative sensory tests [5,6,7], cutaneous axon reflex, autonomic and sudomotor tests [8,9,10]. The aim of the present study was to determine whether quantitative neurovascular function measurements as used to assess diabetic polyneuropathy[3,8,9,10] are sufficiently sensitive to be confounded by other factors which may disturb neurovascular function or affect the axon reflex. For example, it has been long known [11] that workers with prolonged exposure to vibration often have symptoms of paraesthesia, finger numbness and blanching, typical of neurovascular dysfunction [12]. Other factors such as smoking [13] and nicotine [14] may also modify axon reflex responses. Two preliminary reports of the present study have been presented [15,16].

METHODS

Subjects

The subjects were consenting volunteers without exposure to neurotoxic chemicals or vasoactive drugs. Five groups of subjects were tested: (a) healthy controls - males exposed to no vibration or to infrequent use of handyman tools (n=10, mean age 52.3 yrs); (b) diabetic subjects with a mean of 6 years duration (n=10, 2 insulin dependent and 8 non-insulin dependent, mean age 55.7 yrs); (c) maintenance employees exposed to high frequency pneumatic hand tools (n=10, mean age 52.2 yrs); (d) subjects who were not diabetic or exposed to vibrating tools, but were being treated with the ACE-inhibitor enalapril 20 mg daily for hypertension (n=5, mean age 54 yrs); (e) subjects who had smoked more than 10 cigarettes daily for at least 15 yrs (n=10, mean age 51 yrs). All subjects were informed consenting volunteers, and the protocol had approval from the Human Ethics Committee of Monash University and the Alfred Group of Hospitals.

Neurovascular function tests

The neurovascular test battery included the measurement of the axon reflex response by laser Doppler velocimeter [11,17,18] (Periflux Pf1d) evoked on the dorsum of the finger by iontophoresis of acetylcholine 16mC (ACh) in a concentric chamber. The laser Doppler probe-holder and iontophoretic chamber on the finger dorsum are illustrated in Fig 1.

Also recorded were the cutaneous microvascular dilator responses to stimulation of muscarinic receptors by iontophoretic application of pilocarpine 16 mC (PILO) and of the direct nitrodilator sodium nitroprusside 16 mC (SNP). The dose of drug introduced into the epidermis by iontophoresis is given by the product of current (millamps) x time (secs)[17]. Fig 2 depicts diagrammatically the iontophoretic chambers in cross section and illustrates the patterns of flare or dilator response evoked by the test battery of chemical stimuli.

In all subjects the skin temperature of digits was held between 33^0 and 34^0C by a water-perfused jacket around the forearm and wrist during the testing. The dilator responses were measured as change relative to resting flux by means of on-line computer analysis using the 'Perisoft' program (Perimed). Responses were integrated for 4 mins from the end of the iontophoretic application of vasoactive compounds and expressed as Volt.min[15]. The axon reflex nature of the response to ACh was confirmed by its persistence after proximal nerve local anaesthesia (see Fig 1), and its similarity to axon reflex provoked by noxious electrical stimulation of finger skin [15]. Sample records using the 'Perisoft' (Perimed) program are illustrated in Fig 3.

Statistical analysis

Nonparametric analysis of the data with Wicoxon's Signed Rank test , and analysis of variance were performed. The significance level of differences is shown by the P-value.

RESULTS

There was a significant reduction ($P<0.05$) in the size of the ACh-evoked axon reflex responses for both the diabetic subjects (Fig 4a) and the group of subjects exposed to vibration (Fig 4b). The latter also showed a significant reduction ($P<0.05$) in the muscarinic receptor-dependent dilator response to PILO 16mC (Fig 4b), but responses to SNP were not significantly different in these groups.

The group of subjects treated with the ACE-inhibitor enalapril showed a significant reduction in the axon reflexes evoked by ACh 16mC iontophoresis (Fig 5a - $P<0.05$). The group of smokers showed a tendency toward reduction in the endothelium-dependent dilator response to PILO 16mC which was almost significant ($P=0.054$).

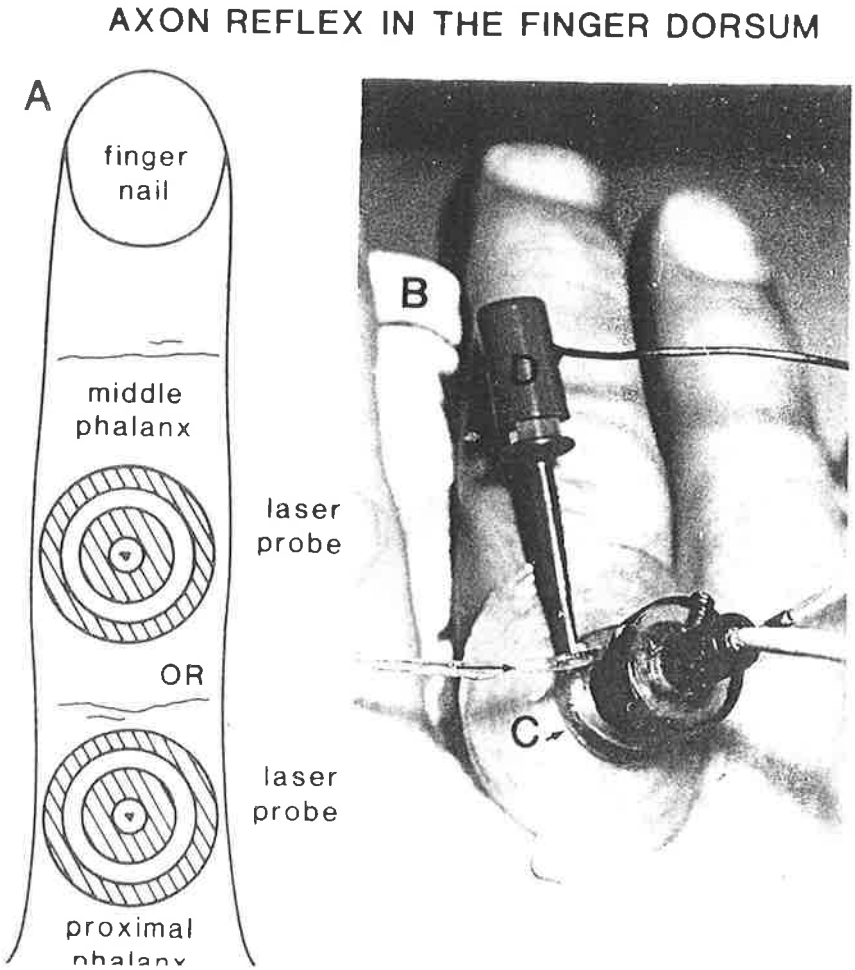


Fig 1 Chemically evoked axon reflex. A: fibres of the laser Doppler probe are at least 5mm distant from the closest edge of the iontophoretic chamber. B: photograph showing the laser Doppler probe held vertically in the curved plastic holder 0.5mm above the skin surface by a perspex collet; in the probe a concentric groove 3mm wide (arrowed at C) served as an iontophoretic chamber. An aqueous 1% solution of drug to be iotophoresed was injected through the inlet tubing (arrow); the electrical connection (D) clipped onto the stainless inlet needle is also seen.

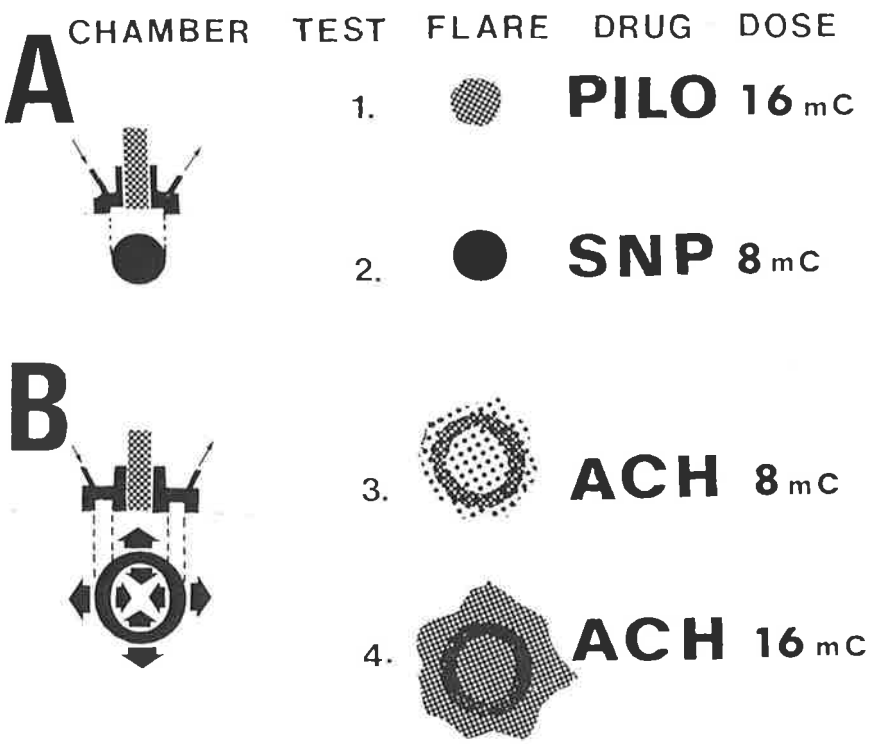


Fig 2 Patterns of vasodilator response. Diagrammatic illutration of perspex probe-holder and direct iontophoretic chamber A, and the flushed spots evoked after iontophoresis of vasoactive compounds onto skin immediately under the laser probe.
The chemicals used in 'direct' chamber A are the muscarinic agonist pilocarpine (PILO) 16mC, and a smooth muscle nitrodilator sodium nitroprusside (SNP) 8mC. In B, the concentric chamber, whose nearest edge is 5mm from the laser probe optical fibres, allows iontophoretic application of acetylcholine(ACh) into skin remote from the recording site. The axon reflex flare evoked by ACh excitation of nociceptors via nicotinic cholinoceptors is shown spreading both centripetally and centrifugally from the area of application. The corresponding axon reflex flare responses, shown as graded in intensity and size, are ACh dose-dependent.

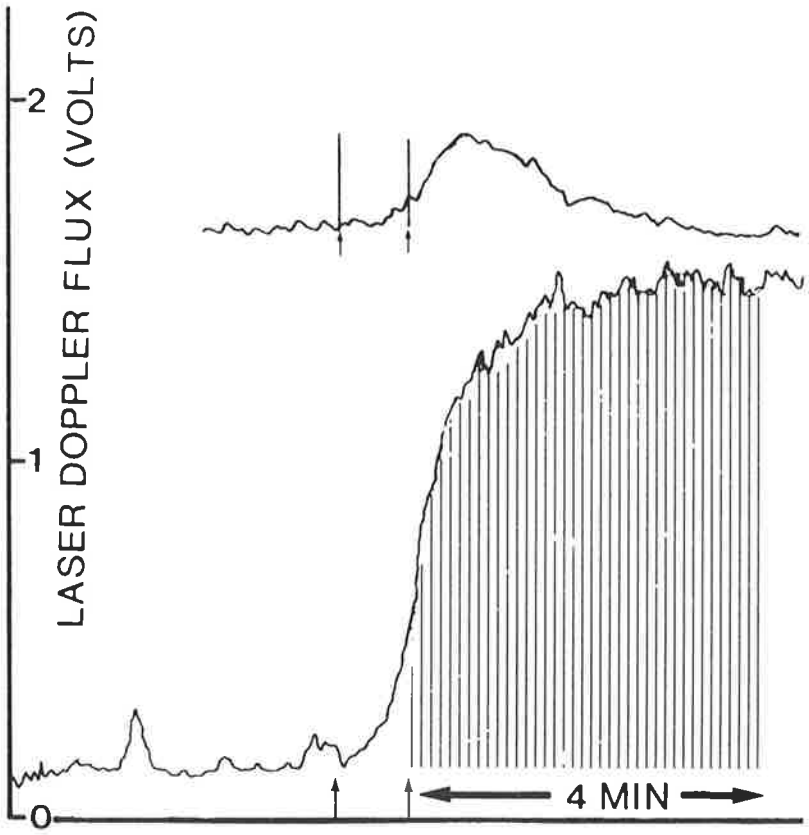


Fig 3 Laser Doppler flux recordings, showing typical response to PILO 16mC, applied from a direct iontophoretic chamber, in the line between the small arrows. The muscarinic receptor-dependent vasodilation is seen rising rapidly to about 1.5V amplitude. The total response measured for 4 min is 6.07 V.min (shaded area), computed by Perisoft programs.
An axon reflex evoked by 16mC ACh is shown (inset) at the same gain for comparison. Iontophoresis between small arrows.

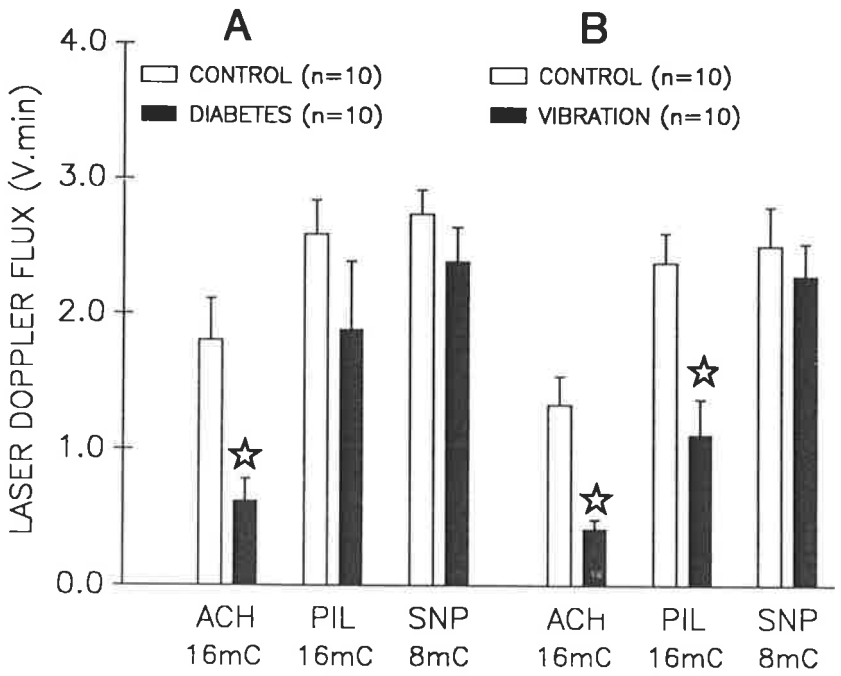


Fig 4 Diabetic versus control subjects. Histograms depicting means + SEM (bars) of neurovascular test responses to concentric application of ACh 16mC, to evoke an axon reflex; also direct application of PILO 16mC and SNP 8mC to evoke muscarinic receptor-dependent and smooth muscle dilator responses, respectively.

In A are shown responses of 10 diabetic subjects (mean age 56 yrs, mean duration of diabetes 6 yrs) and of 10 age-matched controls. B: illustrating responses of 10 subjects exposed to hand-arm vibration (maintenance workers, frequently using various hand tools, n=10, mean age 52 yrs) and 10 age-matched controls. Open columns depict control subject responses in each instance.

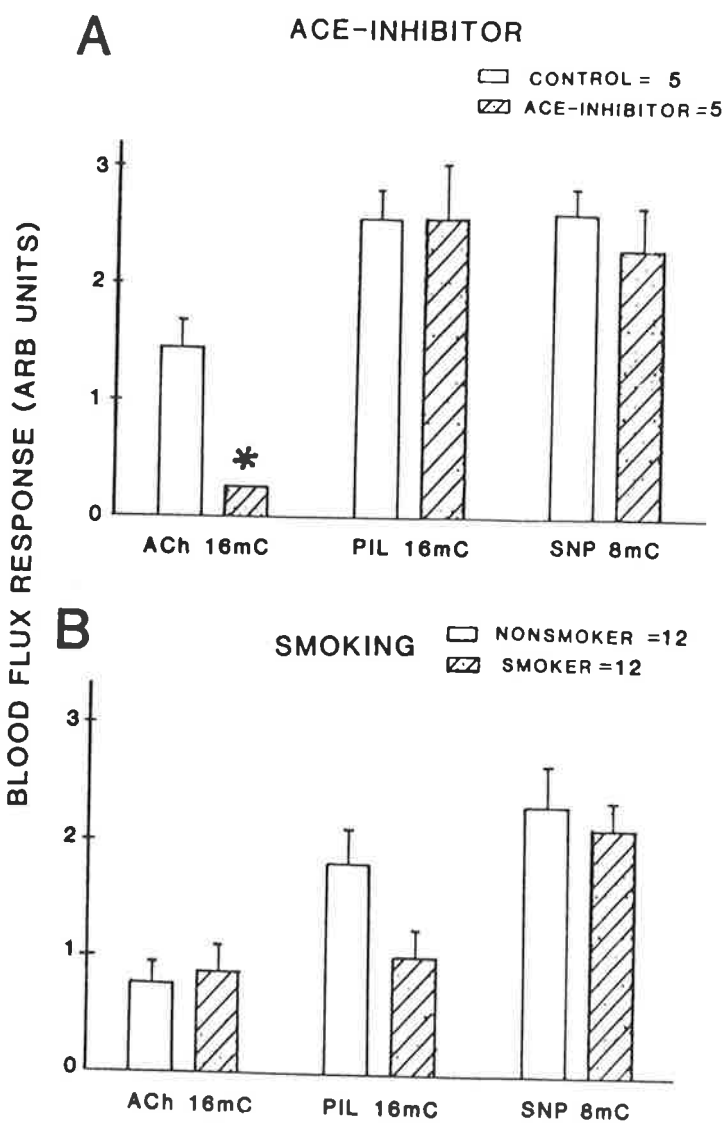


Fig 5 Smoking and ACE inhibitor versus controls. Histograms depict means + SEM (bars) of neurovascular test responses to concentric application of ACh 16mC, to evoke an axon reflex; also direct application of PILO 16mC and SNP 8mC to evoke muscarinic receptor-dependent and smooth muscle dilator responses, respectively.
A: illustrating responses of 5 subjects treated for a mean of 2 months with the angiotensin converting enzyme inhibitor enalapril 10mg bd, and 5 age-matched controls. In B are shown responses of 12 long-term smokers (mean age 51 yrs, mean duration of smoking 16 yrs) and of 12 age-matched controls. Open columns depict control subject responses in each instance.

DISCUSSION

The development of a sensitive method for measuring relative changes in microvascular blood flow was first achieved with the 'Periflux' laser Doppler velocimeter [19] (LDV) and other similar instruments soon became available [17]. Comparisons between absolute blood flow, e.g. measured by microspheres, and the flux voltage signals from LDV's showed good linear relationships for some instruments but no direct 1:1 correspondence [17]. Using the LDV to measure small increases in skin microvascular flux in response to graded electrical [8] and chemical stimuli [9,10,18] enabled neurogenic inflammation and the function of primary afferent nerves and their nociceptors to be studied. The original axon reflex of Bayliss [20] and triple response of Lewis [21] have been re-examined as part of a nocifensor or damage-control system [22]. It has been shown to be present in skeletal muscle [23], bladder and gastrointestinal mucosa [24] and even operates to modulate blood flow in the pia-arachnoid at spinal dorsal root entry zones [17].

In vivo micropharmacology using iontophoresis e.g. with LDV flux measurement, has demonstrated the sequential mechanisms of neuropeptide release from primary sensory nerve endings [24], and endothelium-dependent vasodilatation by smooth muscle relaxation [18]. The marked reduction of neurogenic inflammation in diabetes mellitus [3] has clinical significance e.g. in impaired wound healing and resistance to infection [25]. Similarly, the desensitisation of nociceptor endings by the neurotoxin capsaicin has proven clinically useful in treating some chronic pain states [26], as well as in providing a useful tool with which to explore neurovascular interactions and nociceptor mechanisms [27,28]. The relative quantitative contributions of peptide release from primary afferent nerves, mast cell histamine, other autocoids, tachykinins and prostaglandins to the total inflammatory response are all being redefined using these techniques [29,30]. Interactions with sympathetic efferent nerves which may produce skin vasodilatation [31] are also under close investigation [32].

Our finding of reduction in neurovascular responses to ACh and PILO in subjects with prolonged exposure to hand-arm vibration suggests significant small nerve fibre and perhaps also endothelial dysfunction. This is consonant with recent epidemiological findings [11,12]. These data suggest that this neurovascular test battery could prove a sensitive and useful adjunct to the medical examination for segmental vibration (AS 2763-1988) and for the investigation of Raynaud's phenomena. In considering the pathogenesis of Raynaud's phenomena whether primary or vibration-induced, the most important issue of central regulatory disturbance as compared with peripheral vascular responsiveness remains to be determined. The presence of a functional impairment of nociceptive afferents

which has now been established by quantitative sensory testing [12] has been confirmed by neurovascular function tests [15,16].

Using bretylium iontophoresis [33] to produce sympathetic blockade in skin, our non-invasive neurovascular function studies have indicated that axon reflex flare does not involve any significant participation of sympathetic postganglionic nerves [34]. By contrast, the cholinoceptors appear to have a more dynamic and complex role in axon reflex flare. Chronic smokers, exposed to regular nicotine intake, show upregulation of nicotinic receptors on nociceptive afferents resulting in significantly enhanced axon reflex flare responses [13]. Further, enhancement of ACh-evoked axon reflexes occurs during relatively brief but higher dose exposure in the rat [14], and this is likely to be due to upregulation of the nicotinic ACh-receptors on nociceptive sensory nerves [14]. The present results tested only a small group of smokers, and their nicotine intake from more than 10 cigarettes daily is uncertain, but was probably less than that for subjects in the group described by Hahn [13] who smoked more than 20 cigarettes daily for more than 15 years. Thus it is not totally at variance with the earlier reports that the present results showed only a non-significant trend for enhanced axon reflexes, and the PILO response in the smoking group bordered on significance. This suggests a possible endothelial effect of moderate smoking. Nevertheless, given the reports of Hahn [13] and of Grunfeld *et al* [14], it is possible that smoking may confound the interpretation of neurovascular tests relying on ACh-evoked axon reflexes.

Finally the results of ACE-inhibitor treatment show clearly that this compound, with its effects on the bradykinin system [35], is also readily able to modify ACh-evoked axon reflexes. Therefore caution must be exercised when interpreting the results of axon reflex tests of nociceptive function applied to diabetic subjects [3,5,10].

ACKNOWLEDGEMENTS

The support of the Australian Research Council and National Health and Medical Research Council for this study is gratefully acknowledged. Dr. Lindblad's stay as a visiting scientist at Monash University was supported by a grant from the Swedish Environment Fund and this is also gratefully acknowledged.

REFERENCES

1. Asbury AK, Brown MJ. Clinical and pathological studies of diabetic neuropathies. In: Goto Y, Horiuchi A, Kogure K (eds). Diabetic neuropathy. Amsterdam: Excerpta Medica 1982; 50-57.

2. Dyck PJ, Karnes JL, Daube J, O'Brien P, Service FJ. Clinical and neuropathological criteria for the diagnosis and staging of diabetic polyneuropathy. Brain 1985; 108:861-880.
3. Westerman RA, Widdop RE, Hogan C, Zimmet P. Non-invasive tests of neurovascular function: reduced axon reflex response in diabetes mellitus. Neuroscience Letters 1987; 81:177-182.
4. Westerman RA, Delaney CA, Ivamy-Phillips A, Horowitz M, Roberts A 1989. Concordance between different measures of small sensory and autonomic fibre neuropathy in diabetes mellitus. Clinical and Experimental Neurology 26, 51-63.
5. Consensus Statement: Report and Recommendations of the San Antonio Conference on 8-10 Feb 1988. Diabetes 37:100-104.
6. Jamal GA, Hansen S, Weir AI, Ballantyne JP. An improved automated method for measurement of thermal thresholds. 2. Patients with peripheral neuropathy. Journal of Neurology, Neurosurgery and Psychiatry 1985; 48:361-366.
7. Jamal GA, Hansen S, Weir AI, Ballantyne JP. The neurophysiologic investigation of small fibre neuropathies. Muscle and Nerve 1987; 10:537-545.
8. Westerman RA, Low A, Pratt A *et al.* Electrically evoked skin vasodilatation: a quantitative test of nociceptor function in man. Clinical and Experimental Neurology 1986; 23:81-89.
9. Parkhouse N, Le Quesne PM. Quantitative objective assessment of peripheral nociceptive C-fibre function. Journal of Neurology, Neurosurgery and Psychiatry, 1988; 51:28-34.
10. Benarroch EE, Low PA. The acetylcholine-induced flare response in evaluation of small fiber dysfunction. Annals of Neurology 1991; 29:590-595.
11. Taylor W. Bad vibrations. New Scientist 1989; 127:41-47.
12. Ekenvall L, Nilsson B, Gustavsson P. Temperature and vibration thresholds in vibration syndrome. British Journal of Industrial Medicine 1986; 43:825-829.
13. Hahn A. The effects of smoking on small nerve and blood vessel function in man. Unpublished BSc Hons Thesis. Clayton. Monash University 1990.
14. Grunfeld JA, Tiedemann GJ, Westerman RA. Chronic nicotine exposure enhances cutaneous axon reflexes in the rat. Neuro Report 1991; 2:421-424.
15. Westerman RA, Lindblad L-E, Wajnblum D, Roberts RGD. Reduced neurovascular responses in fingers of manual workers chronically exposed to vibration. Proceedings of the Australian Physiological and Pharmacological Society 1990; 21:51P.
16. Westerman RA, Lindblad L-E, Wajnblum D, Roberts RGD, Delaney CA. Confounding factors in non-invasive tests of neurovascular function in diabetes mellitus. Australian and New Zealand Journal of Medicine 1991; 21 Suppl 1:170.
17. Westerman RA, Widdop RE, Hannaford J *et al.* Laser Doppler velocimetry in the measurement of neurovascular function. Australian Journal of Physical and Engineering Sciences in Medicine 1988; 11:53-66.
18. Westerman RA, Low AM, Widdop RE, Neild TO, Delaney CA. Non-invasive tests of neurovascular functionin human and experimental diabetes mellitus. In: Molinatti GM, Bar RS, Belfiore F, Porta M (eds). Endothelial cell function in diabetic microangiopathy: problems in methodology and clinical aspects. Frontiers in Diabetes. Karger 1990; 9:127-138.

19. Nilsson GE, Tenland T, Oberg PA. Evaluation of laser Doppler flowmetry for measurement of tissue blood flow. IEEE Transactions in Biomedical Engineering, 1980; 27:597-604.
20. Bayliss WM. On the origin from the spinal cord of the vasodilator fibres of the hind limb and on the nature of these fibres. Journal of Physiology, 1901; 26:173-209.
21. Lewis T. The blood vessels of the human skin and their responses. London. Shaw & Sons 1927.
22. Lembeck F. Sir Thomas Lewis' nocifensor system, histamine and substance P - containing primary afferent nerves. Trends in Neurosciences 1983; 6:106-108.
23. Hilton SM, Marshall JM. Dorsal root vasodilatation in cat skeletal muscle. Journal of Physiology 1980; 299:277-288.
24. Szolcsanyi J. Antidromic vasodilatation and neurogenic inflammation. Agents and Actions 1988; 23:4-11.
25. Kjartansson J, Dalsgaard CJ, Jonsson CE. Decreased survival of experimental critical flaps in rats after sensory denervation with capsaicin. Plastic and Reconstructive Surgery 1987; 79:218-223.
26. Westerman RA, Roberts RGD, Westerman DA, Kotzmann RR, Widdop RE, Carter B. Action of topical capsaicin on affected dermatomes in postherpetic neuralgia. Clinical and Experimental Neurology 1988; 25:71-84.
27. Jansco N, Jansco-Gabor A, Szolcsanyi J. Direct evidence for neurogenic inflammation and its prevention by denervation and by pretreatment with capsaicin. British Journal of Pharmacology 1967; 31:138-151.
28. Kenins P, Hurley JV, Bell C. The role of substance P in the axon reflex in the rat. British Journal of Dermatology 1984; 111:551-559.
29. White DM, Helme RD. Release of substance P from peripheral nerve terminals following electrical stimulation of sciatic nerve. Brain Research 1985; 336:27-31.
30. Roberts RGD, Westerman RA, Widdop RE, Kotzmann RR, Payne R. Effect of capsaicin in cutaneous axon reflex dilation in man. Agents and Actions 1992; 35: In press.
31. Blumberg H, Wallin BG. Direct evidence of neurally mediated vasodilatation in hairy skin of human foot. Journal of Physiology 1987; 382:105-121.
32. Westerman RA, Pano I, Rabavilas A, Hahn A, Nunn A, Roberts RGD, Burry H. Reflex sympathetic dystrophy: altered axon reflex and autonomic responses. Clinical and Experimental Neurology 1992; 29: In press.
33. Kellogg DL, Johnson DM, Kosiba WA. Selective abolition of the vasoconstrictor responses in skin by local iontophoresis of bretylium. American Journal of Physiology 1989; 257:H1599-H1606.
34. Roberts RGD, Westerman RA, Lindblad L-E. Sympathetic vasoconstricor nerves and neurogenic inflammation in the skin. Proceedings of the Australian Physiological and Pharmacological Society 1992; 23:31P.
35. Eisen V. Kallikreins and kinins. In: Greaves MW, Shuster S (eds). Pharmacology of the skin I. Berlin. Springer 1989;309-330.

In: Clin Exper Neurol 29: 189-209, (1992)
Edited by M Eadie, C Lander, M Pender

HEREDITARY SENSORY RADICULAR NEUROPATHY: DEFECTIVE NEUROGENIC INFLAMMATION

R. A. Westerman*, A. Block*, A. Nunn†, C. A. Delaney*, A. Hahn*, X. Dennett‡ and R. W. Carr*

International Diabetes Institute* and Department of Prosthetics & Orthotics†, Caulfield General Medical Centre, South Caulfield, and State Neuropathology Service‡, University of Melbourne, Parkville, Victoria

SUMMARY

Hereditary sensory radicular neuropathy exhibits autosomal dominant inheritance with complete penetrance in males and incomplete penetrance in females. Newer tests of small sensory nerve function were used in screening 8 family members aged between 14 and 66 years. All exhibited some frequent features of the disorder with an onset in the 2nd or 3rd decade, foot ulceration, foot callus, loss of pin prick, thermal and light touch sensation, and some reduction in vibration acuity and proprioception in the lower limbs. The hands were involved in 3 of 8, muscle involvement was present in 5 of 8, but deafness was not detected by audiometry. Nerve conduction velocity, sensory action potentials, latency and amplitude, thermal acuity, vibration acuity and axon reflex flares were measured in all patients. One sural nerve biopsy confirmed the presence of peripheral fibre loss in this predominantly sensory neuropathy. Chemically evoked axon reflex tests were used to evaluate the extent of primary sensory nerve fibre involvement. All patients were tested using a Moor MBF 3-D dual channel laser Doppler velocimeter. Acetylcholine or phenylephrine iontophoretically applied as 16mC doses evoked absent or tiny axon reflexes in areas of impaired pin prick sensation. By contrast, direct microvascular dilator responses to nitroprusside (smooth muscle dependent) and acetylcholine (endothelium-dependent) were present but somewhat reduced in areas with defective neurogenic inflammation. These results differ significantly from the responses obtained in age-matched healthy controls ($P<0.05$). Foot pressure analysis was performed for orthoses in 2 affected members with foot ulceration using the Musgrave Footprint system. The utility of these noninvasive measures of small sensory fibre function in family screening is exemplified in this study.

Although the condition of hereditary sensory radicular neuropathy (HSRN) had been described previously under various titles [1,2,3,4,5], a definitive account was provided by Wallace [6] in 1970. In his monograph [6] he reported the results of personal interviews and questionnaires involving more than 400 members of the 'E' family in which at least 42 members were affected. The present study describes the results of tests performed on 8 new subjects with this condition, 3 of whom have a certain kinship with the 'E' family of Wallace [6]. Although the condition exhibits autosomal dominant inheritance, not all family members are affected [6] and therefore screening of younger members of affected families is important. The purpose of this report is to describe the results of newer quantitative tests of small sensory nerve function in these families and to demonstrate the utility of such quantitative tests (vibration & thermal perception threshold [7,8,9], and sudomotor axon reflex [10,11]) in noninvasive screening of putatively affected family members at an earlier age.

This disorder has a variable presentation, usually at puberty or some time later. The first sign is nearly always the occurrence of some injury which leads to a blister or ulcer on the foot, which becomes indolent. At this stage the distal sensory loss may be discovered, but it may precede the initial injury and ulceration. A detailed description is provided by Wallace in pages 13-34 of his monograph [6].

METHODS

The 8 test cases presently studied were aged between 14 and 66 years, 3 having a family tree complete from 1833, on which their position is shown in Fig 1 (modified and extended from Fig 4 of Wallace, with permission). The present test cases were interviewed, examined, and their clinical features and quantitative sensory testing (QST) results were recorded. These results included thermal perception thresholds for warm and cold using a Medelec TTT device [7,8,12], vibration perception thresholds using a Biothesiometer model PVD [9], and axon reflex skin flare evoked by iontophoretic application of acetylcholine [10,11,13] (ACh) or phenylephrine (PE) in a circumferential chamber and measured by a Moor MBF3D laser Doppler flowmeter or a Periflux Pf1d flowmeter. Similarly, direct application of a smooth muscle nitrodilator (sodium nitroprusside - SNP) allowed measurement of microvascular reactivity [14].

Histopathology

A sural nerve biopsy from one affected family member taken at the age of 30 years was fixed in 2.5% phosphate buffered glutaraldehyde, followed by osmication and dehydration through graded acetone prior to embedding in Spurr's (Bio-Rad) resin. Thin sections were examined in a Siemens electron microscope.

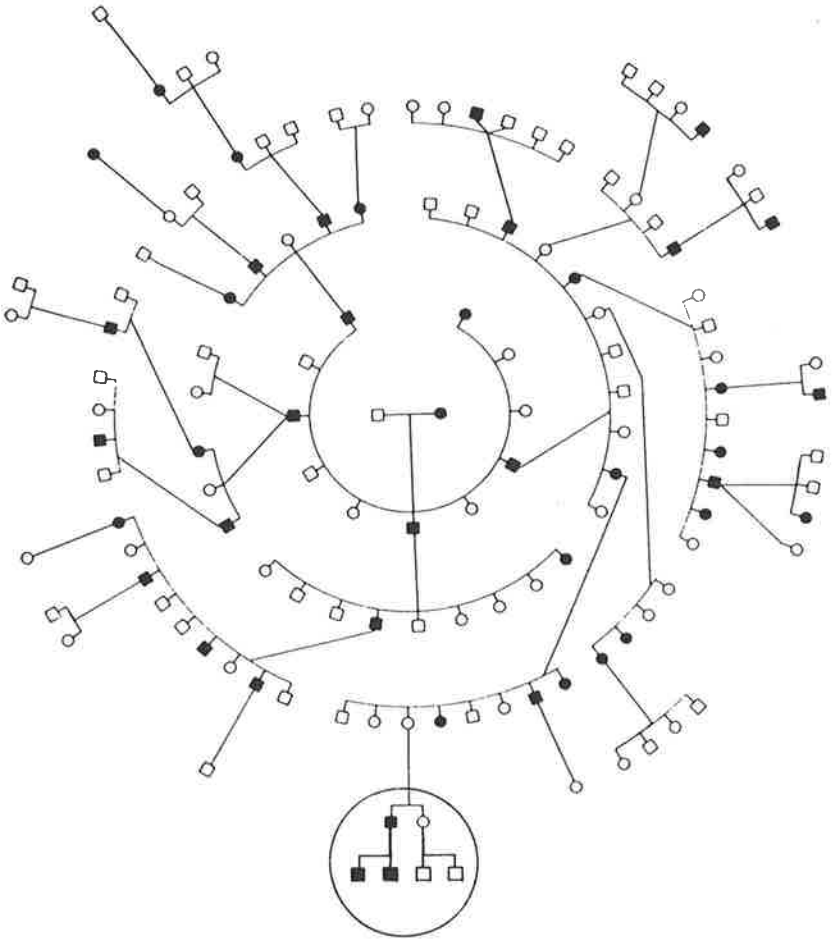


Fig 1 Abbreviated family tree of Wallace's 'E'-family (modified with permission) illustrating inheritance of hereditary sensory radicular neuropathy in 3 of 8 present cases shown in the circle.

Vibration perception threshold

The measurements of vibration perception threshold were made using a hand-held biothesiometer model PVD (Biomedical Instruments, Newbury, Ohio, USA) whose plastic probe vibrated at 100Hz. The linear scale of 0 - 50 arbitrary units reflects the applied voltage which is proportional to the square root of the amplitude of vibration. All measurements were made in a quiet room maintained comfortably warm at 22-24°C. The weight of the instrument was used to maintain a firm but gentle pressure against the following bony prominences(test sites):
elbow - olecranon, ulnar styloid; finger - tip of thumb, 2nd metacarpal head; knee -fibular head, patellar tendon, tibial tuberosity; feet - medial and lateral malleoli of ankle; toes - tip of great toe, first metatarsal head. Subjects were familiarized with the buzzing sensation of the probe against the tip of the thumb and the voltage was gradually increased until the vibration was just felt, and then decreased until it just disappeared. Shoes and socks were removed and then the foot and toe sites were tested in the same way. Variability of this procedure was less than 8% for 4 normal subjects tested repeatedly at the same foot and toe sites over 2 periods of 5 consecutive days (see also Wiles *et al*[7]). Results were expressed individually for each of the 8 subjects and plotted against the age-related norms of Wiles *et al*[7].

Thermal perception threshold

All test subjects had warm and cold thresholds measured on the anterior aspect of the wrist, and on the dorsum of both feet, using a Medelec TTT device. In this automated thermal threshold test[8,9,12], warm or cold stimuli were delivered to the skin surface through a Peltier thermode. The magnitude and duration of the applied current regulated the thermal stimulus and a constant rate of change of temperature (1°C per sec) was provided for each stimulus. Thermal stimuli were presented only during one of 2 time windows shown to the subject by a pair of illuminated light-emitting diodes and the subject indicated in which time period each stimulus occurred by using a switch. The subject's success rate was analysed by the microprocessor using the 'up-down transform rule' to compute the temperature which the subject could detect reliably.

Sudomotor axon reflexes

Subjects were either test cases, or age and gender- matched controls, and were tested seated comfortably in a recliner chair. When the forearm was being tested, the arm was comfortably positioned on the arm of the chair, with its volar surface facing upwards. For testing the foot dorsum, the leg was extended and supported approximately 30cm above the ground. Relative changes in skin blood flow were measured with a laser Doppler velocimeter (Moor MBF3D or Periflux Pf1d). The LDV measured basal blood flow while sitting in a plastic probe-holder that was fixed to the skin by double-sided adhesive discs (3M). A battery powered constant current source (WPI A360) was used to provide a direct (galvanic) current for drug iontophoresis. The drugs were dissolved in distilled water to produce 1% aqueous solutions, and were introduced into the perspex chamber/probe holder by a syringe attached by teflon tubing to the stainless needle/electrode (Fig 2). An indifferent electrode of cotton gauze wet with distilled water was attached to either the wrist or the ankle of the subject. After inserting the laser probe

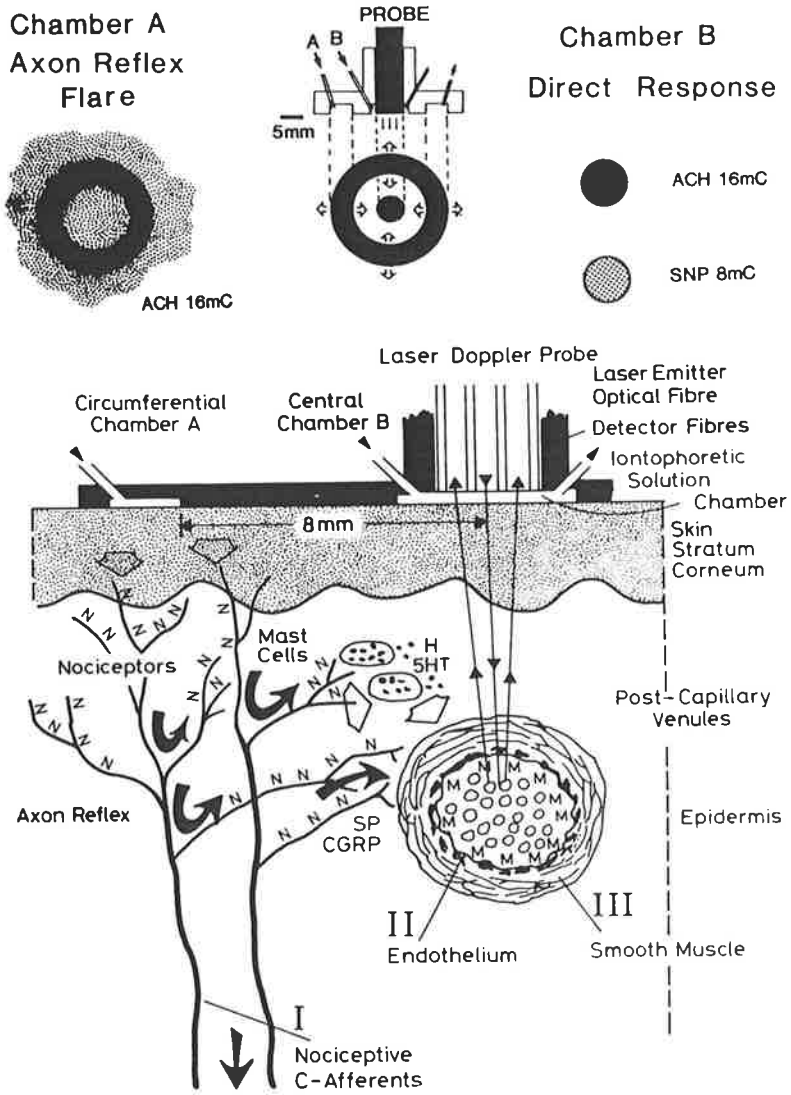


Fig 2 Summarises skin sensory nerve axon reflex mechanisms. Diagrammatic section (top, centre) of laser Doppler probe in perspex holder attached to skin, with outer circumferential iontophoretic chamber A, and central chamber B for direct application of dilator compounds under the laser probe. Polymodal nociceptors, stimulated by ACh iontophoresis, release neuropeptides which excite adjacent targets (nociceptors, mast cells, microvascular endothelium) initiating the inflammatory cascade and the spreading neurogenic flare (shown in loose stipple, above). Direct application of ACh, or sodium nitroprusside (SNP) via chamber B produces the small direct dilator responses illustrated at right by filled circle (ACh), and dense stipple (SNP), respectively.

and recording basal blood flow for one or 2 minutes, an appropriate anodal or cathodal current was applied to introduce the desired ions into the epidermis. The product of DC current level x time defined the dose of drug introduced, and was expressed as total charge transferred in milliCoulombs (with current in mA and duration in sec). The blood flux changes occurred in response to activation of the microvascular endothelium, either by the peptides released by an axon reflex from nociceptive afferents, or by direct endothelial or smooth muscle activation. Flux change was measured as the voltage.time integral by a digitising programme (Sigmascan) during the 4 minutes following iontophoretic application of a drug. A more detailed description of the iontophoretic technique is given in Westerman *et al* [14].

Nerve conduction velocity

For those test cases who had not already had NCV's measured by a clinical neurophysiologist prior to attendance, a Cadwell 5200A portable EMG machine was used to record the median and ulnar sensory nerve conduction velocity, action potential amplitude and latency in the hands from the fingers to the wrist, and in the feet or legs from the medial plantar and sural nerves. Electrode placements and techniques were those of De Lisa and McKenzie [15].

The Musgrave Footprint system

To measure foot pressure distribution with time during repeated steps, subjects walked over 2 footplates each with an FSR (force sensing resistor) containing a grid array of 2048 (64 x 32) pressure sensitive sensors of 5 x 5 mm. These were calibrated individually and mounted in the footprint system using a M-30-K load cell. These were electronically scanned at 68Hz (136,533 sensors/sec), and the data were read into an IBM-AT compatible computer with an 80386 SX/DX or 80486 SX/DX CPU.

The Musgrave Footprint analysis software was convenient to use, and ran under MSDOS (version 3.3 or later). It was possible to export the data in a standard format readable by most commercial spreadsheets (such as Lotus 123, Supercalc, Excel etc), database managers (dBaseV, Foxbase, R-Base etc) and statistical packages (SPSS, MathStat etc). If graphical manipulation was required it was possible to do this via the facilities offered by Windows Version 3.1. A standard Hewlett Packard Paintjet was used for the colour printouts of selected screens. Foot pressures during the cycle were analysed in many different ways. These included comparisons of stance phase (e.g. contact, mid-stance, propulsive phase), foot-ground sequences, centre of load (one or both feet). Single sensors or groups of sensors* could be selected to provide load/time* and % pressure/time* graphs. A 'GRID' array of sensors was selectable providing peak pressure values of each individual sensor as well as the values for each sensor per scan. There was also an option to analyse certain foot statistics on screen (length, width, angles, angle changes, distances).

Examples of asterisked analysis methods are given in Figs 9 and 10.

Statistical methods

Statistical analysis of the data was performed using the SPSS-X student package on an IBM PC 386-compatible microcomputer, and the Sigmaplot 4.0 statistical routines. A nonparametric test (Wilcoxon Signed Rank test) of significance was used to compare neurovascular test results between groups.

RESULTS

Features of hereditary sensory radicular neuropathy

Table 1 provides a summary of the clinical details of the 8 test cases in the present cohort and some features of the condition, while the relative frequency of findings in this group is compared with that of Wallace's group in Fig 3. Concordance was generally good, but in neither of the present families was spina bifida evident, while muscle involvement, proprioceptive loss and impaired vibration acuity were much more evident.

Table 1 Summary of findings present 8 cases

CASE NUMBER	AGE AT ONSET	SEX	FOOT ULCERATION	FOOT CALLUS	LIGHT TOUCH LOSS	TEMPERATURE LOSS	PIN PRICK LOSS	PROPRIOCEPTIVE LOSS	PRESSURE LOSS	VIBRATION SENSE LOSS	HAND INVOLVEMENT	SWEATING INVOLVEMENT	MUSCLE INVOLVEMENT	PROGRESSION	PARAESTHESIA, PAINS	LIGHTENING PAINS	DEAFNESS AUDIOGRAM	SENILE CATARACT	SPORTING ABILITY	DUPUYTREN CONTRACTURE	EYE COLOUR	HEIGHT (cm)	WEIGHT (kg)	AGE WHEN TESTED	ANKLE JERKS	SPINA BIFIDA
1	14	M	-	+	+	+	-	-	+	+	-	+	-	-	+	+	-	-	+	-	HAZEL	183	95	14	-	-
2	14	M	-	+	+	+	+	+	+	+	-	-	+	-	+	-	-	-	+	-	HAZEL	183	65	18	-	-
3	18	M	-	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	+	-	BLUE	182	65	19	-	-
4	15	M	+	+	+	+	+	+	+	+	-	-	+	+	-	+	-	-	+	-	HAZEL	185	76	25	-	-
5	24	M	+	+	+	+	-	-	+	+	+	+	+	+	+	+	-	-	+	-	BROWN	183	125	36	-	-
6	20	M	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	-	-	HAZEL	175	125	44	-	-
7	24	M	+	-	+	+	-	+	+	+	-	+	-	+	+	-	-	-	-	-	GREY	175	82	64	-	-
8	36	F	-	-	+	+	+	+	+	+	+	-	+	-	+	+	-	-	-	-	HAZEL	172	115	41	-	-

Histopathology

In one of the family members a sural nerve biopsy had been performed 12 years before when this patient was undergoing neurological diagnostic investigation of his severe sensory neuropathy. An electronmicroscopic montage of portion of the nerve recently taken from the file block of nerve is shown in Fig 4. Examination with both light and electron microscopy showed a complete absence of myelinated nerve fibres within the nerve fasciculus. Increased amounts of endoneurial collagen were present. There was no significant perineurial thickening.

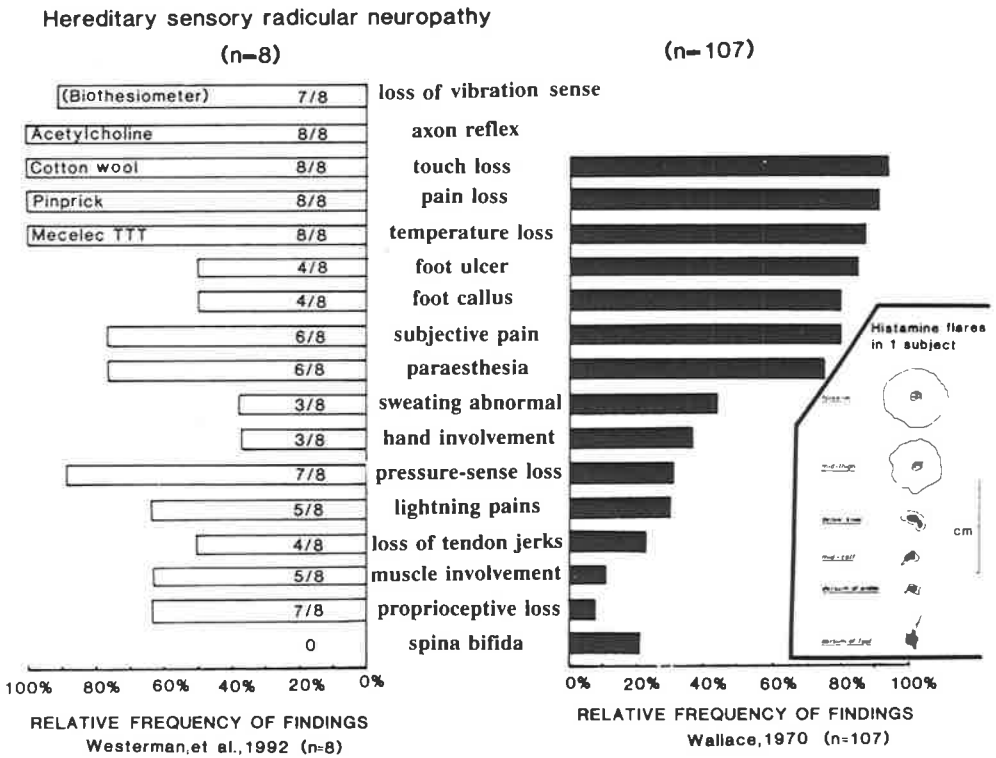


Fig 3 Relative frequency of findings in hereditary sensory radicular neuropathy compared for Wallace[6], and the present cohort of test cases (modified with permission). Inset shows for one subject the decreasing size of axon reflex flares evoked by 0.05ml of 0.1% histamine injected intradermally. Sites from above downwards are on forearm, mid-thigh, below knee, mid-calf, dorsum of ankle, and dorsum of foot.

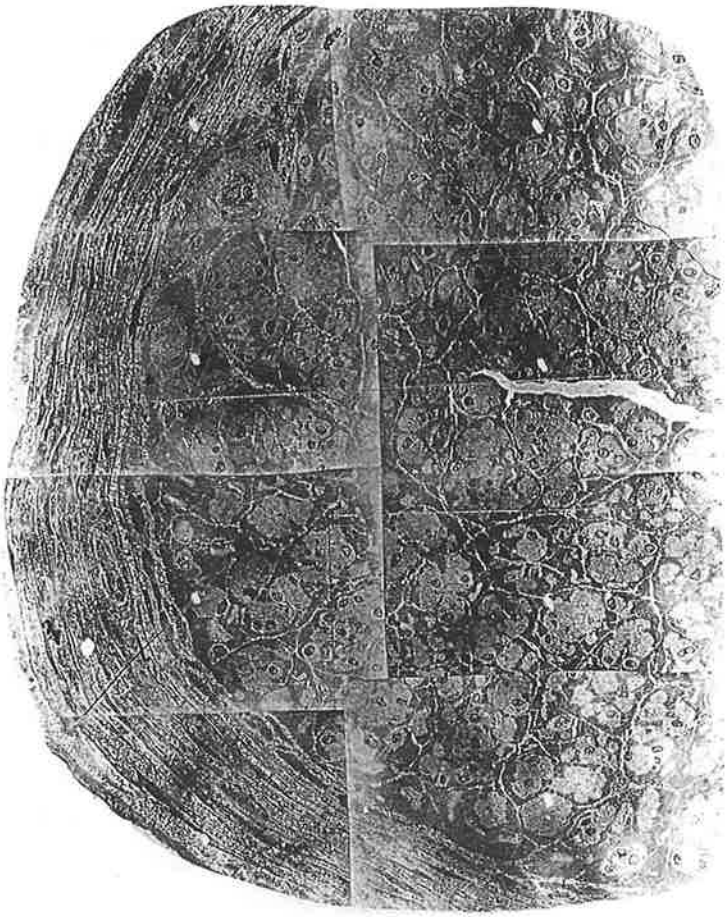


Fig 4 Electronmicrographic montage of sural nerve biopsy from one affected HSRN family member at age 33 years, showing complete absence of myelinated nerve fibres. Calibration bar 10μm. Inset: higher magnification shows marked reduction in unmyelinated nerve fibres. Calibration bar 2μm.

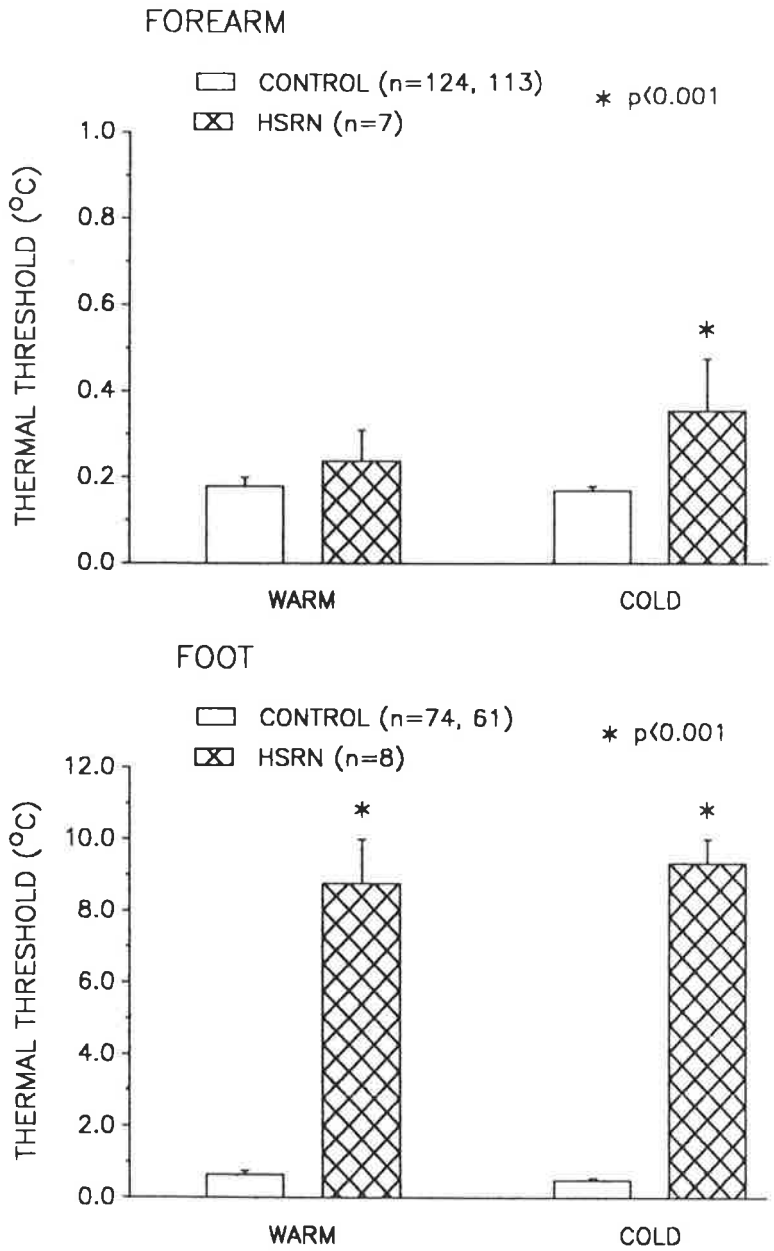


Fig 6 Thermal acuity measured by the Medelec TTT device for the test cohort compared with age and gender matched normal controls. (Note scale change on abscissa between forearm and foot).

Vibration acuity

The vibration sensitivity of the 8 HSRN test cases in this study, measured by means of a Biothesiometer model PVD, is shown for upper and lower limb test sites in Fig 5. Only the youngest 3 (14,19,21 years) of these family members showed vibration perception thresholds conforming closely to the normal range for age (Wiles *et al* [7]) over most of the test sites. Older family members (aged 25 to 66 years) all showed some impaired vibration acuity at the great toe or first metatarsal head (column E) and at the ankle malleoli (column D).

Thermal acuity

Fig 6 shows the sensitivity to warm and cold stimuli applied over the dorsum of the feet and the volar surface of the wrists. Both warm and cold thermal perception thresholds were elevated for all HSRN test cases on the feet, consistent with impaired thermal acuity in this condition.

Chemically evoked axon reflex flare

Typical results for this test of nociceptive sensory nerve function on the foot dorsum of 3 of the 8 HSRN family members are provided in Fig 7. It is evident that the axon reflex dilator responses to ACh and PE are dramatically reduced, while the endothelial responses to ACh applied directly via chamber B (Fig 2) or the smooth muscle responses to direct SNP are larger, but variable. The pooled results of skin vascular reactions to iontophoresis for the 8 family members, and for 8 age and gender matched healthy subjects are shown in histograms (Fig 8). The most significant difference between the groups is the reduction of the ACh-evoked axon reflex in HSRN test cases as compared with controls. The dilator responses to direct ACh and SNP are just significantly reduced for the HSRN group compared with the control group.

Analysis of foot pressures during the step cycle was performed on the Musgrave Footprint system. This is illustrated in Figs 9 and 10 for one affected family member (aged 36 years, body weight 120kg) with foot ulcers and osteomyelitis. His maximum pressures were displayed in a grid array (A) for all sensors in the footplate, and then an area corresponding to the metatarsal heads was selected for enlargement and display (B). From these, 3 high pressure regions were chosen and pressure/time graphs were printed for each of these areas, shown in Fig 9C. In the same subject Fig 10 shows the total load/time plot performed for the left foot in A, with no covering on the foot, and in B when wearing an orthosis. The total load is shown plotted against time for both feet in Fig 10C; for the left foot during walking the peak load during propulsion was 143.3% of bodyweight (120 kg), but for the right foot during corresponding step cycles the peak load propulsion was 98.4% of bodyweight. The further

analysis with centre of load showed a rolling from medial to lateral with the left foot bearing the disproportionate loading during the step cycle. Wearing the orthosis gave evidence of effective load reduction on areas subjected to peak pressure.

DISCUSSION

Consequences of sensory neuropathy

Injury or noxious stimulation of skin or deeper tissues results in excitation of nociceptive afferent nerves leading to pain sensation, behavioural modification and a neurogenic inflammatory response [16,17]. Thus nociceptive sensory nerves should now be considered as sensory and motor in function, both types of responses being nocifensive [18,19].

In normally innervated skin neurogenic inflammation is seen as a pink flare[20], but is abolished by denervation [21,22]. The neuropeptides released in this nocifensor reaction [23,24] aid wound healing [25,26,27], but in patients with severe sensory neuropathy due to causes such as diabetes mellitus [22] or HSRN the protective neurogenic responses are reduced or absent [22]. The progressive sensory loss in HSRN usually results in severe injury and often deformity [6] and this is most evident in the most severely neuropathic family members. Although the condition shows an autosomal dominant inheritance, not all family members are affected, and therefore screening of younger potentially affected family members is important. To this end, genetic linkage studies are in progress elsewhere [28].

The present report describes a small cohort involving 8 members of 2 families. Because the major defects in HSRN appear to relate to the progressive severe sensory neuropathy, conventional and quantitative sensory testing [11] of small and large nerve fibre function was performed. Vibration acuity, which depends upon larger myelinated fibres, was reduced in the older affected family members (Fig 5), which is consistent with the results of the sural nerve biospy (Fig 4) indicating severe involvement of large and small nerve fibres.

Small sensory nerve fibre function

Only clinical tests of small sensory nerve fibre function (pinprick, temperature) were performed by Wallace [6], except for recording skin flares to intradermal injections of 1:1000 histamine in 'several' affected individuals. He interpreted the absence of the normal flare surrounding the wheal as due to degeneration of peripheral processes of the spinal root ganglion neurones. His illustrative figure, reproduced as an inset to Fig 3, graphically demonstrates the absence of neurogenic flare in this case (VI,115). Further, his clinical descriptions of the sensory changes closely resemble those commonly recorded in diabetic sensory neuropathy [29].

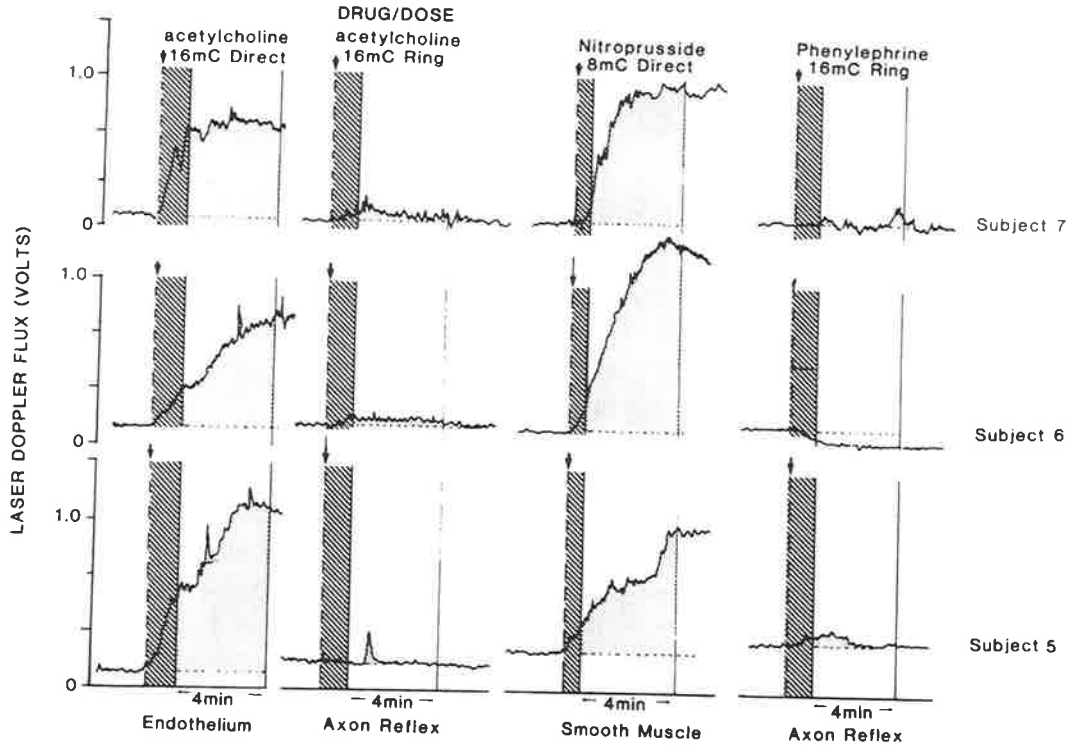


Fig 7 Representative axon reflex and direct dilator responses obtained from the foot dorsa of test Cases 5,6,7. The measurement time after the end of iontophoretic drug application is 4 min (shown on the ordinate) and laser Doppler flux (volts) is given on the abscissa. The iontophoretic period (either 80s or 40s) is shown by cross-hatching, while the total flux change in 4 min is shown by the stippled zone above the resting flux line. Substance iontophoresed, chamber used and dose are shown above, and the target structure is shown below the flux records.

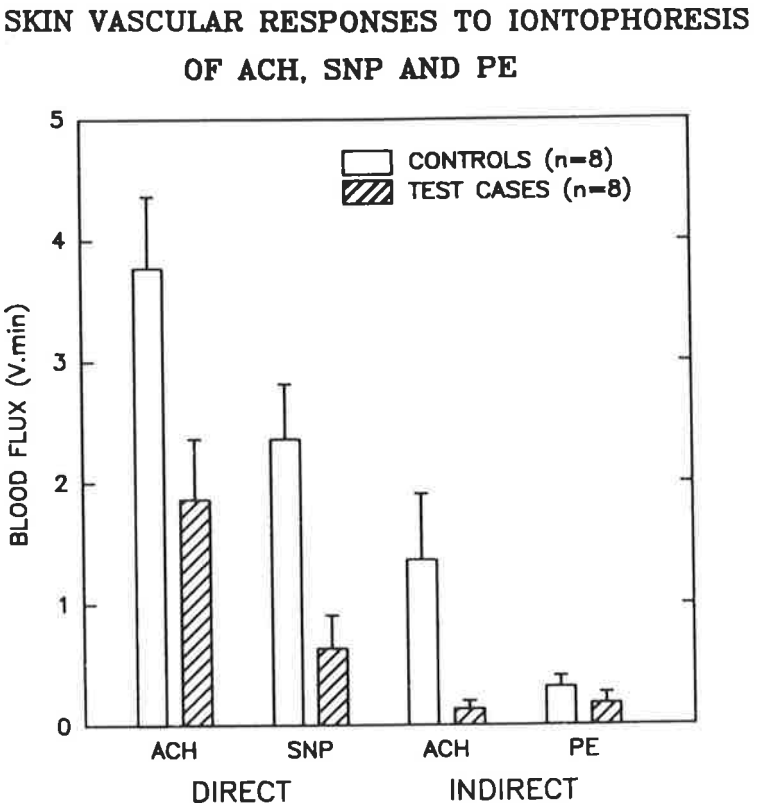


Fig 8 Histograms showing means and standard deviations of the skin vascular reactions on the foot dorsa of all 8 test cases for the drugs applied directly (ACh, SNP) or indirectly in a circumferential chamber to evoke axon reflexes (ACh, PE).

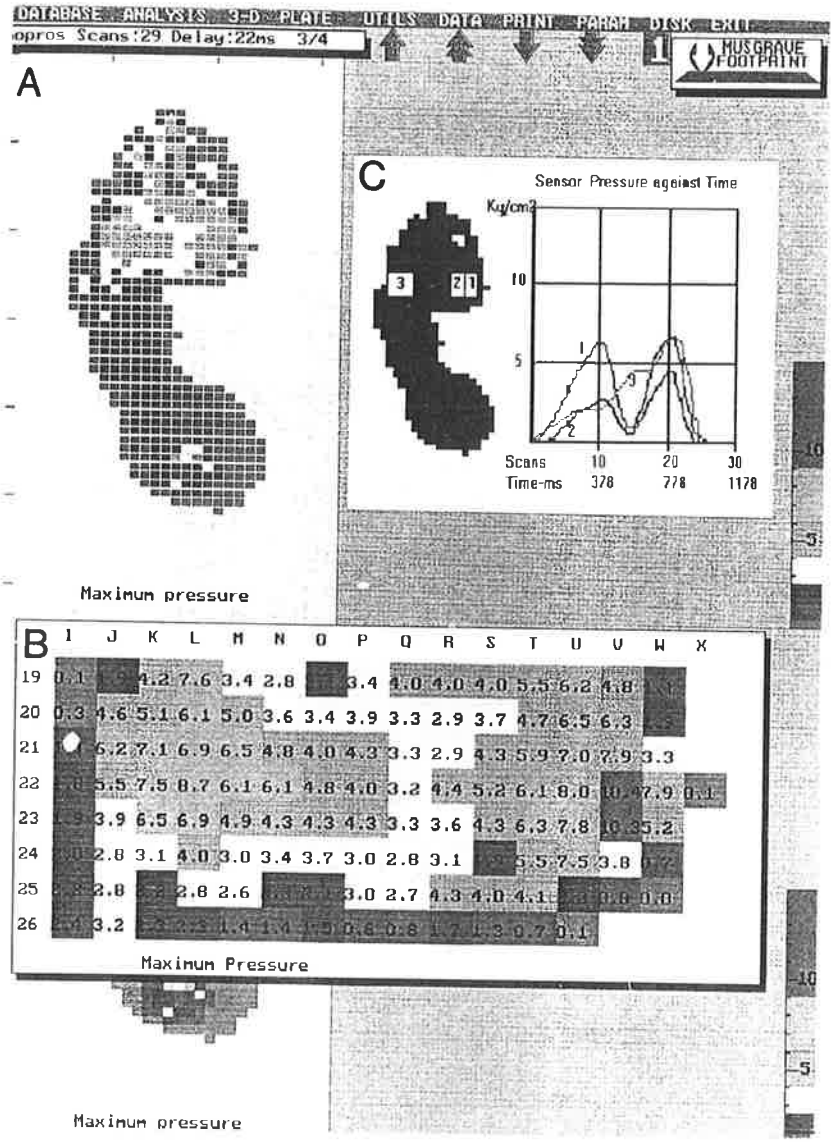


Fig 9 Foot pressure analysis using the Musgrave Footprint.
In one affected family member (aged 36 years, body weight 120kg) who had foot ulcers and osteolysis, foot pressures were recorded for repeated steps without shoes or an orthosis. A grid array of maximum pressures for each sensor is shown in A, with the black rectangle defining the area to be enlarged. In B the selected area for maximum individual sensor pressures is enlarged, with peak pressure values for each sensor being identified and displayed. From these, in the inset C, 3 major high pressure sensor areas are selected and pressure/time graphs displayed for the chosen scans and areas.

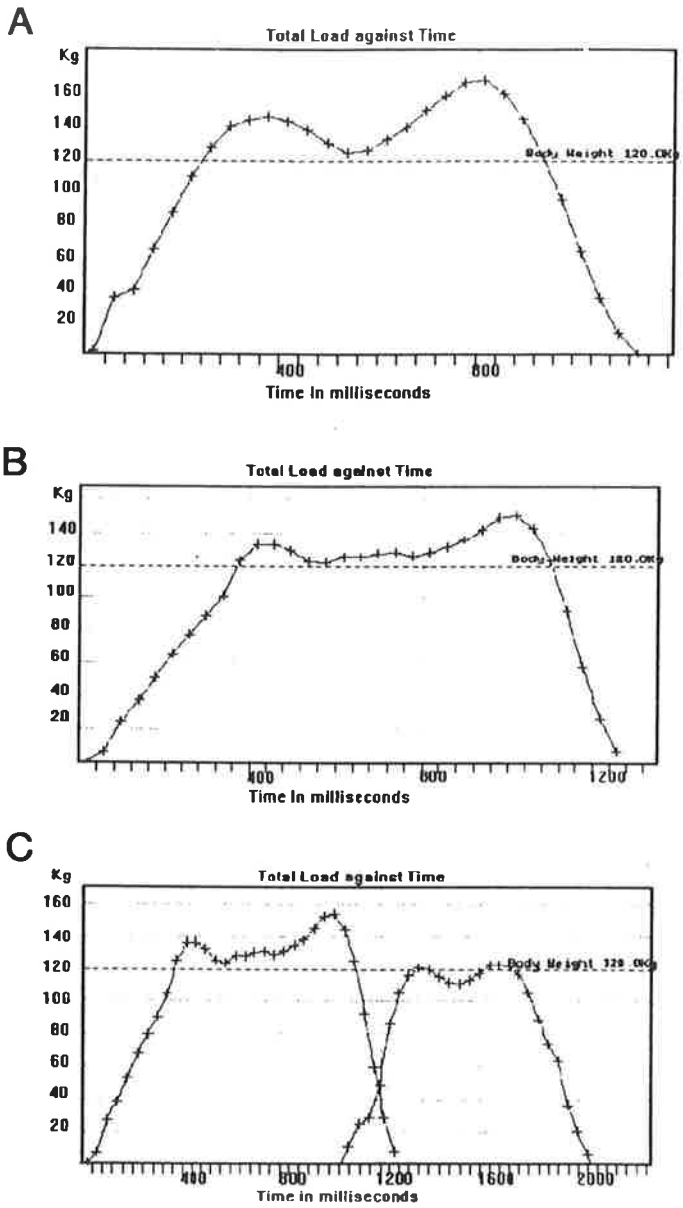


Fig 10 Foot pressure analysis using the Musgrave Footprint.
In the same subject as Fig 9, total load/time displays are shown for the left foot without an orthosis (A) and with an orthosis (B) being worn during the step recording. The pressures versus time plot for both the right and left foot during corresponding step cycles is shown in C. Note that the peak total load during propulsion for the left foot without a prosthesis is 171.9 kg (143.3% of bodyweight), while that for the right foot shows a peak total load of 118kg (98.4% of bodyweight).

The impairment of thermal acuity that was clearly evident in the present cohort, and which was more striking in older (33-66 years) than in younger (14-25 years) family members, is consistent with Wallace's findings. Warm sensation is conveyed by both C-thermal and C-polymodal fibres, so that a greater deficit in warm perception than in cold perception could be expected if the smallest fibres are affected earliest and most severely.

This provided the rationale for the axon reflex testing by iontophoretic application of ACh, which is known to excite nociceptive afferents by nicotinic cholinoceptors on the primary sensory nerves [30]. The results show a striking and significant reaction in the ACh-evoked axon reflex on the dorsum of the foot of affected family members, and this is more severe in the older subjects. A surprising finding was the reduction of the microvascular smooth muscle response to the direct nitrodilator SNP in the HSRN group compared to the age matched controls. This might suggest that expression of the gene defect does not only lead to a progressive loss of dorsal root ganglion cells, particularly of the smallest type, but is also expressed at the microvascular smooth muscle. This is possible because the exact nature of the genetic defect is unknown, but it is not easy to relate it directly to the sensory nerve deficit because the dilator mechanism in the 2 situations is different. Certainly axon reflex dilator responses are endothelium-dependent [13], occurring via nitric oxide release by endothelium. Also, alterations in the number and density of ACh-receptors on target neurones have been demonstrated [30]. However, the mode of action of the nitrodilator SNP appears to be by the release of nitric oxide in smooth muscle, and occurs in the absence of endothelium [31]. By contrast, axon reflexes evoked by neurotransmitters which act on sensory nerves, such as acetylcholine or histamine, trigger excitation of the nociceptor and then a cascade involving release of neuropeptides. These act on the endothelium which in turn relaxes the smooth muscle by release of nitric oxide, and this 'axon reflex' vasodilation does not occur if endothelium is removed or inactivated [13,31]. This smooth muscle dysfunction in HSRN remains to be confirmed in a larger number of cases, and its pathological significance remains unknown.

Screening tests and risk assessment

In terms of screening tests, the 'low-tech' classical test of nociceptive sensation viz. pinprick, as carefully applied by Wallace [6], appears to offer much. Chemical axon reflex tests can be performed either by iontophoresis, as in the present study, or by simple intradermal injection of histamine, as illustrated by Wallace [6]. This is another specific test of nociceptive sensory function and the present results suggest that this test, together with pinprick acuity, could be the most useful early screening tests in younger family members. Quantitative

sensory tests of thermal acuity also showed high concordance, but vibration acuity was less sensitive in the youngest test cases. Family counselling, instruction and explanantion of HSRN is important.

Finally, the risks of foot damage in the neuropathic foot are just as great in HSRN as in severe diabetic neuropathy [32]. Analysis of foot pressure distribution during walking, using instruments such as the Musgrave Footprint system, can greatly assist the prosthetist/orthotist and supervising physician to select the best combination of orthosis, protective footwear, and other techniques such as double-knit socks. The aim is for optimum weight redistribution to avoid dangerous peak pressures with prolonged durations during the step cycle. Foot pressure analysis also provides information to assist in decisions regarding reconstructive surgery or amputation.

In the longer term prospectively, genetic linkage studies are unquestionably the most important ones, and particularly the studies of the Sydney group [28].

ACKNOWLEDGEMENTS

The authors gratefully acknowledge that this research was performed with equipment provided by the Shepherd Foundation, the Clive and Vera Ramaciotti Foundation, the Lillian Cooper Foundation, and the Balwyn Rotary Club. Without this most generous support such detailed quantitative sensory testing would not have been possible. The authors are also most grateful for the continued support and facilities provided by the International Diabetes Institute, and for technical assistance of Mrs Libby Prior, Ms Angela Forsyth in typing and Ms Dianne Clare for illustrations. It is a pleasure to acknowledge the technical skills of Mrs Anna Friedhuber and Dr R A McD Anderson for helpful comments. We are also much indebted to Preston Communications Ltd, suppliers of the Musgrave Footprint system who enable us to analyse the foot pressures of 2 affected family members using borrowed hardware and software. The authors most gratefully acknowledge the analysis skills of Miss Kate Tuck using Musgrave Software.

REFERENCES

1. Hicks EP. Hereditary perforating ulcer of the foot. Lancet, 1922; 1:319-321.
2. Denny-Brown D. Hereditary sensory radicular neuropathy. Journal of Neurology, Neurosurgery and Psychiatry, 1951; 14:237-252.
3. Van Bogaert L. Familial ulcers, mutilating lesions of the extremities, and acro-osteolysis. British Medical Journal 1957; 11:367-371.
4. Dyck PJ, Kennel AJ, Magal IV, Kraybill EN. A Virginia kinship with hereditary sensory neuropathy, peroneal muscular atrophy and pes cavus. Proceedings of the Mayo Clinic 1965; 40:685-694.

5. Pinsky L, Di George AM. Congenital familial sensory neuropathy with anhydrosis. Journal of Pediatrics 1966; 68:1-13.
6. Wallace DC. Hereditary sensory radicular neuropathy. A family study. Glebe, Australasian Medical Publishing Co., 1970.
7. Wiles PG, Pearce SM, Rice PJS, Mitchell JMO. Vibration perception threshold: Influence of age, height, sex and smoking, and calculation of accurate centile values. Diabetic Medicine 1991; 8:157-161.
8. Jamal GA, Hansen S, Weir AI, Ballantyne JP. An improved automated method for measurement of thermal thresholds. 1. Normal subjects. Journal of Neurology, Neurosurgery and Psychiatry 1985; 48:354-360.
9. Jamal GA, Hansen S, Weir AI, Ballantyne JP. An improved automated method for measurement of thermal thresholds. 2. Patients with peripheral neuropathy. Journal of Neurology, Neurosurgery and Psychiatry 1985; 48:361-366.
10. Parkhouse N, Le Quesne PM. Quantitative objective assessment of peripheral nociceptive C-fibre function. Journal of Neurology, Neurosurgery and Psychiatry 1988; 51:28-34.
11. Consensus Statement: Report and Recommendations of the San Antonio Conference on 8-10 Feb, 1988. Diabetes 37:100-104.
12. Jamal GA, Hansen S, Weir AI, Ballantyne JP. The neurophysiologic investigation of small fibre neuropathies. Muscle and Nerve 1987; 10:537-545.
13. Westerman RA, Widdop RE, Low A, Neild TO, Delaney CA. Non-invasive tests of neurovascular function in human and experimental diabetes mellitus. In: Molinatti G, Bar R, Belfiore S, Porta M (Eds). Frontiers in Diabetes: 1989;9:127-138.
14. Westerman RA, Widdop RE, Hannaford J, *et al.* Laser Doppler velocimetry in the measurement of neurovascular function. Australasian Physical & Engineering Sciences in Medicine 1988; 11:53-65.
15. De Lisa JA, McKenzie K. Nerve conduction velocity techniques. New York; Raven Press, 1st edn 1980.
16. Lewis T. The blood vessels of the human skin and their responses. London; Shaw & Sons, 1927.
17. Blumberg H, Wallin BG. Direct evidence of neurally mediated vasodilatation in hairy skin of human foot. Journal of Physiology 1987; 382:105-121.
18. Lewis T. The nocifensor system of nerves and its reactions. I. British Medical Journal 1937; 1:431-434. II. British Medical Journal 1937; 1:491-494.
19. Lembeck F. Sir Thomas Lewis' nocifensor system, histamine and substance P - containing primary afferent nerves. Trends in Neurological Science 1983; 6:106-108.
20. Westerman RA, Magerl FW, Szolcsanyi J, *et al.* Vasodilator axon reflexes. In: Vanhoutte PM (Ed) Vasodilatation: Vascular smooth muscle, peptides, autonomic nerves and endothelium. New York; Raven Press. 1988; 107-112.
21. Jansco N, Jansco-Gabor A, Szolcsanyi J. Direct evidence for neurogenic inflammation and its prevention by denervation and by pretreatment with capsaicin. British Journal of Pharmacology 1967; 31:138-151.

22. Westerman RA, Widdop RE, Hogan C, Zimmet P. Non-invasive tests of neurovascular function: reduced responses in diabetes mellitus. Neuroscience Letters 1987; 81:177-182.
23. Kenins P, Hurley JV, Bell C. The role of substance P in the axon reflex in the rat. British Journal of Dermatology 1984; 111:551-559.
24. White DM, Helme RD. Release of substance P from peripheral nerve terminals following electrical stimulation of sciatic nerve. Brain Research 1985; 336:27-31.
25. Kjartannson J, Dalsgaard C-J. Calcitonin gene-related peptide increases survival of a musculocutaneous critical flap in the rat. European Journal of Pharmacology 1987; 142:355-358.
26. Kjartansson J, Dalsgaard C-J, Jonsson C-E. Decreased survival of experimental critical flaps in rats after sensory denervation with capsaicin. Plastic and Reconstructive Surgery 1987; 79:218-223.
27. Nilsson J, von Euler A, Dalsgaard C-J. Stimulation of connective tissue cell growth by substance P and Neurokinin A. Nature 1985; 315:61-62.
28. Nicholson G. Personal communication, 1992.
29. Benarroch EE, Low PA. The acetylcholine-induced flare response in evaluation of small fiber dysfunction. Annals of Neurology 1991; 29 (6):590-595.
30. Grunfeld JA, Tiedemann GJ, Westerman RA. Chronic nicotine exposure enhances cutaneous axon reflexes in the rat. NeuroReport 1991; 2:421-424.
31. Tare M, Parkington HC, Coleman HA, Neild TO, Dusting GJ. Hyperpolarization and relaxation of arterial smooth muscle caused by nitric oxide derived from the endothelium. Nature 1990; 346:69-71.
32. Boulton AM. The diabetic foot. (1988). Medical Clinics of North America 72:1513-1530.

In: Clin Exper Neurol 29: 210-233, (1992)
Edited by M Eadie, C Lander, M Pender

REFLEX SYMPATHETIC DYSTROPHY: ALTERED AXON REFLEX AND AUTONOMIC RESPONSES

R.A. Westerman*, I. Pano*, A. Rabavilas*, A. Hahn†, A. Nunn†‡, R.G.D. Roberts and H.Burry†

Department of Physiology*, Monash University, Clayton, Vic, and Department of Rehabilitation†, University of Melbourne, Essendon and District Memorial Hospital, Essendon, Vic, and Department of Orthotics, Prosthetics and Amputation Services‡, Caulfield General Medical Centre, Caulfield, Vic.

SUMMARY

Cutaneous axon reflex vasodilation depends on the integrity of C-polymodal nociceptor nerve fibres. It is evoked chemically by iontophoresis of either acetylcholine (ACh) or noradrenaline and agonists, e.g. phenylephrine (PE). In the recent study of Drummond *et al* [16], patients with reflex sympathetic dystrophy (RSD) who exhibited clearly increased sweating of the affected hand or foot had lower plasma noradrenaline levels on the painful side. Therefore the present study used laser Doppler flowmetry (Moor MBF3D) to measure ACh and PE evoked axon reflexes in the skin of 20 healthy consenting controls, 24 consenting lower limb amputees, 24 patients with chronic pain resembling RSD, and in the skin of their respective contralateral unaffected limbs.

Drugs as 1% aqueous solutions were iontophoresed from an annular chamber 5mm distant from the centrally located laser Doppler flow probe, with a constant (DC) current of 0.2mA. In control subjects ACh and PE produced neurogenic inflammatory (axon reflex) responses of 1.44 ± 1.01 and 0.30 ± 0.36 V.min, respectively. When ACh was preceded by PE pretreatment at the same skin site, the axon reflex dilator response (1.80 ± 1.14 V.min) was increased, but not significantly, and the response to PE following ACh pretreatment was significantly increased (0.92 ± 1.19 V.min). Cycle exercise for 20 min at a work rate sufficient to raise the heart rate to 140-160 bpm significantly reduced that ACh-evoked axon reflex to 1.09 ± 1.99 V.min ($p<0.03$) but increased the PE-evoked response to 0.63 ± 0.85 V.min ($p<0.05$). In RSD patients the ACh-evoked axon reflexes were significantly smaller ($p<0.05$) in the skin of the painful limb (0.95 ± 0.60 V.min) compared with the non-affected limb (1.71 ± 2.10 V.min); the latter were not significantly different from the responses in healthy subjects (1.44 ± 1.01 V.min). In painful limbs of RSD subjects the PE-evoked response (0.39 ± 0.65 V.min) was significantly enhanced by ACh pretreatment (0.76 ± 0.21 V.min), but the ACh response after PE-pretreatment was not enhanced.

These data indicate that axon reflexes are evoked in human skin by cholinergic and α-adrenergic agonists and these mechanisms of C-fibre activation show interactions possibly at the receptor level. The altered pattern of chemically evoked axon reflexes in the skin of affected limbs in patients with RSD suggests a peripheral role of the sympathetic innervation in nociception and chronic pain maintenance, not excluding any central effects.

Reflex sympathetic dystrophy (RSD) is a syndrome, or perhaps a group of related syndromes, characterised by pain, usually burning in quality, vasomotor and sudomotor disturbance, dystrophy and, very often, marked hypersensitivity. RSD can occur following surgery, trauma, or certain disease states[1]. Evidence of autonomic involvement includes the clinical features of temperature and colour change, usually redness early, and pallor later with, in some cases blueness[2]; sudomotor changes ; vasomotor instability associated with prolonged capillary refill time[2]; finally, sympathetic blockade to provide pain relief is considered diagnostic[3,4,5] and may be used therapeutically[6,7,8,9]. The trophic changes include swelling, stiffness and sometimes fibrosis; osteoporosis may occur, with trophic changes in skin and nails, and a general marked aversion to use the affected limb[1,2,9].

The pathogenesis of RSD is unknown, but has been reviewed recently[10,11,12]. Various mechanisms have been proposed[2,11,13], including sensitisation of nociceptors[14], which may be associated with an increased sympathetic outflow[2]. However, more recent evidence suggests that vasoconstrictor tone[15], and plasma noradrenaline levels and those of its metabolite dihydroxyphenylglycol (DHPG)[16] are reduced in affected regions. This supports the contrary view, that RSD is associated with decreased sympathetic outflow[16].

This fundamental question deserves clarification because RSD treatment has often included sympathectomy or more recently, regional sympathetic blockade[9]. The present study aims to measure neurovascular function, including nociceptor and autonomic responses in RSD-affected skin using non-invasive techniques[17,18,19]. A preliminary report of this has been presented[20].

METHODS

Subjects

Subjects used in this study included 20 healthy consenting volunteers, and 24 patients with features of RSD who had suffered pain with signs of sympathetic dysfunction for periods between 3 and 72 months (average=19.7

months). Their ages varied from 18 to 67 years with a mean of 43.6 years. Upper limb pain was prominent in 8 of the 24 subjects. In all except 2 cases, pain first started after injury to a limb. In addition, 24 lower limb amputees were tested, at an average of 2.4 months after the amputation.

A full medical examination was conducted on each patient by a rehabilitation physician. Also a general questionnaire regarding medication and exercise, smoking and drinking habits was completed by each patient, as well as a more detailed questionnaire with the assistance of a clinical neuropsychologist. Confidentiality of all records was maintained by using locked files and only placing patient initials on any recorded data.

Eight of the patients underwent chemical sympathectomies during the study, with a return of pain in only one of these subjects. Written voluntary informed consent was given by all patients for the procedures which were approved by the Monash University and Royal Melbourne Hospital Ethics Committees.

Control subjects consisted of healthy consenting volunteers recruited from Monash University, Caulfield General Medical Centre and the Essendon District Memorial Hospital, age-matched as far as possible.

Examination & preparation for recording

Each participant was given a detailed explanation of the procedures involved prior to testing. Subjects with RSD received additional explanation and reassurance that the testing was non-invasive and did not involve any further induction of pain. In RSD subjects, tests were conducted on the painful limb and on corresponding contralateral sites. Subjects who had undergone sympathectomy were tested on the sympathectomised and contralateral non-sympathectomised limbs (where applicable).

The dorsal surfaces of both hands were used as the sites of testing in all control subjects. Test sites in patients were judged on the basis of the site of pain. The hand dorsum was used in 16 patients, the dorsal area of the knee was used in 3 patients, and the foot dorsum/ankle was used in 5. To reduce skin impedance and prevent leakage from iontophoretic chambers, test sites were shaved with an electric razor and cleaned with a 70% isopropyl alcohol swab.

Subjects whose upper limbs were being tested were seated in a comfortable chair with their arms resting on a pillow placed on their lap during testing. Alternatively, lower limb subjects were provided with a seat upon which they rested their legs during testing. Prior to testing, the ambient temperature was

recorded as was the skin temperature at sites on both normal and painful limbs using a 'First-Temp' infrared thermometer in scan mode. Patient testing was first performed on the non-painful side and then, with patient consent, testing continued on the painful side.

Neurovascular tests: laser doppler velocimetry

To test neurovascular function, the skin blood flux was monitored before, during and after iontophoretic application of vasoactive chemicals[21]. The skin blood flux was measured non-invasively using a dual channel Moor MBF3-D laser flow monitor[20,22]. This sensitively measured changes in the local skin microcirculation at 2 different sites simultaneously to reduce variability[23]. A laser probe holder was attached to the skin at the test sites with a double-sided adhesive disc which produced a water-tight seal with the skin. Two types of probe holders (chambers) were used: A - an indirect circumferential chamber (Figs 1,2) was used to produce an axon reflex and spreading flush by nociceptor stimulation distant from the recording laser probe. The direct (central) chamber - B (Figs 1,2) was used to test endothelium and smooth muscle, by measuring changes in microcirculatory activity produced by iontophoretic application of agonists immediately beneath the laser probe.

A concentric chamber combining both the indirect and direct chambers was used in 8 control subjects making possible 2 different (i.e. indirect and direct) iontophoretic applications at the same recording site. All these chambers combined the function of iontophoretic applicator and probe holder for blood flux measurements.

The laser probe used for measuring blood flux was inserted vertically at the centre of the holder (Fig 1) and held 0.55mm from the skin[17]. The laser Doppler velocimeter uses glass fibres to transmit a near infra-red beam (810 nm) to illuminate the tissue and collect the reflected light. Laser light scattered from moving red blood cells and static tissues undergoes a frequency shift according to the Doppler principle. The reflected light then travels back to a photodetector to produce an output which is linearly related to the product of the number of cells and their velocity (i.e. the blood flux). The laser diodes output 1.2mW at their tip and respond almost identically over a range of red blood cell velocities and concentrations[22]. Therefore they can be used to measure dynamic changes at differing sites reliably and safely, sampling an approximate radius of 1.5mm of tissue.

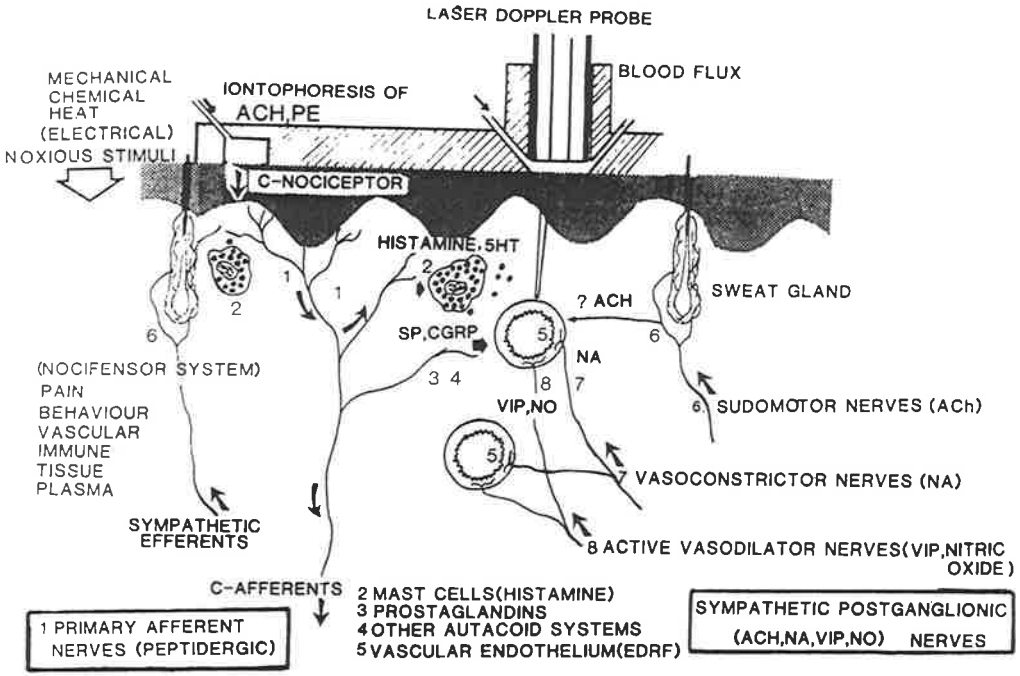


Fig 1 Skin innervation. Diagrammatic cross section through skin and perspex probe holder. The laser Doppler probe monitors blood flux at the central chamber while iontophoresis of ACh or PE from the circumferential chamber occurs. Here chemical stimulation of C-nociceptors (1) activates the cascade involving mast cell degranulation and histamine (2), prostaglandin synthesis (3), other autacoids, tachykinins (4) and the microvascular endothelium (5). Also shown in the diagram, but not usually participating in an axon reflex dilator response[20,25,34] in normal skin are post-ganglionic autonomic sudomotor nerves (6) which are cholinergic, vasoconstrictor noradrenergic nerves (7) and active vasodilator nerves (8) probably peptidergic or nitrergic. These all innervate targets close to nociceptive sensory nerves and so have the potential for abnormal connectivity[4,16] or functional relations[26] after injury. Some nocifensor responses of the primary afferent nerves (containing substance P and calcitonin gene related peptide) are listed at left.

All the responses measured were obtained using the Moor MBF3D laser Doppler velocimeter (wavelength 810 nm). Probes were calibrated weekly against a reference motility standard. This standard used the Brownian motion of polystyrene microspheres in water to produce the reference signals. The Brownian motion is temperature dependent and hence the standard was stored between 20 and 22 degrees Celsius.

Iontophoresis

This is the method by which ionised substances are introduced into the skin to stimulate sensory and autonomic nerves or microvascular structures[21]. The chemicals used to elicit axon reflex responses (neurovascular testing) were the cholinergic and adrenergic agonists, ACh and PE respectively, dissolved as 1% solutions in distilled water[20]. Iontophoresis used a battery-powered iontophoresis unit to provide a 0.2mA direct (galvanic) current to effect the transfer of the drug. To complete the circuit, an indifferent electrode (distilled water-soaked gauze) was placed either on the subject's wrist or around the ankle. The active electrode polarity was dependent on the charge of the drug to be transferred: anodal currents were used for cations in ACh chloride and PE hydrochloride. The total amount of drug ions iontophoresed was given as the total charge in millicoulombs (mC) obtained as the product of duration of application (s) x current (mA)[21]. ACh and PE were iontophoresed for a total of 16mC (0.2mA x 80s)[20]. The current strength was sufficiently small to produce minimal local and absent central effects: only a mild prickling or itching sensation was noticed by some subjects during iontophoresis.

Sodium nitroprusside (SNP), an endothelium-independent nitrodilator, was used as a 1% solution applied from a direct chamber[18] in patients having undergone chemical blocks. A cathodal current repelled the anion into the skin for a total charge of 8mC (0.2mA x 40s). The agonists used had only local effects in these tests and all equipment was fully isolated from the patient and mains power by a safety circuit.

Responses to iontophoresis of ACh, PE and SNP were recorded for a period of 4 minutes from the end of iontophoresis on a DP600 chart recorder (ICI Instruments) - see Fig 2 for typical records. The microvascular dilator responses were measured as the changes in blood flux above the baseline. These were quantified by measuring the voltage-time integral under the curve of each response using the 'Moorsoft' programme or a digitising tablet and 'Sigmascan' software (Jandel).

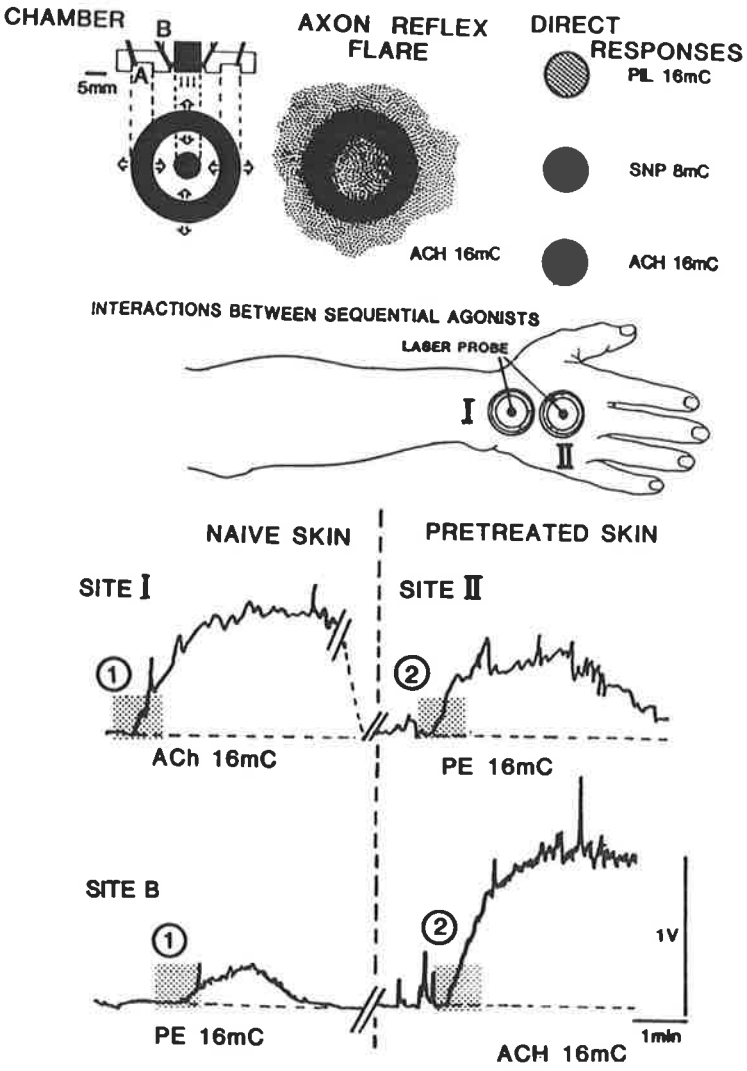


Fig 2 Iontophoretic chambers and response patterns.
Upper left: Perspex probe holder with circumferential chamber (A) and central chamber (B) showing dimensions. Illustrated is an annular dilator response with spreading axon reflex flush. Upper right: illustrates the small circular red responses to endothelial dilators (pilocarpine or acetylcholine) or the nitrodilator sodium nitroprusside (SNP) iontophoresed from the central chamber.
Mid-portion: Shows location of perspex probe holders on the hand dorsum at sites I and II.
Lower portion: Shows blood flux vs time records of interactions between sequential agonists iontophoretically applied at site I, ACh 16mC, then PE 16mC. At Site II, PE 16mC preceded ACh 16mC. Note the enhanced larger size of the axon reflex after pretreatment compared to the corresponding size on naive skin. The stippled zone indicates the period of iontophoresis on the time axis. Calibration bars 1V. and 1 min.

Iontophoretic protocols

The chemicals used, their doses and combinations of iontophoretic applications are shown below for all subjects. It is important to note that each test was performed at the corresponding sites on each side of the body (Figs 2,3,4,5,6,7).

Control subjects: using a perspex circumferential chamber,

1. Site I: 16mC ACh followed by 16mC PE
2. Site II: 16mC PE followed by 16mC ACh

Subjects with RSD and subjects with sympathectomy: as above.

Amputees:

In 25 patients with recent lower limb amputations the battery of iontophoretic tests was performed on denervated skin, and on corresponding normal skin from the contralateral limb (Fig 4A,4B).

Concentric chambers

In these tests, eliciting an axon reflex preceded iontophoresis of an endothelium-independent nitrodilator in the direct compartment when the blood flux returned to baseline. In both amputees and patients with RSD, changes in microcirculatory activity were measured, recorded and compared to direct responses elicited by the same vasodilator (SNP) on naive skin sites (i.e. without axon reflex pretreatment - Fig 4C).

Iontophoresis in these concentric chambers consisted of:

Site I: 16mC axon reflex (ring), then 8mC SNP (direct).
Site II: 8mC SNP (direct).

Exercise and neurovascular function

Of the 20 control subjects, 8 volunteered for an exercise session. Only 2 of the RSD subjects participated in a 6 week exercise program. The 8 control (non-RSD) subjects completed the exercise period consisting of cycling in a stationary position for 15 minutes at a workload of 60% of the estimated maximal heart rate (with a 3 minute warm up and 2 minute cool down period). The pulse was monitored for the duration of the exercise period using a Polar sports-tester which calculated the heart rate every 15 seconds. Iontophoretic tests (see protocol above) were conducted immediately before the exercise period. The tests carried out immediately post exercise consisted of:

Site I: 16mC ACh;
Site II: 16mC PE in direct chambers with no sequential application of agonists. (For results see Fig 3).

Statistical analysis

Data are expressed as the means ± SEM. The significance of differences was calculated by either Student's t-test or non-parametric analysis using Wilcoxon's signed rank test for paired and unpaired data, as appropriate.

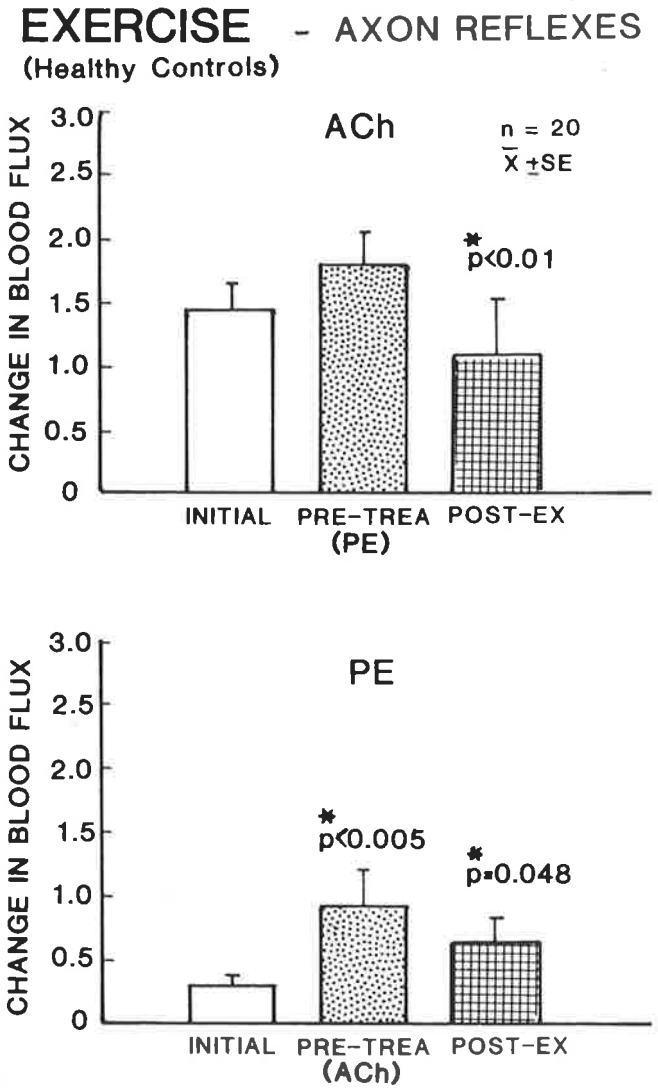


Fig 3 Exercise. Mean laser Doppler blood flux responses to indirect iontophoretic application of (a) ACh 16 mC and (b) PE 16 mC are shown for healthy control subjects who performed exercise (see methods). Initial values pre-exercise are given at left (open columns), the right hand histograms (crossed columns) show blood flux responses to the same chemical stimulus at the end of exercise. Middle histograms (stipple) show the blood flux changes pre-exercise but following sequential application of the other agonist ie the middle (stippled) column in the upper half is the response to ACh after PE treatment and in the lower half, the middle column shows PE axon reflex responses after ACh pretreatment. The right hand column responses post-exercise are not sequential applications, so they should be compared with the left hand column showing initial pre-exercise axon reflex responses. Statistical significance was measured by non-parametric Wilcoxon signed rank test.

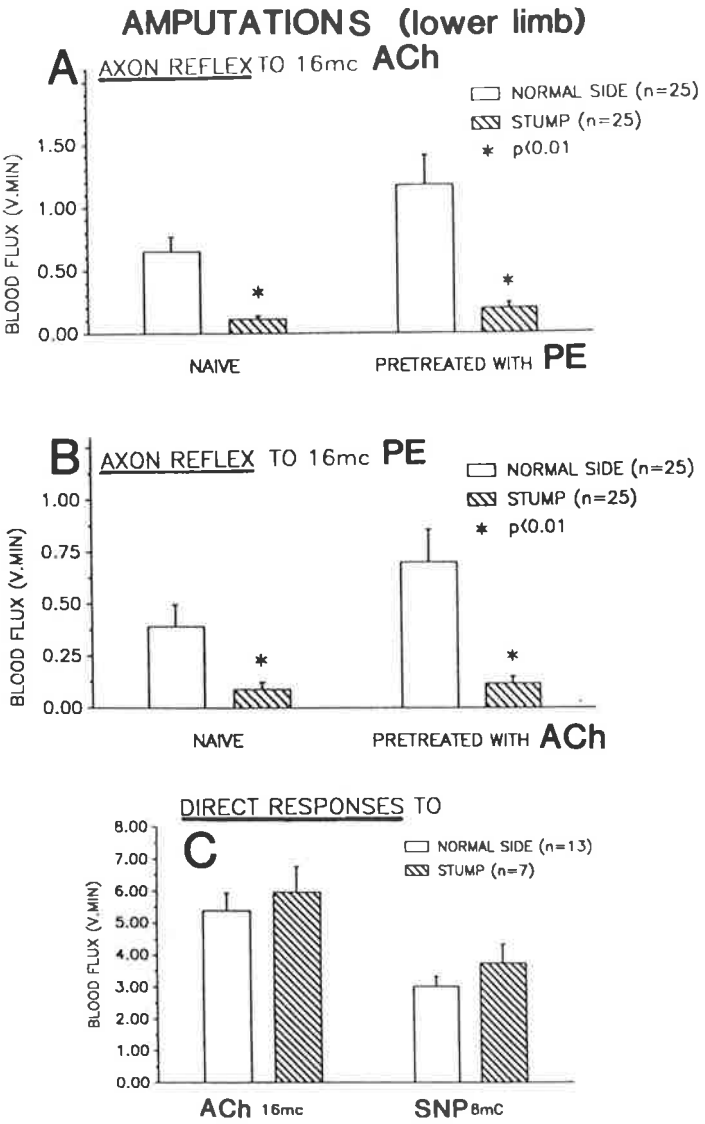


Fig 4 Amputation. Shows mean laser Doppler blood flux responses to iontophoretic application of (A) ACh 16 mC, (B) PE 16 mC onto naive and post-amputation stump skin (left hand columns) on average 2.1 months post-amputation. Sequential application of ACh and PE were also performed, and the pretreated responses are shown in the right hand columns.
In C, a nitrodilator sodium nitroprusside (SNP) 8 mC was applied from the central direct chamber of a dual chamber concentric probe holder. Responses with (left columns) and without (right columns) pretreatment by prior axon reflex evoked from the peripheral ring chamber are shown. Statistical significance according to Wilcoxon's signed rank test is shown by asterisk and P-value.

RESULTS

Control subjects and exercise

The hand dorsum was used to test neurovascular function in control subjects (Fig 2). Axon reflex responses elicited by iontophoretic application of cholinergic and adrenergic agonists were measured.

The sequential application of 2 agonists elicits potentiated axon reflexes (see Fig 2), the second agonist iontophoresed producing an enhanced second axon reflex vasodilatation.

Fig 3 shows the axon reflex response to PE in control/non-RSD subjects which was significantly enhanced by ACh pretreatment, when compared to that elicited on a naive site (ie without pretreatment) (Student's t-test, $t=3.287$, $p<0.01$). By contrast, PE pretreatment seemed to have no significant potentiating effect on ACh axon reflex responses in the control subjects in this study.

Following exercise, a reduced axon reflex to ACh was seen and the attenuation was significant (see Fig 3A). PE on the other hand showed a larger neurovascular response following the 20 min exercise period in control subjects. Potentiation was also significant (see Fig 3) even with the small number of subjects tested.

Amputees

Axon reflexes from normal and denervated skin are shown for amputees in Fig 4. In Fig 4A, on amputation stump skin, responses to either ACh alone, or following PE pretreatment, were both significantly reduced ($p<0.01$) compared to responses obtained from corresponding skin sites on intact limbs in the same subjects. Similarly, in Fig 4B responses to PE, whether alone or after pretreatment by ACh, were significantly reduced when compared with those on corresponding skin sites on the intact limb ($p<0.01$). In Fig 4C, results from concentric chamber tests were used to examine different sequential agonist interactions on naive and pretreated sites. Direct iontophoresis of sodium nitroprusside (SNP) on a naive skin site showed no statistical difference in vascular-dependent vasodilatation when compared with a site pretreated with an axon reflex (Fig 4C).

Reflex sympathetic dystrophy vs healthy control subjects

When compared to healthy control subjects, the ACh axon reflex response on naive skin of RSD-affected subjects was significantly reduced when analysed by the Student's parametric independent t-test ($t=2.779$, $p<0.01$ - see Fig 5). The average of the axon reflex responses elicited by PE in RSD and non-RSD subjects showed no statistically significant difference when both groups were compared.

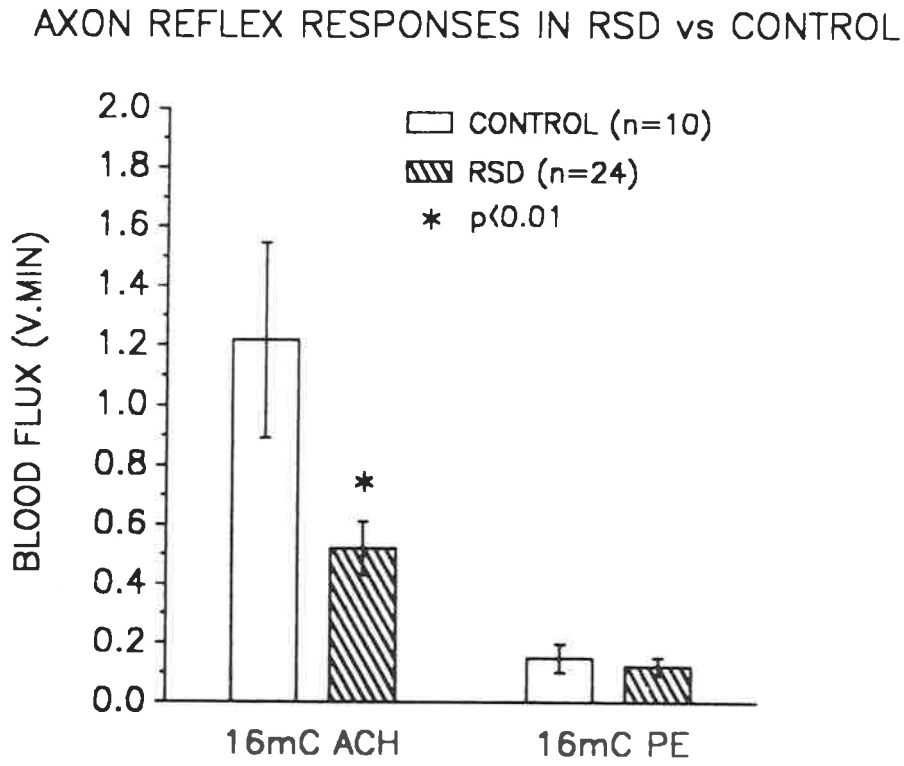


Fig 5. Healthy controls vs subjects with RSD. Mean laser Doppler blood flux responses to indirect iontophoretic application of ACh 16 mC and PE 16 mC onto naive skin are shown for n=10 healthy control subjects (open columns) and for n=24 patients with RSD (hatched columns). Asterisks denote statistical significance with p-values, measured by Wilcoxon's non-parametric signed rank tests.

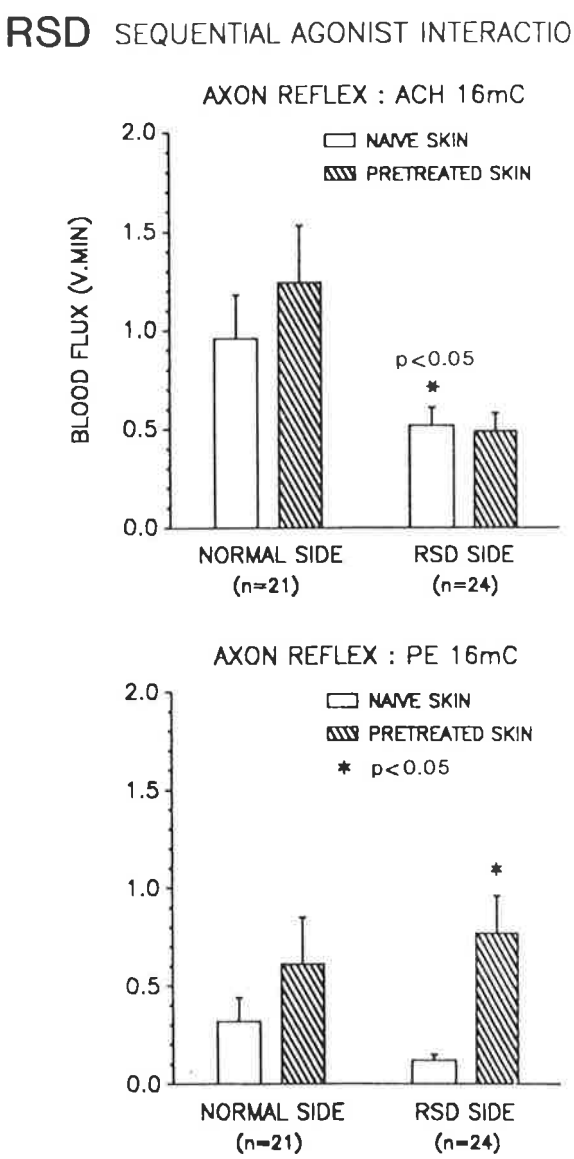


Fig 6 RSD subjects: Normal vs affected side. Mean laser Doppler blood flux responses to indirect iontrophoretic sequential application of agonists ACh 16 mC and PE 16mC as described in Methods. For both upper and lower panels, the initial agonist response, described as naive skin, is shown as open columns and the response after pretreatment with the other agonist is shown cross-hatched. Statistical significance was measured by non-parametric Wilcoxon signed rank tests; P-values are given for the significant comparisons (asterisked).

Fig 6 shows the responses in subjects with RSD comparing the limb of the painful side to that on the non-painful side (contralateral limb). Sequential interations between cholinergic and adrenergic agonists elicited enhanced axon reflexes in all non-RSD skin areas, but not in RSD skin in the same subjects in response to ACh iontophoresis. Difference in subject numbers indicate the patients who suffered bilateral RSD: in these subjects each limb was recorded as a separate entity. Fig 6A shows how pretreatment with PE produced an enhanced ACh response (not statistically significant) in normal skin. However, in RSD skin, the tendency to enhancement was abolished. ACh pretreatment in both normal (contralateral) and RSD skin elicited an enhanced PE axon reflex. Statistically significant PE potentiation by ACh pretreatment ($t=3.538$, $p<0.01$) however, was seen only at sites on RSD skin (Fig 6B).

Pain rating and anxiety level: visual analogue scales

The relation between subject pain rating and duration of RSD syndrome in months showed no statistical significance; however there was a strong tendency for pain to increase with time. This is indicative of the evolution of the syndrome which depicts changes in pain levels throughout the progressive stages.

No clear correlation of anxiety levels with neurovascular responses was found in patients with RSD. However people with maximal anxiety (measured with visual analogue scales) had no blood flux readings greater than 1 V.min, and generally tended to show smaller flux levels.

Skin temperature

Measured by emission thermography, reflex sympathetic dystrophic skin show a wide range of temperatures. However, there was no significant difference between RSD-affected and contralateral normal sites.

Sympathectomy

When compared to normal contralateral limbs, axon reflexes of sympathectomised skin showed a tendency to be reduced (see Fig. 7). The PE axon reflex on sympathectomised skin, as assessed by a Student's t-test, showed a statistically significant reduction ($t=2.185$, $p<0.05$).

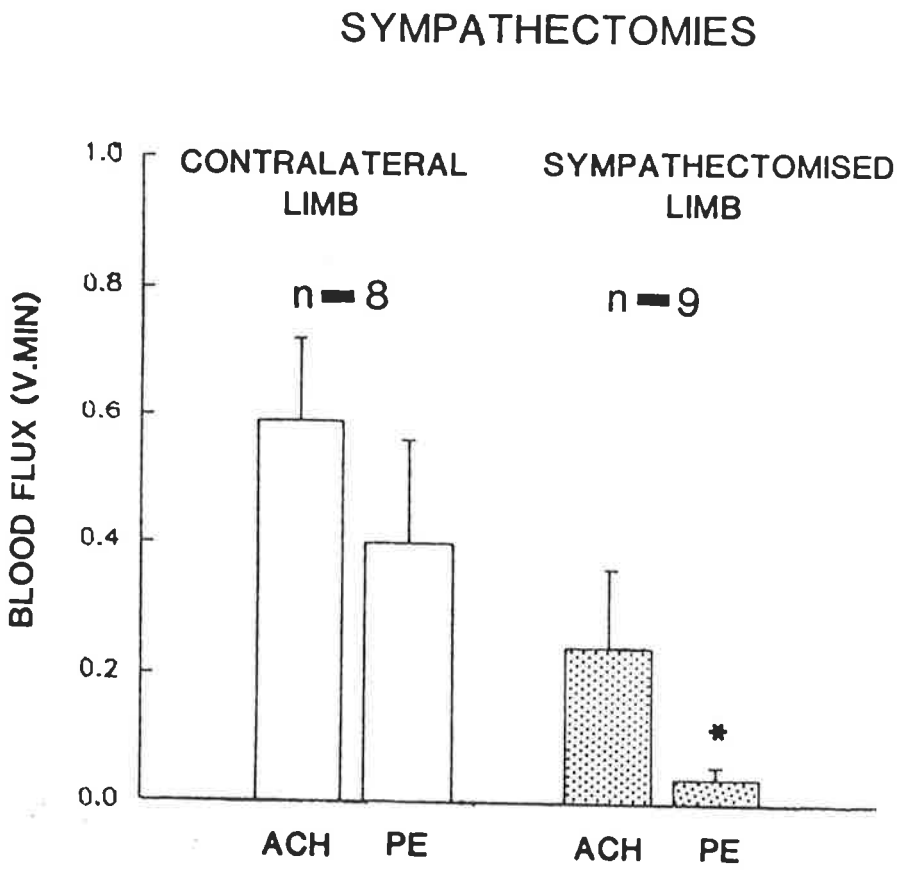


Fig 7 Axon reflex flare responses evoked on sympathectomised skin compared to sites on the contralateral (non-sympathectomised) limb. The chemically blocked limb had a tendency to exhibit reduced responses with that to PE being significantly reduced.

DISCUSSION

The effects of RSD can be devastating if it is inadequately treated. In later stages the affected limb may become almost non-functional, and the persistent pain so distressing that it may destroy the patient's quality of life in every arena - job, employability, family relationships, and recreation. Insufficient is known about the mechanisms underlying RSD, its pathogenesis, how to prevent it, or even how to diagnose and manage it effectively. In this condition of RSD (also termed sympathetically-maintained pain), there are several critical questions unanswered, and these should be discussed in the context of the present axon reflex findings: (1) Is the sympathetic tone to the RSD-affected limb increased or decreased? (2) How do sensory and autonomic nerves contribute to the response to injury? (3) How may the present findings be best interpreted? (4) Is RSD a particular form of disordered healing? (5) What might be causes for such a disorder? (6) What types of therapy are suggested by the foregoing?

Role of sympathetic nervous system in RSD

In RSD, unlike normal injuries, sympathetic activity (either sudomotor or vasomotor) aggravates or prolongs pain in the affected region, while blocking sympathetic outflow to that limb usually reduces or abolishes the pain for some time[2]. The conventional assumption about the warm,dry,red skin in the affected region that occurs early, and which later tends to be cold and sweaty, is that of sympathetic overactivity[2]. This predicates the basis for the common diagnostic procedures and treatment, viz some form of sympathetic blockade[5,6,7,8]. In spite of this view, there is now increasing evidence suggesting the contrary[16]. First, Wallin *et al*[26], using microneurographic recording of sympathetic efferent activity to RSD-affected skin, found qualitatively normal responses to arousal stimuli. Christensen and Hendricksen[15] used proximal nerve block to interrupt sympathetic outflow in 6 patients with RSD and showed a significantly smaller increase of skin blood flow (35%) in the affected hand than on the other side (122%). Rosen and colleagues[27] described reduced nailfold blood flow using laser Doppler velocimetry in 12 patients with RSD compared with that in controls. Drummond *et al*[16] studied venous plasma catecholamine levels in 26 patients with RSD features and found that levels of plasma noradrenaline, and of its metabolite DHPG, were lower in the painful limb. All these findings suggest that sympathetic activity to RSD-affected limbs in the situations described was lower rather than higher than in the unaffected limbs. This is more consistent with the sweating and vasomotor disturbance being due to super-sensitivity to sympathetic neurotransmitters[16].

Sensory and autonomic nerve interactions

The sensory innervation of the skin comprises many unmyelinated fibres including those involved in nociception[28] . Cutaneous axon reflexes depend upon the integrity of C-polymodal nociceptors, and therefore the axon reflex flare[17,18,19] is an index of cutaneous primary sensory neural function. Neurogenic inflammation (antidromic vasodilatation) is mediated by peptide release from sensory nerves[29,30,31] which may be activated by noxious stimuli or injury. Responses initiated by nociceptive sensory nerves have been regarded as protective or nocifensor [32,33] and there is now more evidence about the contributions of sensory nerves to healing[34,35,36,37]. However, not all skin vasodilator responses are evoked by nociceptive sensory nerves, and sympathetic postganglionic nerves are also involved[38,39], which provides potential for distorted relationships with nociceptive sensory nerves after injury[4,11].

Axon reflexes in RSD, exercise, amputation and sympathectomy

The findings in the present study showed that axon reflexes may be evoked in normal and RSD-affected skin by iontophoretic application of ACh, acting via nicotinic acetylcholine receptors on nociceptive afferents. The ACh-evoked axon reflex was significantly reduced in RSD-affected skin compared with normal skin. Although smaller than the ACh-evoked axon reflex dilation, an axon reflex was also evoked by phenylephrine, an alpha-adrenergic-agonist, not previously known to excite nociceptors. PE produces local vasoconstriction (presumably by direct action on vascular smooth muscle/endothelium at the site of iontophoresis), as well as a spreading flare which occurs in skin remote from the site of iontophoretic application. The fact that this response, as well as that to ACh, normally is almost abolished in denervated skin supports its axon reflex nature. Its function in normal skin is unknown.

Interactions between sequential applications of cholinergic and adrenergic agonists are altered in RSD-affected skin. Pretreatment of a skin site with an ACh-evoked axon reflex results in a significantly enhanced PE-evoked axon reflex in both control skin, and in RSD-affected skin.This enhancement may reflect some increased excitability (lowered excitation threshold) of nociceptive afferents involved in the peptide release to evoke the axon reflex dilation. This is in contrast to the ACh-evoked axon reflex on skin pretreated with PE, which is not significantly enhanced in RSD skin. This difference in behaviour of the two 'agonists' ACh and PE on nociceptor excitation remains to be explained, but may reflect their normally different contributions to the activation of nociceptive sensation. The significant increase ($p<0.05$) in the ACh-enhanced PE-evoked axon reflex was reduced by sympathectomy. This suggests that upregulation of adrenoceptors on nociceptive afferent nerves may contribute to the enhanced PE

axon reflex response. The exact nature and extent of the interaction between cholinergic and adrenergic efferents and (peptidergic) nociceptive sensory fibres is unknown, but the present results of these *in vivo* tests are evidence of a significant interaction.

Healthy control subjects exercised on a cycle for 20 min, sufficient to raise heart rate to 140-160 b.p.m, showed significantly reduced ACh-evoked axon reflexes ($p<0.03$) but increased PE-evoked responses ($p<0.05$). The former result may be due to the increased level of blood flux at the steady state exertion level described. This would be likely to reduce the apparent ACh-evoked response from an elevated 'baseline' flux[21].

In amputees, stump skin of the amputated lower limb compared to the normal skin of the contralateral limb showed significantly reduced ACh ($p<0.05$) and PE ($p<0.01$) axon reflex responses. Iontophoresis of the nitrodilator sodium nitroprusside revealed no differences in small blood vessel function. This is consistent with the behaviour of denervated skin, and emphasizes the paucity of nociceptive innervation near the undercut amputation flap even 2 to 2.5 months after the operation. It suggests a more central site for the generation of phantom and stump pain phenomena.

Interpretation of data

Clearly, laser Doppler flowmetry and iontophoretic application of vasoactive compounds allows the study of neurovascular function and nociception in the chronic pain states associated with RSD. The findings are consistent with a decreased sympathetic outflow to the RSD-affected limb inducing noradrenergic hypersensitivity[16], and thus leading to increased nociceptor sensitivity to catecholamines (either neural or humoral in origin), and enhanced PE axon reflexes. Exercise led in the short term to significantly reduced ACh-evoked axon reflexes, and increased PE-evoked responses. This is probably due to the increased circulating catecholamine levels after 20 min of moderate steady-state exercise. These findings suggest that treatment with agents such as adrenergic agonists or exercise may reduce receptor hypersensitivity and thus reduce sympathetically maintained pain.

Is RSD distorted healing?: possible causes

First, altered chemosensitivity of nociceptors is a likely mechanism by which sympathetically maintained pain is enhanced and prolonged. The sweating abnormality in RSD may indicate hypersensitivity to cholinergic or adrenergic neurotransmitters, because both are probably involved in induction of sweating at most limb sites[42]. Furthermore, the nicotinic receptors on peripheral sensory

nerves in the skin are capable of modulation or upregulation[43],as are adrenergic receptors on sympathetic target tissues[44]. Wallin *et al*[45] showed that pain could be rekindled in sympathectomized skin by iontophoresis of noradrenaline. Devor[46] has shown that experimental neuromas in rats develop abnormal chemosensitivity to adrenergic agonists, and a similar process may contribute to the increased pain and allodynia seen around the injury site in RSD. Sato and Perl[47] recently reported enhancement of C-sensory responses to noxious heat by adrenaline or noradrenaline after partial injury to a mixed peripheral nerve. They suggested that nerve injury leads to supersensitivity of α_2-adrenoceptors in adjacent and otherwise normal sensory nerve terminals[47]. Thus a major proposition is that decreased sympathetic neurotransmitter release at and near an injury may modulate (i.e. upregulate) nociceptors and so distort the pain-producing mechanism in RSD[48]. The results of Cline *et al*[49] and experimental models of RSD in animals[50,51] also support this view[48].

Other putative mechanisms by which the recovery from normal neurogenic inflammation may be distorted and prolonged are suggested in the phase 3 pathophysiology summarised in Fig 8. These include readjustments of CNS nociceptive afferent pathways with an enhanced contribution from wide dynamic range (WDR) mechanoreceptors[51]; the possible deficiency of trophic or growth factors essential for repair of injured tissue and regeneration of nerve[52,53;34-37]; and the possible permanent induction of neuronal oncogenes, e.g. C-fos[54], which result in synthesis of new protein within nerve cells throughout the entire nociceptive sensory pathways- a sort of pain memory within the neurones. Thus a number of neuropathic mechanisms may coexist, and may contribute to the different types of pain symptoms shown by patients with RSD or sympathetically maintained pain[48,55].

Management of RSD - sympathetically maintained pain (SMP)

There is much evidence for the possible contributions of the sympathetic nervous system to pain maintenance and enhancement in RSD and SMP[48,55-60]. The recommendation of Bonica[57] is for a specific and aggressive treatment which consists initially of a series of regional sympathetic blockades. Prolonged stellate ganglion block[58] or topical application of clonidine[59] has also been suggested. Based upon the recent findings of Drummond *et al*[16] and the present study, it may also be possible in the future to use adrenergic agonists and appropriate exercise to modulate the hypersensitivity of nociceptors and their afferent pathways. The place of other speculative therapies such as the application of neuropeptides[35,37], nerve growth factors[54], and transcutaneous electrical stimulation of nerves[61] remains unclear.

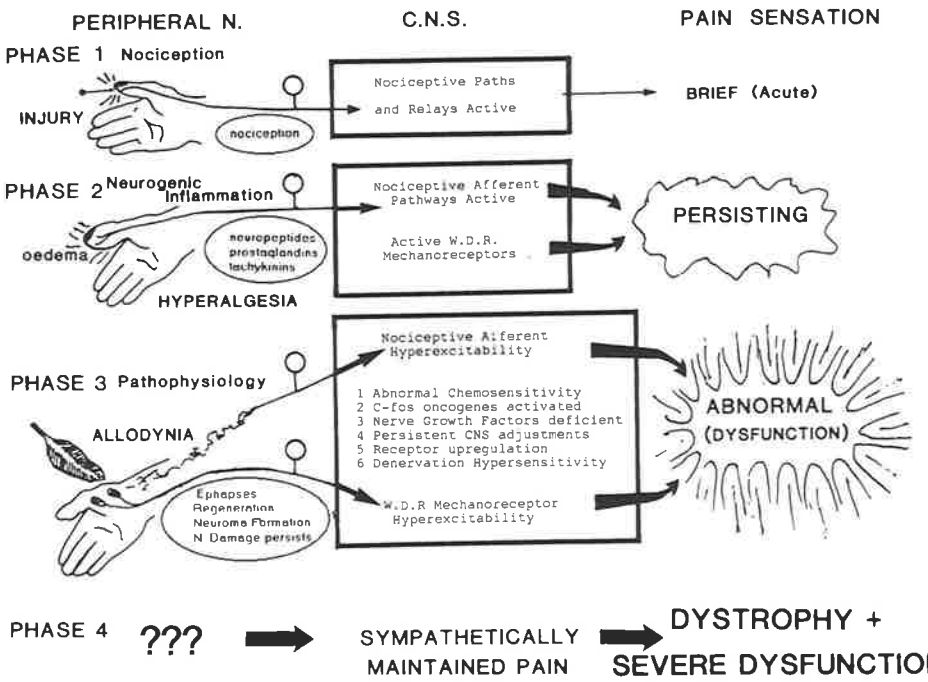


Fig 8 Pathophysiology summary. Phase 1 is normal nociception resulting in normal activation of CNS pathways and the normal acute pain experience. Phase 2 is signalled by development of hyperalgesia accompanying neurogenic inflammation of the injured part; chronic pain persists. In phase 3 various mechanisms may interact: all mechanisms shown in the diagram have been identified in various experimental and clinical models of chronic pain states. Any may contribute to the final progression and deterioration into phase 4 i.e. sympathetically maintained pain (RSD) in which atrophy of skin,bone,joint and nerve may occur with severe loss of function. Relief of pain by regional sympathetic blockade may occur at any stage from phase 2 to 4.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge that this research was performed with equipment provided by the Shepherd Foundation, the Clive and Vera Ramaciotti Foundation, the Lillian Cooper Foundation, and the Balwyn Rotary Club. Without this most generous support such detailed quantitative sensory testing would not have been possible. The assistance of the Professorial Department of Rehabilitation Medicine (University of Melbourne) funded by Workcare was invaluable. The authors are also most grateful for the continued support and facilities provided by the International Diabetes Institute, and for technical assistance of Mrs Libby Prior and Ms Angela Forsyth for typing and Ms Dianne Clare for the illustrations.

REFERENCES

1. Abram S. Incidence-Hypothesis-Epidemiology. In: Stanton-Hicks M (ed). Pain and the sympathetic nervous system. Norwell, Kluwer Academic, 1990; 1-15.
2. Bonica JJ. Causalgia and other reflex sympathetic dystrophies. Advances in Pain Research and Therapy 1979; 3:141-166.
3. Gandhavadi B, Rosen JS, Addison RG. Autonomic pain: features. Postgraduate Medicine 1982; 71:85-90.
4. Jänig W. Causalgia and reflex sympathetic dystrophy: in which way is the sympathetic nervous system involved. Trends in Neurosciences 1985; 11:471-477.
5. Arner S. Intravenous phentolamine test: diagnostic and prognostic use in reflex sympathetic dystrophy. Pain; 46:17-22.
6. Loh N, Nathan PW. Painful peripheral states and sympathetic blocks. Journal of Neurology, Neurosurgery and Psychiatry 1978; 41:664-671.
7. Bonelli S, Conoscente F, Movilia PG, Restelli L, Francucci B, Grossi E. Regional intravenous guanethedine vs stellate ganglion block in reflex sympathetic dystrophies Pain 1983; 16:297-307.
8. Schutzer MD, Gossling MD. The treatment of reflex sympathetic dystrophy syndrome. Journal of Bone and Joint Surgery 1984; 66A:625-629.
9. Hannington-Kiff A. Retrograde intravenous sympathetic target blocks in limbs. In: Stanton-Hicks M (ed). Pain and the sympathetic nervous system. Norwell, Kluwer Academic, 1990; 191-206.
10. Schwartzman RJ, McLellan TL. Reflex sympathetic dystrophy: a review. Archives of Neurology 1987; 44:555-561.
11. Jänig W. Experimental approach to reflex sympathetic dystrophy and related syndromes. Pain 1991; 46:241-245.
12. Schott GD. Mechanisms of causalgia and related clinical conditions. Brain 1986; 109:717-738.
13. Procacci P, Maresca M. Reflex sympathetic dystrophies and algodystrophies: historical and pathogenic considerations. Pain 1987; 31:137-146.
14. Marchettini P, Lacerenza M, Ieracitano N, Canal N. Sensitised nociceptors in reflex sympathetic dystrophies. Functional Neurology 1989; 4:135-141.

15. Christensen K, Hendricksen O. The reflex sympathetic dystrophy syndrome. Scandinavian Journal of Rheumatology 1983; 12:263-267.
16. Drummond PD, Finch PM, Smythe GA. Reflex sympathetic dystrophy: the significance of differing plasma catecholamine concentrations in affected and unaffected limbs. Brain Research 1991; 115:2025-2036.
17. Parkhouse N, Le Quesne PM. Quantitative objective assessment of peripheral nociceptive C-fibre function. Journal of Neurology, Neurosurgery and Psychiatry 1988; 51:28-34.
18. Westerman RA, Widdop RE, Low A, Neild TO, Delaney CA. Non-invasive tests of neurovascular function in human and experimental diabetes mellitus. In: Molinatti G, Bar R, Belfiore S, Porta M (eds). Frontiers in diabetes: Vol 9 Basel; Karger 1989; 127-138.
19. Benarroch EE, Low PA. The acetylcholine-induced flare response in evaluation of small fibre dysfunction. Annals of Neurology 1991; 29:590-595.
20. Roberts RGD, Westerman RA, Hahn A, Pano I, Rabavilas A. Interactions between adrenergic and cholinergic agonists which cause axon reflex vasodilatation. Proceedings of the Australian Physiological and Pharmacological Society 1991; 22:46P.
21. Westerman RA, Widdop RE, Hannaford J *et al.* Laser Doppler velocimetry in the measurement of neurovascular function. Australasian Physical and Engineering Sciences in Medicine 1988; 11:53-65.
22. Obeid AN, Boggett DM, Barnett NJ, Dougherty G, Rolfe P. Depth discrimination in laser Doppler skin blood flow measurements using different lasers. Medical and Biological Engineering and Computing 1988; 26:415-419.
23. Hales JRS, Stephens FRN, Fawcett AA, Daniel K, Sheahan J, Westerman RA, James SB. Observations on a new non-invasive monitor of skin. Clinical and Experimental Physiology and Pharmacology 1989; 16:403-415.
24. Roberts RGD, Westerman RA, Widdop RE, Kotzmann RR, Payne RR. Effect of capsaicin on cutaneous axon reflex vasodilatation in man. Agents and Actions 1992; 35: In press.
25. Blumberg H, Wallin BG. Direct evidence of neurally mediated vasodilatation in hairy skin of human foot. Journal of Physiology 1987; 382:105-121.
26. Wallin BG, Torebjork E, Hallin R. Preliminary observations on the pathophysiology of hyperalgesia in the causalgic pain syndrome. In: Zotterman, Y (ed). Sensory functions of the skin in primates, with special reference to man. Wenner-Gren International Symposium. Oxford, Pergamon Press, 1976; 27:498-502.
27. Rosen L, Ostergren J, Fagrell B, Stranden E. Skin microvascular circulation in the sympathetic dystrophies evaluated by videophotometric capillaroscopy and laser Doppler fluxmetry. European Journal of Clinical Investigation 1988; 18:305-308.
28. Gibbins IL, Vickers JC. Unmyelinated intradermal sensory nerve fibres lacking immuno-reactivity to neuropeptides and neurofilament triplet proteins in the skin of guinea-pigs. Proceedings of the Australian Physiological and Pharmacological Society 1991; 22:90P.

29. Jansco N, Jansco-Gabor A, Szolcsanyi J. Direct evidence for neurogenic inflammation and its prevention by denervation and by pretreatment with capsaicin. British Journal of Pharmacology 1967; 31:138-151.
30. Lembeck F, Holzer P. Substance P as neurogenic mediator of antidromic vasodilation and neurogenic plasma extravasation. Archives of Pharmacology 1979; 310:175-183.
31. Foreman JC. Peptides and neurogenic inflammation. British Medical Bulletin 1987; 43:386-400.
32. Lewis T. The nocifensor system of nerves and its reactions. I. British Medical Journal 1937; 1:431-434. II. British Medical Journal 1937; 1:491-494.
33. Lembeck F. Sir Thomas Lewis' nocifensor system, histamine and substance P - containing primary afferent nerves. Trends in Neurosciences 1983; 6:106-108.
34. Nilsson J, von Euler A, Dalsgaard C-J. Stimulation of connective tissue cell growth by substance P and Neurokinin A. Nature 1985; 315:61-62.
35. Kjartannson J, Dalsgaard C-J. Calcitonin gene-related peptide increases survival of a musculocutaneous critical flap in the rat. European Journal of Pharmacology 1987; 142:355-358.
36. Kjartansson J, Dalsgaard C-J, Jonsson C-E. Decreased survival of experimental critical flaps in rats after sensory denervation with capsaicin. Plastic and Reconstructive Surgery 1987; 79:218-223.
37. Watcher MA, Wheeland RG. The role of topical agents in the healing of full-thickness wounds. Journal of Dermatology Surgery and Oncology 1989; 15:1188-1195.
38. Blumberg H, Wallin BG. Direct evidence of neurally mediated vasodilatation in hairy skin of human foot. Journal of Physiology 1987; 382:105-121.
39. Westerman RA, Blumberg H, Hollerback M, Jager M, Kiss J. Direct micro-neurographic and pharmacological evidence for the existence of sympathetic neurones mediating active vasodilatation in human skin. Proceedings of the Australian Physiological & Pharmacological Society 1986; 17(1):23P.
40. Lewis T. The blood vessels of the human skin and their responses. London; Shaw & Sons 1927.
41. Westerman RA, Widdop RE, Hannaford J, Hogan C, Roberts RGD, Zimmet P. Non-invasive tests of neurovascular function. Clinical and Experimental Neurology 1988; 24:129-137.
42. Shields SA, MacDowell KA, Fairchild SB, Campbell ML. Is mediation of sweating cholinergic, adrenergic or both? A comment on the literature. Psychophysiology; 24:312-319.
43. Grunfeld JA, Tiedemann GJ, Westerman RA. Chronic nicotine exposure enhances cutaneous axon reflexes in the rat. NeuroReport 1991; 2:421-424.
44. Fleming WW, Westfall DP. Adaptive supersensitivity. In: Trendelenburg U, Weiner N, (eds). Catecholamines I. Handbook of Experimental Pharmacology, Vol90/I, Berlin, Springer-Verlag, 1988; 509-559.

45. Wallin BG, Torebjork E, Hallin R. Preliminary observations on the pathophysiology of hyperalgesia in the causalgic pain syndrome. In: Zotterman Y (ed). Sensory functions of the skin in primates, with special reference to man. Wenner-Gren International Symposium. Oxford, Pergamon 1976; 27:498-502.
46. Devor M. Nerve pathophysiology and mechanisms of pain in causalgia. Journal of the Autonomic Nervous System 1983; 7:371-384.
47. Sato J, Perl ER. Adrenergic excitation of cutaneous pain receptors induced by peripheral nerve injury. Science 1991; 251:1608-1610.
48. Jänig W. Experimental approach to reflex sympathetic dystrophy and related syndromes. Pain 1991; 46:2412-2415.
49. Cline MA, Ochoa J, Torebjork HE. Chronic hyperalgesia and skin warming caused by sensitized C-nociceptors. Brain 1989; 112:621-647.
50. Wakisaka S, Kajander KC, Bennett GJ. Abnormal skin temperature and abnormal sympathetic vasomotor innervation in an experimental painful peripheral neuropathy. Pain 1991; 46:299-313.
51. Shyu BC, Olausson B, Huang KH, Widerstrom E, Andersson SA. Effects of sympathetic stimulation on C-fibre responses in rabbit. Acta Physiologica Scandinavica 1989; 137:73-84.
52. Roberts WJ. Hypothesis on the physiological basis for causalgia and related pains. Pain 1986; 24:297-311.
53. Knyazev GG, Knyazeva GB, Tolochko ZA. Trophic functions of primary sensory neurons: are they really local? Neuroscience 1991; 42:555-560.
54. Kilpatrick TJ, Talman PS, Bartlett PF, Nossal GJV. Nerve growth factors: Their role in development and clinical neurology. Australian and New Zealand Journal of Medicine, 1992; In press.
55. Fitzgerald M. C-Fos and the changing face of pain. Trends in Neurosciences 1990; 13:439-440.
56. McMahon SB. Mechanisms of sympathetic pains. British Medical Bulletin 1991; 47:584-600.
57. Bonica JJ. Causalgia and other reflex sympathetic dystrophies. In: JJ Bonica, (ed.) The management of pain. 2nd edn. Philadelphia, Lea & Febinger 1990; 220-243.
58. Todd DP. Prolonged stellate block in treatment of reflex sympathetic dystrophy. Agressologie 1991; 32(5 Spec No.): 281-282.
59. Davis KD, Treede RD, Raja SN, Meyer RA, Campbell JN. Topical applications of clonidine relieve hyperalgesia in patients with sympathetically maintained pain. Pain 1991; 47:309-317.
60. Wahren LK, Torebjork E, Nystrom B. Quantitative sensory testing before and after regional guanethedine block in patients with neuralgia in the hand. Pain 1991; 46:23-30.
61. Kaada B. Promoted healing of chronic ulceration by transcutaneous nerve stimulation (TNS) Vasa 1983; 12:262-269.