INTERLEUKIN-1β AND TUMOUR NECROSIS FACTOR-α AS INDUCERS OF AIRWAY HYPERRESPONSIVENESS AND MICROVASCULAR LEAKAGE

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Declaration

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Publications and presentations[†]

Full publications

Reynolds, P.N., Rice, A.J., Reynolds, A.M., Thornton, A.T., Holmes, M.D., and Scicchitano, R. (1997) Tachykinins contribute to the acute airways response to allergen in sheep actively sensitized to Ascaris suum. *Respirology* **2**, 193-200.

Reynolds, A.M., Reynolds, P., Holmes, M., and Scicchitano, R. (1998) Tachykinin NK_2 receptors predominantly mediate tachykinin-induced contractions in ovine trachea. *European Journal of Pharmacology* **341**, 211-223.

Reynolds, A.M., Holmes, M.D., and Scicchitano, R. (2000) Cytokines enhance airway smooth muscle contractility in response to acetylcholine and neurokinin A. *Respirology* **5**, 153-160.

Rice, A.J., Reynolds, P.N., Reynolds, A.M., Holmes, M.D., and Scicchitano, R. (2001) Tachykinin-induced bronchoconstriction in sheep is NK-1 receptor mediated and exhibits tachyphylaxis. *Respirology* **6**, 113-123.

Reynolds, A.M., Holmes, M.D., Scicchitano, R. (2002) Interleukin-1β and tumour necrosis factor-α increase microvascular leakage in the guinea pig trachea. *Respirology* **7**, 23-28.

[†] Note the candidate was enrolled on a part-time basis from 1994-2002.

Abstracts

Thornton, A.T., Tiivas, K., Rice, A.J., Schilling, S., Reynolds, A.M., Scicchitano, R. A computer system for the continuous measurement of lung resistance in a large animal model of asthma. Proceedings of the annual scientific meeting of the Australian and New Zealand Society of Respiratory Science, Sydney, 1993.‡

Reynolds, A.M., Rice, A., Scicchitano, R., Thornton, A. The effect of intravenous substance P on airway resistance in sheep. Proceedings of the annual scientific meeting of the Australian and New Zealand Society of Respiratory Science, Hamilton Island, 1994.

Reynolds, A.M., Scicchitano, R., Holmes, M.D. NK_2 receptors predominate in the sheep trachea. Proceedings of the annual scientific meeting of the Thoracic Society of Australia and New Zealand, Hobart, 1995. *Australian and New Zealand Journal of Medicine* **25**: 427, 1995.

Reynolds, A.M., Scicchitano, R., Rice, A., Holmes, M. *In Vitro* contractility to tachykinins in ovine trachea: Comparison with *in vivo* effects. Proceedings of the annual scientific meeting of the Australian and New Zealand Society of Respiratory Science, Perth, 1996.

‡ This abstract relates to the development of the *in vivo* sheep model.

Reynolds, A.M, Holmes, M.D., Scicchitano, R. *In vitro,* tachykinin-iduced ovine tracheal smooth muscle contraction is mediated by NK_2 receptors. Proceedings of the annual scientific meeting of the Thoracic Society of Australia and New Zealand, Wellington, New Zealand, 1997.

Reynolds, A.M., Holmes, M.D., Scicchitano, R. Interleukin-1β and tumour necrosis factor-α increase airway smooth muscle contractility, *in vitro*. Proceedings of the European Respiratory Society, Annual Congress, Geneva, Switzerland, 1998. *European Respiratory Journal* **12** (Suppl 28): 115s, 1998.

Reynolds, A.M, Holmes, M.D, Scicchitano, R. Inflammatory cytokines increase airway smooth muscle contractility, *in vitro*. Proceedings of the annual scientific meeting of the Thoracic Society of Australia and New Zealand, Adelaide, 1998. *Respirology* **5**(Suppl): A85, 2000.

Reynolds, A.M., McNamara, T.R., Holmes, M.D., Scicchitano, R. Interleukin-1β and tumour necrosis factor-α synergistically enhance airway smooth muscle (ASM) contractility to acetylcholine, *in vitro*. Proceedings of the World Congress on Lung Health, Florence, 2000. *European Respiratory Journal* **14** (Suppl): 2000, Abstract No. 2678.

Abstract

In asthma the development of airway hyperresponsiveness (AHR) has been linked to the presence of airway inflammation, however, the mechanism by which inflammation leads to AHR remains elusive. There is a school of thought that AHR, *in vivo*, is not due to an increased sensitivity of airway smooth muscle (ASM) to various contractile agonists, but rather, is a property of intact airways which have been structurally changed by chronic inflammation which then allows them to narrow excessively. The primary structural change which is considered to be important in this scenario, is an increase in airway wall thickness. Thickening of the airway wall may result from several mechanisms, one being mucosal oedema.

This thesis is principally an examination of the effects of the pro-inflammatory cytokines, tumour necrosis factor-α (TNFα) and interleukin-1β (IL-1β), on two mechanisms considered to be important in the development of AHR. These being i) an increased contractility of ASM and ii) the development of microvascular leakage (MVL). The former was examined using an ovine model of *in vitro* contractility and the latter examined *in vivo* using an isolated tracheal segment (ITS) in the guinea pig.

Following the detailed characterization of contractile responses of ovine ASM to various agonists, it was determined that TNFα enhances acetylcholine (ACh) contractility in ovine tracheal smooth muscle and that this effect is synergistic in the presence of IL-1β. These studies were then extended to examine mechanisms by which TNFα and IL-1β enhance ASM contractility *in vitro*. In this series of experiments contractile responses to neurokinin A (NKA) and histamine were also examined.

Pre-incubation of ASM with TNFα and IL-1β caused a significant leftward shift in, and an increased in the magnitude of, the concentration-response curves to both ACh and NKA. Whereas, histamine contractility remained unchanged following cytokine incubation. There was no functional alteration to specific M_3 and NK_2 receptors, as reflected by antagonist affinity studies, following cytokine exposure. Neurokinin A contractility in the presence of phosphoramidon indicated that the enhanced contractility following cytokine exposure was not due to a reduction in endogenous neutral endopeptidase activity. Removal of extracellular calcium ions attenuated the contractile response to low concentrations of ACh in control and cytokine pretreated tissue. However, enhanced contractility following TNFα and IL-1β pretreatment was still present. These results demonstrate that ovine tracheal smooth muscle becomes hyperresponsive to ACh and NKA following TNFα and IL-1β exposure, via a mechanism involving intracellular calcium mobilization. In calcium-free Krebs-Henseleit solution the contractile response to histamine in tracheal muscle strips was virtually abolished. This suggests that histamine contractions in ovine ASM are predominantly mediated by extracellular calcium influx. And furthermore, the lack of an enhancement to histamine contractility following cytokine pretreatment appears to reflect agonist-specific mechanistic differences in post-receptor signalling pathways that mediate ovine ASM contraction.

Finally, the effect of a combination of TNFα and IL-1β on airway MVL was examined. Airway MVL was quantitated by determining Evans blue (EB) extravasation in an isolated segment of trachea, in anesthetised mechanically ventilated guinea pigs. The data demonstrate that TNFα and IL-1β aerosol challenge significantly increased EB extravasation compared to saline control challenge.

In summary, IL-1β and TNFα are capable of enhancing ASM contractility to a variety of agonists and inducing MVL. The former mediated by a mechanism involving intracellular calcium mobilization. The results of these studies have important implications in acute exacerbations of asthma, where peribronchial oedema in conjunction with potentiated bronchoconstrictor responses, may induce rapid airway instability by unlinking the airways from the parenchyma, leading to life threatening bronchoconstriction.

Abbreviations

The following abbreviations have been used throughout this thesis.

α	alpha
AC	adenylyl cyclase
ACh	acetylcholine
AHR	airway hyperresponsiveness
ANOVA	analysis of variance
AP-1	activating protein 1
ASM	airway smooth muscle
ATP	adenosine triphosphate
β	beta
BSA	bovine serum albumin
BSL	baseline
°C	degrees centigrade
Ca^{++}	calcium ions
$[Ca^{++}]_i$	cytosolic free calcium ion concentration
$CaCl_2$	calcium chloride
cAMP	cyclic adenosine monophosphate
Carbachol	carbamylcholine chloride
CCRC(s)	cumulative concentration-response curve(s)
C_{dyn}	dynamic compliance
cGMP	cyclic guanosine monophosphate
cm	centimetre

CO_2	carbon dioxide
DAG	diacylglycerol
4-DAMP	4-diphenylacetoxy-N-methyl peperidine methiodide
DMEM	Dulbecco's modified Eagle's medium
EB	Evans blue
EC_{50}	effective concentration to cause 50% of the maximal effect
ECM	extracellular matrix
EGTA	ethyleneglycol-bis (β-aminoethyl ether)N,N,N',N'-tetraacetic acid
E_{max}	maximal effect
ER	endoplasmic reticulum
gm	gram
GM-CSF	granulocyte-macrophage colony stimulating factor
H_2O	water
ID	internal diameter
Ig	immunoglobulin
IL	interleukin
IL-1β	interleukin-1beta
IMVS	Institute of Medical and Veterinary Science
IFN-γ	interferon gamma
IP_3	inositol 1,4,5-triphosphate
IP_3R	inositol triphosphate receptor
ITS	isolated tracheal segment
IV	intravenous
K^+	potassium ions
KCl	potassium chloride
kg	kilogram

K-H	Krebs-Henseleit solution
KH_2PO_4	potassium dihydrogen orthophosphate
L	litre
log	logarithm
M	molar concentration
M_1	muscarinic type 1
M_2	muscarinic type 2
M_3	muscarinic type 3
MABP	mean arterial blood pressure
MAP	mitogen-activated protein
mg	milligram
$MgSO_4.7H_2O$	magnesium sulphate heptahydrate
min(s)	minute(s)
ml(s)	millilitre(s)
MLCK	myosin light chain kinase
ml/hr	millilitres per hour
mmHg	millimetres of mercury
mm	millimetre
mM	millimoles per litre
MVL	microvascular leakage
N_2	nitrogen
NaCl	sodium chloride
$NaHCO_3$	sodium hydrogen carbonate
NANC	non-adrenergic, non-cholinergic
NEP	neutral endopeptidase
NF-κB	nuclear factor kappa B

ng	nanograms
NKA	neurokinin A
NKB	neurokinin B
nm	nanometre
NO	nitric oxide
NPK	neuropeptide K
NPγ	neuropeptide gamma
O_2	oxygen
ΔP	pressure difference
P	pressure
P	probability
pA_2	antagonist affinities
pD_2	apparent agonist affinities
PG	prostaglandin
PIP_2	phosphatidylinositol 4,5-biphosphate
PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PPT	preprotachykinin
P_{TP}	transpulmonary pressure
®	registered trade mark
R_L	lung resistance
SD	standard deviation
SEM	standard error of the mean
SP	substance P

SR	sarcoplasmic reticulum
™	trade mark
TNFα	tumour necrosis factor-alpha
TNFR	tumour necrosis factor receptor
TRAF	tumour necrosis factor receptor associated factor
µg	microgram
µl	microlitre
U/ml	units per millilitre
VIP	vasoactive intestinal peptide
VVO	vesiculo-vacuolar organelles

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Chapter 1 Introduction

1.1 Introduction

Within the last two decades there has been a great deal of interest in the role of sensory nerves in the pathogenesis of asthma. A number of neurotransmitters are co-localized to these nerves including the tachykinins. Barnes in 1986 (Barnes, 1986a) proposed that axonal reflexes are central to the pathogenesis of asthma. He suggested that epithelial damage leads to stimulation of sensory nerves and that antidromic conduction of afferent impulses results in the release of neuropeptides in the airways.

Tachykinins have a number of actions that are relevant to asthma; they are able to constrict bronchial smooth muscle (Martling *et al.*, 1987b), stimulate mucus secretion (Rogers *et al.*, 1989), are potent vasodilators (Piedimonte *et al.*, 1993) and are the putative mediators of neurogenic inflammation (plasma extravasation) (McDonald *et al.*, 1996). Much evidence has accumulated for the involvement of sensory nerves in the pathogenesis of asthma and airway hyperresponsiveness (AHR) (Joos *et al.*, 1994), albeit predominantly from rodent models of allergic asthma (Solway & Leff, 1991). Airway hyperresponsiveness is considered a hallmark of asthma and is defined as an abnormality of the airways that allows them to narrow too easily and to a greater extent than normal airways to a variety of stimuli.

From this background our laboratory felt it was essential to extend this work to an animal model of allergic asthma which we felt is more representative of human asthma.

To this end we established the ovine model of allergic asthma described by Abraham and colleagues (Abraham *et al.*, 1983), which has been well characterized and validated as an experimental model of asthma.

Using this model we have obtained *prima facie* evidence for the involvement of endogenously released tachykinins in the early response to inhaled A*scaris*. We have shown that phosphoramidon enhances and CP-96,345, a specific NK_1 antagonist, inhibits immediate bronchoconstriction following A*scaris* inhalation in sensitized sheep. Pretreatment with capsaicin, which ablates sensory nerves, attenuates antigen-induced bronchoconstriction (Reynolds *et al.*, 1997).

Historically, this thesis arose as a result of these findings, since we postulated that released tachykinins may induce AHR in allergic sheep by potentiating cholinergic mediated contraction by the facilitation of pre-junctional transmitter release. Or possibly, that released tachykinins, by acting directly on bronchial smooth muscle, may demonstrate enhanced bronchoconstrictor responses due to decreased neutral endopeptidase (NEP) activity within the airways, or to a decreased production of epithelial-derived relaxant factors, as a consequence of epithelial disruption, or both. Thus began the examination, both *in vivo* and *in vitro*, of the effect of exogenously applied tachykinins substance P (SP) and neurokinin A (NKA) on ovine airway function in non-allergic sheep with a view to extending the studies in allergic animals. However, due to time constraints the effects in the latter group were not examined.

As early as 1987 it was recognized that the clinical manifestations of asthma including AHR may result from a specific type of airway inflammation (Borish, 1987), however, the mechanism by which inflammation leads to AHR remains elusive. There is a school

of thought that AHR, *in vivo*, is not due to an increase sensitivity of airway smooth muscle (ASM) to various contractile agonists, but rather, is a property of intact airways which have been structurally changed by chronic inflammation which then allows them to narrow excessively. Purported mechanisms include: i) liquid filling of the airway lumen, ii) an increase in mucosal thickness and iii) a decrease in the interdependence between the airway wall and parenchyma (as reviewed in Sterk, 1993a). The latter two may in part result from microvascular fluid extravasation into the airway mucosa. A number of early *in vitro* studies have failed to detect abnormalities in smooth muscle contractility, or to demonstrated a relationship between *in vivo* and *in vitro* AHR (de Jongste *et al.*, 1987b; Cerrina *et al.*, 1986; Goldie *et al.*, 1986; Armour *et al.*, 1984; Armour *et al.*, 1984a; Roberts *et al.*, 1984; Vincenc *et al.*, 1983). Recent evidence however, has demonstrated the presence of *in vitro* AHR in asthmatic airways compared to normal tissue (Bjorck *et al.*, 1992; Cerrina *et al.*, 1989). Thus, there is an apparent intrinsic difference between normal and allergic ASM. Potentially this difference may result from the presence of specific cytokines (as a consequence of inflammation) directly influencing ASM contractility, by affecting signal transduction pathways that mediate ASM contraction. This may be in addition to their inflammatory effects on airway wall structure.

It is now considered that airway inflammation in asthma is a cytokine controlled response rather than simply an antibody mediated one, and with this there has been a rapid exploration of the involvement of cytokines in the development of AHR. It is against this latter background that the ensuing studies undertaken in this thesis were directed. The latter chapters of the thesis are devoted to the detailed examination of the effects of the pro-inflammatory cytokines, tumour necrosis factor-α (TNFα) and interleukin 1-β (IL-1β), in the induction of *in vitro* AHR, and as inducers of

microvascular leakage (MVL), two mechanisms considered to be important in AHR. Although a multitude of cytokines are involved in asthma and airway inflammation, with different cytokines assuming greater importance in different subsets of asthma, TNFα and IL-1β can be considered as an archetypal model in examining these effects. Moreover, the results obtained may provide a unifying mechanism for acquired AHR following industrial irritant exposure and the enhanced AHR observed during exacerbation of asthma e.g. post viral infection and airway instability in sudden fatal asthma.

1.2 Hypothesis and aims

Although the aims of the present thesis appear varied, they are best categorized as general and specific. The general aims document the historic starting point and the development of my technical expertise required to test my hypothesis, while the specific aims outline the studies designed to examine the stated hypothesis. The first general aim arose from my initial interest in AHR and was a collaborative extension of the laboratory's development of the ovine bronchoconstrictor model of allergic asthma. The work described in general aims 2 and 3 was vital in developing the necessary research tools required for characterizing *in vitro* bronchoconstrictor responses in ovine airways.

The general aims of the present study are:

1. To determine the mechanisms involved in the *in vivo* airway response to exogenous SP in non-allergic sheep.
2. To characterize the *in vitro* contractile responses of normal (non-sensitized) sheep ASM to receptor specific tachykinin agonists, ACh, and histamine.

3. To determine the mechanism of action of tachykinins on ASM. That is, whether tachykinins (SP and NKA) act directly on ASM or through endogenous ACh release or indirectly through other mediators e.g. histamine.

Hypothesis

The hypothesis of this study is that cytokines involved in the inflammatory process, namely IL-1β and TNFα specifically alter ASM contractility and MVL. And that, in the former effect, the mechanism involves changes in signal transduction in ASM.

The specific aims of the present study are:

1. To determine whether IL-1β and TNFα, either alone or in combination, can induce *in vitro* AHR to selected agonists (tachykinins (NKA), ACh and histamine) in non-sensitized sheep airways.
2. To elucidate the mechanisms by which TNFα and IL-1β enhance ovine *in vitro* smooth muscle contractility.
3. To determine the *in vivo* effect of IL-1β and TNFα, on tracheal microvascular permeability in the guinea pig.

1.3 Literature review

This review begins with an outline of airway inflammation and the structural remodeling observed in chronic asthma. Following is a summary of the postulated *in vivo* mechanisms by which airways become hyperresponsive in asthma, both mechanical and neural. The latter, due to initial interest, is extended to include a more in-depth review of the mechanisms by which tachykinins may induce AHR. From this the involvement of cytokines in airway inflammation is introduced and includes a brief

discussion of unifying mechanisms that may link the development of AHR in particular subsets of asthma to the presence of specific cytokines. The next section describes similarities and possible convergence of the signal transduction pathways for smooth muscle contraction and pro-inflammatory (TNFα and IL-1β) cytokine cell signaling, together with an up-to-date review of the known *in vitro* effects of TNFα and IL-1β on smooth muscle tone. Finally, is a stand-alone summary discussion of the ovine and guinea pig models of allergic asthma and MVL, respectively.

1.4 Asthma

"Asthma is now defined as a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role, in particular, mast cells, eosinophils, T lymphocytes, neutrophils, and epithelial cells… The inflammation also causes an associated increase in the existing bronchial hyperresponsiveness to a variety of stimuli" (Global Initiative for Asthma, 1997).

Asthma is a common disease which is associated with significant morbidity and mortality. It appears that asthma prevalence is increasing worldwide. Epidemiological studies of asthma prevalence, as determined by questionnaires documenting asthmatics symptoms, have demonstrated a prevalence rate in adults between 25-30% in English speaking countries e.g. Australia, New Zealand, United Kingdom and United States (Beasley *et al.*, 2000). In Europe, depending on country of residence, asthma affects 10-20% of the adult population.

1.4.1 Asthma aetiology

Asthma appears to result from a complex interplay of genetic and environmental factors. Various risk factors have been assigned to the development of asthma; in

particular the presence of allergy or atopy, as documented by the presence of elevated levels of circulating immunoglobulin E (IgE), and AHR. There exists a strong genetic predisposition for the development of atopy (Rona *et al.*, 1997; Sears *et al.*, 1996; Postma *et al.*, 1995), and AHR (van Herwerden *et al.*, 1995). Furthermore, there is a close association between circulating levels of IgE and the development of AHR and asthma in children (Sears *et al.*, 1993; Sears *et al.*, 1991) and asthma in adults (Bodner *et al.*, 1998; Burrows *et al.*, 1989). As well as genetic factors, various childhood environmental factors are associated with an increased risk of developing asthma. These include such things as childhood exposure to cigarette smoke (Weiss *et al.*, 1980), the number of childhood viral infections (Martinez *et al.*, 1995), family size (Rona *et al.*, 1997; Sunyer *et al.*, 1997) and diet (Hodge *et al.*, 1996). Environmental risk factors associated with the development of adult asthma appear to be exposure to airborne contaminants (Anto *et al.*, 1989), cigarette smoking (Bodner *et al.*, 1998) and occupational exposure to various sensitizing agents (Chan-Yeung & Malo, 1995), such as cereal dust, latex and isocyanates.

1.5 Airway hyperresponsiveness

Airway hyperresponsiveness (also termed: bronchial hyperresponsiveness) is considered a hallmark of asthma in which exaggerated airway narrowing occurs in response to a wide variety of non-specific stimuli. The development of AHR has been linked to the presence of airway inflammation (Rogers & O'Connor, 1993; Hogg *et al.*, 1991) however, the mechanism by which inflammation leads to AHR remains elusive. Moreover, it has been suggested that AHR and airway inflammation may be parallel pathophysiological processes rather than causal (Brusasco *et al.*, 1998; Haley & Drazen, 1998; Chapman *et al.*, 1993).

1.6 Inflammation

1.6.1 Overview

An inflammatory response is a normal homeostatic mechanism to body tissue injury. It is a well-orchestrated series of cellular events intended to halt tissue destruction, remove invading matter (particulate or organism) and helps to return normal tissue function (Gauldie, 1991). Inflammation is a localized immune response induced by a variety of causes (e.g. injury, bacteria, trauma, chemical, and heat). The inflammatory response is characterized by dilatation of local blood vessels, increased blood flow and exudation of fluid and plasma proteins into the surrounding tissue and the migration of blood borne leucocytes into the affected tissue (Guyton & Hall, 1996a). This natural process can and does become disturbed resulting in chronic inflammation and permanent changes to tissue.

1.7 Airway inflammation

Early histological evidence of airway inflammation in asthmatics was obtained at autopsy following fatal attacks. The predominant features found were mucus plugging, intraluminal plasma-protein exudate containing eosinophils, epithelial cells and neutrophils, a marked thickening of the airway wall (Carroll *et al.*, 1996; Carroll *et al.*, 1993; Huber & Koessler, 1922), with submucosal oedema and capillary dilatation (Dunnill, 1960), and an increase in smooth muscle fibre number (hyperplasia) and an increase in smooth muscle fibre size (hypertrophy) (Heard & Hossain, 1973). In addition, epithelial shedding, eosinophil infiltration beneath a grossly thickened basement membrane and goblet cell hyperplasia were observed (Dunnill *et al.*, 1969; Dunnill, 1960; Messer *et al.*, 1960).

With advances in microscopy, immunohistochemical staining and the standardization of morphometric counting techniques (Bai *et al.*, 1994; James *et al.*, 1988), it has become apparent that airway inflammation is a principal feature associated with asthma even in patients with mild disease (Beasley *et al.*, 1989; Jeffery *et al.*, 1989). Further characterization of inflammation in the large and small airways has been made possible using flexible fibre-optic bronchoscopy in conjunction with endobronchial biopsy and bronchoalveolar lavage, and more recently sputum induction. Inflammatory cells are found in the lamina propria, bronchial smooth muscle and submucosa of asthmatic airways (refer Figure 1.1 for normal airway wall structure). These cells include eosinophils, T-lymphocytes, mast cells, macrophages, basophils and neutrophils (Ammit *et al.*, 1997; Hamid *et al.*, 1997; Faul *et al.*, 1997; Carroll *et al.*, 1997a; Sur *et al.*, 1993). Numerous studies have documented an association between inflammatory cellular infiltrate and airway function (Duddridge *et al.*, 1993; Laitinen *et al.*, 1992; Gibson *et al.*, 1990; Corrigan *et al.*, 1988). However, very few have demonstrated a strong relationship between inflammatory intensity and asthma disease severity, perhaps the exception being a study by Bousquet and coworkers, which demonstrated a significant relationship between eosinophil numbers in the airway mucosa and asthma severity (Bousquet *et al.*, 1990). This lack of association between cellular infiltrate and disease severity may reflect the quiescent state of the inflammatory cells at the time of sampling, i.e. there may be a stronger association observed if the active state of the inflammatory cells is determined. This is perhaps best exemplified by the finding of Hamid and associates, where they found a larger number of eosinophils in the outer airway wall area than the inner, however, a greater number of activated eosinophils were found in the inner wall area (Hamid *et al.*, 1997).

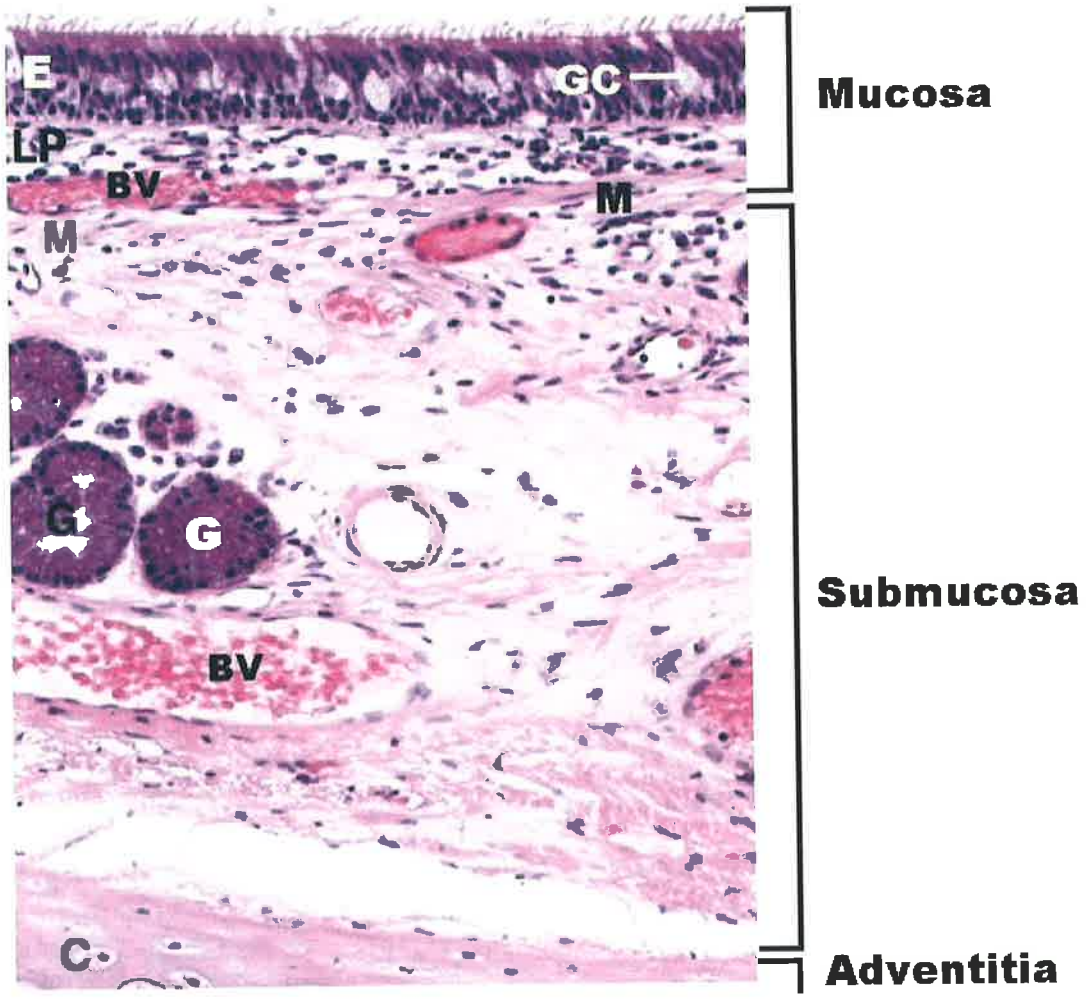


Figure 1.1 Basic structure of a normal bronchial wall (H&E stain). E, epithelium; GC, goblet cell; LP, lamina propria; BV, blood vessel; M, smooth muscle layer; G, mucous glands; C, cartilage. Adapted and reproduced with permission from Young & Heath (2000).

Possibly the most striking common morphometric feature of the airway in chronic severe asthma is the pronounced airway wall thickening (Carroll *et al.*, 1996; Carroll *et al.*, 1993; Kuwano *et al.*, 1993; Takizawa & Thurlbeck, 1971; Dunnill *et al.*, 1969). The increased thickness has been linked to chronic inflammation which results in various structural changes within the airways, termed "airway remodeling". An alteration in both muscular and non-muscular structures is seen, with airways smaller than 2 mm also being affected.

1.7.1 Structural changes in asthmatic airways

1.7.1.1 Airway epithelium

A pseudo-stratified columnar epithelium lines the airway consisting of ciliated and non-ciliated cells with a few mucus-secreting goblet cells, tightly attached to a basement membrane (Figure 1.2). The epithelium forms an interface between the environment and the remainder of the respiratory tract (Montefort *et al.*, 1992). Biopsy specimens of asthmatic airways demonstrate epithelial disruption, such that the columnar cells are shed leaving the basal cells intact (Jeffery *et al.*, 1989; Laitinen *et al.*, 1985). The extent of epithelial shedding is both variable and patchy, and can be seen in mild asthma (Beasley *et al.*, 1989; Laitinen *et al.*, 1985). Epithelial shedding is thought to result from a disruption of interepithelial adhesion molecules between the suprabasal and basal cell layers (Montefort *et al.*, 1993). This may be related to inflammatory cell migration from microvessels beneath the bronchial epithelium with accompanying oedema (plasma extravasation) (Pilewski & Albelda, 1993) and / or the release of cytotoxic substances, such as cationic proteins, proteases and oxygen radicals from

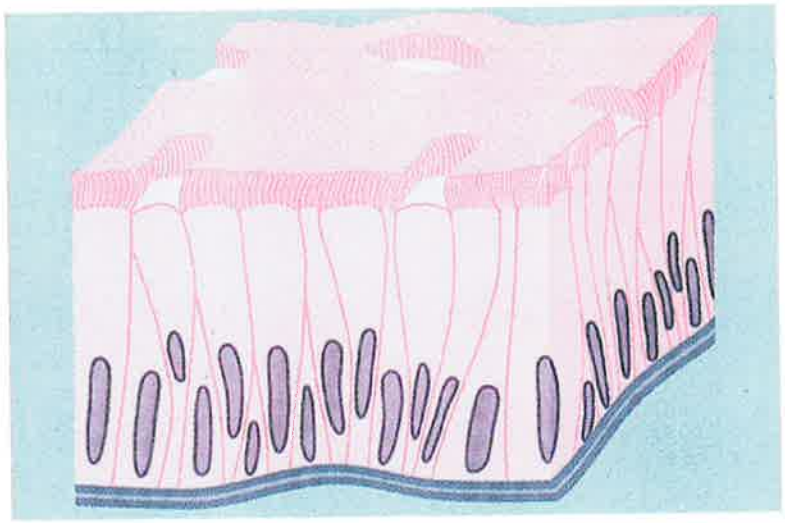


Figure 1.2a Diagram of pseudo-stratified columnar ciliated epithleium, which gives the impression that there is more than one layer of cells. However, all cells rest on the basement membrane, with their nuclei disposed at different levels, giving the illusion of stratification.

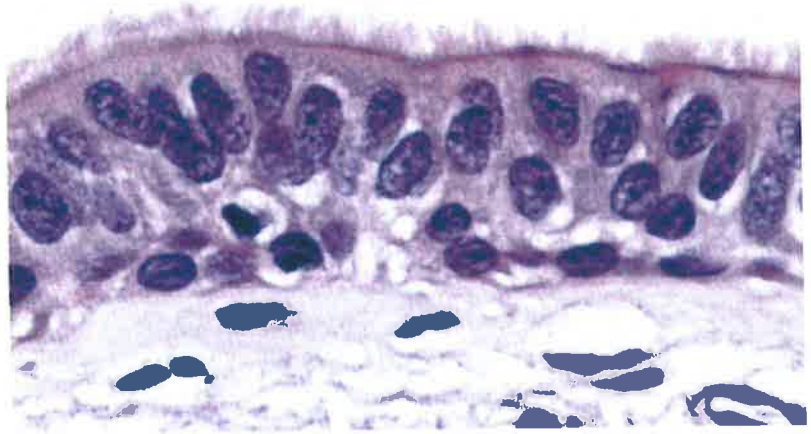


Figure 1.2b Haematoxylin and eosin (H&E) micrograph of respiratory epithelium. Reproduced with permission from Young & Heath (2000).

inflammatory cells within the airway mucosa (Bousquet *et al.*, 1994; Coyle *et al.*, 1994; Ohashi *et al.*, 1992; Montefort *et al.*, 1992). Another alteration to the epithelium associated with inflammation in asthma is goblet cell hyperplasia (Laitinen *et al.*, 1985).

1.7.1.2 Subepithelial structures

In asthmatic airways there is thickening of the reticular layer (lamina reticularis) of the epithelial basement membrane, often termed sub-epithelial fibrosis. This being due to deposits of collagen, types I, III and V, and fibronectin (Jeffery *et al.*, 1989; Roche *et al.*, 1989), which is in contrast to the type IV collagen component found in normal airways. The deposition of these compounds has been attributed to the increased presence of myofibroblasts, associated with inflammation, within the subepithelial area (Brewster *et al.*, 1990).

There exists throughout the submucosa of the bronchial tree a network of discrete longitudinal bundles being quite distinct from the epithelial basement membrane and subepithelial collagen layer. These bundles contain elastic fibres, collagen and myofibroblast matrix. In asthma (fatal and non-fatal) the longitudinal bundles become hypertrophied, with a decrease in elastin and an increased presence of collagen and myofibroblasts (Carroll *et al.*, 2000).

1.7.1.3 Mucous glands

Submucosal mucous glands are distributed throughout the cartilaginous airways and are responsible for producing most of the mucus found in large airways. In normal adults these glands occupy approximately 12% of the airway wall (Matsuba & Thurlbeck, 1972) and are known to double in cases of fatal asthma (Carroll *et al.*, 1993). Submucosal gland hyperplasia is thought to contribute to the excessive mucus

production observed in fatal asthma. Considerable mucus impaction is seen in the airways at autopsy in fatal asthma (Dunnill, 1960), which may result from a decrease in mucociliary clearance during exacerbative episodes of asthma (O'Riordan *et al.*, 1992), possibly by ciliary dysfunction induced by inflammatory mediators (Wanner *et al.*, 1996), increased mucus viscosity (Pavia *et al.*, 1985) and ciliated epithelium disruption (Laitinen *et al.*, 1985).

1.7.1.4 Airway smooth muscle

In normal adults, approximately 3% of the bronchial wall is occupied by smooth muscle, this increases to 20% in bronchioles (Matsuba & Thurlbeck, 1972). In asthmatic patients a three fold increase in ASM bulk is observed in both proximal and distal airways, (Carroll *et al.*, 1993; Saetta *et al.*, 1991; Ebina *et al.*, 1990; Dunnill *et al.*, 1969). Notwithstanding others have reported no increase in ASM content of the larger airways in asthmatics (Thomson *et al.*, 1996). Where increased muscle mass has been demonstrated morphometric studies demonstrate no clear mechanism at play. It appears that the increase in smooth muscle results from both hyperplasia and hypertrophy with striking differences between individuals. Ebina and colleagues have demonstrated two patterns of ASM thickening. One group of patients demonstrate muscle hyperplasia in the larger more proximal airways, while in the second group hypertrophy prevails throughout the airways, being more marked in the distal airways (Ebina *et al.*, 1993). Despite these structural differences, it is evident that in fatal asthma muscle mass is increased in all airways greater than 2-4 mm in diameter compared with non-fatal asthma (Carroll *et al.*, 1993; Kuwano *et al.*, 1993). Of note however, is that these changes in smooth muscle are quite variable and may prove unpredictable as to a fatal outcome in individual patients (Roche, 1998).

1.7.1.5 Bronchial circulation

The bronchial circulation arises from bronchial and intercostal arteries from the aorta. These vessel branches anastomose freely with each other forming a dense vascular plexus in the peribronchial space. They give rise to small arterioles which penetrate the mucosal layer where they form a second submucosal plexus. This occurs throughout the airways from the trachea to terminal airways (Deffebach *et al.*, 1987). The presence of a thickened oedematous airway mucosa containing dilated and congested blood vessels in fatal asthma has long been documented (Dunnill, 1960). However, only recently has the importance of such a finding to the pathophysiology of asthma and bronchial hyperresponsiveness been recognized (Mitzner *et al.*, 1995).

Angiogenesis is the process by which new blood vessels emerge from existing endothelial lined vessels. This is a normal process required for growth, development, wound healing and inflammation. An increase in airway vasculature can occur by two processes, sprouting angiogenesis (the proliferation of endothelial cells to form new blood vessels) or microvascular enlargement (where endothelial cells proliferate resulting in an enlargement of the vessel circumference). This latter mechanism results in larger vessels than original, which appear dilated or congested, without being more numerous (Busse *et al.*, 1999). It is not known which mechanism of angiogenesis are involved for increasing airway wall thickness in asthma. However, it is clearly evident from a number of recent morphometric studies that there is both an increase in bronchial submucosal vessel number (Orsida *et al.*, 1999; Li & Wilson, 1997) and vascular volume (Carroll *et al.*, 1997b; Kuwano *et al.*, 1993).

1.7.1.6 Extracellular matrix

The extracellular matrix (ECM) provides structural support within various tissues, and until recently was thought to be a relatively inert structure within the airways (Spurzem, 1996). The ECM is a complex network of macromolecules produced and secreted by tissue mesenchymal cells (e.g. epithelial and endothelial cells, fibroblasts and smooth muscle). The ECM consists of fibrous proteins such as collagen and elastin, and adhesive proteins (fibronectin, tenascin and laminin) in a polysaccharide gel containing several proteoglycans and glycosaminoglycans, such as decorin, biglycan, versican and hyaluronan (Bianco *et al.*, 1990; Zimmermann & Ruoslahti, 1989). Hyaluronan has received particular attention in recent times, its presence, confers specific viscoelastic properties (e.g. lubrication, hydration and filtering effects) (Fraser *et al.*, 1997) to the ECM, and facilitates cell migration and proliferation during injury and repair (Knudson & Knudson, 1993; Laurent & Fraser, 1992). Increased levels of hyaluronan are found in the BAL fluid from asthmatics, with the magnitude strongly correlating with disease severity (Bousquet *et al.*, 1991).

It is becoming apparent that the changes in ECM are intricately involved in airway wall remodeling in asthma. Moreover, the cell types present, including inflammatory infiltrate (eosinophils, macrophages, lymphocytes, mast cells and neutrophils), their activation state, and the mediators released or secreted acutely affect its composition. The ECM is now known to be a dynamic structure, establishing an equilibrium balance between synthesis and degradation of its components. Matrix metalloproteinases (MMP) and their tissue inhibitors (tissue inhibitors of metalloproteinase (TIMP)) aid in this homeostasis (Birkedal-Hansen *et al.*, 1993). Matrix metalloproteinases are members of a family of preolytic enzymes that degrade ECM. It is postulated that there may be an imbalance between MMP and their inhibitors in asthma resulting in abnormal

tissue repair (Mautino *et al.*, 1999a). Alterations in MMP and TIMP balance have been documented in asthmatic patients. In particular increased levels of MMP-9 and TIMP-1 have been observed in lavage fluid and bronchial biopsy specimens (Lemjabbar *et al.*, 1999; Mautino *et al.*, 1999b; Hoshino *et al.*, 1998; Mautino *et al.*, 1997). Furthermore, matrix metalloproteinases have been implicated in epithelial fibrosis (Hoshino *et al.*, 1998), angiogenesis (Stetler-Stevenson, 1999; Sang, 1998) and ASM proliferation (Rajah *et al.*, 1999; Johnson & Knox, 1999; Foda *et al.*, 1999).

1.8 Airway inflammation and airway hyperresponsiveness

Airway hyperresponsiveness is defined as an abnormality of the airways that allows them to narrow too easily and to a greater extent than normal airways to a variety of inhaled non-specific stimuli. The stimuli may be either chemical or physical, and are classed as direct or indirect. Direct stimuli (e.g. cholinergic agonists, histamine, leukotriene D_4) act directly on ASM via specific receptors. Physical and chemical agents such as exercise, hypertonic saline, adenosine and sulphur dioxide are indirect stimuli as they induce airway narrowing by stimulating inflammatory cells and/or sensory nerves to release bronchoconstrictor mediators (Joos *et al.*, 1993).

The underlying abnormality in asthma is a specific type of airway inflammation, and the development of AHR is thought to be due to the inflammation. However, the mechanism by which airway inflammation leads to AHR remains unclear. One body of evidence suggests that AHR is an *in vivo* phenomenon and results from airway wall remodeling including the presence of airway wall oedema. In this model the contractility of the ASM does not need to be enhanced *per se*, that is, the ASM is normal. The other theory is that ASM contractility is enhanced for a variety of reasons

including the effects of inflammatory cell-derived mediators. There is evidence to support both postulates.

Airway hyperresponsiveness is not unique to asthma, and is seen in patients with other chronic inflammatory diseases such as chronic obstructive pulmonary disease (Klein & Salvaggio, 1966), cystic fibrosis (van Haren *et al.*, 1992), sarcoidosis (Laitinen *et al.*, 1983), chronic bronchitis (Enarson *et al.*, 1987) and bronchiectasis (Varpela *et al.*, 1978). Furthermore, asthmatic symptoms can occur without AHR (Stanescu & Frans, 1982) and AHR has been observed in subjects without any demonstrable inflammatory changes (Power *et al.*, 1993).

It has been universally concluded that, in moderate to severe asthmatics, the failure to demonstrate a plateau or maximal effect in *in vivo* bronchoprovocation dose-response studies (Woolcock *et al.*, 1984), equates to excessive airway narrowing. This is thought to be related to an increase in ASM shortening (i.e. the ASM is allowed to contract to a greater extent) for a given stimulus (Moreno *et al.*, 1986). Mechanisms which determine excessive degrees of airway narrowing include: 1) an increase in mucosal thickness, 2) a decrease in the interdependence between the airway wall and parenchyma, 3) liquid filling of the airway lumen and 4) an enhanced contractility of ASM itself (Moreno *et al.*, 1986).

1.8.1 Mucosal thickening

Elaborate mathematical modeling based on morphometric data of asthmatic airways and a geometric model of the human tracheobronchial tree has suggested that changes in airway wall thickness will dramatically accentuate airway narrowing (Wiggs *et al.*, 1992; Moreno *et al.*, 1986). Anatomically the airway wall can be divided into three

areas: 1) an inner wall i.e. tissue between the luminal surface and the innermost layer of smooth muscle. This area consists of epithelium, basement membrane, lamina reticularis and loose connective tissue containing mucous glands and the submucosal microvasculature; 2) the smooth muscle layer; and 3) the outer wall area consisting of tissue between the outermost layer of smooth muscle and the surrounding lung parenchyma i.e. the adventia (Pare & Bai, 1996).

Wiggs and colleagues demonstrated that the enhanced airway narrowing seen in asthma and chronic obstructive pulmonary disease can be explained mathematically by loss of elastic recoil and an increase in inner airway wall thickness (Wiggs *et al.*, 1992). Both of which have been clearly demonstrated in asthmatic subjects (Carroll *et al.*, 1993; Kuwano *et al.*, 1993; Woolcock & Read, 1968). Furthermore, Wiggs *et al* demonstrated that the dynamics of airway narrowing is further exaggerated if the inner airway wall thickening is allowed to encroach into the lumen, while only inducing minimal changes to baseline airways resistance (Wiggs *et al.*, 1992).

Schematically the airway lumen is often depicted as a smooth surface however, histological sections demonstrate small folds in the mucosa (Figure 1.3). Following ASM contraction these folds become more prominent as muscular contraction forces all the structures within the muscle layer to buckle. Mucosal folding prevents the airway from collapsing by providing a load against which the muscle must contract, however, this load is decreased if fewer folds are present allowing greater ASM shortening. The observation that there are fewer folds in asthmatic airways (Wiggs *et al.*, 1994) has lead several groups to examine what physical aspects of airway wall structure might influence the mucosal buckling or folding pattern (Wiggs *et al.*, 1997; Lambert, 1991). Modeling by Lambert *et al* demonstrated that the pressure required to buckle an

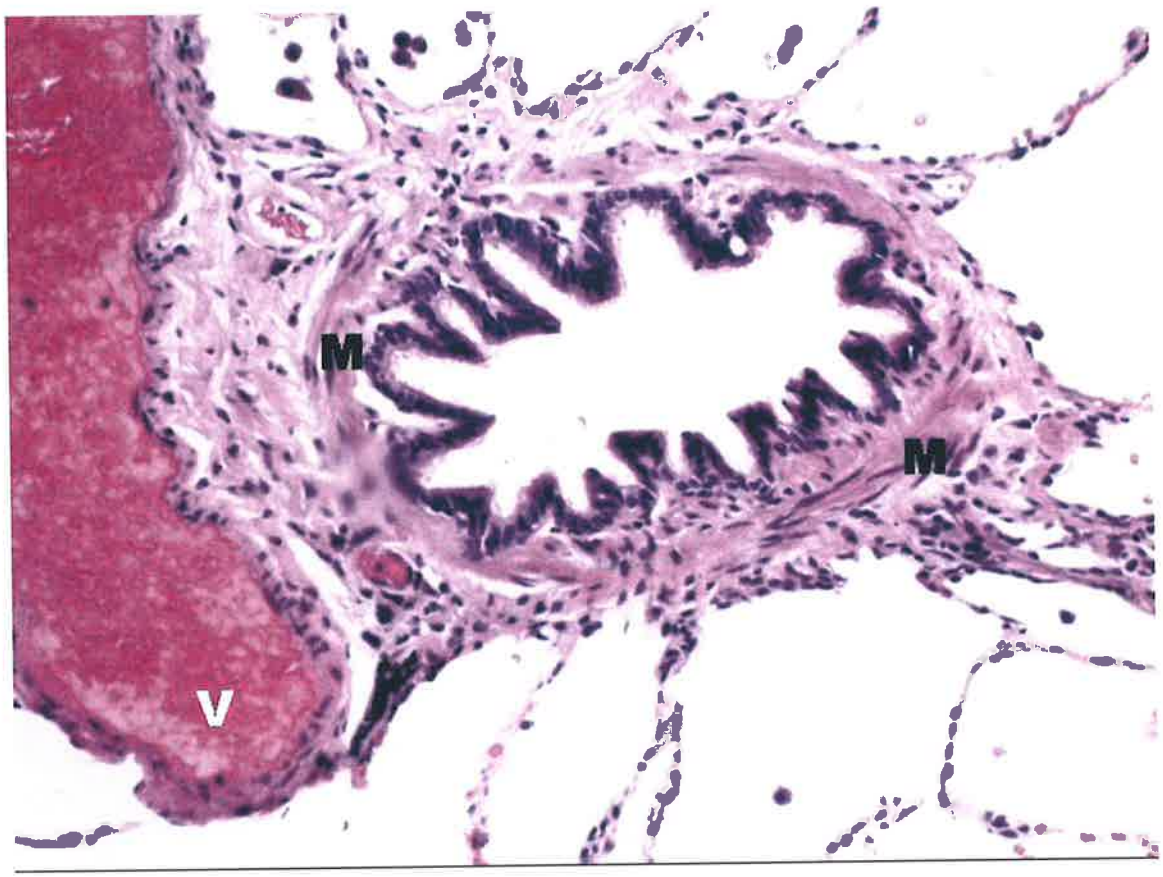


Figure 1.3 Micrograph of a normal bronchiole (H&E stain) illustrating the pattern mucosal folding. M, smooth muscle layer; V, vein accompanying the bronchiole. Adapted and reproduced with permission from Young & Heath, (2000).

artificial airway (elastic tube) was dependent on the number of folds created which in turn determined the propensity for luminal narrowing. That is a mucosal buckling pattern with fewer folds requires less pressure and has a greater tendency towards airway narrowing (Lambert, 1991). Wiggs and colleagues using a more sophisticated bilayer cylindrical model further developed this concept. This group demonstrated that the most critical factor which influenced the buckling pattern (number of folds) of their two layer model was the thickness or stiffness of the inner layer. A thickened or stiff inner layer resulted in a folding pattern with fewer circumferential folds allowing the tube to narrow to a greater extent before the folds push against one another and determine the degree of luminal narrowing (Wiggs *et al.*, 1997). A thickening or stiffening or the tube's inner layer was likened to the subepithelial collagen layer observed in asthmatic airways due to inflammatory remodeling processes, which consequently would lead to a reduced number of mucosal folds and enhanced airway narrowing, i.e. a mechanism for AHR.

1.8.2 Airway-parenchymal interdependence

In normal airways, the calibre of the airway is determined by the balance between the inward force of the contracting ASM and the tethering action of the lung parenchymal elements, which exert an outward force on the airway, opposing airway narrowing. During *in vivo* bronchoconstriction, ASM contracts against an intrinsic load applied by the elastic elements of the lung parenchyma. Normally this intrinsic load impedes ASM shortening and prevents excessive airway narrowing (Robinson *et al.*, 1992). That is, there is interdependence between the airway wall and lung parenchyma in maintaining airway calibre. Macklem hypothesized that an increase in adventitial wall area external to the smooth muscle, as a consequence of inflammation, would result in a decrease or "uncoupling" of the airway-parenchymal interdependence (Macklem, 1990). That is,

the intrinsic load (the outward force) applied by the surrounding parenchyma is diminished allowing the ASM to contract to a greater extent, in response to a stimulus, until the inward and outward forces equalize. He further speculated that oedema accompanying airway inflammation might uncouple the airway from the parenchyma to such an extent that the ASM may contract sufficiently as to completely occlude the airway.

Morphometric analysis of asthmatic airways have demonstrated thickened outer wall areas (Carroll *et al.*, 1993; Kuwano *et al.*, 1993) with increased inflammatory cell infiltrate (Saetta *et al.*, 1991), increased adventitial vascular area (Kuwano *et al.*, 1993) and increased ECM depsoition (Roberts, 1995). Moreover, in cases of fatal asthma there is a significant increase in outer wall area compared to non-fatal asthma (Carroll *et al.*, 1993).

Pare and colleagues have summarized the physiological consequences of mathematically changing airway wall dimensions on airway function. Simply by increasing the inner airway wall area form 20% to 40% (which by itself produces a small increase in airway resistance) and imposing a physiologically acceptable amount of ASM shortening (30%) then airway resistance of the thickened airway increases tenfold above that of the "normal airway" (Moreno *et al.*, 1986), Figure 1.4. Similarly if one assigns an increase to the outer adventitial wall area then for a given degree of ASM shortening, the change or uncoupling of airway-parenchymal interdependence leads to an increase in airway resistance six times greater than that of the "normal model airway" (Pare & Bai, 1995), Figure 1.5. Finally, and possibly most importantly, their mathematical model demonstrated that an increase in ASM mass was the most significant abnormality responsible for the increased resistance in response to a

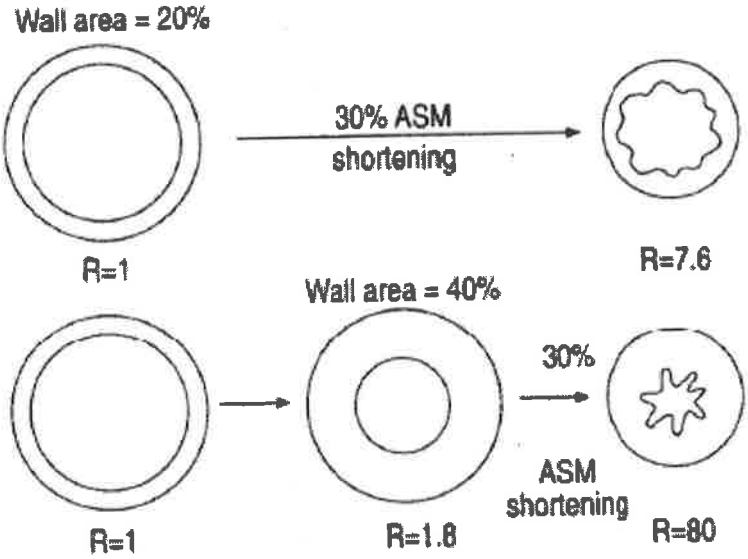


Figure 1.4 Diagrammatic representation of the effect of inner wall thickening on airway resistance. ASM, airway smooth muscle. When 30% ASM shortening occurs, the airway resistance (R) increases from an arbitary value of 1 to 7.6. An increase in wall area of 40% produces a small increase in baseline R to 1.8, but in the presence of a thickened wall, 30% ASM shortening increases R to 80 times that of baseline. Reproduced from Pare & Bai (1996).

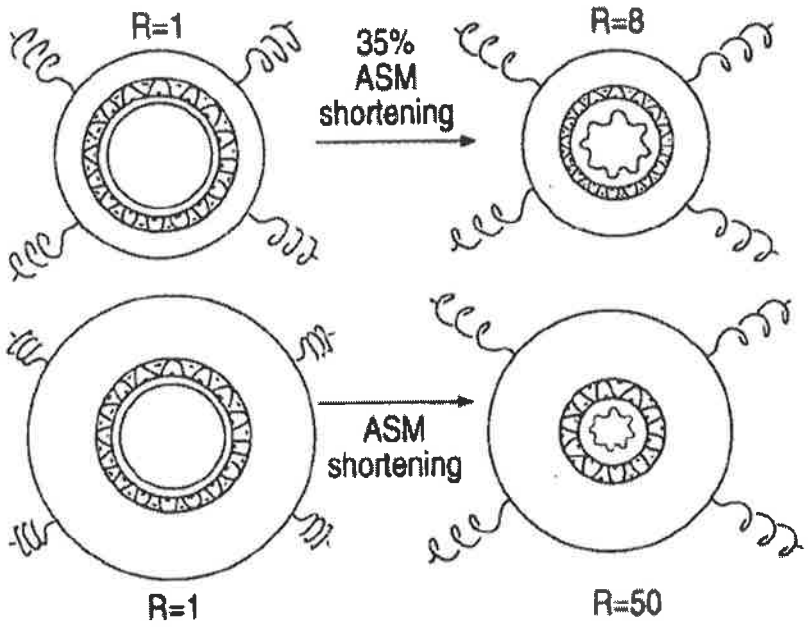


Figure 1.5 Effect of adventitial wall thickening on airway resistance. The normal airway is given a resistance (R) value of 1. When the airway smooth muscle (ASM) is stimulated to contract, shortening occurs until the stress in the surrounding lung parenchyma (springs) prevents further shortening, resulting in an R of 8. With adventital thickening (outer wall area) the stress in the surrounding parenchyma is decreased and greater ASM shortening is possible before parenchymal recoil prevents further airway narrowing. Ultimately leading to enhanced airway narrowing with an R of 50. Adapted and reproduced from Pare & Bai (1995).

bronchoconstricting stimulus seen in asthma (Lambert *et al.*, 1993). This group further demonstrated that a greater ASM mass *per se*, would generate a greater total force for a given (normal) degree of muscle stimulation resulting in greater luminal narrowing. This airway narrowing would be greatly enhanced by mucosal and adventitial wall thickening known to accompany ASM hyperplasia (Carroll *et al.*, 1993; Kuwano *et al.*, 1993; Saetta *et al.*, 1991; Ebina *et al.*, 1990; Dunnill *et al.*, 1969). A prominent feature of asthmatic airways compared to control data is the increase in ASM cross-sectional area which is doubled in fatal asthma compared to non-fatal asthma (Carroll *et al.*, 1993; Kuwano *et al.*, 1993; Heard & Hossain, 1973).

1.8.3 Microvascular leakage

Aside from the vascular remodeling within the airway wall, i.e. the increased number and size of blood vessels (see above) as a mechanism for AHR, MVL of plasma from these abundant vessels and cell migration induced by various inflammatory mediators will cause airway wall oedema. A detailed discussion of mechanisms which control 'basal' permeability, and associated permeability coefficients, of water and small solutes is beyond the scope of this introduction, however, these topics have been extensively reviewed by Michel and Curry (Michel & Curry, 1999).

Vascular leakage of plasma is a principal sign of acute inflammation. The importance of airway vascular leakage in asthma has only been appreciated over the last fifteen years (Persson, 1991; Persson, 1986), despite the earlier findings of plasma proteins in sputum, mucus plugs and bronchoalveolar lavage fluid from asthmatics (Lam *et al.*, 1985; Heilpern & Rebuck, 1972; Dunnill, 1960). Vascular leakage of macromolecules is an active process under physiological and pharmacological control (Majno *et al.*, 1969), and is generally referred to as increased vascular permeability (Michel & Curry,

1999). Experimental evidence for inflammation induced increases in microvascular permeability in the airway was described using dyes which bound to plasma proteins (Saria & Lundberg, 1983). At any one time, airway plasma extravasation will be determined by the permeability of the airway microvasculature and by microvascular pressure, the latter will vary with changes in airway blood flow and vascular resistance (Persson, 1987). While the degree of airway wall oedema will be governed by the rate of net fluid extravasation, lymphatic drainage and transepithelial liquid transport into the airway lumen (Wanner, 1989). Movement of the exudate within the interstitium is affected by hydrostatic pressure gradients, ultimately leading to the bulk movement of plasma into the airway lumen (Persson *et al.*, 1996; Persson *et al.*, 1990). This appears to be a unidirectional flux of macromolecules as airway luminal absorption (inward permeability) of small and large solutes into the mucosa is unaffected (Erjefalt & Persson, 1991; Greiff *et al.*, 1991).

Ultrastructural studies of microvascular beds have demonstrated that inflammatory mediator induced plasma extravasation occurs in post capillary venules through large gaps between endothelial cells (McDonald, 1994; Majno *et al.*, 1969). These intercellular gaps are produced by endothelial cell contraction (McDonald, 1994; Joris *et al.*, 1987). While delayed inflammatory responses to mild thermal burns or injury involves vascular leakage from both capillaries and venules (Cotran & Majno, 1964). Recent investigations using electron microscopy and serial section reconstructioning techniques have suggested that increased vascular permeability also occurs via a transcellular route involving vesiculo-vacuolar organelles (VVO – interconnecting grapelike clusters of vesicles and vacuoles) (Baluk *et al.*, 1997; Feng *et al.*, 1996). These VVO occur more frequently and take up macromolecules upon stimulation of endothelial cells by various mediators such as vascular endothelial growth factor

(VEGF, earlier know as vascular permeability factor), histamine and serotonin (Feng *et al.*, 1996). The induction of increased vascular permeability by various inflammatory mediators appears to be a transient phenomenon with maximal effects being obtained with 1-10 minutes with a return to basal levels usually within 30 minutes (Baluk *et al.*, 1997; Michel & Kendall, 1997; Evans *et al.*, 1989).

In addition to the more permanent structural changes in the airway wall, increased microvascular permeability will induce further immediate and significant oedematous changes to the airway wall. This in turn will amplify bronchoconstrictor responses. For example, oedema in the submucosa by interacting with the abnormal proteoglycan deposits (versican and biglycan) beneath the epithelium (Huang *et al.*, 1999; Roberts, 1995), could lead to increased stiffness or turgor of the subepithelial layer causing a further decrease in the number of mucosal folds following smooth muscle activation (Wiggs *et al.*, 1997). Liquid accumulation within an already thickened vascular submucosa would subsequently increase inner wall area, while advential oedema would result in further decoupling of the airway wall from parenchymal attachments. All of which, together or separately, would result in a far greater degree of luminal narrowing during ASM contraction (Pare & Bai, 1996; Wiggs *et al.*, 1992; Hogg *et al.*, 1987), than would otherwise occur in the absence of increased microvascular permeability.

1.8.4 Airway luminal liquid

Mucus hypersecretion is a common finding in the inflamed asthmatic airway. James and Carroll have examined the extent to which mucus may contribute to airway narrowing and resistance during bronchoconstriction. They demonstrated that airway resistance can increase dramatically, up to 700-fold, if mucus discharge, inner airway

wall thickening and a modest degree of ASM shortening occurs simultaneously (James & Carroll, 1995).

The passage of plasma exudate resulting from increased microvascular permeability of the submucosal capillaries into the airway lumen has been documented for some time (Dunnill, 1960). As ASM contracts the mucosa folds forming ridges that protrude into the airway lumen. Luminal oedema will rapidly fill the small pockets or interstices that form between the epithelial projection reducing luminal cross-sectional area and increasing airway resistance (Yager *et al.*, 1989). Airway oedema containing plasma proteins will lead to the disruption of mucociliary clearance, and possibly enhancing mucus plugging, by decreasing ciliary beat frequency (Sanderson & Sleigh, 1981) and increasing mucus viscosity (List *et al.*, 1978). Furthermore, luminal plasma exudate will also react with pulmonary surfactant causing it to become dysfunctional (Seeger *et al.*, 1985). Besides the well recognized role of surfactant in maintaining open alveoli, another major physiological role is in maintaining narrow conducting airway patency (Enhorning *et al.*, 1995). Plasma proteins are known to inhibit surfactant function (Seeger *et al.*, 1985) leading to increased surface tension thereby making small airways unstable and prone to collapse (Yap *et al.*, 1994).

In addition to these physiological effects of enhanced MVL, the exuded plasma is capable of initiating other biological effects. Extravasated plasma is rich in proteins capable of activating protein cleavage cascades, cytokine production, inflammatory cell recruitment, cellular proliferation, and sensory neural pathways (Persson *et al.*, 1996).

1.8.5 Airway smooth muscle contractility

The early failure to demonstrate a relationship between *in vivo* and *in vitro* AHR (de Jongste *et al.*, 1987b; Cerrina *et al.*, 1986; Armour *et al.*, 1984; Armour *et al.*, 1984a; Roberts *et al.*, 1984; Vincenc *et al.*, 1983), has lead to the suggestion that AHR is an *in vivo* phenomenon due to airway wall remodeling, rather than an abnormality of the intrinsic properties of the ASM itself. These studies used tissue obtained from "normal" areas of lungs following carcinoma resection, usually from smokers with or without bronchial hyperresponsiveness. These studies concentrated on trying to detect increased ASM contractility to direct stimuli e.g. histamine and methacholine, invariably demonstrating no difference, although relaxation characteristics appeared abnormal (Cerrina *et al.*, 1986; Goldie *et al.*, 1986). Recently, it has been established that carcinoma resected ASM is hyporesponsive to cholinergic agonists (Armour *et al.*, 1996). However, isolated reports of increased ASM contractility from asthmatic subjects to various spasmogens have been reported (Bjorck *et al.*, 1992; Cerrina *et al.*, 1989; de Jongste *et al.*, 1987a; Schellenberg & Foster, 1984).

1.8.6 Passive sensitization

There is now accumulating evidence suggesting that sensitization alters ASM biochemistry and contractility. Passive sensitization involves the incubation of ASM with sensitizing serum containing high concentrations of IgE, which then confers airway responsiveness to specific allergens and hyperresponsiveness to non-specific agonists, such as histamine (Black *et al.*, 1989) and NKA (Ben-Jebria *et al.*, 1993), the latter caused by a serum factor other than elevated IgE (Watson *et al.*, 1997). Passive sensitization of human ASM induces non-specific *in vitro* hyperresponsiveness possibly via alterations in calcium ion (Ca^{++}) mobilization (Marthan *et al.*, 1992; Black *et al.*, 1989) and/or enhanced protein kinase C (PKC) activation (Rossetti *et al.*, 1995).

Furthermore, demonstrable changes in the contractile apparatus have been detected in sensitized ASM. Airway smooth muscle from sensitized dogs, mice and passively sensitized human bronchial smooth muscle exhibit increased maximal shortening velocity and capacity to shorten (Mitchell *et al.*, 1997; Fan *et al.*, 1997; Antonissen *et al.*, 1979), despite no change in isometric force generation. The mechanism appears to involve an increase in smooth muscle cell myosin light chain kinase (MLCK) content (Ammit *et al.*, 2000; Jiang *et al.*, 1992).

In vitro hyperresponsiveness, as measured by isometric contractions, can be induced by antigen exposure, bacterial endotoxin or viral infection (Molimard *et al.*, 1998; Colasurdo *et al.*, 1995; Folkerts & Nijkamp, 1990; Saban *et al.*, 1987), by the addition of activated inflammatory cell supernatants and inflammatory products to organ baths (Anticevich *et al.*, 1996; Folkerts *et al.*, 1992), and *in vitro* exposure to various oxidizing agents (Marthan *et al.*, 1996). Others have shown that passive sensitization of human bronchi *in vitro*, enhanced smooth muscle contractility and decreased relaxation responses (Villanove *et al.*, 1993; Ben-Jebria *et al.*, 1993; Black *et al.*, 1989).

The link between the inflammatory process and the induction of AHR, as demonstrated by an increased contractility *in vitro* has been further strengthened by these recent findings. Ammit *et al.* detected a significantly greater number of mast cells within the smooth muscle of sensitized bronchi compared to non-sensitized bronchi (Ammit *et al.*, 1997). Furthermore, Johnson and colleagues demonstrated that mast cell tryptase potentiated histamine contractility, *in vitro*, in sensitized but not in non-sensitized bronchi (Johnson *et al.*, 1997), while another research group has shown human mast cell tryptase can induce *in vitro* hyperresponsiveness to histamine in non-sensitized human

bronchi, by tryptase-induced mast cell migration from the epithelium to the submucosa and smooth muscle layer (Berger *et al.*, 1999).

1.9 Airway hyperresponsiveness and neural innervation

Another factor besides airway inflammation and airway wall remodeling that may be responsible for the individual variability in AHR seen in asthmatics is autonomic dysfunction, i.e. an alteration in neuronal function.

For many years it was thought that the autonomic control of mammalian ASM was provided by contractile and relaxant innervation by cholinergic and adrenergic nerves, respectively. However, with the advent of electrical field stimulation techniques another type of innervation that was neither adrenergic nor cholinergic was demonstrated in a variety of species, including man (Davis *et al.*, 1982; Richardson & Beland, 1976). An extensive historic review of these findings is provided by Ellis and Undem (Ellis & Undem, 1994). Earlier coined as the 'third' nervous system (Laitinen & Laitinen, 1987; Barnes, 1984), but has since been defined as non-adrenergic, non-cholinergic or NANC innervation.

Autonomic nerves, in addition to their regulatory role of ASM tone, regulate other aspects of airway function such as mucus secretion, blood flow, microvascular permeability and inflammatory cell activation and migration (Basbaum *et al.*, 1990; Maclagan, 1987; Richardson & Webber, 1987; Barnes, 1987a; Barnes, 1986b).

1.9.1 Adrenergic innervation

The adrenergic system involves sympathetic nerves, circulating catecholamines, and α- and β-adrenoceptors. Sympathetic pathways from the spinal cord to specific effector

organs comprise a preganglionic and postganglionic neuron (Refer Figure 1.6). The preganglionic fibres leave the spinal cord and synapse with postganglionic fibres in one of the many ganglia of the paravertebral sympathetic ganglion chains. The postganglionic fibres then travel to the various organs they control. Some preganglionic sympathetic nerve fibres pass, without synapsing, to the adrenal medullae. There the nerves end and upon stimulation cause the secretion of adrenaline and noradrenaline into the blood stream. Within the sympathetic nervous system postganglionic fibres arising from the cervical ganglia enter the lung at the hilus, accompanying the vagus nerves (Guyton & Hall, 1996b).

The adrenergic system is generally considered 'inhibitory' as its predominant function is to relax ASM, an effect mediated by β-adrenoceptor stimulation (Barnes, 1986b), while ASM α-adrenoceptor function *per se*, is considered to have only a minor role in bronchomotor tone (Goldie, 1990; Zaagsma *et al.*, 1987). Due to the sparse sympathetic innervation in human ASM (Laitinen *et al.*, 1985b; Richardson & Beland, 1976), it has generally been concluded that adrenergic inhibitory nerves do not have a major role in controlling ASM tone (Barnes, 1995). Adrenergic nerves, however, are often associated with airway ganglia, bronchial vessels and submucosal glands (Widdicombe, 1993; Goldie, 1990; Laitinen, 1985a). Therefore, the effect of α-adrenoceptor activation on other airway structures such as, blood vessels, mucous glands, nerve transmission and mast cells are possibly more likely to influence airway calibre (Barnes, 1992; Goldie, 1990). Physiological studies have suggested sympathetic neurotransmitter release (noradrenaline) at airway ganglia inhibits parasympathetic neurotransmission through the ganglia (Baker *et al.*, 1983) (Figure 1.6).

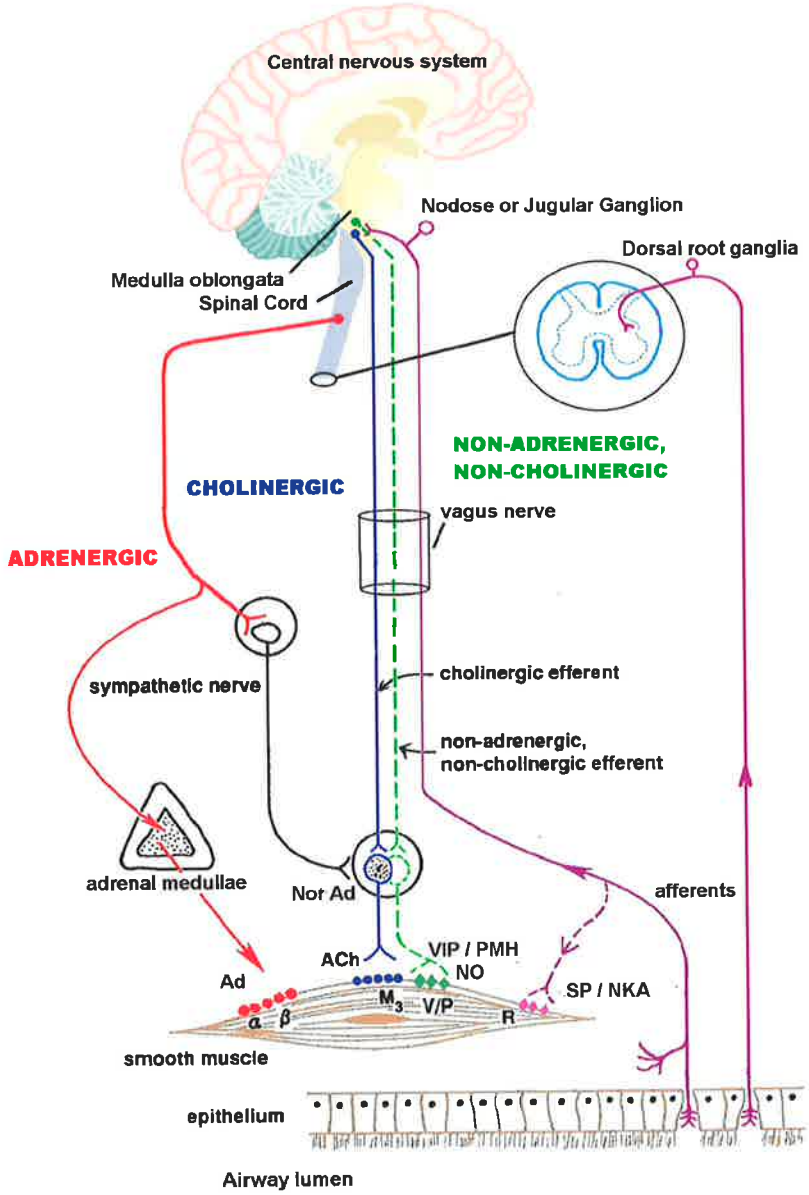


Figure 1.6 Schematic diagram illustrating the adrenergic, cholinergic and non-adrenergic, non-cholinergic (NANC, dashed lines) nervous systems innervating ASM. Release of neurotransmitters adrenaline (Ad) and acetylcholine (ACh) stimulate specific α, β and M_3 receptors on ASM. In the NANC system the preganglionic relaxant fibres arise from nuclei within the medulla oblongata, travelling in the vagus nerves synapsing with postganglionic fibres in the airway ganglia. Upon activation these efferent nerves release vasoactive intestinal peptide (VIP), peptide histidine methionine (PHM) and nitric oxide (NO), that activate specific relaxant receptors (V/P) on ASM. The vagus nerves also carry capsaicin-sensitive, tachykinin-containing afferent fibres which synapse in brain stem nuclei and whose cell bodies are found in the nodose and jugular ganglia. Similar afferents also arise from the spinal cord and their cell bodies are located in the dorsal root ganglia. Stimulation of the collateral branches of these sensory afferents causes the antidromic release of neurokinins such as SP and NKA. Adapted from Casale (1991), Ellis & Undem (1994), Young & Heath (2000)(Young & Heath, 2000; Ellis & Undem, 1994).

β-adrenoceptor dysfunction has been implicated as a mechanism for AHR in asthma. Several studies in man and animals have demonstrated a decreased relaxation response in allergic ASM compared to control tissue (Song *et al.*, 1997; Villanove *et al.*, 1993; Ben-Jebria *et al.*, 1993; Wills-Karp & Gilmour, 1993a). Moreover, polymorphisms of the β-adrenoreceptor have been documented and appears to acts as disease modifiers of asthma rather than having a causative role. Different polymorphisms have been associated with elevated IgE levels (Dewar *et al.*, 1997), nocturnal asthma and bronchial hyperreactivity (Liggett, 2000). The latter two effects being related to the expression of specific polymorphisms that subsequently alter β-receptor regulation (Liggett, 2000).

1.9.2 Cholinergic innervation

Cholinergic innervation is considered 'excitatory' playing an important role in maintaining airway tone and in mediating acute bronchoconstriction. Cholinergic nerves are most often associated with the parasympathetic nervous system. Approximately 75% of all parasympathetic nerve fibres are in the vagus nerves (passing to the entire thoracic and abdominal region of the body). In the parasympathetic system preganglionic fibres pass uninterrupted to their effector organ, and within the organ walls synapse with postganglionic nerves. Within the respiratory system, cholinergic nerves originating centrally from the respiratory centre of the medulla oblongata, travel down the vagus nerve synapsing in parasympathetic ganglia located in airway walls (Refer Figure 1.6). Short post-ganglionic fibres travel from the ganglia to ASM and glands (Laitinen & Laitinen, 1987). Cholinergic innervation is greater in larger airways than in peripheral airways (Barnes *et al.*, 1983). Vagal stimulation leads to acetylcholine (ACh) release from nerve terminals, activation of muscarinic receptors on ASM and glands with resultant bronchoconstriction and mucus secretion.

Thus far, five subtypes of muscarinic receptors (M_1-M_5) have been genetically determined (Jones, 1993), however, only three are considered to be important in regulating airway tone (Eglen *et al.*, 1994; Barnes, 1989). M_1 receptors which are located on parasympathetic ganglia facilitate vagal transmission (Kilbinger *et al.*, 1993; Barnes, 1992), Figure 1.7, while M_2 receptors are located on post-ganglionic cholinergic nerve endings where they function as "autoreceptors" (ten Berge *et al.*, 1996; Minette & Barnes, 1990). Stimulation of these M_2 receptors results in a decrease in ACh release. M_2 autoreceptors have been demonstrated in man (ten Berge *et al.*, 1996; Patel *et al.*, 1995; Minette *et al.*, 1989), dog (Brichant *et al.*, 1990), guinea pig (Fryer & Wills-Karp, 1991) and rat (Aas & Maclagan, 1990). Dysfunctional M_2 receptors have been demonstrated in various animal models of AHR. For instance enhancement of vagally-mediated bronchoconstrictor responses, due to a loss of M_2 receptor function, has been demonstrated following virus infection (Fryer *et al.*, 1994), ozone exposure (Schultheis *et al.*, 1994), and antigen challenge (ten Berge *et al.*, 1995; Fryer & Wills-Karp, 1991). Furthermore, asthmatic subjects demonstrate M_2 receptor dysfunction *in vivo* (Minette *et al.*, 1989) and *in vitro* (Song *et al.*, 1997). These observations may explain, in part, the increased AHR seen after ozone exposure (Golden *et al.*, 1978), and in exacerbative episodes of asthma following viral infection (Folkerts *et al.*, 1998; Sterk, 1993b) and antigen exposure (Boulet *et al.*, 1983; Cockcroft *et al.*, 1977b).

The receptors responsible for ASM contraction are of the M_3 subtype, although M_2 receptors are found on smooth muscle (Eglen *et al.*, 1994). Stimulation of M_3 receptors results in phosphoinositide hydrolysis with the formation of inositol triphosphate and

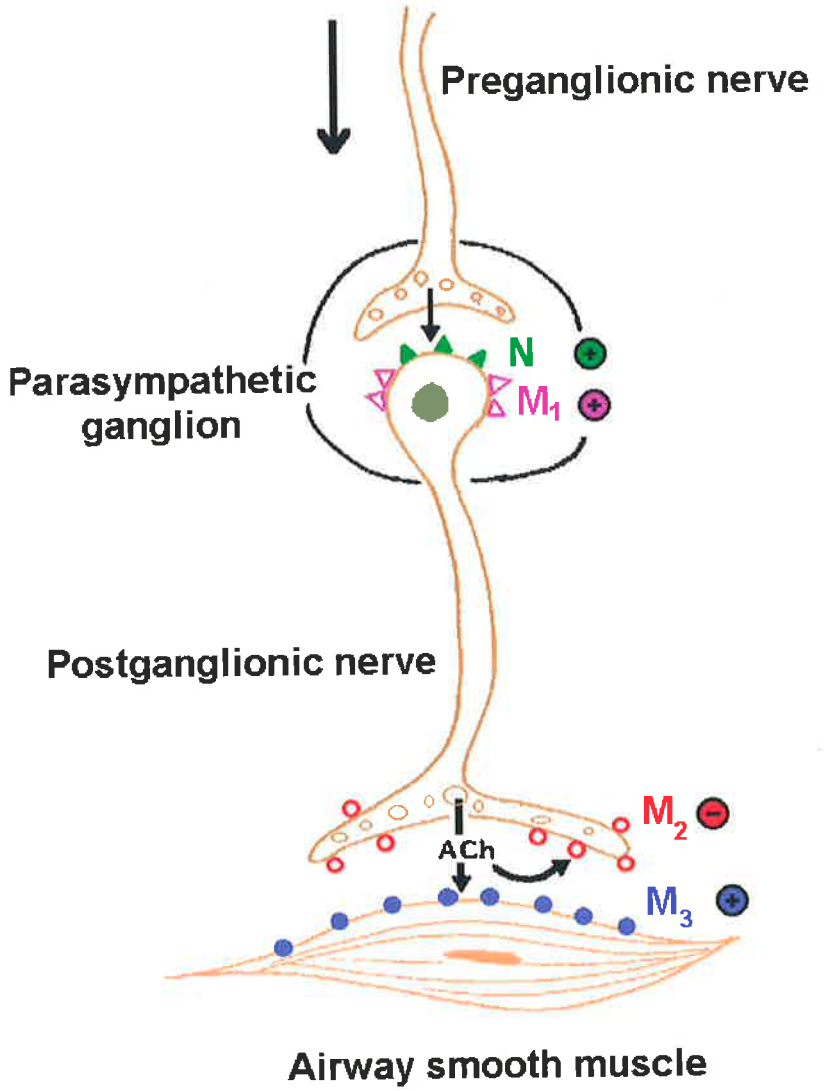


Figure 1.7 Muscarinic subtypes in airways. Ganglionic neurotransmission is mediated by nicotinic (N) receptors, but muscarinic M_1 receptors may have a facilitatory role. In postganglionic nerves M_2 receptors inhibit acetylcholine (ACh) release, which acts on M_3 receptors on airway smooth muscle. Thereby reducing the effect of ACh on airway M_3 receptors. Adapted from Barnes (1992).

resultant Ca^{++} release from intracellular stores, thereby initiating contraction (Chilvers *et al.*, 1994). The exact function of ASM M_2 receptors remains unclear (Fryer & Jacoby, 1998; Eglen *et al.*, 1994). Smooth muscle M_2 receptor stimulation leads to inhibition of the adenylyl cyclase (AC) intracellular messenger system which is involved in smooth muscle relaxation (Schramm & Grunstein, 1992). Therefore, although M_2 receptors are not involved in ASM contraction *per se*, they appear to inhibit relaxation responses mediated by AC, such as β-adrenoceptor mediated relaxation (Fryer & Jacoby, 1998; Eglen *et al.*, 1994; Barnes, 1987b). M_3, and a smaller proportion of M_1, receptors are also localized to submucosal glands (Mak & Barnes, 1990).

1.9.3 Afferent innervation

The parasympathetic nervous system also consists of vagal afferent or sensory fibres within the airway mucosa, that mediate a number of protective sensory mechanisms. Vagal afferent nerves arise from large airways and travel to the medulla, and include myelinated fibres from slowly adapting stretch receptors and rapidly adapting irritant receptors. Stimulation of the latter irritant fibres causes reflex mediated tachypnoea, cough, bronchoconstriction, laryngeal constriction and mucus secretion (Solway & Leff, 1991). The majority of sensory nerves are unmyelinated C-afferent fibres which travel in the vagus to the medulla and whose neurons are located in the upper thoracic dorsal ganglion and the nodose and jugular ganglia (Lundberg *et al.*, 1984a) (Refer Figure 1.6). Two populations of C-fibre endings have been described (pulmonary and bronchial) based on their circulatory accessibility following intravenous injection of stimulating agents (Coleridge & Coleridge, 1984). Sensory nerves innervate the airway epithelium, smooth muscle, glandular structures and vasculature (Solway & Leff, 1991; Laitinen, 1985a; Lundberg *et al.*, 1984a).

1.9.4 Non-adrenergic, non-cholinergic innervation

With respect to ASM tone, NANC neural responses may induce either relaxation (inhibitory, i-NANC) or contraction (excitatory, e-NANC). Initially it was thought that this was a discrete neural system, but it now appears that NANC effects are mediated by the release of neurotransmitters (e.g. neuropeptides and nitric oxide) from the classical autonomic nerves (Barnes, 1992; Ellis & Undem, 1992; Lundberg *et al.*, 1987).

1.9.4.1 Inhibitory NANC system

Presently the general consensus is that i-NANC innervation of ASM is a component of the parasympathetic system (Barnes, 1995; Ellis & Undem, 1994; Stretton, 1991). That is, i-NANC bronchodilator responses result from the release of co-localized bronchodilator peptides, vasoactive intestinal peptide (VIP) and peptide histidine methionine or peptide histidine isoleucine (in humans and animals, respectively) from cholinergic nerves (Palmer & Barnes, 1987; Uddman & Sundler, 1987; Laitinen *et al.*, 1985c), and the synthesis and release of nitric oxide, upon cholinergic nerve stimulation (Ellis & Undem, 1992) (Figure 1.6). Nitric oxide (NO) is synthesized via nitric oxide synthatase, which has been shown to be co-localized with VIP in airway cholinergic nerves (Fischer & Hoffmann, 1996). Activation of the i-NANC system (i.e. co-release/synthesis of VIP and NO) is thought to act as a "braking mechanism" in maintaining bronchomotor tone. In human and animal *in vitro* studies both VIP and NO have demonstrated bronchodilator actions (Ellis & Undem, 1992; Li & Rand, 1991; Palmer *et al.*, 1986), and both are able to influence cholinergic neural transmission by decreasing ACh release from cholinergic nerve endings (Ward *et al.*, 1993; Belvisi *et al.*, 1993). Since a defect in the i-NANC system has been demonstrated in allergic cats (Miura *et al.*, 1992), it was postulated that this defect might occur in asthma. Although

no such defect has been observed in mild asthma (Lammers *et al.*, 1989), an absence of VIP-immunoreactive nerves has been described in asthmatic lungs (Ollerenshaw *et al.*, 1989). Furthermore, VIP exhibits potent anti-inflammatory properties in the lung. These include inhibition of lymphocyte proliferation, monocyte and macrophage activation, mast cell mediator release, and reduces acute oedematous lung injury (Said, 1991).

1.9.4.2 Excitatory NANC system

In addition to the inhibitory i-NANC efferent system in the airways, there is also a NANC afferent nervous system that protects the airways against inhaled irritants, particles and over-inflation (stretch) (Solway & Leff, 1991; Lundberg *et al.*, 1983). As previously mentioned activation of these e-NANC nerves provides an important homeostatic role in airway function. This is achieved by activating respiratory protective reflexes such as cough and bronchoconstriction (Solway & Leff, 1991).

Excitatory-NANC responses are mediated by the release of tachykinins as a result of antidromic stimulation of C-fibres in the afferent nervous system (Figure 1.6). These fibres are classified as capsaicin-sensitive, tachykinin-containing sensory nerves. Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) is the irritant compound of chilli peppers and has been used extensively in the study of these nerves (Holzer, 1988; Martling, 1987a; Lundberg *et al.*, 1984; Papka *et al.*, 1984; Lundberg *et al.*, 1983a).

1.9.4.2.1 Tachykinins

Tachykinins are a group of small peptides (approximately 10 amino acids); they derive their name from their ability to cause rapid contraction of a variety of smooth muscle

types. Structurally tachykinins are characterized by a common C-terminal amino acid sequence of Phe-X-Gly-Leu-Met, where **X** is an aliphatic or aromatic amino acid and the C-terminal methionine is amidated (Figure 1.8). To date, most of the attention relating to the role of neural mechanisms in asthma has been directed to SP. Substance P is an eleven amino acid peptide, originally isolated from equine brain and intestine by Von Euler and Gaddum in 1931 (Von Euler & Gaddum, 1931), and shown to have vasoactive and smooth muscle contracting properties.

Five tachykinin peptides have been identified in mammalian neural tissue, SP, NKA, neurokinin B (NKB), and the amino terminal extended forms of NKA; neuropeptide K (NPK) and neuropeptide gamma (NPγ) (Solway & Leff, 1991; Helke *et al.*, 1990). Two genes encode the mammalian tachykinins, one gene encodes only NKB (preprotachykinin (PPT-II)). The other gene, the PPT-I is responsible, for encoding SP, and NKA, and its extended forms. Substance P, NKA and its derivatives arise by alternative splicing of mRNA derived from the PPT-I gene and post-translational modification of the PPT-A (Helke *et al.*, 1990). Usually a number of tachykinins co-localize to the same nerve often with calcitonin-gene related peptide, although the latter is not a tachykinin, and may be released concomitantly by the same stimulus (Lundberg *et al.*, 1987; Martling, 1987a; Hua *et al.*, 1985; Lundberg *et al.*, 1985). A large number of agents and stimuli are able to cause the release of endogenous neuropeptides due to their ability to stimulate sensory nerves (Solway & Leff, 1991). These include (electrical) vagal nerve stimulation (Lundberg *et al.*, 1983), hyperventilation (Ray *et al.*, 1988), airway hypocapnia (Reynolds & McEvoy, 1989), leukotrienes (Solway & Leff, 1991), inhalation of hypertonic saline (Pedersen *et al.*, 1998), sulphur dioxide (Hajj *et al.*, 1996), bradykinin, histamine, ether, capsaicin and cigarette smoke (Lundberg *et al.*, 1991; Lundberg & Saria, 1983b).

Peptide Sequence of the Mammalian Tachykinins

Substance P Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH2

Neurokinin A His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH2

Neuropeptide K Asp-Ala-Asp-Ser-Ser-Ile-Glu-Lys-Gln-Val-Ala-Leu-Leu-Lys-Ala-Leu-Tyr-Gly-His-Gly-Gln-Ile-Ser-His-Lys-Arg-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH2

Neuropeptide γ Asp-Ala-Gly-His-Gly-Gln-Ile-Ser-His-Lys-Arg-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH2

Neurokinin B Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH2

Figure 1.8 Peptide sequence of the mammalian tachykinins, showing homologous sequences at the caboxy termini, the regions responsible for interaction with tachykinin receptors.

1.9.4.2.2 Tachykinin Receptors

In mammalian tissues, the tachykinins exert their effects principally by interaction of their C-terminal ends with specific receptors (Mussap *et al.*, 1993; Frossard & Advenier, 1991). A classification system was proposed in 1986, which is based on the relative affinities of the individual tachykinin for the receptors. Tachykinin receptors are designated NK_1, NK_2 or NK_3 with SP, NKA and NKB as the preferred endogenous ligands respectively. However because of the common C-terminal structure, there is some cross-reactivity of each of the ligands for each of the receptors. Structurally, the receptors consist of an extracellular amino terminus, seven hydrophobic transmembrane helices with alternating extracellular and cytoplasmic loops and a cytoplasmic carboxy terminus (Nakanishi *et al.*, 1993; Gerard *et al.*, 1993; Burbach & Meijer, 1992) (Figure 1.9). This basic structure of the tachykinin receptors places them in the super-family of guanine nucleotide binding proteins (G-protein) linked receptors, which also includes the adrenergic, rhodopsin and muscarinic receptors (Venter & Fraser, 1990). Second messenger responses described include activation of a G_s-protein leading to activation of AC, with subsequent conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) (Dessauer *et al.*, 1996; Birnbaumer & Brown, 1990), and the activation of a G_q-protein leading to activation of phospholipase C (PLC), with the conversion of phosphatidylinositol 4,5-biphosphate (PIP_2) to inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) (and an increase in intracellular Ca^{++} from internal reticular stores via the inositol triphosphate receptor (IP_3R) (Hughes *et al.*, 1990; Harden, 1990). There are some non-receptor mediated effects (particularly with SP) which involve the interaction of the N-terminal end with inflammatory cells, e.g. polymorphonuclear leukocytes, lymphocytes, mast cells and neutrophils (Maggi, 1997; Frossard & Advenier, 1991).

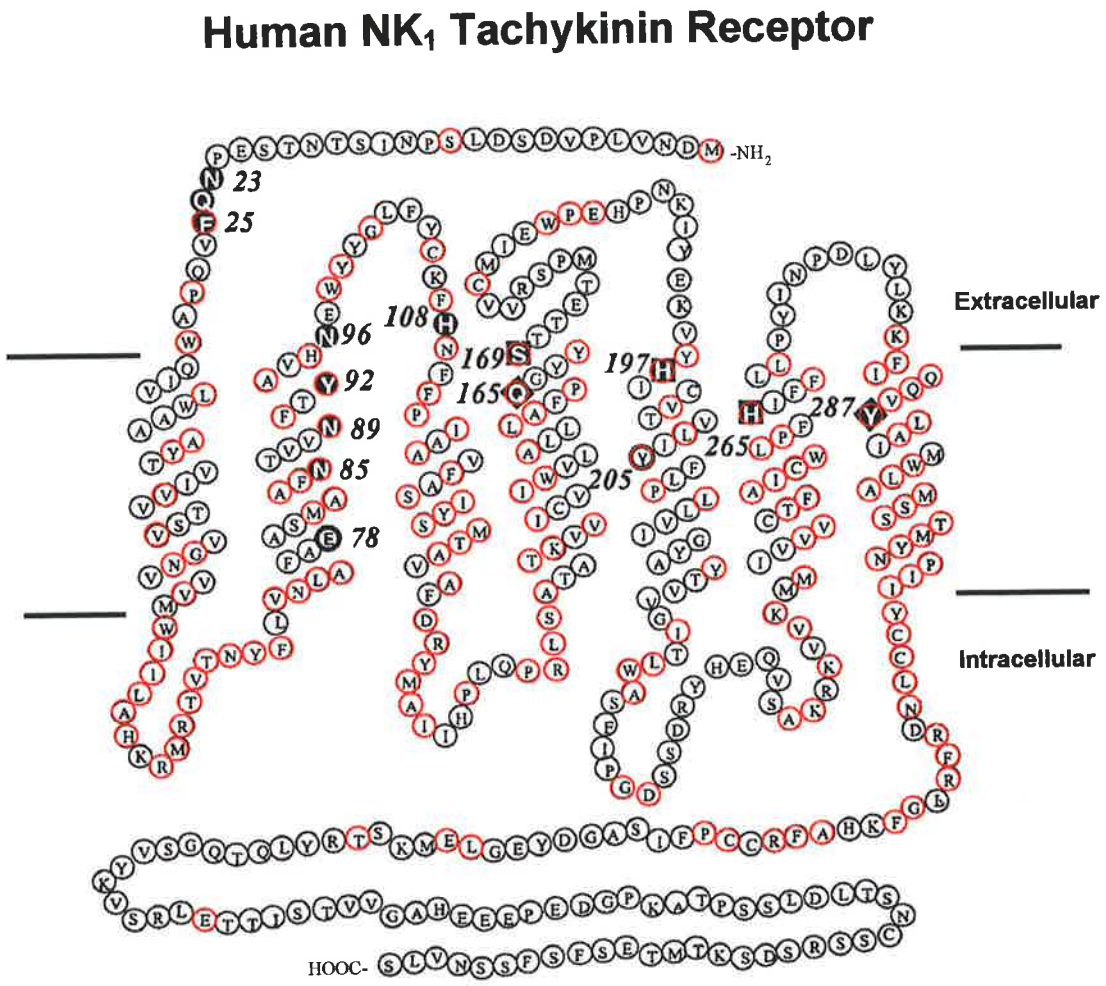


Figure 1.9 Schematic representation of the human NK_1 tachykinin receptor. Red circles indicate homology between NK_1, NK_2 and NK_3 receptors. Filled circles indicate amino acids involved in peptide binding. Filled squares indicate amino acids involved in non-peptide antagonist binding. Diamonds indicate both peptide and antagonist binding. Residues 78 and 205 are important for receptor activation. Adapted from Fong *et al.* (1995).

1.9.4.2.3 Axon reflex

In 1986 Barnes put forward a hypothesis that suggested a physiological role for sensory nerves in the pathogenesis of asthma and AHR, Figure 1.10. He proposed that epithelial desquamation, a prominent feature of asthmatic airways (Laitinen *et al.*, 1985), allows stimulation of sensory nerves, (by mediators and other products of inflammation), followed by antidromic conduction of afferent impulses resulting in the local release of tachykinins in the airways (Barnes, 1986a). Thus resulting in bronchoconstriction, neurogenic inflammation and inflammatory cell infiltrates.

1.9.4.2.4 Biological actions

Tachykinins have a variety of biological effects in the lung that mimic many features of asthma, (Joos *et al.*, 1994; Solway & Leff, 1991) (Table 1.1). Historically it has been thought that this involvement has related to the physiological effects of tachykinins in the airways. Many experiments have demonstrated that tachykinin-containing nerves have an effector function in the airways in addition to their sensory role. Tachykinins or antidromic stimulation of these nerves *ex vivo* and *in vivo* causes vasodilatation, plasma extravasation, leukocyte infiltration, and mucus hyper-secretion (features which characterize the inflammatory response at any mucosal surface) as well as broncho-constriction. Most of this evidence has been obtained from animal studies (in particular, rodents). Tachykinins are able to constrict bronchial smooth muscle (Joos & Pauwels, 1990; Maggi *et al.*, 1989; Fine *et al.*, 1989; Uchida *et al.*, 1987; Gerard, 1987; Sekizawa *et al.*, 1987a), stimulate mucus secretion (Borson *et al.*, 1987), are potent vasodilators (Constantine *et al.*, 1991; Laitinen *et al.*, 1987; Hua *et al.*, 1984), are mediators of

Axon Reflex Hypothesis

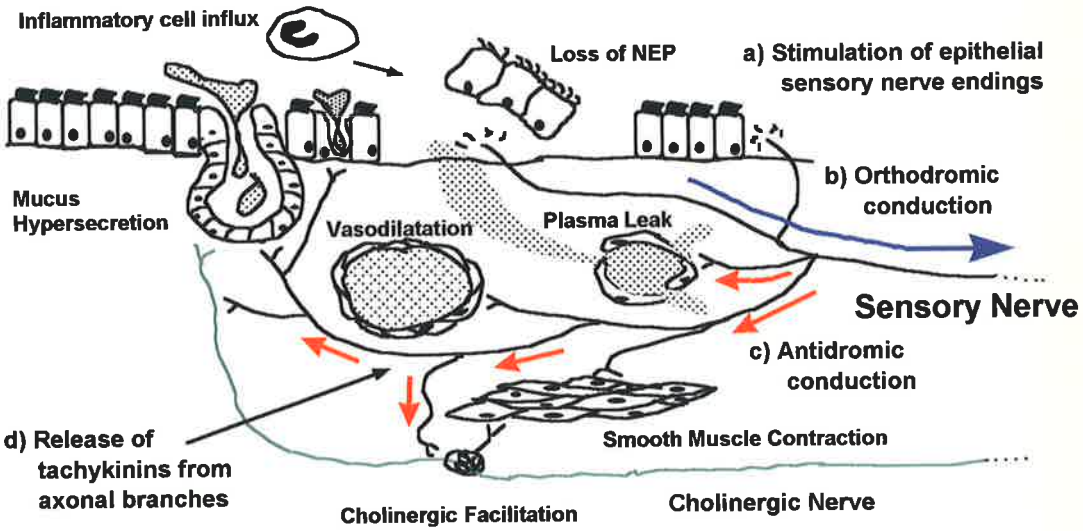


Figure 1.10 The above schematic, adapted from Barnes (1986a), illustrates a proposed mechanism for the interaction of sensory fibres with airway structures in the pathogenesis of asthma. Stimulation of airway sensory nerves (a) leads not only to the orthodromic conduction of impulses centrally for the mediation of sensory responses (blue arrow, b) but also to antidromic conduction of impulses down axonal branches (red arrows, c) with the resultant release of tachykinins into the surrounding tissue (d). Thus tachykinins may exert a number of local responses including smooth muscle contraction, increased vascular permeability and vasodilatation, mucus secretion and stimulation of inflammatory cells. Epithelial loss, as seen in asthma, may further potentiate tachykinin effects due to a loss of NEP and reduced tachykinin degradation. All of these responses are of potential importance in the pathogenesis of airways inflammation.

Table 1.1 Major biological effects of tachykinins relevant to asthma *

Tachykinin response	Receptor type
Bronchoconstriction	NK_2 (NK_1)
Vasodilatation	NK_1
Plasma extravasation	NK_1
Mucus secretion	NK_1
Facilitate cholinergic neurotransmission	NK_1 (NK_2)
Inflammatory cells	
T Lymphocyte proliferation	NK_1
Alveolar macrophage stimulation	NK_2
Fibroblast proliferation	NK_1, NK_2
Neutrophil activation	NK_1

* Compiled from Solway & Leff (1991), Frossard & Advenier (1991), Lundberg (1995)(Lundberg, 1995; Frossard & Advenier, 1991). Parentheses indicate receptors of less importance.

plasma extravasation (Belvisi *et al.*, 1989; Rogers *et al.*, 1988; McDonald, 1988) and enhance cholinergic neurotransmission (Belvisi *et al.*, 1994; Watson *et al.*, 1993; Hall *et al.*, 1989).

In man, *in vitro* studies demonstrate that NKA mediates bronchial smooth muscle contraction via NK_2 receptors (Joos & Pauwels, 1990; Naline *et al.*, 1989). *In vivo* inhalation studies, employing very large concentrations of SP and NKA, demonstrate dose-dependent bronchoconstriction, with NKA being more potent than SP and asthmatics being more responsive than normals (Cheung *et al.*, 1993; Cheung *et al.*, 1992; Crimi *et al.*, 1988; Joos *et al.*, 1987) suggesting NK_2 receptor activation. However, the contractile effects of SP and NKA are reduced by nedocromil sodium, suggesting that indirect mechanisms such as stimulation of inflammatory cells and/or nerves are involved (Crimi *et al.*, 1992; Joos *et al.*, 1989a; Crimi *et al.*, 1988). Furthermore, SP inhalation in asthmatics pretreated with anticholinergic or antihistamine agents demonstrates that the bronchoconstrictor effect partially involves a cholinergic mechanism but not histamine release (Crimi *et al.*, 1990). Substance P and NKA appear not to have an effect on cholinergic neurotransmission in human bronchi *in vitro*, unless in the presence of a potassium ion (K^+) channel blocker (Black *et al.*, 1990b). Substance P stimulates mucus secretion, an effect which has been studied on human bronchi *in vitro* (Rogers *et al.*, 1989).

1.9.4.2.5 Neurogenic inflammation

In recent years, there has been a great deal of interest in the concept of neurogenic inflammation within the airway. Neurogenic inflammation is a term used to describe a group of local responses that increase vascular permeability, plasma extravasation and

oedema formation, that occur following sensory nerve stimulation and tachykinin release (Jancso *et al.*, 1967). Historically, the concept arose from studies in the skin, where evidence suggested the skin's wheal and flare response involved sensory nerve activation and axonal reflexes (Holzer, 1988; Foreman & Jordan, 1983). Airway neurogenic inflammation has been extensively demonstrated in various animals species (McDonald *et al.*, 1996; Piedimonte *et al.*, 1993; Lotvall *et al.*, 1991; Belvisi *et al.*, 1989; Borson *et al.*, 1989; Lundberg *et al.*, 1984), but its relevance to human asthma remains uncertain (Barnes, 1995; Karlsson, 1993). This may be a reflection of the sparse distribution of SP-immunoreactive nerves and tachykinin receptors in human airways compared with rodent airways (Walsh *et al.*, 1994; Bowden & Gibbins, 1992; Martling, 1987a).

1.9.4.2.6 Pro-inflammatory effects of tachykinins

Researchers have provided extensive evidence that SP, and in some instances NKA, modulate inflammation and immunity. Pro-inflammatory effects include neutrophil and eosinophil chemotaxis (Wiedermann *et al.*, 1993), neutrophil activation (Wozniak *et al.*, 1989), pulmonary mast cell degranulation (Cross *et al.*, 1997; Heaney *et al.*, 1995), and increased immunoglobulin production by lymphocytes (Scicchitano *et al.*, 1988). Substance P leads to the release of chemotactic activity for neutrophils from airway epithelial cells (Von Essen *et al.*, 1992) and triggers the adhesion of neutrophils to venules (Baluk *et al.*, 1995) and epithelium (DeRose *et al.*, 1994). *In vitro,* SP is known to upregulate pro-inflammatory cytokine expression, such as IL-1β and TNFα in allergic tissue (Okamoto *et al.*, 1993). Moreover, tachykinins demonstrate important proliferative effects, including the proliferation (and migration) of lung fibroblasts (Harrison *et al.*, 1995) and airway epithelial cells (Kim *et al.*, 1995); the proliferation of

B lymphocytes (Laurenzi *et al.*, 1989), smooth muscle cells (Noveral & Grunstein, 1995), endothelial cells and angiogenesis (Wiedermann *et al.*, 1996; Fan *et al.*, 1993). Whether the endogenous release of tachykinins contributes to AHR as a consequence of inflammatory cell activation and migration is uncertain. However, a study by Cheung *et al.* in asthmatic subjects demonstrated enhanced airway narrowing 24 hours post SP inhalation (Cheung *et al.*, 1994), which they attributed to changes in airway geometry i.e. airway wall thickening secondary to inflammatory cellular infiltration (Pare & Bai, 1995; Moreno *et al.*, 1986).

Numerous studies support the view that sensory nerve function might be altered in asthma. Evidence from histochemical studies at autopsy, suggest that SP-containing nerves are more common in asthmatic than in control subjects (Ollerenshaw *et al.*, 1991). Although this observation has not been confirmed in biopsy specimens (Howarth *et al.*, 1991), the later findings of increased SP levels detected in bronchoalveolar lavage fluid (Nieber *et al.*, 1992) and sputum (Tomaki *et al.*, 1995) from asthmatics, suggests an increased release of sensory neuropeptides in asthma. Furthermore, the observation of decreased SP content in asthmatic lungs compared to normals has been suggested to reflect both an augmented release and degradation of SP (Lilly *et al.*, 1995). Recently, studies have also documented possible changes in neurokinin receptor expression in asthma. Increased mRNA transcripts for both NK_1 (Adcock *et al.*, 1993) and NK_2 (Bai *et al.*, 1995) receptors have been detected in lung tissue from asthmatics compared with controls. This may be in response to the enhanced local release of tachykinins and/or a cytokine-induced increase in gene transcription (Adcock *et al.*, 1994).

1.9.4.2.7 Tachykinin degradation

Once released, tachykinins are subject to rapid degradation by endopeptidase, the most important being NEP (EC 3.4.24.11) (Martling, 1987a). This membrane-bound protease has been identified immunohistochemically in several sites within the lung, including epithelium, submucosal glands, nerves, blood vessels and ASM (Nadel & Borson, 1991). Other peptidases, such as angiotensin-converting enzyme, kininase II, aminopeptidases and acetylcholinesterase, exhibit activity against tachykinins but play a less important role (Solway & Leff, 1991; Skidgel *et al.*, 1984). It has been proposed that a loss of NEP may promote airway inflammation and AHR (Nadel, 1990). Conditions that are known to decrease NEP activity in animals include chronic antigen exposure (Lilly *et al.*, 1994), ozone (Murlas *et al.*, 1992), toluene diisocyanate (Sheppard *et al.*, 1988), cigarette smoke (Dusser *et al.*, 1995), viral infections (Borson *et al.*, 1989), and IL-1β exposure (Tsukagoshi *et al.*, 1995a). Decreased NEP activity has been observed in man following ozone exposure (Hazbun *et al.*, 1993). An effect that may be partly responsible for acute ozone-induced changes in AHR demonstrated *in vitro* (Marthan *et al.*, 1996), and *in vivo* (Molfino *et al.*, 1991; Folinsbee & Horvath, 1986; Silverman, 1979).

Given the *in vitro* findings in animal studies that demonstrated enhanced contractile responses to exogenous SP and NKA following epithelial removal (Fine *et al.*, 1989; Frossard *et al.*, 1989; Sekizawa *et al.*, 1987a), it has been speculated that the shedding of the epithelium that occurs in asthma (Laitinen *et al.*, 1985) may result in a loss of airway NEP activity and heightened AHR. Differences in NEP activity between mild asthmatics and control subjects were assessed using a selective NEP inhibitor, thiorphan (Jeng *et al.*, 1989). Neutral endopeptidase inhibition enhanced bronchoconstrictor responses to NKA, however, this occurred to the same degree in those with asthma as

those without, suggesting that there was no fundamental difference in NEP activity between asthmatics and normals (Cheung *et al.*, 1993). Moreover, the lack of an effect of NEP inhibition on baseline lung function in asthmatics (Crimi *et al.*, 1994; Cheung *et al.*, 1993), has been seen as evidence indicating that tachykinins are not involved in maintaining basal airway tone (Joos *et al.*, 1994; Karlsson, 1993).

1.9.4.2.8 Sensory nerve hyperresponsiveness

Since the introduction of bronchoprovocation tests using specific receptor agonists (i.e. direct acting agents) such as histamine and methacholine (Juniper *et al.*, 1978; Cockcroft *et al.*, 1977a), it is now apparent that asthmatics also bronchoconstrict to a variety of indirect stimuli that have little or no effect in healthy subjects. For example, bradykinin (Fuller *et al.*, 1987), sulphur dioxide (Sheppard *et al.*, 1980), water (Anderson *et al.*, 1983) and adenosine (Cushley *et al.*, 1983) cause bronchoconstriction in asthmatic patients. Following on from evidence obtained in animal experiments demonstrating that these very same stimuli activate sensory nerves resulting in neuropeptide release (Spina, 1998b), it has been hypothesized that in asthmatics there is heightened irritability of sensory afferent nerve endings compared to healthy individuals, thereby contributing to AHR. That is, chronic airway inflammation results in hyperalgesia of sensory afferents, akin to that demonstrated in the skin (Spina, 1998b; Barnes, 1996b; Karlsson, 1993). Hyperalgesia is the sensitization of sensory nerves, resulting in a lowering of the threshold for activation to thermal, chemical and mechanical stimuli.

Various inflammatory mediators known to induce hyperalgesia in the skin, include bradykinin (Manning *et al.*, 1991), 15-hydroperoxyeicosatetraenoic acid (Levine *et al.*,

1986), prostaglandins (Ferreira & Nakamura, 1979), leukotrienes (Levine *et al.*, 1984), neuropeptides (Nakamura-Craig & Gill, 1991), platelet activating factor (Bonnet *et al.*, 1981), IL-1β (Ferreira *et al.*, 1988) and nerve growth factor (Woolf *et al.*, 1994), many of which are mediators of asthma (Barnes *et al.*, 1998). While activation of these hyperalgesic afferents in skin results in pain, it has been suggested that within the lower airways, pain may manifest as cough and chest tightness (Barnes, 1996a). Interestingly, perceived chest tightness and discomfort is increased in asthmatics following broncho-constriction induced by indirect agonists such as adenosine and metabisulphite compared with methacholine challenge (Marks *et al.*, 1996). And furthermore, hyperalgesia has been demonstrated in cases of allergic rhinitis as the pollen season progresses (Greiff *et al.*, 1995).

In chronic stable asthma the ineffectiveness of anticholinergic agents suggests cholinergic mechanisms do not play a major role in controlling airway function, however, their benefit in exacerbations of asthma suggests that cholinergic mechanisms become more important. This may be due to dysfunction of muscarinic autoreceptors, a reduction in the braking mechanism of NO or via enhanced sensory reflex activation (Barnes, 1996a). Although there is evidence of an increase in afferent function of sensory nerves in asthma, the evidence for augmented efferent responses such as neurogenic inflammation (Barnes, 1996b) and tachykinin-induced bronchoconstriction (Ichinose *et al.*, 1996; Fahy *et al.*, 1995b) is lacking. Inhaled capsaicin in healthy and asthmatic subjects only causes transient bronchoconstriction (Hansson *et al.*, 1992; Collier & Fuller, 1984) via a cholinergic pathway (Fuller *et al.*, 1985), while the bronchoconstrictor effects of capsaicin on isolated human bronchi are equivocal (Hulsmann & De, 1996; Honda *et al.*, 1991; Lundberg *et al.*, 1983a). However, the role of neuropeptide efferent functions on inflammatory and immune cells may assume a

greater importance in chronic disease. That is, the chronic release of neuropeptides in the airways from sensory nerves by antidromic stimulation may enhance inflammatory cell infiltrate and cellular proliferation, thereby contributing to airway wall remodeling and hyperresponsiveness (Spina, 1996; Barnes, 1996a).

1.10 Airway inflammation and cytokines

As previously mentioned asthma manifests as a disease characterized functionally by airflow limitation and AHR. Histologically, the changes seen are those of chronic inflammation involving many cell types such as eosinophils, neutrophils, lymphocytes, mast cells, fibroblasts, epithelial and endothelial cells.

1.10.1 Cytokines and T-lymphocytes

There is increasing evidence that lymphocytes, in particular T-lymphocytes may be involved in the initiation and the persistence of airway inflammation in asthma (Kon & Kay, 1999a; Jeffery, 1994; Corrigan *et al.*, 1988) by virtue of their presence and activation within the airway mucosa (Howarth *et al.*, 1994). Lymphocytes mediate immune responses within the body in response to foreign proteins. Basically, mature T-lymphocytes are subdivided into 2 distinct subsets, helper T cells (T_H) and T cytotoxic/suppressor ($T_{C/S}$) cells on the basis of the surface expression of CD4 and CD8 antigen, respectively. $CD8^+$ cells are generally activated by intracellular pathogens such as viruses in conjunction with major histocompatibility complex class I molecules (MHC class I), while $CD4^+$ cells recognize and are activated by foreign antigens such as allergens and are processed in association with MHC Class II on the cell surface of specialized antigen presenting cells e.g. dendritic cells (Hamid & Minshall, 2000). Recently much attention has been paid to $CD4^+$ cells in the pathogenesis of asthma because of their ability to drive antigen-specific inflammation and regulate IgE

synthesis. Effector functions of $CD4^+$ and $CD8^+$ cells are mediated by the secretion of cytokines. Cytokines are an extensive group of extracellular signaling proteins (hormones) that regulate inflammatory and immune reactions. Cytokine actions may be autocrine, influencing the cell that produced it; paracrine, influencing cells within close proximity of its release; or in endocrine manner by exerting more distant systemic effects.

Historically, $CD4^+$ T_H cells were further characterized into two subsets T_{H1} and T_{H2} based on the secreted cytokine profile of murine lymphocytes (Mosmann *et al.*, 1986). Murine T_{H1} cells expressed interferon gamma (IFN-γ), tumour necrosis factor-β (TNF-β) and IL-2; T_{H2} cells preferentially expressed IL-4, IL-5 and IL-10, and both classes produced IL-3 and granulocyte-macrophage colony stimulating factor (GM-CSF) (Borish & Rosenwasser, 1996). T_{H1} and T_{H2} differentiate from a common precursor naïve T_{H0} cell during a 'commitment' process, where IL-4 influences the differentiation to T_{H2} cells (Seder *et al.*, 1992) and IL-12 to T_{H1} cell type (Manetti *et al.*, 1993). However, the T_H subclasses of human lymphocytes are less well defined (Romagnani, 1992). Human T_{H1} cells produce IFN-γ and TNF-β; T_{H2} cells, IL-4, IL-5 and IL-9, while both types produce IL-2, IL-3, IL-10, IL-13 and GM-CSF (Borish & Rosenwasser, 1996). In addition to the above-mentioned T_H-cell cytokines, other cells within the airways (both mesenchymal and inflammatory) are able to produce an extensive array of cytokines. These cell types and associated cytokine effects considered relevant to asthma have recently been extensively reviewed (Hamid & Minshall, 2000; Chung & Barnes, 1999) and are best summarized in Table 1.2

It is less clear whether polarization of T_H cells to T_{H1} or T_{H2} subtypes occurs in sheep. In ruminants most research has concentrated on cytokine responses in response to

Table 1.2 Summary of cytokines, source and effects relevant to asthma.

Cytokine	Level [a]	Major effects	BHR [b]	Cellular sources
IL-1β	↑	Epithelial and T cell activation, mucin secretion, T_{H2} growth factor, B cell growth factor, neutrophil chemoattractant	↑	Macrophages, epithelium
IL-2	↔ / ↑	T-cell proliferation, induces eosinophilia		T cells (T_{H0}), eosinophils
IL-3	↑	↑ Proliferation and differentiation of myeloid progenitors		T cells (T_{H1} / T_{H2}), mast cells
IL-4	↑	↑ IgE, eosinophil recruitment (by VCAM-1 induction), T_{H2}-type switching, ↑ Eosinophil growth.		T cells (T_{H0} / T_{H2}), mast cells, eosinophils
IL-5	↑	Eosinophil differentiation, activation and survival, ↓ Apoptosis, ↓ T_{H2}	↑	T cells (T_{H2}), mast cells, eosinophils, epithelial cells
IL-6	↑ *	↑ IgE, ↑ ASM proliferation and hypertrophy, T-cell growth factor, B-cell growth factor		Macrophages, eosinophils, T_{H2}, epithelial and mast cells
IL-7	?	Lymphocyte proliferation, B-cell growth factor		
IL-8	↑	Neutrophil chemoattractant, recruits primed eosinophils	↑	Eosinophils, epithelial and smooth muscle cells,
IL-9	↑	Mast cell proliferation and differentiation, T-cell growth factor, ↑ IgE	↑	T_{H2} cells
IL-10	↑ †	Inhibits macrophage and lymphocyte activation, ↓ eosinophil survival, ↓ T_{H1} and T_{H2} cells, ↑ mast cell growth	↓	Lymphocytes (T_{H1} / T_{H2}), macrophages
IL-11	↑ ‡	Airway remodeling, activates fibroblasts, ↓ macrophage activation, B-cell growth factor	↑	Eosinophils, epithelial, lung stromal, and ASM cells
IL-12	↓	Induces T_{H1}-type differentiation, ↓ IgE, ↑ IFN-γ production by T cells		Macrophages, lymphocytes
IL-13	↑	↑ IgE and VCAM-1, ↓ IFN-γ and IL-12, activates eosinophils, ↓ apoptosis		Basophils, CD4 and CD8 T and epithelial cells
IL-14	?	B-cell growth factor		
IL-15	?	T-cell growth factor, induces eosinophilia		
IL-16	↑	CD4 T-cell and eosinophil chemoattractant		Epithelial cells
IL-17	?	Activates macrophage, fibroblasts, epithelial and endothelial cells, ↑ T-cell proliferation		
IL-18	↓	Induces IFN-γ production from T_{H1} cells, activates NK cells and monocytes, ↓ IgE		Macrophages, epithelial cells
IFN-γ	↔	↓ IgE, activates epithelial cells, endothelial cells, alveolar macrophages and monocytes, ↓ T_{H2}	↓	Lymphocytes
GM-CSF	↑	Eosinophil survival, proliferation and differentiation of myeloid progenitors	↑	Epithelial cells, T lymphocytes (T_{H1} / T_{H2})
TNFα	↑	↑ Granulocyte recruitment, primes inflammatory cells, activates epithelial, endothelial and antigen presenting cells, ↑ fibroblast proliferation,	↑	Macrophages, epithelial cells
TGF-β	↑	↑ Fibroblast collagen deposition, ↓ T-cell proliferation, ↓ ASM proliferation, chemoattractant for monocytes, fibroblasts and mast cells		Activated eosinophils, macrophages, fibroblasts, epithelial cells
SCF	↑	↑ Mast cell proliferation and differentiation, ↑ VCAM-1 on eosinophils		Macrophages, epithelial cells
PDGF	↑	↑ Fibroblast, ↑ ASM and epithelial cell proliferation, ↑ collagen		Eosinophils, epithelial cells
IGF-1	↔	Mitogen for fibroblasts and smooth muscle cells		Epithelial and ASM cells

[a] level of expression in asthmatics; [b] effect on bronchial hyperresponsiveness; ↑ increased; ↓ decreased; ↔ normal; BHR = bronchial hyperresponsiveness; IL = interleukin; IFN = interferon; GM-CSF = granulocyte-macrophage colony stimulating factor; TNF = tumour necrosis factor; TGF = transforming growth factor; SCF = stem cell factor; PDGF = platelet derived growth factor; NK = natural killer; T_H cells = T helper cells; VCAM = vascular adhesion molecule; ASM = airway smooth muscle; IGF = insulin-like growth factor. *Reported to be increased in serum. † Increased only in nonatopic subjects. ‡ Increased in severe asthma. Compiled from references Chung & Barnes (1999), Hamid & Minshall (2000), Holgate *et al.* (2000), Soussi-Gounni *et al.* (2001).

infectious diseases and adjuvants in immunization therapy, rather than allergy. In these species γδ T cells are more numerous and play a prominent role in infection than do $CD8^+$ cells. Stimulation of γδ T cells results in a T_{H1} type response with the expression of IL-2, INFγ and TNFα (Wood & Seow, 1996).

In extrinsic (allergic or atopic) asthma the T-cell cytokine profile is suggestive of a T_{H2}-like phenotype (Robinson *et al.*, 1992) and the development of AHR (Robinson *et al.*, 1993), expressing IL-3, IL-4, IL-5, IL-10, IL-13 and GM-CSF (Chung & Barnes, 1999). Furthermore, in intrinsic (non-atopic) asthma and in cases of occupational asthma due to isocyanates for example (Lemiere *et al.*, 1996), the T-cell activation and cytokine profile observed in bronchial biopsy and lavage specimens are similar to those seen in extrinsic asthma (Humbert *et al.*, 1996a; Bentley *et al.*, 1992). The T_{H2} pattern of cytokine expression is associated with several pathophysiological features of asthma, for example, over-production of IgE, mast cell proliferation and eosinophilia.

1.10.2 Cytokines and IgE expression

Human genetic linkage studies have demonstrated significant relationships between AHR and elevated IgE (atopy) (Postma *et al.*, 1995), and the high-affinity IgE receptor (van Herwerden *et al.*, 1995). IgE production is dependent on IL-4 (Del Prete *et al.*, 1988) and IL-13 (McKenzie *et al.*, 1993) which causes B-lymphocytes to switch to IgE production via the activation of specific receptors and signal transduction pathways (Oettgen & Geha, 2001), and is enhanced by IL-5, IL-10 and TNFα (Hamid & Minshall, 2000; Pene *et al.*, 1988). A major source of IL-4 mRNA in atopic and non-atopic individuals is $CD4^+$ T cells and to a lesser extent $CD8^+$ T cells (Ying *et al.*, 1997; Robinson *et al.*, 1992). Elevated IgE levels are observed following acute viral infections and toluene diisocyanate exposure (Tee *et al.*, 1998), which may result from

the ability of IL-4 to switch activated $CD8^+$ T cells to a T_{H2}-like phenotype of cytokine expression (Anderson & Coyle, 1994). IgE exerts its biological effects via coupling to high-affinity (FcεRI) and low-affinity (FcεRII or CD23) receptors. Mast cells, basophils, macrophages and eosinophils are able to express the FcεRI and/or FcεRII cell surface receptors (Holgate, 1997). Moreover, recent studies indicate that FcεRII and FcεRI are up-regulated in ASM (Hakonarson *et al.*, 1999) and epithelial cells (Campbell *et al.*, 1998) in asthma, respectively. Viruses, IgE, IL-4 and IL-13 are known to upregulate the expression of either FcεRI or FcεRII receptors (Grunstein *et al.*, 2001; MacGlashan *et al.*, 1998; Abbas *et al.*, 1996; Barnes, 1994). Furthermore, the level of FcεRI expression in bronchial biopsies is indistinguishable between intrinsic and extrinsic asthma (Humbert *et al.*, 1996b). Therefore, a unifying mechanism for AHR in intrinsic asthma, viral infection and reactive airways dysfunction syndrome (Brooks *et al.*, 1998) may result from a local increase in IgE levels and FcεRI and FcεRII expression, leading to enhanced localized 'allergic responses' via cell-cell interaction and cytokine release within the airways, without demonstrable atopy.

1.10.3 Cytokines and mast cells

Mast cell proliferation is influenced by a combination of cytokines which include IL-3, IL-4, IL-10, and nerve growth factor (Thompson-Snipes *et al.*, 1991; Matsuda *et al.*, 1991; Kirshenbaum *et al.*, 1989). Two types of human mast cells have been described based on their neutral protease content, the MC_T (tryptase-positive, chymase negative) and MC_{TC} (tryptase positive, chymase positive) with the MC_T being the most prominent in human airway mucosa (Schwartz, 1992). Increased mast cell numbers have been observed in the airway mucosa of patients with asthma of varying etiology (Koshino *et al.*, 1996; di Stefano *et al.*, 1993). Moreover, a significantly greater number of mast

cells are found within ASM of allergic individuals compared to non-allergic subjects (Ammit *et al.*, 1997).

There has been a resurgence of interest in the mast cell in the pathogenesis of airway inflammation and asthma (Rossi & Olivieri, 1997; Polosa, 1995). While mast cells degranulate via IgE coupling to FcεRI, mediator release can also occur by non-IgE dependent stimuli, such as bradykinin, neuropeptides (Cross *et al.*, 1997; Heaney *et al.*, 1995), and nerve stimulation (Bienenstock *et al.*, 1991). For many years the importance of the mast cell in asthma centered on its involvement in the acute allergic bronchoconstrictor response mediated via the release of histamine, prostaglandins and leukotrienes (Lazarus, 1987). More recently, attention has been paid to the effect of tryptase on ASM function. Tryptase has been shown to enhance *in vitro* smooth muscle contractility to histamine in human and canine bronchi (Berger *et al.*, 1999; Johnson *et al.*, 1997; Sekizawa *et al.*, 1989). However, it is now clearly evident that mast cells also secrete a variety of preformed and newly synthesized cytokines (IL-3, IL-4, IL-5, IL-6, IL-8, IL-13, GM-CSF and TNFα) (Bradding & Holgate, 1996) which in turn play a major role in the prolonged inflammatory phase following allergen exposure (Wasserman, 1994). For example, the release of IL-1β, TNFα and IL-4 induce recruitment of neutrophils and eosinophils from the mucosal microvasculature via processes involving the upregulation of E-selectin, intercellular cell adhesion molecule (ICAM-1), vascular cell adhesion molecule (VCAM-1) and very late appearing antigen (VLA-4) (Smith *et al.*, 1993).

1.10.4 Cytokines and eosinophils

IL-5 is a potent and selective chemoattractant for eosinophils (Iwama *et al.*, 1992). It is involved in eosinophil proliferation (Campbell *et al.*, 1987), and their enhanced survival

and cytotoxicity (Saito *et al.*, 1988). In addition, IL-3 and GM-CSF enhance eosinophil activation and survival (Rothenberg *et al.*, 1988; Owen *et al.*, 1987). IL-5 has been shown to cause airway eosinophilia and AHR (Foster *et al.*, 1996). Supportive evidence for a pathogenic role of cytokines in allergy induced AHR has been provided by models in which AHR has been specifically attenuated by neutralizing antibodies to specific cytokines. Anti-IL-5 can prevent AHR and eosinophil accumulation following antigen challenge in guinea pigs (van Oosterhout *et al.*, 1993), mice (Hamelmann *et al.*, 1997) and monkeys (Mauser *et al.*, 1995), however, following viral infection anti-IL-5 prevents AHR but not the inflammatory cell infiltrate (Schwarze *et al.*, 1997; van Oosterhout *et al.*, 1995). Moreover, other studies in various animal models demonstrate that eosinophil infiltration and the development of AHR are two distinct processes, such that the presence of eosinophils alone is not sufficient to alter airway contractile responses (Vargaftig, 1997; Lefort *et al.*, 1996; Lilly *et al.*, 1996; Eum *et al.*, 1995).

1.11 Asthma and pro-inflammatory cytokines; IL-1β and TNFα

Although a multitude of cytokines are involved in asthma (Table 1.2) and airway inflammation, with different cytokines assuming greater importance in different subsets of asthma, TNFα and IL-1β will be considered as the cytokines of interest in asthma and AHR, in this thesis.

Over the last decade it has been well recognized that asthma is characterized by increased cytokine production (Bradding *et al.*, 1994; Robinson *et al.*, 1992). Bradding and colleagues have demonstrated a sevenfold increase in the number of mast cells staining for TNFα in the asthmatic biopsies compared to controls. Moreover, significantly higher protein levels of TNFα and IL-1β have been found in bronchial

biopsy and lavage fluid from symptomatic atopic and non-atopic asthmatics compared to asymptotic subjects (Ackerman *et al.*, 1994; Broide *et al.*, 1992), suggesting a further upregulation of these two cytokines, at least, in acute asthmatic exacerbations. Furthermore, IL-1β and TNFα immunoreactivity is also increased in bronchial specimens of subjects with toluene diisocyanate-induced asthma (Maestrelli *et al.*, 1995).

Tumour necrosis factor-α is an ubiquitous cytokine in the development of inflammatory responses, and is being postulated as having a fundamental role in airway inflammation and acquired AHR (Shah *et al.*, 1995; Kips *et al.*, 1993a). Tumour necrosis factor-α is a powerful pro-inflammatory cytokine, it can augment inflammatory cellular influx in the bronchial mucosa. Tumour necrosis factor-α is chemoattractant for neutrophils and monocytes (Ming *et al.*, 1987), it upregulates the expression of adhesion molecules, promoting transendothelial migration of inflammatory cells (Smith *et al.*, 1993) and transepithelial migration of neutrophils, the latter by inducing the production of IL-8 (Smart & Casale, 1994). Tumour necrosis factor-α has profibrotic actions leading to tissue remodeling and is a powerful proliferative agent. It is capable of inducing angiogenesis, the proliferation of fibroblasts (Piguet *et al.*, 1990), ASM (Amrani *et al.*, 1996; Stewart *et al.*, 1995) and it enhances ECM production (Schwingshackl *et al.*, 1999; Sampson *et al.*, 1992).

Of its many reported actions, IL-1β is capable of inducing fibroblast proliferation (Schmidt *et al.*, 1982), ASM hyperplasia (De *et al.*, 1995), increasing fibronectin and collagen synthesis (Dinarello & Savage, 1989), inducing airway neutrophilia (Tsukagoshi *et al.*, 1994) and in rat skin, eosinophil accumulation, an effect mediated

by IL-8 production (Sanz *et al.*, 1995). All these effects are relevant to airways inflammation and airway remodeling.

Interleukin-1β and TNFα are co-localized in mast cells and macrophages and are released from these cells following IgE receptor stimulation of high-affinity (FcεRI) and low-affinity (FcεRII), respectively (Gosset *et al.*, 1999; Gordon *et al.*, 1990) and may be considered as "first wave" cytokines. Following this a second wave response occurs mediated by selectins, adhesion molecules, and chemokines (e.g. IL-8), which amplify the inflammatory response leading to a secondary influx of cells. Tumour necrosis factor-α and IL-1β are potent inducers of IL-8 production in epithelial, endothelial and ASM cells (John *et al.*, 1998; Cromwell *et al.*, 1992; Standiford *et al.*, 1990).

1.12 Airway hyperresponsiveness and pro-inflammatory cytokines; IL-1β and TNFα

The development of AHR following the inhalation of irritant stimuli and viral infections has been well established. Recent research has shown that following ozone exposure, toluene diisocyanate inhalation and viral infection there is an increased production of cytokines which include IL-1β, TNFα and IL-8 (Maestrelli *et al.*, 1995; Basha *et al.*, 1994; Pendino *et al.*, 1994). Furthermore, respiratory viruses have been shown to increase a variety of cytokines and prominent among these is IL-8 (Johnston, 1995). Occupational asthma results suggest exposure to occupational agents increases release of cytokines such as TNFα (Siracusa *et al.*, 1992).

Tumour necrosis factor-α has been implicated as a principal mediator of AHR by the use of neutralizing antibodies in rodent models of allergic asthma (Renzetti *et al.*,

1996). Moreover, *in vivo* AHR has been demonstrated in animals and normal human subjects following administration of IL-1β (Tsukagoshi *et al.*, 1994), TNFα (Thomas *et al.*, 1995; Kips *et al.*, 1992; Wheeler *et al.*, 1990) and IL-8 (Xiu *et al.*, 1995), and is often associated with airway neutrophilia (Thomas *et al.*, 1995; Xiu *et al.*, 1995; Kips *et al.*, 1992). Whether this is a direct effect on ASM, or due to changes in airway wall dimensions by inflammatory remodeling, by the release of other mediators such as arachidonic acid metabolites from inflammatory cells (Roubin *et al.*, 1987), by increased MVL or by a combination of these events, remains to be determined. This thesis will specifically address whether TNFα and IL-1β, either alone or in combination can enhance ovine ASM contractility *in vitro*?

1.13 TNFα and IL-1β signal transduction pathways

Two receptor types have been described for each of the pro-inflammatory cytokines, IL-1β and TNFα. For IL-1β the type I receptor (IL-1R1) and a type II receptor (IL-1R2). The IL-1R1 is involved in signal transduction while IL-1R2 is not. The latter receptor being involved in homeostatic mechanisms acting as a decoy for excess IL-1β (Colotta *et al.*, 1994). In the case of TNFα, the two receptors are TNFR-1(p55) and TNFR-II(p75). Both receptors are involved in signal transduction, with soluble forms of both (p55 and p75) being described as acting as inhibitors of TNFα mediated responses (Nophar *et al.*, 1990). The TNFRp55 mediates most of TNFα effects such as apoptosis, fibroblast proliferation and adhesion molecule expression (Tartaglia & Goeddel, 1992), while effects mediated by TNFRp75 relate to T-cell development, activation and apoptosis (Erickson *et al.*, 1994).

The signal transduction pathways for these pro-inflammatory cytokines have not been fully elucidated. Tumour necrosis factor-α and IL-1β mediate similar biological effects by activating the same set of intracellular transcription factors, the two most prominent being nuclear factor κB (NF-κB) and activating protein 1 (AP-1) (Eder, 1997). These factors, are then capable of inducing the production of many proteins such as cell-adhesion molecules, cytokines, inducible enzymes and growth factors, involved in immune and inflammatory processes, some of which are common to asthma (Barnes & Adcock, 1998b).

Evidence suggests that IL-1β and TNFα signal transduction is associated with the activation of the TNF receptor associated factors (TRAF), TRAF-6 and TRAF-2, respectively, with convergence to mitogen-activated protein (MAP) kinase cascades. Activation of the various MAP kinase cascades, in turn, leads to the induction of transcription factors such as, NF-κB and AP-1 (Eder, 1997). Moreover, recent studies have revealed that other signal transduction pathways mediated by TNFp55 activation, involve the activation of G-binding proteins (Yanaga *et al.*, 1992) and PLC (Machleidt *et al.*, 1996), while in ASM cells stimulation of TNFp55 or CD 40, a member of the TNF receptor superfamily containing TNF-p55 and p75 receptors (Banchereau *et al.*, 1994), results in signaling events involving tyrosine kinases (Lazaar *et al.*, 1998), enhanced Ca^{++} mobilization (Amrani *et al.*, 1996) and phosphoinositide accumulation (Amrani *et al.*, 1997). All of which are similar to the signaling pathways involved in ASM contraction (Chilvers *et al.*, 1994; Schramm & Grunstein, 1992). Therefore, can one definitively establish whether TNFα and IL-1β alter ASM contractility by mechanisms involving intracellular signaling?

1.14 Signal transduction pathways in airway smooth muscle

Airway smooth muscle contraction and relaxation involve agonist-receptor interactions with specific G-proteins, which are coupled to second-messenger pathways that mediate changes in airway tone. Three main types of G-proteins have been described G_s, G_i and G_q. G_s and G_i produce stimulation and inhibition of the enzyme AC, respectively. It is thought that a bi-directional control operates on PLC, however, the $G_{q(i)}$ protein has yet to be identified (Chilvers *et al.*, 1994). G-proteins exist as heterotrimeric complexes of α, β and γ subunits, the latter two as a tightly bound complex (Hepler & Gilman, 1992). Multiple G_α subunits have been described and classified according to their homology and sensitivity to pertussis and cholera toxins (Kaziro *et al.*, 1991).

1.14.1 Smooth muscle contraction

Upon agonist-receptor binding, the G_q-protein dissociates where the free $G_{q\alpha}$ subunit stimulates PLC resulting in the hydrolysis of PIP_2 to IP_3 (Schramm & Grunstein, 1992). Inositol 1,4,5-triphosphate then binds to specific IP_3R on intracellular Ca^{++} stores (such as the sarcoplasmic reticulum (SR)) leading to increased levels of cytosolic free Ca^{++}. The level of accumulation of IP_3 is regulated by metabolizing enzymes. In particular, inositol 3′-kinase, which phosphorylates IP_3 to inositol $(1,3,4,5)P_4$, with elevated intracellular levels demonstrated during sustained contractile responses (Chilvers *et al.*, 1994). Inositol $(1,3,4,5)P_4$ is a putative second messenger regulating Ca^{++} influx and/or intracellular translocation (Schramm & Grunstein, 1992). Four Ca^{++} bind calmodulin, resulting in the activation of MLCK (Jiang & Stephens, 1994), which in turn phosphorylates myosin leading to an increase in myosin activity, cross-linking of the cellular contractile elements (myosin and actin) and finally, contraction (Sweeney, 1998), Figure 1.11.

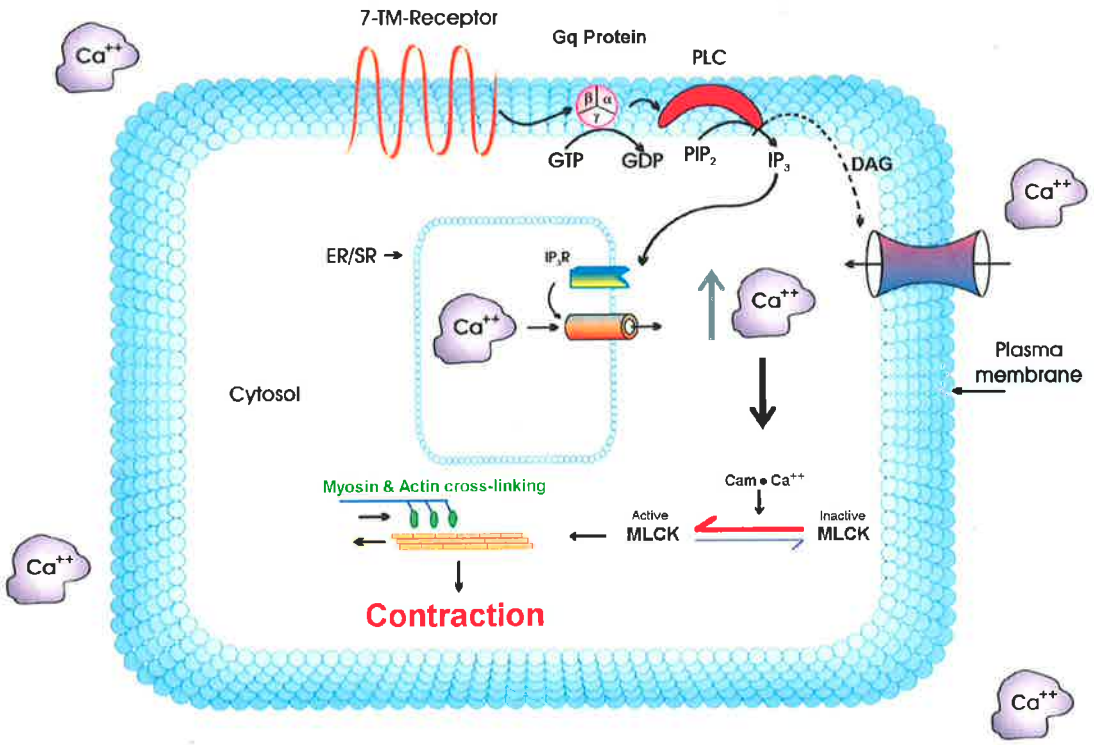


Figure 1.11 Schematic diagram illustrating pathways involved in airway smooth muscle contraction. Following agonist (e.g. acetylcholine) binding to a specific 7 transmembrane domain receptor (7-TM-Receptor) in the cell membrane, the associated G_q protein (heterotrimeric complex of α, β and γ subunits) dissociates to a βγ dimer and a free α subunit. This α subunit activates phospholipase C (PLC) which in turn catalyses the hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP_2). This results in the formation of inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 via activation of IP_3 receptors (IP_3R) on the endoplasmic or sarcoplasmic reticulum (ER/SR) induces the release of calcium ions (Ca^{++}) stored in these intracellular organelles. DAG in addition to activating protein kinase C (not shown) may also stimulate Ca^{++} entry (dotted line). The free cytosolic Ca^{++} associates with calmodulin (Cam) causing a conformational change resulting in an ability to interact with inactive myosin light chain kinase (MLCK) rendering MLCK active. Activated MLCK phosphorylates myosin and in the presence of actin and activated myosin-ATPase (not shown) provides energy for cyclic cross-linking of contractile elements and thus contraction.

Concomitant with the formation of the second messenger IP_3 is the generation of DAG which activates PKC (Schramm & Grunstein, 1992). Signal transduction pathways involving the activation of PKC mediate many physiological responses, apart from smooth muscle contraction, for example cell secretion, gene expression and cell proliferation (Blackshear *et al.*, 1988; Nishizuka, 1988). The multiplicity of PKC may result from its many described isoforms, many of which are found in ASM, PKC α, β1, β2, δ, ε, μ, ξ, ζ and θ (Webb *et al.*, 1997; Donnelly *et al.*, 1995). The mobilization of Ca^{++} and activation of PKC play important roles in the initiation and modulation of intensity of ASM contraction (Schramm & Grunstein, 1992).

Cellular responses are also mediated by the phospholipase A_2 (PLA_2) signal transduction pathway, which involves activation of various PLA_2 isoforms that hydrolyze membrane phospholipids to generate arachidonic acid and lysophospholipids (Lin *et al.*, 1992; Axelrod, 1990). The liberated arachidonic acid is then converted to a number of compounds, including prostaglandins, thromboxanes and leukotrienes, which are able to induce smooth muscle contraction and cell proliferation.

1.14.2 Smooth muscle relaxation

In ASM, Figure 1.12, relaxation is mediated by receptors, such as the β_2-adrenoreceptors, coupled to AC via G_s-proteins. Stimulation of these receptors results in the release of the $G_{s\alpha}$ subunit, which then activates AC. This enzyme system catalyses the breakdown of ATP to cAMP. The accumulation of cAMP then activates protein kinase A (PKA) leading to relaxation by phosphorylating key cellular substrates (Knox & Tattersfield, 1995). Protein kinase A via phosphorylating specific intracellular proteins leads to the inhibition of PIP_2 hydrolysis, increased uptake of free Ca^{++} by

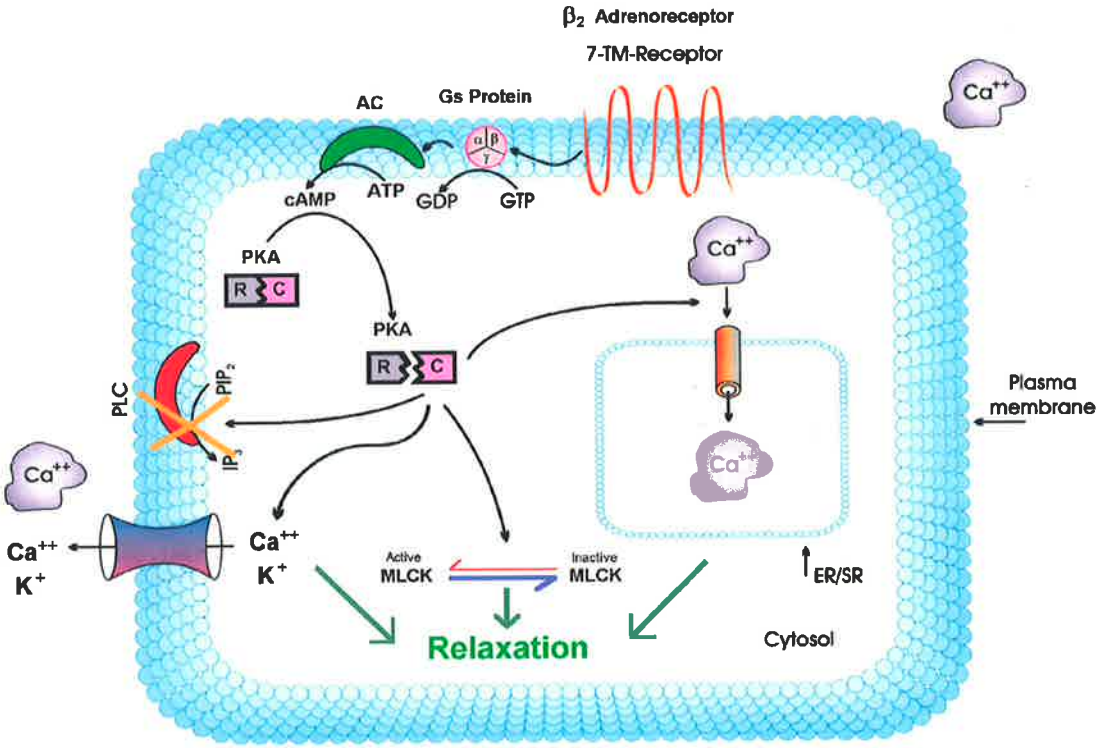


Figure 1.12 Schematic representation of the pathways involved in airway smooth muscle relaxation. Following agonist binding to a specific 7 transmembrane domain receptor (7-TM-Receptor, e.g. β_2 Adrenoreceptor) in the cell membrane, the associated G_s protein dissociates freeing the α subunit from $\beta\ \gamma$. The $G_{s\alpha}$ activates adenylyl cyclase (AC), catalyzing the breakdown of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). cAMP in turn activates protein kinase A (PKA), causing the dissociation of the catalytic subunit (C) from the regulatory subunit (R). The catalytic subunit is able to phosphorylate a number of key targets within the cell, leading to the inhibition of phosphatidylinositol 4,5-biphosphate (PIP_2) hydrolysis, increased Ca^{++} uptake by intracellular stores (ER/SR), inhibition of myosin light chain kinase (MLCK) activation, and the activation of membrane ion channels (e.g. Ca^{++}-dependent potassium (K^+) channels) and transporters thereby promoting Ca^{++} extrusion. The net effects of these actions being reduced levels of cytosolic Ca^{++} and phosphorylation (inactivation) of MLCK, all of which ultimately lead to relaxation.

intracellular stores, inactivation of MLCK and activation of K^+ channels resulting in K^+ efflux, which ultimately leads to relaxation (Knox & Tattersfield, 1995; Torphy, 1994; Black *et al.*, 1990).

Also of note is that other agonists such as nitric oxide and atrial natriuretic peptide induce ASM relaxation. This signal transduction pathway involves guanylyl cyclase via the formation of cyclic guanosine monophosphate (cGMP), which in turn activates a cGMP-dependent protein kinase (PKG) (Torphy, 1994). Furthermore, cGMP at high concentrations can activate PKA (Turner *et al.*, 1994), thus demonstrating convergence of the two bronchodilator pathways. Cyclic AMP and cGMP are hydrolyzed by phosphodiesterase, of which seven have been described (Beavo, 1995). Phosphodiesterase 3, 4 and 5 are important in ASM relaxation responses where inhibition of these isoenzymes results in bronchodilatation, as a result of increased intracellular cAMP and cGMP. Receptors linked to G_i rather than G_s of the AC system, for example airway M_2 receptors, inhibit AC and reduce cAMP formation (Knox & Tattersfield, 1995).

1.15 TNFα, IL-1β and airway smooth muscle function

It is generally accepted that ASM, itself, is an active participant in airway inflammatory processes: by actively proliferating, being capable of producing multiple cytokines and chemokines, and expressing a variety of cell surface receptors and adhesion molecules. Many of the signal transduction pathways involved in these cellular responses share and converge with those responsible for the muscle's contractile phenotype (extensively reviewed by Barnes (Barnes, 1998a) and Panettieri (Panettieri, Jr., 1998)). Thus it is now apparent that cytokines liberated during inflammatory responses may directly

influence ASM contractility, by affecting signal transduction pathways that mediate ASM contraction.

In recent years, most attention has focused on the mechanism of action of IL-1β and TNFα on the ASM relaxation process. Studies have demonstrated an impairment in β-adrenoreceptor function following incubation of isolated ASM to either TNFα or IL-1β, such that the relaxation responses of β-agonists on pre-contracted ASM is diminished (Hakonarson *et al.*, 1996; Koto *et al.*, 1996; Munakata *et al.*, 1996; Wills-Karp *et al.*, 1993b). On further examination this effect was attributed to functional antagonism of relaxation occurring between the inhibitory receptor G_i-proteins and the stimulatory β-adrenoreceptor G_s-proteins coupled to the AC system (Hotta *et al.*, 1999; Hakonarson *et al.*, 1996; Koto *et al.*, 1996; Hirata *et al.*, 1994). That is, AC is under dual regulation by a stimulatory G_s protein and an inhibitory G_i protein (Schramm & Grunstein, 1992). Isolated ASM exposed to IL-1β and TNFα has been shown to increase $G_{i\alpha}$ protein expression (the G-protein subunit that is involved in inhibiting AC) (Hotta *et al.*, 1999; Hakonarson *et al.*, 1996; Koto *et al.*, 1996; Hirata *et al.*, 1994), while $G_{s\alpha}$ expression remains unchanged (Hotta *et al.*, 1999; Shore *et al.*, 1997; Hirata *et al.*, 1994). The increased expression of $G_{i\alpha}$ protein with no change in $G_{s\alpha}$ levels enhances the inhibitory pathway, leading to lower AC activity and impaired relaxation.

In animal studies the effects of TNFα and IL-1β on *in vitro* contractility are limited with varying results, this may reflect the different methodologies employed. In each study, differing concentrations and time periods were used for the exposure of ASM preparations to the individual cytokines. Several research groups have demonstrated increased *in vitro* contractility to cholinergic agonists following either TNFα or IL-1β

pretreatment (Pennings *et al.*, 1998; Hakonarson *et al.*, 1996), others have not (Koto *et al.*, 1996; Munakata *et al.*, 1996; Kips *et al.*, 1993; Wills-Karp *et al.*, 1993b). In isolated human airways, the induction *in vitro* of hyperresponsiveness by TNFα has been demonstrated to electrical field stimulation but not to contractile agonists *per se* (Anticevich *et al.*, 1995). Furthermore, Amrani and colleagues demonstrated, in cultured tracheal smooth muscle cells, that agonist-induced increases in cytosolic free Ca^{++} were enhanced by TNFα pretreatment (Amrani *et al.*, 1997; Amrani *et al.*, 1995b).

To date, the effects of IL-1β on isolated ASM contractility has more often demonstrated a decrease in the sensitivity of smooth muscle to contractile agonists rather than an increase, an effect involving the production of a relaxant factor from the airway epithelium (Munakata *et al.*, 1996; Tamaoki *et al.*, 1994).

1.16 Synergism; TNFα and IL-1β

Cytokines exert a diversity of actions, some being complicated by synergistic and antagonistic interactions. Synergism between IL-1β and TNFα has been described in a wide variety of physiological responses, for example the induction of haemodynamic shock (Okusawa *et al.*, 1988), enhanced IL-8 secretion from ASM (John *et al.*, 1998), the production of nitric oxide in peripheral blood (Kosaka *et al.*, 1994), enhanced transforming growth factor-β production (Phan *et al.*, 1992) and up-regulation of intracellular adhesion molecule expression in endothelial cell (Hashimoto, 1994). Moreover, recent studies have demonstrated synergism between TNFα and IL-1β in modifying the rabbit airway relaxant and contractile responses by an impairment of β-adrenoreceptor coupled relaxation (Hakonarson *et al.*, 1996), down-regulating airway M_2 receptor expression (Haddad *et al.*, 1996) and the development of lung oedema in a

rat model (Wesselius *et al.*, 1995). Although the latter study assessed gross vascular leakage, as assessed by lung wet-to-dry weight ratios, it begs the question - what are the effects of TNFα and IL-1β on airway microvascular permeability?

1.17 Fatal asthma and pro-inflammatory cytokines

Postmortem studies of fatal asthma have provided much of the evidence regarding airway inflammation and airway wall remodeling in clinical asthma. Histological examination has repeatedly demonstrated epithelial shedding, inflammatory cell infiltration beneath a grossly thickened basement membrane, goblet cell hyperplasia (Dunnill *et al.*, 1969; Dunnill, 1960; Messer *et al.*, 1960), marked airway wall thickening (Huber & Koessler, 1922) and smooth muscle hyperplasia and hypertrophy (Heard & Hossain, 1973).

Published evidence suggests that the occurrence of fatal asthma is in patients with severe asthma, and can be categorized as either slow or sudden onset (Strunk, 1993). The former allows affected individuals time to seek assistance, however, this may be postponed for a number of reasons e.g. a disregard of symptom severity, and/or socio-economic factors leading to inadequate medical treatment (Molfino & Slutsky, 1994), while sudden-onset involves the rapid progression of airway obstruction with the possibility of death occurring within minutes.

Many investigators have defined sudden-onset fatal asthma as death within 1-2 hours after the onset of the attack, and slow onset fatalities occurring within 3 hours to days. Using this chronological scale sudden-onset fatal asthma presents histologically as a distinct entity from slow onset fatal asthma. Compared to slow onset fatal asthma, the airway submucosa in sudden death asthma contains an increased numbers of

neutrophils, both intravascular and extravasated, mucous gland hyperplasia and reduced eosinophil numbers (Carroll *et al.*, 1996; Sur *et al.*, 1993).

Moreover, patients in acute respiratory failure i.e. status asthmaticus of sudden-onset demonstrate airway neutrophilia, in excess of what may be induced by mechanical ventilation, and significantly elevated levels of IL-8, TNFα and IL-1β (Tillie-Leblond *et al.*, 1999; Lamblin *et al.*, 1998). A similar scenario of neutrophilia mediated by elevated IL-8 levels has also been observed by Fahy and colleagues in patients suffering acute severe asthma (Fahy *et al.*, 1995a).

More often than not the catastrophic event that results in sudden-onset fatal asthma is not known, however, it may sometimes result from infection, a massive allergen or irritant exposure (Messer *et al.*, 1960). Numerous human and animal studies have characterized the inflammatory response following such triggers. Ozone (Tsukagoshi *et al.*, 1995; Basha *et al.*, 1994; Holtzman *et al.*, 1983) or toluene diisocyanate inhalation (Fabbri *et al.*, 1991; Sheppard *et al.*, 1986), viral infection (Wang & Forsyth, 2000; van Oosterhout *et al.*, 1995) and endotoxin-contaminated allergen (Hunt *et al.*, 1994) or endotoxin (Kubo *et al.*, 1994) are associated with an airway neutrophilic response, and where measured increased IL-8 production (Basha *et al.*, 1994).

Immunohistochemical analysis of lymphocyte populations in cases of sudden-onset fatal asthma demonstrate a preponderance of $CD8^+$ compared to $CD4^+$ cells in peribronchial tissue, being the inverse of that found in stable asthma (Faul *et al.*, 1997). This possibly suggests that different immunopathological mechanism are at play, or perhaps may reflect the fact that $CD8^+$ cells are generally activated by endotoxin (Forestier *et al.*, 2000) and intracellular pathogens such as viruses (Kon & Kay, 1999b).

Apart from the structural components contributing to an increased airway wall thickness found in fatal asthma i.e. smooth muscle, mucous glands, cartilage (Carroll *et al.*, 1993) and subepithelial structures (Carroll *et al.*, 2000; Roche *et al.*, 1989), non-structural components determining airway wall thickness, such as widening of endothelial gap junctions and oedema fluid, are increased in cases of fatal asthma of short duration compared to that in slow-onset fatal asthma (Carroll *et al.*, 1997b). Furthermore, the inflammatory infiltrate is increased in pulmonary arterial walls of sudden fatal asthma compared to non-asthma controls, and appears to be polarized to the vascular adventitia adjacent to airways (Saetta *et al.*, 1991).

Pathological examination of the airways in cases of sudden-onset fatal asthma has demonstrated, in certain individuals, airways that are devoid of mucus, i.e. "empty airways" suggesting a neural mechanism at play. Possibly, neutrophil derived products released during the acute neutrophilic response may enhance ACh release from parasympathetic nerve endings (Anticevich *et al.*, 1996; Hughes *et al.*, 1993; Daniel & O'Byrne, 1991), and/or sensory nerve hyperalgesia as a result of increased IL-1β and TNFα (Ferreira *et al.*, 1993; Ferreira *et al.*, 1988). Therefore, an enhanced bronchoconstrictor response, in the presence of airway wall oedema, may result in excessive ASM shortening to such a degree as to completely occlude the airway (James *et al.*, 1989). One then wonders whether the co-release of TNFα and IL-1β may provide the fatal combination of enhanced ASM contractility with a concomitant increase in MVL?

1.18 Animal models

As with all non-human mammalian species, differences in some aspects of airway

physiology and anatomy from man will occur (Summarized in Table 1.3). There are limitations, however, to the use of human tissue for *in vitro* studies, including poor suitability as tissue is often obtained from lung carcinoma resection specimens and may be hyporesponsive (Armour *et al.*, 1996). Therefore, it is necessary at some stage to use experimental animal models if detailed information on the role of cytokines in airway MVL and AHR are to be obtained.

1.18.1 Asthma

Numerous animal species have been used as experimental models of asthma including monkeys, sheep, dogs, rats, rabbits and guinea pigs (as reviewed in Wanner & Abraham, 1982). The use of such animal models has greatly increased the understanding of the mechanisms involved in the pathogenesis and pathophysiology of asthma. Ideally, the responses of animal models should reflect similar pathology and physiology to human disease (refer Table 1.4 for detailed comparative physiology and histology). The sheep *in vivo* model of antigen-induced bronchoconstriction is well characterized. In sensitized sheep, A*scaris suum* inhalation results in immediate bronchoconstriction, hypoxia, gas trapping (Wanner *et al.*, 1979) and in some animals the development of a late reaction (6-8 hours post challenge) (Abraham *et al.*, 1983), responses which are representative of human asthma (Cockcroft *et al.*, 1977b; McFadden & Lyons, 1968; Woolcock & Read, 1966). Both immediate (early) and late responder sheep develop AHR at approximately two hours post challenge (Soler *et al.*, 1989) but only the latter group develops prolonged hyperresponsiveness which can be found at 24 hours (Lanes *et al.*, 1986).

Pharmacologically similar mediators to those implicated in asthma (Barnes *et al.*, 1998) such as histamine, leukotrienes, platelet activating factor, oxygen radicals and adhesion

Table 1.3 Comparative morphology and biology of several animals and man.

	Human	**Sheep**	**Dog**	**Monkey**
Pleura	Thick	Thick	Thin	Thin
Lobes	Right lung - 3 Left lung – 2	Right lung – 4 or 5 Left lung - 3	Right lung – 4 Left lung - 3	
Lobulation	Imperfect	Well developed	Absent	Absent
Interlobar connective tissue	Extensive Partially surrounds many lobules	Extensive Completely surrounds many lobules	Little if any	Little
Bronchi	Divide internally No apical bronchi	Main bronchus divides internally Apical bronchus on right side *	Main bronchus divides outside lung No apical bronchus	
Bronchioles	Several generations	Several generations	Fewer generations	Commonly only one
Terminal bronchioles (TB)	TB end in respiratory bronchioles	TB end in very short respiratory bronchioles or alveolar ducts	TB end in respiratory bronchioles	TB end in respiratory bronchioles
Respiratory Bronchioles	Several generations	Single short generation	Several generations	Several generations
Collateral ventilation	Present Poorly developed	Present Poorly developed	Present Well developed	Present Well developed
Arterial supply to pleura	Bronchial artery	Bronchial artery	Pulmonary artery	Pulmonary artery
Bronchial artery – Pulmonary artery Shunts	Present	Present	Not demonstrated	Not demonstrated

Compiled from references Olson & Wardle 1992, Magno (1990), Terry *et al.* (1987), Tyler (1983), Wanner & Abraham (1982) and May (1970).

* Refer Figure 2.4 for a diagram of the apical right bronchus in the sheep lung.

Table 1.4 Comparison of antigen challenge on airway physiology and histology in human asthma and allergic animal models.

	Human	**Sheep**	**Guinea pig**	**Dog**	**Monkey**
Airway resistance	Increased	Increased	Increased	Increased	Increased
Total lung capacity	Increased	?	Decreased	Unchanged	Unchanged
Functional residual capacity	Increased or unchanged	Increased	Increased	Unchanged	Unchanged
Residual volume	Increased	?	Increased (?)	Unchanged	Unchanged
Elastic recoil	Decreased or unchanged	Unchanged	Unchanged	Unchanged	Unchanged
Reproducibility of antigen challenge	Reproducible	Reproducible	Variable	Variable	?
Demonstrate late asthmatic response	Yes	Yes	Yes	Yes	No
Antigen challenge increases AHR	Yes	Yes	Yes	Yes	Yes
Changes in airway morphology					
Submucosal gland hypertrophy	+	+	-	-	-
Smooth muscle hypertrophy	+	-	+	-	-
Subepithelial fibrosis	+	+/-	-	-	-
Cellular infiltrates	+	+	+	+	+

Compiled from references Carroll *et al.* (2000), Hamid *et al.* (1997), Kaneko *et al.* (1996), Mauser *et al.* (1995), Becker *et al.* (1995), Akutsu *et al.* (1995), Carroll *et al.* (1993), Chen *et al.* (1991a), Chen *et al.* (1991b), Wanner & Abraham, (1982) and Souhrada & Dickey, (1976).

molecules (Abraham *et al.*, 1999; Lansing *et al.*, 1993; Tomioka *et al.*, 1989; Abraham, 1988; Lanes *et al.*, 1986) have been implicated in antigen-induced bronchoconstriction and the development of the late asthmatic response. Furthermore, different pharmacological agents such as antihistamines, cromoglycate, nedocromil sodium and corticosteroids have similar actions in preventing early and late asthmatic reactions and AHR in sheep (Abraham *et al.*, 1987; Delehunt *et al.*, 1984; Abraham *et al.*, 1981a) and man (Crimi *et al.*, 1989; O'Byrne *et al.*, 1987).

As in man, allergic sheep compared to non-allergic sheep, demonstrate AHR to both direct and indirect inhaled stimuli (Joos *et al.*, 1993), such as aerosols of methacholine (Fujimoto *et al.*, 1995), carbachol (Soler *et al.*, 1989), histamine (Ahmed *et al.*, 1983), bradykinin (Abraham *et al.*, 1991) and chemical irritants, such as nitric acid (Abraham *et al.*, 1982) and sulphur dioxide (Abraham *et al.*, 1981b). Furthermore, *in vitro* AHR to carbachol and histamine, has been demonstrated in allergic sheep models (Wagner *et al.*, 1985; Mirbahar & Eyre, 1982).

Following antigen challenge sheep develop a cellular inflammatory response with increased numbers of eosinophils, neutrophils and macrophages in bronchoalveolar lavage fluid (Fujimoto *et al.*, 1995; Bosse *et al.*, 1987). Demonstrably similar inflammatory cell populations are found in the bronchoalveolar lavage of allergic individuals (Basha *et al.*, 1994; Corrigan & Kay, 1991). Histologically, allergic hyperresponsive sheep demonstrate an increased tendency for airway wall eosinophilia, an increased number of secretory granules in airway mast cells (Chen *et al.*, 1991a) and submucosal gland hyperplasia (Chen *et al.*, 1991b), morphological findings that are consistent with human asthma (Hamid *et al.*, 1997; Carroll *et al.*, 1993).

The *in vivo* sheep model has other advantages; lung resistance (R_L) can be measured in conscious animals, therefore, avoiding the confounding effects of mechanical ventilation and anaesthetic agents, and longitudinal studies can be performed in the same animal.

Animal models of allergic bronchoconstriction have been criticized because they do not usually demonstrate baseline increases in R_L analogous to chronic asthma in man, however, Bosse and colleagues reported that repeated and prolonged (more than nine months) antigen aerosol challenge in sheep resulted in increased baseline R_L (Bosse *et al.*, 1987).

The advent of genetically manipulated species, such as transgenic and gene knockout mice, with their well-characterized immune system, is contributing to the understanding of the immunopathology of allergic disease. Various research groups are "dissecting out" the complex role cytokines play in the development of airway inflammation and hyperresponsiveness. For example, various genetically manipulated murine models have demonstrated that eosinophil infiltration and the development of AHR are two distinct processes and that the presence of eosinophils alone is not sufficient to alter airway responsiveness (Pauwels *et al.*, 1997; Lefort *et al.*, 1996; Eum *et al.*, 1995). Research in these murine models can therefore, lead to the development of innovative concepts and therapeutically beneficial cytokine antagonists and agonists. These can then be further tested in larger animal models representative of human asthma and, in turn, man.

1.18.2 Microvascular permeability

Animal models of airway MVL have largely focused on the accumulation of oedema within the guinea pig trachea (Evans *et al.*, 1988b; Evans *et al.*, 1987), with a majority of studies concerning Evans blue (EB) extravasation into this airway tissue. The measurement of EB leakage has become a standard technique to detect microvascular permeability and compares favourably with radiolabelled albumin in that respect (Rogers *et al.*, 1989a; Saria & Lundberg, 1983).

I have developed, in the guinea pig, a technique for isolating a section of trachea *in vivo* (Reynolds *et al.*, 1992), similar to that described by other workers using larger species (Deffebach *et al.*, 1989; Kirsch *et al.*, 1988). The particular advantage of this isolated section of trachea is that it allows prolonged exposure of a segment of airway to various stimuli i.e. inhaled cytokines and different physiological gases without causing adverse systemic effects. And furthermore, using this preparation it has been determined that the amount of tracheal EB extravasation in animals exposed to an isocapnic gas was comparable to EB extravasation data obtained by other workers in their respective control guinea pigs (Thompson *et al.*, 1987; Gordon *et al.*, 1987).

By using this *in vivo* isolated tracheal segment (ITS) model, I wished to determine the capability of TNFα and IL-1β as inducers of airway MVL in non-allergic animals.

1.19 Summary

Thus what began as an examination of the role of sensory nerves and tachykinins in allergic asthma and AHR has now developed into an examination of TNFα and IL-1β as inducers of *in vitro* AHR, and MVL *in vivo*.

Asthma is a disease characterized by reversible airways obstruction, a specific type of airway inflammation, AHR (Global Initiative for Asthma, 1997), epithelial damage (Laitinen *et al.*, 1985) and increased cytokine production (Robinson *et al.*, 1993; Broide *et al.*, 1992). Airway hyperresponsiveness is an abnormality of the airways that allows them to narrow easily and excessively to inhaled bronchoconstrictor stimuli. The stimuli may be either chemical or physical, and classed as direct or indirect. Direct stimuli (e.g. cholinergic agonists, histamine, leukotriene D_4) act directly on ASM via specific receptors. Physical and chemical agents such as antigen, exercise, hypertonic saline, adenosine and sulphur dioxide are indirect stimuli as they induce airway narrowing by stimulating inflammatory cells and/or sensory nerves (Joos *et al.*, 1993). The development of AHR has been linked to the presence of airway inflammation (Hogg *et al.*, 1991) and the increased exposure of airway sensory nerve endings in damaged epithelium to irritant stimuli (Barnes, 1991). However, the mechanism by which inflammation leads to AHR remains elusive.

In normal airways the degree of induced airway narrowing is determined by the balance between the inward force of the contracting ASM and the tethering action of lung parenchymal elements, which exert an outward force on the airway opposing airway narrowing (Robinson *et al.*, 1992). That is, there is an interdependence between airway wall and lung parenchyma in maintaining airway calibre. In inflamed airways, airway wall dimensions are increased, which changes the balance between the inward and outward acting forces, such that there is an uncoupling of the interdependence between the airway and the parenchyma, resulting in greater airway narrowing for a given stimulus (Mitchell & Gray, 1996). The primary structural change which is considered to be important in the induction of AHR, is an increase in airway wall thickness (Wiggs *et al.*, 1992). Thickening of the airway may result from cellular infiltration (Saetta *et*

al., 1991), deposition of connective tissue (Carroll *et al.*, 2000), thickened basement membrane (Roche *et al.*, 1989), hypertrophy and hyperplasia of ASM (Ebina *et al.*, 1993), vascular distension (Carroll *et al.*, 1997b) and oedema formation (Persson, 1991). It has been postulated that oedema accompanying airway inflammation may uncouple the airway from the parenchyma to such an extent that ASM contraction can be so severe as to completely occlude the airway (Macklem, 1990). Other mechanisms which determine excessive degrees of airway narrowing include, an enhanced contractility of ASM itself, and liquid filling of the airway lumen (Sterk, 1993a).

A number of early studies have looked for abnormalities in smooth muscle contractility *in vitro*, invariably demonstrating no difference (de Jongste *et al.*, 1987b; Armour *et al.*, 1984; Roberts *et al.*, 1984; Vincenc *et al.*, 1983). This has lead some to speculate that an increase in contractility of ASM seems an unlikely mechanism for the development of AHR *in vivo*. More recent studies have demonstrated *in vitro* AHR between normal and asthmatic tissue (Bjorck *et al.*, 1992; Cerrina *et al.*, 1989). *In vitro* hyper-responsiveness can be induced by antigen exposure (Colasurdo *et al.*, 1995) or viral infection (Saban *et al.*, 1987), by the addition of activated inflammatory cell supernatants and inflammatory products to organ baths (Johnson *et al.*, 1997; Anticevich *et al.*, 1996). Others have shown that passive sensitization of human bronchi *in vitro*, enhanced smooth muscle contractility and decreased relaxation responses (Villanove *et al.*, 1993; Ben-Jebria *et al.*, 1993; Black *et al.*, 1989). Demonstrable changes in the contractile apparatus have been detected in sensitized ASM (Ammit *et al.*, 2000; Rao *et al.*, 1991). These results indicate a link between the inflammatory process and the induction of AHR *in vitro*. However, it seems unlikely that any single cell, mediator or physiological mechanism is wholly responsible for the development of AHR.

It is now considered that airway inflammation in asthma is a cytokine controlled response rather than simply an antibody mediated one (Chung & Barnes, 1999). There is now a rapid exploration of the involvement of cytokines in the development of AHR. Cytokines are an extensive group of extracellular protein hormones that regulate inflammatory and immune reactions. Cytokines are synthesized and release on cell activation and induce formation of other cytokines in an amplification cascade.

Tumour necrosis factor-α and IL-1β are ubiquitous cytokines in the development of inflammatory responses, with TNFα being postulated as having a fundamental role in airway inflammation and acquired AHR (Shah *et al.*, 1995; Kips *et al.*, 1993a). Both IL-1β and TNFα are upregulated in asthma and exacerbations of asthma (Ackerman *et al.*, 1994; Broide *et al.*, 1992).

The development of AHR following the inhalation of irritant stimuli (Paggiaro *et al.*, 1993; Folinsbee & Horvath, 1986) and viral infections (Sterk, 1993b) has been well established. Recent studies have shown that following ozone exposure, toluene diisocyanate inhalation and viral infection there is an increased production of cytokines which include IL-1β, TNFα and IL-8 (Johnston, 1995; Maestrelli *et al.*, 1995; Basha *et al.*, 1994). And furthermore, these very same stimuli are associated with the upregulation of IgE and IgE receptors (FcεRI or FcεRII) (Grunstein *et al.*, 2001; Tee *et al.*, 1998; MacGlashan *et al.*, 1998).

Interleukin-1β and TNFα are co-localized in mast cells and macrophages and are released from these cells and lymphocytes following IgE receptor stimulation (Gosset *et*

al., 1999; Gordon *et al.*, 1990) and may be considered as "first wave" cytokines. Following this a second wave response occurs mediated by selectins, adhesion molecules, and chemokines, one of which being IL-8. Therefore, a unifying mechanism for *in vitro* ASM hyperresponsiveness following passive sensitization (Black *et al.*, 1989), ozone (Marthan *et al.*, 1996) and viral exposure (Folkerts *et al.*, 1992) may be the localized release of TNFα and IL-1β, following IgE receptor stimulation, from resident mast cells (Ammit *et al.*, 1997) and lymphocytes (Berger *et al.*, 1998) in airway preparations affecting smooth muscle contractility. Moreover, *in vivo* AHR has been demonstrated in animals and normal human subjects following administration of TNFα (Thomas *et al.*, 1995; Kips *et al.*, 1992; Wheeler *et al.*, 1990), IL-1β (Tsukagoshi *et al.*, 1994) and IL-8 (Xiu *et al.*, 1995).

Evidence suggests that cytokine cell signaling is mediated by the activation of certain G-binding proteins (Yanaga *et al.*, 1992), PLC (Machleidt *et al.*, 1996), enhanced Ca^{++} mobilization (Amrani *et al.*, 1996) and phosphoinositide accumulation (Amrani *et al.*, 1997), which are similar to the signaling pathways involved in ASM contraction (Chilvers *et al.*, 1994; Schramm & Grunstein, 1992). Thus cytokines might directly influence ASM contractility, by affecting signal transduction pathways that mediate ASM contraction.

Although some information is available regarding the effect of TNFα and IL-1β on ASM relaxation suggesting a disruption of the relaxation signal transduction pathway (Hirata *et al.*, 1994; Wills-Karp *et al.*, 1993b) the effects of TNFα and IL-1β on *in vitro* contractility are limited and inconclusive. *In vitro* induction of AHR by TNFα has been demonstrated in isolated human airways to electrical field stimulation but not to

contractile agonists per se (Anticevich *et al.*, 1995), whereas in animals studies the effect of TNFα in augmenting contractile responses has been varied (Pennings *et al.*, 1998; Kips *et al.*, 1993; Wills-Karp *et al.*, 1993b). In cultured human ASM cells prior exposure to TNFα augments the transient increases in cytosol free calcium ion concentration ($[Ca^{++}]_i$) induced by contractile agonists (Amrani *et al.*, 1995b). To date, the effect of IL-1β on isolated ASM contractile responses have demonstrated no change or a decrease in contractility rather than an increase (Munakata *et al.*, 1996; Tamaoki *et al.*, 1994; Wills-Karp *et al.*, 1993b).

Evidence is emerging that both the epithelium and endothelium are major sources of pro-inflammatory cytokines (Holgate *et al.*, 2000; Nilsen *et al.*, 1998; Spina, 1998a; Pober & Cotran, 1990). Once released they provide a cytokine gradient to guide leukocyte migration across the endothelium to damaged or infected areas (i.e. sites of inflammation). This gradient would result in significantly high local concentrations of cytokines which may in turn upregulate the responsiveness of adjacent ASM and/or cause airway mucosal oedema. Very few studies have examined the effect of specific cytokines on ASM contractility and as inducers of MVL.

Although a multitude of cytokines are involved in asthma (Table 1.2) and airway inflammation, with different cytokines assuming greater importance in different subsets of asthma, TNFα and IL-1β will be the cytokines of choice in examining their effects on two mechanisms considered to be important in AHR. These are; i) increased contractility of ASM and ii) the development of MVL. The latter may be an important mechanism during acute exacerbation of asthma, where peribronchial oedema may induce rapid airway instability by unlinking the airways form the parenchyma, leading to life-threatening bronchoconstriction (Macklem, 1990).

If one can show that cytokines contribute to the development and expression of AHR and MVL in the experimental animals chosen, it may ultimately lead to new improved therapeutic initiatives for the treatment of asthma.

Chapter 2 Methods

2.1 Animal ethics

The experimental procedures followed in all the animal studies outlined in this thesis were approved by the Animal Ethics Committees of the University of Adelaide and the Institute of Medical and Veterinary Science (IMVS), Adelaide, South Australia, Australia.

2.2 Sheep *in vivo* experiments

2.2.1 Animals

Australian merino ewes aged 12-18 months were used for these studies. They were housed in the IMVS and fed food and water *ad libitum*. The number of animals assigned to the various experimental and control groups were based on previously published studies using the ovine model of allergic asthma (Abraham *et al.*, 1991; Wanner *et al.*, 1979).

2.2.2 Measurement of lung resistance

The measurement of lung resistance using a computer assisted model has been described previously from our laboratory (Reynolds *et al.*, 1997) and is derived from the method of Abraham *et al.* (Abraham *et al.*, 1983). Sheep were fasted overnight prior to each experiment. Unsedated sheep were restrained in a sling in the prone position with their head immobilized. Topical nasal anaesthesia was achieved with a 10 %w/v lignocaine spray (Xylocaine, Astra Pharmaceuticals, Sydney) followed by a 2% Xylocaine gel (Astra). A 100 cm oesophageal balloon catheter (Jaeger, Wuerzburg, Germany) was introduced through one nasal passage and placed in the stomach. The

catheter was withdrawn slowly so that its final position was in the lower third of the oesophagus, as shown by a negative pressure deflection with clearly discernible cardiac oscillations during inspiration. The sheep were intubated transnasally with a cuffed endotracheal tube (Portex™, 7.5 mm internal diameter (ID)) using a flexible fibreoptic bronchoscope (Pentax, Japan). Pleural pressure was estimated from the oesophageal balloon catheter, and airway pressure in the trachea was measured using a side hole catheter (2.0 mm ID) introduced through the endotracheal tube and positioned with the hole 5 cm distal to the tip of the endotracheal tube. Transpulmonary pressure (P_{TP}) was determined using the pressure difference (ΔP) between tracheal pressure and oesophageal pressure as measured by a differential pressure transducer (Gaeltec Ltd., Neomedix Systems, Sydney). Flow through the endotracheal tube was measured using a pneumotachograph (Fleisch No.1, Dyna Sciences, Blue Bell, PA) heated to 37°C which was in line with a separate differential pressure transducer (see Figure 2.1).

While the animals were breathing spontaneously, the signals of flow and pressure were recorded by a 486SX personal computer (Protech Equipment, Adelaide) for breath by breath analysis using software written specifically for the project (Mr. K. Tiivas, Thoracic Medicine, Royal Adelaide Hospital, Adelaide). The program allowed the on-line display of R_L, dynamic compliance (C_{dyn}), tidal volume and breath frequency (Figure 2.2). Dynamic compliance and tidal volume were obtained by integration of the flow signal received from the pneumotachograph. Lung resistance (cm $H_20.L^{-1}$.sec) was calculated as P_{TP} / Δ Flow, where $P_{TP} = \Delta P - 1 / C_{dyn} \times$ Volume, using the mid tidal volume technique (Cook *et al.*, 1957; Mead & Whittenberger, 1953). A schematic representation of the signal analysis is shown in Figure 2.3.

Reported values of R_L were calculated by taking the mean value of at least 5

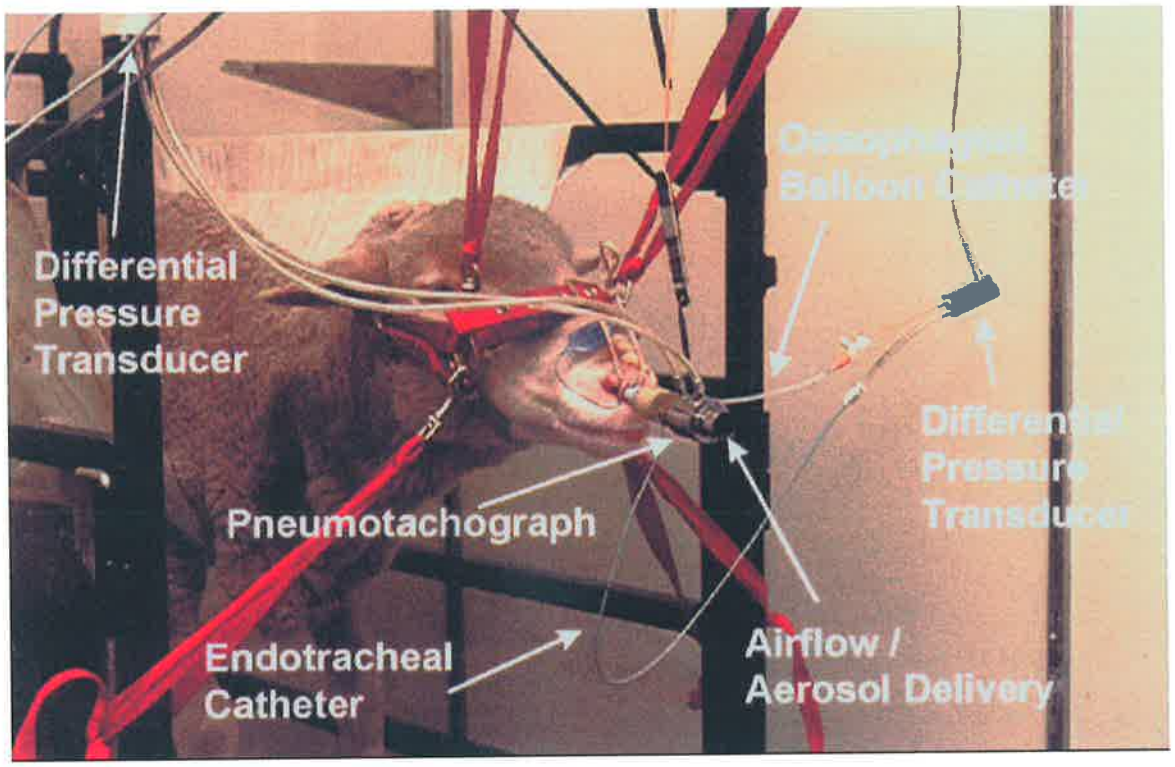


Figure 2.1 Catheter and transducer placement for the *in vivo* measurement of lung resistance in sheep.

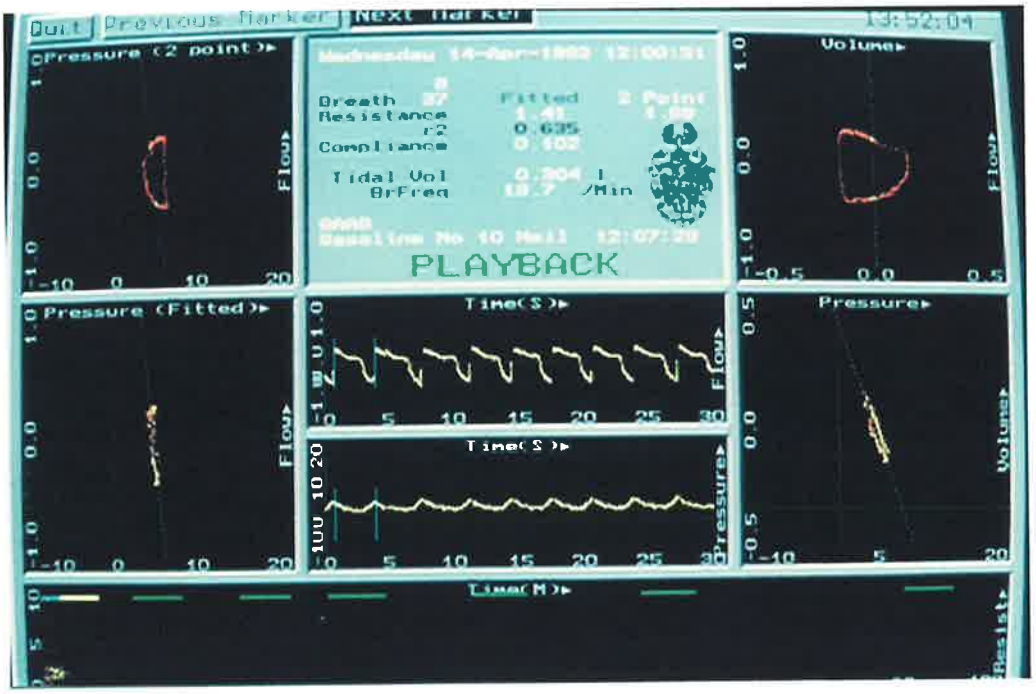


Figure 2.2 On-line computer screen analysis of lung resistance measurements in conscious sheep.

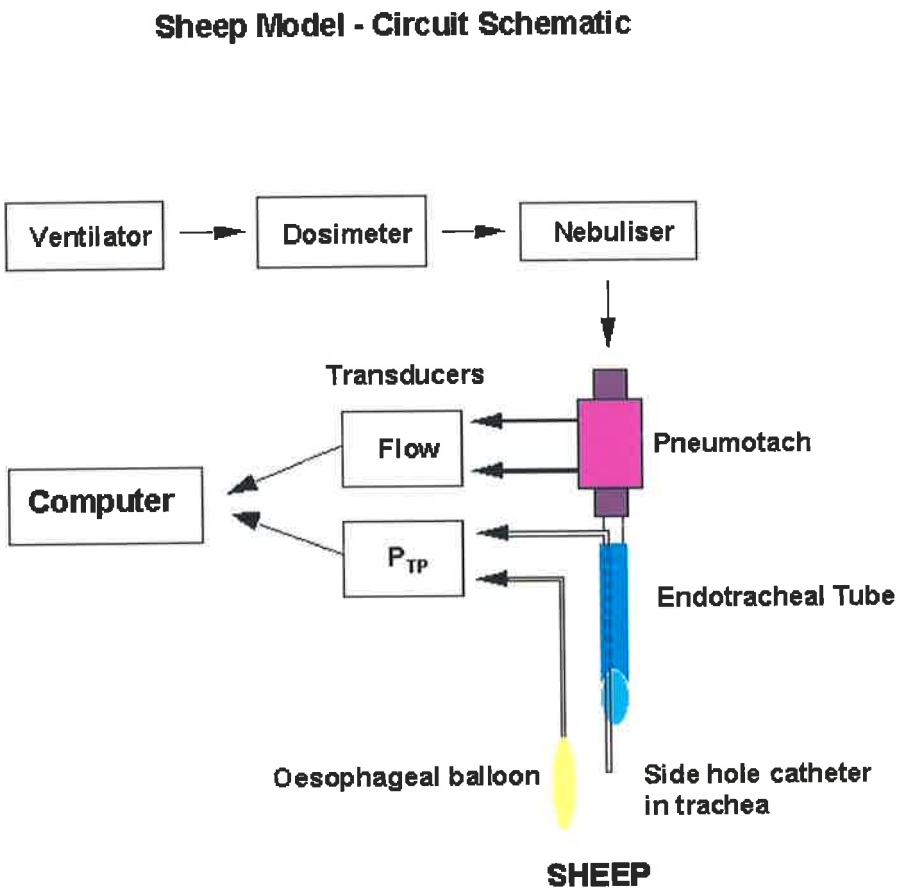


Figure 2.3 Circuit schematic for the analysis of lung resistance in sheep.

consecutive breaths. The software program contained a number of algorithms to identify artefacts caused by swallowing or animal movement. Breaths were deemed acceptable if both tidal volume and breath frequency were within 20% of the previous breath, P_{TP} was positive and the start and end of the breath were clearly delineated. Colour coding of the breath signals on the computer screen allowed for immediate real time monitoring of the quality of the data being collected (Figure 2.2), thus allowing the immediate determination of any technical problems such as blockage of catheters with mucus. A novel aspect of this computerized method is the real time breath by breath computer analysis of R_L and the algorithms used to identify spurious breaths. This approach greatly simplified resistance calculations, enabled immediate recognition of the plateau in resistance during bronchial challenges and eliminated the potential for operator bias.

2.2.3 Aerosol delivery

Aerosols were delivered using a ventilator and nebulizer (Bird™ Mark 14, Palm Springs, CA). During aerosolisation the ventilator was set to deliver a tidal volume of 350 mls using a 3% CO_2 in air mixture, at a rate of 20 breaths per minute. Preliminary studies demonstrated that nebulization with air alone resulted in a period of apnoea (due to hypocapnia). The nebulizing jet was delivered via a custom made dosimeter (Biomedical Engineering, Royal Adelaide Hospital, Adelaide), which nebulized for one second per breath, delivering 7.2 µl per breath. This aerosol delivery system has been shown to induce reproducible bronchoconstrictor responses in conscious sheep (Reynolds *et al.*, 1997).

2.2.4 Vascular access

Venous access for drug delivery was obtained via a permanent indwelling 16 gauge

catheter (Intracath™, Tuta Laboratories, Lane Cove, Australia) placed in the left jugular vein. In some animals an arterial catheter was placed in the right carotid artery for measurement of blood pressure. Catheters were placed under general anaesthesia at least seven days prior to commencement of experiments. Venous catheters were flushed daily with heparinized saline. Arterial catheters were connected to a pressurized infusion bag containing 500 mls heparinized saline (1 U/ml), which was delivered at a rate of 2 mls/hr at a pressure of 120 mmHg. For measurement of blood pressure the arterial line was connected to a disposable pressure transducer (Transpac® IV, Abbott Critical Care Systems, Sligo, Ireland) and signals were recorded on a polygraph (Neotrace 600 ZEF, Neomedix Systems, Sydney). Mean arterial blood pressure (MABP) was calculated as diastolic +((systolic - diastolic)/3).

2.3 Sheep *in vitro* experiments

2.3.1 General

Adult merino ewes were killed using a captive bolt device (Supercash Mark-II, Accles and Shelvoke Ltd., UK) thereby avoiding possible confounding effects of anaesthetic agents on ASM contractility. Tissues from four to nine animals were used in each experimental group. Their lungs were excised *en bloc* and placed in ice-cold oxygenated buffered Krebs-Henseleit (K-H) solution of the following composition (in mM): 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25.0 $NaHCO_3$ and 11 glucose (BDH AnalaR®, MERCK Pty. Ltd., Kilsyth, Australia) (pH 7.4) for transportation to the laboratory. Experiments were either commenced within 1.5 hours of excision or the tissue was stored in fresh ice-cold oxygenated buffered K-H solution at 4°C for subsequent use within 36 hours. Preliminary studies revealed no change in contractility to ACh or carbachol after this period of time (Chapter 4).

2.3.2 Preparation of airway smooth muscle

Strips of tracheal smooth muscle (3-8 mm wide x 20-25 mm long), bronchial rings (3-4mm ID, 2-3mm long) and parenchymal strips (15-20mm long x 3-5mm wide x 3-5 mm thick) were dissected from sheep lungs as shown in Figure 2.4.

2.3.2.1 Tracheal smooth muscle strips

The trachea was excised and placed immediately in fresh ice-cold aerated K-H solution. Single tracheal rings 3-8 mm in width were then cut in a transverse orientation from the section of trachea above the origin of the right upper lobe (see Figure 2.5). Then the connective tissue and most of the cartilage was removed under ice-cold K-H solution. Strips of epithelium-intact tracheal smooth muscle with cartilaginous insertions were dissected from the posterior portion of each trachea ring according to the method of Murphy *et al.* (Murphy *et al.*, 1989). A steel hook was then inserted into each end of the tracheal smooth muscle and adjacent cartilage (Lulich *et al.*, 1976) for mounting in the tissue organ baths (see below, Figure 2.8).

2.3.2.2 Bronchial rings

For bronchial smooth muscle preparations a section of lung from the left side (Figure 2.4) was removed and placed in fresh ice-cold aerated K-H solution. Bronchial ring preparations containing cartilage (2-4 mm ID and 2-3 mm in length) were then dissected free from the surrounding parenchyma under ice-cold aerated K-H solution in a similar fashion to that described (Armour *et al.*, 1988). Care was taken to obtain bronchial rings of similar dimensions rather than the same airway generation as the latter has been shown to be an inadequate classification for studying bronchial reactivity *in vitro* (Chitano *et al.*, 1993). The bronchial rings were supported longitudinally through the

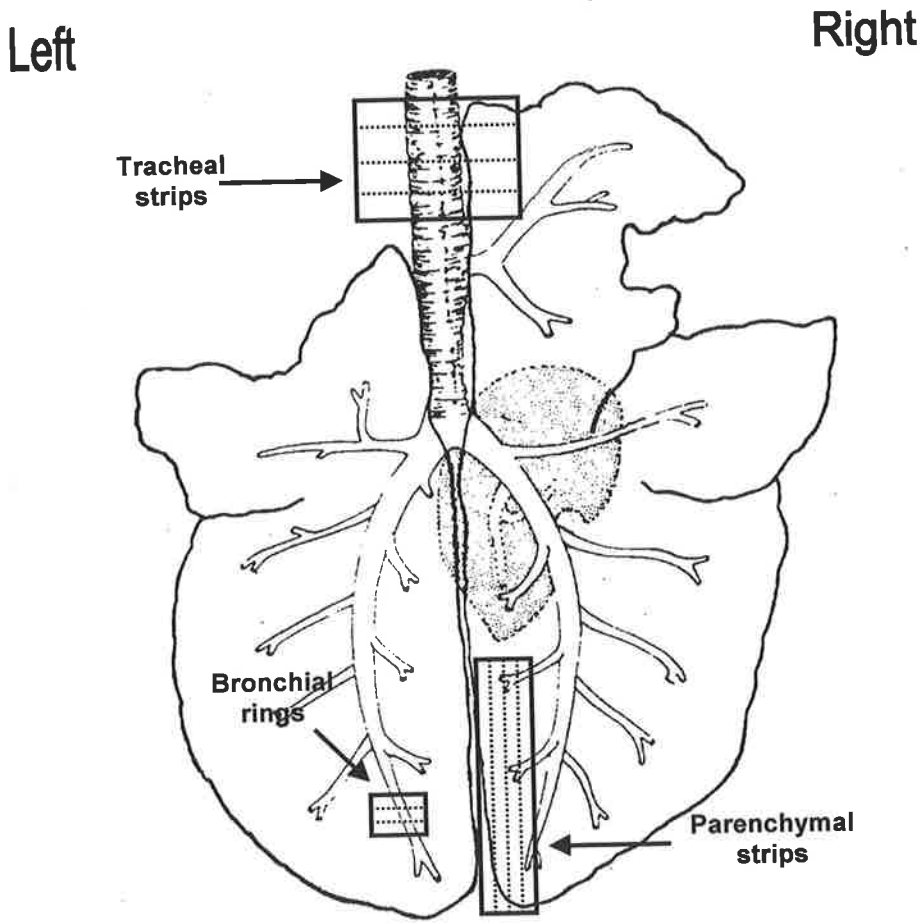


Figure 2.4 Diagrammatic representation of the sheep lung showing the areas of trachea, bronchus and parenchyma from which airway smooth muscle preparations were taken.

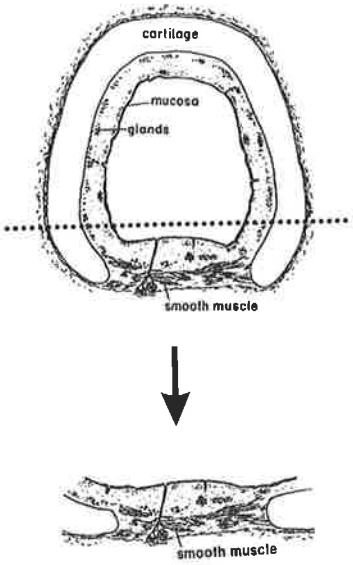


Figure 2.5 Diagram representing a single cartilaginous tracheal ring being dissected further to produce the tracheal smooth muscle strip.

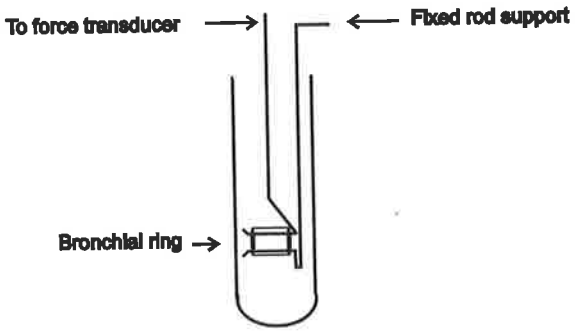


Figure 2.6 Schematic representation of a bronchial ring preparation mounted in an organ bath.

lumen by triangular stainless steel wire supports (Hulsmann & de Jongste, 1993) for mounting in the organ baths (see Figure 2.6).

2.3.2.3 Parenchymal lung strips

Parenchymal lung strips were obtained using methods similar to those previously described for large animals (Wagner *et al.*, 1985; Kleinstiver & Eyre, 1979; Lulich *et al.*, 1976). The lower section of the right lobe (Figure 2.4) was excised and placed in a dissecting dish containing fresh ice-cold aerated K-H solution. Using a scalpel, three parallel incisions were made about 5 mm apart, each extending about 4 cm. The longitudinal axis of the parenchymal strip was cut parallel to the lung margin, approximately 5 mm from the margin, thus giving two thin wedge sections of peripheral lung. From each thin wedge section 3 parallel incisions were made about 3 mm apart extending the full length of the wedge (see Figure 2.7). The central 2 cm section of each strips was identified and excised, giving two paired parallel parenchymal strips, just below the pleural surface with approximated dimensions of 20mm x 5mm x 3mm. For all parenchymal samples, strips were chosen from sites that had a macroscopically normal appearance and contained no visible bronchi or major blood vessels. A steel hook was then inserted into each end of the parenchymal lung strip for mounting in the organ baths (see Figure 2.8).

2.3.3 Protocol for *in vitro* isometric contraction

The ASM preparations were mounted on steel hooks, the lower hook was attached to a fixed support rod and the upper hook, via a fine gold chain, to a Grass FT03 force-displacement transducer (Grass Instruments, Qunicy, MA). The latter was

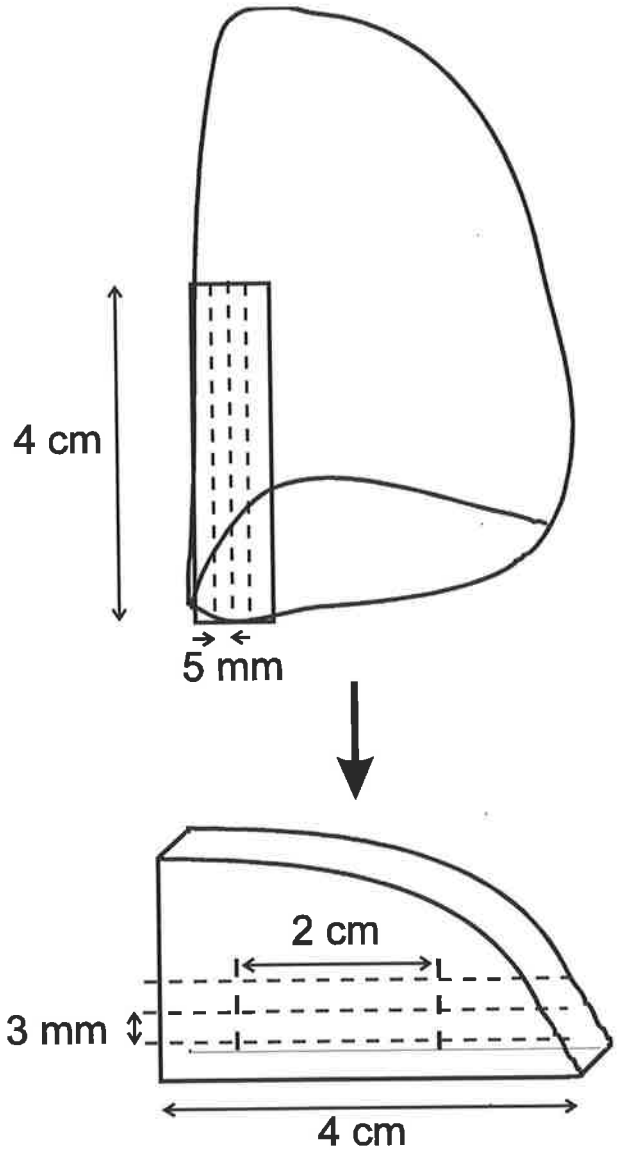


Figure 2.7 Diagram illustrating the preparation of isolated parenchymal lung strips from ovine lungs.

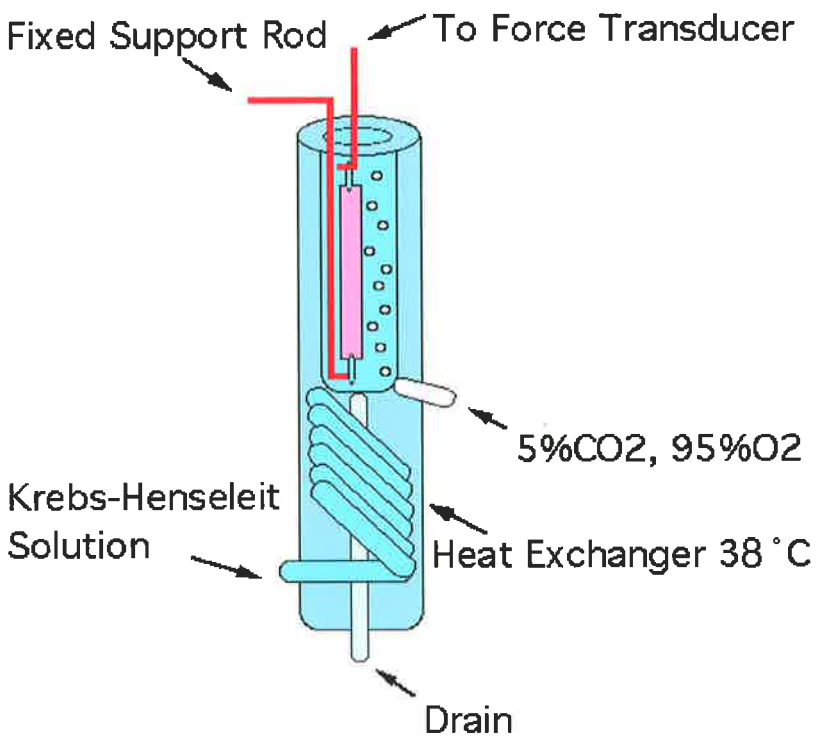


Figure 2.8 Schematic representation of the Harvard™ glass water-jacketed organ baths.

mounted on a rack and pinion clamp so that resting muscle length and therefore resting tension could be optimized (Mitchell *et al.*, 1989).

The tissues were suspended under tension in 20 ml siliconized, water-jacketed, glass organ baths (Harvard Apparatus, South Natick, MA) in K-H solution continuously bubbled with 95% O_2: 5% CO_2 and maintained at 38°C (Figure 2.8). The ASM segments were allowed to equilibrate in the water baths for 90 mins prior to the addition of agonists. During the equilibration period the bath solution was changed every 20 mins and resting load adjusted to maintain optimal tension throughout the equilibration period. Changes in ASM tension were measured isometrically and continuously recorded on polygraph (Neotrace 600 ZEF, Neomedix Systems, Sydney, Australia).

Whenever possible paired tissue samples were studied in duplicate. Adjacently cut tissue sections were considered as a pair. When studies were performed in duplicate the next adjacent paired segments were used, see Figure 2.9.

2.3.3.1 Cumulative concentration-response curves

At the end of the equilibration period, when a stable baseline tension was achieved, a reference contractile response to 10^{-3} M ACh was obtained. Once this contraction had reached a plateau, the tissues were washed every 20 mins until baseline tone was re-established (usually 60-90 mins). Cumulative concentration-response curves (CCRCs) were then obtained for the various agonists (see Figure 2.10A). This was achieved by the stepwise addition in increasing concentrations of agonist to the organ bath (in half-logarithmic increments) once the response to the preceding concentration had reached a plateau (Van Rossum, 1963). Cumulative concentration-response curves were conducted to an assigned maximal agonist concentration. The maximal concentrations

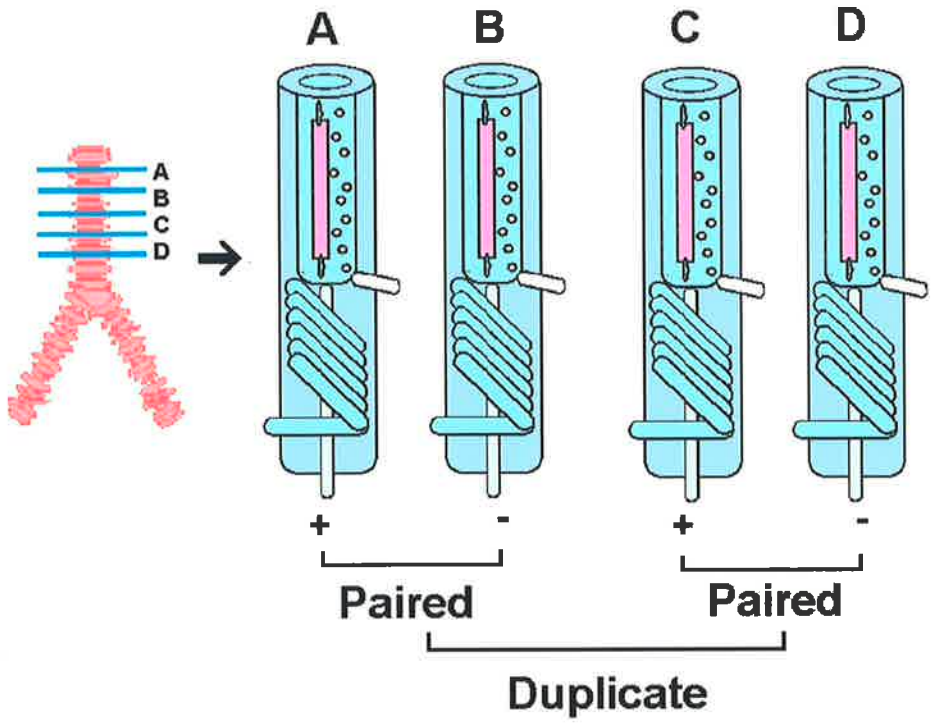


Figure 2.9 Schematic representation of paired tissue samples in duplicate, for tracheal strip, bronchial ring and parenchymal strip preparations.

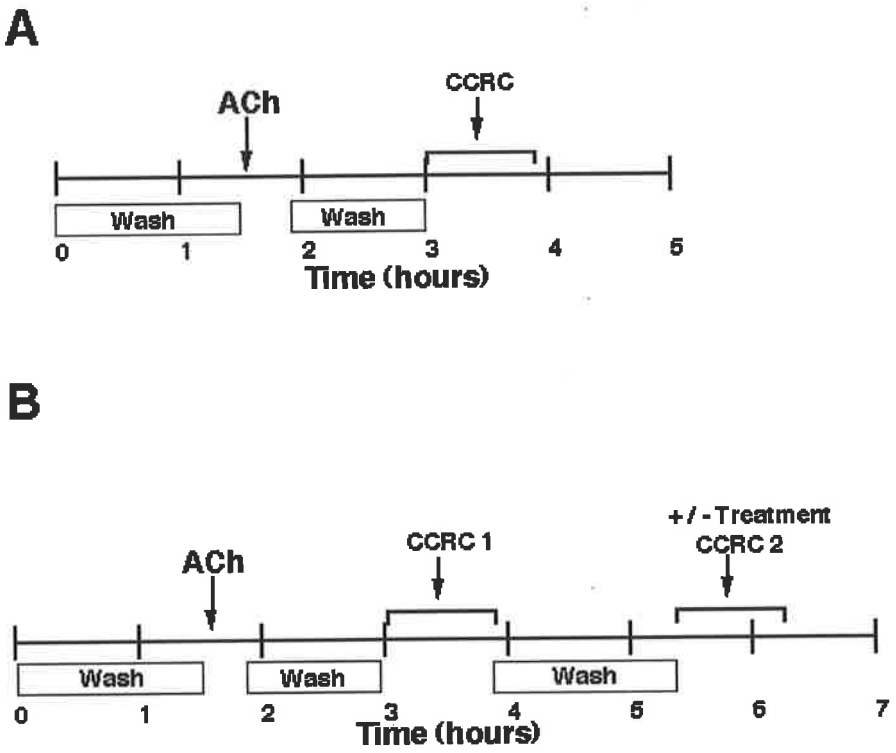


Figure 2.10 Time plot of sequences used in performing CCRC A) single CCRC and B) successive CCRCs.

being 10^{-3} M for ACh, $3x10^{-5}$ M for carbachol. With regard to tachykinins, CCRCs were stopped at 10^{-5}M, and at $3x10^{-6}$ M for NPγ, due to the cost factor involved in achieving higher concentrations in the large 20 ml organ baths required to accommodate tracheal smooth muscle strips.

2.3.3.2 Cumulative concentration-responses with neutral endopeptidase inhibition

In this series of experiments, following the reference ACh contraction, the NEP inhibitor, phosphoramidon (10^{-5} M, final bath concentration) (Hudgin *et al.*, 1981), was added to the organ bath of one of the paired tissues, the other receiving no treatment.

2.3.3.3 Consecutive cumulative concentration-response curves

In some series of experiments two successive CCRCs were obtained for an agonist. This enabled each tissue to act as its own control and provided an opportunity to detect the possible confounding effect of tachyphylaxis for each agonist under investigation. Following the first CCRC the tissues were washed until stable baseline tone was re-established (90-120 mins) after which a second CCRC was performed (see Figure 2.10B).

2.3.3.4 Cumulative concentration-response curves for agonists in the presence of specific antagonists

In this series of experiments two successive CCRCs were obtained for the agonist studied. Following the first CCRC the tissues were washed until stable baseline tone was re-established (90-120 mins). At this point, one of the paired tissues received the relevant specific antagonist, the other no treatment. Following a 30 min incubation period the second CCRC was performed.

2.3.3.5 Cumulative concentration-response curves in calcium-free K-H solution

For these experiments, the contractile responses to contractile agonists were elicited in the absence of extracellular Ca^{++}. Following a preconditioning ACh exposure the tissues were washed twice (at 15 min intervals) with K-H solution from which the Ca^{++} had been omitted and to which 10^{-3} M ethyleneglycol-bis (β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) had been added. Subsequently, the bathing solution was changed to one with no Ca^{++} and no EGTA and the tissues were washed three times with this solution. When baseline tone was stable in all tissues, CCRCs to agonists were obtained. After completing the response curves the tissues were washed every 20 mins with Ca^{++} containing K-H solution until baseline tone was re-established (at least 60 mins). When achieved, a reference contractile response to 10^{-3} M ACh was obtained.

2.3.4 Analysis of results

2.3.4.1 Cumulative concentration-response curves

In each tracheal smooth muscle preparation, the maximal effect (E_{max}) was the maximal tension generated in milligrams (mg) for each agonist, at the highest agonist concentration studied. For each tissue sample the E_{max} was also normalized to a reference contraction of 10^{-3} M ACh (E_{max} %ACh) or for wet tissue weight ((E_{max} (mg)/weight (mg)). A CCRC was then constructed relating the cumulative concentration of the agonist to the response. EC_{50} values were calculated as the agonist concentration that caused 50% of the maximal effect at the highest concentration studied, provided that the CCRC was sigmoidal rather than linear at the maximal concentrations.

Apparent affinities pD_2 values (defined as the negative log of the agonist concentration that caused 50% of maximal effect, -log EC_{50}) were calculated, by interpolation from the CCRC for each experiment. In cases when duplicate tissues were studied, a mean CCRC was constructed for each experiment and a geometric mean EC_{50} calculated.

To determine the rank order of potency of the tachykinins studied and to detect any significant shift in the CCRC following NEP inhibition an EC_{25} or EC_{10} (defined as the molar concentration of agonist required to induce a 25% or 10% contraction of that produced by 10^{-3} M ACh, respectively) was calculated. The EC_{25} or EC_{10} was an arbitrarily determined response which was on the exponential portion of the CCRC, a calculation that has been used for decades by other researcher groups (McKay *et al.*, 1992; Tomioka *et al.*, 1991; Black *et al.*, 1981). Results are expressed as mean ± standard error of the mean (SEM) (n, number of animals). EC_{50}, EC_{25} and EC_{10} values are given as geometric mean values with 95% confidence limits.

2.3.4.2 Consecutive cumulative concentration-response curves

In experiments in which two consecutive CCRCs were obtained, the contractile responses to each concentration of agonist in the first CCRC were expressed as a percent of the maximal response (E_{max}) in that airway preparation. Contractile responses in the second CCRC were expressed as a percentage of the E_{max} of the first CCRC.

The antagonist affinities values (pA_2) for specific antagonists with their respective agonist were calculated as follows. Firstly, the EC_{50} values for the first and second (antagonist present) CCRCs were determined. Then a dose ratio for each muscle strip was calculated as the EC_{50} for agonist in the presence of a given concentration of

antagonist divided by the EC_{50} in the absence of the antagonist. Schild plot regressions (Arunlakshana & Schild, 1959) were made by plotting log (dose ratio - 1) for the agonist vs. -log antagonist concentration. Slopes were determined by least square linear regression analysis of data points from each muscle strip experiment. The pA_2 value was determined as the intercept of this regression line with the abscissa (Arunlakshana & Schild, 1959). The competitive nature of antagonism was also verified using the Schild plot method.

2.4 Guinea pig *in vivo* experiments

2.4.1 Animals

Adult albino Dunkin Hartley guinea pigs, bred in closed colonies, were obtained from the IMVS Gilles Plains field station. Experiments were performed on guinea pigs of either sex weighing 758±109 (SD) gm.

2.4.2 Surgical preparation

Guinea pigs were housed in the IMVS and fed food and water *ad libitum*. On the day of the experiments guinea pigs were first anaesthetized by intraperitoneal pentobarbital sodium 40 mg/kg (Nembutal®) and placed in the supine position on a heated blanket, under a 100-Watt heating lamp to maintain body temperature between 37-38°C. Body temperature was monitored using a CIG series 80 temperature monitor (CIG, Sydney, Australia) connected to a rectal probe (Yellow springs Instrument Co., Yellow Springs, OH). The abdomen and throat were shaved and the midline anaesthetized intradermally with lignocaine hydrochloride (Xylocaine®). Following a midline incision, the trachea was then exposed by blunt dissection, just before it enters the thorax and cannulated with a tight fitting 12 gauge plastic cannula (Dwellcath™, Tuta Laboratories, Lane Cove, Australia) through a longitudinal incision (approx. 3-5mm) without ligation to

minimize disruption to the tracheal microcirculation. This cannula was connected to a small animal ventilator (Model 665A, Harvard Apparatus Co., South Natick, MA) and fixed volume cycled ventilation commenced (30 strokes/min, tidal volume 8 mls/kg, positive end-expiratory pressure 2 cm H_2O), without air leakage, with conditioned air (Figure 2.11). That is, compressed air which has been heated and humidified (see Section 2.4.3 Conditioning of inspiratory gases). Airway pressure was continuously monitored by a side-port connection from the tracheal cannula to a pressure transducer (Transpac® IV, Abbott Critical Care Systems, Sligo, Ireland) and recorded on a polygraph (Neotrace 600 ZEF, Neomedix Systems, Sydney, Australia). The animals were hyperventilated with twice the tidal volume (e.g. sighing) by manually blocking the outflow of the ventilator every 20 minutes to improve lung function by preventing atelectasis and slow lung collapse (Widdicombe & Davies, 1983; Niemeier & Bingham, 1972). During the course of the experiments, measurements of C_{dyn} were made to monitor and deterioration in airway function. Dynamic compliance measurements were not taking within a five minute period of the sighing manoeuvre. Dynamic compliance was calculated by dividing tidal volume by the difference between end-inspiratory and end-expiratory airway pressure.

The external jugular vein was cannulated using a 20 gauge catheter (Insyte™, Becton Dickinson, Sandy, UTAH), and tied securely with black braided silk suture (4/0 Ethicon™, Johnson&Johnson, Sydney, Australia). The animals were heparinized (150U/kg, heparin sodium) and this catheter kept patent by filling its dead space with heparinized saline (150U/ml) and used subsequently for EB (50 mg/ml) administration (see below). A second fine cannula (Insyte™, 20 gauge) was passed through the anterior tracheal wall into the tracheal lumen just below the larynx, and connected to a

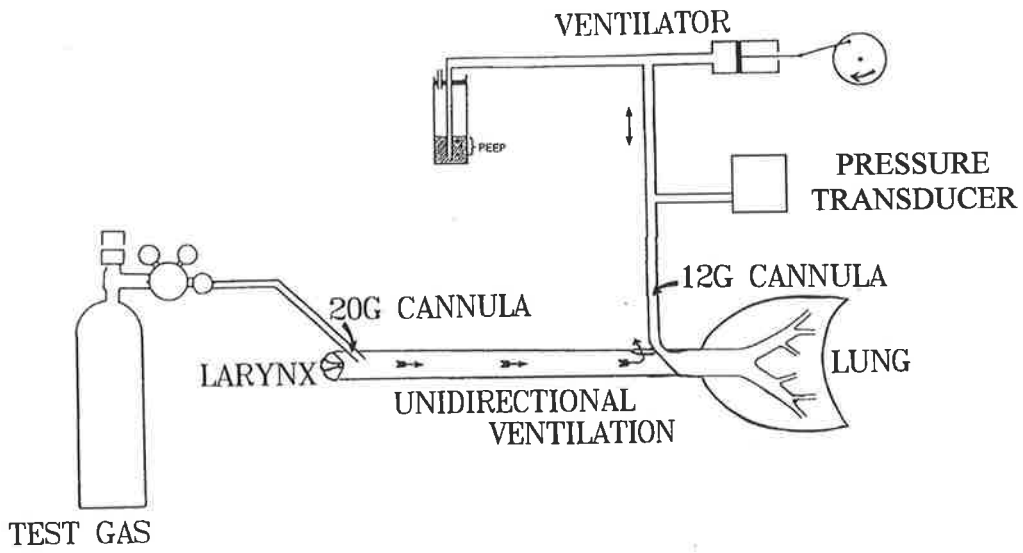


Figure 2.11 Experimental set-up for studies measuring microvascular leakage in the guinea pig isolated tracheal segment.

heated and humidified isocapnic normoxic gas source, as previously described (Reynolds *et al.*, 1992). This ITS was ventilated unidirectionally with the isocapnic gas at a constant flow (200 mls/min) with gas escaping freely just before the distal cannula (Figure 2.11). Flow rate was measured continuously using a precision bore flow meter (No. FP 1/8-08-G-5/81; Fischer & Porter, Workington, UK) connected in series with a compressed gas source. Reverse gas flow through the ITS was prevented by occluding the animal's nose and mouth with a rubber mask.

2.4.3 Conditioning of inspiratory gases

Conditioned air used for the ventilator inspirate was heated and humidified by bubbling compressed air through a series of heated (38°C) water chambers and through inspiratory tubing that was warmed throughout its length by insulated heating tape (Reynolds & McEvoy, 1988). Temperature at the airway opening was measured using an in-line thermistor (Yellow Springs 400). Inspired gas water content was determined before the experiments by directing a large gas sample of known volume from the ventilator through anhydrous calcium chloride ($CaCl_2$) (BDH AnalaR®, MERCK Pty. Ltd., Kilsyth, Australia) desiccating tubes. The isocapnic, normoxic gas used in tracheal ventilation was similarly conditioned. Humidity was then calculated from the change in weight of the drying tubes, the sampled volume and the temperature measured at the respective sampling ports (Lange & Forker, 1969). Temperature and humidity for the ventilator inspirate and unidirectional isocapnic gas were 35°C: 92% and 34°C: 85%, respectively.

2.4.4 Measurement of plasma extravasation

Vascular leakage was determined by EB extravasation (Rogers *et al.*, 1989a; Saria & Lundberg, 1983). Immediately prior to unidirectional ventilation of the ITS, EB

(50 mg/kg) was injected intravenously. Following 1 hour of unidirectional ventilation the animal was given a lethal intravenous injection of pentobarbital sodium. The chest was opened and the heart exposed. A plastic cannula (Dwellcath™, 12 gauge) was passed through a left ventriculotomy into the ascending aorta, and a glass cannula inserted into the right atrium, via the right ventricle. The ventricles were cross-clamped and whole body perfusion commenced with phosphate buffered saline (PBS) (pH 7.40) at a perfusion pressure of 100 cm H_2O for 2.5 minutes to remove intravascular dye. The tracheal segment (approx. 2 cm) was excised and dissected free of the esophagus and connective tissue. The middle third of this segment was then excised, blotted dry on filter paper (Whattman® No.1, Maidstone, UK) and its weight recorded using an electronic balance (Model HF300G, A&D Co., Ltd., Tokyo, Japan). Evans blue was extracted in 2 mls formamide (Sigma Chemical, St Louis, MO, USA) at 60°C for 24 hours in stoppered tubes. A 200 µl aliquot was taken from each sample and loaded onto a 96 flat bottom well tissue culture plate (Linbro®, Flow Laboratories, McLean, Virginia) (see Figure 2.12). Evans blue content in each well was determined by light absorbance (optical density) at 630nm using a MR7000 microplate reader (Dynatech, Guernsey, Channel Islands). The EB concentration in each sample was then calculated by linear interpolation from a standard curve of EB in formamide (0.25-10 µg/ml). Tracheal EB content was expressed as µg/gram wet tissue weight.

2.4.5 Aerosol delivery

Aerosols were generated from a Ventstream® (Medic-Aid, Paghan, Sussex, UK) jet nebulizer powered at 7L/min, with 5%CO_2: 95%O_2 (mass median diameter 3µm, per manufacturer's specifications). The nebulizer was attached to a 1 metre silastic tube (6 mm ID) which was connected to the 20 gauge tracheal cannula. Nebulizer output was determined at the end port of the silastic tube by directing saline aerosol through a

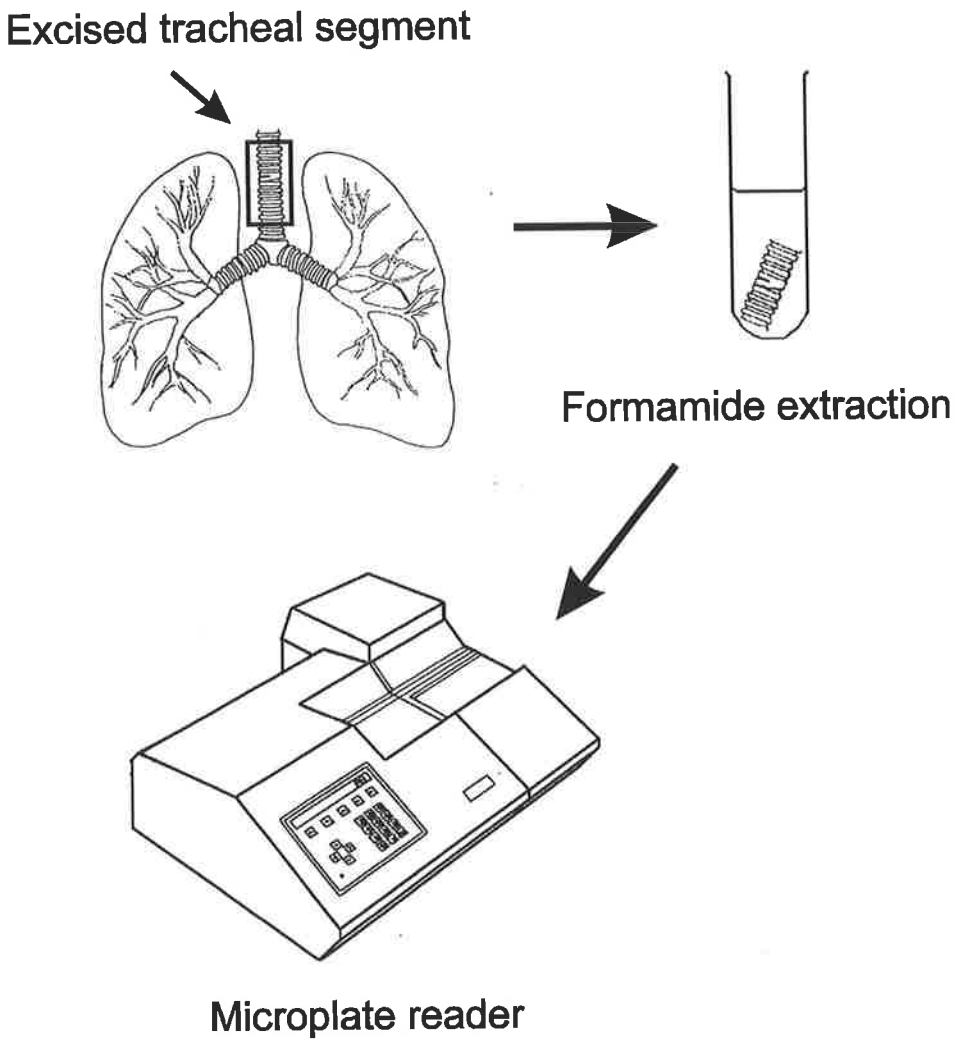


Figure 2.12 Schematic diagram for the Evans blue extraction process from guinea pig trachea.

desiccating column for a specified time and determining the weight gain. Output was determined to be 17±1.0 μl per minute.

2.4.6 Experimental groups

Tracheal EB extravasation was examined in 4 groups of animals. To determine baseline airway MVL in the tracheal segment EB content was measured without aerosol challenge in five animals. At the beginning of each aerosol challenge experiment, the nebulizer bowl was filled with 5 mls of either saline (diluent), histamine or IL-1β and TNFα. In the control group for aerosol challenge, the ITS (n=5) was subjected to an aerosol challenge of saline (diluent). To test whether a combination of IL-1β (10 ng/ml) and TNFα (100 ng/ml) induced EB extravasation, the ITS was exposed to an aerosol of these cytokines (n=5). As a positive control the ITS was exposed to histamine as an aerosol ($5x10^{-2}$ M) (n=3). All aerosol challenges were for one minute.

2.5 Statistical analysis

2.5.1 Sheep *in vivo* experiments

All statistical analyses were performed using repeated measures Analysis of Variance (ANOVA). Significant differences between group means were determined using Fisher's Protected Least Significant Difference analysis (Welkowitz *et al.*, 1982). The significance level was set at P less than 0.05 for all analyses. The Fisher's Protected Least Significant Difference test requires the performance of the ANOVA and calculation of an F ratio which provide protection against multiple t-test comparisons. This protected t procedure provides control and balance between Type I and Type II errors test, where a Type I error is to falsely reject the null hypothesis, that is failing to detect a real difference and a Type II error occurs when the null hypothesis is falsely

accepted. The Fisher's test also protects against small deviations in group variances provided sample sizes are equal and treatment group numbers are small (less than 6) as the case in the *in vivo* studies. (Welkowitz *et al.*, 1982).

2.5.2 Sheep *in vitro* experiments

For the preliminary *in vitro* studies of ovine smooth muscle contractility (Chapter 4), the between and within sheep variability of the contractile response to the agonists studied was determined by a two-way ANOVA with single observations (Tallarida *et al.*, 1988). Similarly for the viability studies, between sheep and between day variance was determined by a two-way ANOVA with single observations. Values were considered significant at $P < 0.05$ level. The Cochran's statistic was used as a preliminary test to check for homogeneity of group variances (Kirk, 1995; Godfrey, 1985).

Statistical analyses were performed using two-tailed paired and unpaired Student's *t*-test to detect significant differences in mean CCRCs between control and treated tissue. Values were considered significant at $P < 0.05$ level.

2.5.3 Guinea pig *in vivo* experiments

Again the Cochran's statistic was used as a preliminary test to check for homogeneity of group variances (Kirk, 1995; Godfrey, 1985). Mean values for each treatment group were analyzed by one-way ANOVA. When an F value indicated a significant difference comparisons between group means were performed using the Bonferroni t statistic (Kirk, 1995). An ANOVA for randomized block design was used to test separately for significant changes in C_{dyn} over time (Wallenstein *et al.*, 1980). A P value less than 0.05 was considered significant.

For ANOVA and multiple comparison tests one assumes homogeneity of variances in the different treatment groups. Although the ANOVA is robust with respect to violation of this assumption exception arise in cases of unequal variances with unequal sample sizes (Kirk, 1995). In the present guinea pig study comparisons were made between several groups in which the animal sample size was small and unequal. Therefore the Cochran's *C* statistic was used to establish that the experiment groups exhibited equal variances in order to minimize the experimental Type I error rate.

2.6 Reagents

2.6.1 Sheep *in vivo* experiments

Substance P (Auspep, Melbourne, Australia) was dissolved in 10% ethanol / 0.1% bovine serum albumin (BSA) (Sigma Chemical, St Louis, MO) in sterile saline (Abraham *et al.*, 1991) to a concentration of 1 mg/ml and stored at -70°C. Phosphoramidon (Sigma) was dissolved in sterile water to a stock concentration of 1 mg/ml then aliquotted and stored at -20 °C. CP-96,345 (donated by Pfizer Pharmaceuticals, New York, NY) was made to a stock concentration of 100 mg/ml in sterile PBS (pH 7.40), stored at -20°C, then diluted to a dose of 0.1 mg/kg in 10 mls of normal saline on the day of the experiment. Atropine, (Astra Pharmaceuticals, Sydney, Australia) was diluted in 10 mls 0.9% NaCl to a dose of 0.1 mg/kg and delivered intravenously by a 10 min infusion. Sodium cromoglycate (10 mg/ml solution, Fisons, UK) was used as supplied, 4 mls being delivered by inhalation. Indomethacin (Sigma) was dissolved in 0.8% sodium bicarbonate to a working concentration of 10 mg/ml and administered at a dose of 2 mg/kg intravenously. Histamine acid phosphate (Sigma) was dissolved in 0.9% NaCl to various working concentrations on the day of the experiment and administered intravenously. Pyrilamine (Sigma) was dissolved in 10

mls sterile water to a total dose of 2 mg/kg body weight on the day of the experiment. Neurokinin A (Auspep) was dissolved in 10% ethanol / 0.1% BSA in sterile saline to a stock concentration of 1 mg/ml and stored at -70°C. Doses of SP and NKA at $5x10^{-6}$ mol/kg were made up in 10% ethanol / 0.1% BSA in sterile saline on the day of the experiment.

2.6.2 Sheep *in vitro* experiments

The K-H solution was made up (in batches) to a volume of 10 litres by the addition of the following quantities of the specific chemicals:

Chemical	Chemical composition	Weight (grams)
Sodium chloride	NaCl	69.2
Potassium Chloride	KCl	3.50
Magnesium sulphate heptahydrate	$MgSO_4.7H_2O$	2.90
Potassium dihydrogen orthophosphate	KH_2PO_4	1.66
Sodium hydrogen carbonate	$NaHCO_3$	21
Glucose	D-Glucose	20
Calcium chloride	$CaCl_2$	*

* Calcium chloride was made up as a 1 molar stock solution, from which 25 mls was added to the 10 litre K-H mixture. For the Ca^{++}-free K-H solution, $CaCl_2$ was omitted and replaced with 25 mls of 1 molar NaCl.

Stock solutions of SP, NKA, NPγ, [Sar^9,$Met(O_2)^{11}$]-SP, GR94800 (AUSPEP, Melbourne, Australia), acetylcholine chloride, carbamylcholine chloride (carbachol), phosphoramidon, atropine sulphate, pyrilamine, EGTA (Sigma Chemical Co., St Louis,

MO), and 4 diphenylacetoxy-N-methyl peperidine methiodide (4-DAMP) (ICN Biomedicals, Aurora, OH, USA), were made up in distilled water and aliquots kept at -70°C. While stock solutions of NKB, [Nle^{10}]-NKA(4-10) and senktide (AUSPEP, Melbourne, Australia) were prepared in 40% dimethylsulfoxide (BDH AnalaR®) and stored at -70°C. Histamine acid phosphate (BDH AnalaR®, MERCK Pty. Ltd. Kilsyth, Australia) stock solution was made up in distilled water and aliquots kept at 4°C. For the calcium-free Krebs studies serial dilutions of ACh and histamine were prepared in calcium-free K-H.

On each study day, serial dilutions of agonists and antagonists were prepared in K-H solution and kept on ice for the duration of the experiment. All compounds were added to the bath in volumes not exceeding 0.5% of the total bath volume. Plastic vials and pipette tips were used to store and handle the tachykinin peptides and other agents. Organ baths were coated with Coatasil®, a silicone treatment for glass (AJAX Chemicals, Sydney, Australia).

2.6.2.1 Tracheal tissue incubation experiments

Stock solutions of human recombinant TNFα, IL-1β (Promega, Madison, WI, USA) were prepared in sterile water and aliquots kept at -20°C. For the incubation studies differing concentrations of IL-1β and TNFα were added to Dulbecco's modified Eagle's medium (DMEM) (Gibco. BRL, Gaithersburg, MD), supplemented with antibiotics (penicillin 100 U/ml and streptomycin 100 *u*g/ml, Sigma).

2.6.3 Guinea pig *in vivo* experiments

Nembutal® (Rhône Mérieux, Pinkenba, Qld., Australia); saline (0.9% sodium chloride, BP) and Xylocaine® (0.5%, Astra Pharmaceuticals, Sydney, Australia); heparin sodium BP (Davis Bull Laboratories, Mulgrave, VIC, Australia); EB and formamide (Sigma Chemical Co., St Louis, MO); histamine acid phosphate (BDH AnalaR®, MERCK Pty. Ltd. Kilsyth, Australia); $CaCl_2$ (dried, 8-24 mesh, AJAX Chemicals, Sydney, Australia); human recombinant TNFα, IL-1β (Promega, Madison, WI, USA). Stock solutions of TNFα, IL-1β were prepared in sterile water and aliquots kept at -20°C (histamine kept at 4°C). On each study day, dilutions were prepared in sterile saline and kept on ice for the duration of the experiment. Plastic vials and pipette tips were used to store and handle the reagents. Compressed gases: ventilator inspirate, medical air; isocapnic-normoxic gas, 5% CO_2: 21% O_2: 74% N_2; nebulizer gas, 5% CO_2: 95% O_2 (Air Liquide, Melbourne, Australia).

Chapter 3 The effect of exogenous tachykinins on lung resistance in conscious sheep

3.1 Introduction

Numerous animal species have been used as experimental models of asthma including monkeys, sheep, dogs, rats, rabbits and guinea pigs (Wanner & Abraham, 1982). None of these is completely representative of human asthma. The sheep *in vivo* model of antigen-induced bronchoconstriction has been well characterized and validated as an experimental model of asthma (Abraham *et al.*, 1988a; Abraham *et al.*, 1983). As previously discussed in the introduction, the cellular events in the airways, the timing of the early and late asthmatic responses and development of AHR after allergen challenge in sheep, appear to be quantitatively and qualitatively similar to those in man (Soler *et al.*, 1991; Abraham *et al.*, 1988a) while prolonged AHR occurs in dual responders (Lansing *et al.*, 1993). Furthermore, pharmacologically similar mediators have been implicated in antigen-induced bronchoconstriction and the development of the late asthmatic response to those implicated in human asthma (Barnes *et al.*, 1998). Animals challenged for long periods of time increase their baseline R_L akin to chronic asthma (Bosse *et al.*, 1987). Histologically, allergic hyperresponsive sheep demonstrate an increased tendency for airway wall eosinophilia, an increased number of secretory granules in airway mast cells (Chen *et al.*, 1991a) and submucosal gland hyperplasia (Chen *et al.*, 1991b), morphological findings that are consistent with human asthma (Hamid *et al.*, 1997; Carroll *et al.*, 1993).

In addition, the *in vivo* sheep model has other advantages, in that R_L can be measured in conscious animals, therefore, avoiding the confounding effects of mechanical ventilation and anaesthetic agents, and longitudinal studies can be performed in the same animal.

In allergic airway responses, such as asthma, there may be hyperalgesia of sensory nerves, resulting in increased airway inflammation and obstruction as a result, in part, of the local effects of tachykinins in the airways (Spina, 1998b; Barnes, 1996b; Karlsson, 1993). Several studies, particularly in the guinea pig and the rat, have suggested an important role for tachykinins in the mediation of airway responses. However, the importance of tachykinins in non-rodent mammals *in vivo* is less clear (Karlsson, 1993). In our laboratory we have established the ovine model of allergic bronchoconstriction (Abraham *et al.*, 1983) and found evidence for a role for C-fibre afferents and tachykinins in the mediation of the acute response to allergen (Reynolds *et al.*, 1997). In that study three experimental interventions were used: capsaicin desensitization, NEP inhibition by phosphoramidon and NK_1 receptor blockade by CP-96,345. Following these interventions allergic bronchoconstrictor responses were attenuated, augmented and diminished, respectively. As a result of these observations, it was decided to examine the effect of exogenously applied tachykinins in naïve non-allergic sheep, with the intention to examine how these responses may be altered in allergic sheep.

In this chapter, in collaboration with Dr Paul Reynolds, we examine *in vivo,* the mechanism of tachykinin-induced airway responses by exogenously applied tachykinins. We investigate the time course of the bronchoconstriction response to intravenous SP, then examine the effect of several pharmacological agents on SP-induced bronchoconstriction, including the NEP inhibitor phosphoramidon (Hudgin

et al., 1981), the NK_1 receptor antagonist CP-96,345 (Snider *et al.*, 1991), atropine, sodium cromoglycate, indomethacin and the histamine H_1-receptor antagonist, pyrilamine. We also compared the SP-induced airway response to that induced by NKA. These studies help to expand the understanding of tachykinin physiology *in vivo* in a large animal model that is potentially of more significance for the understanding of human responses than the widely reported rodent models.

3.2 Methods

3.2.1 Animals

Australian merino ewes aged 12-18 months were used for these studies. They were housed in the IMVS and fed food and water *ad libitum*. Detailed methodologies for the measurement of lung resistance, aerosol delivery and surgical preparation are given in Chapter 2 - Methods (Sections 2.2.2, 2.2.3 and 2.2.4, respectively).

3.2.2 Drugs

All drugs, compounds and solutions used in the following experiments are listed, prepared and used according to the methods described in Chapter 2 - Methods, Section 2.6.1, Reagents.

3.2.3 Tachykinin administration

Three methods of tachykinin administration were evaluated in preliminary studies - inhalation, bolus intravenous injection and intravenous infusion. SP administered by inhalation (to a maximal dose of 144 μg, 20 breaths of a 1 mg/ml solution) did not result in any significant increase in R_L, even after phosphoramidon (288 μg inhaled) administration. Subsequently, SP was given either by bolus injections ($5x10^{-6}$ mol/kg) or by a short infusion (0.01 mg/kg total dose made up in 2 mls diluent, at a rate of

1 ml/min over 2 minutes). Neurokinin A was also given by bolus intravenous injection ($5x10^{-6}$ mol/kg). In all cases intravenous lines were first primed with diluent (10% ethanol / 0.1% BSA in normal saline) to reduce the adherence of neuropeptides to the plastic tubing.

3.2.4 Experimental protocol

3.2.4.1 Effect of intravenous SP on R_L and arterial blood pressure

The airways response to a bolus dose of SP ($5x10^{-6}$ mol/kg IV) and short infusion of SP (0.01 mg/kg IV over 2 mins) was assessed in 6 sheep. Lung resistance was monitored at baseline and continuously for 15 mins from the onset of SP administration. In 5 sheep the blood pressure response was measured concurrently with the airway response.

3.2.4.2 Assessment of tachyphylaxis

The effect of repeated SP infusions given on the same day was examined in 4 sheep. Following an initial infusion (time = 0), repeat infusions were given at 120, 180 and 210 mins, thus the effects of SP infusions separated by 30, 60 and 120 mins were assessed. Lung resistance was measured at baseline (5 mins) and for 15 mins from the onset of the infusion.

3.2.4.3 Assessment of day to day reproducibility

Reproducibility of the response to a single daily SP infusion was determined over 3 consecutive days in 6 sheep. Lung resistance was measured as for the tachyphylaxis studies.

3.2.4.4 Modulation of SP effect with drugs

The effect of five drug interventions was assessed in the same 6 sheep used for the

reproducibility studies. Each drug study consisted of two SP challenges on consecutive days. One of the two challenges was designed as a control, and whereby SP administration was preceded by the administration of the relevant diluent for the active drug under investigation. For the phosphoramidon and sodium cromoglycate studies, the diluent control was 40 breaths of normal saline. For the CP-96,345 and atropine studies the control was a 10 ml bolus injection of normal saline, and for the indomethacin study the control was a 10 ml bolus injection of 0.8% sodium bicarbonate in normal saline.

For the control and active drug challenges, R_L was measured initially for 5 mins to establish a stable baseline. Active drug or diluent was then administered and R_L was measured 20 mins later to ensure there was no significant shift in R_L from the original baseline. SP was then administered by infusion while R_L was monitored continuously for 15 mins. The peak R_L was determined, and expressed as a percentage of the R_L measurement taken immediately prior to the commencement of the SP infusion. The drugs given were: phosphoramidon (244 μg inhaled), CP-96,345 (0.1 mg/kg IV), atropine (0.1 mg/kg IV), indomethacin (2 mg/kg IV) and sodium cromoglycate (40 mg inhaled). The drug studies were performed in a random order at weekly intervals over a 5 week period.

3.2.4.5 Effect of pyrilamine on substance P-induced increases in R_L

In a separate set of experiments, the effect of the anti-histamine pyrilamine on SP-induced increase in R_L was assessed in 3 sheep. The ability of pyrilamine to block the bronchoconstrictor response to histamine was first assessed. Histamine was administered intravenously in a dose response fashion to a maximum cumulative dose of 18.75 μg/kg. Two days later, the anti-histamine pyrilamine was administered

intravenously (2 mg/kg) 20 minutes before histamine administration. One week later the R_L response to a bolus injection of SP ($5x10^{-6}$ mol/kg) was measured, then, two days after this, pyrilamine (2 mg/kg IV) was given 20 mins prior to SP. In each case R_L was monitored at baseline for 5 mins and continuously for 15 mins from the administration of each of the agents used.

3.2.4.6 Comparison between the R_L response to SP and NKA

The R_L response to equimolar bolus doses of SP and NKA ($5x10^{-6}$ mol/kg) were compared in four sheep. SP and NKA responses were measured on separate days to avoid confounding by tachyphylaxis.

3.2.5 Data Analysis

Data are presented as means ± SEM expressed either as an absolute value (cm $H_2O.L^{-1}$.sec) or as a percent of baseline R_L. All statistical analyses were performed using a repeated measures ANOVA. Significant differences between group means was determined using Fisher's Protected Least Significant Difference analysis (Welkowitz *et al.*, 1982). The significance level was set at $P < 0.05$ for all analyses.

3.3 Results

3.3.1 Time course of airways response to SP

In preliminary studies, SP was administered by inhalation up to a maximal concentration of 1 mg/ml (total dose 144 µg) in four sheep, Figure 3.1. No significant effect on R_L was seen with this dose of SP. Mean increase in R_L (± SEM, as a percent of baseline) following SP inhalation was 49.3 ± 23.4%. Intravenous administration of SP was then evaluated. The airways response to a bolus intravenous dose of SP ($5x10^{-6}$ mol/kg) was transient, lasting approximately 5 breaths (Figure 3.2). In an effort

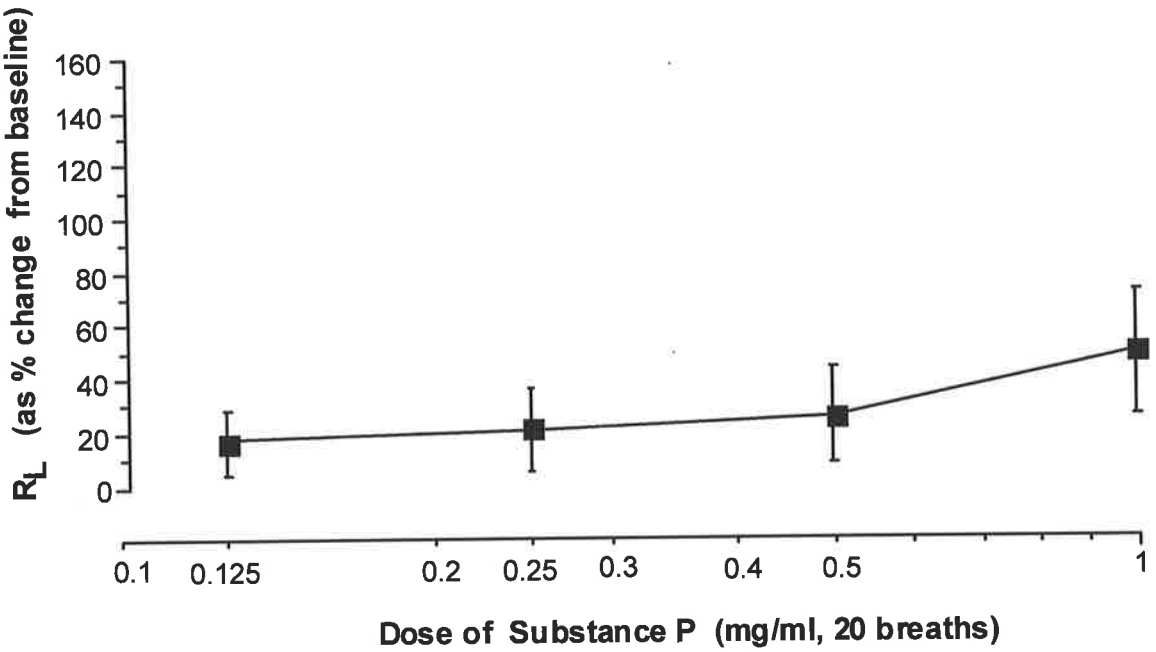


Figure 3.1 The airway response to aerosol SP. Responses are expressed as a percentage change from baseline. Values represent mean ± SEM, n=4 animals.

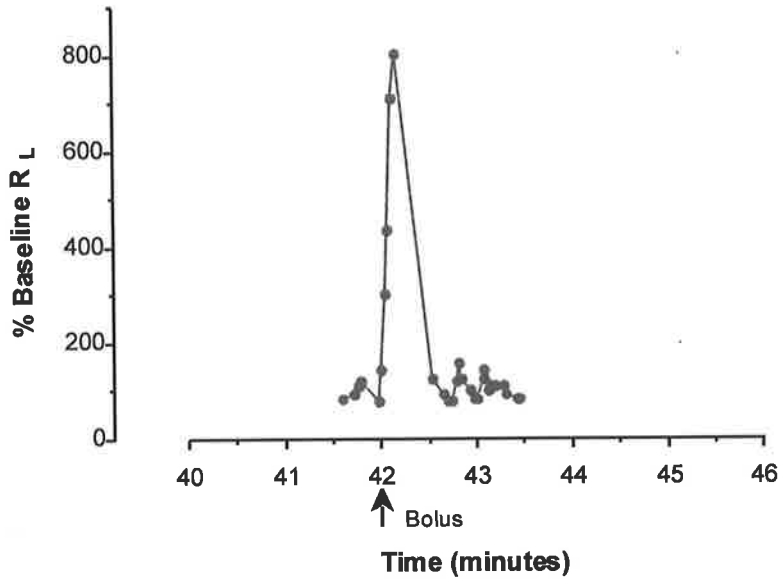


Figure 3.2 Breath by breath time course of R_L, in an individual sheep, following a two minute intravenous infusion of SP ($5x10^{-6}$ mol/kg). Responses are expressed as a percentage of baseline.

to induce a more prolonged effect, we assessed the response to a 2 min infusion of SP. There was minimal prolongation of the response however, and R_L had returned to baseline by the completion of the 2 min infusion (Figure 3.3). Mean baseline R_L (± SEM) prior to SP infusion was 1.72 ± 0.24 (cm $H_2O.L^{-1}.sec$), (n = 6 sheep). No significant change in MABP was seen.

3.3.2 Tachyphylaxis

Substance P infusions at t = 0 and t = 120 produced significant increases in R_L above baseline (Figure 3.4). However, compared with the initial SP infusion (t = 0), there were significant attenuation in the bronchoconstrictor responses following subsequent infusions of SP at t = 120, t = 180 and t = 210. Moreover, the observed increase in R_L to SP infusions at t =180 and 210 minutes were not significantly different from baseline. Mean baseline R_L (± SEM) prior to t=0 SP infusion was 1.83±0.13 (cm $H_2O.L^{-1}.sec$), (n = 4 sheep). During the course of this study we assessed whether SP was having any medium term effect on R_L by comparing the immediate pre-SP infusion R_L values at each infusion time point over the 3.5 hour duration of the study. No change in baseline values was observed. Mean pre-infusion R_L (as a % baseline R_L, ± SEM) at t = 120, 180 and 210 minutes were 96.0 ± 3%, 89.5 ± 4% and 90.3 ± 7%, respectively.

3.3.3 Day to day reproducibility

Although there was significant inter-sheep variability in the response to infused SP, there was no significant differences in the responses of individual sheep to SP infusions administered at 24 hour intervals over a 3 day period (Figure 3.5). There was also no significant change in the absolute baseline R_L value for individual sheep over the course of the 3 day reproducibility study (data not shown). These findings indicated that drug intervention studies conducted at 24 hour intervals would be unlikely to be confounded

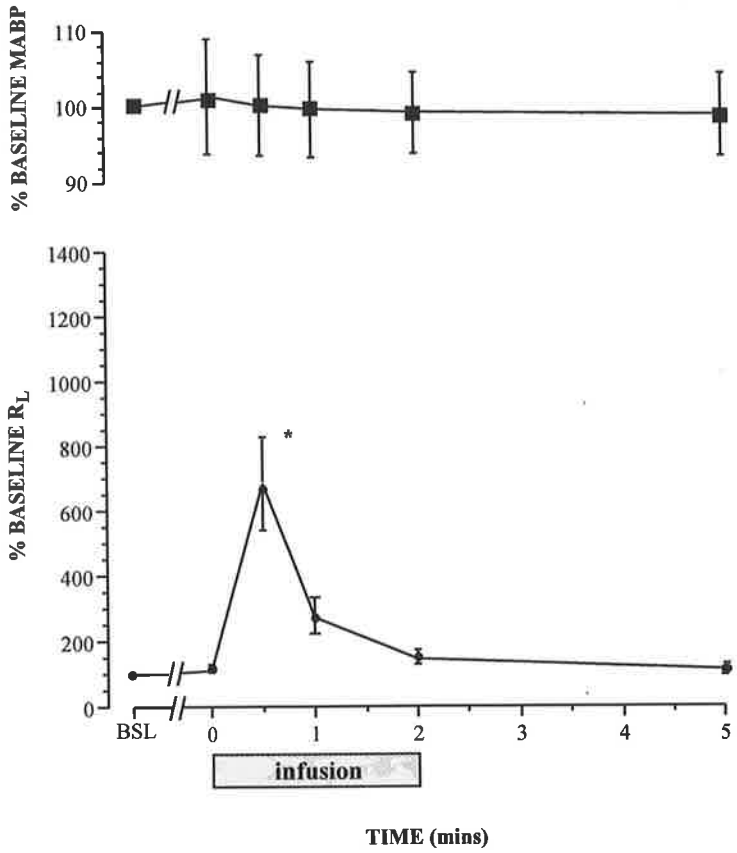


Figure 3.3 Time course of R_L (n = 6 sheep) and mean arterial blood pressure (MABP) (n = 5 sheep) following a two minute intravenous infusion of SP (0.01 mg/kg). Responses are expressed as a percentage of baseline (BSL). Values represent mean ± SEM. * $P < 0.05$ versus baseline.

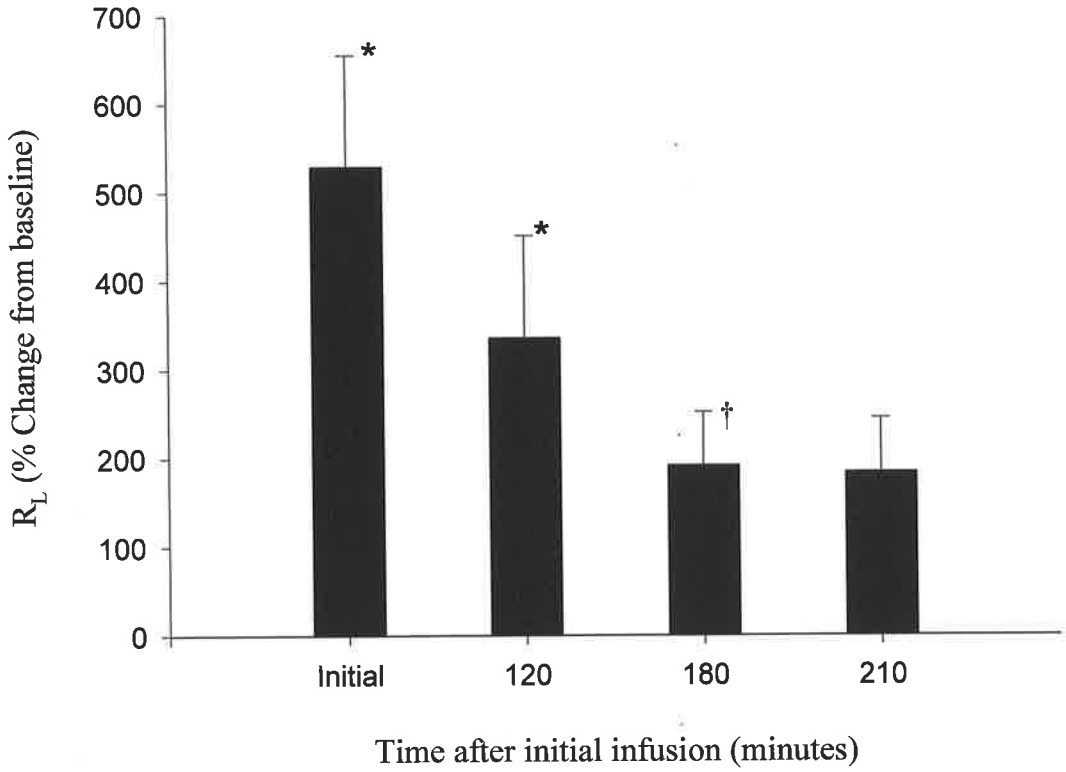


Figure 3.4 Effect of repeated infusions of SP on R_L. Shaded bars represent peak R_L response following two minute infusions of SP (0.01 mg/kg). After the initial SP infusion (t = 0), repeat infusion were separated by 120, 60 and 30 minutes (i.e. t = 120, 180 and 210 minutes, respectively). All values represent mean ± SEM, expressed as a percentage of the baseline at t = 0 in four sheep. t is time in minutes post initial SP infusion. * $P < 0.05$ versus pre-infusion baseline. † $P < 0.05$ versus peak R_L following initial SP infusion (t = 0).

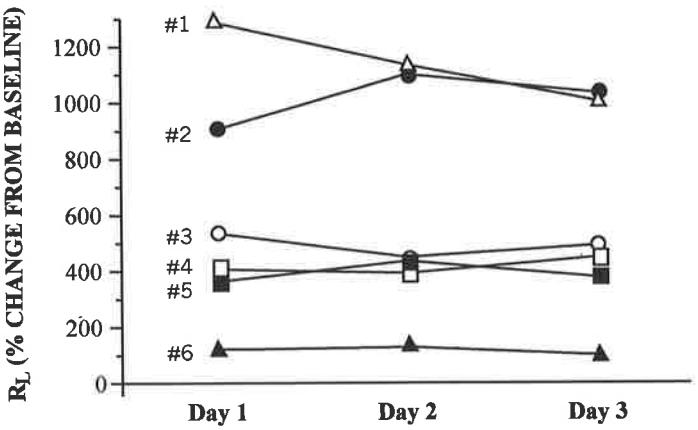


Figure 3.5 Individual R_L responses to intravenous SP (0.01 mg/kg), in six sheep, over three consecutive days. Values represent percent change in R_L from each day's baseline.

by either spontaneous variation in the response of individual sheep, tachyphylaxis due to the previous SP dose, or shifts in baseline R_L (either spontaneous or due to SP).

3.3.4 Drug interventions

There was no significant difference between initial baseline R_L measurements and the measurements taken 20 mins after either active drug or diluent administration. Lung resistance values after diluent control or drug are shown in Table 3.1. In all of the diluent control challenges, SP produced a significant rise in R_L (Figure 3.6). The response to SP was completely abolished by CP-96,345 and atropine (Figure 3.6). Both indomethacin and sodium cromoglycate caused a slight diminution in the responses to SP but these were not statistically significant. Of particular note is that pretreatment with phosphoramidon significantly increased the peak response to SP but had no effect on the duration of the response (Figure 3.7).

3.3.5 Effect of pyrilamine

Pretreatment with pyrilamine blocked the R_L response to histamine but had no effect on the response to SP (Figure 3.8).

3.3.6 Effect of SP compared to NKA

At a dose of $5x10^{-6}$ mol/kg SP significantly increased R_L, whereas at the equivalent dose, NKA caused only a slight increase in R_L which was not significantly different to baseline (Figure 3.9). Pre-challenge R_L (± SEM) for the four sheep on each SP and NKA challenge day were 1.72 ± 0.22 and 2.23 ± 0.34 (cm $H_2O.L^{-1}$.sec), respectively, and were not statistically different.

Table 3.1 Raw baseline R_L data obtained for intervention studies.

Intervention Study	Pre-SP Baseline R_L [a]	
	After Diluent	After Pretreatment
Phosphoramidon	1.62 ± 0.12	1.86 ± 0.25
CP-96,345	2.47 ± 0.26	2.14 ± 0.18
Atropine	1.87 ± 0.08	1.67 ± 0.19
Sodium Cromoglycate	2.08 ± 0.28	1.89 ± 0.16
Indomethacin	2.24 ± 0.17	2.74 ± 0.31

[a] Values are mean R_L ± SEM (cm $H_2O.L^{-1}.sec$).

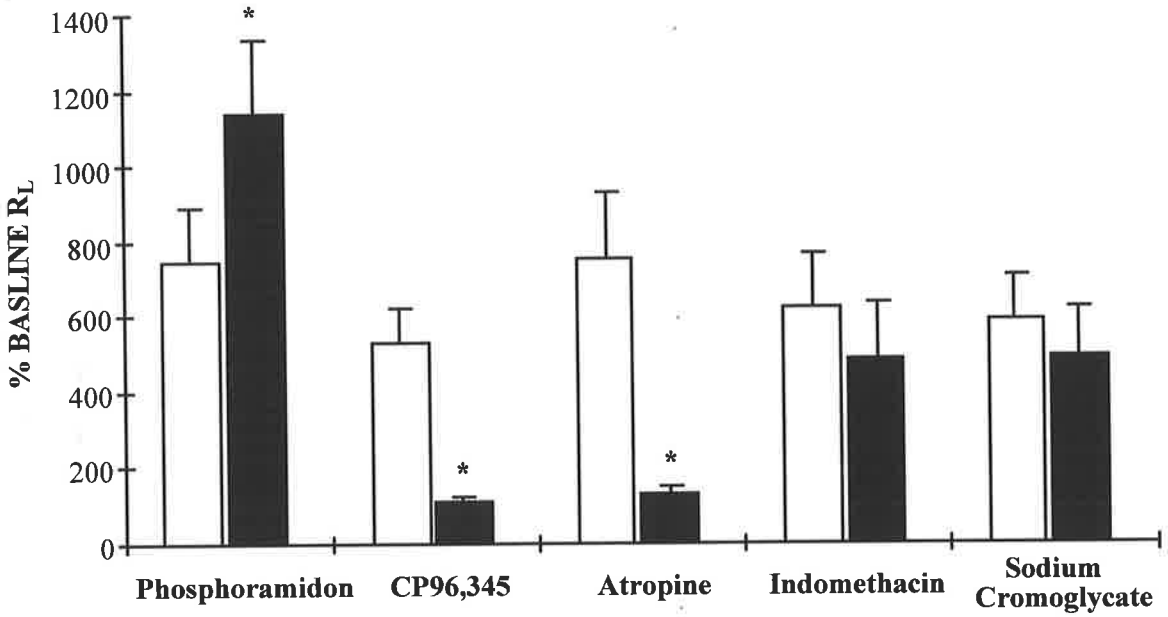


Figure 3.6 Effect of pharmacological intervention on the airway response to infused SP (0.01 mg/kg). Open bars: response to SP following administration of respective diluent. Shaded bars: response to SP following active drug administration. Please refer to methods for specific doses and diluents. Values are mean ± SEM, in six sheep, and expressed as a percentage of R_L immediately prior to SP infusion. * Significantly different from diluent control $P < 0.05$.

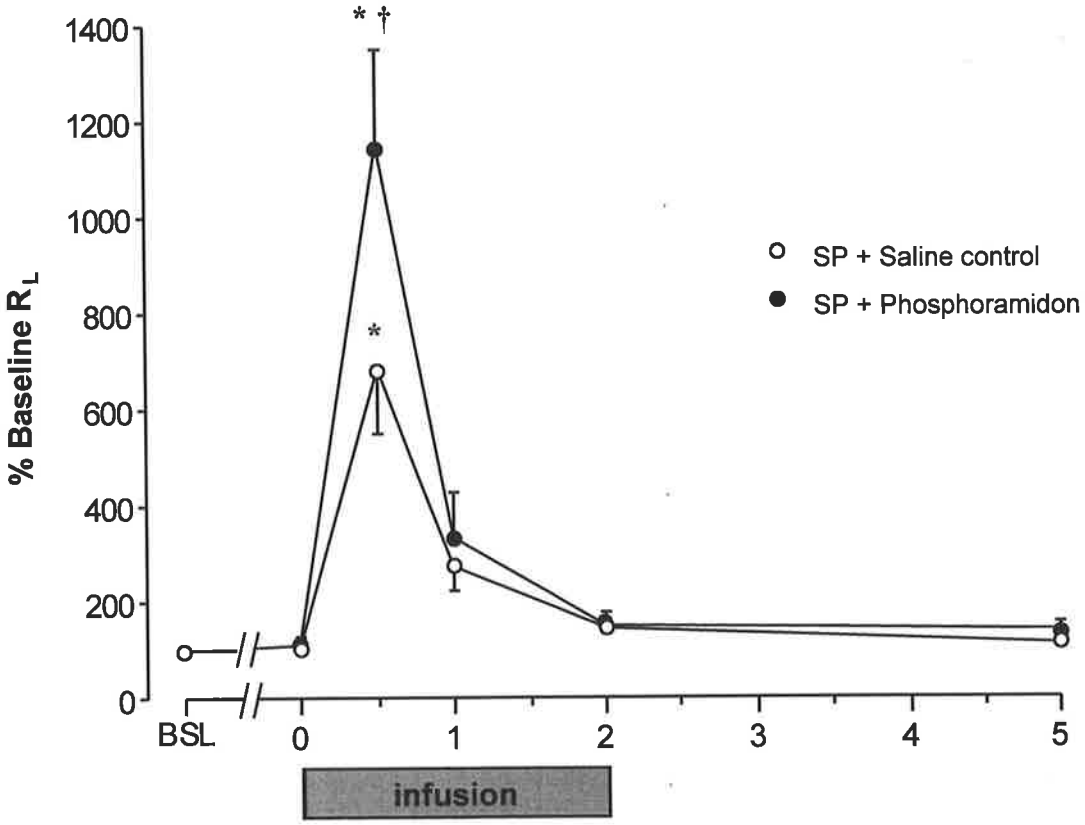


Figure 3.7 Time course of the response to SP infusion on the R_L following control (saline) pre-treatment (○) or pre-treatment with phosphoramidon (●). Values are mean ± SEM for 6 sheep. * $P < 0.05$ vs baseline, † $P < 0.05$ vs peak R_L response in control challenge.

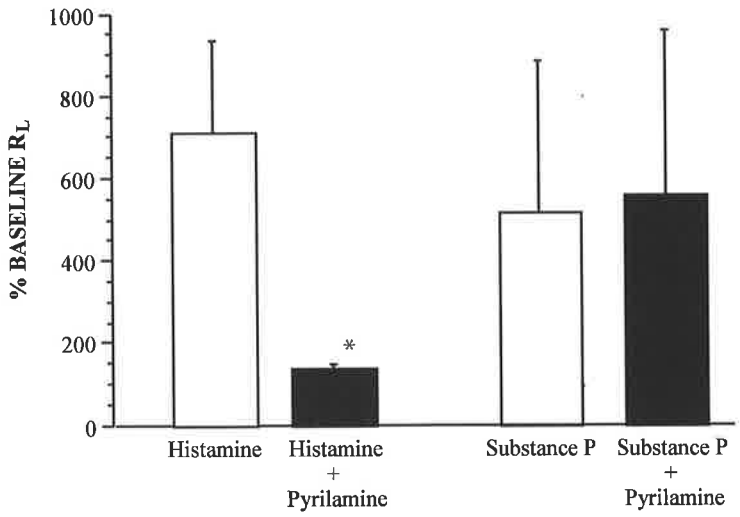


Figure 3.8 Effect of pyrilamine (2mg/kg, IV) on R_L changes induced by histamine (10μg/kg, IV) and substance P (5×10^{-6} mol/kg, IV). Values represent mean ± SEM in three sheep and are expressed as a percentage of baseline. Open bar: R_L response following intravenous diluent (0.9% NaCl). Closed bar: R_L response after IV pyrilamine. * $P < 0.05$ versus control challenge.

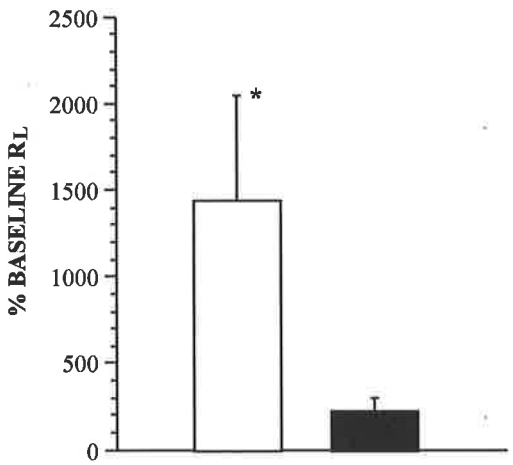


Figure 3.9 Comparison between equimolar doses (5 x 10^{-6} mol/kg, IV) of SP and NKA in four sheep. Open bar: SP. Closed bar: NKA. Values are mean ± SEM, expressed as a percentage prechallenge R_L. * $P < 0.05$ versus baseline.

3.4 Discussion

The experiments conducted in this chapter demonstrate that intravenous SP increases R_L in sheep and also characterize this response in a number of ways. Unlike previous reports (Corcoran & Haigh, 1992; Constantine *et al.*, 1991) we did not find that SP had any significant effect on blood pressure. Therefore it is unlikely that the responses described were confounded by reflex responses to hypotension. Although the effect of inhaled SP was investigated in preliminary experiments, like Abraham *et al* no significant effect was seen (Abraham *et al.*, 1991). It seems unlikely that these negative results were due to inadequate doses of inhaled SP, as the concentrations we employed are known to induce bronchoconstriction in asthmatics rather than healthy subjects (Joos *et al.*, 1987). Therefore, the lack of a bronchoconstrictor effect may reflect differences in endopeptidase activity in ovine airways (Abraham *et al.*, 1991).

The response to bolus intravenous SP was transient lasting for less than one minute, with the peak effect typically lasting only 5 breaths, this response being similar to that observed by others following bolus intravenous SP in sheep (Corcoran & Haigh, 1992) (Parsons *et al.*, 1992). Possible mechanisms for the transient nature of this response include the induction of tachyphylaxis and rapid SP degradation.

Very short half-lives for intravascular SP have been documented in various species ranging from 0.5 to 1.6 minutes (Schaffalitzky *et al.*, 1986; Yeo *et al.*, 1984). Furthermore, vascular responses to infused SP in man have demonstrated very short biological half-life with the rapid development of tachyphylaxis (McEwan *et al.*, 1988). In an effort to distinguish between these possibilities we examined the effect of SP delivered by infusion. The use of a computerised measurement of R_L, which enables

continuous on-line measurements, allows one to reliably measure the time course of transient airway responses.

When SP was delivered by infusion, there was overall prolongation of the airway response, but no significant increase in duration of the peak R_L. The return of R_L to baseline while the infusion was still running clearly indicated a rapid tachyphylaxis to SP, rather than rapid degradation of SP alone. This finding is in accord with *in vitro* studies of Ca^{++} mobilization responses to SP and with vascular permeability responses in rat blood vessels, but has not previously been reported for airways responses (Bowden *et al.*, 1994; McMillian *et al.*, 1987). This effect was further examined by performing repeated infusions on the same day, which demonstrated that tachyphylaxis to the effect of infused SP persisted for at least 2 hours after an initial infusion. At 24 hours however, there was no evidence of persistent tachyphylaxis. These findings clearly have important implications for the design of studies examining the effects of drugs or other interventions on the response to SP, as results will be confounded by tachyphylaxis if insufficient time is allowed between baseline and post-intervention SP doses. For example, one report examined the effect of atropine on SP responses only 20 minutes after the index SP dose and only one hour after a SP dose response study (Corcoran & Haigh, 1992). No comment was made regarding tachyphylaxis in that study, although the transient nature of the response to SP was similar to the present study. The effect of atropine on SP induced airway responses they reported was therefore likely to have been confounded by tachyphylaxis.

Possible mechanisms for the development of tachyphylaxis include receptor phosphorylation and second messenger uncoupling (Garland *et al.*, 1996; Sanders & LeVine, 1996). NK_1 receptor internalisation, a mechanism which has been

demonstrated by direct visualisation in endothelial cells (Bowden *et al.*, 1994), appears less likely to play a role (Sanders & LeVine, 1996). The present findings suggest that the induction of tachyphylaxis is likely to be a very powerful mechanism for limiting the effects of endogenously released tachykinins.

Inhaled phosphoramidon enhanced the increase in R_L due to SP, a finding in agreement with several studies (Inoue *et al.*, 1997; Sheppard *et al.*, 1988), including those in humans (Cheung *et al.*, 1992). In light of the rapid tachyphylaxis to intravenous SP, the finding that inhibition of NEP was able to enhance the airway response indicates that NEP degradation of SP occurs extremely rapidly, clearly having a significant effect before the development of tachyphylaxis. This observation that the duration of the response to SP in the presence of NEP inhibition was not altered may reflect partial inhibition of NEP by phosphoramidon, whether the inhaled dose of phosphoramidon was adequate to completely inhibit NEP activity remains to be determined. However, the present dosage was based on previous *in vivo* sheep studies conducted in our laboratory (Reynolds *et al.*, 1997) and by others (Abraham *et al.*, 1991). Or alternatively may reflect SP is being degraded by other peptidases such as angiotensin-converting enzyme, kininase II, aminopeptidases and acetylcholinesterase which are known to exhibit activity against tachykinins but to a lesser extent than NEP (Skidgel *et al.*, 1984).

It has been proposed that a reduction in NEP (due to epithelial damage) may occur in the airways of asthmatics and hence potentiate SP effects in this disease. Several studies in animal models have shown an increase in airway responsiveness following exposure to noxious agents coincides with a reduction in NEP (Nadel & Borson, 1991), and that anti-inflammatory corticosteroids increase NEP production (Sont *et al.*, 1997;

Borson & Gruenert, 1991). However, studies reported to date have not demonstrated evidence for decreased NEP activity in asthmatics, although severe asthmatics have not been assessed (Cheung *et al.*, 1993). The present findings in sheep indicate that NEP is a powerful and rapid inhibitor of SP, and suggest that the sheep model may be useful to further examine the importance of NEP in the modulation of airway responses.

The tachykinin receptor subtype responsible for the effect of SP was characterized by examining the relative potency of SP and NKA, and by using the NK_1 specific antagonist, CP-96,345 (Snider *et al.*, 1991). The much greater potency of SP, and the inhibition seen with CP-96,345, indicates predominantly an NK_1 receptor mediated response. The R_L response to SP was also abolished by atropine, suggesting that the effect seen was largely due to the stimulation of cholinergically mediated broncho-constriction. While no formal attempt in this series of experiments was made to determine the exact mechanism by which SP mediates cholinergic bronchoconstriction Corcoran and colleagues demonstrated that in sheep intravenous SP induced broncho-constriction was possibly mediated via myelinated vagal afferent fibres (Corcoran & Haigh, 1995). *In vitro* studies in rabbit and guinea pig airways have shown that SP administration increases both pulmonary parasympathetic neurotransmission at the ganglia and ACh release from post-ganglionic nerve terminals innervating ASM (Armour *et al.*, 1991; Hall *et al.*, 1989; Tanaka & Grunstein, 1986) via NK_1 receptor activation (Belvisi *et al.*, 1994; Watson *et al.*, 1993). Of note is that in isolated human bronchi NKA potentiates cholinergic neural responses via a prejunctional mechanism, in the presence of a K^+ channel blocker (Black *et al.*, 1990b). Therefore, SP binding to NK_1 receptors on post-ganglionic cholinergic nerves may mediate the effects of SP observed in this chapter. Evidence for cholinergic mediation of the airway response to SP has also been demonstrated in humans where ipratroprium bromide reduced the

airway response to inhaled SP in subjects with asthma (Crimi *et al.*, 1990). In view of these cholinergic mechanisms mediating SP induced bronchoconstriction *in vivo*, it is also tempting to speculate that tachyphylaxis to SP may result from the released ACh activating pre-junctional cholinergic M_2 autoreceptors which provide a "braking mechanism" for vagally mediated ACh release (Pendry, 1993). However, this remains to be determined.

The possibility that SP may have other indirect effects on R_L was also examined. Other potential mechanisms for the induction of bronchoconstriction by SP include the stimulation of histamine release from pulmonary mast cells, and the production of bronchoconstricting prostaglandins. Substance P also has important effects on vascular permeability (Holzer, 1988) and stimulation of inflammatory cells including neutrophils (Wozniak *et al.*, 1989), eosinophils (Wiedermann *et al.*, 1993) and lymphocytes (Scicchitano *et al.*, 1988). Although the sheep had a total of four SP infusions over a 3.5 hour period during the tachyphylaxis study, no change in baseline R_L was seen. Therefore a significant effect on lung resistance due to changes in vascular calibre or the induction of oedema is unlikely. In addition, although the reproducibility studies were not designed primarily to assess cellular influx, it is interesting to note that there was no change in baseline R_L nor an increase in bronchial reactivity to SP over the three days of this study, to suggest that a significant influx of inflammatory cells had taken place. However, this remains to be determined either by histological sections or bronchoalveolar lavage.

Substance P increases histamine release from both human (Cross *et al.*, 1997; Heaney *et al.*, 1995) and guinea pig (Lilly *et al.*, 1995) pulmonary mast cells and the release of histamine from guinea pig lung can be blocked by specific NK_1 and NK_2 receptor

antagonists (Lilly *et al.*, 1995). The question of histamine release was specifically addressed in the present study by using the H_1 receptor antagonist, pyrilamine. It was found that pyrilamine had no effect on the airway response to SP, thus implying that histamine release was not involved in SP-induced bronchoconstriction. This is in agreement with Crimi *et al* who showed that the acute bronchoconstrictor response to inhaled SP in humans was not blocked by the antihistamine astemazole (Crimi *et al.*, 1990). During these experiments it was also noted that the bronchoconstriction in response to exogenous histamine reached a sustained plateau for at least several minutes, which was quite different to the transient response to SP.

There is some evidence that SP-induced prostaglandin production may be important in airway responses. SP has been shown to induce the production of both bronchoconstricting (PGD_2, $PGF_{2\alpha}$) (Ingenito *et al.*, 1991) and bronchodilating (PGE_2) (Devillier *et al.*, 1992) prostaglandins under different circumstances. To investigate any role for SP-induced prostaglandin production, indomethacin was used to inhibit prostaglandin synthesis. Despite indomethacin pretreatment at doses known to be biologically active towards airway responses in allergic sheep (Lanes *et al.*, 1986), no significant effect on the response to SP was seen, suggesting prostaglandin production was not important in the immediate airway response.

Studies in humans have shown that nedocromil sodium reduces the response to inhaled NK-A, which is the predominant bronchoconstricting tachykinin in human airways (Crimi *et al.*, 1992; Joos *et al.*, 1989b). The mechanism of this effect is unclear but there is some evidence that nedocromil may inhibit sensory nerve activation (Chung, 1996; Armour *et al.*, 1991). There is also evidence that sodium cromoglycate may inhibit tachykinin action in the airways (Yamawaki *et al.*, 1997; Chung, 1996). In view

of these reports, the effect of sodium cromoglycate, at biologically active doses (Abraham *et al.*, 1981a), on SP-induced bronchoconstriction was investigated, however, no significant effect was seen.

In conclusion, the findings of the studies performed in this chapter indicate that intravenous SP causes transient bronchoconstriction in sheep, that this is an NK_1 receptor mediated response acting via cholinergic mechanisms, and that this response is rapidly abolished by SP degradation and the induction of tachyphylaxis. No evidence was found for histamine or prostaglandin involvement in the acute response, or any evidence to suggest a delayed increase in R_L, which may implicate vascular or inflammatory cell responses.

The *in vivo* effects of SP in sheep appear at variance with those observed in humans. In man it is generally perceived that *in vivo* tachykinin-induced bronchoconstriction is via a direct effect on ASM predominantly mediated by NK2 receptors, as NKA is far more potent than SP. However, on closer scrutiny of the human data it is apparent that very large doses of NKA were required to induce bronchoconstriction, being 20-fold greater than that for SP (Evans *et al.*, 1988a). Furthermore, *in vivo* NKA-induced broncho-constriction is partly inhibited by nedocromil sodium (Crimi *et al.*, 1992; Joos *et al.*, 1989b) and antimuscarinic agents (Crimi *et al.*, 1990; Joos *et al.*, 1988), both indicating an indirect effect. *In vitro,* tachykinins contract human bronchi predominantly via NK_2 receptors with NKA being more potent than SP (Advenier *et al.*, 1992; Naline *et al.*, 1989; Black *et al.*, 1988). Therefore, in order to determine whether these apparent discrepancies result from significant species differences between human and ovine ASM formal *in vitro* contractility studies were conducted. The results of which form a major component of this thesis.

Chapter 4 Ovine airway smooth muscle contractility: Preliminary *in vitro* studies

4.1 Introduction

Few contractile studies have been conducted on ovine ASM. Therefore it was necessary to undertake a series of preliminary experiments to determine the optimal length or resting load to which the ovine ASM preparations should be subjected for studies of isometric contraction as performed in this thesis. In addition the *in vitro* viability and reproducibility of the contractile responses needed to be determined, as there is virtually no information available on these aspects of ovine smooth muscle contractility.

4.2 Methods

4.2.1 General

Lungs were obtained from merino ewes as described in Chapter 2 - Methods, Section 2.3.1. Tissues from three to five animals were used in each experimental group.

4.2.2 Preparation of airway smooth muscle

Strips of tracheal smooth muscle (3-8 mm x 20-25 mm), bronchial rings (3-4mm ID, 2-3mm long) and parenchymal strips (15-20mm x 3-5mm x 3-5 mm) were prepared for *in vitro* studies as described in Chapter 2 - Methods, Section 2.3.2.

4.2.3 Length tension studies

Preliminary studies were conducted on tracheal strips, bronchial rings and parenchymal

lung strips to determine the optimal resting tension for each tissue type. Optimal length (the length at which maximal contractile force is elicited for a given agonist) was determined from length-tension studies. This was achieved by mounting the ASM preparations on steel hooks, the lower hook was attached to a fixed support rod and the upper hook, via a fine gold chain, to a Grass FT03 force-displacement transducer (Grass Instruments, Qunicy, MA). The latter was mounted on a rack and pinion clamp so that resting muscle length and therefore resting tension could be optimized (Mitchell *et al.*, 1989).

The tracheal strips, bronchial rings and parenchymal lung strips were suspended under initial tensions of 1 gm, 0.5 gm and 1 gm, respectively, in organ baths as described in Chapter 2 - Methods, Section 2.3.3. The tissue segments were allowed to equilibrate in the water baths for 15 minutes prior to performing length-tension studies for each tissue. This was achieved by obtaining intermittent contractile responses to 100 mM KCl (final bath concentration). Once the contraction to KCl had reached a plateau, the tissues were washed until baseline tone was re-established. The load of each tissue was then increased by a one gram increment, after which another contractile response to KCl was obtained. This procedure was repeated until responses to KCl were maximal and reproducible. Changes in ASM tension were measured isometrically and continuously recorded on polygraph. For these length-tension studies six tissue samples from each area of the tracheobronchial tree were obtained from three animals.

4.2.4 Measurement of the reproducibility of ovine airway smooth muscle in response to contractile agonists

This series of experiments was designed to determine the between and within sheep variability of contractile responses to various agonists in normal ovine tracheal smooth

muscle strip, bronchial ring and parenchymal lung strip preparations. Three ASM preparations were obtained from each of the three regions of the lung (trachea, bronchi and parenchyma) from each animal studied. All tissue preparations were mounted and suspended under an appropriate tension (see length tension results below) in siliconized, heated organ baths as described in Chapter 2 - Methods, Section 2.3.3.

At the end of the equilibration period, when a stable baseline tension was achieved, a reference contractile response to 10^{-3} M ACh was obtained. Once this contraction had reached a plateau, the tissues were washed every 20 mins until baseline tone was re-established (usually 60-90 mins) (Figure 4.1). Cumulative concentration-response curves were obtained for ACh, carbachol and NKA in tracheal smooth muscle strips and for ACh in bronchial ring and parenchymal strips (Figure 4.1), as described in Chapter 2 - Methods, Section 2.3.3.1. Following the CCRC each tissue was then blotted for one minute on filter paper (Whattman® No.1, Maidstone, UK) and its weight recorded using an electronic balance (Model HF300G, A&D Co., Ltd., Tokyo, Japan).

4.2.5 Measurement of ovine tracheal smooth muscle viability in response to contractile agonists

This series of experiments was designed to determine the viability of the contractile responses to ACh, carbachol and NKA in normal ovine tracheal smooth muscle strips following storage (up to 50 hours) in ice cold K-H buffered solution. By design, viability studies were not conducted on bronchial or parenchymal preparations as all subsequent contractility studies using these preparations were conducted within 8 hours of obtaining the tissue.

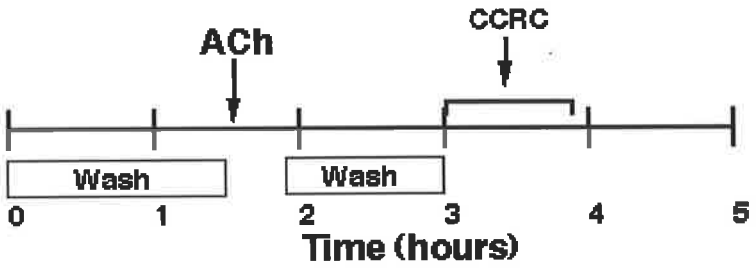


Figure 4.1 Time sequence adopted in performing CCRC.

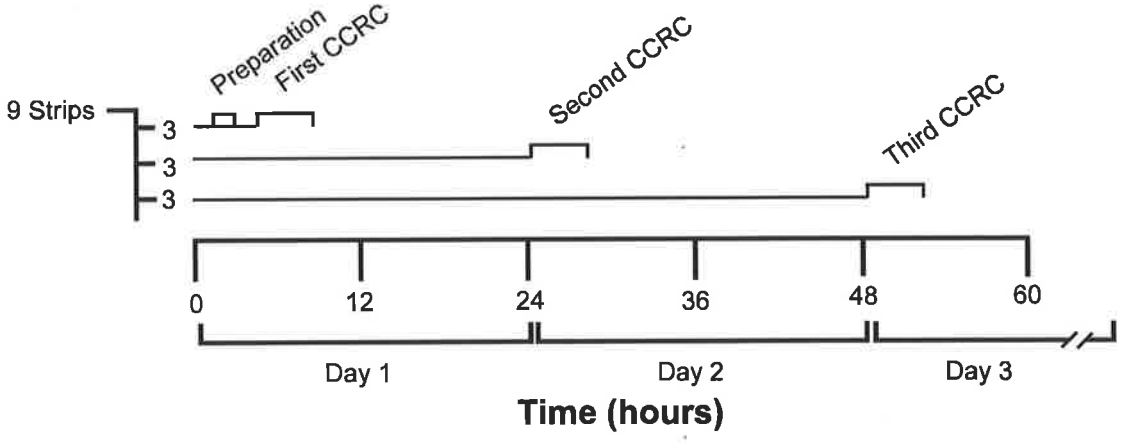


Figure 4.2 Schematic illustrating the design of the tissue viability study.

For each agonist studied nine tracheal smooth muscle strips from each animal were prepared for *in vitro* studies of isometric contraction. Cumulative concentration-response curves were obtained within 4-6 hours of excision. For each animal three muscle strips were studied on Day 1 while the remaining six strips were stored in a large volume (100 mls) of aerated (5% CO_2: 95% O_2) ice-cold K-H solution. From these six, three strips were studied on Day 2, while the last 3 strips remained in storage, and were studies on Day 3, (see Figure 4.2), with the exception of NKA. Due to the cost factor involved in using tachykinin agonists, NKA viability experiments were conducted over a two day period. The K-H solution used in the storage process was exchanged every 12 hours with 100 mls of fresh ice-cold aerated K-H solution.

On each study day a single complete CCRC was obtained for each tracheal strip as outlined in Figure 4.1. Following each CCRC, every tissue was blotted and weighed as above.

4.2.6 Analysis of results

4.2.6.1 Length tension studies

The response to 100 mM KCl during the length-tension studies were expressed as a percentage of the highest response at any load (Jongejan *et al.*, 1988).

4.2.6.2 Cumulative concentration-response curves

In each tracheal smooth muscle preparation, the E_{max} was calculated as the maximal tension generated in milligrams (mg) for each agonist, at the highest agonist concentration studied. For each tissue sample the E_{max} was also normalized to a reference contraction of 10^{-3} M ACh (E_{max} %ACh) and for wet tissue weight ((E_{max} (mg)/weight (mg)). A CCRC was then constructed relating the cumulative

concentration of the agonist to the response. From these CCRCs E_{max} and pD_2 values were calculated as described in Chapter 2 - Methods, Section 2.3.4.1.

4.2.7 Statistical analysis

The Cochran's *C* statistic was used as a preliminary test to check for homogeneity of group variances (Kirk, 1995; Godfrey, 1985). The between and within sheep variability of the contractile response to the agonists studied was determined by a two-way ANOVA with single observations (Tallarida *et al.*, 1988). Similarly for the viability studies, between sheep and between day variance was determined by the two-way ANOVA. Values were considered significant at $P < 0.05$ level.

4.2.8 Drugs

All drugs, compounds and solutions including the K-H used in the following experiments are listed, prepared and used according to the methods described in Chapter 2 - Methods, Section 2.6.2, Reagents.

4.3 Results

4.3.1 Length tension studies

The effect of increasing the resting tension of ovine ASM (tracheal, bronchial and parenchymal) on contractility to KCl (100 mM) is shown in Figure 4.3. The optimal length for isometric contraction for tracheal smooth muscle was achieved by equilibrating the tissue against a load of between 2.5 and 3.5 grams. The optimal resting tension for bronchial rings was determined to be between 4 and 5 grams, while that for parenchymal strip preparations was between 3.5 and 4.5 grams (Figure 4.3).

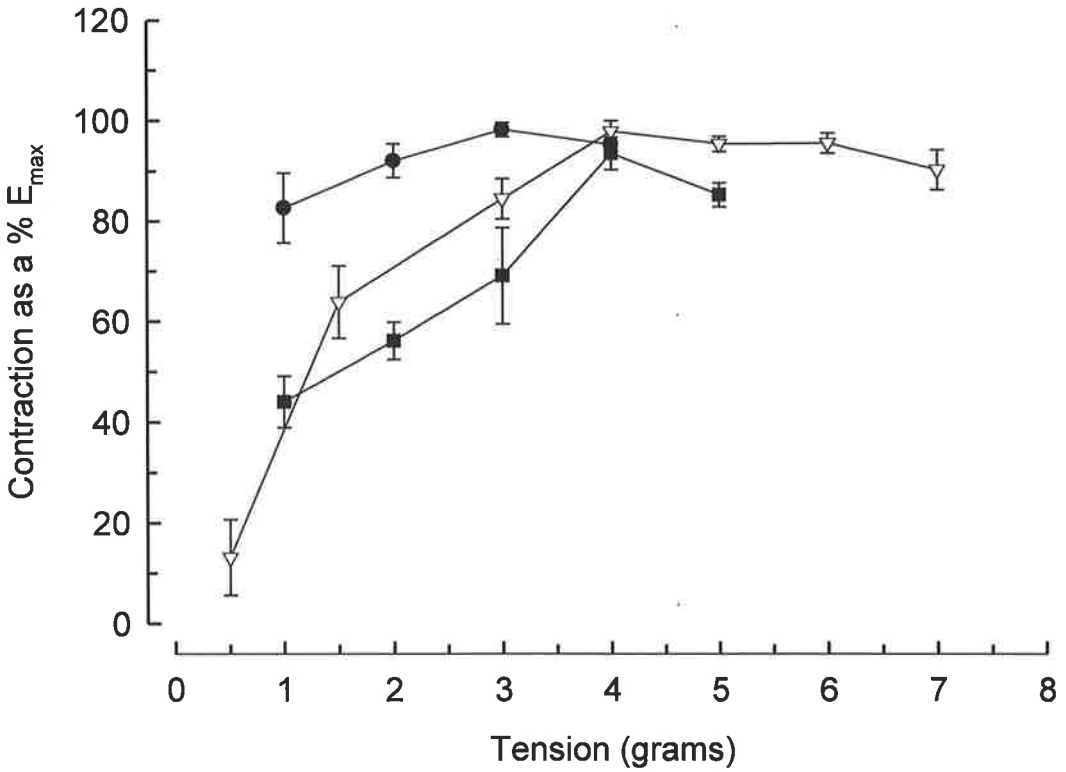


Figure 4.3 Maximal response to KCl (100 mM) in ovine tracheal strips (●), bronchial rings (▽) and parenchymal strips (■) with increasing load. Contractile responses are expressed as a percentage of the maximal response obtained at any given load. Values are mean ± SEM for n=6 tissue samples at each load.

4.3.2 Reproducibility of ovine airway smooth muscle contractility

The values of pD_2, E_{max}, E_{max}%ACh and E_{max} (mg)/weight (mg) for tracheal muscle strips are summarized in Table 4.1. Each number represents the value for each strip to ACh, carbachol, and NKA for each animal studied. Each tissue preparation was exposed to one agonist only.

The preliminary analysis using the Cochran's *C* statistic demonstrated that there was homogeneity among group variances, which allowed the two-way ANOVA with single observations to be applied to the data. The outcomes of which are displayed in Table 4.2 as *F*-ratios. A significant between sheep variation of E_{max} to ACh *F*-ratio was observed when the E_{max} was expressed as the absolute force generated. However, this significant variation was obviated when E_{max} to ACh was normalized for tissue weight and to a reference contraction of 10^{-3} M ACh. All other *F*-ratios demonstrated no significant between or within sheep variation in contractile responses to the agonists studied. To further illustrate the reproducibility of contractile responses in ovine tracheal smooth muscle, mean CCRC for each animal to each agonist studied are depicted in Figures 4.4 A-B, and 4.5 for ACh, carbachol and NKA, respectively. In these figures the error bars for each animal indicate intra-sheep variability, while the close proximity of the graphs of each animal is indicative of inter-sheep variability.

The reproducibility of contractile responses to ACh was also examined in bronchial and parenchymal preparations. Values of pD_2, E_{max}, E_{max}%ACh and E_{max} (mg)/weight (mg) for bronchial rings and parenchymal strips are summarized in Table 4.3 and Table 4.4, respectively. Each number represents the value for each tissue preparation to ACh, for each animal studied. Again, each tissue preparation was exposed to one agonist only.

Table 4.1 Reproducibility of contractile responses to ACh, carbachol and NKA in tracheal smooth muscle strips.

	pD_2			E_{max} (mg)			E_{max} (mg)/ weight (mg)			E_{max} %ACh		
	A1	A2	A3	A1	A2	A3	A1	A2	A3	A1	A2	A3
Sheep 1	4.89	5.15	5.30	38900	57200	51500	292	430	505	117	112	101
Sheep 2	5.40	5.10	5.40	48650	61150	62750	389	430	441	116	109	109
Sheep 3	5.40	5.57	5.54	77550	82310	85630	289	280	354	111	106	105
Sheep 4	4.96	5.15	5.55	61680	68930	51710	303	292	252	93	108	109
	C1	C2	C3	C1	C2	C3	C1	C2	C3	C1	C2	C3
Sheep 1	7.23	7.30	7.39	56410	52460	67650	242	446	422	128	134	124
Sheep 2	7.34	7.39	7.30	72470	66530	71320	426	387	438	134	136	142
Sheep 3	7.52	7.48	7.41	69080	56570	74320	512	393	564	123	109	134
Sheep 4	7.15	7.25	7.41	74160	80600	62490	508	486	579	110	119	142
	N1	N2	N3	N1	N2	N3	N1	N2	N3	N1	N2	N3
Sheep 1	7.28	6.96	6.70	18500	27488	14825	218	250	185	71	98	61
Sheep 2	7.00	6.64	5.72	24562	28275	22825	292	214	204	95	82	84
Sheep 3	6.40	6.60	6.10	26500	23250	35375	182	153	205	78	79	84
Sheep 4	6.46	6.70	6.74	18575	29000	25662	151	207	183	85	86	76
Sheep 5	5.89	7.00	6.46	24450	33000	27250	175	229	189	59	93	83

Each value represents the measurement obtained for each individual tracheal smooth muscle strip. Abbreviations: A1-3; ACh contractile response in three muscle strips. C1-3; carbachol response for each muscle strip. N1-3; response to NKA in three strips. pD_2; –log EC_{50}. E_{max}; maximal contractile response expressed as force generated in mg. E_{max} (mg)/weight (mg); E_{max} normalized for wet tissue weight. E_{max}%ACh; E_{max} expressed as % of reference contraction to 10^{-3} M ACh.

Table 4.2 ANOVA results for the reproducibility studies of contractile responses to ACh, carbachol and NKA in tracheal smooth muscle strips.

	F-ratios											
	pD_2			E_{max} (mg)			E_{max} (mg)/ weight (mg)			E_{max} %ACh		
Source of Variation	A	C	N	A	C	N	A	C	N	A	C	N
Between sheep	2.86	2.83	1.07	14.10*	1.39	1.37	4.50	2.82	1.38	0.69	1.43	0.32
Within sheep	3.03	0.59	1.43	2.82	0.32	1.68	1.49	1.49	0.29	0.23	1.74	1.12

Each figure represents the *F*-ratio from the two-way ANOVA with single observations. Abbreviations: A; ACh contractile response. C; carbachol response. N; response to NKA. pD_2; –log EC_{50}. E_{max}; maximal contractile response expressed as force generated in mg. E_{max} (mg)/weight (mg); E_{max} normalized for wet tissue weight. E_{max}%ACh; E_{max} expressed as % of reference contraction to 10^{-3} M ACh. * Denotes a significant *F*-ratio at the $P < 0.05$ level.

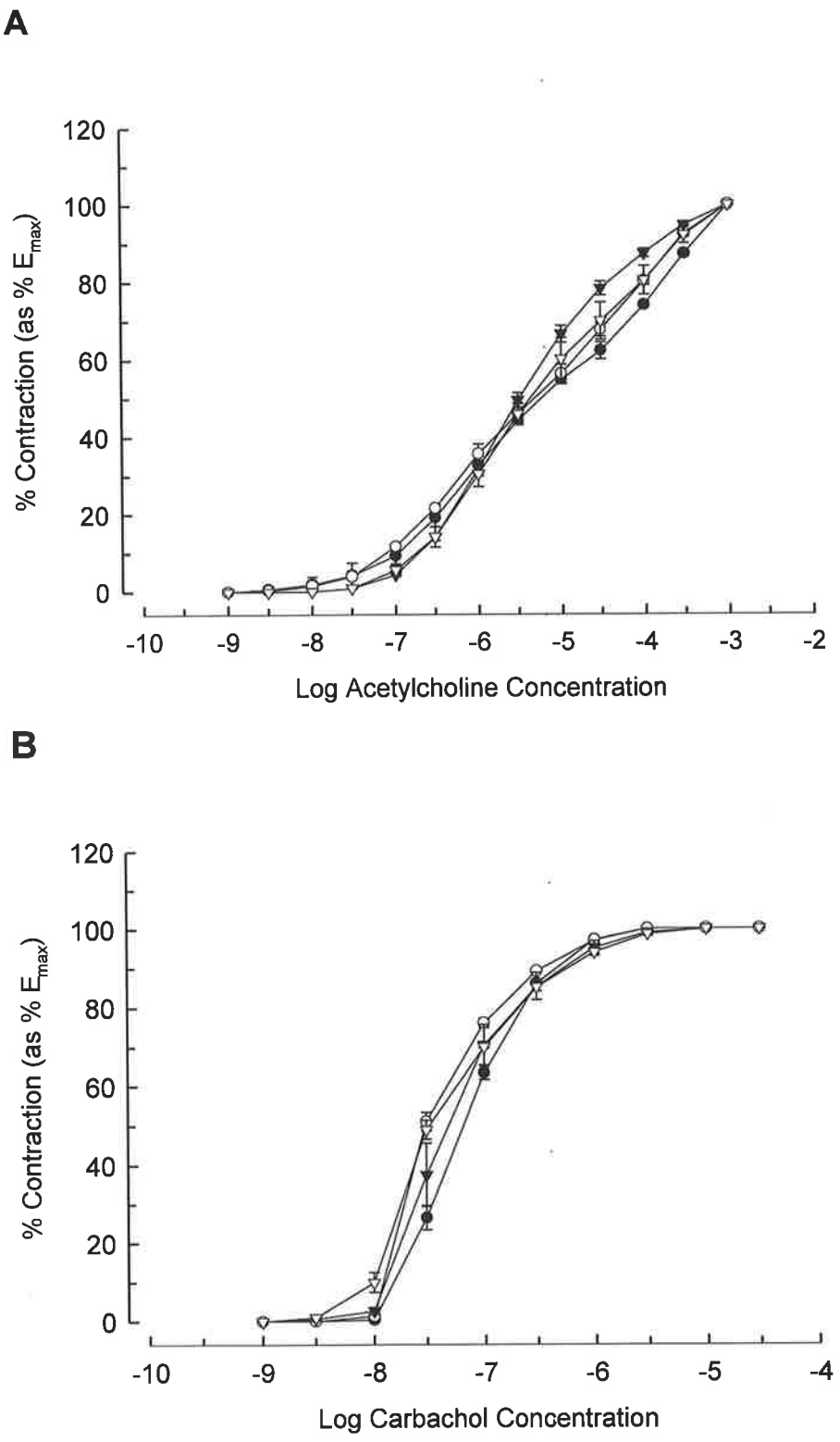


Figure 4.4 Mean cumulative concentration-response curves for ACh (A) and carbachol (B) in four sheep (●○▼▽). Points represent the mean values from three tracheal muscle strips for each animal and are expressed as a percent of the maximal response E_{max}. Vertical bars indicate SEM.

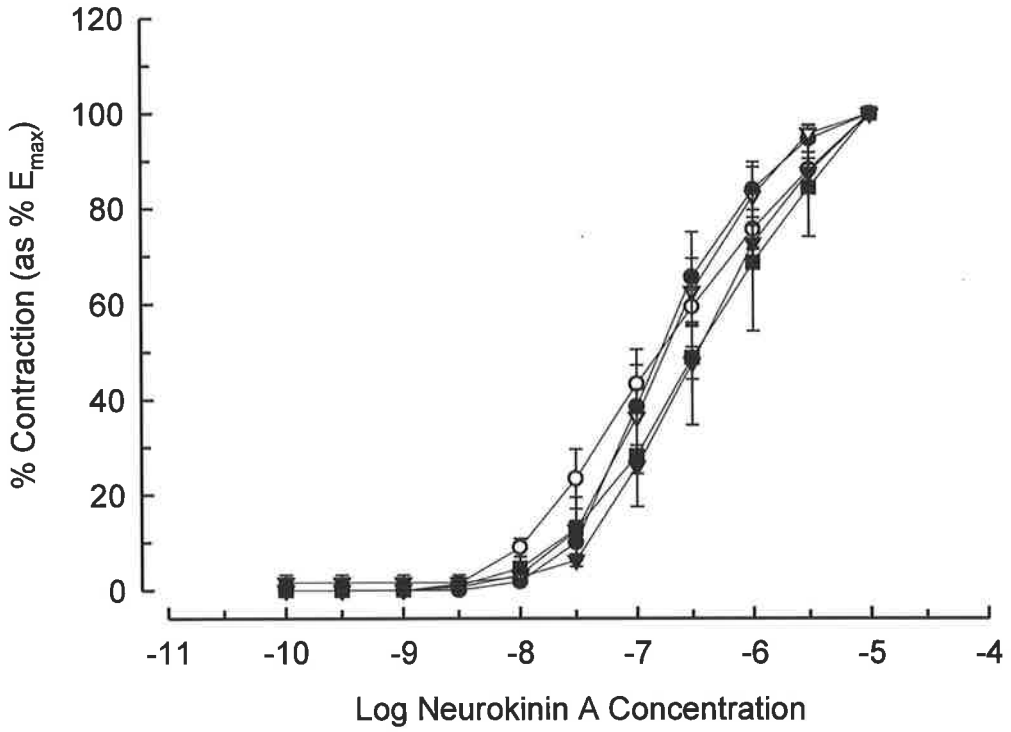


Figure 4.5 Mean cumulative concentration-response curves for NKA in five sheep (●○▼▽■). Points represent the mean values from three tracheal muscle strips for each animal and are expressed as a percent of the maximal response E_{max}. Vertical bars indicate SEM.

Table 4.3 Reproducibility of contractile responses to ACh in bronchial rings.

	pD_2			E_{max} (mg)			E_{max} (mg)/ weight (mg)			E_{max} %ACh		
	A1	A2	A3	A1	A2	A3	A1	A2	A3	A1	A2	A3
Sheep 1	4.10	4.74	5.00	2905	5735	6045	31.2	66.7	70.3	77	93	98
Sheep 2	4.96	5.03	5.10	2295	2555	2830	38.9	40.6	44.9	75	83	92
Sheep 3	5.10	5.33	5.05	3640	3575	3620	42.2	43.6	42.1	101	99	98
Sheep 4	4.63	5.10	5.20	4885	5055	3145	38.7	51.2	73.6	104	92	98

Table 4.4 Reproducibility of contractile responses to ACh in parenchymal strips

	pD_2			E_{max} (mg)			E_{max} (mg)/ weight (mg)			E_{max} %ACh		
	A1	A2	A3	A1	A2	A3	A1	A2	A3	A1	A2	A3
Sheep 1	4.92	5.08	4.64	920	1230	835	5.7	7.6	4.4	61	83	58
Sheep 2	4.96	4.60	4.93	1300	1820	1265	7.6	10.7	12.3	65	90	60
Sheep 3	4.70	4.94	5.00	1080	1375	985	6.6	8.4	5.3	69	88	93
Sheep 4	4.51	5.34	5.29	1460	1680	1790	7.8	7.7	9.6	82	97	103

Each value represents the measurement obtained for each individual tissue preparation. Abbreviations: A1-3; ACh contractile response in three parenchymal strips. pD_2; –log EC_{50}. E_{max}; maximal contractile response expressed as force generated in mg. E_{max} (mg)/weight (mg); E_{max} normalized for wet tissue weight. E_{max}%ACh; E_{max} expressed as % of reference contraction to 10^{-3} M ACh.

The Cochran's *C* statistic demonstrated homogeneity among group variances for both bronchial and parenchymal tissues, allowing analysis by the two-way ANOVA with single observations. The outcomes of which are displayed as *F*-ratios in Table 4.5. A significant between and within sheep variation of E_{max} to ACh in parenchymal strips was observed when the E_{max} was expressed as an absolute force in mg. Again, this significant variation was obviated when E_{max} to ACh was normalized for tissue weight and to a reference contraction of 10^{-3} M ACh. All other *F*-ratios demonstrated no significant between or within sheep variation ACh contractility. The mean CCRC for each animal to ACh for bronchial and parenchymal tissue are displayed graphically in Figures 4.6 A and B, respectively.

The viability data of ovine tracheal smooth muscle to contractile agonists, ACh, carbachol and NKA are shown in Table 4.6. Viability for ACh and carbachol was assessed over three days while that for NKA over two. Once again group variances demonstrated homogeneity. Significant between sheep and between day variability was demonstrated (*F*-ratio) for contractile responses to ACh only when expressed as an absolute force (Table 4.7). This variability was removed when E_{max} was normalized to a reference contraction of ACh and for wet tissue weight. Contractile responses to carbachol and NKA demonstrated no between sheep or between day variability. The effect of tracheal smooth muscle storage in ice-cold oxygenated K-H solution on the mean CCRC to ACh, carbachol and NKA are shown in Figures 4.7 A-B, and 4.8, respectively. No significant changes in the CCRC were observed between days 1 to 3 for ACh (Figure 4.7A) and carbachol (Figure 4.7B). With respect to NKA a slight but non-significant leftward shift in the mean CCRC between day 1 and 2 was seen (pD_2 values, Table 4.7). When a two-tailed paired Student's *t*-test was used to compare the

Table 4.5 ANOVA results for the reproducibility of contractile responses to ACh in bronchial rings and parenchymal lung strips

	F-Ratios							
	pD_2		E_{max} (mg)		E_{max} (mg)/ weight (mg)		E_{max} %ACh	
Source of Variation	A BR	A PS	A BR	A PS	A BR	A PS	A BR	A PS
Between sheep	3.31	0.29	2.61	11.07 *	1.40	3.57	2.90	4.18
Within sheep	3.72	0.62	0.55	5.91 *	3.23	0.94	0.92	3.97

Each figure represents the *F*-ratio from the two-way ANOVA with single observations. Abbreviations: A BR and A PS; ACh contractile response in bronchial rings and parenchymal strips, respectively. pD_2; –log EC_{50}. E_{max}; maximal contractile response expressed as force generated in mg. E_{max} (mg)/weight (mg); E_{max} normalized for wet tissue weight. E_{max}%ACh; E_{max} expressed as % of reference contraction to 10^{-3} M ACh. * Denotes a significant *F*-ratio at the $P < 0.05$ level.

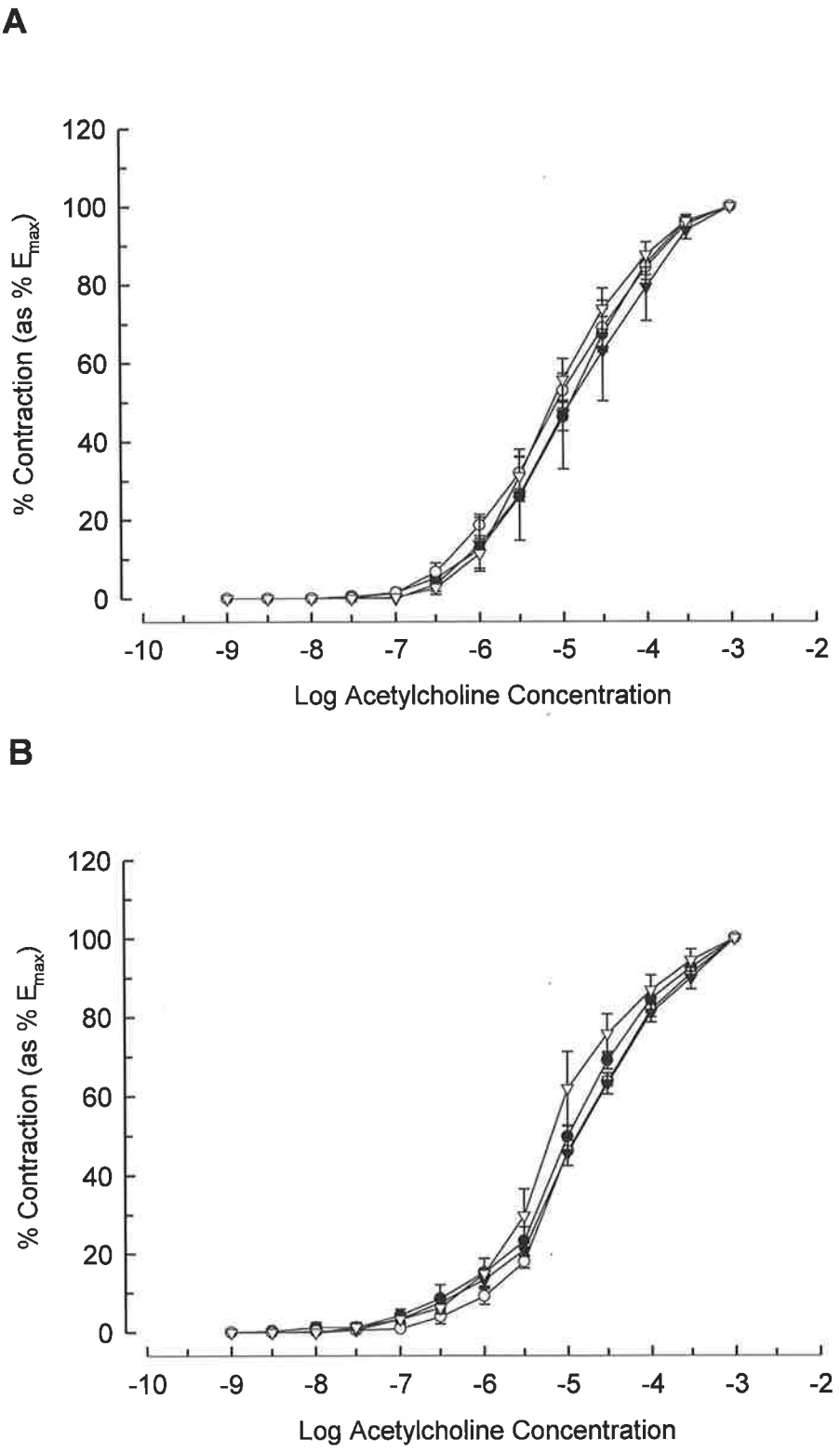


Figure 4.6 Mean cumulative concentration-response curves for acetylcholine in bronchial rings (A) and parenchymal strips (B) in four sheep (●○▼▽). Points represent the mean values from three tissue preparations for each animal and are expressed as a percent of the maximal response E_{max}. Vertical bars indicate SEM.

Table 4.6 Viability of tracheal smooth muscle in response to ACh, carbachol and NKA following storage.

	pD_2			E_{max} (mg)			E_{max} (mg)/ weight (mg)			E_{max} %ACh		
	A D1	A D2	A D3	A D1	A D2	A D3	A D1	A D2	A D3	A D1	A D2	A D3
Sheep 1	5.11	5.35	5.33	49200	38867	36050	409	233	217	110	104	92
Sheep 2	5.30	5.23	5.21	57517	24333	30017	420	194	240	111	105	109
Sheep 3	5.50	5.52	5.64	81830	61560	65093	308	448	342	107	113	110
Sheep 4	5.22	5.11	5.60	60773	45407	58067	282	202	263	103	96	93
	C D1	C D2	C D3	C D1	C D2	C D3	C D1	C D2	C D3	C D1	C D2	C D3
Sheep 1	7.31	6.95	6.97	59440	62120	64113	370	366	402	129	128	137
Sheep 2	7.34	7.40	7.22	70107	69777	77530	417	398	361	137	138	141
Sheep 3	7.47	7.52	7.21	66673	71903	47983	489	466	337	122	116	143
Sheep 4	7.27	7.60	7.20	72417	54097	53097	524	431	349	124	114	119
	N D1	N D2		N D1	N D2		N D1	N D2		N D1	N D2	
Sheep 1	6.98	7.40		20271	33562		218	156		77	85	
Sheep 2	6.45	6.70		25220	27387		237	179		87	91	
Sheep 3	6.37	7.03		28375	14183		180	280		80	55	
Sheep 4	6.63	6.79		24412	32130		180	221		82	77	
Sheep 5	6.45	6.32		28233	37403		198	179		78	73	

Each value represents the mean value obtained from three tracheal smooth muscle strips per animal. Abbreviations: A D1-D3; ACh contractile responses for days 1, 2 and 3. C D1-D3; carbachol response for days 1, 2 and 3. N D1-D2; response to NKA for days 1 and 2. pD_2; –log EC_{50}. E_{max}; maximal contractile response expressed as force generated in mg. E_{max} (mg)/weight (mg); E_{max} normalized for wet tissue weight. E_{max}%ACh; E_{max} expressed as % of reference contraction to 10^{-3} M ACh.

Table 4.7 ANOVA results for the viability of tracheal smooth muscle responses to ACh, carbachol and NKA following storage.

	F-Ratios											
	pD_2			E_{max} (mg)			E_{max} (mg)/ weight (mg)			E_{max} %ACh		
Source of Variation	A	C	N	A	C	N	A	C	N	A	C	N
Between sheep	3.03	2.91	4.08	15.81*	1.12	0.59	0.91	0.89	0.25	3.07	4.66	1.52
Between days	1.56	2.67	4.26	10.60*	0.49	0.57	1.36	3.02	0.00	1.64	2.85	0.65

Each figure represents the *F*-ratio from the two-way ANOVA with single observations. Abbreviations: A, C and N; ACh contractile responses in tracheal strips to ACh, carbachol and NKA, respectively. pD_2; –log EC_{50}. E_{max}; maximal contractile response expressed as force generated in mg. E_{max} (mg)/weight (mg); E_{max} normalized for wet tissue weight. E_{max}%ACh; E_{max} expressed as % of reference contraction of 10^{-3} M ACh. * Denotes a significant difference at the $P < 0.05$ level.

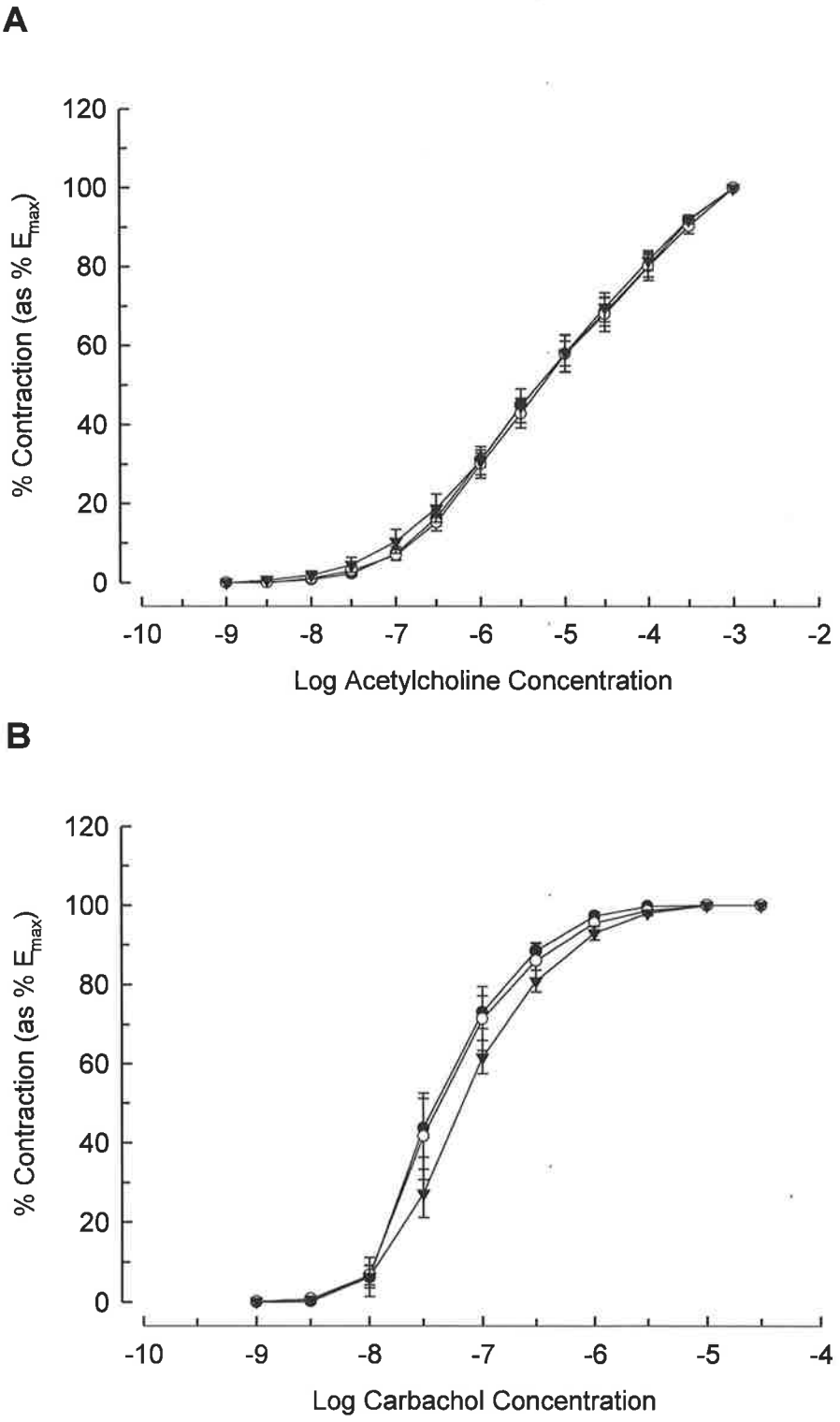


Figure 4.7 Mean cumulative concentration-response curves for acetylcholine (A) and carbachol (B) for Day 1 (●), Day 2 (○) and Day 3 (▼). Points represent the mean values from three tracheal muscle strips from each of four sheep and are expressed as a percent of the maximal response E_{max}. Vertical bars indicate SEM.

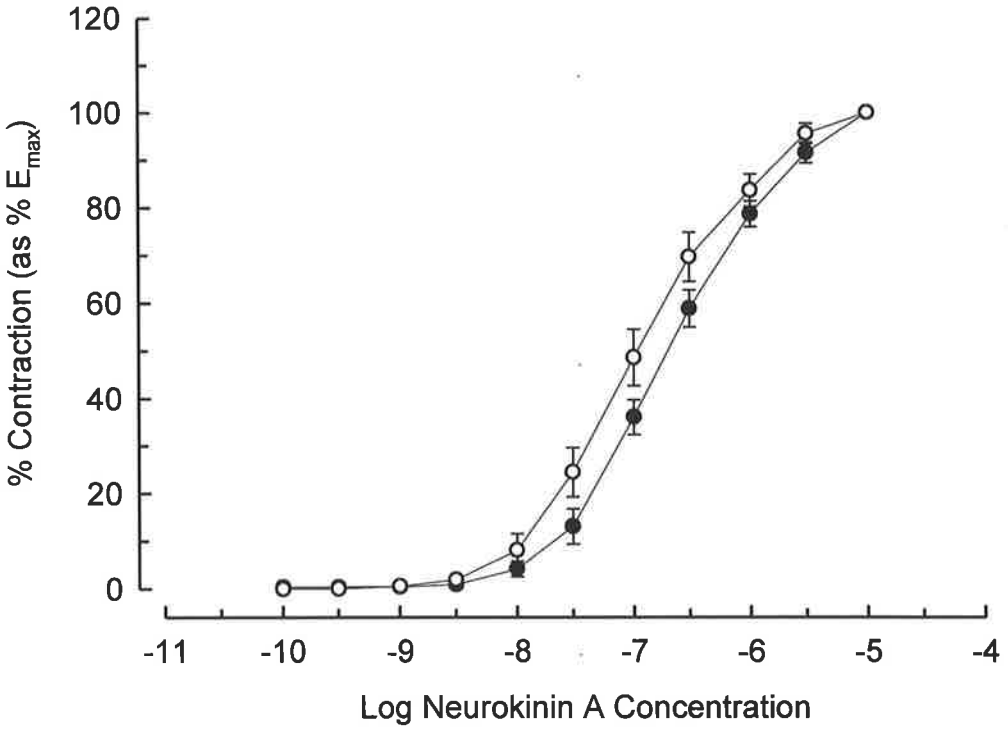


Figure 4.8 Mean cumulative concentration-response curves for neurokinin A for Day 1 (●) and Day 2 (○). Points represent the mean values from three tracheal muscle strips from each of five sheep and are expressed as a percent of the maximal response E_{max}. Vertical bars indicate SEM. Differences are not significant at any point (Paired Student's *t*-test $P < 0.05$).

mean results for the CCRC to NKA between days 1 and 2, again, no significant differences were found (Figure 4.8).

4.4 Discussion

The maximal isometric force generated in smooth muscle preparations is dependent on the resting tension or load applied to the muscle (Hulsmann & de Jongste, 1993; Stephens, 1978). From the length-tension studies described in this chapter, the optimal load required for ovine tracheal smooth muscle strips was between 2.5 and 3.5 grams, while that for bronchial ring preparations was between 4 and 5 grams. These values for tracheal and bronchial smooth muscle preparations are in agreement with those found by others (Abraham *et al.*, 1996; Jackowski *et al.*, 1993; Sheller & Brigham, 1982). In ovine parenchymal strip preparations the load required to produce maximal isometric force was between 3.5 and 4.5 grams, again similar to published values for this species (Sheller & Brigham, 1982).

I showed contractile responses to ACh, carbachol and NKA in tracheal smooth muscle strips demonstrated little within and between sheep variability. Values for pD_2, E_{max} (mg)/weight (mg) and E_{max}%ACh demonstrated no significant variability in contractile response to the agonists studied. Of note is that the ANOVA revealed a significant between sheep variability in E_{max} to ACh, however, this variability was not seen when the E_{max} was normalized to either wet tissue weight or a reference contraction, thus indicating that the between sheep variation is related to the size of the tracheal strip. The between and within sheep variability in contraction to ACh was also assessed in bronchial rings and parenchymal strips. In these tissue preparations the ANOVA results only demonstrated significant within and between sheep variability in the E_{max} to ACh in parenchymal strips. Again following normalization of the E_{max} (maximal force

generated) to either tissue weight or to a reference contraction the variability was obviated.

Reproducible measurements of pD_2, E_{max} (mg)/weight (mg) and E_{max}%ACh for ACh and carbachol can be obtained up to 54 hours following lung excision demonstrating prolonged viability. With respect to NKA-induced contractions, tracheal ASM remains viable up to 30 hours post excision. However, significant between sheep and between day variability in the absolute isometric force generated to ACh was observed. Again this variability was eliminated when contractions were normalized.

The consistent finding of variability in the maximal force generated for ACh in ovine strip preparations (tracheal and parenchymal) would seem to indicate that E_{max} for ACh is dependent on strip size. A significant relationship has been demonstrated between smooth muscle volume and maximal isometric force generation in bronchial strip preparations (Armour *et al.*, 1988).

4.4.1 Summary

Reproducible contractile responses to various agonists can be obtained in ovine ASM preparations provided E_{max} is normalized to wet tissue weight or to a reference contraction, thus avoiding any confounding effects introduced by non-uniform tissue size. Furthermore, ovine tracheal smooth muscle strips remain viable for several days following storage in oxygenated buffered K-H solution. Given the minimal between and within sheep variability in ASM responsiveness, the ovine smooth muscle preparation provides a sensitive model in which detectable changes in muscle contractile properties may be possible following pharmacological intervention.

Chapter 5 Characterization of tachykinin contractile responses in ovine airway smooth muscle

5.1 Introduction

As previously mentioned, it is generally perceived that in man tachykinins induce bronchoconstriction (both *in vivo* and *in vitro*) by a direct effect on ASM, being mediated by NK_2 receptors, as NKA is far more potent than SP (Advenier *et al.*, 1992; Naline *et al.*, 1989; Black *et al.*, 1988; Evans *et al.*, 1988a). However, on closer scrutiny of the published human *in vivo* data it is apparent that very large doses of NKA were required to induce bronchoconstriction, being 20-fold greater than that for SP. Moreover, later human *in vivo* studies indicated that NKA-induced bronchoconstriction involved indirect mechanisms, being partly inhibited by nedocromil sodium (Crimi *et al.*, 1992; Joos *et al.*, 1989b) and antimuscarinic agents (Crimi *et al.*, 1990; Joos *et al.*, 1988).

In view of the above, the *in vivo* effects of SP in sheep (as described in Chapter 3) may not be at variance with those observed in man, as first thought. Therefore, in order to determine whether these apparent discrepancies result from significant species differences between human and ovine ASM, it was necessary to undertake more extensive well-controlled, *in vitro* studies in naïve animals, to elucidate the mechanisms of action of tachykinins on ovine ASM. Although the contractile actions of tachykinins, *in vitro*, have been studied extensively in many species (Black *et al.*, 1990a; Naline *et al.*, 1989; Devillier *et al.*, 1988), there has been no work reported on isolated sheep

airways.

Firstly, the *in vitro* contractile response of normal ovine tracheal, bronchial and parenchymal smooth muscle were examined to the tachykinin peptides: SP; NPγ; NKA; NKB; tachykinin NK_1-specific agonist, [Sar^9,$Met(O_2)^{11}$]-SP; NK_2 receptor agonist, [Nle^{10}]-NKA(4-10); NK_3 agonist, [Succinyl-Asp^6,Me-Phe^8]-SP(6-11) (senktide), as well as ACh, carbachol, and histamine. Secondly, further experiments were conducted to determined whether SP, NKA and [Sar^9,$Met(O_2)^{11}$]-SP act directly on ovine ASM or through endogenous ACh release (Yau & Youther, 1982), or indirectly via other mediators such as histamine (Lilly *et al.*, 1995; Joos *et al.*, 1988a).

5.2 Methods

5.2.1 General

Lungs were obtained from merino ewes as described in Chapter 2 - Methods, Section 2.3.1. Tissues from three to six animals were used in each experimental group.

5.2.2 Preparation of airway smooth muscle

Strips of tracheal smooth muscle (3-8 mm wide x 20-25 mm long), bronchial rings (3-4mm ID, 2-3mm long) and parenchymal strips (15-20mm long x 3-5mm wide x 3-5 mm thick) were prepared for *in vitro* studies as described in Chapter 2 - Methods, Section 2.3.2.

The ASM preparations were prepared for cumulative concentration-response studies as described in Chapter 2 - Methods, Section 2.3.3. These studies were commenced within 24 hours of excision. Preliminary studies revealed no change in agonist contractility after this period of time. All airway preparations were suspended under

tension at loads found to provide an optimal length for eliciting maximal contractile responses in ovine airways (See Chapter 4 - Preliminary experiments).

5.2.3 Effects of agonists

Cumulative concentration-response curves were obtained for ACh, carbachol, histamine, the tachykinin peptides: SP, NPγ, NKA, NKB, and the tachykinin NK_1, NK_2 and NK_3 receptor-specific agonists; $[Sar^9,Met(O_2)^{11}]$-SP, $[Nle^{10}]$-NKA(4-10) and senktide, respectively.

5.2.4 Effect of neutral endopeptidase inhibition

Cumulative concentration-response curves were obtained for SP, NPγ, NKA, $[Sar^9,Met(O_2)^{11}]$-SP, $[Nle^{10}]$-NKA(4-10) in the absence and presence of phosphoramidon (10^{-5} M, final bath concentration), as described in Chapter 2 - Methods, Section 2.3.3.2.

5.2.5 Effect of atropine, and pyrilamine on substance P, $[Sar^9,Met(O_2)^{11}]$-SP and neurokinin A contractility

To establish whether tachykinin-induced smooth muscle contraction was by a direct effect, by the liberation of histamine or via a cholinergic mechanism, we compared SP, NKA and $[Sar^9,Met(O_2)^{11}]$-SP contractility in the presence and absence of atropine (10^{-6} M), and SP-induced contraction in the presence of the histamine H_1-receptor antagonist, pyrilamine (10^{-6} M). In addition, the effectiveness of the atropine and pyrilamine blockade was confirmed by obtaining CCRCs for ACh and histamine in the presence and absence of their respective antagonist, as described in Chapter 2 - Methods, Section 2.3.3.4.

For this series of experiments two successive CCRCs were obtained for each agonist (SP, NKA, [Sar^9,$Met(O_2)^{11}$]-SP, ACh and histamine), as described in Chapter – 2 Methods, Section 2.3.3.3.

5.2.6 Analysis of results

Cumulative concentration-response curves for each tissue specimen studied was obtained from the tensions generated in response to the addition of each concentration of agonist. From these CCRCs E_{max}, pD_2, EC_{50}, EC_{25} and EC_{10} values were calculated as described in Chapter 2 - Methods, Section 2.3.4.

Statistical analyses were performed using two-tailed paired and unpaired Student's *t*-test. Values were considered significant at $P < 0.05$ level.

5.2.7 Reagents

All drugs and solutions used in the experiments described in this chapter are listed in Chapter 2 - Methods, Section 2.6.2 – Reagents. On each study day, serial dilutions were prepared in K-H solution and kept on ice for the duration of the experiment. Plastic vials and pipette tips were used to store and handle the tachykinin peptides and other agents. Organ baths were coated with the silicone coating product, Coatasil ®.

5.3 Results

5.3.1 Effects of agonists

5.3.1.1 Trachea

In the first series of experiments the contractile responses to several non-peptide agonists and tachykinins were studied. The CCRCs are shown in Figures 5.1A and B. Carbachol was the most active non-peptide agonist studied having the highest E_{max} of

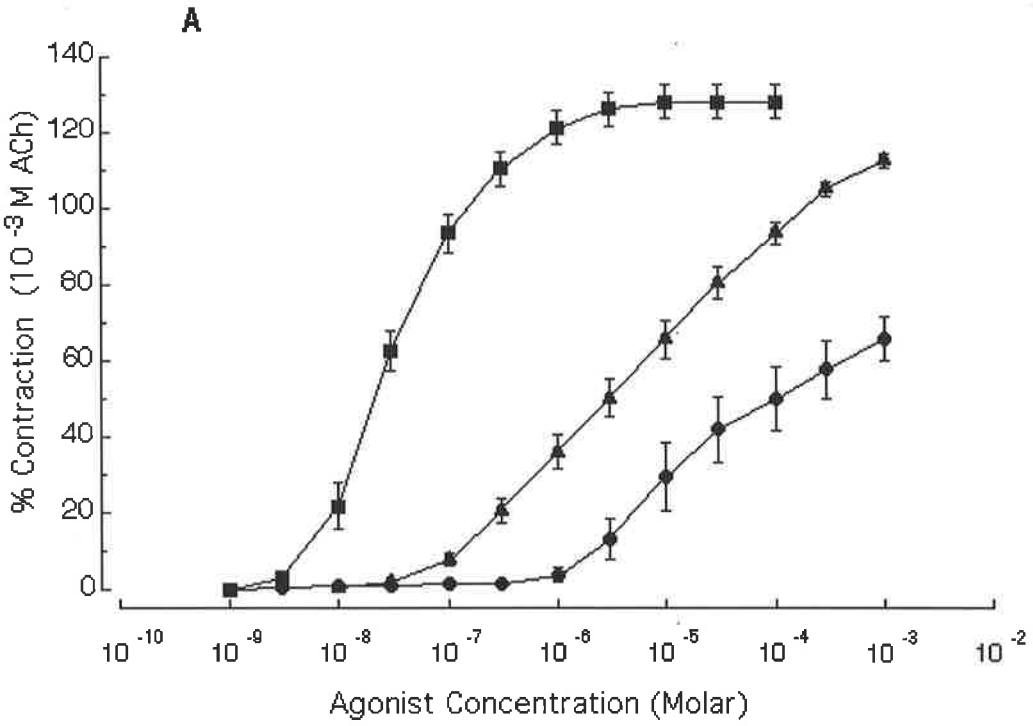


Figure 5.1A Mean cumulative concentration-response curves for carbachol (■), acetylcholine (▲), and histamine (●) on ovine isolated tracheal smooth muscle. Mean responses are from 6 animals and are expressed as a percentage of the reference contraction to 10^{-3} M ACh. Vertical bars indicate SEM.

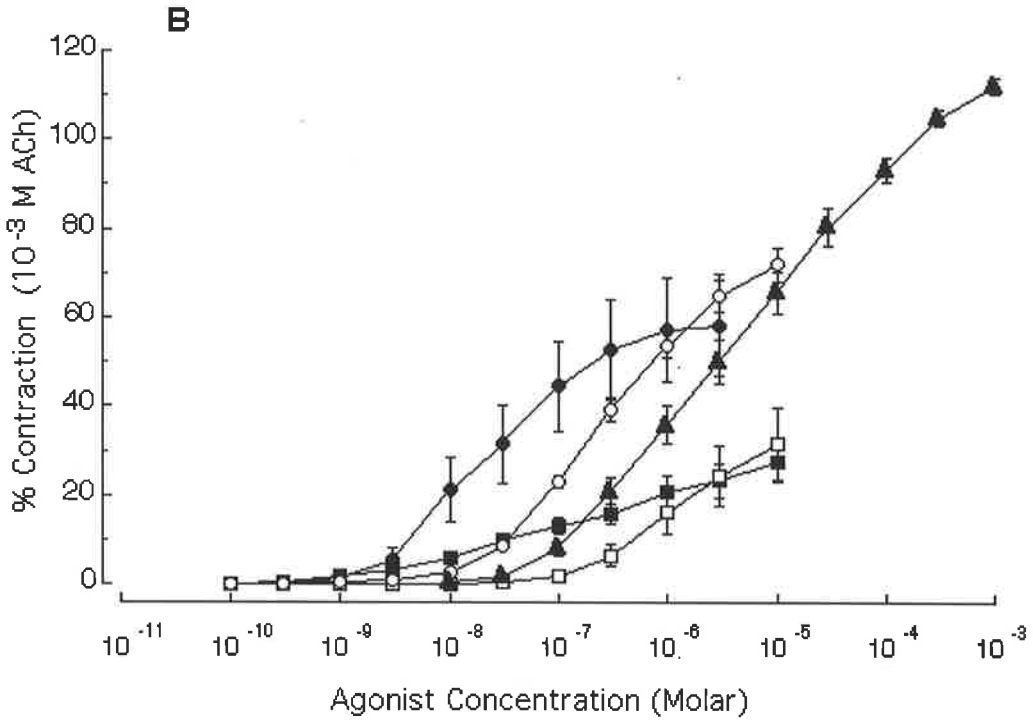


Figure 5.1B Mean cumulative concentration-response curves for neurokinin A (○), neuropeptide gamma (●), substance P (■), neurokinin B (❑), and acetylcholine (▲) on ovine isolated tracheal smooth muscle. Mean responses are from 6 animals and are expressed as a percentage of the reference contraction to 10^{-3} M ACh. Vertical bars indicate SEM.

128.1 ± 4.5% of the reference contraction to 10^{-3} M ACh, and a pD_2 value of 7.49 ± 0.04 (Table 5.1). Acetylcholine had a higher potency and E_{max} than histamine at the concentrations studied (Figure 5.1A, Table 5.1).

All the tachykinins studied produced concentration dependent contractions in ovine tracheal smooth muscle, however, maximal contractile responses were not always achieved for each tachykinin at the concentrations used. Therefore, EC_{50} and pD_2 values could not be determined for all tachykinins. An EC_{25} value, however, was calculated for each tachykinin and a rank order of potency established (Table 5.2), this being NPγ > NKA > SP ≥ NKB. NPγ was approximately 10 times more potent than NKA, pD_2 values 7.92 ± 0.10 and 6.67 ± 0.08, respectively ($P<0.05$, unpaired *t*-test, Table 5.1). At 3 x 10^{-6} M, similar efficacies for NPγ and NKA were observed, E_{max} 58.4 ± 11.7% ACh and 65.0 ±3 .7% ACh, respectively, (Figure 5.1B). Substance P and NKB had similar E_{max} values at 10^{-5} M (Table 5.1) and potencies as reflected in the calculated EC_{25} values (Table 5.2).

5.3.1.2 Bronchus

The CCRCs to carbachol, ACh and the tachykinins, SP, NKA and B in bronchial rings are shown in Figures 5.2. Carbachol and ACh were the most active non-peptide agonists studied in bronchial tissue having similar E_{max} values (Table 5.3), with carbachol being more potent than ACh with pD_2 values being 7.06 and 4.85, respectively. Histamine was non-responsive at the concentrations studied (Table 5.3). Of the tachykinin agonists studied in bronchial ring segments, significant contractile responses were obtained to SP and the NK_1 specific agonist, [Sar^9,$Met(O_2)^{11}$]-SP, while NKA and NKB produced a relatively weak response at the highest concentration used

Table 5.1 Mean maximal effect, geometric mean EC_{50} and apparent affinities for various agonists in ovine tracheal smooth muscle.

Agonist	n	E_{max} [a]	EC_{50} (M)		Mean pD_2 (±SEM)
Carbachol	6	128.1 ± 4.5	3.23×10^{-8}	(2.52×10^{-8} , 4.14×10^{-8})	7.49 ± 0.04
Acetylcholine	6	112.4 ± 2.0	4.93×10^{-6}	(2.06×10^{-6} , 1.18×10^{-5})	5.31 ± 0.15
Histamine	6	65.6 ± 5.9	1.18×10^{-5}	(4.55×10^{-6} , 3.05×10^{-5})	4.93 ± 0.16
Neuropeptide gamma	6	58.4 ± 11.7	1.19×10^{-8}	(6.45×10^{-9} , 2.21×10^{-8})	7.92 ± 0.10
Neurokinin A	6	72.0 ± 3.8	2.16×10^{-7}	(1.32×10^{-7} , 3.53×10^{-7})	6.67 ± 0.08
$[Nle^{10}]$-NKA(4-10)	6	45.3 ± 5.7	5.45×10^{-7}	(2.96×10^{-7} , 1.01×10^{-6})	6.26 ± 0.10
Substance P	6	27.4 ± 4.0	NC		NC
$[Sar^{9},Met(O_2)^{11}]$-SP	4	11.4 ± 2.8	9.82×10^{-9}	(5.30×10^{-9} , 1.82×10^{-8})	8.01 ± 0.08
Neurokinin B	6	31.4 ± 8.4	NC		NC

[a] Mean E_{max} ± SEM were expressed as a percentage of the response to 10^{-3} M acetylcholine. n= number of sheep. pD_2 values were calculated as -log EC_{50}. Geometric mean EC_{50} values and 95% confidence limits (are shown in parenthesis). NC, not calculated as agonist contractile response at 10^{-5} M still linear.

Table 5.2 Geometric mean EC_{25} [a] values for tachykinins in ovine tracheal smooth muscle.

Agonist	n	EC_{25} (M)	95% Confidence Limits
Neuropeptide gamma	6	2.67×10^{-8} [b]	5.32×10^{-9} , 1.34×10^{-7}
Neurokinin A	6	1.16×10^{-7} [b]	9.10×10^{-8} , 1.47×10^{-7}
Substance P	6	2.48×10^{-6} [b]	5.87×10^{-7} , 1.05×10^{-5}
Neurokinin B	6	3.32×10^{-6} [c]	1.05×10^{-6} , 1.05×10^{-5}

[a] The concentration of agonist at which a contractile response equal to 25% of that induced by 10^{-3} M acetylcholine is attained (see Methods this chapter). Values are geometric mean EC_{25} values and 95% confidence limits. [b] Significantly different from each other (Unpaired *t*-test), $P < 0.05$. [c] Not significantly different from SP EC_{25} (Unpaired *t*-test), $P < 0.83$. n = number of sheep.

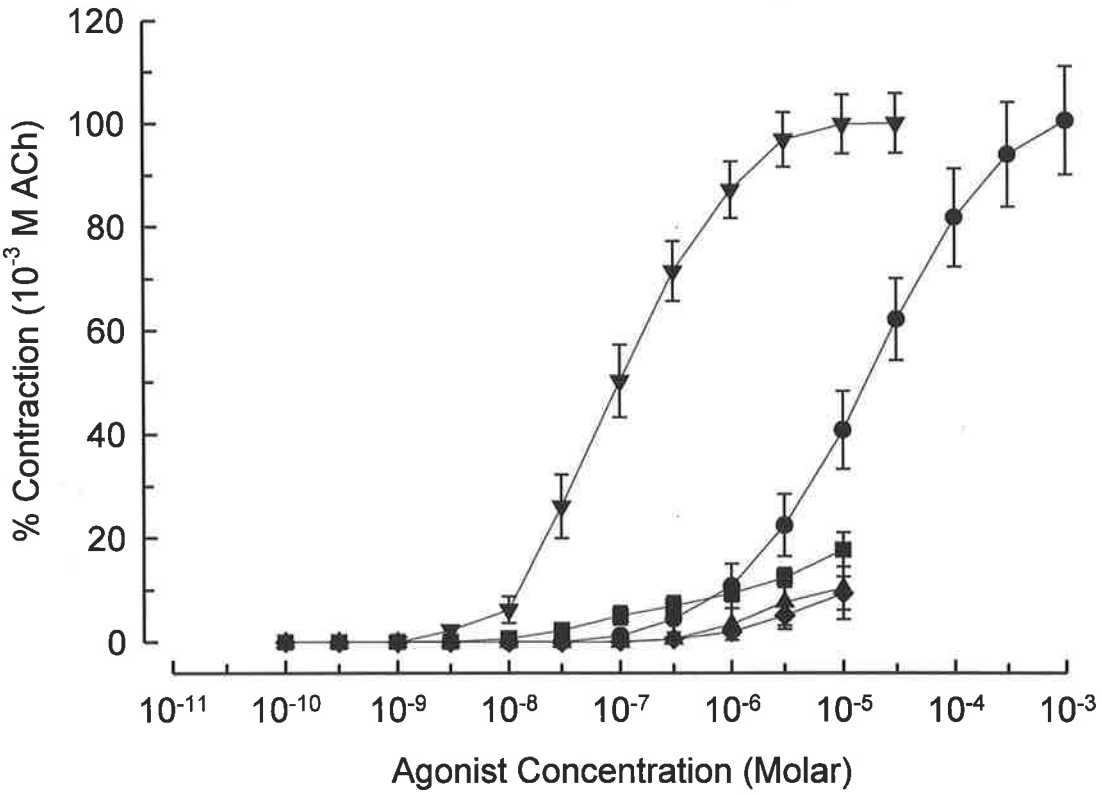


Figure 5.2 Mean cumulative concentration-response curves for carbachol (▼), acetylcholine (●), substance P (■), neurokinin A (◆), and neurokinin B (▲) on ovine isolated bronchial smooth muscle. Mean responses are from 3-6 animals and are expressed as a percentage of the reference contraction to 10^{-3} M ACh. Vertical bars indicate SEM.

Table 5.3 Mean maximal effect, geometric mean EC_{50} and apparent affinities for various agonists in ovine bronchial smooth muscle.

Agonist	n	E_{max} [a]	EC_{50} (M)		Mean pD_2 (±SEM)
Carbachol	6	100.0 ± 5.8	8.77×10^{-8}	(4.18×10^{-8} , 1.84×10^{-7})	7.06 ± 0.13
Acetylcholine	6	100.5 ± 10.5	1.40×10^{-5}	(5.45×10^{-6} , 3.62×10^{-5})	4.85 ± 0.16
[Sar^9,$Met(O_2)^{11}$]-SP	4	22.6 ± 4.2	1.13×10^{-7}	(3.09×10^{-8} , 4.10×10^{-7})	6.95 ± 0.22
Substance P	6	17.8 ± 3.3	NC		NC
Neurokinin A	6	9.4 ± 3.2	NC		NC
Neurokinin B	3	10.4 ± 6.1	NC		NC
[Nle^{10}]-NKA(4-10)	3	1.0 ± 0.5	ND		ND
Senktide	3	0.8± 0.7	ND		ND
Histamine	4	0.0 ± 0.0	ND		ND

[a] Mean E_{max} ± SEM were expressed as a percentage of the response to 10^{-3}M acetylcholine. n= number of sheep. pD_2 values were calculated as -log EC_{50}. Geometric mean EC_{50} values and 95% confidence limits (are shown in parenthesis). NC, not calculated as agonist contractile response at 10^{-5}M still linear. ND, not determined due to low agonist activity at 10^{-5} M in this tissue.

(10^{-6} M). The specific NK_2 and NK_3 receptor agonists, [Nle^{10}]-NKA(4-10) and senktide, were inactive in ovine bronchial rings.

5.3.1.3 Parenchyma

Carbachol, ACh and histamine produced concentration dependent contractions in ovine parenchymal strip preparation, Figure 5.3. In this preparation histamine was the most potent agonist studied, having the highest E_{max} of 112.7 ± 12.1% ACh (10^{-3} M), and pD_2 value of 6.04 ± 0.06 (Table 5.4). Acetylcholine and carbachol demonstrated similar potencies (pD_2 values, Table 5.4), however, ACh demonstrated a higher E_{max} than carbachol when expressed as a percentage of the reference contraction to 10^{-3} M ACh (Figure 5.3, Table 5.4). Parenchymal strips were unresponsive to the tachykinins studied Table 5.4. Therefore, EC_{50} and pD_2 values to these agonists could not be determined in this tissue.

5.3.2 Effect of neutral endopeptidase inhibition

5.3.2.1 Trachea

Phosphoramidon did not cause any significant change in baseline tension. Neutral endopeptidase inhibition increased the amplitude of contractions to SP and NKA. Phosphoramidon (10^{-5} M) produced a leftward shift in the CCRC for SP, NKA and NPγ compared to control tracheal smooth muscle strips (Figures 5.4A - C, respectively). This leftward shift, however, was only statistically significant for SP and NKA as reflected in the lower EC_{25} values (Table 5.5). Phosphoramidon did not change the amplitude of contraction or shift the CCRC for the tachykinin selective agonists, [Sar^9,$Met(O_2)^{11}$]-SP nor [Nle^{10}]-NKA(4-10), Figures 5.4D and 5.4E, respectively. Senktide was relatively inactive in tracheal smooth muscle with E_{max} values in the

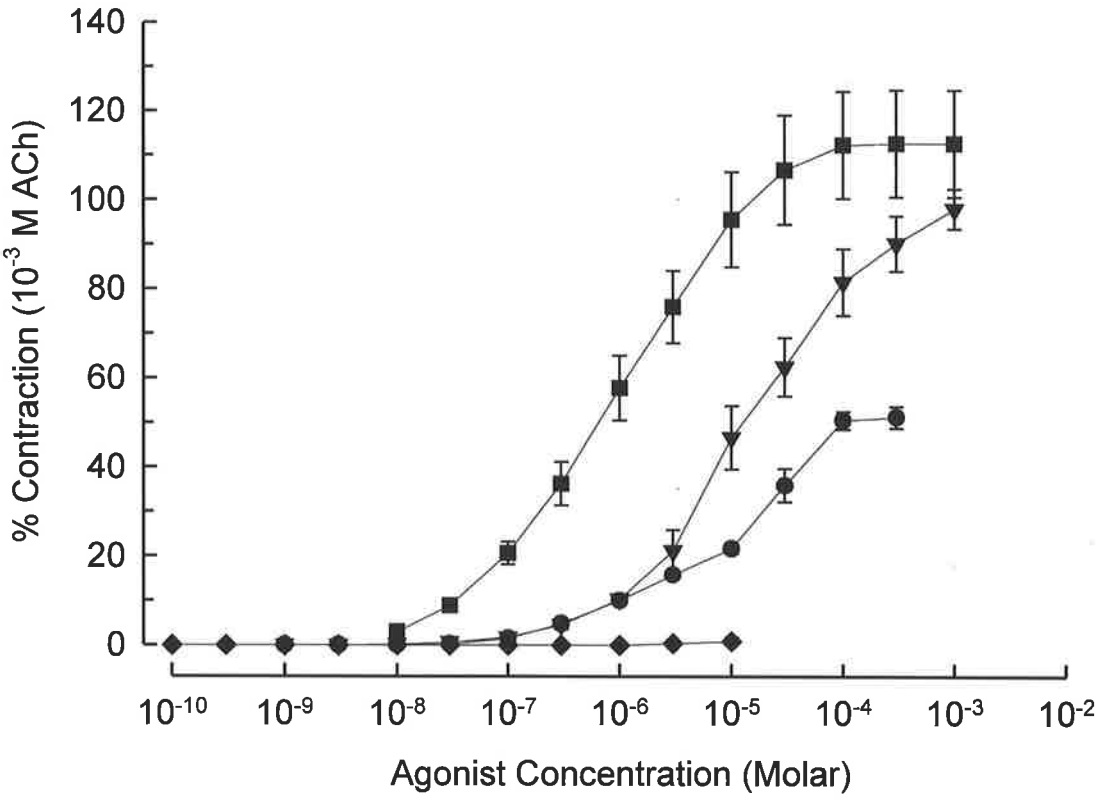


Figure 5.3 Mean cumulative concentration-response curves for carbachol (●), acetylcholine (▼), histamine (■), and substance P (◆) on ovine isolated parenchymal smooth muscle strips. Mean responses are from 3-6 animals and are expressed as a percentage of the reference contraction to 10^{-3} M ACh. Vertical bars indicate SEM.

Table 5.4 Mean maximal effect, geometric mean EC_{50} and apparent affinities for various agonists in ovine parenchymal smooth muscle strips.

Agonist	n	E_{max} [a]	EC_{50} (M)		Mean pD_2 (±SEM)
Carbachol	3	51.2 ± 2.4	1.26×10^{-5}	(9.27×10^{-6} , 1.71×10^{-5})	4.90 ± 0.03
Acetylcholine	6	98.0 ± 4.5	1.44×10^{-5}	(8.42×10^{-6} , 2.45×10^{-5})	4.84 ± 0.09
Histamine	6	112.7 ± 12.1	9.03×10^{-7}	(6.43×10^{-7} , 1.27×10^{-6})	6.04 ± 0.06
[Sar^9,$Met(O_2)^{11}$]-SP	6	2.7 ± 0.9	ND		ND
Substance P	3	0.9 ± 0.8	ND		ND
Neurokinin A	6	0.5 ± 0.3	ND		ND
Neurokinin B	3	0.9 ± 0.5	ND		ND
[Nle^{10}]-NKA(4-10)	3	2.2 ± 1.1	ND		ND
Senktide	3	3.6 ± 0.7	ND		ND

[a] Mean E_{max} ± SEM were expressed as a percentage of the response to 10^{-3}M acetylcholine. n= number of sheep. pD_2 values were calculated as -log EC_{50}. Geometric mean EC_{50} values and 95% confidence limits (are shown in parenthesis). ND, not determined due to low agonist activity at 10^{-5} M in this tissue.

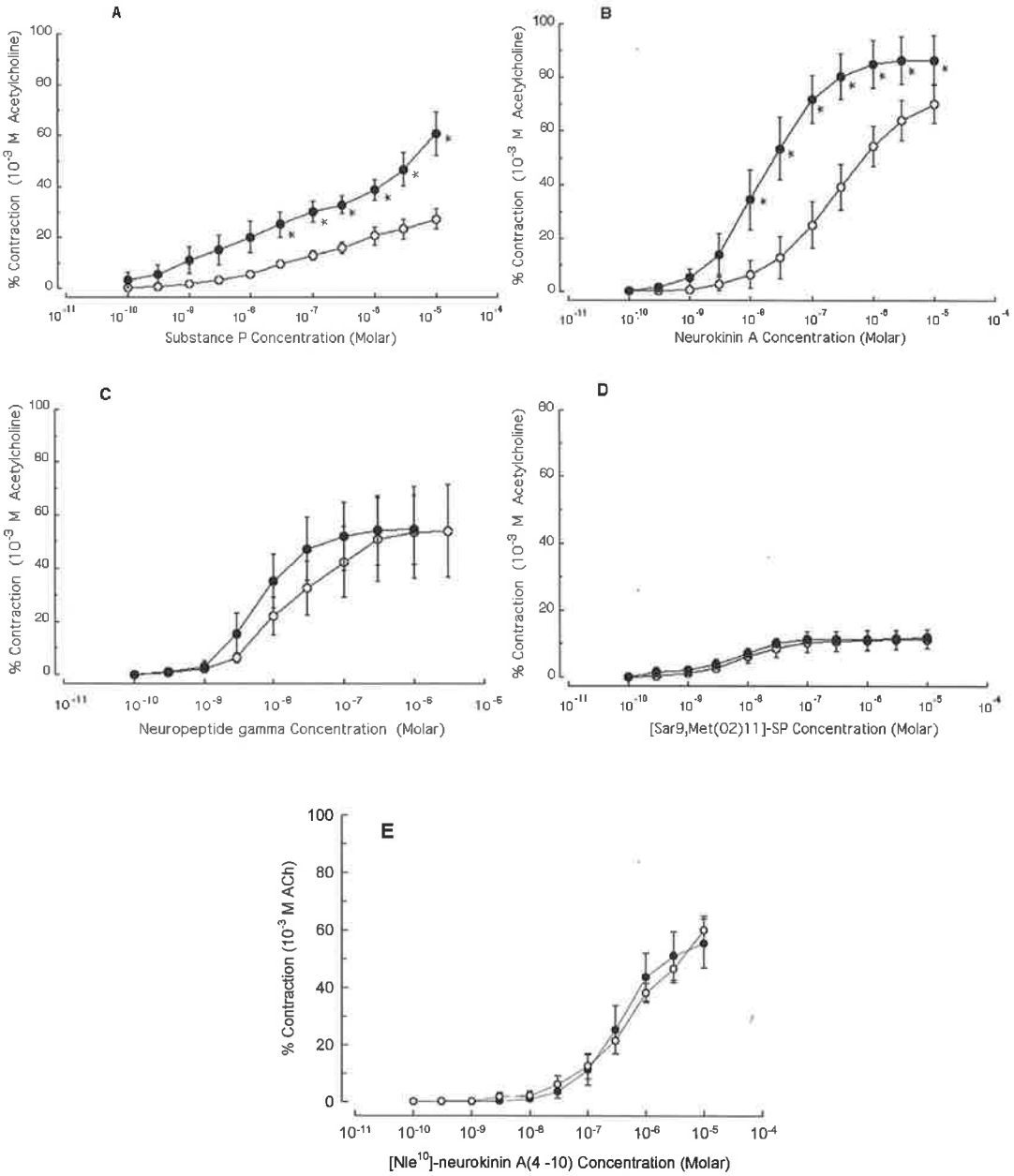


Figure 5.4 Cumulative concentration-response curves in tracheal muscle strips for substance P (A, n=6); neurokinin A (B, n=5); neuropeptide gamma (C, n=4); [Sar^9,$Met(O_2)^{11}$]-SP (D, n=4); and [Nle^{10}]-neurokinin A(4-10) (E, n=3) in the absence (○) and presence (●) of phosphoramidon 10^{-5}M. Mean responses are expressed as a percentage of the reference contraction to 10^{-3} M ACh. Vertical bars indicate SEM. * Denotes a statistically significant ($P < 0.05$, paired *t*-test) difference between tissues treated with and without phosphoramidon.

Table 5.5 Geometric mean EC_{25} [a] and 95% Confidence Limits for tachykinins in the absence and presence of phosphoramidon (10^{-5}M) in tracheal smooth muscle.

Agonist	n	EC_{25} (M) [a] Control	EC_{25} (M) [a] Phosphoramidon
Substance P	6	2.10×10^{-6} (5.62×10^{-7} , 7.84×10^{-6})	2.17×10^{-8} (1.61×10^{-9} , 2.94×10^{-7})*
Neurokinin A	5	1.10×10^{-7} (1.98×10^{-8} , 6.10×10^{-7})	6.97×10^{-9} (1.37×10^{-9} , 3.54×10^{-8})*
Neuropeptide gamma	4	2.72×10^{-8} (3.20×10^{-9} , 2.30×10^{-7})	7.35×10^{-9} (9.86×10^{-10} , 5.40×10^{-8})
$[Nle^{10}]$-NKA(4-10)	3	3.48×10^{-7} (8.17×10^{-8} , 1.48×10^{-6})	3.17×10^{-7} (3.70×10^{-8} , 2.71×10^{-6})
$[Sar^{9},Met(O_2)^{11}]$-SP	4	NC	NC

[a] The concentration of agonist at which a contractile response equal to 25% of that induced by 10^{-3} M acetylcholine is attained (see Methods). Values are geometric mean EC_{25} values and 95% confidence limits (shown in parenthesis). * Significantly different from control (Paired *t*-test), $P < 0.05$. NC, EC_{25} not calculated due to low agonist activity at 10^{-5} M in this tissue.

absence and presence of phosphoramidon being 0.4 ± 0.2% and 1.6 ± 0.4% ACh, respectively.

5.3.2.2 Bronchus and parenchyma

As a result of the low activity of tachykinins on ovine bronchial and parenchymal tissue (Table 5.3 and Table 5.4, respectively) the effect of neuropeptide inhibition phosphoramidon on SP contractility was examined only. Once again, phosphoramidon did not cause any significant change in baseline tension in bronchial ring or parenchymal strip preparations. Neutral endopeptidase inhibition with phosphoramidon (10^{-5} M) increased the amplitude of contractions, and produced a significant 69-fold leftward shift in the CCRC for SP in bronchial rings. EC_{10} and (95% confidence intervals) for SP in bronchial rings with and without phosphoramidon were, 1.08×10^{-8} (3.25×10^{-9}, 3.58×10^{-8}) and 7.48×10^{-7} (2.66×10^{-7}, 2.09×10^{-6}), respectively ($P < 0.05$). However, there was no significant increase in the maximal response to SP with phosphoramidon. E_{max} for SP, in the presence and absence of phosphoramidon (10^{-5} M), was 25.7 ± 6.1% and 17.8 ± 3.3% ACh, respectively, Figure 5.5. In the presence of phosphoramidon a plateau in the CCRC for SP was observed (Figure 5.5), thus enabling an EC_{50} determination, this being 3.79×10^{-8} with 95% confidence limits between 2.77×10^{-8} and 5.18×10^{-8}. Phosphoramidon did not change the amplitude of contraction for SP in parenchymal strips. E_{max} values for SP, obtained in parenchymal strips, in the presence and absence of phosphoramidon (10^{-5} M), were 1.90 ± 1.0% and 0.9 ± 0.8% ACh, respectively.

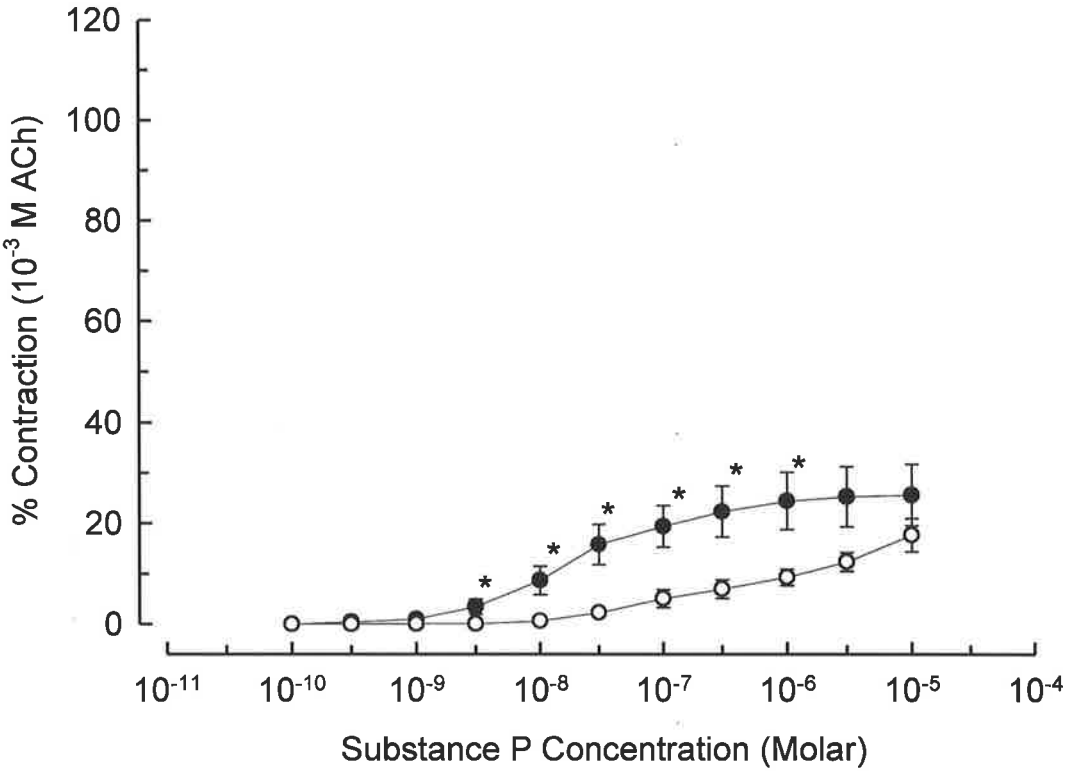


Figure 5.5 Cumulative concentration-response curves for substance P, obtained in ovine bronchial rings (n=6 sheep), in the absence (○) and presence (●) of phosphoramidon 10^{-5}M. Mean responses are expressed as a percentage of the reference contraction to 10^{-3} M ACh. Vertical bars indicate SEM. * Denotes a statistically significant difference between tissues treated with and without phosphoramidon ($P < 0.05$, paired *t*-test).

5.3.3 The effect of specific NK_1, NK_2 and NK_3 receptor agonists

5.3.3.1 Trachea

The contractile effects of the specific tachykinin NK_1, NK_2 and NK_3 receptor agonists [Sar^9,$Met(O_2)^{11}$]-SP, [Nle^{10}]-NKA(4-10) and senktide are shown in Figures 5.6A - C, respectively. In each graph contractile responses to the corresponding endogenous tachykinin is also shown. The specific tachykinin NK_1 agonist (Figure 5.6A) is relatively weak in ovine tracheal smooth muscle with an E_{max} at 10^{-5} M of only 11.4 ± 2.8% ACh compared with SP. The selective tachykinin NK_2 agonist, [Nle^{10}]-NKA(4-10) elicited significant contractions in tracheal smooth muscle strips, however, this again was lower than that seen with NKA (Table 5.1, Figure 5.6B). Senktide was inactive in the tracheal smooth muscle preparation with an E_{max} of 0.5 ± 0.3% ACh compared with NKB (E_{max} 31.4 ± 8.4% ACh, Figure 5.6C).

5.3.3.2 Bronchus and parenchyma

As previously mentioned tachykinins appear to be relatively inactive in bronchial and parenchymal tissue compared to the trachea. To best illustrate this point Figure 5.7 depicts the contractile effects of the specific tachykinin NK_1, NK_2 and NK_3 receptor agonists, in bronchial ring preparations, together with their corresponding endogenous tachykinin, Figures 5.7A, B and C, respectively. The specific tachykinin NK_1 agonist (Figure 5.7A) elicited significant contractions in ovine bronchial rings with an E_{max} at 10^{-5} M of 22.6 ± 4.2% ACh being of similar magnitude to that obtained with SP, Table 5.3. The selective tachykinin NK_2 agonist, [Nle^{10}]-NKA(4-10) and the NK_3 agonist, senktide were inactive in bronchial preparations with E_{max} values of 1.04 ± 0.5% and 0.7 ± 0.7% ACh, respectively (Figure 5.7B and C).

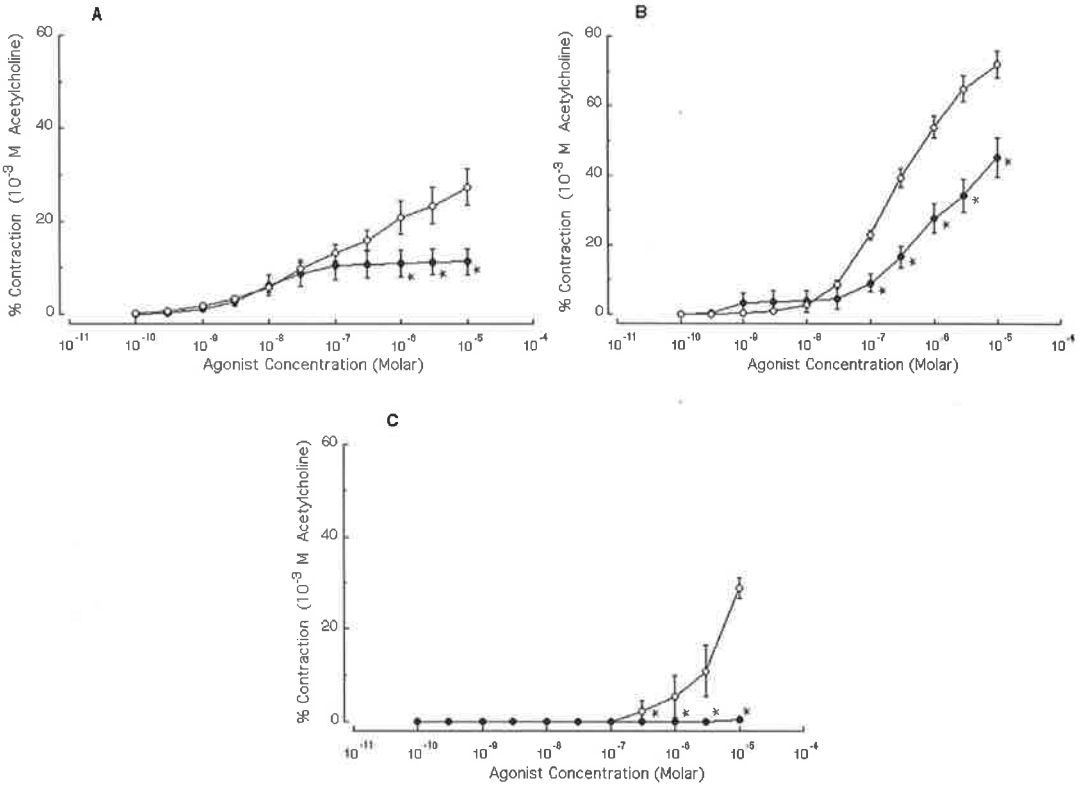


Figure 5.6 (A) Effect of the tachykinin NK_1 receptor specific agonist, [Sar^9,$Met(O_2)^{11}$]-SP (●, n=4) and substance P (○, n=6) on isolated tracheal strips. (B) Effect of the tachykinin NK_2 receptor specific agonist, [Nle^{10}]-NKA(4-10) (●, n=4) and neurokinin A (○, n=6) on isolated tracheal strips. (C) Effect of the tachykinin NK_3 receptor specific agonist, senktide (●, n=3) and neurokinin B (○, n=6) on isolated tracheal strips. Mean responses are expressed as a percentage of the reference contraction to 10^{-3}M ACh. Vertical bars indicate SEM. * Denotes a statistically significant ($P < 0.05$, unpaired *t*-test) difference between treatment groups.

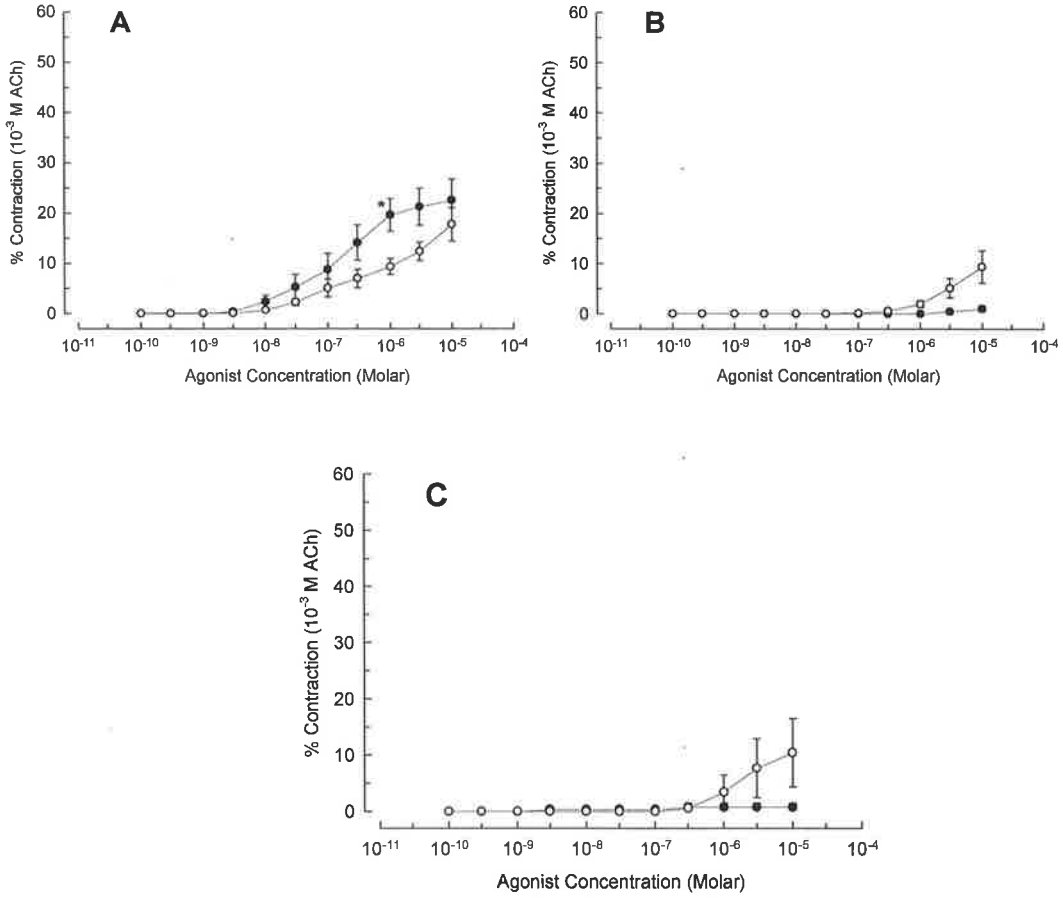


Figure 5.7 (A) Effect of the tachykinin NK_1 receptor specific agonist, [Sar^9,$Met(O_2)^{11}$]-SP (●, n=6) and substance P (○, n=6) on isolated bronchial rings. (B) Effect of the tachykinin NK_2 receptor specific agonist, [Nle^{10}]-NKA(4-10) (●, n=3) and neurokinin A (○, n=6) on isolated bronchial rings. (C) Effect of the tachykinin NK_3 receptor specific agonist, senktide (●, n=3) and neurokinin B (○, n=3) on isolated bronchial rings. Mean responses are expressed as a percentage of the reference contraction to 10^{-3}M ACh. n equals the number of sheep. Vertical bars indicate SEM. * Denotes a statistically significant ($P < 0.05$, unpaired *t*-test) difference between treatment groups.

Contractility studies for SP, NKA, NKB and the specific tachykinin NK_1, NK_2 and NK_3 receptor agonists demonstrated these agonists to be relatively weak or inactive in ovine parenchymal strips (Table 5.4).

5.3.4 Assessment of tachyphylaxis

5.3.4.1 Trachea

In order to determine if ACh, SP, NKA, [Sar^9,$Met(O_2)^{11}$]-SP and histamine demonstrated tachyphylaxis, successive CCRCs were obtained for each agonist separated by 90-100 min. Results for the consecutive CCRCs for ACh, SP, NKA, [Sar^9,$Met(O_2)^{11}$]-SP and histamine are shown in Figures 5.8A - E, respectively. No significant decrease in E_{max} or shift in the CCRC for ACh, SP and NKA was seen, compared with the first CCRC (Figures 5.8A-C, Table 5.6). Substance P, NKA and ACh, did not exhibit tachyphylaxis. In contrast a tachyphylactic response to successive [Sar^9,$Met(O_2)^{11}$]-SP and histamine CCRCs was observed in tracheal smooth muscle (Figures 5.8D and E, Table 5.6).

5.3.4.2 Bronchus

As in the trachea, similar studies to assess tachyphylaxis were conducted on bronchial smooth muscle. Since NKA demonstrated weak activity and histamine was inactive in the bronchial ring preparation, tachyphylaxis experiments to these agonists were not performed. No tachyphylaxis was observed in the contractile response to ACh, SP or [Sar^9,$Met(O_2)^{11}$]-SP, Figures 5.9A - C, respectively. No significant differences were seen in the E_{max} and EC_{50} values between the first and second CCRC for each agonist studied, Table 5.7.

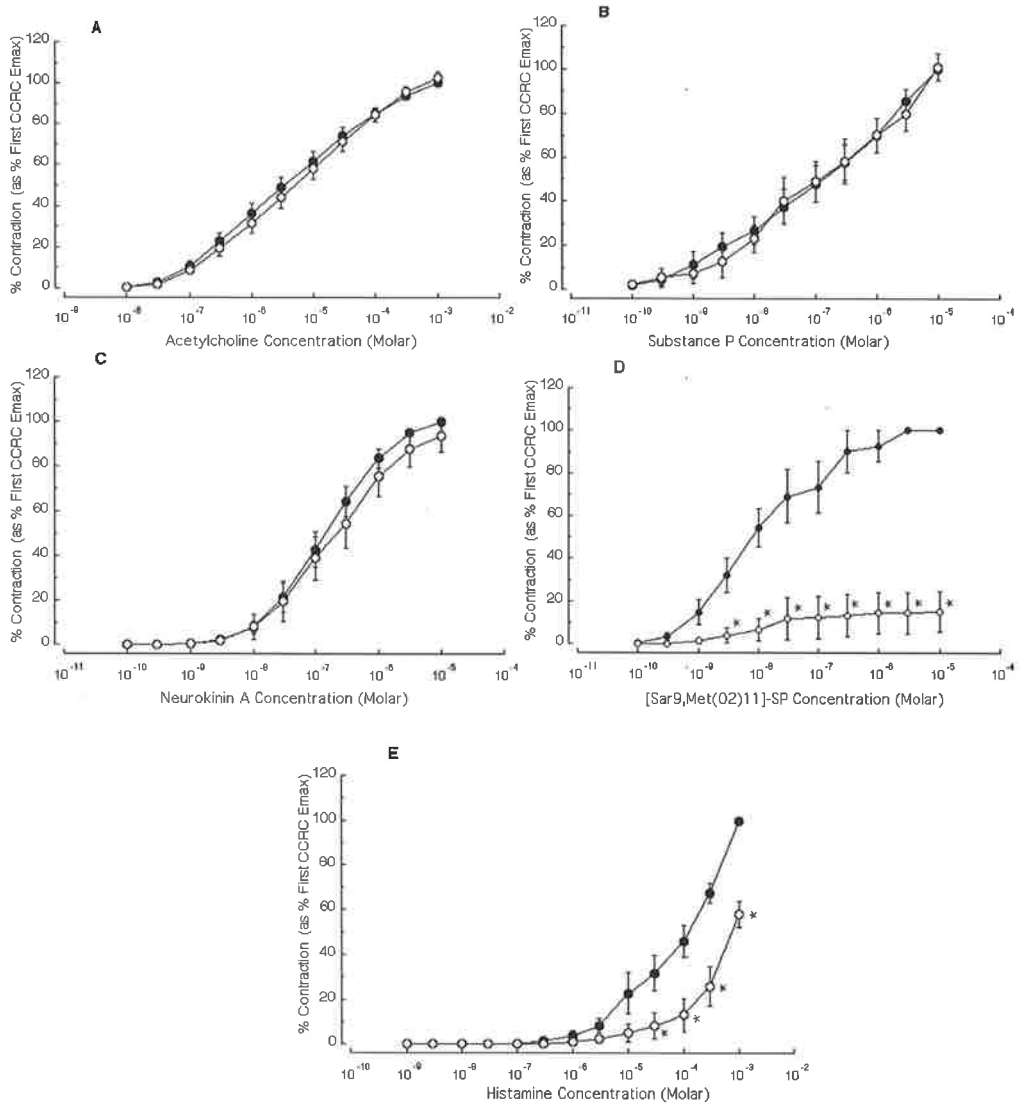


Figure 5.8 Mean cumulative concentration-response curves for acetylcholine (A, n=6); substance P (B, n=6); neurokinin A (C, n=6); [Sar^9,$Met(O_2)^{11}$]-SP (D, n=4); and histamine (E, n=3). (●) and (○), first and second cumulative concentration-response curves respectively, in the same tracheal strip. Contractile responses are expressed as a percent of the maximal response in the first cumulative concentration-response curve (First CCRC E_{max}). Vertical bars indicate SEM. * Denotes a statistically significant difference ($P < 0.05$, paired *t*-test) from first cumulative concentration-response curves.

Table 5.6 Mean maximal effect (E_{max}) and mean EC_{50} values for agonists of first and second cumulative concentration-response curves (CCRC) in ovine tracheal smooth muscle strips.

		E_{max} [a]	EC_{50} [b] (M)	
Agonist	n	Second CCRC	First CCRC	Second CCRC
Acetylcholine	6	102.4 ± 3.0	3.15×10^{-6} (1.03×10^{-6}, 9.62×10^{-6})	4.57×10^{-6} (1.70×10^{-6}, 1.23×10^{-5})
Substance P	6	101.1 ± 6.1	1.92×10^{-7} (5.14×10^{-8}, 6.78×10^{-7})	1.50×10^{-7} (2.67×10^{-8}, 8.48×10^{-7})
Neurokinin A	6	93.9 ± 7.3	1.39×10^{-7} (5.34×10^{-8}, 3.63×10^{-7})	2.10×10^{-7} (4.63×10^{-8}, 9.48×10^{-7})
Histamine	3	58.0 ± 6.0*	7.53×10^{-5} (1.78×10^{-5}, 3.17×10^{-4})	6.86×10^{-4} (2.96×10^{-4}, 1.59×10^{-3})*
$[Sar^{9},Met(O_2)^{11}]$-SP	4	15.3 ± 9.4*	9.87×10^{-9} (1.70×10^{-9}, 5.73×10^{-8})	NC

[a] Values for maximal contractile response (E_{max}) are expressed as mean ± SEM as a percentage of the maximal contraction achieved in the first cumulative concentration-response curve. [b] Geometric mean EC_{50} values and 95% confidence limits (in parenthesis) were calculated from each individual experiment. n, is the number of animals used per group. * $P < 0.05$, significantly different from first cumulative concentration-response curve (paired *t*-test). NC, not calculated due to low activity at 10^{-5}M.

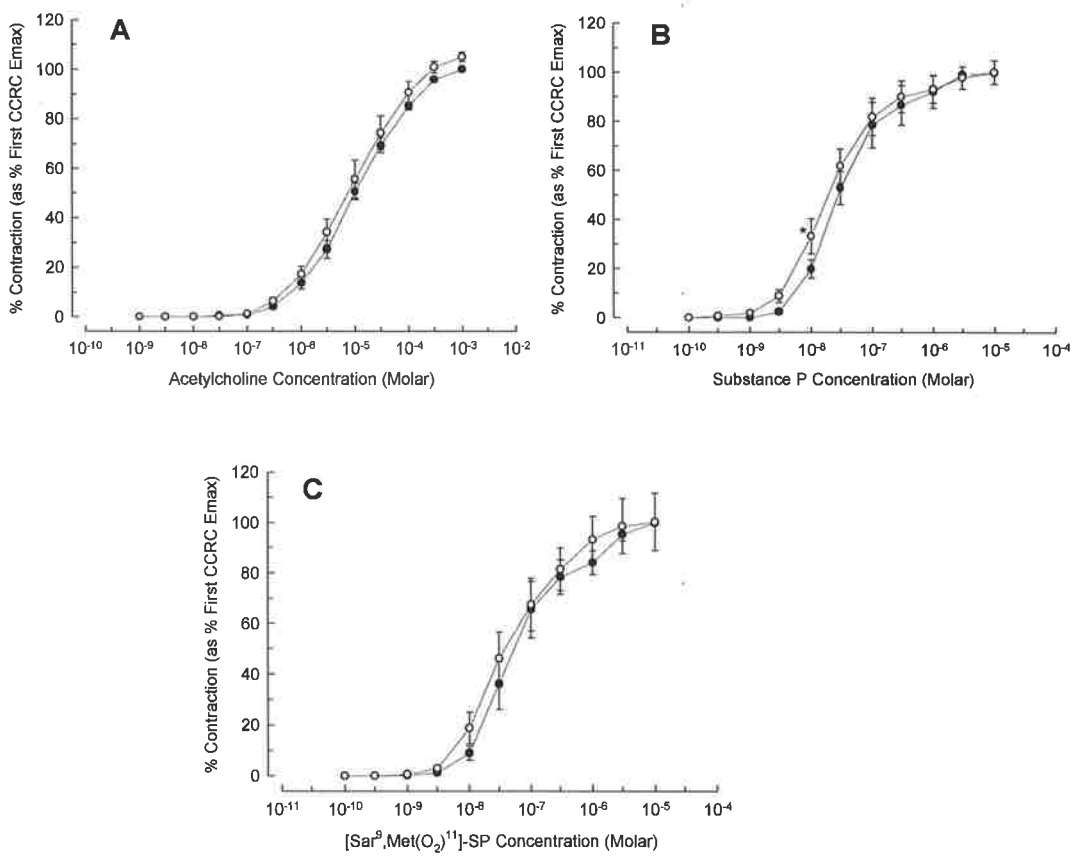


Figure 5.9 Mean cumulative concentration-response curves for acetylcholine (A, n=5); substance P (B, n=6); and [Sar^9,$Met(O_2)^{11}$]-SP (C, n=3). (●) and (○), first and second cumulative concentration-response curves respectively, in the same bronchial ring. Contractile responses are expressed as a percent of the maximal response in the first cumulative concentration-response curve (First CCRC E_{max}). In the case for substance P, each cumulative concentration-response curve was determined in the presence of phosphoramidon (10^{-5}M). Vertical bars indicate SEM. * Denotes a statistically significant difference ($P < 0.05$, paired *t*-test) from first cumulative concentration-response curves.

Table 5.7 Mean maximal effect (E_{max}) and mean EC_{50} values for agonists of first and second cumulative concentration-response curves (CCRC) in ovine bronchial smooth muscle.

		E_{max} [a]	EC_{50} [b] (M)	
Agonist	n	Second CCRC	First CCRC	Second CCRC
Acetylcholine	5	105.0 ± 1.9	9.43×10^{-6} (6.41×10^{-6}, 1.39×10^{-6})	7.43×10^{-6} (2.55×10^{-6}, 2.17×10^{-5})
Substance P[§]	6	100.0 ± 4.8	2.81×10^{-8} (1.39×10^{-8}, 5.70×10^{-8})	1.98×10^{-8} (9.12×10^{-9}, 4.30×10^{-8})
$[Sar^{9},Met(O_2)^{11}]$-SP	3	100.3 ± 11.4	5.35×10^{-8} (8.72×10^{-9}, 3.28×10^{-7})	3.50×10^{-8} (6.26×10^{-9}, 1.96×10^{-7})

[a] Values for maximal contractile response (E_{max}) are expressed as mean ± SEM as a percentage of the maximal contraction achieved in the first cumulative concentration-response curve. [b] Geometric mean EC_{50} values and 95% confidence limits (in parenthesis) were calculated from each individual experiment. n, is the number of animals used per group. § In the case for substance P, each cumulative concentration-response curve was determined in the presence of phosphoramidon (10^{-5} M).

5.3.4.3 Parenchyma

Contractility studies to document tachyphylaxis to SP, [Sar^9,$Met(O_2)^{11}$]-SP or NKA in parenchymal strips were not performed due to the low activity demonstrated for tachykinins in this tissue (Table 5.4). However, tachyphylaxis studies for ACh and histamine were undertaken. The graphical results for these studies are shown in Figure 5.10. As observed in tracheal tissue consecutive CCRCs to histamine in parenchymal strips did demonstrate tachyphylaxis. There was a significant diminution in E_{max} and a two-fold rightward shift of the second CCRC compared to the first, whereas successive CCRCs to ACh did not demonstrate any change, Table 5.8.

5.3.5 Effect of atropine and pyrilamine on substance P, [Sar^9,$Met(O_2)^{11}$]-SP and neurokinin A contractility

5.3.5.1 Trachea

In the absence of tachyphylaxis, each tissue's contractile response can be normalised to a standard contraction (i.e., the maximal effect obtained in the first CCRC) and thus allows for direct comparisons between tissues receiving different treatments. Atropine at 10^{-6} M did not reduce the magnitude of SP and NKA induced contractions, nor did it affect the potency of these agonists (Figures 5.11A and B, Table 5.9). Adequate atropine blockade was achieved as evidenced by the significant 285-fold rightward shift of the ACh CCRC in the presence of 10^{-6} M atropine, Table 5.9, Figure 5.11C.

Since consecutive CCRCs for [Sar^9,$Met(O_2)^{11}$]-SP exhibited tachyphylaxis (Figure 5.8D) the effect of atropine on [Sar^9,$Met(O_2)^{11}$]-SP contractility was examined in paired tissue samples. In these experiments atropine (10^{-6} M) was added to one of the paired tissues while the other bath received no treatment. The presence of atropine significantly attenuated the contractile response to the tachykinin NK_1 agonist,

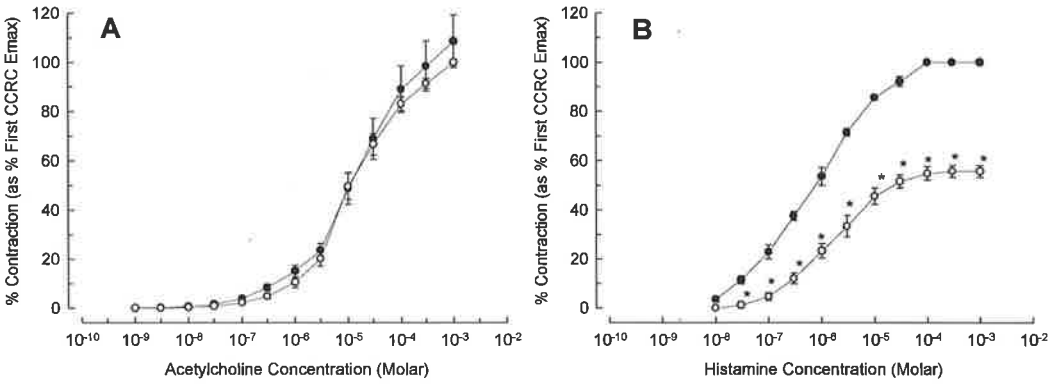


Figure 5.10 Mean cumulative concentration-response curves for acetylcholine (A, n=6); and histamine (B, n=3). (●) and (○), first and second cumulative concentration-response curves respectively, in the same parenchymal strip. Contractile responses are expressed as a percent of the maximal response in the first cumulative concentration-response curve (First CCRC E_{max}). Vertical bars indicate SEM. * Denotes a statistically significant difference ($P < 0.05$, paired *t*-test) from first cumulative concentration-response curves.

Table 5.8 Mean maximal effect (E_{max}) and mean EC_{50} values for agonists of first and second cumulative concentration-response curves (CCRC) in ovine parenchymal strips.

		E_{max} [a]	EC_{50} [b] (M)	
Agonist	n	Second CCRC	First CCRC	Second CCRC
Acetylcholine	6	108.6 ± 10.6	1.15×10^{-5} (6.35×10^{-6}, 2.08×10^{-5})	1.31×10^{-5} (9.23×10^{-6}, 1.86×10^{-5})
Histamine	3	55.7 ± 2.4*	7.18×10^{-7} (3.52×10^{-7}, 1.46×10^{-6})	1.62×10^{-6} (7.16×10^{-7}, 3.43×10^{-6})*

[a] Values for maximal contractile response (E_{max}) are expressed as mean ± SEM as a percentage of the maximal contraction achieved in the first cumulative concentration-response curve. [b] Geometric mean EC_{50} values and 95% confidence limits (in parenthesis) were calculated from each individual experiment. n, is the number of animals used per group. * $P < 0.05$, significantly different from first cumulative concentration-response curve (paired *t*-test).

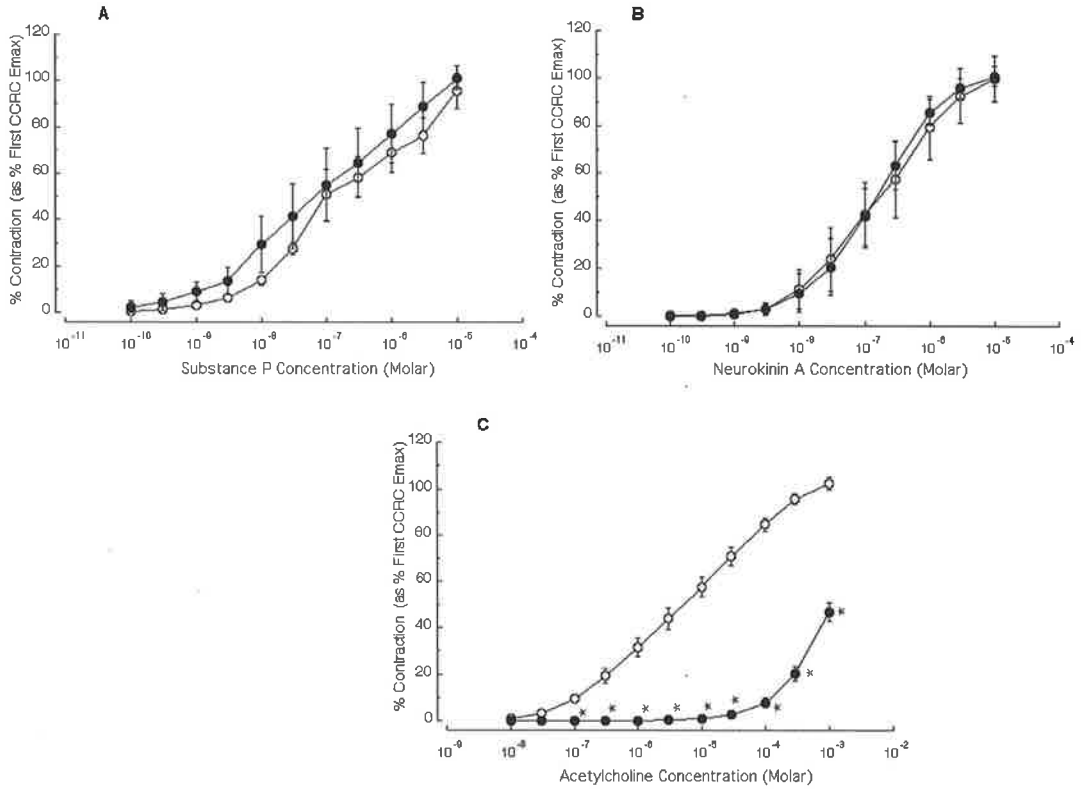


Figure 5.11 Mean second cumulative concentration-response curves in tracheal smooth muscle for (A) substance P (n=4), (B) neurokinin A (n=4), (C) acetylcholine (n=6) in the absence (○) and presence (●) of 10^{-6}M atropine. Contractile responses are expressed as a percent of the maximal response in the first cumulative concentration-response curve (First CCRC E_{max}). Vertical bars indicate SEM. * Denotes a statistically significant difference ($P < 0.05$, paired *t*-test) between control and atropine treated tissue.

Table 5.9 Mean maximal effect (E_{max}) and mean EC_{50} values for agonists in the second cumulative concentration-response curve in control and atropine treated tracheal strips.

		E_{max} [a]		EC_{50} [b] (M)	
Agonist	n	Control	Atropine	Control	Atropine
Acetylcholine	6	102.4 ± 3.0	46.0 ± 4.6*	4.50×10^{-6} (1.75×10^{-6}, 1.16×10^{-5})	1.15×10^{-3} (7.09×10^{-4}, 1.85×10^{-3})*
Substance P	4	95.7 ± 7.6	101.2 ± 5.3	1.20×10^{-7} (6.08×10^{-8}, 2.38×10^{-7})	9.39×10^{-8} (6.21×10^{-9}, 1.42×10^{-6})
Neurokinin A	4	100.1 ± 9.6	100.9 ± 4.2	1.52×10^{-7} (2.52×10^{-8}, 9.20×10^{-7})	1.26×10^{-7} (2.47×10^{-8}, 6.40×10^{-7})

[a] Values for maximal contractile response (E_{max}) for second cumulative concentration-response curve are expressed as mean ± SEM as a percentage of the maximal contraction achieved in the first cumulative concentration-response curve.

[b] Geometric mean EC_{50} values and 95% confidence limits (in parenthesis) were calculated from the mean EC_{50} values of the second cumulative concentration-response curve in each individual experiment. n, is the number of animals used per group. * $P < 0.05$, significantly different between control and atropine treated tissue (paired *t*-test).

[Sar^9,$Met(O_2)^{11}$]-SP (Figure 5.12); E_{max} being 8.3 ± 2.8% and 2.0 ± 1.3% of 10^{-3} M ACh, in the absence and presence of atropine, respectively ($P < 0.05$).

Pyrilamine, an histamine H_1-receptor antagonist, had no affect on the CCRC for SP (Figure 5.13A). In order to establish that adequate histamine receptor antagonism was achieved with pyrilamine (10^{-6} M), CCRCs for histamine were performed in paired tissue samples, since consecutive CCRCs for histamine exhibited tachyphylaxis (Figure 5.8E). In this series of experiments pyrilamine was added to one of the paired tissues 30 mins prior to obtaining the CCRC for histamine. Figure 5.13B documents that 10^{-6} M pyrilamine was sufficient to significantly antagonise histamine contractility in ovine tracheal smooth muscle, causing a significant 47-fold rightward shift in the CCRC ($P < 0.05$ paired *t*-test). The additions of atropine and pyrilamine to the organ baths did not cause any significant change in baseline tone.

5.3.5.2 Bronchus

Due to the apparent lack of NK_2 receptor activity in ovine bronchial tissue, the effect of atropine on NKA contractility in bronchial rings was not studied. However, the presence of atropine on NK_1 receptor mediated responses (SP and [Sar^9,$Met(O_2)^{11}$]-SP) in bronchial rings was examined. Atropine had no significant effect on SP or [Sar^9,$Met(O_2)^{11}$]-SP response curves (Figure 5.14A and B), with E_{max} and EC_{50} values of the second CCRC being similar to those measured in the first (Table 5.10). Again atropine blockade to cholinergic responses in bronchial tissue was achieved. Atropine at 10^{-6} M produced a significant 224-fold rightward shift of the CCRC to ACh in bronchial rings (Figure 5.14C and Table 5.10). Although histamine did not contract bronchial rings (Table 5.3), the effect of pyrilamine on SP contractions was examined and, not surprisingly had no effect (Figure 5.15 and Table 5.11).

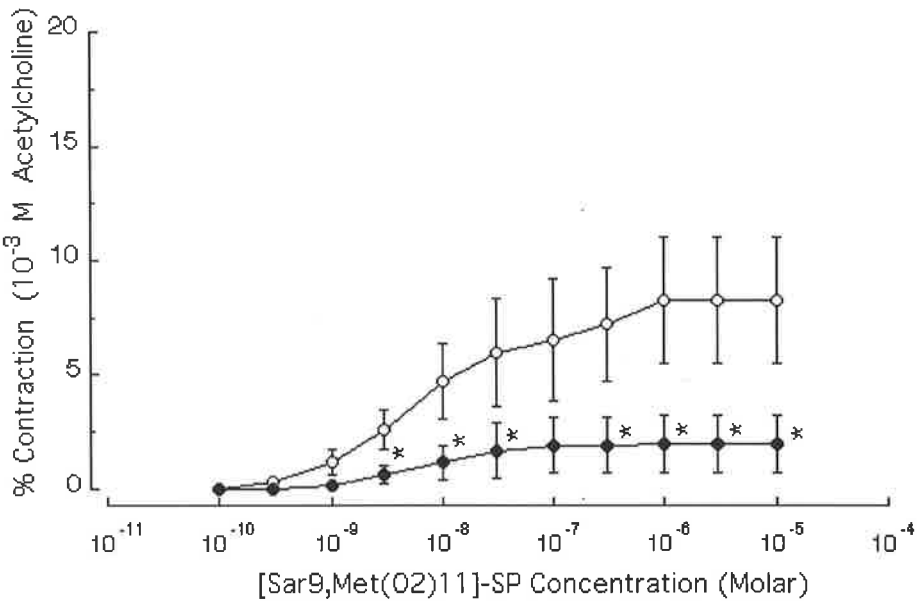


Figure 5.12 Effect of the absence (○) and presence (●) of 10^{-6}M atropine on [Sar^{9},$Met(O_2)^{11}$]-SP contractions in paired tracheal smooth muscle strips. Points represent mean values from 4 animals. Contractile responses are expressed as a percentage of the reference contraction to 10^{-3}M ACh. Vertical bars indicate SEM. * Denotes a statistically significant difference ($P < 0.05$, paired *t*-test) between control and atropine treated tissue.

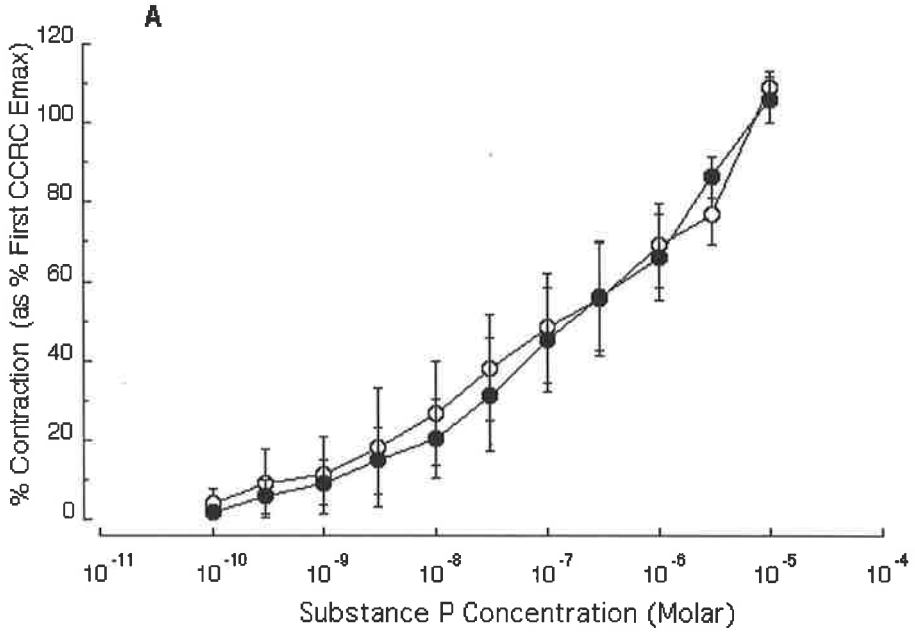


Figure 5.13A Mean second cumulative concentration-response curves for substance P (n=3) in the absence (○) and presence (●) of 10^{-6}M pyrilamine. Contractile responses are expressed as a percent of the maximal response in the first cumulative concentration-response curve (First CCRC E_{max}). Vertical bars indicate SEM.

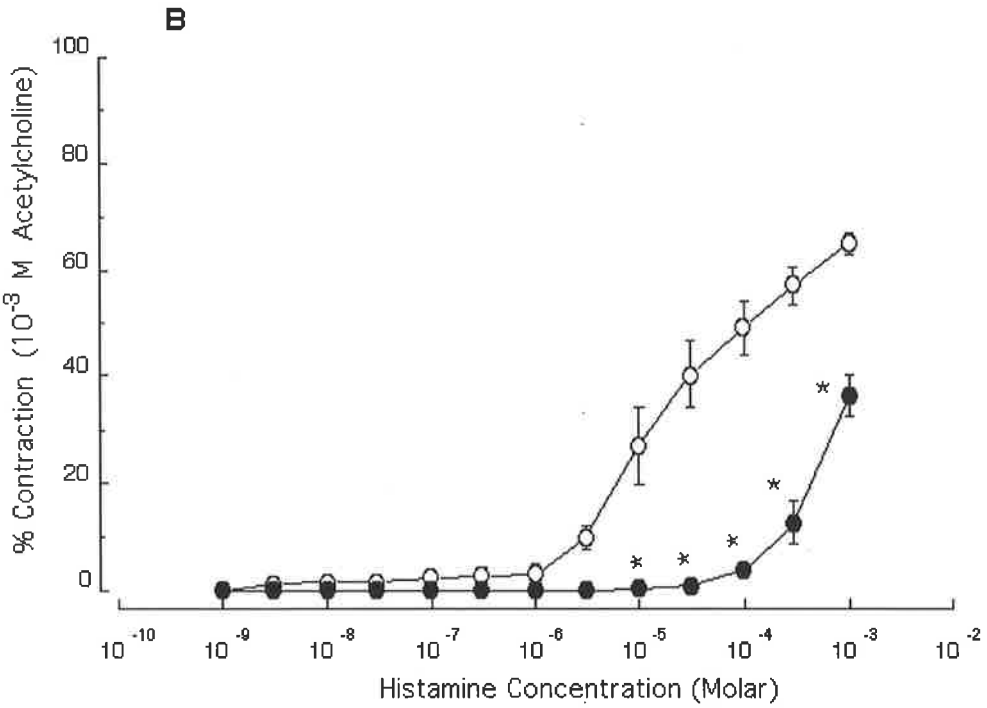


Figure 5.13B Effect of the absence (○) and presence (●) of 10^{-6}M pyrilamine on histamine contractions in paired tracheal smooth muscle strips. Points represent mean values from 3 animals. Contractile responses are expressed as a percentage of the reference contraction to 10^{-3}M ACh. Vertical bars indicate SEM. * Denotes a statistically significant difference ($P < 0.05$, paired *t*-test) between control and pyrilamine treated tissue.

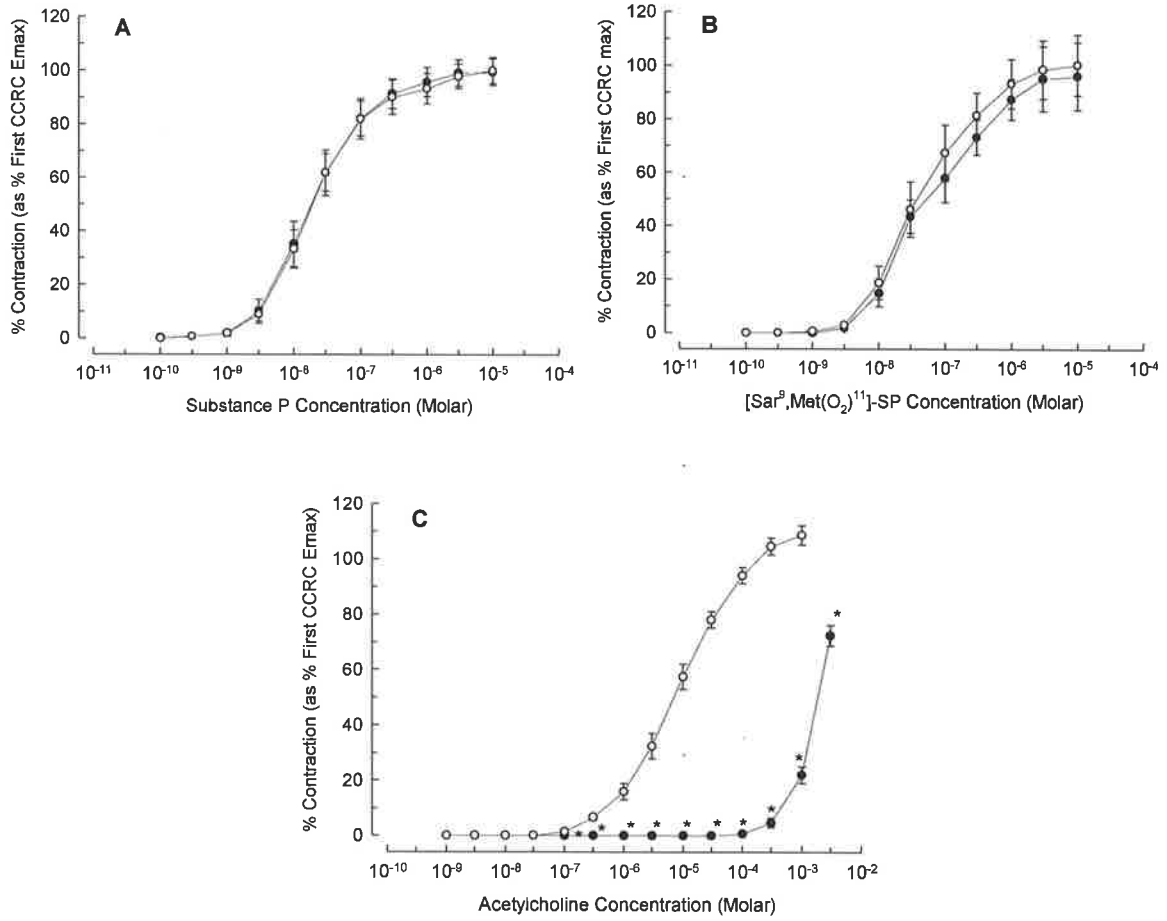


Figure 5.14 Mean second cumulative concentration-response curves in bronchial smooth muscle for (A) substance P (n=6), (B) [Sar^9,$Met(O_2)^{11}$]-SP (n=6), (C) acetylcholine (n=6) in the absence (○) and presence (●) of 10^{-6}M atropine. Contractile responses are expressed as a percent of the maximal response in the first cumulative concentration-response curve (First CCRC E_{max}). In the case for substance P, each cumulative concentration-response curve was determined in the presence of phosphoramidon (10^{-5}M). Vertical bars indicate SEM. * Denotes a statistically significant difference ($P < 0.05$, paired *t*-test) between control and atropine treated tissue.

Table 5.10 Mean maximal effect (E_{max}) and mean EC_{50} values for agonists in the second cumulative concentration-response curve in control and atropine treated bronchial rings.

		E_{max} [a]		EC_{50} [b] (M)	
Agonist	n	Control	Atropine	Control	Atropine
Acetylcholine	6	108.8 ± 3.5	72.5 ± 3.8†*	7.89×10^{-6} (4.31×10^{-6}, 1.45×10^{-5})	1.78×10^{-3} (1.47×10^{-3}, 2.16×10^{-3})*
Substance P§	6	100.0± 4.8	99.4 ± 4.9	1.98×10^{-8} (9.12×10^{-9}, 4.30×10^{-8})	1.90×10^{-8} (9.12×10^{-9}, 3.97×10^{-8})
[Sar^9,$Met(O_2)^{11}$]-SP	3	100.3 ± 11.4	96.2 ± 11.4	3.50×10^{-8} (6.26×10^{-9}, 1.96×10^{-7})	4.37×10^{-8} (3.03×10^{-8}, 6.30×10^{-8})

[a] Values for maximal contractile response (E_{max}) for second cumulative concentration-response curve are expressed as mean ± SEM as a percentage of the maximal contraction achieved in the first cumulative concentration-response curve.

[b] Geometric mean EC_{50} values and 95% confidence limits (in parenthesis) were calculated from the mean EC_{50} values of the second cumulative concentration-response curve in each individual experiment. † E_{max} at 3 $x10^{-3}$ M ACh. § In the case for substance P, each cumulative concentration-response curve was determined in the presence of phosphoramidon (10^{-5} M). n, is the number of animals used per group. * $P < 0.05$, significantly different between control and atropine treated tissue (paired *t*-test).

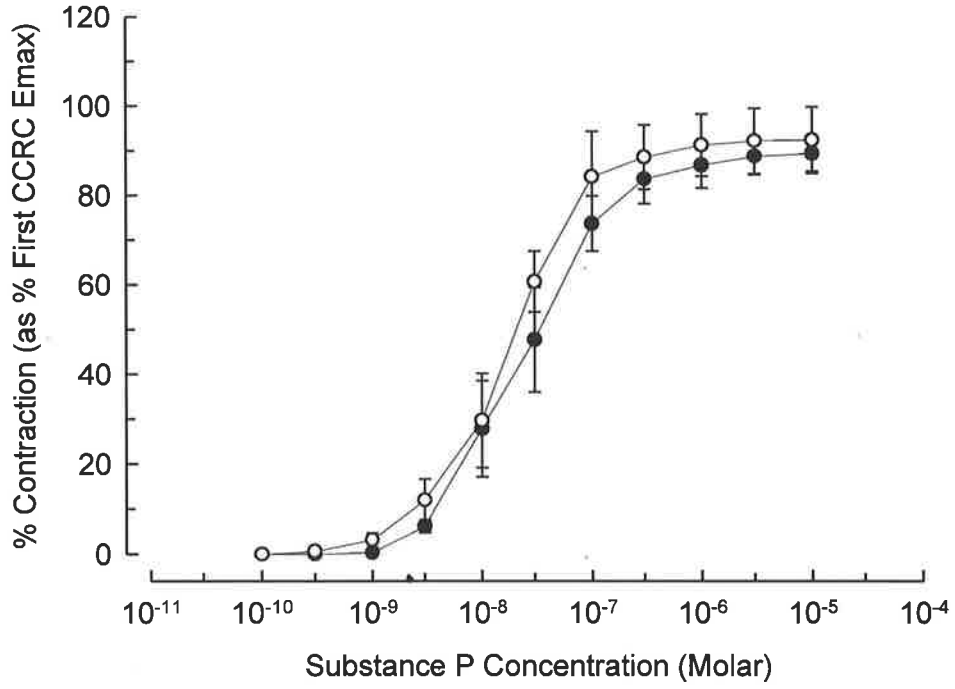


Figure 5.15 Mean second cumulative concentration-response curves in bronchial rings for substance P (n=3) in the absence (○) and presence (●) of 10^{-6}M pyrilamine. Contractile responses are expressed as a percent of the maximal response in the first cumulative concentration-response curve (First CCRC E_{max}). In the case for substance P, each cumulative concentration-response curve was determined in the presence of phosphoramidon (10^{-5}M). Vertical bars indicate SEM. Differences are not significant at any point (paired *t*-test, $P < 0.05$).

Table 5.11 Mean maximal effect (E_{max}) and mean EC_{50} values for agonists in control and pyrilamine (10^{-6}M) treated bronchial rings and parenchymal strips.

		E_{max}		EC_{50} (M)	
Agonist	n	Control	Pyrilamine	Control	Pyrilamine
Bronchial rings					
Substance P[§]	3	92.4± 7.41 [a]	89.4 ± 3.9 [a]	1.69×10^{-8} (6.65×10^{-9}, 4.30×10^{-8}) [b]	2.65×10^{-8} (3.16×10^{-9}, 2.26×10^{-7}) [b]
Parenchymal strips					
Histamine	3	114.9 ± 10.7 [c]	97.0± 8.7 [c]	8.80×10^{-7} (6.45×10^{-7}, 1.20×10^{-6})	1.66×10^{-5} (6.08×10^{-6}, 4.55×10^{-5})*

[a] Values for maximal contractile response (E_{max}) for second cumulative concentration-response curve are expressed as mean ± SEM as a percentage of the maximal contraction achieved in the first cumulative concentration-response curve. [b] Geometric mean EC_{50} values and 95% confidence limits (in parenthesis) were calculated from the mean EC_{50} values of the second cumulative concentration-response curve in each individual experiment. [c] E_{max} values are expressed as a percentage of the reference contraction to 10^{-3}M acetylcholine. § In the case for substance P, each cumulative concentration-response curve was determined in the presence of phosphoramidon (10^{-5} M). n, is the number of animals used per group. * $P < 0.05$, significantly different between control and atropine treated tissue (paired *t*-test).

5.3.5.3 Parenchyma

When conducting the experiments investigating the effects of atropine and pyrilamine on tachykinin mediated responses in parenchymal tissue, the first series of experiments undertaken were those examining the effectiveness of atropine and pyrilamine concentrations on ACh and histamine contractility, respectively. Following these studies, the next series involved determining the contractile properties of tachykinins in ovine parenchymal strips. The results of which demonstrated that tachykinins and tachykinin receptor specific agonists have little contractile activity in parenchymal tissue (Table 5.4). In view of this, however, the results of the atropine and pyrilamine studies have been included for completeness. Once again adequate antagonism of ACh by 10^{-6} M atropine was demonstrated. In the presence of atropine E_{max} (at 3 x 10^{-3} M ACh) for second CCRC expressed as a percentage of the maximal contraction achieved in the first CCRC was reduced to 38.7 ± 7.8% compared to 85.6 ± 2.3% in control parenchymal strips. Atropine also resulted in a 2.44 log unit rightward shift of the CCRC for ACh in parenchymal strips, Figure 5.16, as reflected in EC_{25} values (95% confidence limits) in the absence and presence of atropine, 5.44 x 10^{-6} (2.31 x 10^{-6}, 1.28 x 10^{-5}) and 1.51 x 10^{-3} (6.02 x 10^{-4}, 3.78 x 10^{-3}), respectively, ($P < 0.05$, paired *t*-test).

Since consecutive CCRCs for histamine in parenchymal strips exhibited tachyphylaxis (Figure 5.10B), the effect of pyrilamine (10^{-6} M) on histamine contractility was examined in paired tissue samples. For this series of experiments pyrilamine was added to one of the paired strips 30 mins prior to obtaining the CCRC for histamine. Figure 5.17 documents that 10^{-6} M pyrilamine was sufficient to significantly antagonize histamine contractility in ovine parenchymal preparation, causing a significant 19-fold rightward shift in the CCRC ($P < 0.05$ paired *t*-test) (Table 5.11).

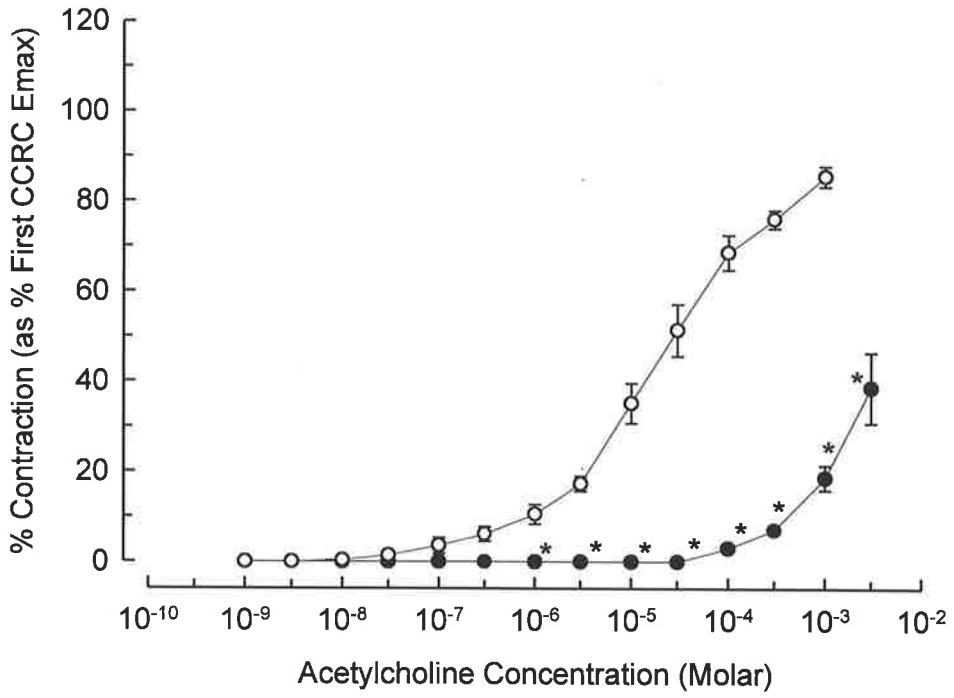


Figure 5.16 Mean second cumulative concentration-response curves in parenchymal smooth muscle for acetylcholine (n=3) in the absence (○) and presence (●) of 10^{-6}M atropine. Contractile responses are expressed as a percent of the maximal response in the first cumulative concentration-response curve (First CCRC E_{max}). Vertical bars indicate SEM. * Denotes a statistically significant difference ($P < 0.05$, paired *t*-test) between control and atropine treated tissue.

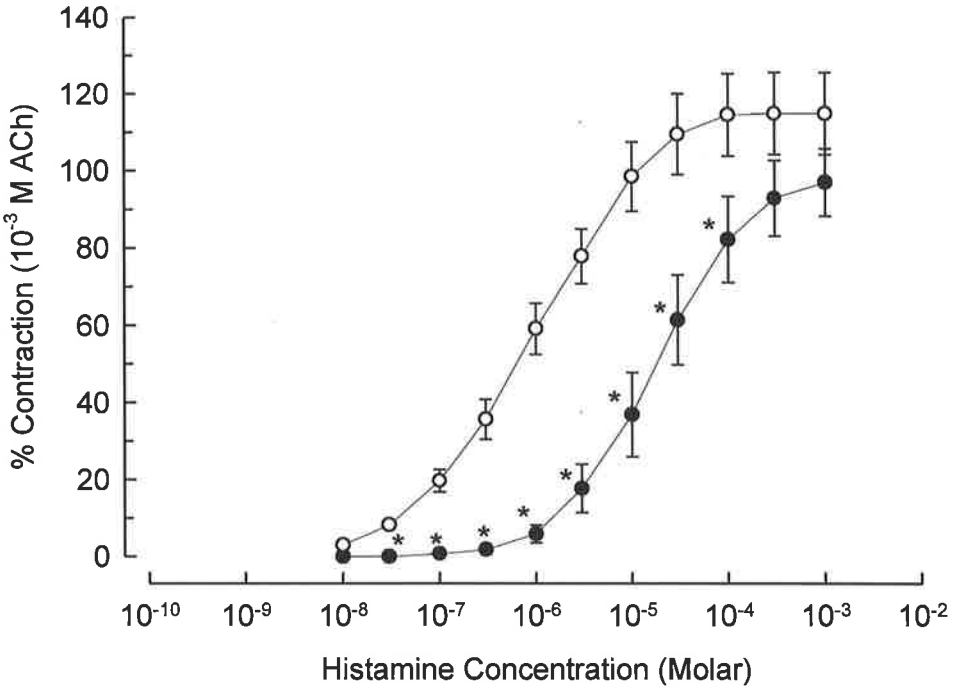


Figure 5.17 Effect of the absence (○) and presence (●) of 10^{-6}M pyrilamine on histamine contractions in paired parenchymal smooth muscle strips. Points represent mean values from 6 animals. Contractile responses are expressed as a percentage of the reference contraction to 10^{-3}M ACh. Vertical bars indicate SEM. * Denotes a statistically significant difference ($P < 0.05$, paired t-test) between control and pyrilamine treated tissue.

5.3.6 Regional differences

A summary of pD_2, E_{max} (mg), E_{max} (mg)/wt (mg) and E_{max}%ACh for the various agonists in ovine tracheal, bronchial and parenchymal smooth muscle preparations are shown in Table 5.12. Regional differences in cholinergic contractile responses were observed throughout the three lung regions. This regional difference being more evident with respect to carbachol. There was a decrease in sensitivity (pD_2 values) to both ACh and carbachol from proximal to distal airways. This was in contrast to histamine, where pD_2 values for histamine in parenchymal tissue were significantly higher than those measured in tracheal tissue. There were significant regional differences in the absolute magnitude of contraction (E_{max} (mg), E_{max} (mg)/wt (mg)). For each contractile agonist the maximal tension generated was significantly higher in tracheal tissue compared to bronchial which in turn was significantly higher than in the parenchymal preparation, with the exception of [Sar^9,$Met(O_2)^{11}$]-SP. [Sar^9,$Met(O_2)^{11}$]-SP was equally effective in tracheal and bronchial smooth muscle but less effective on parenchymal strips. Histamine was equally as effective as ACh in parenchymal tissue.

Of the tachykinins, NKA demonstrated significant activity in the trachea with significantly less and no activity in bronchial and parenchymal tissue, respectively. In tracheal muscle strips SP, in the presence of phosphoramidon, demonstrated equipotency with NKA and induced contractile forces that were of similar magnitude to those induced by NKA. Following NEP inhibition a significant regional sensitivity to SP was observed with significantly higher pD_2 values demonstrated in bronchial rings compared to tracheal strips. Furthermore, with phosphoramidon, SP in bronchial preparations was equipotent and equally effective as the specific NK_1 receptor agonist [Sar^9,$Met(O_2)^{11}$]-SP.

Table 5.12 Regional differences in E_{max} and pD_2 values throughout the ovine tracheobronchial tree.

Preparation	pD_2	E_{max} (mg)	E_{max}(mg)/wt(mg)	E_{max} %ACh	n
Carbachol					
Tracheal strip	7.49 (0.04)	38625 (1379)	396.7 (12.9)	128.0 (4.5)	6
Bronchial ring	7.06 (0.13)*	5053 (601)*	37.3 (3.9)*	100.0 (5.8)*	6
Parenchymal strip	4.90 (0.03)*†	950 (180)*†	5.5 (1.0)*†	51.2 (2.4)*†	3
Acetylcholine					
Tracheal strip	5.31 (0.15)	36540 (3962)	340.2 (36.8)	112.4 (2.0)	6
Bronchial ring	4.85 (0.16)	6754 (1862)*	54.5 (10.5)*	100.5 (10.5)	6
Parenchymal strip	4.84 (0.09)*	1832 (279)*†	12.3 (2.1)*†	98.0 (4.5)*	6
Histamine					
Tracheal strip	4.93 (0.17)	24692 (2600)	204.9 (27.7)	65.6 (5.9)	6
Bronchial ring	ND	ND	ND	ND	3
Parenchymal strip	6.04 (0.06)*	1989 (376)*	13.7 (2.9)*	112.7 (12.1)*	6
Neurokinin A					
Tracheal strip	6.67 (0.08)	24513 (1806)	169.6 (4.8)	72.0 (3.8)	6
Bronchial ring	NC	525 (196)*	6.4 (2.3)*	9.4 (3.2)*	6
Parenchymal strip	ND	12.5 (8.0)*†	0.1 (0.1)*†	0.5 (0.3)*†	6
Substance P					
Tracheal strip	NC	7870 (999)	67.2 (9.2)	27.4 (4.0)	6
Bronchial ring	NC	1019 (278)*	15.4 (2.9)*	17.7 (3.3)	6
Parenchymal strip	ND	19 (19)*†	0.1 (0.1)*†	0.9 (0.9)*†	3
Substance P §					
Tracheal strip	6.64 (0.19)	18052 (2868)	154.0 (24.1)	60.7 (8.6)	6
Bronchial ring	7.42 (0.05)*	2179 (437)*	28.7 (6.1)*	25.7 (6.1)*	6
Parenchymal strip	ND	52 (36)*†	0.4 (0.3)*†	1.9 (1.1)*†	3
[Sar^9,$Met(O_2)^{11}$]-SP					
Tracheal strip	8.01 (0.08)	3832 (1316)	34.5 (12.1)	11.4 (2.8)	4
Bronchial ring	6.95 (0.22)*	2163 (466)	18.7 (4.1)	22.6 (4.2)	6
Parenchymal strip	ND	67 (23)*†	0.5 (0.1)*†	2.7 (0.9)*†	6

pD_2 values were calculated as -log EC_{50}. SEM are shown in parenthesis. E_{max}; maximal contractile response expressed as force generated in mg. E_{max} (mg)/weight (mg); E_{max} normalized for wet tissue weight. E_{max}%ACh; E_{max} expressed as % of reference contraction to 10^{-3} M ACh. n= number of sheep. * Denotes a significant difference from value in the trachea (unpaired *t*-test) $P < 0.05$. † Denotes a significant difference from value in the bronchus (unpaired *t*-test) $P < 0.05$. NC, not calculated as agonist contractile response at 10^{-5} M still linear. ND, not determined due to low agonist activity at 10^{-5} M in this tissue.

5.4 Discussion

The results of this chapter demonstrate that, NPγ, SP, NKA and NKB contract ovine tracheal smooth muscle in a concentration-dependent manner. The rank order of potency was NPγ > NKA > SP ≥ NKB. The contractile responses to SP and NKA were increased by inhibition of NEP. In bronchial ring segments significant contractile responses were only obtained to SP and the specific NK_1 receptor agonist, whereas, NKA and NKB were relatively inactive. While in parenchymal tissue, the ability of tachykinins to induce any contractile was negligible. This lack of an effect of tachykinins has been demonstrated in isolated canine bronchial segments (McKay *et al.*, 1992) and parenchymal tissue in the guinea pigs (Uchida *et al.*, 1987; Foreman *et al.*, 1985) and mouse (Brandolini *et al.*, 2000). In view of the marked regional sensitivity to tachykinins in sheep airways, most of the discussion will pertain to tachykinin contractility in ovine tracheal and bronchial preparations.

In tracheal smooth muscle tachykinin-induced contraction *in vitro* appears to be predominantly mediated by tachykinin NK_2 receptors, although, tachykinin NK_1 receptors also appear to be involved. Substance P and NKA contractions were not mediated by cholinergic mechanisms. However, a significant cholinergic component was present in [Sar^9,$Met(O_2)^{11}$]-SP induced tracheal smooth muscle contraction, while NK_1 receptor mediated contraction in ovine bronchial smooth muscle was not cholinergically mediated. Repeated exposure of tracheal smooth muscle to either ACh, SP or NKA did not exhibit tachyphylaxis although tachyphylaxis was observed to [Sar^9,$Met(O_2)^{11}$]-SP and histamine. No tachyphylaxis was observed in contractile responses in bronchial rings to ACh, SP or [Sar^9,$Met(O_2)^{11}$]-SP. Tachyphylaxis to histamine was demonstrated in parenchymal strips. In the case of SP, the contractile

responses in tracheal and bronchial preparations were not mediated indirectly through histamine release (Lilly *et al.*, 1995; Joos *et al.*, 1988a; Foreman & Jordan, 1983).

This study is the first to characterize the contractile effects of NPγ, SP, NKA, and NKB throughout the ovine tracheobronchial tree. Of the tachykinins studied in tracheal smooth muscle, NPγ was the most potent which has also been found for isolated airways from rabbits (Black *et al.*, 1992), guinea pigs (Burcher *et al.*, 1991b) and human bronchi (Qian *et al.*, 1994; Burcher *et al.*, 1991a). For NPγ, NKA and SP, (in the absence of phosphoramidon) the rank order of potency parallels that seen in human, rabbit, and guinea pig airways (Qian *et al.*, 1994; Black *et al.*, 1992; Black *et al.*, 1990a; Warner *et al.*, 1990; Naline *et al.*, 1989; Advenier *et al.*, 1987), summarized in Table 5.13. Furthermore, the results show that NPγ and NKA are more potent contractile agonists in tracheal smooth muscle than ACh and histamine.

Contractile responses to SP and NKA in tracheal strips were sensitive to the inhibition of endopeptidase by phosphoramidon. Phosphoramidon produced a non-uniform increase in the potency of SP, NKA and NPγ (93-fold, 16-fold and 4-fold leftward shift of the EC_{25}, respectively). However, the change for NPγ failed to reach significance.

The reason for the apparent lack of potentiation by phosphoramidon with NPγ is unclear. Differences in the magnitude of augmentation following NEP inhibition have been observed in guinea pig and human bronchi, where SP and NKA enhancement is greater than that for NPγ (Qian *et al.*, 1994; Warner *et al.*, 1990; Naline *et al.*, 1989), indicating SP and NKA are more sensitive to NEP degradation. Furthermore, the potentiating effect of NEP inhibition for a given tachykinin is more pronounced in

Table 5.13 Comparison of mean pD_2 for various agonists found in ovine, human, guinea pig and rabbit, tracheal [a] and bronchial [b] tissue.

	Mean pD_2				
Agonist	Present study	Human	Other sheep	Guinea pig	Rabbit
Carbachol	7.49 [a]	6.26 [b, c]	7.59 [a, n]	6.86 [a, c]	6.99 [a, d]
Acetylcholine	5.31 [a]	5.53 [b, g]	5.37 [a, j]	5.89 [a, c]	6.43 [a, i]
Histamine	4.93 [a]	5.07 [b, k]	ND	6.05 [a, c]	4.99 [a, d]
Neurokinin A	6.67 [a]	6.99 [b, l]	ND	8.18 [a, h]	7.33 [a, i]
[Nle^{10}]-NKA(4-10)	6.26 [a]	6.22 [b, l]	ND	7.25 [a, h]	-
Neuropeptide gamma [p]	8.21 [a]	8.35 [b, m]	ND	8.74 [b, o]	8.80 [a, f]
Substance P	NC	4.92 [b, l]	ND	6.03 [a, h]	5.60 [a, e]
Neurokinin B	NC	Inactive [b, l]	ND	6.83 [a, h]	-

[a] tracheal smooth muscle [b] bronchial smooth muscle [c] Advenier et al. (1987) [d] Armour et al. (1985)

[e] Black et al. (1990a) [f] Black et al. (1992) [g] Cerrina et al. (1989) [h] Devillier et al. (1988)

[i] Goroumaru-Shinkai et al. (1992) [j] Jackowski et al. (1993) [k] Knight et al. (1990) [l] Naline et al. (1989)

[m] Qian et al. (1994) [n] Tomioka et al. (1991) [o] Zeng et al. (1994)

[p] In each case, the pD_2 was determined in the presence of phosphoramidon (10^{-5} M). ND, not previously determined in this species. NC, not calculated due to low agonist activity at 10^{-5} M in this tissue.

smaller airways than in tracheal tissue (Goroumaru-Shinkai *et al.*, 1992; Black *et al.*, 1990a). Therefore, the lack of potentiation for NPγ we observed in tracheal smooth muscle may reflect regional distribution of NEP and the susceptibility of specific tachykinins to NEP degradation. The lack of enhancement of the [Sar^9,$Met(O_2)^{11}$]-SP contractile response by phosphoramidon was not surprising since SP-analogues substituted in position 9 are relatively insensitive to NEP inactivation (Devillier *et al.*, 1988). Neutral endopeptidase inhibition in bronchial tissue also significantly increases the potency of SP.

The contractile response to tachykinins in ovine tracheal smooth muscle appears to be predominantly mediated by tachykinin NK_2 receptors as evidenced by the significant contraction observed with the specific tachykinin NK_2 receptor agonist, [Nle^{10}]-NKA(4-10). However, tachykinin NK_1 receptors appear to participate, as the tachykinin NK_1 specific agonist, [Sar^9,$Met(O_2)^{11}$]-SP, did induce some contraction in tracheal smooth muscle. The lack of a contractile effect of senktide indicates tachykinin NK_3 receptors are not functionally present on ovine tracheal smooth muscle. The higher E_{max} for NKA compared to [Nle^{10}]-NKA(4-10) at equimolar concentrations is likely to be due to non-specific binding of NKA to tachykinin NK_1 receptors. However, in bronchial rings the predominant tachykinin receptor responsible for tachykinin induced contraction is the NK_1 receptor. The specific NK_2 receptor agonist, [Nle^{10}]-NKA(4-10), was a weak agonist on bronchial tissue, whereas, SP contractions, in the presence of phosphoramidon, were of similar magnitude and exhibited equipotency to the contractile responses elicited by [Sar^9,$Met(O_2)^{11}$]-SP.

The present tracheal smooth muscle data demonstrate that, *in vitro*, SP and NKA induced contractions are not mediated by a cholinergic mechanism, since their effect

remained unchanged in the presence of atropine. An important finding of this present study is that, in the trachea, the contractile response to the highly selective tachykinin NK_1 agonist [Sar^9,$Met(O_2)^{11}$]-SP was atropine sensitive and exhibited tachyphylaxis. This may reflect the activation of NK_1 facilitatory receptors at parasympathetic ganglia (Belvisi *et al.*, 1994) or on post-ganglionic nerves (Watson *et al.*, 1993). In ovine trachea, parasympathetic ganglia are concentrated between tracheal cartilaginous rings with post-ganglionic nerves passing into the trachealis muscle, these ganglia become less abundant in small bronchi, being located near bronchial divisions (Smith & Taylor, 1971).

In isolated human, guinea pig and hamster airways SP-induced contractions are reported to be via a direct effect, as antihistamines and antimuscarinic agents (atropine) have no effect on the contractile response (Maggi *et al.*, 1989; Uchida *et al.*, 1987; Lundberg *et al.*, 1983a). However, in isolated ferret trachea SP-induced contractions appear to be partially mediated by a cholinergic mechanism (Sekizawa *et al.*, 1987a; Sekizawa *et al.*, 1987b), while in the rabbit there are conflicting data for either a direct or indirect effect of tachykinins on ASM (Colasurdo *et al.*, 1995; Armour *et al.*, 1991; Tanaka & Grunstein, 1984). In all those studies SP, which lacks specificity, was the agonist used. The present data, using specific tachykinin NK_1 and NK_2 agonists show that the effect of SP on tracheal smooth muscle can be partitioned into two mechanisms. Firstly, SP acts via tachykinin NK_1 receptors associated with postganglionic nerves in tracheal smooth muscle, resulting in the release of ACh, and secondly by a direct effect on tracheal smooth muscle tachykinin NK_2 receptors.

It would appear that in *in vitro* studies the apparent absence or presence of a cholinergic mechanism in species is due to the different methodologies employed, which result in

changes to the relative contribution of the tachykinin NK_1 and NK_2 effects of SP on tracheal smooth muscle. In the rabbit, Tanaka and Grunstein (Tanaka & Grunstein, 1984) used small doses of SP (4-8 x 10^{-8} M) on tracheal smooth muscle which preferentially activate prejunctional tachykinin NK_1 receptors on cholinergic nerves rather than the smooth muscle tachykinin NK_2 receptors (Cook *et al.*, 1990) thus accentuating the cholinergic component. Armour and colleagues (Armour *et al.*, 1991), used higher doses of SP (10^{-5} M) which would tend to mask the small cholinergic effect of SP in rabbit tracheal smooth muscle via prejunctional tachykinin NK_1 receptors because of the overriding predominant effect of SP on tachykinin NK_2 receptors. Furthermore, in rabbit bronchial smooth muscle the atropine sensitive component disappeared indicating that SP-induced contractions were mediated by a direct action on both tachykinin NK_1 and NK_2 receptors (Cook *et al.*, 1990). Cholinergic pathways appear to be less important in smaller airways as cholinergic receptor density and sensitivity decrease from proximal to distal airways (Armour *et al.*, 1985; Barnes *et al.*, 1983), while tachykinin NK_1 receptor sensitivity and density increases from larger to smaller airways in the rabbit (Black *et al.*, 1992; Black *et al.*, 1990a) and guinea pig (Carstairs & Barnes, 1986).

Also of note is that in early studies on human bronchial smooth muscle that tachykinin-induced contractions were said to be solely mediated by tachykinin NK_2 receptors (Advenier *et al.*, 1992; Naline *et al.*, 1989), however, with the advent of more specific NK_1 receptor agonists a later study by the same authors, revealed NK_1 receptor-mediated contractions in small diameter human bronchial preparations. Moreover, they demonstrated that the contractile responses were small in magnitude, subject to tachyphylaxis and mediated by prostanoids (Naline *et al.*, 1996).

The present *in vitro* results demonstrate that the predominant action of SP and NKA is to induce contraction in tracheal smooth muscle via a direct effect on tachykinin NK_2 receptors, while in bronchial muscle SP induced contraction is via a direct mechanism involving NK_1 receptors with no cholinergic component. However, in *in vivo* sheep studies, SP administered intravenously is a more potent bronchoconstrictor than NKA, and the airway response is cholinergically mediated (Rice *et al.*, 2001; Parsons *et al.*, 1992; Corcoran & Haigh, 1992). It is postulated that, *in vivo*, exogenous tachykinins induce bronchoconstriction by the activation of NK_1 receptors on cholinergic nerves resulting in the prejunctional release of ACh from postganglionic nerves (Belvisi *et al.*, 1994; Watson *et al.*, 1993), and by the release of mast cell mediators, in addition to any direct contractile effect on ASM. Furthermore, the relative contributions of each of these mechanisms to the overall bronchoconstrictor response appears to differ between species (Joos *et al.*, 1994).

From the *in vivo* sheep studies, tachykinin-induced bronchoconstriction appears to be predominantly mediated by a tachykinin NK_1-receptor cholinergic mechanism given the tachykinin NK_1 antagonist and atropine sensitivity of the observed bronchoconstriction (Results, Chapter 3) (Rice *et al.*, 2001). The lack of a direct tachykinin NK_2-mediated bronchoconstrictor effect to SP *in vivo*, may simply reflect the relatively small amounts of SP reaching tachykinin tracheal NK_2 and bronchial NK_1 smooth muscle receptors, since a significant proportion of both the aerosol and intravascular SP doses would be inactivated by NEP and angiotensin-converting enzyme (Joos *et al.*, 1994). Both the present *in vitro* data and the *in vivo* results of Chapter 3 clearly demonstrate that SP induced ASM contraction was not mediated by mast cell histamine release (Lilly *et al.*, 1994; Joos *et al.*, 1988a), since the antihistamine, pyrilamine, was without effect.

The use of two consecutive CCRCs has been suggested as the most valid method for comparing the effect of different treatments on contractile responses. Contractions are normalized to a standard contraction, thus eliminating the confounding effects of variability in the actual magnitude of contraction (Black *et al.*, 1989; Marthan *et al.*, 1987). Using this experimental approach we have demonstrated, *in vitro*, that SP and NKA induced ASM contractions do not exhibit tachyphylaxis. The absence of tachyphylaxis to SP and NKA has also been observed in isolated hamster, ferret and guinea pig airways (Ireland *et al.*, 1991; Maggi *et al.*, 1989; Sekizawa *et al.*, 1987b), however, there is conflicting evidence for tachyphylaxis in rabbit airways (Armour *et al.*, 1991; Cook *et al.*, 1990; Tanaka & Grunstein, 1984). This discrepancy once again, is most likely due to the differences in experimental design (see above) which would change the relative contributions of the tachykinin NK_1 cholinergic effect (which does exhibit tachyphylaxis) in tracheal smooth muscle contractions induced by SP.

There is evidence that SP can influence airway responses by the production of both bronchoconstricting and bronchodilating prostaglandin from the airway epithelium (Devillier *et al.*, 1992). In view of the *in vivo* results of Chapter 3 where indomethacin pretreatment had no significant effect on the SP bronchoconstrictor response, indomethacin studies were not performed here. Therefore, it remains to be determined whether the effect cyclo-oxygenase inhibition by indomethacin, or epithelial desquamation can alter the *in vitro* contractility to tachykinins.

The results of this chapter also describe ASM responses to the "more classic" contractile agonists, carbachol, ACh and histamine. In ovine tracheal smooth muscle the rank order of potency was determined as carbachol > ACh > histamine. This being similar to that reported for isolated human bronchi (Knight *et al.*, 1990; Cerrina *et al.*,

1989; Advenier *et al.*, 1987) and rabbit trachea (Goroumaru-Shinkai *et al.*, 1992; Armour *et al.*, 1985). The pD_2 values for ACh and carbachol reported in this study are similar to those reported by others for ovine trachea (Jackowski *et al.*, 1993; Tomioka *et al.*, 1991). The reversal in potency rank observed between tracheal and parenchymal tissue for cholinergic agonists and histamine (i.e. histamine > carbachol = ACh) is in agreement with that documented by others in ovine (Kleinstiver & Eyre, 1979; Eyre, 1969), rabbit (Armour *et al.*, 1985) and guinea pig (Drazen & Schneider, 1978) airways.

The lack of a contractile effect for histamine in ovine bronchus has been well documented (Eyre, 1975; Eyre, 1973; Eyre, 1969). Furthermore, regional differences in histamine contractility throughout the tracheobronchial tree have been documented in other species such as the cat (Lulich *et al.*, 1976; Eyre, 1973), rabbit (Armour *et al.*, 1985; Fleisch & Calkins, 1976) and monkey (Nagai *et al.*, 1992). Whereas histamine is a weak to inactive contractile agonist in tracheal muscle, in bronchial segments histamine possesses significant contractile activity. The possible mechanism responsible for this effect is a greater abundance of H_2 and H_3 receptors mediating relaxation rather than H_1 contractile receptors (Kenakin & Beek, 1982; Chand *et al.*, 1979; Eyre, 1973).

Ovine tracheal and parenchymal smooth muscle did exhibit tachyphylaxis to histamine, a phenomenon that has been shown both, *in vivo* and *in vitro* in dogs (Antol *et al.*, 1988), However, in human isolated airways there are conflicting data (Knight *et al.*, 1995; Knight *et al.*, 1992; Black *et al.*, 1989). In human *in vitro* studies where histamine tachyphylaxis has been demonstrated, the mechanism involves stimulation of epithelial H_2-receptors (Knight *et al.*, 1992) with the subsequent release of the bronchodilating prostanoids, PGE_2 and prostacyclin (Knight *et al.*, 1995). Moreover the

absence of tachyphylaxis to histamine in isolated human airways appears to be associated with non-steroidal anti-inflammatory treatment in that patient group (Knight *et al.*, 1995). (Marthan *et al.*, 1987)*In vivo*, human subjects have demonstrated tachyphylaxis to inhaled histamine (Manning *et al.*, 1987), however, an absence of tachyphylaxis to histamine inhalation has also been observed (Ruffin *et al.*, 1981) and has been attributed to corticosteroid use in this group of subjects. The lack of a tachyphylactic effect to histamine in isolated ovine smooth muscle reported by Eyre (Eyre, 1969) may be explained by the fact that his study simply used single bolus doses of agonist whereas, I conducted formal cumulative concentration-response studies. This latter approach allows for a more detailed assessment of agonist efficacy and potency, without failing to detect tachyphylaxis.

The use of the lung parenchymal strip as an ASM preparation to study small airway pharmacology, has over the years gone in and out of favour (Hulsmann & de Jongste, 1993; Eyre & Mirbahar, 1981; Lulich *et al.*, 1976). However, it has been extensively used in studies of human (Finney *et al.*, 1984) and animal (both large and small) lung function (Armour *et al.*, 1985; Kleinstiver & Eyre, 1979; Drazen & Schneider, 1978). The present findings of marked regional difference between central tracheal muscle and parenchymal strip preparations are in accordance with those demonstrated by others (Armour *et al.*, 1985; Wagner *et al.*, 1985; Drazen & Schneider, 1978). Moreover, it is well recognized here, and by others that the parenchymal strip is not a "pure" ASM preparation, as other non airway contractile elements are present, such as small peripheral blood vessels (Finney *et al.*, 1984; Drazen & Schneider, 1978) and contractile interstitial cells (Kapanci *et al.*, 1974). Therefore, its use as a model ASM has been highly questioned (Hulsmann & de Jongste, 1993; Eyre & Mirbahar, 1981), in particular in the study of sympathomimetic drugs on airway muscle function due to the

non-airway components. For example, whether noradrenaline contracts or relaxes parenchymal strips is dependent on the ratio of vascular muscle to ASM (Hulsmann & de Jongste, 1993). Furthermore, the absence of contractile responses to tachykinins observed in the present study may reflect the net effect of tachykinin-induced contraction and relaxation of the airway and vascular smooth muscle present in the parenchymal preparation.

Overall, tissue sensitivity as reflected by the pD_2 values for the various contractile agonists studied in ovine tracheal smooth muscle are in closer agreement to those determined in isolated human bronchi (Qian *et al.*, 1994; Knight *et al.*, 1990; Naline *et al.*, 1989; Cerrina *et al.*, 1989) than for rabbit and guinea pig ASM (Goroumaru-Shinkai *et al.*, 1992; Devillier *et al.*, 1988; Advenier *et al.*, 1987) (Table 5.13). Thereby indicating that the ovine tracheal smooth muscle preparation may prove a useful model to study airway smooth muscle pharmacology in response to inflammatory mediators.

Chapter 6 Effect of IL-1β and TNFα on acetylcholine contractility in ovine tracheal smooth muscle

6.1 Introduction

Over the last decade it has been well recognized that asthma is characterized by increased cytokine production (Bradding *et al.*, 1994; Robinson *et al.*, 1992). Significantly higher protein levels of TNFα and IL-1β have been found in bronchial biopsy and lavage fluid from symptomatic atopic and non-atopic asthmatics compared to asymptomatic subjects (Ackerman *et al.*, 1994; Broide *et al.*, 1992), suggesting an upregulation of these two cytokines, at least in acute asthmatic exacerbations. Although it is likely a number of cytokines are able to induce AHR and different cytokines may assume greater importance in different subsets of asthma, TNFα and IL-1β will be the cytokines of choice to study this phenomenon.

Interleukin-1β and TNFα are co-localised in mast cells and macrophages and are released following IgE receptor stimulation (Gosset *et al.*, 1999; Gordon *et al.*, 1990). Recently, Ammit and colleagues (Ammit *et al.*, 1997) have shown that significant numbers of mast cells reside in ASM of asthmatics. Once released, these cytokines would result in high localised tissue concentrations, which in turn may upregulate the responsiveness of adjacent ASM. In view of this possibility, very few studies have examined the effect of IL-1β and TNFα on the contractile mechanism of ASM. This may simply reflect the earlier opinions that there is no difference in contractile property

of ASM between normal and asthmatic subjects (Armour *et al.*, 1984a; Roberts *et al.*, 1984; Vincenc *et al.*, 1983).

In vivo AHR has been demonstrated in sheep following TNFα infusion (Wheeler *et al.*, 1990). Whether this AHR results from a direct effect of the cytokine on ASM contractility, or via changes in airway wall structure leading to exaggerated airway narrowing, was not determined. In other animal studies the effects of TNFα and IL-1β on *in vitro* smooth muscle contractility are limited with conflicting results (Pennings *et al.*, 1998; Kips *et al.*, 1993; Wills-Karp *et al.*, 1993b). With this in mind, experiments were designed to determine if TNFα and IL-1β could increase ASM contractility, *in vitro*.

6.2 Methods

6.2.1 General

Tracheal smooth muscle strips were obtained from merino ewes as described in Chapter 2 - Methods, Section 2 3.1. Tissues from nine animals were used in each experimental group. Strips of tracheal smooth muscle (3-5 mm x 20-25 mm) were dissected from the posterior portion of the trachea above the origin of the right upper lobe. (Refer Chapter 2 – Methods, Section 2.3.2.1.)

6.2.2 Tissue incubation with TNFα and IL-1β

The isolated tracheal smooth muscle strips were incubated for 18 hours at room temperature in DMEM, supplemented with antibiotics (penicillin 100 U/ml and streptomycin 100 *u*g/ml, Sigma), with differing concentrations of IL-1β and TNFα, in

an atmosphere of 5% CO_2: 95% O_2. Paired tissue samples were incubated in medium alone under the same conditions and used as controls (Figure 6.1).

Following incubation the tracheal smooth muscle strips were prepared for cumulative concentration-response studies as described in Chapter 2 - Methods, Section 2.3.3. These studies were commenced within 24 hours of excision. The tracheal strips were suspended under tension between 1.5 and 2.5 gms.

6.2.3 Effect of incubation with TNFα and IL-1β on ACh contractility

At the end of the equilibration period, each tracheal segment was exposed to 10^{-4} M ACh for 1 min, after which, the tissues were washed every 20 mins until baseline tension was re-established. Cumulative concentration-response curves were then obtained for ACh. Once the cumulative concentration-responses were obtained, the tissues were washed every 20 mins until baseline tone was re-established (usually 60-90 mins). When achieved, a reference contraction to 10^{-3} M ACh was obtained.

This series of experiments were designed to determine whether IL-1β and/or TNFα affect ACh contractility in ovine tracheal smooth muscle and whether synergism exists. A 'chequer-board' series of experiments was conducted (Figure 6.2), employing a range of cytokine concentrations, considered biologically effective (Wills-Karp *et al.*, 1993b) (Horie *et al.*, 1996; Munakata *et al.*, 1996; Tamaoki *et al.*, 1994), alone and in combination.

6.2.4 Analysis of results

In each tracheal smooth muscle preparation, contractile responses to each ACh concentration studied was expressed as a percentage of the contractile response to

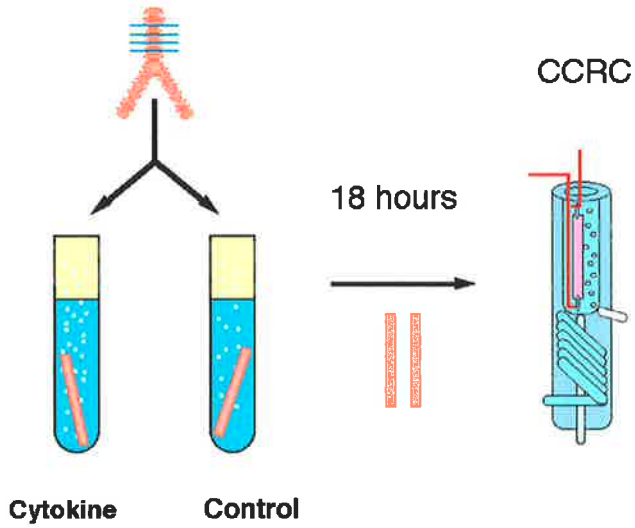


Figure 6.1 Schematic representation of experiment protocol, for tissue incubations and cumulative dose response studies.

TNFα ng/ml

IL-1β ng/ml	0	10	100	500
0				
1				
10				
100				

Figure 6.2 Chequer-board array of cytokine concentrations

ACh (10^{-3} M). Cumulative concentration-response curves were constructed and from which E_{max} and EC_{50} values calculated as described in Chapter 2 – Methods, Section 2.3.4.1.

Statistical analyses were performed using two-tailed paired Student's *t*-test. Values were considered significant at $P < 0.05$ level.

6.2.5 Reagents

Stock solutions of human recombinant TNFα, IL-1β (Promega, Madison, WI, USA), and ACh, (Sigma Chemical, St Louis, MO, USA) were prepared in sterile water and aliquots kept at -20°C. On each study day, serial dilutions of ACh were prepared in K-H solution and kept on ice for the duration of the experiment. Plastic vials and pipette tips were used to store and handle all reagents.

6.3 Results

6.3.1 Effect of TNFα and IL-1β incubation on ACh contractility

The effect of incubating ovine tracheal muscle strips with differing cytokine concentrations alone or in combination on ACh contractility is shown in Figure 6.3. This figure was compiled to allow easy recognition of the effect of cytokine concentrations on ASM contractility. Note, for closer inspection, each graph is displayed in a larger format in Appendix A.

Incubation with IL-1β alone, at all concentrations used, had no effect on ASM contractility to ACh. E_{max} and EC_{50} values were not significantly different between control and IL-1β treated tissue, Table 6.1 and Table 6.2, respectively. When tracheal strips were pre-incubated with TNFα only, a significant increase in E_{max} was observed

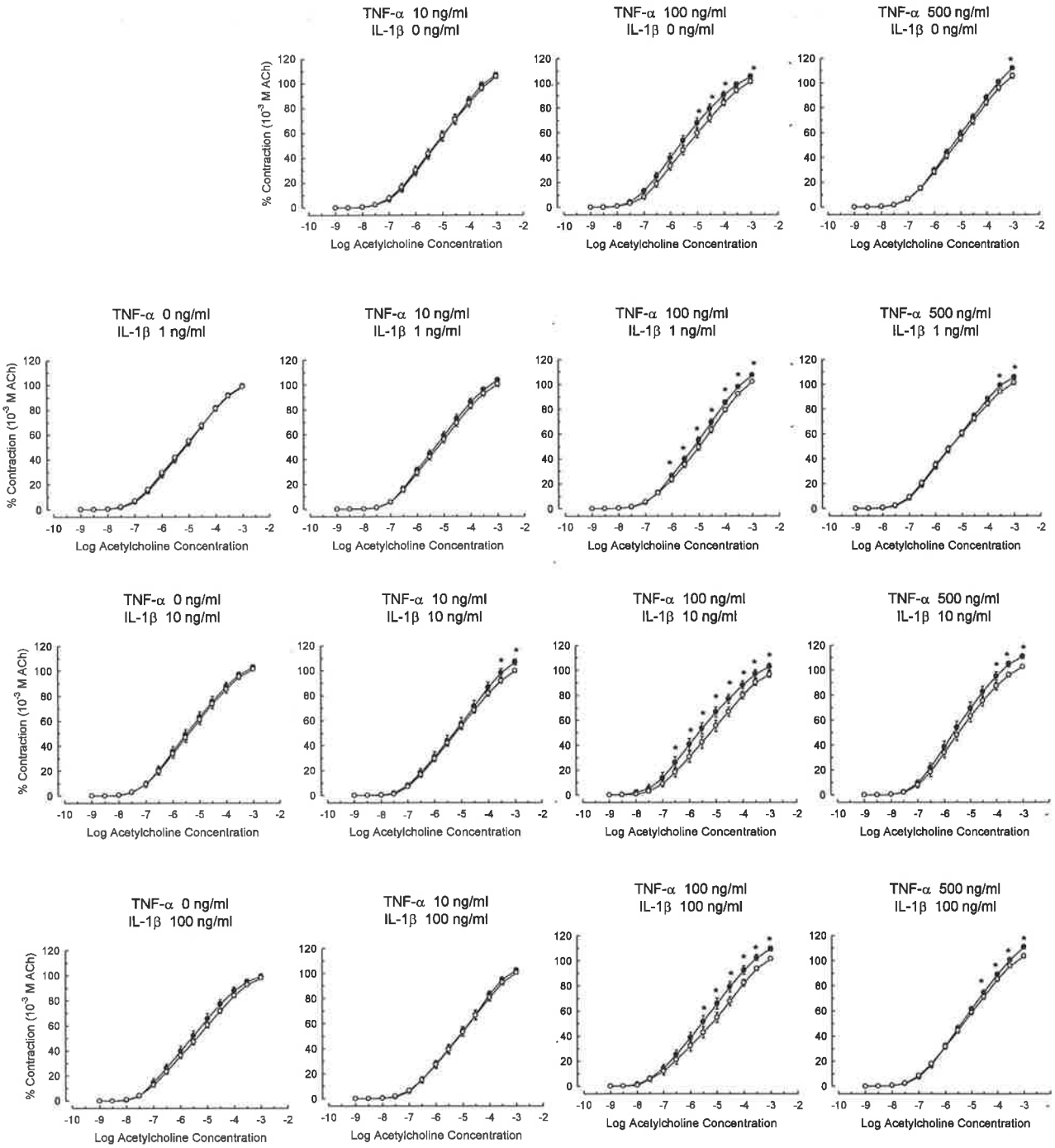


Figure 6.3 Mean cumulative concentration response curve for acetylcholine on tracheal smooth muscle strips in control (○) and cytokine pretreated tissue (●) (note different IL-1β and TNFα). Mean responses are from 9 animals, expressed as a percentage of the reference contraction to 10^{-3} M ACh. Vertical bars indicate SEM. * Denotes a statistically significant difference between control and cytokine treated tissues ($P < 0.05$, paired *t*-test).

Table 6.1 Mean maximal effect (E_{max}) for acetylcholine in control and cytokine treated ovine tracheal strips.

IL-1β Concentration			TNFα Concentration			
			0 ng	10 ng	100 ng	500 ng
	0 ng	Control		106.0 ± 1.7	101.6 ± 1.9	105.9 ± 2.2
		Treatment		107.2 ± 1.7	105.6 ± 1.7 $P < 0.05$	112.0 ± 1.2 $P < 0.05$
	1 ng	Control	98.9 ± 1.1	100.7 ± 2.2	102.4 ± 1.2	101.5 ± 1.6
		Treatment	99.6 ± 1.1	104.1 ± 1.1	107.8 ± 1.2 $P < 0.01$	106.0 ± 1.8 $P < 0.05$
	10 ng	Control	101.6 ± 1.4	99.9 ± 1.4	96.8 ± 2.3	102.9 ± 0.8
		Treatment	103.2 ± 1.6	106.8 ± 2.4 $P < 0.01$	102.7 ± 2.9 $P < 0.05$	111.0 ± 1.7 $P < 0.001$
	100 ng	Control	98.1 ± 1.1	100.5 ± 1.1	101.5 ± 1.1	103.8 ± 1.6
		Treatment	99.5 ± 1.4	102.4 ± 1.6	109.6 ± 1.8 $P < 0.0005$	111.0 ± 1.6 $P < 0.01$

Mean E_{max} ± SEM expressed as a percentage of the response to 10^{-3} M acetylcholine. Mean responses are from 9 animals.

P values shown when significant differences were obtained (Paired *t*-test).

Table 6.2 Mean EC_{50} † values for acetylcholine in control and cytokine treated ovine tracheal strips.

			TNFα Concentration			
			0 ng	10 ng	100 ng	500 ng
IL-1β Concentration	0 ng	Control		6.21×10^{-6} (2.95×10^{-6}, 1.31×10^{-6})	4.50×10^{-6} (2.51×10^{-6}, 8.07×10^{-6})	7.69×10^{-6} (5.46×10^{-6}, 1.08×10^{-5})
		Treatment		7.25×10^{-6} (3.97×10^{-6}, 1.32×10^{-5})	2.67×10^{-6} (1.28×10^{-6}, 5.57×10^{-6})	7.62×10^{-6} (4.85×10^{-6}, 1.20×10^{-5})
	1 ng	Control	5.99×10^{-6} (3.94×10^{-6}, 9.10×10^{-6})	6.10×10^{-6} (3.72×10^{-6}, 1.00×10^{-5})	1.11×10^{-5} (6.55×10^{-6}, 1.87×10^{-5})	3.95×10^{-6} (2.22×10^{-6}, 7.05×10^{-6})
		Treatment	6.91×10^{-6} (4.12×10^{-6}, 1.16×10^{-5})	5.86×10^{-6} (3.39×10^{-6}, 1.01×10^{-5})	8.90×10^{-6} (5.73×10^{-6}, 1.38×10^{-5})	4.52×10^{-6} (2.65×10^{-6}, 7.71×10^{-6})
	10 ng	Control	4.15×10^{-6} (2.04×10^{-6}, 8.45×10^{-6}	5.08×10^{-6} (3.71×10^{-6}, 9.03×10^{-6})	5.07×10^{-6} (2.09×10^{-6}, 1.23×10^{-5})	3.91×10^{-6} (1.90×10^{-6}, 8.02×10^{-6})
		Treatment	3.86×10^{-6} (1.82×10^{-6}, 8.19×10^{-6})	7.05×10^{-6} (3.53×10^{-6}, 1.41×10^{-5})	2.41×10^{-6} * (1.01×10^{-6}, 5.73×10^{-6})	3.60×10^{-6} (1.69×10^{-6}, 7.68×10^{-6})
	100 ng	Control	3.53×10^{-6} (2.22×10^{-6}, 5.62×10^{-6})	7.56×10^{-6} (4.09×10^{-6}, 1.39×10^{-5})	6.13×10^{-6} (2.89×10^{-6}, 1.30×10^{-5})	5.52×10^{-6} (3.89×10^{-6}, 7.84×10^{-6})
		Treatment	2.47×10^{-6} (1.27×10^{-6}, 4.81×10^{-6})	7.90×10^{-6} (4.07×10^{-6}, 1.53×10^{-5})	3.69×10^{-6} (1.71×10^{-6}, 7.97×10^{-6})	7.26×10^{-6} (3.46×10^{-6}, 1.52×10^{-5})

† Geometric mean EC_{50} values and 95% confidence limits (in parenthesis). Mean responses are from 9 animals.

* $P < 0.05$, significantly different between control and cytokine treated tissue (paired *t*-test).

at concentration of 100 ng/ml and 500 ng/ml (Table 6.1). However, TNFα by itself did not produce a significant leftward shift in the CCRC to ACh as reflected by the EC_{50} values (Table 6.2).

When TNFα and IL-1β were used in combination a more consistent hyperresponsive effect on ACh contractility was observed. Pre-incubation with a combination of IL-1β and TNFα at concentrations ≥ 1ng/ml and ≥ 100 ng/ml, respectively, significantly increased E_{max}, Table 6.1. However, only the combination of 10 ng/ml IL-1β and 100 ng/ml TNFα caused a significant leftward shift in the CCRC to ACh, Table 6.2. Furthermore, incubations with 100 ng/ml TNFα in combination with IL-1β demonstrated synergism in the ability of TNFα to induce smooth muscle hyper-responsiveness to ACh, Table 6.3. For example, using an arbitrary scale for the number of significant differences in the CCRC between control and cytokine treated tissue it can be seen that 100 ng/ml TNFα induced some changes in the CCRC. The number of significant differences increase with the addition of IL-1β to the incubation media, despite no demonstrable differences in the CCRC with IL-1β incubation alone.

6.4 Discussion

The development of AHR has been linked to the presence of airway inflammation (Hogg *et al.*, 1991). However, the mechanism by which inflammation leads to AHR remains elusive. Tumour necrosis factor-α is a ubiquitous cytokine in inflammation, and has been postulated to have a fundamental role in airway inflammation in asthma and the development of AHR (Shah *et al.*, 1995; Kips *et al.*, 1993a). Whether the

Table 6.3 Summary of the significant differences in the cumulative concentration response curves to acetylcholine in control and cytokine treated ovine tracheal smooth muscle strips.

		TNF-α			
		0 ng	**10 ng**	**100 ng**	**500 ng**
IL-1β	**0 ng**			++	+
	1 ng			++++	+
	10 ng		+	+++++	++
	100 ng			+++	++

\+ Arbitrary scale for the number of statistically significant differences in the cumulative concentration response curves for cytokine treated smooth muscle and their respective controls.

observed increase in AHR, following *in vivo* exposure to TNFα and IL-1β (Thomas *et al.*, 1995; Tsukagoshi *et al.*, 1994; Wheeler *et al.*, 1990) is due to an increased sensitivity of ASM itself to various contractile agonists, or via changes in airway wall structure leading to exaggerated airway narrowing, is not known. Potentially both mechanisms may co-exist.

In animal studies the effects of TNFα and IL-1β on *in vitro* contractility are limited with varying results (Pennings *et al.*, 1998; Hakonarson *et al.*, 1996; Koto *et al.*, 1996; Munakata *et al.*, 1996; Tamaoki *et al.*, 1994; Kips *et al.*, 1993). Whereas, in isolated human airways, *in vitro* induction of AHR by TNFα has been demonstrated to electrical field stimulation but not to contractile agonists *per se* (Anticevich *et al.*, 1995). Moreover, in a more recent study by the same group, TNFα was shown to potentiate the contractile response of human bronchial rings to ACh. However, they did not observe any effect with 1L-1β, either alone or in combination with TNFα (Sukkar *et al.*, 2001). It is widely accepted that IL-1β and TNFα are co-localised and released upon cell activation (Gordon *et al.*, 1990), and act synergistically to mediate various inflammatory responses (John *et al.*, 1998; Hakonarson *et al.*, 1996; Wesselius *et al.*, 1995; Kondepudi & Johnson, 1993). Given this premise, tracheal smooth muscle strips were co-incubated with TNFα and/or IL-1β.

The results of the series of experiments conducted in this chapter clearly demonstrate that normal ASM becomes hyperresponsive to ACh following combined exposure to IL-1β and TNFα, while ASM hyperresponsiveness was observed in tissue incubated with 100 ng/ml TNFα, synergism was demonstrated when IL-1β was included in the incubation media. Co-incubation with IL-1β (10 ng/ml) and TNFα (100 ng/ml)

increased both the sensitivity (decreased EC_{50}) and responsiveness (increased E_{max}) of ASM to ACh. Furthermore, the observed effective concentration of TNFα and IL-1β of the present study are in agreement with cytokine concentrations found by others to be considered maximally effective in various biological systems (Hakonarson *et al.*, 1996; Horie *et al.*, 1996; Anticevich *et al.*, 1995; Tamaoki *et al.*, 1994; Wills-Karp *et al.*, 1993b). The present data also add to the literary evidence that cytokines do induce cholinergic AHR, *in vitro* (Pennings *et al.*, 1998; Hakonarson *et al.*, 1996; Anticevich *et al.*, 1995). The failure therefore, of several recent studies to demonstrate enhanced muscarinic smooth muscle contractility may simply reflect isolated airways being exposed to a single cytokine rather than in combination (Koto *et al.*, 1996; Munakata *et al.*, 1996; Anticevich *et al.*, 1995; Wills-Karp *et al.*, 1993b).

Although, in the present study, no attempt has been made to elucidate the mechanisms by which cytokines induce AHR in normal ovine tracheal smooth muscle, enhanced contractility may result from an alteration in or increased number of contractile cell surface receptors (Mak *et al.*, 2000; Katsunuma *et al.*, 1999). There is increasing evidence that the proliferative actions of TNFα are mediated via specific TNFα-p55 receptors on ASM and that the activation of these receptors augments the transient increases in concentration of $[Ca^{++}]_i$ induced by contractile agonists (Amrani *et al.*, 1996; Amrani *et al.*, 1995a). Acetylcholine mediates contractions via M_3 smooth muscle receptors, and involves agonist-stimulated hydrolysis of PIP_2 to the second messenger IP_3. This leads to increased levels of $[Ca^{++}]_i$, activation of MLCK, resulting in cross-linking of the cellular contractile elements and contraction (Chilvers *et al.*, 1994; Schramm & Grunstein, 1992). Therefore, it is tempting to speculate that the observed increase in smooth muscle contractility may be due to "functional augmentation" of the contractile process, that is, cytokines may enhance post-receptor

intracellular signalling mechanisms which regulate ASM contraction. Nevertheless, the present data demonstrate that TNFα enhances ACh contractility in ovine tracheal smooth muscle and that this effect is synergistic in the presence of IL-1β. An examination of possible mechanisms involved in this induced *in vitro* hyper-responsiveness forms the basis of the following chapter.

Chapter 7 Cytokines enhance airway smooth muscle contractility in response to ACh and NKA

7.1 Introduction

The early failure to demonstrate a relationship between *in vivo* and *in vitro* AHR (de Jongste *et al.*, 1987b; Cerrina *et al.*, 1986; Armour *et al.*, 1984; Armour *et al.*, 1984a; Roberts *et al.*, 1984; Vincenc *et al.*, 1983), has lead to the suggestion that AHR is an *in vivo* phenomenon due to airway wall remodeling, rather than an abnormality of the intrinsic properties of the ASM itself. However, *in vitro* hyperresponsiveness, as measured by isometric contractions, can be induced by antigen exposure, bacterial endotoxin or viral infection (Molimard *et al.*, 1998; Colasurdo *et al.*, 1995; Folkerts & Nijkamp, 1990; Saban *et al.*, 1987), by the addition of activated inflammatory cell supernatants and inflammatory products to organ baths (Anticevich *et al.*, 1996; Folkerts *et al.*, 1992), and *in vitro* exposure to various oxidizing agents (Marthan *et al.*, 1996). There is now accumulating evidence suggesting that sensitization alters ASM biochemistry and contractility (Ammit *et al.*, 2000; Jiang *et al.*, 1992; Rao *et al.*, 1991). Passive sensitization of human ASM induces *in vitro* AHR possibly via alterations in Ca^{++} mobilization (Marthan *et al.*, 1992; Black *et al.*, 1989) and/or enhanced PKC activation (Rossetti *et al.*, 1995).

While mast cells degranulate via IgE coupling to FcεRI, mediator release can also occur by non-IgE dependent stimuli, such as bradykinin, neuropeptides (Cross *et al.*, 1997; Heaney *et al.*, 1995), and nerve stimulation (Bienenstock *et al.*, 1991). For many years

the importance of the mast cell in asthma centered on its involvement in the acute allergic bronchoconstrictor response mediated via the release of histamine, prostaglandins and leukotrienes (Lazarus, 1987). More recently attention has been paid to the effect of tryptase on ASM function. Tryptase has been shown to enhance *in vitro* smooth muscle contractility to histamine in human and canine bronchi (Berger *et al.*, 1999; Johnson *et al.*, 1997; Sekizawa *et al.*, 1989). However, it is now clearly evident that mast cells also secrete a variety of preformed and newly synthesized cytokines (Bradding & Holgate, 1996) which in turn play a major role in the prolonged inflammatory phase following allergen exposure (Wasserman, 1994).

In the preceding chapter it was found that *in vitro* hyperresponsiveness could be induced in normal ovine tracheal smooth muscle following incubation with IL-1β and TNFα. Recent evidence suggests that IL-1β and TNFα cell signalling is mediated by the activation of certain protein kinases, phosphoinositide hydrolysis and increases in $[Ca^{++}]_i$ (Lazaar *et al.*, 1998; De *et al.*, 1996; Amrani *et al.*, 1995b) which are similar to the signalling pathways involved in ASM contraction (Chilvers *et al.*, 1994; Schramm & Grunstein, 1992). Thus it may be postulated that the observed *in vitro* hyper-responsiveness to ACh may involve alterations in intracellular signal transduction pathways (Amrani & Panettieri, 1998). Therefore, the experiments of the previous chapter have been extended to examine the effects of combined cytokines on *in vitro* ASM contractility to NKA and histamine, and to investigate possible mechanisms that may be involved in the induced *in vitro* hyperreactivity.

7.2 Methods

7.2.1 General

Tracheal smooth muscle strips were obtained from merino ewes as described in

Chapter 2 - Methods, Section 2.3.1. Strips of tracheal smooth muscle (3-5 mm x 20-25 mm) were dissected from the posterior portion of the trachea above the origin of the right upper lobe.

7.2.2 Tissue incubation

The isolated tracheal smooth muscle strips were incubated for 18 hours at room temperature in DMEM, supplemented with antibiotics (penicillin 100 U/ml and streptomycin 100 *u*g/ml, Sigma), with a combination of IL-1β (10 ng/ml) and TNFα (100 ng/ml), in an atmosphere of 5% CO_2: 95% O_2 gas. Paired tissue samples were incubated in medium alone under the same conditions and used as controls (Figure 7.1). Whenever possible paired tissue samples were studied in duplicate (Chapter 2 - Methods, Figure 2.9).

Following incubation the tracheal smooth muscle strips were prepared for cumulative concentration-response studies as described in Chapter 2 - Methods, Section 2.3.3. These studies were commenced within 24 hours of excision. The tracheal strips were suspended under tension between 1.5 and 2.5 gms.

7.2.3 Effects of agonists

At the end of the equilibration period, each tracheal segment was exposed to 10^{-4} M ACh for 1 min, after which, the tissues were washed every 20 mins until baseline tension was re-established. Cumulative concentration-response curves were then obtained for ACh, NKA and histamine. Once the cumulative concentration-responses were obtained, the tissues were washed every 20 mins until baseline tone was re-established (usually 60-90 mins). When achieved, a reference contraction to 10^{-3} M ACh was obtained.

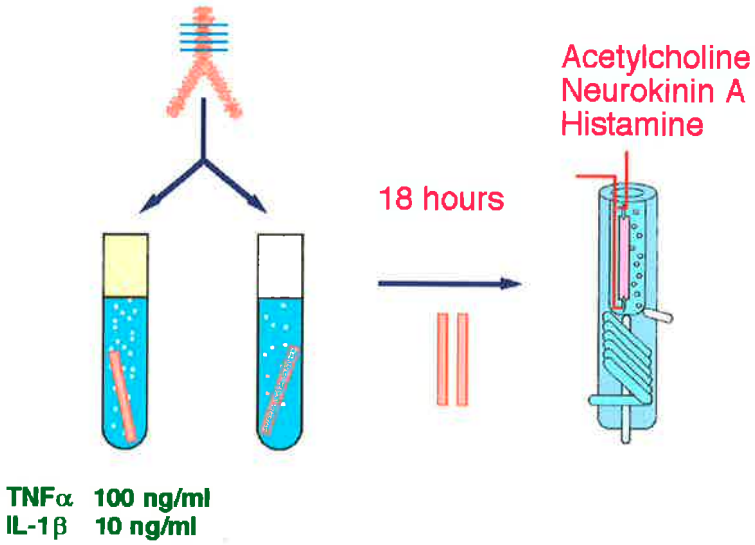


Figure 7.1 Schematic representation of experiment protocol, for tissue incubations and cumulative dose response studies to acetylcholine, neurokinin A and histamine.

7.2.4 Effect of specific M_3 and NK_2 receptor antagonists on ACh and NKA contractility

To establish whether the cytokine enhanced muscle contractility to ACh and NKA may result from a functional alteration to specific M_3 and NK_2 receptors, a series of experiments was conducted using specific M_3 and NK_2 receptor antagonists. The aim of which was to determine if cytokine exposure produced a change in the pA_2 values for the specific antagonist, possibly reflecting a functional change in the M_3 and NK_2 receptor characteristics. In this series of experiments two successive CCRCs were obtained for each agonist. It has previously been shown in Chapter 5 that successive cumulative concentration responses ACh and NKA do not exhibit tachyphylaxis (Reynolds *et al.*, 1998).

Concentration-response curves to ACh were first obtained. After these control measurements, the strips were washed and allowed to return to their resting tension. A second concentration-response curve to ACh was then performed in the presence of 4-DAMP (a specific M_3 receptor antagonist, 10^{-9}, 10^{-8}, 10^{-7} M). Each strip was exposed to only one concentration of the antagonist. At least 90 mins were allowed to elapse between the first and the second concentration-response curve. Similar experiments were performed to NKA, except that phosphoramidon (10^{-6} M) was added to the bath 30 mins before the first and second concentration-response curve. The second concentration-response curve to NKA was performed in the presence of the specific NK_2 receptor antagonist, GR94800 (McElroy *et al.*, 1992) ($3x10^{-9}$, 10^{-8}, $3x10^{-8}$ M). Antagonist contact time was 40 mins for 4-DAMP and GR94800.

7.2.5 Effect of neutral endopeptidase inhibition

In this series of experiments, when baseline tension was re-established following the preconditioning ACh exposure, the NEP inhibitor, phosphoramidon (10^{-5} M, final bath concentration), was added to the organ baths. After a 30 min incubation period, cumulative concentration-responses were obtained for NKA.

7.2.6 Calcium-free Krebs-Henseleit solution

For these experiments, the contractile responses to contractile agonists were elicited in the absence of extracellular Ca^{++} as described in Chapte 2 - Methods, Section 2.3.3.5.

7.2.7 Analysis of results

In each tracheal smooth muscle preparation, contractile responses to each agonist concentration studied was expressed as a percentage of the contractile response to ACh (10^{-3} M) except for the M_3 and NK_2 receptor antagonist experiments (see below). From these CCRCs E_{max}, EC_{50}, and pD_2 values calculated as described in Chapter 2 - Methods, Section 2.3.4.1. The pA_2 values for GR94800 (NK_2 antagonist) and 4-DAMP (M_3 antagonist) with NKA and ACh as the respective agonist were calculated as described in Chapter 2 - Methods, Section 2.3.4.2 - Consecutive cumulative concentration-response curves. Statistical analyses were performed using two-tailed paired Student's *t*-test. Values were considered significant at $P < 0.05$ level.

7.2.8 Reagents

All drugs, compounds and solutions including the K-H used in the following experiments are listed, prepared and used according to the methods described in Chapter 2 - Methods, Section 2.6.2 Reagents.

7.3 Results

7.3.1 Effects of agonists

Pre-incubation with TNFα and IL-1β caused a significant leftward shift in, and an increase in the magnitude of, the concentration-response curves to both ACh and NKA (Figure 7.2A-B, respectively). Whereas, histamine contractility remained unchanged following TNFα and IL-1β incubation (Figure 7.3). E_{max} with and without incubation with cytokines for ACh was 104.4% ± 2.7% (± SEM) and 96.3% ±2.5%, respectively, ($P < 0.001$) (Figure 7.2A), while that for NKA was 58.1% ± 5.6% and 50.4% ± 4.7%, respectively ($P < 0.01$) (Figure 7.2B). E_{max} with and without incubation with cytokines for histamine was 45.2% ± 8.3% and 42.8% ±8.7%, respectively. There was a significant leftward shift in the CCRC to ACh and NKA compared to their respective control strips as reflected in the lower EC_{50} values (Table 7.1) and higher pD_2 values. Mean pD_2 values for ACh and NKA with and without cytokines were 5.66 ± 0.16 vs 5.35 ± 0.13, ($P < 0.001$), and 7.17 ± 0.12 vs 6.93 ± 0.13 ($P < 0.01$), respectively.

7.3.2 Effect of specific M_3 and NK_2 receptor antagonists on ACh and NKA contractility

Both antagonists tested produced a concentration-dependent rightward shift of their respective agonist CCRCs. Figure 7.4 shows the antagonism of GR94800, at various concentrations, to NKA in control (A) and cytokine (B) treated muscle strips. While Figure 7.5 demonstrates the progressive rightward shift of ACh in the presence of the M_3 antagonist, 4-DAMP, on control (A) and cytokine exposed (B) tissue. The generated Schild plots for GR94800 antagonism to NKA in control and cytokine exposed smooth muscle are shown in Figure 7.6. There was no significant difference in the slopes between control and treated tissue being -1.15 (95% confidence limits -1.79, -0.52) and -1.23 (95% confidence limits -1.79, -0.75), respectively. Schild plots for the

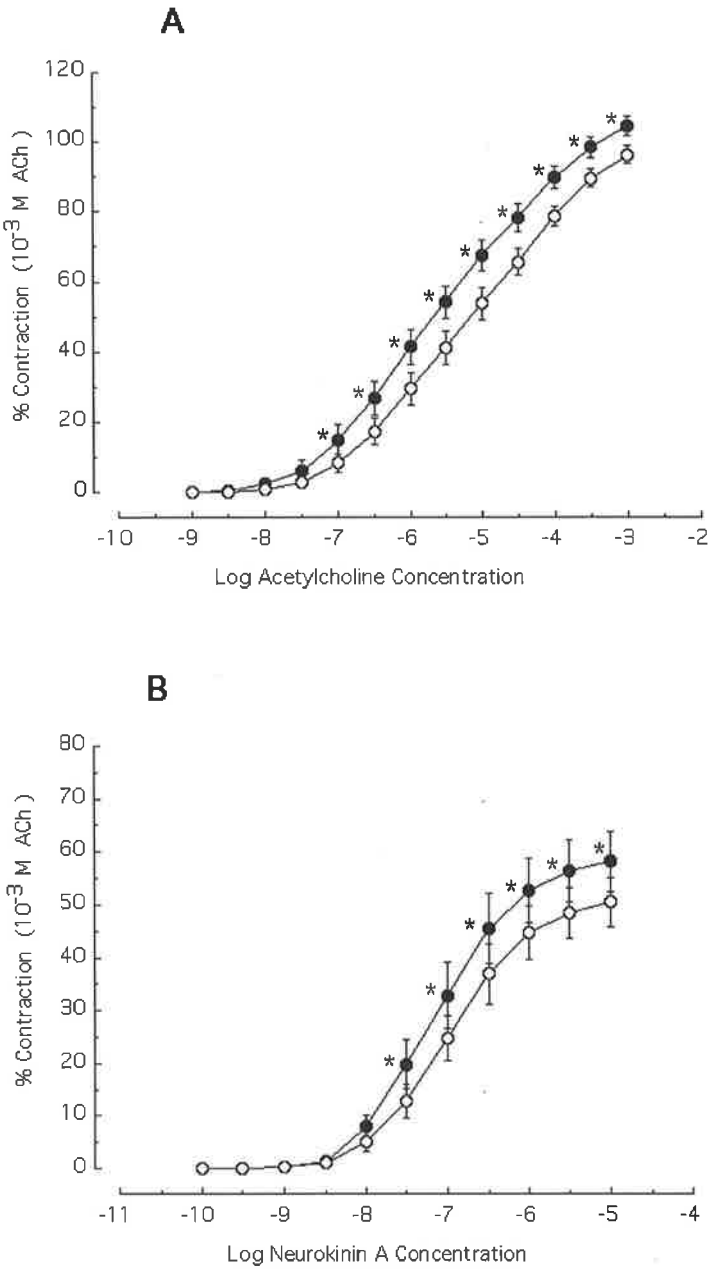


Figure 7.2 Mean cumulative concentration-response curves for acetylcholine (A) and neurokinin A (B) in control (○) and cytokine pretreated (IL-1β; 10 ng/ml, TNFα; 100 ng/ml) tracheal smooth muscle (●). Mean responses are from 9 animals, expressed as a percentage of the reference contraction to 10^{-3} M ACh. Vertical bars indicate SEM. * Denotes a statistically significant difference between control and cytokine treated tissues ($P < 0.05$, paired *t*-test).

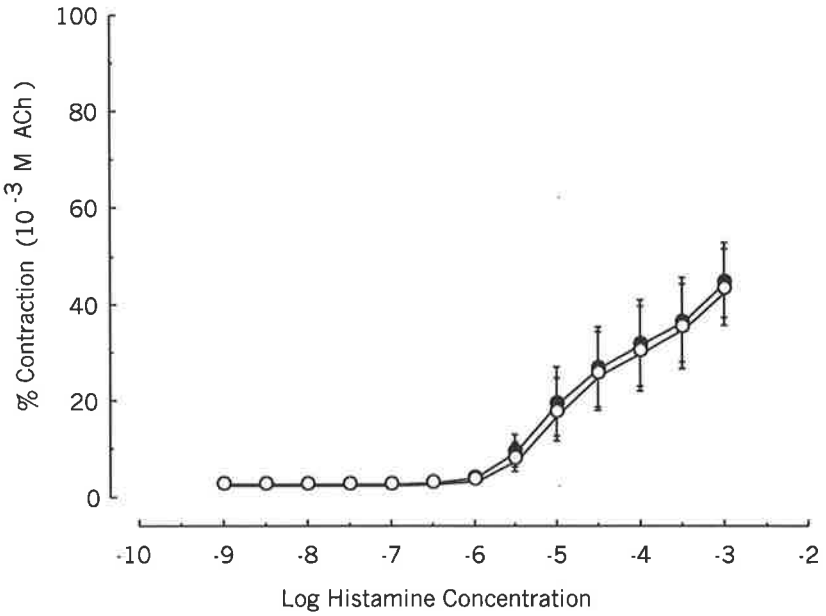


Figure 7.3 Mean cumulative concentration-response curves for histamine in control (○) and cytokine pretreated (IL-1β; 1 ng/ml, TNFα; 100 ng/ml) smooth muscle (●). Mean responses are from 7 animals, expressed as a percentage of the reference contraction to 10^{-3} M ACh. Vertical bars indicate SEM.

Table 7.1 Mean EC_{50} values for agonists in control and cytokine treated ovine tracheal strips.

Agonist	n	EC_{50}† (M) Control	EC_{50} †(M) TNFα, IL-1β
Acetylcholine	9	4.46×10^{-6} (2.24×10^{-6} , 8.88×10^{-6})	2.20×10^{-6} * (9.20×10^{-7} , 5.25×10^{-6})
Neurokinin A	9	1.17×10^{-7} (5.73×10^{-8} , 2.40×10^{-7})	6.75×10^{-8} * (3.62×10^{-8} , 1.26×10^{-7})
Histamine	7	NC	NC

† Geometric mean EC_{50} values and 95% confidence limits (in parenthesis). n = number of animals. NC, not calculated as agonist contractile response at 10^{-3} M still linear. $P < 0.01$, significantly different between control and cytokine treated tissue (paired *t*-test).

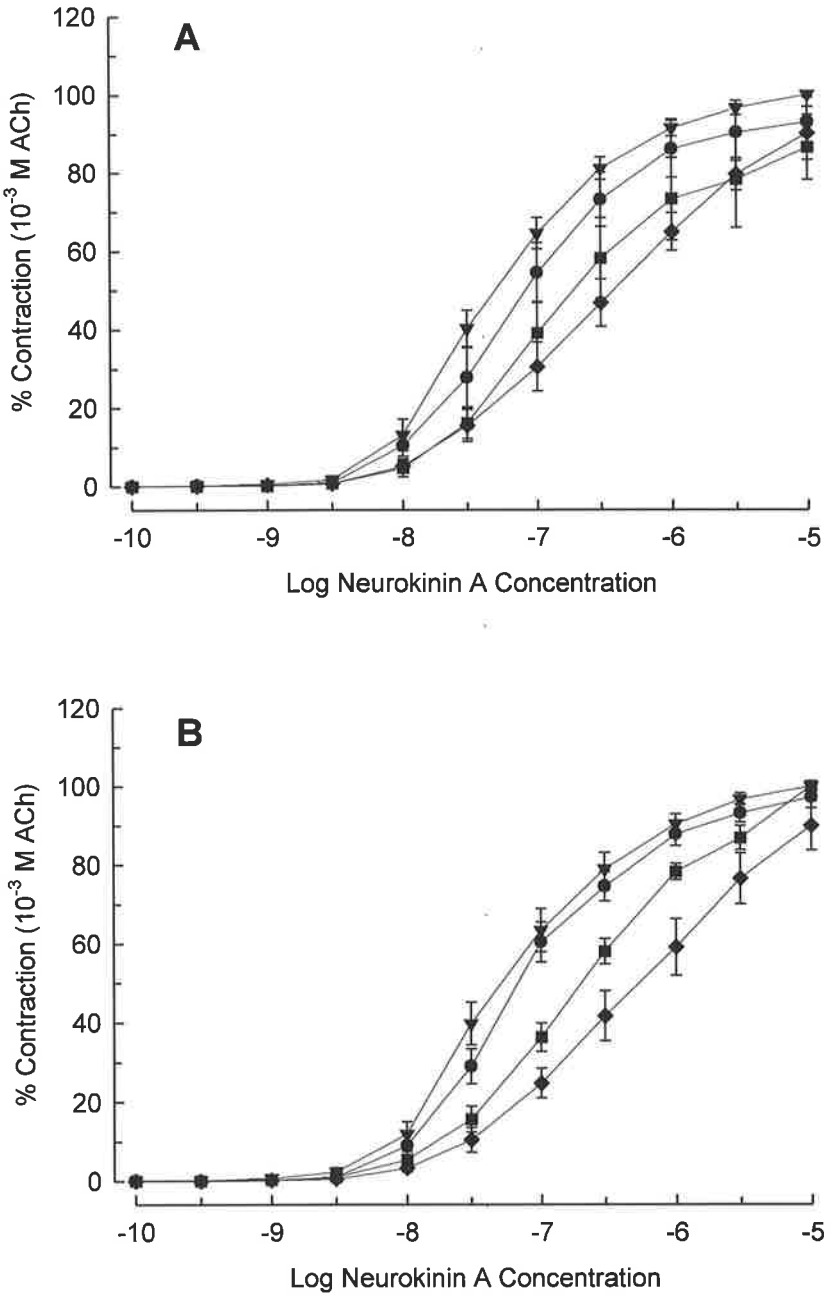


Figure 7.4 Mean cumulative concentration-response curves for NKA (in the presence of 10^{-6} M phosphoramidon) in the absence (▼) and presence of increasing concentration of the NK_2 antagonist, GR94800, (●) $3x10^{-9}$ M; (■) 10^{-8} M; (◆) $3x10^{-8}$ M, in control (A) and cytokine pretreated (B) tracheal smooth muscle strips. Each value is the mean of five animals. Vertical bars indicate SEM.

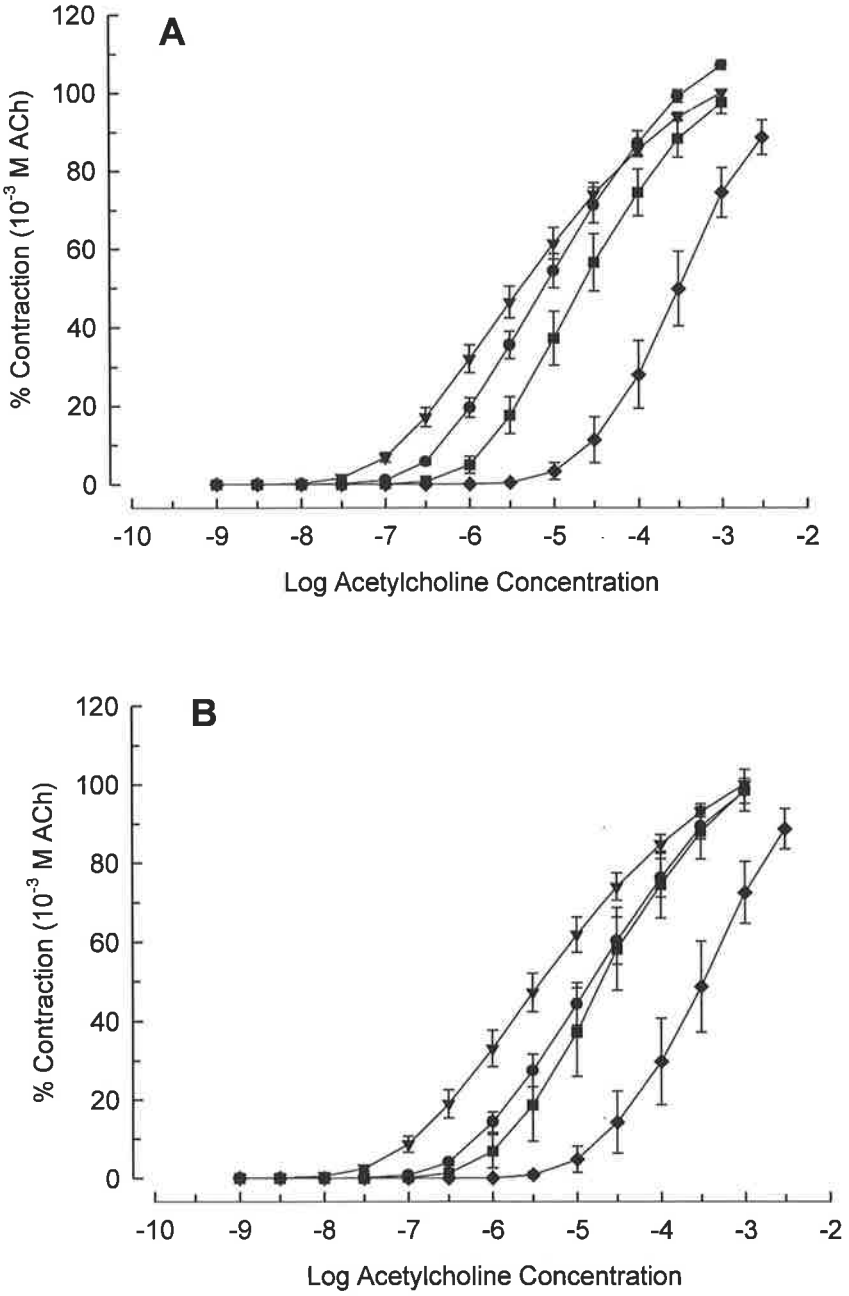


Figure 7.5 Mean cumulative concentration-response curves for ACh in the absence (▼) and presence of increasing concentration of the M_3 antagonist, 4-DAMP, (●) 10^{-9} M; (■) 10^{-8} M; (◆) 10^{-7} M, in control (A) and cytokine pretreated (B) tracheal smooth muscle strips. Each value is the mean of five animals. Vertical bars indicate SEM.

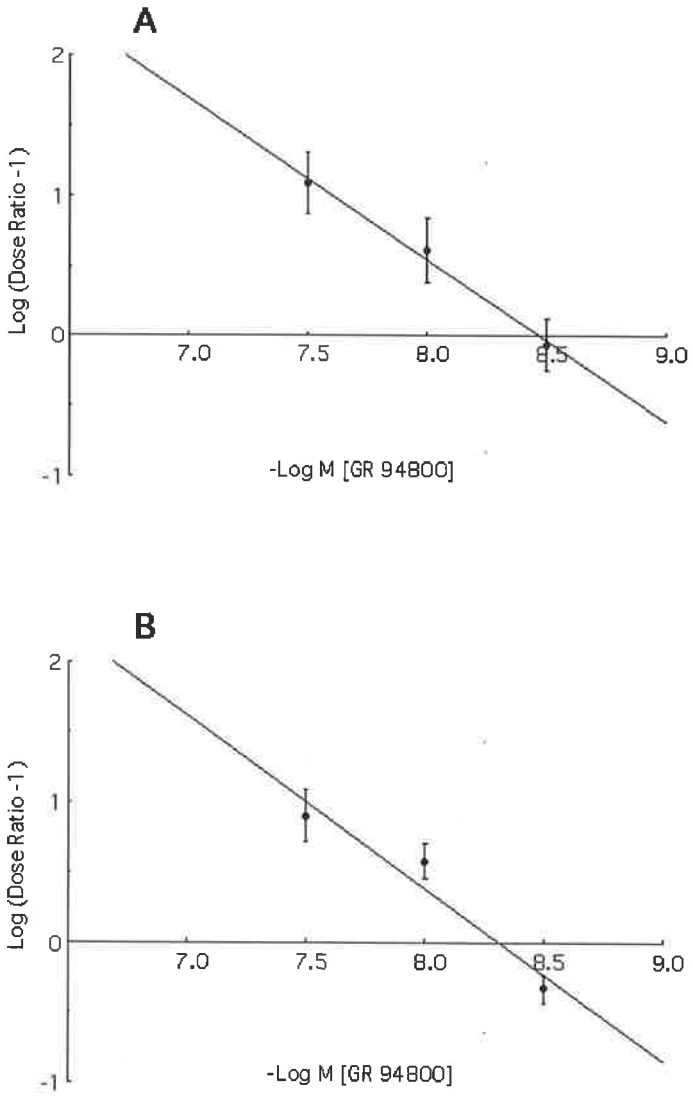


Figure 7.6 Schild plots relative to the antagonism of the contractile responses to neurokinin A (in the presence of phosphoramidon 10^{-6} M) in ovine isolated tracheal strips by GR94800 in A) control and B) TNFα, IL-1β pretreated tissue. Each value is the mean of five animals. Vertical bars indicate SEM.

M_3 antagonist 4-DAMP to ACh are depicted in Figure 7.7. Once again no significant difference in slope was observed. The respective slopes for control and cytokine exposure were -0.90 (95% confidence limits -1.07, -0.73) and -0.86 (95% confidence limits -0.97, -0.76).

Both 4-DAMP and GR94800 exerted competitive antagonism as slopes of the Schild plots were not significantly different from unity. Data in Table 7.2 gives the respective pA_2 values for the M_3 and NK_2 receptor antagonists in control and cytokine pretreated tracheal smooth muscle. Antagonist affinities remained unchanged with cytokine exposure.

7.3.3 Effect of neutral endopeptidase inhibition

Phosphoramidon did not cause any significant change in baseline tension. In the presence of phosphoramidon, the increased contractility to NKA following cytokine pretreatment was still apparent. E_{max} for NKA, in the presence of phosphoramidon (10^{-5} M), was 59.9 ± 5.6% and 72.6 ± 6.8% for control and cytokine pretreated tissue, respectively ($P < 0.05$), Figure 7.8.

7.3.4 Calcium-free K-H solution

Figure 7.9 compares the CCRC for ACh in tracheal smooth muscle in the presence and absence of extracellular Ca^{++}. The contractile response to low concentration of ACh is attenuated with the omission of extracellular Ca^{++}. However, enhanced contractility to ACh in ASM following TNFα and IL-1β pretreatment was still evident following removal of extracellular Ca^{++} (Figure 7.10). E_{max} for ACh, in calcium-free K-H, was 73.2 ± 4.71% and 80.0 ± 4.7% for control and cytokine pretreated tissue, respectively ($P < 0.01$), Figure 7.10. In calcium-free K-H solution the contractile response to

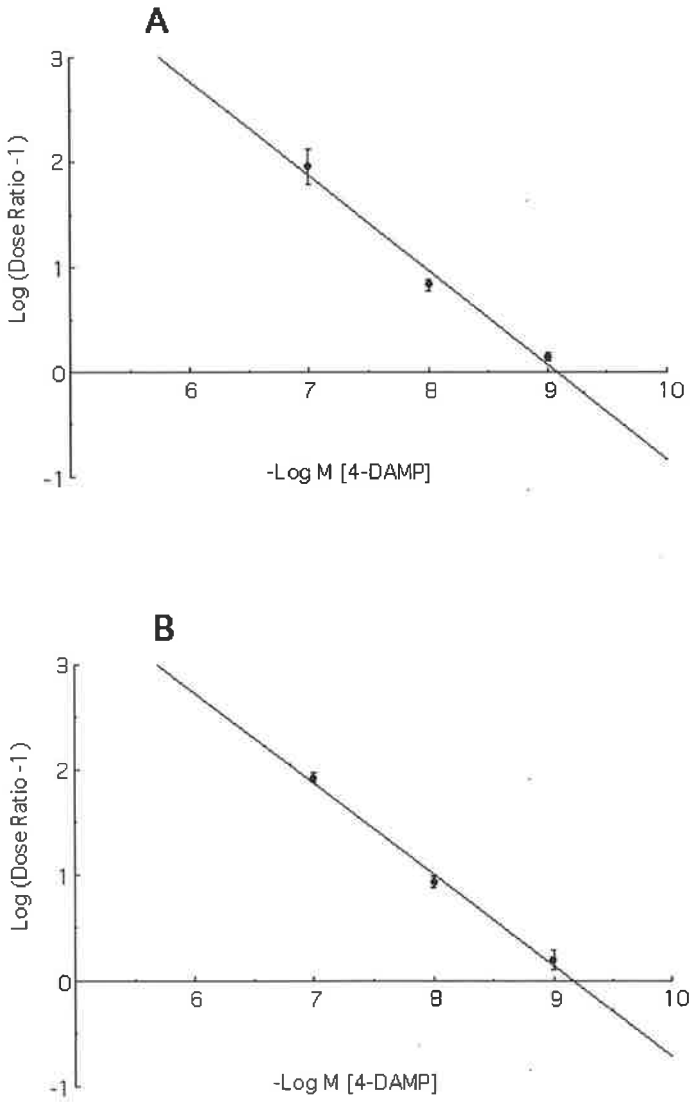


Figure 7.7 Schild plots relative to the antagonism of the contractile responses to acetylcholine in ovine isolated tracheal strips by 4-DAMP in A) control and B) TNFα, IL-1β pretreated tissue. Each value is the mean of five animals. Vertical bars indicate SEM.

Table 7.2 pA_2 values for 4-DAMP and GR94800 to contractions produced by acetylcholine and neurokinin A, respectively, in control and cytokine treated (IL-1β; 10 ng/ml, TNFα; 100 ng/ml) ovine tracheal muscle strips.

		pA_2	
Agonist	Antagonist	Control	TNFα, IL-1β
Acetylcholine	4-DAMP	9.09 ± 0.12	9.18 ± 0.05
Neurokinin A	GR94800	8.47 ± 0.10	8.32 ± 0.07

Each value represents the mean ± SEM from five animals.

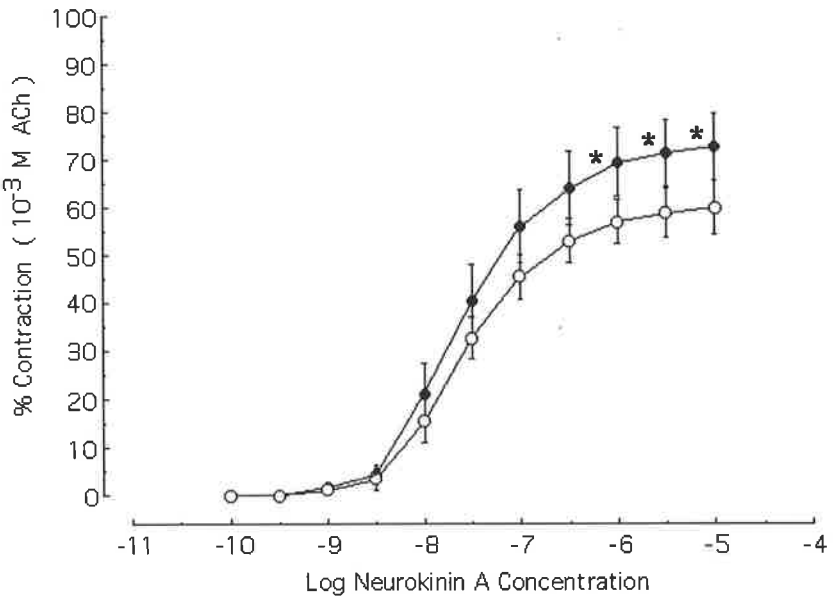


Figure 7.8 Cumulative concentration-response curves for neurokinin A in the presence of phosphoramidon 10^{-5} M (n=5) in control (○) and cytokine pretreated (IL-1β; 10 ng/ml, TNFα; 100 ng/ml) smooth muscle (●). Mean responses are expressed as a percentage of the reference contraction to 10^{-3} M ACh. Vertical bars indicate SEM. * Denotes a statistically significant ($P < 0.05$, paired *t*-test) difference between control and cytokine treated tissues.

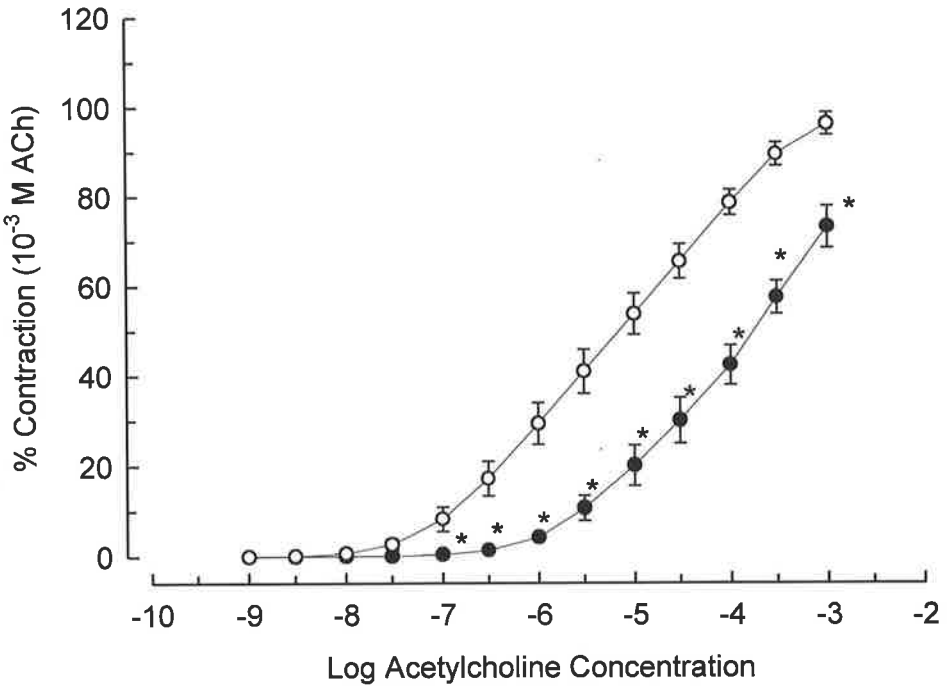


Figure 7.9 Mean cumulative concentration response curves for acetylcholine in the presence (○), n=9 and absence of extracellular calcium in ovine tracheal smooth muscle strips (●), n=7. Mean responses are expressed as a percentage of the reference contraction to 10^{-3} M ACh. Vertical bars indicate SEM. * Denotes a statistically significant difference between the two groups ($P < 0.05$, unpaired *t*-test).

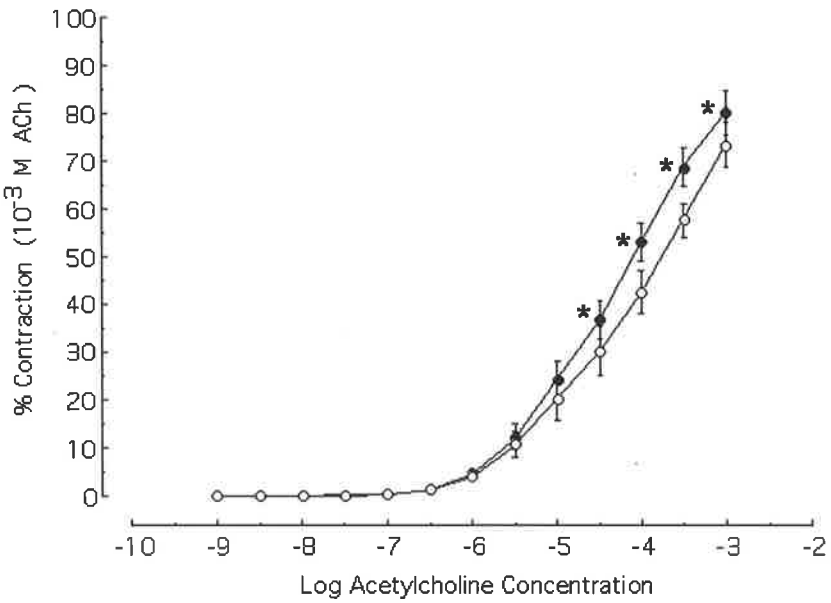


Figure 7.10 Effect of calcium-free K-H solution on the cumulative concentration response curves for acetylcholine in control (○) and cytokine pretreated (IL-1β; 10 ng/ml, TNFα; 100 ng/ml) ovine tracheal smooth muscle strips (●), n = 7. Mean responses are expressed as a percentage of the reference contraction to 10^{-3} M ACh. Vertical bars indicate SEM. * Denotes a statistically significant difference between control and treatment groups ($P < 0.05$, paired *t*-test).

histamine was virtually abolished (Figure 7.11) with the E_{max} for histamine in calcium-free K-H being reduced by 87% compared to that observed in the presence of Ca^{++}.

7.4 Discussion

As previously mentioned asthma manifests as a disease characterized functionally by airflow limitation and AHR. Histologically, the changes seen are those of chronic inflammation involving many cell types such as eosinophils, neutrophils, lymphocytes, mast cells, fibroblasts, epithelial and endothelial cells. Cytokines are an extensive group of extracellular signaling proteins (hormones) that regulate inflammatory and immune reactions. Cytokine actions may be autocrine, influencing the cell that produced it; paracrine, influencing cells within close proximity of its release; or in endocrine manner by exerting more distant systemic effects.

While it is well recognized that IL-1β and TNFα are co-localised in mast cells and macrophages and are released from these cells and lymphocytes following IgE receptor stimulation (Gosset *et al.*, 1999; Ohno *et al.*, 1990; Gordon *et al.*, 1990), these pro-inflammatory cytokines can also be released, by a variety of stimuli, from the airway epithelium (Holgate *et al.*, 2000; Mills *et al.*, 1999; Spina, 1998a) and ASM (Hakonarson *et al.*, 1997). Therefore, IL-1β and TNFα may be considered as "first wave" cytokines resulting in highly concentrated levels within the airway wall, which may in turn alter ASM function.

In view of this possibility, however, more attention has focused on the mechanism of action of these cytokines on the ASM relaxation process. Researchers have demonstrated functional antagonism between the inhibitory muscarinic M_2 receptor

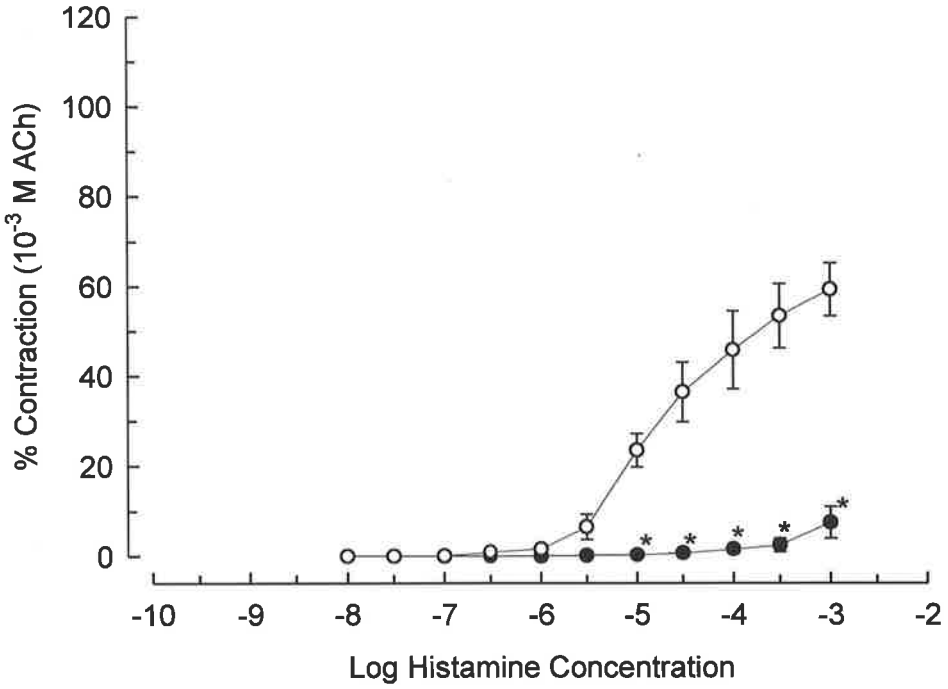


Figure 7.11 Mean cumulative concentration response curves for histamine in the presence (○), n=3 and absence of extracellular calcium in ovine tracheal smooth muscle strips (●), n=3. Mean responses are expressed as a percentage of the reference contraction to 10^{-3} M ACh. Vertical bars indicate SEM. * Denotes a statistically significant difference between the two groups ($P < 0.05$, paired *t*-test).

G_I-proteins and the stimulatory β-adrenoreceptor G_s-proteins coupled to the AC system which mediates smooth muscle relaxation (Hotta *et al.*, 1999; Hakonarson *et al.*, 1996; Koto *et al.*, 1996; Hirata *et al.*, 1994). That is, AC is under dual regulation by a stimulatory G_s protein and an inhibitory G_i protein (Schramm & Grunstein, 1992). In these studies both IL-1β and TNFα have been shown to increase $G_{i\alpha}$ protein expression (the G-protein subunit that is involved in inhibiting AC) (Hotta *et al.*, 1999; Hakonarson *et al.*, 1996; Koto *et al.*, 1996; Hirata *et al.*, 1994), while G_s expression remains unchanged (Hotta *et al.*, 1999; Shore *et al.*, 1997). The increased expression of G_{ia} protein with no change in G_s levels would enhance the inhibitory pathway, leading to lower AC activity and impaired relaxation.

To the best of my knowledge no study has examined cholinergic M_3 receptor or tachykinin NK_2 receptor mediated ASM contractions using a combination of cytokines. The data clearly demonstrate that normal ASM becomes hyperresponsive to ACh (confirming the results of the previous chapter) and NKA following combined exposure to IL-1β and TNFα. These cytokines increased both the responsiveness (increased E_{max}) and sensitivity (decreased pD_2) of ASM to these agonists. Moreover, this study is the first to demonstrate that cytokines enhance smooth muscle contractility to tachykinins, *in vitro*.

The exact mechanism by which these cytokines are able to induce smooth muscle hyperresponsiveness in ovine tracheal smooth muscle is unclear. It may be argued that the enhanced contractility observed to ACh and NKA may result from an alteration in receptor function. The present study using specific M_3 and NK_2 receptor antagonists has demonstrated that cytokine pretreatment did not alter receptor binding characteristics as pA_2 values were the same in control and cytokine exposed tissues.

Furthermore, the pA_2 values for 4-DAMP are similar to those described in human, rat and canine airways (Chiba *et al.*, 1998; Watson *et al.*, 1995; Brichant *et al.*, 1990) and for GR94800 are similar to those described in guinea pig ASM using a specific NK_2 agonist (Zeng & Burcher, 1994). Cytokines may increase the expression of cell surface receptors. This appears unlikely, since several groups using different species have shown that incubation of tracheal tissue with IL-1β or TNFα either causes a decrease or no change in muscarinic (Hotta *et al.*, 1999; Hakonarson *et al.*, 1996; Amrani *et al.*, 1995b) or β-adrenoreceptor binding (Wills-Karp *et al.*, 1993b).

The enhanced contraction seen with NKA was not due to decreased activity of NEP, the principal enzyme responsible for the inactivation of tachykinins (Martling, 1987a). In the presence of NEP inhibition by phosphoramidon, cytokine-induced hypercontractility to NKA was still present, suggesting that endogenous endopeptidase levels were not decreased. Moreover, one might expect tissues co-incubated with IL-1β and TNFα would have increased NEP activity due to the resident fibroblasts in the tracheal segment (Kondepudi & Johnson, 1993).

Acetylcholine and NKA mediate contractions via M_3 and NK_2 smooth muscle receptors, respectively, and involve agonist-stimulated hydrolysis of PIP_2 to the second messenger IP_3 (Chilvers *et al.*, 1994; Grandordy *et al.*, 1988). This leads to increased levels of $[Ca^{++}]_i$, activation of MLCK, resulting in cross-linking of the cellular contractile elements and contraction (Schramm & Grunstein, 1992). Experiments conducted in calcium-free K-H solution were designed to determine whether the cytokine-induced enhanced contractility might result from altered internal Ca^{++} mobilization in tracheal smooth muscle. The protocol employed for the removal of extracellular Ca^{++} using a chelating agent (EGTA) has been extensively used by others

(McKay *et al.*, 1991; Creese & Denborough, 1981; Kirkpatrick *et al.*, 1975b), and shown to be sufficient in the removal of extracellular Ca^{++}. The method allows partitioning of the contractile response between that dependent on extracellular Ca^{++} influx and that resulting from release of Ca^{++} from intracellular stores.

The present results demonstrate that tracheal smooth muscle contractions to low concentrations of ACh rely on the influx of extracellular Ca^{++} while those to higher concentrations predominantly rely on intracellular Ca^{++} mobilization. The finding of a diminished contractile response, in control tissues, to ACh especially at low agonists concentrations in calcium-free K-H is not surprising as this has previously been described by others in bovine and guinea pig tracheal smooth muscle (Creese & Denborough, 1981; Kirkpatrick *et al.*, 1975b). Depletion of extracellular Ca^{++} abolished contractile responses mediated by histamine in ovine tracheal smooth muscle. This observation suggests that histamine contractions in ovine ASM are predominantly mediated by extracellular Ca^{++} influx. This is in agreement with other research groups who have also demonstrated markedly reduced responses to histamine in the absence of Ca^{++} in human (Black *et al.*, 1986), guinea pig (Creese & Denborough, 1981) and bovine (Kirkpatrick, 1975a) ASM. Although, the effect of extracellular Ca^{++} removal on NKA contractility was not studied, others have reported that transmembrane Ca^{++} influx has a minor role only in tachykinin-induced contractions in ASM (Matran *et al.*, 1988).

Of particular interest in the present study is that smooth muscle hyperresponsiveness to ACh in cytokine treated tissue was maintained despite extracellular Ca^{++} removal, thus providing evidence that inflammatory cytokines induce *in vitro* hyperresponsiveness in normal ASM via a mechanism involving intracellular Ca^{++} mobilization. Amrani and

colleagues demonstrated, in cultured tracheal smooth muscle cells, that agonist-induced increases in $[Ca^{++}]_i$ were enhanced by TNFα and IL-1β pretreatment (Amrani *et al.*, 1997; Amrani *et al.*, 1995b). This taken together with the present results suggests that potentiated Ca^{++} mobilization resulting from cytokine exposure does indeed translate to an enhanced contractile response following agonist stimulation. The lack of an enhancement to histamine contractility following cytokine pretreatment appears to reflect agonist-specific mechanistic differences in post-receptor signalling pathways that mediate ASM contraction (Chilvers *et al.*, 1994).

Although, the observed cytokine-induced hyperresponsiveness may appear rather minimal, one would expect this to have a much greater effect on airway narrowing *in vivo*. Airway smooth muscle *in vivo*, is said to be loaded auxotonically, where ASM shortens against an increasing load (Mitchell *et al.*, 1989). Mitchell and colleagues (Mitchell *et al.*, 1989) have shown that normal ASM under auxotonic conditions has an increased capacity to shorten, that is, the muscle is more sensitive to contractile agonists especially at low concentration. Therefore, one would predict that the observed cytokine-induced increase in isometric force, for a given dose of agonists, would produce even greater ASM shortening *in vivo*. This, in the absence of airway wall thickening, would significantly increase airway resistance (Pare & Bai, 1996). Moreover, enhanced ASM shortening, in the presence of airway wall thickening and oedema, (produced as a consequence of inflammation), is capable of increasing airways resistance by an order of magnitude (Pare & Bai, 1996).

In summary, the data obtained in this chapter demonstrate that ovine tracheal smooth muscle becomes hyperresponsive to ACh and NKA following TNFα and IL-1β exposure, via a mechanism involving intracellular Ca^{++} mobilization. The significance

of our finding is that, in acute exacerbations of asthma the release of preformed TNFα and IL-1β may lead to enhanced airway narrowing resulting from endogenously released agonists (e.g. ACh and NKA). And furthermore, in the presence of airway wall thickening and oedema, this enhanced contractility may be so severe as to uncouple the airway from the parenchyma to such an extent that the smooth muscle contraction may completely occlude the airway, a phenomenon observed in sudden-onset fatal asthma (Sur *et al.*, 1993).

Chapter 8 Interleukin-1β and TNFα increase microvascular leakage in the guinea pig trachea

8.1 Introduction

In asthma the development of AHR has been linked to the presence of airway inflammation (Hogg *et al.*, 1991). There is a school of thought that AHR *in vivo* is not due to an increased sensitivity of ASM to various contractile agonists, but rather, is a property of intact airways which have been structurally changed by chronic inflammation which then allows them to narrow excessively (Pare & Bai, 1995). The primary structural change which is considered to be important in this scenario, is an increase in airway wall thickness (Wiggs *et al.*, 1992). Thickening of the airway may result from cellular infiltration (Saetta *et al.*, 1991), deposition of connective tissue (Roberts, 1995), thickened basement membrane (Roche *et al.*, 1989), hypertrophy and hyperplasia of ASM (Ebina *et al.*, 1993), vascular distension (Li & Wilson, 1997; Carroll *et al.*, 1997b) and oedema formation (Wilson, 2000). It has been postulated that oedema accompanying airway inflammation may 'uncouple' the airway from the parenchyma to such an extent that ASM contraction can be so severe as to completely occlude the airway (Macklem, 1990).

The results of the preceding two chapters clearly demonstrate that IL-1β and TNFα act synergistically to enhance smooth muscle contractility to particular agonists, by affecting signal transduction pathways that mediate ASM contraction. Thus providing

supportive evidence that ASM, itself, can become hyperresponsive as a consequence of inflammation (i.e. in the presence of cytokines). Nevertheless, it remains to be determined if the pro-inflammatory cytokines, IL-1β and TNFα can also affect airway microvascular permeability.

TNFα and IL-1β are ubiquitous cytokines in the development of inflammatory responses. Vascular leakage and leukocyte migration are central features of the inflammatory process. In inflammation leukocyte migration is a complex sequential process, involving leukocyte-endothelial tethering and rolling, in post-capillary venules, followed by leukocyte activation, leading to cell adhesion and flattening against the vascular wall. Ultimately resulting in leukocyte diapedesis (transendothelial migration) (Wagner & Roth, 2000). This complex sequence results from the timed orchestrated activation of numerous adhesion molecules, e.g. selectins, integrins and their respective counter-ligands (Wagner & Roth, 2000).

Both IL-1β and TNFα upregulate the expression of adhesion molecules, promoting transendothelial and transepithelial migration of inflammatory cells, such as neutrophils (Bochner, 1997; Smith *et al.*, 1993). Although, TNFα and IL-1β are not themselves chemotactic for neutrophils, their exposure to certain cells can induce the production of chemoattractants such as IL-8, a potent chemokine for neutrophils. For example, TNFα and IL-1β are potent inducers of IL-8 production in epithelial (Cromwell *et al.*, 1992; Standiford *et al.*, 1990), endothelial (Smart & Casale, 1994; Kuijpers *et al.*, 1992) and ASM cells (John *et al.*, 1998).

Although it is well recognized that both TNFα and IL-1β are upregulated in acute

exacerbations of asthma (Ackerman *et al.*, 1994; Broide *et al.*, 1992), virtually no data exist regarding the effects of these cytokines on the airway microvasculature. With this in mind I examined the effect of IL-1β and TNFα on airway MVL, *in vivo*.

An *in vivo* method for isolating a tracheal segment was developed which allowed prolonged exposure of this segment to various agents without causing adverse systemic effects. Vascular leakage was determined by the extravasation of intravenously injected EB, an azo dye, which binds avidly to circulating albumin (Saria & Lundberg, 1983) and has been shown to correlate with the extravasation of radiolabelled albumin in guinea pig airways (Rogers *et al.*, 1989a).

8.2 Methods

8.2.1 Animals

Experiments were performed on Hartley strain guinea pigs of either sex weighing 758±109 (SD) gm (n=18 animals). Detailed methodologies for surgical preparation, the conditioning of inspiratory gases, measurement of plasma extravasation and aerosol delivery are given in Chapter 2 - Methods (Sections 2.4.1, 2.4.2, 2.4.3 and 2.4.4, respectively).

8.2.2 Experimental groups

All animals used in this study underwent the same surgical procedure (see Chapter 2, Section 2.4.1) and were subjected to 1 hour of unidirectional tracheal ventilation with a conditioned isocapnic gas. Tracheal EB extravasation was examined in 4 groups of animals, as detailed in Chapter 2 – Methods, Section 2.4.6 Experimental groups.

To determine the adequacy of artificial ventilation, arterial blood gas samples were taken in selected animals. Prior to lethal intravenous injection of pentobarbital sodium, an arterial blood gas sample was taken by cardiac puncture using a self-filling arterial sampler (PICO™70, Radiometer Copenhagen, Denmark), containing 60 IU of electrolyte balanced heparin. Samples were stored in ice water and analyzed (ABL 520, Radiometer, Copenhagen, Denmark) within 30 mins for pH, $PaCO_2$ and PaO_2. Blood-gas values were measured at 37°C and corrected for the animal's body temperature (Kelman & Nunn, 1966).

Cannulation of the carotid artery to monitor systemic arterial blood pressure was avoided so as not to disturb the tissue above and around the ITS. However, left ventricular systolic pressures were obtained at the end of each experiment to determine if gross vasoconstrictor or vasodilator responses had occurred during the experimental intervention. Blood pressure was obtained by left ventricular puncture using a 19 gauge needle (Becton Dickinson) connected to a vinyl catheter filled with heparinized saline (150U/ml). This was connected to a disposable Transpac® pressure transducer and signals recorded on a Neotrace polygraph.

8.2.3 Reagents

All drugs, compounds and solutions used in the following experiments are listed, prepared and used according to the methods described in Chapter 2 - Methods, Reagents - Section 2.6.3.

8.2.4 Statistical analysis

Data are presented as the mean ± SEM. All statistical analyses performed in this series of experiments are described in Chapter 2 - Methods, Section 2.5.3.

8.3 Results

Figure 8.1 shows the standard curve for EB concentration and optical density measured at 630nm using the MR7000 microplate reader (Dynatech, Guernsey, Channel Islands). Linear regression analysis of the plotted points produced the linear equation $y = 0.0402x + 0.0009$, with an r^2 value of 1.

There was no difference in baseline values for body weight, body temperature and C_{dyn} between any experimental group (Table 8.1). The effect of aerosol cytokine exposure on tracheal EB extravasation is shown in Figure 8.2. Tumour necrosis factor-α and IL-1β aerosol challenge significantly increased EB extravasation (28.9±1.6 μg/gm wet tissue, mean ± SEM) compared to saline challenge (13.8±3.0 μg/gm; $P < 0.05$). Tracheal dye extravasation following isocapnic ventilation, without aerosol challenge, was not significantly different from saline challenged animals (17.5±2.9 and 13.8±3.0 μg/gm, respectively). Histamine aerosol significantly increased tracheal EB extravasation (50.1±4.8 μg/gm; $P < 0.05$) compared to the saline challenged trachea. When the ITS is ventilated with an hypocapnic gas (0% CO_2: 21% O_2: 79% N_2) significant EB extravasation occurs (52.3 ± 2.0 mg/gm, $P < 0.05$ compared with isocapnic ventilation, without aerosol challenge) (Reynolds *et al.*, 1992).

Changes in C_{dyn}, for each experimental group, with continuous mechanical ventilation are shown in Figure 8.3. There was no significant change in C_{dyn} with respect to time within each treatment group or between groups. Arterial blood gas values for the selected animals following two hours of mechanical ventilation are shown in Table 8.2. No significant difference in left ventricular systolic blood pressure was observed between treatment groups (Table 8.3).

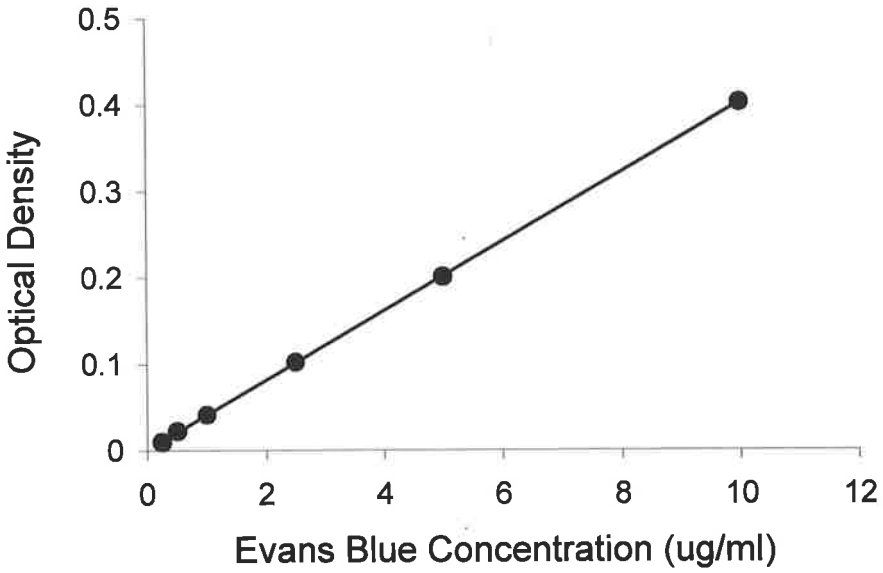


Figure 8.1. Standard curve for Evans blue in formamide. The concentrations of Evans blue were 0.25, 0.5, 1.0, 2.5, 5.0, and 10 μg/ml.

Table 8.1. Body weight, baseline body temperature and baseline C_{dyn} for guinea pigs in each experimental group.

Group	n	Body weight (gm)	Body temp (°C)	C_{dyn} (ml/cmH_2O)
Isocapnic	5	790 ± 53	36.7 ± 0.4	0.68 ± 0.05
Saline	5	716 ± 32	37.4 ± 0.4	0.61 ± 0.05
IL-1β,TNFα	5	792 ± 70	37.6 ± 0.4	0.67 ± 0.03
Histamine	3	716 ± 19	37.8 ± 0.9	0.62 ± 0.01

Values are mean ± SEM; n equals number of guinea pigs.

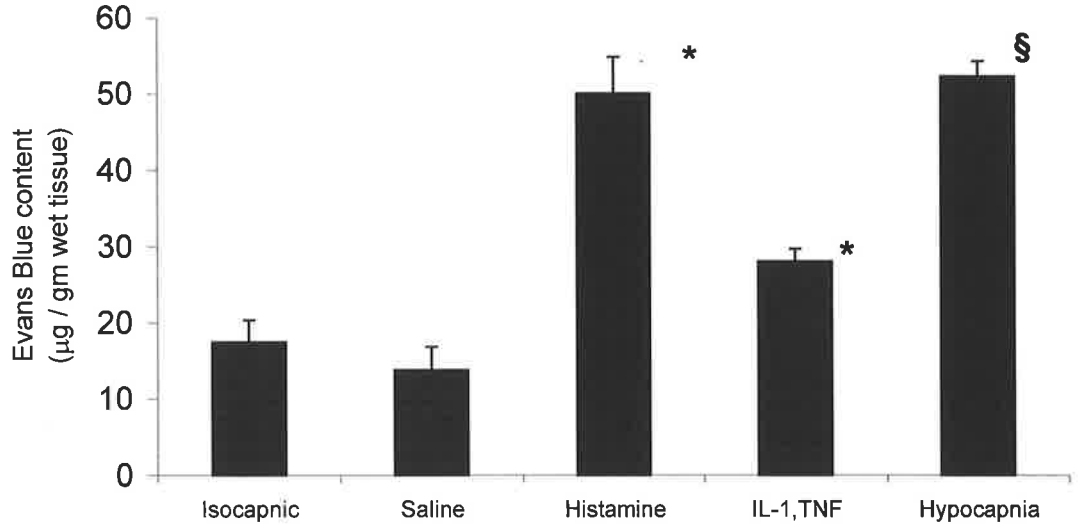


Figure 8.2. Tracheal Evans blue content following isocapnic ventilation and aerosol challenge with saline (n=5), IL-1β (10 ng/ml) and TNFα (100 ng/ml) (n=5), and histamine (n=3). Values are mean ± SEM. * Significant difference between saline aerosol challenge. ($P < 0.05$, Bonferroni t statistic). § Tracheal Evans blue content following hypocapnic ventilation, data from Reynolds *et al.* (1992), demonstrating deleterious effect on microvasculature by ventilating with 0% CO_2.

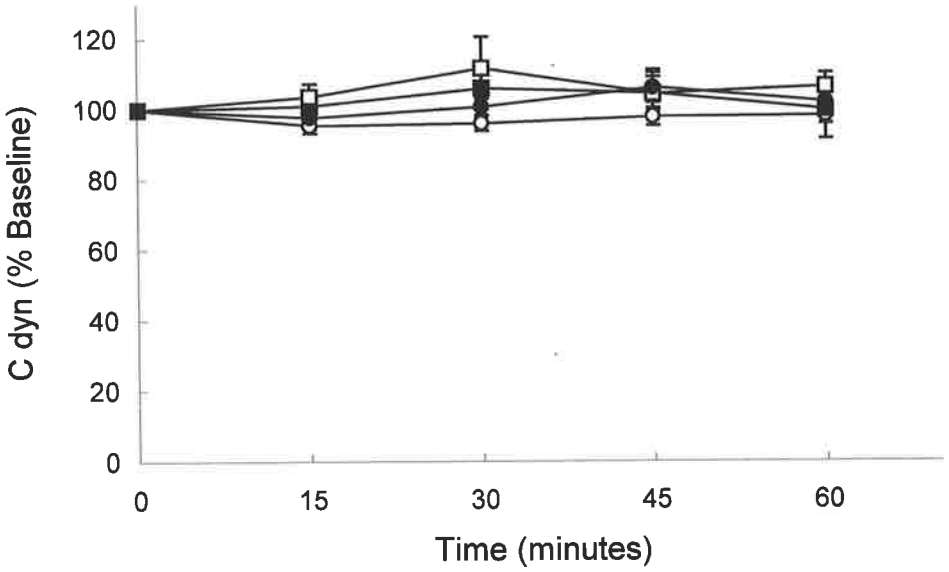


Figure 8.3. Temporal changes in C_{dyn} with continuous mechanical ventilation. Readings taken every 15 minutes following isocapnic ventilation (○, n=5) and aerosol challenge with saline (■, n=5); IL-1β (10 ng/ml) and TNFα (100 ng/ml) (●, n=5); and histamine ($5x10^{-2}$ M) (□, n=3). Values are mean ± SEM.

Table 8.2. Arterial blood gas parameters for anaesthetized, mechanically ventilated guinea pigs.

n	pH	$PaCO_2$ (mmHg)	PaO_2 (mmHg)
5	7.43 ± 0.03	36.5 ± 2.7	85.5 ± 5.6

Values are mean ± SEM; n equals number of guinea pigs.

Table 8.3. Left ventricular systolic blood pressure.

Treatment Group	n	Systolic pressure (mmHg)
Isocapnic	5	55 ± 6
Saline	5	57 ± 6
IL-1β,TNFα	5	62 ± 4
Histamine	3	58 ± 4

Values are mean ± SEM; n equals number of guinea pigs.

8.4 Discussion

Virtually no data exist on the effect of IL-1β and TNFα on airway MVL. That which are available deal primarily with animal models of acute respiratory distress syndrome (Koh *et al.*, 1996; Wesselius *et al.*, 1995) and inflammatory brain disease (Anthony *et al.*, 1997). The two former studies assessed gross vascular leakage across the lung's alveolar compartment rather than the airway microvasculature. More recent studies have examined the effect of TNFα on permeability cultured endothelial cell monolayers (van Griensven *et al.*, 1999; Mark & Miller, 1999). While these studies have demonstrated enhanced permeability, they were considered representative models of blood-brain barrier and ischemia-reperfusion injury.

It is widely accepted that IL-1β and TNFα are co-localized in mast cells and macrophages, are released following IgE-receptor stimulation (Gosset *et al.*, 1999; Barnes, 1994) and act synergistically to mediate various inflammatory responses (Hakonarson *et al.*, 1996; Wesselius *et al.*, 1995). Furthermore, a variety of stimuli induce the synthesis and release of IL-1β and TNFα in airway epithelial cells (Polito & Proud, 1998; Spina, 1998a). Once released they provide a cytokine gradient to guide leukocyte migration across the endothelium to sites of inflammation. This gradient would result in significantly high local concentrations of cytokines that may in turn upregulate the responsiveness of vascular endothelium causing mucosal oedema. With this in mind, it was decided to challenge the ITS with an aerosol of these two inflammatory cytokines in order to replicate local cytokine release, and determine their effect on vascular permeability in the extensive subepithelial microvascular plexus. The cytokine concentrations used in the present study (TNFα (100 ng/ml); IL-1β (10 ng/ml)

were based on what may be considered maximally effective in various biological systems as reflected by the results obtained in Chapter 6, and described by others (Hakonarson *et al.*, 1996; Horie *et al.*, 1996; Tamaoki *et al.*, 1994; Kondepudi & Johnson, 1993; Wills-Karp *et al.*, 1993b).

Previously, I have developed, in the guinea pig, a technique for isolating a section of trachea *in vivo* (Reynolds *et al.*, 1992), similar to that described by other workers using larger species (Deffebach *et al.*, 1989; Kirsch *et al.*, 1988). However, in those studies the tracheal segment was superfused with physiological solutions, while the present technique maintains a more natural air/epithelium interface. The advantage of this model is that gas composition within an ITS can be altered without affecting pulmonary gas exchange, and therefore, in the present study the effects of aerosols on airway microvascular function can be examined in the absence of confounding bronchoconstrictor or systemic effects.

To my knowledge the present study is the first to demonstrate that an aerosol of IL-1β and TNFα induces airway MVL in the guinea pig trachea. No significant difference in EB extravasation between isocapnic ventilated and saline-challenged, isocapnic ventilated tracheal segments was observed, indicating that aerosol challenge by itself does not induce MVL. The fact that histamine aerosol induced EB extravasation indicates that the microcirculation of the tracheal segment following surgery was sufficiently intact to allow changes in protein extravasation to occur. The degree of airway MVL induced by aerosol histamine challenge was in accordance with that described by Inoue and colleagues (Inoue *et al.*, 1997).

At any one time the extent of EB extravasation in the trachea will be determined by the permeability of the tracheal microvasculature and by tracheal microvascular pressure (Persson, 1987). The latter will vary with changes in tracheal blood flow and vascular resistance. It has been established that in the skin, vasodilators potentiate plasma exudation by increasing local blood flow (Brain & Williams, 1985) and vasoconstrictors attenuate the vascular response by decreasing blood flow at the inflamed site (Beets & Paul, 1980). From these early studies it was generally concluded that decreases and increases in systemic blood pressure equated to a respective increase or decrease in regional blood flow within the tracheal circulation, and thereby influenced MVL. However, several studies have shown that vasodilators and vasoconstrictors are unable to potentiate or attenuate plasma exudation within the airways (Takahashi *et al.*, 1993; Lotvall *et al.*, 1992; Svensson *et al.*, 1992; Rogers *et al.*, 1988). Moreover, in a recent study, by measuring tracheal mucosal blood flow directly, Cui and coworkers demonstrated that an increase in systemic blood pressure led to increased tracheal blood flow and potentiated plasma exudation in the guinea pig trachea (Cui *et al.*, 1999).

In the present study, the changes in EB extravasation could have been due to changes in vascular permeability, microvascular pressure, or both. Aortic blood pressure is considered the most important inflow pressure for the tracheobronchial circulation and may provide some information on possible hydrostatic pressure changes in the tracheal microcirculation. In the present guinea pig preparation cannulation of the carotid artery to monitor systemic arterial blood pressure was not performed, however, left ventricular systolic pressures were measured. Systolic blood pressure, in all treatment groups, was similar to those reported in conscious chronically instrumented guinea pigs (Hosenpud *et al.*, 1983). Therefore, it would appear that the observed increase in MVL following

cytokine treatment resulted from a change in vascular permeability rather than an increase in tracheal microvascular pressure or blood flow.

As the upper airways were bypassed by the surgical protocol, thereby eliminating the natural mechanisms of heating and humidification during inspiration (Hanna & Scherer, 1986), conditioned inspiratory gases were used to prevent deleterious changes in lung function that occur with airway drying and cooling (Ray *et al.*, 1991; Freed *et al.*, 1987). The degree of heating and humidification employed was comparable to that measured in the upper trachea and carina regions of human and canine lungs (Ray *et al.*, 1989; Gilbert *et al.*, 1987). The use of conditioned inspirates has been shown by others not to increase EB extravasation in the guinea pig airway (Garland *et al.*, 1991), and confirmed by my previous study (Reynolds *et al.*, 1992).

No significant changes in C_{dyn} were observed in any treatment group throughout the experimental procedure, illustrating the stability of the experimental technique and the benefit of examining airway MVL without any confounding systemic effects. For example, histamine-induced bronchoconstriction following aerosol challenge was avoided. Our baseline values for C_{dyn} in mechanically ventilated guinea pigs are in agreement with those reported by others (Lotvall *et al.*, 1990). Adequacy of mechanical ventilation was confirmed by the arterial blood gas measurements. Body temperature corrected values were within the normal ranges for both conscious (Hart *et al.*, 1984) and anaesthetized guinea pigs (Brown *et al.*, 1989; Bar-Ilan & Marder, 1980). Arterial blood gas values were corrected for temperature (Kelman & Nunn, 1966) due to the temperature difference between the arterial blood sample and the arterial blood gas analyzer.

Evans blue binds avidly to circulating albumin (Saria & Lundberg, 1983) and has been used extensively to quantify plasma extravasation in the guinea pig airway (Rogers *et al.*, 1989a). Evidence suggests that mediators of MVL act directly on the endothelium of post-capillary venules by contracting endothelial cells thereby opening intercellular gap junctions leading to MVL (McDonald, 1994; Joris *et al.*, 1987; Majno *et al.*, 1969). The exact mechanism by which these cytokines are able to induce MVL in the guinea pig trachea remains to be determined.

Vascular permeability is dependent on intracellular Ca^{++} mobilization and the activation of a cascade of calcium-dependent mechanisms downstream from this initial rise in $[Ca^{++}]_i$ (Michel & Curry, 1999). That is phosphorylation of myosin light chains by MLCK, increased tension developed by actin-myosin interactions and the formation of intercellular gaps by endothelial cell contraction (McDonald, 1994; Joris *et al.*, 1987).

Research by others has demonstrated that Ca^{++} mobilization from internal stores is a critical event in the initiation of microvascular permeability (Chetham *et al.*, 1999), and the magnitude of the initial peak in the $[Ca^{++}]_i$ is proportional to the increase in permeability (Michel & Curry, 1999). Furthermore, it has also been observed that leukocyte-endothelium interactions, in cultured endothelial cell monolayers, induce transient increases in $[Ca^{++}]_i$ and are associated with increased permeability (Huang *et al.*, 1993).

Dermal injection of IL-1β and TNFα in the rat, induce vascular leakage in post-capillary venules (Yi & Ulich, 1992) with the accumulation of intra-and peri-vascular neutrophils. Therefore it is tempting to speculate, in conjunction with my results obtained in Chapter 7, that tracheal exposure to TNFα and IL-1β may enhance internal

Ca^{++} mobilization in endothelial cells, disrupt endothelial cell-to-cell contacts (Del Maschio *et al.*, 1996), thereby increasing vascular permeability with plasma extravasation. Histological examination of the isolated tracheal segment following IL-1β and TNFα exposure may have provided evidence for such a mechanism.

In summary, the present study demonstrates that the pro-inflammatory cytokines, TNFα and IL-1β induce significant MVL in the guinea pig trachea. The significance of this finding is that, in acute exacerbations of life threatening asthma the release of preformed TNFα and IL-1β from resident mast cells in the smooth muscle of small airways (Carroll *et al.*, 2001) may induce neutrophil transmigration with plasma extravasation (Carroll *et al.*, 1996) leading to an increase in airway wall mucosal thickness. This being in addition to the long term proliferative actions of plasma borne mediators on ASM (Roth *et al.*, 2000) and its surrounding ECM (Johnson *et al.*, 2000) by repeated episodic plasma extravasation.

An acute increase in airway wall mucosal thickness by itself may have little effect on airway resistance (Hogg *et al.*, 1987), however, in the presence of smooth muscle contraction will induce profound airway narrowing (Pare & Bai, 1995). This enhanced narrowing may be so severe as to completely occlude the airway, a phenomenon observed in sudden-onset fatal asthma (Sur *et al.*, 1993).

Chapter 9 Discussion and future directions

9.1 General discussion

Advances in the understanding of the pathophysiology of asthma and AHR have occurred by employing various animal models, leading to the development of innovative concepts and new therapeutic initiatives that can be applied to man. However, as with all non-human species, differences in some aspects of airway physiology and biochemistry from man will occur.

In our laboratory we have established the ovine model of allergic bronchoconstriction described by Abraham and colleagues. Using this model we found evidence for a role for C-fibre afferents and tachykinins in the mediation of the acute response to allergen. Following that study, a systematic *in vivo* study of the effects of exogenously applied tachykinins on ovine bronchoconstrictor responses was undertaken. This study begins this thesis where the findings indicate that intravenous SP causes transient broncho-constriction in sheep, that this is an NK_1 receptor mediated response acting via cholinergic mechanisms, given the sensitivity of the SP bronchoconstrictor response to an NK_1 antagonist and atropine (Rice *et al.*, 2001). And furthermore, the bronchoconstrictor response is rapidly abolished by SP degradation and the induction of tachyphylaxis. No evidence was found for histamine or prostaglandin involvement in the acute response, or any evidence to suggest a delayed increase in R_L, which may implicate vascular or inflammatory cell responses.

The *in vivo* effects of SP in sheep appear at variance with those observed in humans. In man it is generally perceived that *in vivo* tachykinin-induced bronchoconstriction is via

a direct effect on ASM predominantly mediated by NK_2 receptors, as NKA is far more potent than SP. However, on closer scrutiny of the published human data it is apparent that very large doses of NKA were required to induce bronchoconstriction, being much greater than that for SP (Evans *et al.*, 1988a). Furthermore, *in vivo* NKA-induced bronchoconstriction is partly inhibited by nedocromil sodium (Crimi *et al.*, 1992; Joos *et al.*, 1989b) and antimuscarinic agents (Crimi *et al.*, 1990; Joos *et al.*, 1988), both indicating an indirect effect. *In vitro,* tachykinins contract human bronchi predominantly via NK_2 receptors with NKA being more potent than SP (Advenier *et al.*, 1992; Naline *et al.*, 1989; Black *et al.*, 1988). Therefore, in order to determine whether these apparent discrepancies result from significant species differences between human and ovine ASM, formal *in vitro* contractility studies were conducted.

Studies of *in vitro* ASM contractility provide valuable information on airway pharmacology (Hulsmann & de Jongste, 1993). However, for this information to be useful in determining significant differences between control tissue and that subjected to various pharmacological interventions, formal baseline studies are required to assess the reproducibility of contractile responses to various agonists i.e. the within and between sheep variation. From the length-tension studies conducted in this thesis, the optimal load required for ovine tracheal smooth muscle strips was between 2.5 and 3.5 grams, while that for bronchial ring preparations was between 4 and 5 grams. These values are in agreement with those determined by others in isolated ovine airways (Abraham *et al.*, 1996; Jackowski *et al.*, 1993; Sheller & Brigham, 1982). In ovine parenchymal strip preparations the load required to produce maximal isometric force was between 3.5 and 4.5 grams, again similar to published values for this species (Sheller & Brigham, 1982).

Furthermore, the results obtained in Chapter 4 provide an insight into the reproducibility of contractile responses in ovine ASM and the viability of the ovine tracheal smooth muscle preparation. Reproducible contractile responses, both within and between sheep, to various agonists were obtained in ovine ASM preparations provided E_{max} is normalized to wet tissue weight or to a reference contraction, thus avoiding any confounding effects introduced by non-uniform tissue size. Significant relationships have also been demonstrated by others between smooth muscle volume and maximal isometric force generation in bronchial strip preparations (Armour *et al.*, 1988). Moreover, ovine tracheal smooth muscle strips remain viable for several days following storage in cold oxygenated buffered K-H solution. Given the minimal between and within sheep variability in ASM responsiveness, the ovine smooth muscle preparation provides a sensitive model in which detectable changes in contractile properties are possible following pharmacological intervention.

In ovine tracheal smooth muscle tachykinin-induced contraction *in vitro* appear to be predominantly mediated by tachykinin NK_2 receptors, although, tachykinin NK_1 receptors also appear to be involved. Substance P and NKA contractions did not exhibit tachyphylaxis and were not mediated by cholinergic mechanisms. However, a significant cholinergic component was present in $[Sar^9,Met(O_2)^{11}]$-SP induced tracheal smooth muscle contractions and exhibited tachyphylaxis. This may reflect the activation of NK_1 facilitatory receptors at parasympathetic ganglia (Belvisi *et al.*, 1994) or on post-ganglionic nerves (Watson *et al.*, 1993). In ovine trachea, parasympathetic ganglia are concentrated between tracheal cartilaginous rings and post-ganglionic nerves pass into the trachealis muscle, the ganglia become less abundant in small bronchi, being located near bronchial divisions (Smith & Taylor, 1971).

In bronchial ring segments significant contractile responses were only obtained to SP and the specific NK_1 receptor agonist, whereas NKA and NKB were relatively inactive. The NK_1 receptor mediated contractions in bronchial smooth muscle were neither tachyphylactic nor cholinergically mediated. In parenchymal tissue the ability of tachykinins to induce any contractile response was negligible.

This regional shift in mechanisms mediating tachykinin-induced ASM contractions in sheep may at first appear at variance with human *in vitro* data. Early studies on human bronchial smooth muscle demonstrated that tachykinin-induced contractions were solely mediated by tachykinin NK_2 receptors (Advenier *et al.*, 1992; Naline *et al.*, 1989), however, with the advent of more specific NK_1 receptor agonists a later study by the same authors, revealed NK_1 receptors mediated contractions in small diameter human bronchial preparations. Moreover, their later study demonstrated that the contractile responses were small in magnitude, subject to tachyphylaxis and were mediated by prostanoids (Naline *et al.*, 1996). Marked regional sensitivity to tachykinins has been demonstrated by others in isolated canine bronchial segments (McKay *et al.*, 1992) and parenchymal tissue in the guinea pig (Uchida *et al.*, 1987; Foreman *et al.*, 1985) and mouse (Brandolini *et al.*, 2000).

In light of the present *in vitro* results, the lack of a direct NK_2 mediated broncho-constrictor effect to SP *in vivo*, may simply reflect the relatively small amounts of SP reaching tracheal NK_2 and bronchial NK_1 smooth muscle receptors, since a significant proportion of both the aerosol and intravascular SP doses would be inactivated by NEP and angiotensin-converting enzyme (Joos *et al.*, 1994). Moreover, intravascular SP may induce bronchoconstriction by principally activating NK_1 in abundant tracheal parasympathetic ganglia, ultimately resulting in the prejunctional release of ACh from

postganglionic nerves (Belvisi *et al.*, 1994; Watson *et al.*, 1993), before the SP is inactivated by NEP. The observed tachyphylaxis to SP *in vivo* may result from the released ACh activating pre-junctional cholinergic M_2 autoreceptors providing a "braking mechanism" for vagally mediated ACh release (Pendry, 1993).

Overall, tissue sensitivity as reflected by the pD_2 values for the various contractile agonists studied in ovine tracheal smooth muscle are in closer agreement to those determined in isolated human bronchi (Qian *et al.*, 1994; Knight *et al.*, 1990; Naline *et al.*, 1989; Cerrina *et al.*, 1989) than for rabbit and guinea pig ASM (Goroumaru-Shinkai *et al.*, 1992; Devillier *et al.*, 1988; Advenier *et al.*, 1987) (Table 5.13). Given the considerable similarity between ovine and human ASM pharmacology, in the absence of available human tissue, the ovine tracheal smooth muscle preparation would be an ideal animal model for examining the effect of pharmacological interventions on bronchoconstrictor responses.

It is well recognized here, and by others that airway preparations of bronchial rings, tracheal and parenchymal strips do not represent "pure" ASM preparations, as other non-contractile and non-airway contractile elements are present, such as cartilaginous tissue, small peripheral blood vessels (Drazen & Schneider, 1978),(Finney *et al.*, 1984) contractile interstitial cells (Kapanci *et al.*, 1974), and alveolar ducts (Drazen & Schneider, 1978). An abundance of cartilaginous structure in the airway may underestimate the contractile force generated depending on the stimuli (Chitano *et al.*, 1993), whereas the presence and distribution of non-airway contractile elements may lead to inconsistent responses within and between species (Hulsmann & de Jongste, 1993). However, given these confounding effects studies using these airway preparations provide valuable additions to our current knowledge of airway function.

Following the *in vitro* characterization of contractile responses in ovine airways there was a shift in interest of this thesis from the role of tachykinins in AHR and inflammation to a study of the mechanisms by which pro-inflammatory cytokines induce AHR. As previously stated, the development of AHR has been linked to the presence of airway inflammation (Hogg *et al.*, 1991). However, the mechanism by which inflammation leads to AHR remains elusive. The remainder of this thesis is devoted to an examination of the effects of the pro-inflammatory cytokines, TNFα and IL-1β, on two mechanisms considered to be important in the development of AHR. Namely, i) an increased contractility of ASM and ii) the development of MVL. Although it is likely a number of cytokines are able to induce AHR and different cytokines may assume greater importance in different subsets of asthma, TNFα and IL-1β will be the cytokines of choice to study this phenomenon.

Tumour necrosis factor-α is a ubiquitous cytokine in inflammation, and has been postulated to have a fundamental role in airway inflammation in asthma and the development of AHR (Shah *et al.*, 1995; Kips *et al.*, 1993a). *In vivo* AHR has been demonstrated in sheep following TNFα infusion (Wheeler *et al.*, 1990). Whether this AHR results from a direct effect of the cytokine on ASM contractility, or via changes in airway wall structure leading to exaggerated airway narrowing was not determined. Using the considerable expertise gained thus far in my studies of bronchoconstrictor responses in ovine airways, experiments were designed to examine the effect of TNFα and IL-1β on ovine ASM contractility, *in vitro*. A range of concentrations for both IL-1β and TNFα were used, based on what may be considered to be effective in various biological systems (Hakonarson *et al.*, 1996; Horie *et al.*, 1996; Anticevich *et al.*, 1995;

Tamaoki *et al.*, 1994; Wills-Karp *et al.*, 1993b). The results of this series of experiments clearly demonstrate that TNFα enhances ACh contractility in ovine tracheal smooth muscle and that this effect is synergistic in the presence of IL-1β, which by itself has no effect on ASM contractility. Synergism becomes apparent when optimised concentrations of TNFα are in excess (10 to 100-fold) of those for IL-1β.

In subsequent investigations in this thesis, attention was directed towards elucidating the mechanisms by which TNFα and IL-1β enhance ovine *in vitro* smooth muscle contractility. Once again ovine tracheal strips were incubated in media containing IL-1β and TNFα at concentrations considered to be maximally effective. Following incubation the strips were prepared for *in vitro* studies of isometric contraction in response to the cumulative addition of ACh, NKA and histamine. Pre-incubation with TNFα and IL-1β caused an increase in both the responsiveness (increased E_{max}) and sensitivity (decreased pD_2) of ASM to ACh and NKA, whereas, histamine contractility remained unchanged following cytokine incubation. Neurokinin A contractility in the presence of phosphoramidon indicated that the enhanced contractility following cytokine exposure was not due to a reduction in endogenous NEP activity.

The enhanced contractility to ACh and NKA may result from an alteration in M_3 or NK_2 receptor function or an increase in expression of these cell surface receptors. In the case of the former, the present study demonstrated no differences in M_3 and NK_2 receptor antagonist affinity values between control and cytokine treated tissue. As for cytokine treatment increasing cell receptor numbers, several research groups have demonstrated that incubation of tracheal smooth muscle IL-1β or TNFα either caused a decrease or no change in muscarinic receptors (Hąkonarson *et al.*, 1996). Thus the

occurrence of either mechanism being responsible for enhanced muscle contractility appears unlikely.

Removal of extracellular Ca^{++} attenuated the contractile response to low concentrations of ACh in control and cytokine pretreated tissue. However, enhanced contractility following TNFα and IL-1β pretreatment was still present, providing evidence that the mechanism involves an augmentation in intracellular Ca^{++} mobilization. The observation of diminished contractile responses to ACh, especially at low agonists concentrations, in calcium-free K-H is not surprising as this has previously been described by others in bovine and guinea pig tracheal smooth muscle (Creese & Denborough, 1981; Kirkpatrick *et al.*, 1975b).

In calcium-free K-H solution the contractile response to histamine in tracheal muscle strips was virtually abolished. This suggests that histamine contractions in ovine ASM are predominantly mediated by extracellular Ca^{++} influx, an observation that has been made in other species including man (Black *et al.*, 1986; Creese & Denborough, 1981; Kirkpatrick, 1975a). Furthermore, the lack of an enhancement to histamine contractility following cytokine pretreatment appears to reflect agonist-specific differences in post-receptor signalling pathways that mediate ovine ASM contraction.

The present results seem at variance to a recent study by Sukkar and colleagues (Sukkar *et al.*, 2001). In their study co-incubation of isolated human bronchi with TNFα and IL-1β abolished the *in vitro* AHR observed following TNFα incubation, rather than demonstrating synergism. Although these differences may be species related, variations in methodologies are apparent. In the present study isolated ASM preparations were obtained from healthy sheep in the absence of anaesthetic agents. Whereas for the

human study, bronchial tissue (albeit non-sensitised) was obtained from lung transplantation and surgical resections for lung carcinoma, the latter known to be hyperresponsive to cholinergic agonists (Armour *et al.*, 1996). Furthermore, volatile anaesthetics are known to attenuate increases in $[Ca^{++}]_i$ evoked by contractile agonists predominantly by attenuating Ca^{++} release from the SR rather than extracellular Ca^{++} influx (Karaki *et al.*, 1997). Differing cytokine concentrations were employed. Whether human ASM incubated with higher concentrations of TNFα produced further hyperresponsiveness was not evident. Perhaps valuable nutrients required to elicit synergism were missing as the human ASM was incubated in a physiological salt solution rater than in tissue culture medium. Therefore, it remains to be determined whether these differences in the induction of *in vitro* AHR following cytokine incubation are species, disease or methodologically related.

The results of this thesis clearly demonstrate that IL-1β and TNFα act synergistically to enhance smooth muscle contractility to particular agonists, by affecting signal transduction pathways that mediate ASM contraction. Nevertheless, it remains to be determined whether pro-inflammatory cytokines can also affect airway microvascular permeability. This may be important during acute exacerbation of asthma, where peribronchial oedema may induce rapid airway instability by unlinking the airways from the parenchyma, leading to life threatening bronchoconstriction (Macklem, 1990). With this in mind, an isolated section of guinea pig trachea was challenge with an aerosol of TNFα and IL-1β in order to replicate local cytokine release, and determine their effect on vascular permeability in the extensive subepithelial microvascular plexus.

In this surgical mode, EB extravasation was quantitated in the ITS, in anesthetised mechanically ventilated guinea pigs. The advantage of this model is that it allows

prolonged exposure of this segment to various agents without causing adverse systemic effects. The present study is the first to demonstrate that an aerosol of IL-1β and TNFα induces airway MVL in the guinea pig trachea. However, due to time constraints the mechanism by which this occurs was not determined. Vascular permeability is dependent on intracellular Ca^{++} mobilization and the activation of a cascade of calcium-dependent mechanisms downstream from this initial rise in $[Ca^{++}]_i$ (Michel & Curry, 1999), ultimately leading to increased tension developed by actin-myosin interactions and the formation of intercellular gaps by endothelial cell contraction (McDonald, 1994; Joris *et al.*, 1987). Furthermore, others have demonstrated that Ca^{++} mobilization from internal stores is a critical event in the initiation of microvascular permeability (Chetham *et al.*, 1999), and the magnitude of the initial rise in $[Ca^{++}]_i$ is proportional to the increase in permeability (Michel & Curry, 1999). Therefore, in view of the results obtained in the ASM contractility studies, it is tempting to speculate that tracheal exposure to TNFα and IL-1β may enhance internal Ca^{++} mobilization in endothelial cells, triggering intercellular gap formation, and thereby increasing microvascular permeability with plasma extravasation.

The findings of the present thesis clearly demonstrate that the pro-inflammatory cytokines, TNFα and IL-1β, modulate two aspects of airway function considered to be important in the development of AHR. These being i) enhancing ASM contractility and ii) increasing MVL, and that in the former the mechanism involves an augmentation in intracellular Ca^{++} mobilization. The above effects being in addition to any diminution in ASM relaxation responses (Hakonarson *et al.*, 1996; Koto *et al.*, 1996; Munakata *et al.*, 1996; Wills-Karp *et al.*, 1993b). While the simultaneous effect of these pro-inflammatory cytokines on AHR and MVL was not determined *in vivo*, functional

correlates between the degree of plasma extravasation and AHR have been documented in both human and animal studies (Kimura *et al.*, 1992; Van de Graaf *et al.*, 1991).

Although the heightened *in vitro* contractility appears minimal, one would predict even greater ASM shortening *in vivo*, (and hence airway narrowing) due to the ASM being loaded auxotonically. And while an acute increase in airway wall mucosal thickness by itself has little effect on airway resistance (Hogg *et al.*, 1987), in the presence of smooth muscle contraction will induce profound airway narrowing (Pare & Bai, 1995). The ability of TNFα and IL-1β to induce MVL is of potential clinical significance in cases of sudden-onset fatal asthma. In these individuals there is much accumulated evidence to suggest an involvement of the microvasculature in the fatal outcome. In sudden death asthma the airway submucosa contains increased numbers of neutrophils, both intravascular and extravasated, (Carroll *et al.*, 1996; Sur *et al.*, 1993) with the inflammatory infiltrate being polarized to the vascular adventitia adjacent to airways (Saetta *et al.*, 1991). Therefore, the localized release of TNFα and IL-1β in acute severe exacerbations of asthma (Tillie-Leblond *et al.*, 1999) may induce marked airway wall oedema that uncouples the airway from the parenchyma to such an extent that ASM contraction can be so severe as to completely occlude the airway (Macklem, 1990), a phenomenon often observed in sudden-onset fatal asthma (Sur *et al.*, 1993).

9.2 Future directions

In ASM contraction, agonist-receptor binding results in the hydrolysis of PIP_2 to IP_3 which in turn binds to specific IP_3R on intracellular Ca^{++} stores (such as the SR) leading to the release of Ca^{++} and ultimately contraction (Schramm & Grunstein, 1992). In the contractile compartment of smooth muscle cells, $[Ca^{++}]_i$ is primarily determined by the

balance between the release of Ca^{++} from the SR and the re-uptake via the sarco-endoplasmic reticulum Ca^{++}-ATPase pump (SERCA) (Karaki *et al.*, 1997).

Of particular significance in the present study is the finding that TNFα and IL-1β enhance ASM responsiveness to agonists that induce contraction via a mechanism not solely dependent on extracellular Ca^{++} influx. It had long been suspected that an alteration in Ca^{++} mobilization may mediate AHR, principally by enhanced Ca^{++} influx (Black *et al.*, 1989; Middleton, 1984). The present results demonstrate that cytokine-induced AHR involves an alteration in intracellular Ca^{++} mobilization, possibly by either an enhanced release of Ca^{++} from the SR or a sustained elevation in $[Ca^{++}]_i$.

Amrani and colleagues demonstrated, in cultured tracheal smooth muscle cells, that agonist-induced increases in $[Ca^{++}]_i$ were enhanced by TNFα and IL-1β pretreatment (Amrani *et al.*, 1997), and in the case of TNFα, this augmentation was mediated by increased IP_3 accumulation (Amrani *et al.*, 1997) and a decreased Ca^{++} re-uptake by SERCA pumps (Amrani *et al.*, 1996). While a variety of physiological responses are mediated by signal transduction pathways that involve the release of Ca^{++} following IP_3 binding to specific IP_3R on the SR, little is know about IP_3R regulation. Therefore, it is tempting to speculate that IL-1β and TNFα may also affect intracellular Ca^{++} mobilization by altering IP_3R function.

The IP_3R is encoded by 3 different genes resulting in the production of 3 receptor subtypes or isoforms, (IP_3R-1, IP_3R-2 and IP_3R-3). The relative abundance or expression of each receptor subtype appears to be tissue- and development-specific (Wojcikiewicz, 1995; Newton *et al.*, 1994). The IP_3R subtypes form a heterotetrameric

protein complex in the SR membrane, which act as Ca^{++} channels (Monkawa *et al.*, 1995) (Figure 9.1). While all receptor subtypes exhibit similar specificity for inositol phosphates, each differs in its affinity for IP_3 (Newton *et al.*, 1994). More recent studies have demonstrated that the ability of IP_3 to release Ca^{++} from intracellular stores is determined by the differential expression of the IP_3R subtypes in the heterotetrameric IP_3R complex (Miyakawa *et al.*, 1999; Wojcikiewicz & Luo, 1998).

Therefore, one possible mechanism for the enhanced intracellular Ca^{++} mobilization following TNFα and IL-1β exposure may result from their ability to modulate the differential expression of IP_3R subtypes within the cell. This aspect is perhaps best studied by employing molecular biology techniques. Using quantitative reverse transcriptase-polymerase chain reaction techniques, experiments can be conducted in cultured cells of interest, such as ASM and vascular endothelial cells, to determine the effect of IL-1β and TNFα incubation, alone or in combination, on the differential expression of IP_3R-1, IP_3R-2 and IP_3R-3 mRNA. Follow these studies, Western blot analysis using specific antibodies against the IP_3R-1, IP_3R-2 and IP_3R-3 proteins, should be performed to confirm that any change in the differential expression IP_3R subtypes mRNA, induced by these cytokines, does in deed translate to an altered expression at the protein level.

Presently, the physiological significance of an alteration in the differential expression of IP_3R subtypes remains to be determined, however, it is thought that it may modulate intracellular Ca^{++} release (Miyakawa *et al.*, 1999). Therefore, the above studies should be extended to include functional studies to characterize the effect of an altered IP_3R subtype expression, induced by cytokine treatment, on intracellular Ca^{++} releasability. Calcium ion mobilization studies first involve loading the intracellular Ca^{++} stores by a

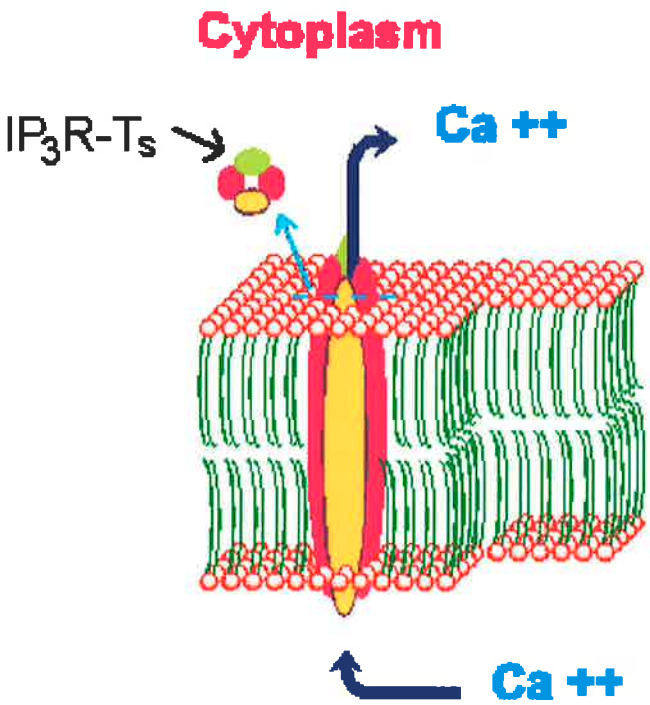


Figure 9.1 Diagrammatic representation of the inositol triphosphate receptor, illustrating the Ca^{++} channel formed by a combination of the three IP_3R subtypes (IP_3R-Ts) into an heterotetrameric complex.

brief incubation period with either radiolabelled $^{45}Ca^{++}$ or fluorescent-labeled Ca^{++}, such as Fura-2 or Indol-1. Then exposing the cells to agonists of interest, in the present case IP_3 the intracellular agonist for the IP_3R, and finally determining the amount of labeled Ca^{++} released into the cytoplasm. Loading intracellular stores with $^{45}Ca^{++}$ being preferable since fluorescent monitoring of $[Ca^{++}]_i$ is limited by various factors such as, interference in the fluorescent signal by endogenous fluorescent substances and rapid leakage of Fura-2 from the cell (Karaki *et al.*, 1997).

Following $^{45}Ca^{++}$ loading in cells of interest (e.g. cultured ASM and vascular endothelial cells of known IP_3R subtype expression) formal dose-response studies of IP_3 concentration on Ca^{++} release would be conducted. The results of which would provide evidence as to whether an alteration in the differential expression of IP_3R subtypes confers a change in sensitivity of the IP_3 receptor to release Ca^{++} from intracellular stores. In view of the results of the present thesis, such an increase in sensitivity of the IP_3R to the second messenger IP_3, in both vascular and ASM cells may result in the augmentation of agonist-induced responses, including increased MVL and enhanced smooth muscle contractility. Moreover, in an asthmatic this may predispose the individual, during acute exacerbations, to exaggerated life threatening broncho-constrictor responses following exposure to a variety of stimuli. However, this remains to be established.

Bibliography

Aas, P. & Maclagan, J. (1990). Evidence for prejunctional M2 muscarinic receptors in pulmonary cholinergic nerves in the rat. *British Journal of Pharmacology* **101**, 73-76.

Abbas, A.K., Murphy, K.M. & Sher, A. (1996). Functional diversity of helper T lymphocytes. *Nature* **383**, 787-793.

Abraham, W.M. (1988). The role of leukotrienes in allergen-induced late responses in allergic sheep. *Annals of the New York Academy of Sciences* **524**, 260-270.

Abraham, W.M., Abraham, M.K. & Ahmed, T. (1996). Protective effect of heparin on immunologically induced tracheal smooth muscle contraction in vitro. *International Archives of Allergy & Immunology* **110**, 79-84.

Abraham, W.M., Ahmed, A., Cortes, A., Soler, M., Farmer, S.G., Baugh, L.E. & Harbeson, S.L. (1991). Airway effects of inhaled bradykinin, substance P, and neurokinin A in sheep. *Journal of Allergy & Clinical Immunology* **87**, 557-564.

Abraham, W.M., Ahmed, A., Sabater, J.R., Lauredo, I.T., Botvinnikova, Y., Bjercke, R.J., Hu, X., Revelle, B.M., Kogan, T.P., Scott, I.L., Dixon, R.A., Yeh, E.T. & Beck, P.J. (1999). Selectin blockade prevents antigen-induced late bronchial responses and airway hyperresponsiveness in allergic sheep. *American Journal of Respiratory & Critical Care Medicine* **159**, 1205-1214.

Abraham, W.M., Delehunt, J.C., Yerger, L. & Marchette, B. (1983). Characterization of a late phase pulmonary response after antigen challenge in allergic sheep. *American Review of Respiratory Disease* **128**, 839-844.

Abraham, W.M., Kim, C.S., King, M.M., Oliver, W.J. & Yerger, L. (1982). Effects of nitric acid on carbachol reactivity of the airways in normal and allergic sheep. *Archives of Environmental Health* **37**, 36-40.

Abraham, W.M., Oliver, W.J., King, M.M., Yerger, L. & Wanner, A. (1981a). Effect of pharmacologic agents on antigen-induced decreases in specific lung conductance in sheep. *American Review of Respiratory Disease* **124**, 554-558.

Abraham, W.M., Oliver, W.J., Welker, M.J., King, M.M., Wanner, A. & Sackner, M.A. (1981b). Differences in airway reactivity in normal and allergic sheep after exposure to sulfur dioxide. *Journal of Applied Physiology* **51**, 1651-1656.

Abraham, W.M., Sielczak, M.W., Wanner, A., Perruchoud, A.P., Blinder, L., Stevenson, J.S., Ahmed, A. & Yerger, L.D. (1988a). Cellular markers of inflammation in the airways of allergic sheep with and without allergen-induced late responses. *American Review of Respiratory Disease* **138**, 1565-1571.

Abraham, W.M., Stevenson, J.S., Chapman, G.A., Tallent, M.W. & Jackowski, J. (1987). The effect of nedocromil sodium and cromolyn sodium on antigen-induced responses in allergic sheep in vivo and in vitro. *Chest* **92**, 913-917.

Ackerman, V., Marini, M., Vittori, E., Bellini, A., Vassali, G. & Mattoli, S. (1994). Detection of cytokines and their cell sources in bronchial biopsy specimens from asthmatic patients. Relationship to atopic status, symptoms, and level of airway hyperresponsiveness. *Chest* **105**, 687-696.

Adcock, I.M., Peters, M., Gelder, C., Shirasaki, H., Brown, C.R. & Barnes, P.J. (1993). Increased tachykinin receptor gene expression in asthmatic lung and its modulation by steroids. *Journal of Molecular Endocrinology* **11**, 1-7.

Adcock, I.M., Shirasaki, H., Gelder, C.M., Peters, M.J., Brown, C.R. & Barnes, P.J. (1994). The effects of glucocorticoids on phorbol ester and cytokine stimulated transcription factor activation in human lung. *Life Sciences* **55**, 1147-1153.

Advenier, C., Naline, E., Drapeau, G. & Regoli, D. (1987). Relative potencies of neurokinins in guinea pig trachea and human bronchus. *European Journal of Pharmacology* **139**, 133-137.

Advenier, C., Naline, E., Toty, L., Bakdach, H., Emonds-Alt, X., Vilain, P., Breliere, J.C. & Le Fur, G. (1992). Effects on the isolated human bronchus of SR 48968, a potent and selective nonpeptide antagonist of the neurokinin A (NK2) receptors. *American Review of Respiratory Disease* **146**, 1177-1181.

Ahmed, T., Krainson, J.P. & Yerger, L.D. (1983). Functional depression of H2 histamine receptors in sheep with experimental allergic asthma. *Journal of Allergy & Clinical Immunology* **72**, 310-320.

Akutsu, I., Kojima, T., Kariyone, A., Fukuda, T., Makino, S. & Takatsu, K. (1995). Antibody against interleukin-5 prevents antigen-induced eosinophil infiltration and bronchial hyperreactivity in the guinea pig airways. *Immunology Letters* **45**, 109-116.

Ammit, A.J., Armour, C.L. & Black, J.L. (2000). Smooth-muscle myosin light-chain kinase content is increased in human sensitized airways. *American Journal of Respiratory & Critical Care Medicine* **161**, 257-263.

Ammit, A.J., Bekir, S.S., Johnson, P.R., Hughes, J.M., Armour, C.L. & Black, J.L. (1997). Mast cell numbers are increased in the smooth muscle of human sensitized isolated bronchi. *American Journal of Respiratory & Critical Care Medicine* **155**, 1123-1129.

Amrani, Y., Krymskaya, V., Maki, C. & Panettieri, R.A.J. (1997). Mechanisms underlying TNF-alpha effects on agonist-mediated calcium homeostasis in human airway smooth muscle cells. *American Journal of Physiology* **273**, L1020-L1028

Amrani, Y., Magnier, C., Enouf, J., Wuytack, F. & Bronner, C. (1995a). Ca2+ increase and Ca(2+)-influx in human tracheal smooth muscle cells: role of Ca2+ pools controlled by sarco-endoplasmic reticulum Ca(2+)-ATPase 2 isoform. *British Journal of Pharmacology* **115**, 1204-1210.

Amrani, Y., Martinet, N. & Bronner, C. (1995b). Potentiation by tumour necrosis factor-alpha of calcium signals induced by bradykinin and carbachol in human tracheal smooth muscle cells. *British Journal of Pharmacology* **114**, 4-5.

Amrani, Y., Panettieri, R.A.J., Frossard, N. & Bronner, C. (1996). Activation of the TNF alpha-p55 receptor induces myocyte proliferation and modulates agonist-evoked calcium transients in cultured human tracheal smooth muscle cells. *American Journal of Respiratory Cell & Molecular Biology* **15**, 55-63.

Amrani, Y. & Panettieri, R.A.J. (1998). Cytokines induce airway smooth muscle cell hyperresponsiveness to contractile agonists. *Thorax* **53**, 713-716.

Anderson, G.P. & Coyle, A.J. (1994). TH2 and 'TH2-like' cells in allergy and asthma: pharmacological perspectives. *Trends in Pharmacological Sciences* **15**, 324-332.

Anderson, S.D., Schoeffel, R.E. & Finney, M. (1983). Evaluation of ultrasonically nebulised solutions for provocation testing in patients with asthma. *Thorax* **38**, 284-291.

Anthony, D.C., Bolton, S.J., Fearn, S. & Perry, V.H. (1997). Age-related effects of interleukin-1 beta on polymorphonuclear neutrophil-dependent increases in blood-brain barrier permeability in rats. *Brain* **120**, 435-444.

Anticevich, S.Z., Hughes, J.M., Black, J.L. & Armour, C.L. (1995). Induction of human airway hyperresponsiveness by tumour necrosis factor-alpha. *European Journal of Pharmacology* **284**, 221-225.

Anticevich, S.Z., Hughes, J.M., Black, J.L. & Armour, C.L. (1996). Induction of hyperresponsiveness in human airway tissue by neutrophils--mechanism of action. *Clinical & Experimental Allergy* **26**, 549-556.

Anto, J.M., Sunyer, J., Rodriguez-Roisin, R., Suarez-Cervera, M. & Vazquez, L. (1989). Community outbreaks of asthma associated with inhalation of soybean dust. Toxicoepidemiological Committee. *New England Journal of Medicine* **320**, 1097-1102.

Antol, P.J., Gunst, S.J. & Hyatt, R.E. (1988). Histamine tachyphylaxis in canine airways despite prostaglandin synthesis inhibition. *Journal of Applied Physiology* **65**, 1944-1949.

Antonissen, L.A., Mitchell, R.W., Kroeger, E.A., Kepron, W., Tse, K.S. & Stephens, N.L. (1979). Mechanical alterations of airway smooth muscle in a canine asthmatic model. *Journal of Applied Physiology* **46**, 681-687.

Armour, C.L., Black, J.L. & Berend, N. (1985). Histamine and carbachol contractile responses in proximal and distal airways of the rabbit. *Clinical & Experimental Pharmacology & Physiology* **12**, 19-23.

Armour, C.L., Black, J.L., Berend, N. & Woolcock, A.J. (1984). The relationship between bronchial hyperresponsiveness to methacholine and airway smooth muscle structure and reactivity. *Respiration Physiology* **58**, 223-233.

Armour, C.L., Diment, L.M. & Black, J.L. (1988). Relationship between smooth muscle volume and contractile response in airway tissue. Isometric versus isotonic measurement. *Journal of Pharmacology & Experimental Therapeutics* **245**, 687-691.

Armour, C.L., Johnson, P.R., Alouan, L.A. & Black, J.L. (1991). Substance P-induced contraction of rabbit airways: mechanism of action. *Journal of Applied Physiology* **70**, 813-817.

Armour, C.L., Johnson, P.R. & Black, J.L. (1988). Potentiation of contraction of rabbit airway smooth muscle by some cyclooxygenase products. *Prostaglandins* **35**, 959-968.

Armour, C.L., Johnson, P.R. & Black, J.L. (1991). Nedocromil sodium inhibits substance P-induced potentiation of cholinergic neural responses in the isolated innervated rabbit trachea. *Journal of Autonomic Pharmacology* **11**, 167-172.

Armour, C.L., Lazar, N.M., Schellenberg, R.R., Taylor, S.M., Chan, N., Hogg, J.C. & Pare, P.D. (1984a). A comparison of in vivo and in vitro human airway reactivity to histamine. *American Review of Respiratory Disease* **129**, 907-910.

Armour, C.L., McKay, K.O., Johnson, P.R., Glanville, A.R. & Black, J.L. (1996). Does the disease state influence the responsiveness of human airways studied in vitro? *Journal of Applied Physiology* **80**, 2211-2216.

Arunlakshana, O. & Schild, H.O. (1959). Some quantitative uses of drug antagonists. *British Journal of Pharmacology* **14**, 48-58.

Axelrod, J. (1990). Receptor-mediated activation of phospholipase A2 and arachidonic acid release in signal transduction. *Biochemical Society Transactions* **18**, 503-507.

Bai, A., Eidelman, D.H., Hogg, J.C., James, A.L., Lambert, R.K., Ludwig, M.S., Martin, J., McDonald, D.M., Mitzner, W.A. & Okazawa, M. (1994). Proposed nomenclature for quantifying subdivisions of the bronchial wall. *Journal of Applied Physiology* **77**, 1011-1014.

Bai, T.R., Zhou, D., Weir, T., Walker, B., Hegele, R., Hayashi, S., McKay, K., Bondy, G.P. & Fong, T. (1995). Substance P (NK1)- and neurokinin A (NK2)-receptor gene expression in inflammatory airway diseases. *American Journal of Physiology* **269**, L309-L317

Baker, D.G., Basbaum, C.B., Herbert, D.A. & Mitchell, R.A. (1983). Transmission in airway ganglia of ferrets: inhibition by norepinephrine. *Neuroscience Letters* **41**, 139-143.

Baluk, P., Bertrand, C., Geppetti, P., McDonald, D.M. & Nadel, J.A. (1995). NK1 receptors mediate leukocyte adhesion in neurogenic inflammation in the rat trachea. *American Journal of Physiology* **268**, L263-L269

Baluk, P., Hirata, A., Thurston, G., Fujiwara, T., Neal, C.R., Michel, C.C. & McDonald, D.M. (1997). Endothelial gaps: time course of formation and closure in inflamed venules of rats. *American Journal of Physiology* **272**, L155-L170

Banchereau, J., Bazan, F., Blanchard, D., Briere, F., Galizzi, J.P., van Kooten, C., Liu, Y.J., Rousset, F. & Saeland, S. (1994). The CD40 antigen and its ligand. *Annual Review of Immunology* **12**, 881-922.

Bar-Ilan, A. & Marder, J. (1980). Acid base status in unanesthetized, unrestrained guinea pigs. *European Journal of Physiology* **384**, 93-97.

Barnes, P.J. (1984). The third nervous system in the lung: physiology and clinical perspectives. *Thorax* **39**, 561-567.

Barnes, P.J. (1986a). Asthma as an axon reflex. *Lancet* **1**, 242-245.

Barnes, P.J. (1986b). Neural control of human airways in health and disease. *American Review of Respiratory Disease* **134**, 1289-1314.

Barnes, P.J. (1987a). Cholinergic control of airway smooth muscle. *American Review of Respiratory Disease* **136**, S42-S45

Barnes, P.J. (1987b). Inflammatory mediator receptors and asthma. *American Review of Respiratory Disease* **135**, S26-S31

Barnes, P.J. (1989). Muscarinic receptor subtypes: implications for lung disease. *Thorax* **44**, 161-167.

Barnes, P.J. (1991). Neuropeptides and asthma. *American Review of Respiratory Disease* **143**, S28-S32

Barnes, P.J. (1992). Modulation of neurotransmission in airways. *Physiological Reviews* **72**, 699-729.

Barnes, P.J. (1994). Cytokines as mediators of chronic asthma. *American Journal of Respiratory & Critical Care Medicine* **150**, S42-S49

Barnes, P.J. (1995). Is asthma a nervous disease? The Parker B. Francis Lectureship. *Chest* **107**, 119S-125S.

Barnes, P.J. (1996a). Neuroeffector mechanisms: the interface between inflammation and neuronal responses. *Journal of Allergy & Clinical Immunology* **98**, S73-S81.

Barnes, P.J. (1996b). What is the role of nerves in chronic asthma and symptoms? *American Journal of Respiratory & Critical Care Medicine* **153**, S5-S8.

Barnes, P.J. (1998a). Pharmacology of airway smooth muscle. *American Journal of Respiratory & Critical Care Medicine* **158**, S123-S132.

Barnes, P.J. & Adcock, I.M. (1998b). Transcription factors and asthma. *European Respiratory Journal* **12**, 221-234.

Barnes, P.J., Basbaum, C.B. & Nadel, J.A. (1983). Autoradiographic localization of autonomic receptors in airway smooth muscle. Marked differences between large and small airways. *American Review of Respiratory Disease.* **127**, 758-762.

Barnes, P.J., Chung, K.F. & Page, C.P. (1998). Inflammatory mediators of asthma: an update. *Pharmacological Reviews* **50**, 515-596.

Basbaum, C.B., Madison, J.M., Sommerhoff, C.P., Brown, J.K. & Finkbeiner, W.E. (1990). Receptors on airway gland cells. *American Review of Respiratory Disease* **141**, S141-S144.

Basha, M.A., Gross, K.B., Gwizdala, C.J., Haidar, A.H. & Popovich, J., Jr. (1994). Bronchoalveolar lavage neutrophilia in asthmatic and healthy volunteers after controlled exposure to ozone and filtered purified air. *Chest* **106**, 1757-1765.

Beasley, R., Crane, J., Lai, C.K. & Pearce, N. (2000). Prevalence and etiology of asthma. *Journal of Allergy & Clinical Immunology* **105**, 466-472.

Beasley, R., Roche, W.R., Roberts, J.A. & Holgate, S.T. (1989). Cellular events in the bronchi in mild asthma and after bronchial provocation. *American Review of Respiratory Disease* **139**, 806-817.

Beavo, J.A. (1995). Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms. *Physiological Reviews* **75**, 725-748.

Becker, A.B., Black, C., Lilley, M.K., Bajwa, K., Ford-Hutchinson, A.W., Simons, F.E. & Tagari, P. (1995). Antiasthmatic effects of a leukotriene biosynthesis inhibitor (MK-0591) in allergic dogs. *Journal of Applied Physiology* **78**, 615-622.

Beets, J.L. & Paul, W. (1980). Actions of locally administered adrenoceptor agonists on increased plasma protein extravasation and blood flow in guinea-pig skin. *British Journal of Pharmacology* **70**, 461-467.

Belvisi, M.G., Miura, M., Stretton, D. & Barnes, P.J. (1993). Endogenous vasoactive intestinal peptide and nitric oxide modulate cholinergic neurotransmission in guinea-pig trachea. *European Journal of Pharmacology* **231**, 97-102.

Belvisi, M.G., Patacchini, R., Barnes, P.J. & Maggi, C.A. (1994). Facilitatory effects of selective agonists for tachykinin receptors on cholinergic neurotransmission: evidence for species differences. *British Journal of Pharmacology* **111**, 103-110.

Belvisi, M.G., Rogers, D.F. & Barnes, P.J. (1989). Neurogenic plasma extravasation: inhibition by morphine in guinea pig airways in vivo. *Journal of Applied Physiology* **66**, 268-272.

Ben-Jebria, A., Marthan, R., Rossetti, M. & Savineau, J.P. (1993). Effect of passive sensitization on the mechanical activity of human isolated bronchial smooth muscle induced by substance P, neurokinin A and VIP. *British Journal of Pharmacology* **109**, 131-136.

Bentley, A.M., Maestrelli, P., Saetta, M., Fabbri, L.M., Robinson, D.S., Bradley, B.L., Jeffery, P.K., Durham, S.R. & Kay, A.B. (1992). Activated T-lymphocytes and eosinophils in the bronchial mucosa in isocyanate-induced asthma. *Journal of Allergy & Clinical Immunology* **89**, 821-829.

Berger, P., Compton, S.J., Molimard, M., Walls, A.F., N'Guyen, C., Marthan, R. & Tunon-De-Lara, J.M. (1999). Mast cell tryptase as a mediator of hyperresponsiveness in human isolated bronchi. *Clinical & Experimental Allergy* **29**, 804-812.

Berger, P., Walls, A.F., Marthan, R. & Tunon-De-Lara, J.M. (1998). Immunoglobulin E-induced passive sensitization of human airways: an immunohistochemical study. *American Journal of Respiratory & Critical Care Medicine* **157**, 610-616.

Bianco, P., Fisher, L.W., Young, M.F., Termine, J.D. & Robey, P.G. (1990). Expression and localization of the two small proteoglycans biglycan and decorin in developing human skeletal and non-skeletal tissues. *The Journal of Histochemistry and Cytochemistry* **38**, 1549-1563.

Bienenstock, J., MacQueen, G., Sestini, P., Marshall, J.S., Stead, R.H. & Perdue, M.H. (1991). Mast cell/nerve interactions in vitro and in vivo. *American Review of Respiratory Disease* **143**, S55-S58.

Birkedal-Hansen, H., Moore, W.G., Bodden, M.K., Windsor, L.J., Birkedal-Hansen, B., DeCarlo, A. & Engler, J.A. (1993). Matrix metalloproteinases: a review. *Critical Reviews in Oral Biology and Medicine* **4**, 197-250.

Birnbaumer, L. & Brown, A.M. (1990). G proteins and the mechanism of action of hormones, neurotransmitters, and autocrine and paracrine regulatory factors. *merican Review of Respiratory Disease* **141**, S106-S114.

Bjorck, T., Gustafsson, L.E. & Dahlen, S.E. (1992). Isolated bronchi from asthmatics are hyperresponsive to adenosine, which apparently acts indirectly by liberation of leukotrienes and histamine. *American Review of Respiratory Disease* **145**, 1087-1091.

Black, J., Armour, C., Johnson, P. & Vincenc, K. (1986). The calcium dependence of histamine, carbachol and potassium chloride-induced contraction in human airways in vitro. *European Journal of Pharmacology* **125**, 159-168.

Black, J.L., Armour, C.L., Johnson, P.R., Alouan, L.A. & Barnes, P.J. (1990). The action of a potassium channel activator, BRL 38227 (lemakalim), on human airway smooth muscle. *American Review of Respiratory Disease* **142**, 1384-1389.

Black, J.L., Diment, L.M., Alouan, L.A., Johnson, P.R., Armour, C.L., Badgery-Parker, T. & Burcher, E. (1992). Tachykinin receptors in rabbit airways--characterization by functional, autoradiographic and binding studies. *British Journal of Pharmacology* **107** , 429-436.

Black, J.L., Diment, L.M., Armour, C.L. & Johnson, P.R. (1990a). Distribution of substance P receptors in rabbit airways, functional and autoradiographic studies. *Journal of Pharmacology and Experimental Therapeutics* **253**, 381-385.

Black, J.L., French, R.J. & Mylecharane, E.J. (1981). Receptor mechanisms for 5-hydroxytryptamine in rabbit arteries. *British Journal of Pharmacology* **74**, 619-626.

Black, J.L., Johnson, P.R., Alouan, L. & Armour, C.L. (1990b). Neurokinin A with K+ channel blockade potentiates contraction to electrical stimulation in human bronchus. *European Journal of Pharmacology* **180**, 311-317.

Black, J.L., Johnson, P.R. & Armour, C.L. (1988). Potentiation of the contractile effects of neuropeptides in human bronchus by an enkephalinase inhibitor. *Pulmonary Pharmacology* **1**, 21-23.

Black, J.L., Marthan, R., Armour, C.L. & Johnson, P.R. (1989). Sensitization alters contractile responses and calcium influx in human airway smooth muscle. *Journal of Allergy & Clinical Immunology* **84**, 440-447.

Blackshear, P.J., Nairn, A.C. & Kuo, J.F. (1988). Protein kinases 1988: a current perspective. *The Federation of American Societies for Experimental Biology Journal* **2**, 2957-2969.

Bochner, B.S. (1997). Cellular adhesion and its antagonism. *Journal of Allergy & Clinical Immunology* **100**, 581-585.

Bodner, C.H., Ross, S., Little, J., Douglas, J.G., Legge, J.S., Friend, J.A. & Godden, D.J. (1998). Risk factors for adult onset wheeze: a case control study. *American Journal of Respiratory & Critical Care Medicine* **157**, 35-42.

Bonnet, J., Loiseau, A.M., Orvoen, M. & Bessin, P. (1981). Platelet-activating factor acether (PAF-acether) involvement in acute inflammatory and pain processes. *Agents & Actions* **11**, 559-562.

Borish, L. (1987). The inflammatory theory of asthma. *Immunol Invest* **16**, 501-532.

Borish, L. & Rosenwasser, L.J. (1996). Update on cytokines. *Journal of Allergy & Clinical Immunology* **97**, 719-734.

Borson, D.B., Brokaw, J.J., Sekizawa, K., McDonald, D.M. & Nadel, J.A. (1989). Neutral endopeptidase and neurogenic inflammation in rats with respiratory infections. *Journal of Applied Physiology* **66**, 2653-2658.

Borson, D.B., Corrales, R., Varsano, S., Gold, M., Viro, N., Caughey, G., Ramachandran, J. & Nadel, J.A. (1987). Enkephalinase inhibitors potentiate substance P-induced secretion of 35SO4-macromolecules from ferret trachea. *Experimental Lung Research* **12**, 21-36.

Borson, D.B. & Gruenert, D.C. (1991). Glucocorticoids induce neutral endopeptidase in transformed human tracheal epithelial cells. *American Journal of Physiology* **260**, L83-L89

Bosse, J., Boileau, R. & Begin, R. (1987). Chronic allergic airway disease in the sheep model: functional and lung-lavage features. *Journal of Allergy & Clinical Immunology* **79**, 339-344.

Boulet, L.P., Cartier, A., Thomson, N.C., Roberts, R.S., Dolovich, J. & Hargreave, F.E. (1983). Asthma and increases in nonallergic bronchial responsiveness from seasonal pollen exposure. *Journal of Allergy & Clinical Immunology* **71**, 399-406.

Bousquet, J., Chanez, P., Lacoste, J.Y., Barneon, G., Ghavanian, N., Enander, I., Venge, P., Ahlstedt, S., Simony-Lafontaine, J. & Godard, P. (1990). Eosinophilic inflammation in asthma. *New England Journal of Medicine* **323**, 1033-1039.

Bousquet, J., Chanez, P., Lacoste, J.Y., Enander, I., Venge, P., Peterson, C., Ahlstedt, S., Michel, F.B. & Godard, P. (1991). Indirect evidence of bronchial inflammation assessed by titration of inflammatory mediators in BAL fluid of patients with asthma. *Journal of Allergy & Clinical Immunology* **88**, 649-660.

Bousquet, J., Chanez, P., Vignola, A.M., Lacoste, J.Y. & Michel, F.B. (1994). Eosinophil inflammation in asthma. *American Journal of Respiratory & Critical Care Medicine* **150**, S33-S38

Bowden, J.J., Garland, A.M., Baluk, P., Lefevre, P., Grady, E.F., Vigna, S.R., Bunnett, N.W. & McDonald, D.M. (1994). Direct observation of substance P-induced internalization of neurokinin 1 (NK1) receptors at sites of inflammation. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 8964-8968.

Bowden, J.J. & Gibbins, I.L. (1992). Relative density of substance-P immunoreactive (SP-IR) nerve fibres in the tracheal epithelium of a range of species. *The Federation of American Societies for Experimental Biology Journal* **6**, A1276

Bradding, P. & Holgate, S.T. (1996). The mast cell as a source of cytokines in asthma. *Annals of the New York Academy of Sciences* **796**, 272-281.

Bradding, P., Roberts, J.A., Britten, K.M., Montefort, S., Djukanovic, R., Mueller, R., Heusser, C.H., Howarth, P.H. & Holgate, S.T. (1994). Interleukin-4, -5, and -6 and tumor necrosis factor-alpha in normal and asthmatic airways: evidence for the human mast cell as a source of these cytokines. *American Journal of Respiratory & Critical Care Medicine* **10**, 471-480.

Brain, S.D. & Williams, T.J. (1985). Inflammatory oedema induced by synergism between calcitonin gene-related peptide (CGRP) and mediators of increased vascular permeability. *British Journal of Pharmacology* **86**, 855-860.

Brandolini, L., Asti, C., Ruggieri, V., Intilangelo, A., Pellegrini, L., Chiusaroli, R., Caselli, G.F. & Bertini, R. (2000). Lipopolysaccharide-induced lung injury in mice. II. Evaluation of functional damage in isolated parenchyma strips. *Pulmonary Pharmacology & Therapeutics* **13**, 71-78.

Brewster, C.E., Howarth, P.H., Djukanovic, R., Wilson, J., Holgate, S.T. & Roche, W.R. (1990). Myofibroblasts and subepithelial fibrosis in bronchial asthma. *American Journal of Respiratory & Critical Care Medicine* **3**, 507-511.

Brichant, J.F., Warner, D.O., Gunst, S.J. & Rehder, K. (1990). Muscarinic receptor subtypes in canine trachea. *American Journal of Physiology* **258**, L349-L354

Broide, D.H., Lotz, M., Cuomo, A.J., Coburn, D.A., Federman, E.C. & Wasserman, S.I. (1992). Cytokines in symptomatic asthma airways. *Journal of Allergy & Clinical Immunology* **89**, 958-967.

Brooks, S.M., Hammad, Y., Richards, I., Giovinco-Barbas, J. & Jenkins, K. (1998). The spectrum of irritant-induced asthma: sudden and not-so-sudden onset and the role of allergy. *Chest* **113**, 42-49.

Brown, J.N., Thorne, P.R. & Nuttall, A.L. (1989). Blood pressure and other physiological responses in awake and anesthetized guinea pigs. *Laboratory Animal Science* **39**, 142-148.

Brusasco, V., Crimi, E. & Pellegrino, R. (1998). Airway hyperresponsiveness in asthma: not just a matter of airway inflammation. *Thorax* **53**, 992-998.

Burbach, J.P.H. & Meijer, O.C. (1992). The Structure of Neuropeptide Receptors. *European Journal of Pharmacology* **227**, 1-18.

Burcher, E., Alouan, L.A., Johnson, P.R. & Black, J.L. (1991a). Neuropeptide gamma, the most potent contractile tachykinin in human isolated bronchus, acts via a 'non-classical' NK2 receptor. *Neuropeptides* **20**, 79-82.

Burcher, E., Mussap, C.J., Geraghty, D.P., McClure-Sharp, J.M. & Watkins, D.J. (1991b). Concepts in characterization of tachykinin receptors. *Annals of the New York Academy of Sciences* **632**, 123-136.

Burrows, B., Martinez, F.D., Halonen, M., Barbee, R.A. & Cline, M.G. (1989). Association of asthma with serum IgE levels and skin-test reactivity to allergens. *New England Journal of Medicine* **320**, 271-277.

Busse, W., Elias, J., Sheppard, D. & Banks-Schlegel, S. (1999). Airway remodeling and repair. *American Journal of Respiratory & Critical Care Medicine* **160**, 1035-1042.

Campbell, A.M., Vachier, I., Chanez, P., Vignola, A.M., Lebel, B., Kochan, J., Godard, P. & Bousquet, J. (1998). Expression of the high-affinity receptor for IgE on bronchial epithelial cells of asthmatics. *American Journal of Respiratory Cell & Molecular Biology* **19**, 92-97.

Campbell, H.D., Tucker, W.Q., Hort, Y., Martinson, M.E., Mayo, G., Clutterbuck, E.J., Sanderson, C.J. & Young, I.G. (1987). Molecular cloning, nucleotide sequence, and expression of the gene encoding human eosinophil differentiation factor (interleukin 5). *Proc.Natl.Acad.Sci U.S.A.* **84**, 6629-6633.

Carroll, N., Carello, S., Cooke, C. & James, A. (1996). Airway structure and inflammatory cells in fatal attacks of asthma. *European Respiratory Journal* **9**, 709-715.

Carroll, N., Elliot, J., Morton, A. & James, A. (1993). The structure of large and small airways in nonfatal and fatal asthma. *American Review of Respiratory Disease* **147**, 405-410.

Carroll, N., Mutavdzic, S. & James, A. (2001). Mast cell degranulation is related to asthma severity and varies in different airway wall compartments in asthma. *Respirology* **6**, A21

Carroll, N.G., Cooke, C. & James, A. (1997a). The distribution of eosinophils and lymphocytes in the large and small airways of asthmatics. *European Respiratory Journal* **10**, 292-300.

Carroll, N.G., Cooke, C. & James, A.L. (1997b). Bronchial blood vessel dimensions in asthma. *American Journal of Respiratory & Critical Care Medicine* **155**, 689-695.

Carroll, N.G., Perry, S., Karkhanis, A., Harji, S., Butt, J., James, A.L. & Green, F.H. (2000). The airway longitudinal elastic fiber network and mucosal folding in patients with asthma. *American Journal of Respiratory & Critical Care Medicine* **161**, 244-248.

Carstairs, J.R. & Barnes, P.J. (1986). Autoradiographic mapping of substance P receptors in lung. *European Journal of Pharmacology* **127**, 295-296.

Cerrina, J., Labat, C., Haye-Legrande, I., Raffestin, B., Benveniste, J. & Brink, C. (1989). Human isolated bronchial muscle preparations from asthmatic patients: effects of indomethacin and contractile agonists. *Prostaglandins* **37**, 457-469.

Cerrina, J., Le, R., Labat, C., Raffestin, B., Bayol, A. & Brink, C. (1986). Comparison of human bronchial muscle responses to histamine in vivo with histamine and isoproterenol agonists in vitro. *American Review of Respiratory Disease* **134**, 57-61.

Chan-Yeung, M. & Malo, J.L. (1995). Occupational asthma. *New England Journal of Medicine* **333**, 107-112.

Chand, N., Eyre, P. & DeRoth, L. (1979). Relaxant action of histamine on rabbit trachea: possible existence of third histamine receptor subtype. *Research Communications in Chemical Pathology & Pharmacology* **23**, 211-221.

Chapman, I.D., Foster, A. & Morley, J. (1993). The relationship between inflammation and hyperreactivity of the airways in asthma. *Clinical & Experimental Allergy* **23**, 168-171.

Chen, W., Alley, M.R. & Manktelow, B.W. (1991a). Airway inflammation in sheep with acute airway hypersensitivity to inhaled Ascaris suum. *International Archives of Allergy & Applied Immunology* **96**, 218-223.

Chen, W., Alley, M.R. & Manktelow, B.W. (1991b). Morphological and morphometric studies of the airways of sheep with acute airway hypersensitivity to inhaled Ascaris suum. *International Journal of Experimental Pathology* **72**, 543-551.

Chetham, P.M., Babal, P., Bridges, J.P., Moore, T.M. & Stevens, T. (1999). Segmental regulation of pulmonary vascular permeability by store-operated Ca2+ entry. *American Journal of Physiology* **276** , L41-L50

Cheung, D., Bel, E.H., Den Hartigh, J., Dijkman, J.H. & Sterk, P.J. (1992). The effect of an inhaled neutral endopeptidase inhibitor, thiorphan, on airway responses to neurokinin A in normal humans in vivo. *American Review of Respiratory Disease* **145**, 1275-1280.

Cheung, D., Timmers, M.C., Zwinderman, A.H., Den Hartigh, J., Dijkman, J.H. & Sterk, P.J. (1993). Neutral endopeptidase activity and airway hyperresponsiveness to neurokinin A in asthmatic subjects in vivo. *American Review of Respiratory Disease* **148**, 1467-1473.

Cheung, D., van der Veen, H., Den Hartigh, J., Dijkman, J.H. & Sterk, P.J. (1994). Effects of inhaled substance P on airway responsiveness to methacholine in asthmatic subjects in vivo. *Journal of Applied Physiology* **77**, 1325-1332.

Chiba, Y., Sakai, H. & Misawa, M. (1998). Characterization of muscarinic receptors in rat bronchial smooth muscle in vitro. *Research communications in molecular pathology and pharmacology* **102**, 205-208.

Chilvers, E.R., Lynch, B.J. & Challiss, R.A. (1994). Phosphoinositide metabolism in airway smooth muscle. *Pharmacology & Therapeutics* **62**, 221-245.

Chitano, P., Sigurdsson, S.B., Halayko, A.J. & Stephens, N.L. (1993). Relevance of classification by size to topographical differences in bronchial smooth muscle response. *Journal of Applied Physiology* **75**, 2013-2021.

Chung, K.F. (1996). Effects of nedocromil sodium on airway neurogenic mechanisms. *Journal of Allergy & Clinical Immunology* **98**, S112-S116

Chung, K.F. & Barnes, P.J. (1999). Cytokines in asthma. *Thorax* **54**, 825-857.

Cockcroft, D.W., Killian, D.N., Mellon, J.J. & Hargreave, F.E. (1977a). Bronchial reactivity to inhaled histamine: a method and clinical survey. *Clinical Allergy* **7**, 235-243.

Cockcroft, D.W., Ruffin, R.E., Dolovich, J. & Hargreave, F.E. (1977b). Allergen-induced increase in non-allergic bronchial reactivity. *Clin Allergy* **7**, 503-513.

Colasurdo, G.N., Loader, J.E., Graves, J.P. & Larsen, G.L. (1995). SP-induced contraction of airway smooth muscle in normal and allergen-sensitized rabbits: mechanism of action. *Journal of Applied Physiology* **78**, 428-432.

Coleridge, J.C. & Coleridge, H.M. (1984). Afferent vagal C fibre innervation of the lungs and airways and its functional significance. *Reviews of Physiology Biochemistry & Pharmacology* **99**, 1-110.

Collier, J.G. & Fuller, R.W. (1984). Capsaicin inhalation in man and the effects of sodium cromoglycate. *British Journal of Pharmacology* **81**, 113-117.

Colotta, F., Dower, S.K., Sims, J.E. & Mantovani, A. (1994). The type II 'decoy' receptor: a novel regulatory pathway for interleukin 1. *Immunol Today* **15**, 562-566.

Constantine, J.W., Lebel, W.S. & Woody, H.A. (1991). Inhibition of tachykinin-induced hypotension in dogs by CP-96,345, a selective blocker of NK-1 receptors. *Naunyn-Schmiedebergs Archives of Pharmacology* **344**, 471-477.

Cook, C.D., Sutherland, J.M., Segal, S., Cherry, R.B., Mead, J., McIlroy, M.B. & Smith, C.A. (1957). Studies of respiratory physiology in the newborn infant. III. Measurements of mechanics of respiration. *Journal of Clinical Investigation* **36**, 440-448.

Cook, J.A., Brunner, S.L. & Tanaka, D.T. (1990). Neurokinin receptors mediating substance P-induced contraction in adult rabbit airways. *American Journal of Physiology.* **258**, L99-106.

Corcoran, B.M. & Haigh, A.L. (1992). The effect of tachykinins on sheep bronchomotor tone. *Experimental Physiology* **77**, 471-479.

Corcoran, B.M. & Haigh, A.L. (1995). Effect of vagotomy and vagal cooling on bronchoconstrictor response to substance P in sheep. *Respir Physiol* **102**, 71-77.

Corrigan, C.J., Hartnell, A. & Kay, A.B. (1988). T lymphocyte activation in acute severe asthma. *Lancet* **1**, 1129-1132.

Corrigan, C.J. & Kay, A.B. (1991). The roles of inflammatory cells in the pathogenesis of asthma and of chronic obstructive pulmonary disease. *American Review of Respiratory Disease* **143**, 1165-118.

Cotran, R.S. & Majno, G. (1964). The delayed and prolonged vascular leakage in inflammation. I. Topography of the leaking vessels after thermal injury. *American Journal of Pathology* **45**, 261-281.

Coyle, A.J., Uchida, D., Ackerman, S.J., Mitzner, W. & Irvin, C.G. (1994). Role of cationic proteins in the airway. Hyperresponsiveness due to airway inflammation. *American Journal of Respiratory & Critical Care Medicine* **150**, S63-S71

Creese, B.R. & Denborough, M.A. (1981). Sources of calcium for contraction of guinea-pig isolated tracheal smooth muscle. *Clinical & Experimental Pharmacology & Physiology* **8**, 175-182.

Crimi, E., Brusasco, V. & Crimi, P. (1989). Effect of nedocromil sodium on the late asthmatic reaction to bronchial antigen challenge. *Journal of Allergy & Clinical Immunology* **83**, 985-990.

Crimi, N., Palermo, F., Oliveri, R., Palermo, B., Polosa, R. & Mistretta, A. (1992). Protection of nedocromil sodium on bronchoconstriction induced by inhaled neurokinin A (NKA) in asthmatic patients. *Clinical & Experimental Allergy* **22**, 75-81.

Crimi, N., Palermo, F., Oliveri, R., Palermo, B., Vancheri, C., Polosa, R. & Mistretta, A. (1988). Effect of nedocromil on bronchospasm induced by inhalation of substance P in asthmatic subjects. *Clinical Allergy* **18**, 375-382.

Crimi, N., Palermo, F., Oliveri, R., Palermo, B., Vancheri, C., Polosa, R. & Mistretta, A. (1990). Influence of antihistamine (astemizole) and anticholinergic drugs (ipratropium bromide) on bronchoconstriction induced by substance P. *Annals of Allergy.* **65**, 115-120.

Crimi, N., Palermo, F., Oliveri, R., Polosa, R., Magri, S. & Mistretta, A. (1994). Inhibition of neutral endopeptidase potentiates bronchoconstriction induced by neurokinin A in asthmatic patients. *Clinical & Experimental Allergy* **24**, 115-120.

Cromwell, O., Hamid, Q., Corrigan, C.J., Barkans, J., Meng, Q., Collins, P.D. & Kay, A.B. (1992). Expression and generation of interleukin-8, IL-6 and granulocyte-macrophage colony-stimulating factor by bronchial epithelial cells and enhancement by IL-1 beta and tumour necrosis factor-alpha. *Immunology* **77**, 330-337.

Cross, L.J., Heaney, L.G. & Ennis, M. (1997). Histamine release from human bronchoalveolar lavage mast cells by neurokinin A and bradykinin. *Inflammation Research* **46**, 306-309.

Cui, Z.H., Arakawa, H., Kawikova, I., Skoogh, B.E. & Lotvall, J. (1999). Relationship between systemic blood pressure, airway blood flow and plasma exudation in guinea-pig. *Acta Physiologica Scandinavica* **165**, 121-127.

Cushley, M.J., Tattersfield, A.E. & Holgate, S.T. (1983). Inhaled adenosine and guanosine on airway resistance in normal and asthmatic subjects. *Br J Clin Pharmacol* **15**, 161-165.

Daniel, E.E. & O'Byrne, P. (1991). Effect of inflammatory mediators on airway nerves and muscle. *American Review of Respiratory Disease* **143**, S3-S5

Davis, C., Kannan, M.S., Jones, T.R. & Daniel, E.E. (1982). Control of human airway smooth muscle: in vitro studies. *Journal of Applied Physiology: Respiratory, Environmental & ExercisePhysiology* **53**, 1080-1087.

de Jongste, J.C., Mons, H., Bonta, I.L. & Kerrebijn, K.F. (1987a). In vitro responses of airways from an asthmatic patient. *European Journal of Respiratory Disease* **71**, 23-29.

de Jongste, J.C., Mons, H., Van Strik, R., Bonta, I.L. & Kerrebijn, K.F. (1987b). Comparison of human bronchiolar smooth muscle responsiveness in vitro with histological signs of inflammation. *Thorax* **42**, 870-876.

De, S., Zelazny, E.T., Souhrada, J.F. & Souhrada, M. (1995). IL-1 beta and IL-6 induce hyperplasia and hypertrophy of cultured guinea pig airway smooth muscle cells. *Journal of Applied Physiology* **78**, 1555-1563.

De, S., Zelazny, E.T., Souhrada, J.F. & Souhrada, M. (1996). Role of phospholipase C and tyrosine kinase systems in growth response of human airway smooth muscle cells. *American Journal of Physiology* **270**, L795-L802

Deffebach, M.E., Charan, N.B., Lakshminarayan, S. & Butler, J. (1987). The bronchial circulation. Small, but a vital attribute of the lung. *American Review of Respiratory Disease* **135**, 463-481.

Deffebach, M.E., Salonen, R.O., Webber, S.E. & Widdicombe, J.G. (1989). Cold and hyperosmolar fluids in canine trachea: vascular and smooth muscle tone and albumin flux. *Journal of Applied Physiology* **66**, 1309-1315.

Del Maschio, A., Zanetti, A., Corada, M., Rival, Y., Ruco, L., Lampugnani, M.G. & Dejana, E. (1996). Polymorphonuclear leukocyte adhesion triggers the disorganization of endothelial cell-to-cell adherens junctions. *Journal of Cell Biology* **135**, 497-510.

Del Prete, G., Maggi, E., Parronchi, P., Chretien, I., Tiri, A., Macchia, D., Ricci, M., Banchereau, J., De Vries, J. & Romagnani, S. (1988). IL-4 is an essential factor for the IgE synthesis induced in vitro by human T cell clones and their supernatants. *Journal of Immunology* **140**, 4193-4198.

Delehunt, J.C., Yerger, L., Ahmed, T. & Abraham, W.M. (1984). Inhibition of antigen-induced bronchoconstriction by methylprednisolone succinate. *Journal of Allergy & Clinical Immunology* **73**, 479-483.

DeRose, V., Robbins, R.A., Snider, R.M., Spurzem, J.R., Thiele, G.M., Rennard, S.I. & Rubinstein, I. (1994). Substance P increases neutrophil adhesion to bronchial epithelial cells. *Journal of Immunology* **152**, 1339-1346.

Dessauer, C.W., Posner, B.A. & Gilman, A.G. (1996). Visualizing signal transduction: receptors, G-proteins, and adenylate cyclases [published erratum appears in *Clinical Science* 1997 Feb;92(2):223]. *Clinical Science* **91**, 527-537.

Devillier, P., Acker, G.M., Advenier, C., Marsac, J., Regoli, D. & Frossard, N. (1992). Activation of an epithelial neurokinin NK-1 receptor induces relaxation of rat trachea through release of prostaglandin E2. *Journal of Pharmacology & Experimental Therapeutics* **263**, 767-772.

Devillier, P., Advenier, C., Drapeau, G., Marsac, J. & Regoli, D. (1988). Comparison of the effects of epithelium removal and of an enkephalinase inhibitor on the neurokinin-induced contractions of guinea-pig isolated trachea. *British Journal of Pharmacology* **94**, 675-684.

Dewar, J.C., Wilkinson, J., Wheatley, A., Thomas, N.S., Doull, I., Morton, N., Lio, P., Harvey, J.F., Liggett, S.B., Holgate, S.T. & Hall, I.P. (1997). The glutamine 27 beta2-adrenoceptor polymorphism is associated with elevated IgE levels in asthmatic families. *J Allergy Clin.Immunol.* **100**, 261-265.

di Stefano, A., Saetta, M., Maestrelli, P., Milani, G., Pivirotto, F., Mapp, C.E. & Fabbri, L.M. (1993). Mast cells in the airway mucosa and rapid development of occupational asthma induced by toluene diisocyanate. *American Review of Respiratory Disease* **147**, 1005-1009.

Dinarello, C.A. & Savage, N. (1989). Interleukin-1 and its receptor. *Crit Rev Immunol* **9**, 1-20.

Donnelly, R., Yang, K., Omary, M.B., Azhar, S. & Black, J.L. (1995). Expression of multiple isoenzymes of protein kinase C in airway smooth muscle. *American Journal of Respiratory Cell & Molecular Biology* **13**, 253-256.

Drazen, J.M. & Schneider, M.W. (1978). Comparative responses of tracheal spirals and parenchymal strips to histamine and carbachol in vitro. *Journal of Clinical Investigation* **61**, 1441-1447.

Duddridge, M., Ward, C., Hendrick, D.J. & Walters, E.H. (1993). Changes in bronchoalveolar lavage inflammatory cells in asthmatic patients treated with high dose inhaled beclomethasone dipropionate. *European Respiratory Journal* **6**, 489-497.

Dunnill, M.S. (1960). The pathology of asthma, with special reference to changes in the bronchial mucosa. *Journal of Clinical Pathology* **13**, 27-33.

Dunnill, M.S., Massarella, G.R. & Anderson, J.A. (1969). A comparison of the quantitative anatomy of the bronchi in normal subjects, in status asthmaticus, in chronic bronchitis, and in emphysema. *Thorax* **24**, 176-179.

Dusser, D.J., Lacroix, H., Desmazes-Dufeu, N., Mordelet-Dambrine, M. & Roisman, G.L. (1995). Nedocromil sodium reduces cigarette smoke-induced bronchoconstrictor hyperresponsiveness to substance P in the guinea-pig. *European Respiratory Journal* **8**, 47-52.

Ebina, M., Takahashi, T., Chiba, T. & Motomiya, M. (1993). Cellular hypertrophy and hyperplasia of airway smooth muscles underlying bronchial asthma. A 3-D morphometric study. *American Review of Respiratory Disease* **148**, 720-726.

Ebina, M., Yaegashi, H., Chiba, R., Takahashi, T., Motomiya, M. & Tanemura, M. (1990). Hyperreactive site in the airway tree of asthmatic patients revealed by thickening of bronchial muscles. A morphometric study. *American Review of Respiratory Disease* **141**, 1327-1332.

Eder, J. (1997). Tumour necrosis factor alpha and interleukin 1 signalling: do MAPKK kinases connect it all? *Trends Pharmacol Sci* **18**, 319-322.

Eglen, R.M., Reddy, H., Watson, N. & Challiss, R.A. (1994). Muscarinic acetylcholine receptor subtypes in smooth muscle. *Trends in Pharmacological Sciences* **15**, 114-119.

Ellis, J.L. & Undem, B.J. (1992). Inhibition by L-NG-nitro-L-arginine of nonadrenergic-noncholinergic-mediated relaxations of human isolated central and peripheral airway. *American Review of Respiratory Disease* **146**, 1543-1547.

Ellis, J.L. & Undem, B.J. (1994). Pharmacology of non-adrenergic, non-cholinergic nerves in airway smooth muscle. *Pulmonary Pharmacology* **7**, 205-223.

Enarson, D.A., Vedal, S., Schulzer, M., Dybuncio, A. & Chan-Yeung, M. (1987). Asthma, asthmalike symptoms, chronic bronchitis, and the degree of bronchial hyperresponsiveness in epidemiologic surveys. *American Review of Respiratory Disease* **136**, 613-617.

Enhorning, G., Duffy, L.C. & Welliver, R.C. (1995). Pulmonary surfactant maintains patency of conducting airways in the rat. *American Journal of Respiratory & Critical Care Medicine* **151**, 554-556.

Erickson, S.L., de Sauvage, F.J., Kikly, K., Carver-Moore, K., Pitts-Meek, S., Gillett, N., Sheehan, K.C., Schreiber, R.D., Goeddel, D.V. & Moore, M.W. (1994). Decreased sensitivity to tumour-necrosis factor but normal T-cell development in TNF receptor-2-deficient mice. *Nature* **372**, 560-563.

Erjefalt, I. & Persson, C.G. (1991). Allergen, bradykinin, and capsaicin increase outward but not inward macromolecular permeability of guinea-pig tracheobronchial mucosa. *Clinical & Experimental Allergy* **21**, 217-224.

Eum, S.Y., Haile, S., Lefort, J., Huerre, M. & Vargaftig, B.B. (1995). Eosinophil recruitment into the respiratory epithelium following antigenic challenge in hyper-IgE mice is accompanied by interleukin 5- dependent bronchial hyperresponsiveness. *Proc.Natl.Acad.Sci U.S.A.* **92**, 12290-12294.

Evans, T.W., Chung, K.F., Rogers, D.F. & Barnes, P.J. (1987). Effect of platelet-activating factor on airway vascular permeability: possible mechanisms. *Journal of Applied Physiology* **63**, 479-484.

Evans, T.W., Dixon, C.M., Clarke, B., Conradson, T.B. & Barnes, P.J. (1988a). Comparison of neurokinin A and substance P on cardiovascular and airway function in man. *British Journal of Clinical Pharmacology* **25**, 273-275.

Evans, T.W., Rogers, D.F., Aursudkij, B., Chung, K.F. & Barnes, P.J. (1988b). Inflammatory mediators involved in antigen-induced airway microvascular leakage in guinea pigs. *American Review of Respiratory Disease* **138**, 395-399.

Evans, T.W., Rogers, D.F., Aursudkij, B., Chung, K.F. & Barnes, P.J. (1989). Regional and time-dependent effects of inflammatory mediators on airway microvascular permeability in the guinea pig. *Clinical Science* **76**, 479-485.

Eyre, P. (1969). The pharmacology of sheep tracheobronchial muscle: a relaxant effect of histamine on the isolated bronchi. *British Journal of Pharmacology.* **36**, 409-417.

Eyre, P. (1973). Histamine H2-receptors in the sheep bronchus and cat trachea: the action of burimamide. *British Journal of Pharmacology* **48**, 321-323.

Eyre, P. (1975). Comparisons of reactivity of pulmonary vascular and airway smooth muscle of sheep and calf to tryptamine analogues, histamine, and antigen. *American Journal of Veterinary Research* **36**, 1081-1084.

Eyre, P. & Mirbahar, K.B. (1981). Is the lung parenchyma strip a true airway preparation? *Agents Actions* **11**, 173-176.

Fabbri, L.M., Maestrelli, P., Saetta, M. & Mapp, C.E. (1991). Airway inflammation during late asthmatic reactions induced by toluene diisocyanate. *American Review of Respiratory Disease* **143**, S37-S38

Fahy, J.V., Kim, K.W., Liu, J. & Boushey, H.A. (1995a). Prominent neutrophilic inflammation in sputum from subjects with asthma exacerbation. *Journal of Allergy & Clinical Immunology* **95**, 843-852.

Fahy, J.V., Wong, H.H., Geppetti, P., Reis, J.M., Harris, S.C., Maclean, D.B., Nadel, J.A. & Boushey, H.A. (1995b). Effect of an NK1 receptor antagonist (CP-99,994) on hypertonic saline-induced bronchoconstriction and cough in male asthmatic subjects. *American Journal of Respiratory & Critical Care Medicine* **152**, 879-884.

Fan, T., Yang, M., Halayko, A., Mohapatra, S.S. & Stephens, N.L. (1997). Airway responsiveness in two inbred strains of mouse disparate in IgE and IL-4 production. *American Journal of Respiratory & Critical Care Medicine* **17**, 156-163.

Fan, T.P., Hu, D.E., Guard, S., Gresham, G.A. & Watling, K.J. (1993). Stimulation of angiogenesis by substance P and interleukin-1 in the rat and its inhibition by NK1 or interleukin-1 receptor antagonists. *British Journal of Pharmacology* **110**, 43-49.

Faul, J.L., Tormey, V.J., Leonard, C., Burke, C.M., Farmer, J., Horne, S.J. & Poulter, L.W. (1997). Lung immunopathology in cases of sudden asthma death. *European Respiratory Journal* **10**, 301-307.

Feng, D., Nagy, J.A., Hipp, J., Dvorak, H.F. & Dvorak, A.M. (1996). Vesiculo-vacuolar organelles and the regulation of venule permeability to macromolecules by vascular permeability factor, histamine, and serotonin. *Journal of Experimental Medicine* **183**, 1981-1986.

Ferreira, S.H., Lorenzetti, B.B., Bristow, A.F. & Poole, S. (1988). Interleukin-1 beta as a potent hyperalgesic agent antagonized by a tripeptide analogue. *Nature* **334**, 698-700.

Ferreira, S.H., Lorenzetti, B.B., Cunha, F.Q. & Poole, S. (1993). Bradykinin release of TNF-alpha plays a key role in the development of inflammatory hyperalgesia. *Agents & Actions* **38 Spec No**, C7-C9

Ferreira, S.H. & Nakamura, M. (1979). I - Prostaglandin hyperalgesia, a cAMP/Ca2+ dependent process. *Prostaglandins* **18**, 179-190.

Fine, J.M., Gordon, T. & Sheppard, D. (1989). Epithelium removal alters responsiveness of guinea pig trachea to substance P. *Journal of Applied Physiology* **66**, 232-237.

Finney, M.J., Berend, N. & Black, J.L. (1984). Cholinergic responses in the human lung parenchymal strip: a structure-function correlation. *European Journal of Respiratory Diseases* **65**, 447-455.

Fischer, A. & Hoffmann, B. (1996). Nitric oxide synthase in neurons and nerve fibers of lower airways and in vagal sensory ganglia of man. Correlation with neuropeptides. *American Journal of Respiratory & Critical Care Medicine* **154**, 209-216.

Fleisch, J.H. & Calkins, P.J. (1976). Comparison of drug-induced responses of rabbit trachea and bronchus. *Journal of Applied Physiology* **41**, 62-66.

Foda, H.D., George, S., Rollo, E., Drews, M., Conner, C., Cao, J., Panettieri, R.A.J. & Zucker, S. (1999). Regulation of gelatinases in human airway smooth muscle cells: mechanism of progelatinase A activation. *American Journal of Physiology* **277**, L174-L182

Folinsbee, L.J. & Horvath, S.M. (1986). Persistence of the acute effects of ozone exposure. *Aviat.Space.Environ.Med* **57**, 1136-1143.

Folkerts, G., Busse, W.W., Nijkamp, F.P., Sorkness, R. & Gern, J.E. (1998). Virus-induced airway hyperresponsiveness and asthma. *American Journal of Respiratory & Critical Care Medicine* **157**, 1708-1720.

Folkerts, G. & Nijkamp, F.P. (1990). Parainfluenza-3 infection of the guinea pig airways induces respiratory airway hyperreactivity in vitro. *Agents & Actions* **30**, 261-263.

Folkerts, G., Verheyen, A., Janssen, M. & Nijkamp, F.P. (1992). Virus-induced airway hyperresponsiveness in the guinea pig can be transferred by bronchoalveolar cells. *Journal of Allergy & Clinical Immunology* **90**, 364-372.

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

Foreman, J.C., Shelly, R. & Webber, S.E. (1985). Contraction of guinea-pig lung parenchymal strips by substance P and related peptides. *Archives Internationales de Pharmacodynamie et de Therapie* **278**, 193-206.

Forestier, C., Deleuil, F., Lapaque, N., Moreno, E. & Gorvel, J.P. (2000). Brucella abortus lipopolysaccharide in murine peritoneal macrophages acts as a down-regulator of T cell activation. *Journal of Immunology* **165**, 5202-5210.

Foster, P.S., Hogan, S.P., Ramsay, A.J., Matthaei, K.I. & Young, I.G. (1996). Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. *Journal of Experimental Medicine* **183**, 195-201.

Fraser, J.R., Laurent, T.C. & Laurent, U.B. (1997). Hyaluronan: its nature, distribution, functions and turnover. *Journal of Internal Medicine* **242**, 27-33.

Freed, A.N., Wang, D. & Menkes, H.A. (1987). Dry air-induced constriction: effects of pharmacological intervention and temperature. *Journal of Applied Physiology* **62**, 1794-1800.

Frossard, N. & Advenier, C. (1991). Tachykinin receptors and the airways. *Life Sciences* **49**, 1941-1953.

Frossard, N., Rhoden, K.J. & Barnes, P.J. (1989). Influence of epithelium on guinea pig airway responses to tachykinins: role of endopeptidase and cyclooxygenase. *Journal of Pharmacology and Experimental Therapeutics* **248**, 292-298.

Fryer, A.D. & Jacoby, D.B. (1998). Muscarinic receptors and control of airway smooth muscle. *American Journal of Respiratory & Critical Care Medicine* **158**, S154-S160

Fryer, A.D. & Wills-Karp, M. (1991). Dysfunction of M2-muscarinic receptors in pulmonary parasympathetic nerves after antigen challenge. *Journal of Applied Physiology* **71**, 2255-2261.

Fryer, A.D., Yarkony, K.A. & Jacoby, D.B. (1994). The effect of leukocyte depletion on pulmonary M2 muscarinic receptor function in parainfluenza virus-infected guinea-pigs. *British Journal of Pharmacology* **112**, 588-594.

Fujimoto, K., Kubo, K., Shinozaki, S., Okada, K., Matsuzawa, Y., Kobayashi, T. & Sugane, K. (1995). Neutrophil elastase inhibitor reduces asthmatic responses in allergic sheep. *Respiration Physiology* **100**, 91-100.

Fuller, R.W., Dixon, C.M. & Barnes, P.J. (1985). Bronchoconstrictor response to inhaled capsaicin in humans. *Journal of Applied Physiology* **58**, 1080-1084.

Fuller, R.W., Dixon, C.M., Cuss, F.M. & Barnes, P.J. (1987). Bradykinin-induced bronchoconstriction in humans. Mode of action. *American Review of Respiratory Disease* **135**, 176-180.

Garland, A., Ray, D.W., Doerschuk, C.M., Alger, L., Eappon, S., Hernandez, C., Jackson, M. & Solway, J. (1991). Role of tachykinins in hyperpnea-induced bronchovascular hyperpermeability in guinea pigs. *Journal of Applied Physiology* **70**, 27-35.

Garland, A.M., Grady, E.F., Lovett, M., Vigna, S.R., Frucht, M.M., Krause, J.E. & Bunnett, N.W. (1996). Mechanisms of desensitization and resensitization of G protein-coupled neurokinin1 and neurokinin2 receptors. *Molecular Pharmacology* **49**, 438-446.

Gauldie, J. (1991). Acute phase response. In *The Encyclopaedia of Human Biology* (Dulbecco, R., ed.), Academic Press, San Diego, pp. 23-35.

Gerard, N.P. (1987). Characterization of substance P contractile activity on isolated guinea pig lung tissues. *Journal of Pharmacology & Experimental Therapeutics* **243**, 901-906.

Gerard, N.P., Bao, L., Xiao-Ping, H. & Gerard, C. (1993). Molecular aspects of the tachykinin receptors. *Regulatory Peptides* **43**, 21-35.

Gibson, P.G., Dolovich, J., Girgis-Gabardo, A., Morris, M.M., Anderson, M., Hargreave, F.E. & Denburg, J.A. (1990). The inflammatory response in asthma exacerbation: changes in circulating eosinophils, basophils and their progenitors. *Clinical & Experimental Allergy* **20**, 661-668.

Gilbert, I.A., Fouke, J.M. & McFadden, E.R.J. (1987). Heat and water flux in the intrathoracic airways and exercise-induced asthma. *Journal of Applied Physiology* **63**, 1681-1691.

Global Initiative for Asthma. (1997). *Guidelines for the Diagnosis and Management of Asthma. Expert Panel Report 2.* NHLBI/WHO Workshop Report.

Godfrey, K. (1985). Statistics in practice. Comparing the means of several groups. *New England Journal of Medicine* **313**, 1450-1456.

Golden, J.A., Nadel, J.A. & Boushey, H.A. (1978). Bronchial hyperirritability in healthy subjects after exposure to ozone. *American Review of Respiratory Disease* **118**, 287-294.

Goldie, R.G. (1990). Receptors in asthmatic airways. *American Review of Respiratory Disease* **141**, S151-S156

Goldie, R.G., Spina, D., Henry, P.J., Lulich, K.M. & Paterson, J.W. (1986). In vitro responsiveness of human asthmatic bronchus to carbachol, histamine, beta-adrenoceptor agonists and theophylline. *Br J Clin Pharmacol* **22**, 669-676.

Gordon, J.R., Burd, P.R. & Galli, S.J. (1990). Mast cells as a source of multifunctional cytokines. *Immunol Today* **11**, 458-464.

Gordon, T., Milligan, S.A., Levin, J., Thompson, J.E., Fine, J.M. & Sheppard, D. (1987). Apparent effect of catalase on airway edema in guinea pigs. Role of endotoxin contamination. *American Review of Respiratory Disease* **135**, 854-859.

Goroumaru-Shinkai, M., Yamamoto, R., Funayama, N. & Takayanagi, I. (1992). Regional differences of the contractile responses to acetylcholine and neurokinin A in rabbit airway: heterogeneous distribution of the metabolic enzymes. *General Pharmacology* **23**, 1057-1061.

Gosset, P., Tillie-Leblond, I., Oudin, S., Parmentier, O., Wallaert, B., Joseph, M. & Tonnel, A.B. (1999). Production of chemokines and proinflammatory and antiinflammatory cytokines by human alveolar macrophages activated by IgE receptors. *Journal of Allergy & Clinical Immunology* **103**, 289-297.

Grandordy, B.M., Frossard, N., Rhoden, K.J. & Barnes, P.J. (1988). Tachykinin-induced phosphoinositide breakdown in airway smooth muscle and epithelium: relationship to contraction. *Molecular Pharmacology* **33**, 515-519.

Greiff, L., Erjefalt, I., Wollmer, P., Pipkorn, U. & Persson, C.G. (1991). Effects of histamine, ethanol, and a detergent on exudation and absorption across guinea pig airway mucosa in vivo. *Thorax* **46**, 700-705.

Greiff, L., Svensson, C., Andersson, M. & Persson, C.G. (1995). Effects of topical capsaicin in seasonal allergic rhinitis. *Thorax* **50**, 225-229.

Grunstein, M.M., Hakonarson, H., Hodinka, R.L., Maskeri, N., Kim, C. & Chuang, S. (2001). Mechanism of cooperative effects of rhinovirus and atopic sensitization on airway responsiveness. *American Journal of Physiology Lung Cell Mol Physiol* **280**, L229-L238

Guyton, A.G. & Hall, J.E. (1996a). Resistance of the body to infection: 1. Leukocytes, granulocytes, the monocyte-macrophage system and inflammation. In *Textbook of medical physiology* (AnonymousW. B. Saunders, Philadelphia, pp. 435-443.

Guyton, A.G. & Hall, J.E. (1996b). The autonomic nervous system; the adrenal medulla. In *Textbook of medical physiology* (AnonymousW. B. Saunders, Philadelphia, pp. 769-781.

Haddad, E.B., Rousell, J., Lindsay, M.A. & Barnes, P.J. (1996). Synergy between tumor necrosis factor alpha and interleukin 1beta in inducing transcriptional down-regulation of muscarinic M2 receptor gene expression. Involvement of protein kinase A and ceramide pathways. *J Biol Chem* **271**, 32586-32592.

Hajj, A.M., Burki, N.K. & Lee, L.Y. (1996). Role of tachykinins in sulfur dioxide-induced bronchoconstriction in anesthetized guinea pigs. *Journal of Applied Physiology* **80**, 2044-2050.

Hakonarson, H., Carter, C., Kim, C. & Grunstein, M.M. (1999). Altered expression and action of the low-affinity IgE receptor FcepsilonRII (CD23) in asthmatic airway smooth muscle. *Journal of Allergy & Clinical Immunology* **104**, 575-584.

Hakonarson, H., Herrick, D.J., Serrano, P.G. & Grunstein, M.M. (1996). Mechanism of cytokine-induced modulation of beta-adrenoceptor responsiveness in airway smooth muscle. *Journal of Clinical Investigation* **97**, 2593-2600.

Hakonarson, H., Herrick, D.J., Serrano, P.G. & Grunstein, M.M. (1997). Autocrine role of interleukin 1beta in altered responsiveness of atopic asthmatic sensitized airway smooth muscle. *Journal of Clinical Investigation* **99**, 117-124.

Haley, K.J. & Drazen, J.M. (1998). Inflammation and airway function in asthma: what you see is not necessarily what you get. *American Journal of Respiratory & Critical Care Medicine* **157**, 1-3.

Hall, A.K., Barnes, P.J., Meldrum, L.A. & Maclagan, J. (1989). Facilitation by tachykinins of neurotransmission in guinea-pig pulmonary parasympathetic nerves. *British Journal of Pharmacology* **97**, 274-280.

Hamelmann, E., Oshiba, A., Loader, J., Larsen, G.L., Gleich, G., Lee, J. & Gelfand, E.W. (1997). Antiinterleukin-5 antibody prevents airway hyperresponsiveness in a murine model of airway sensitization. *American Journal of Respiratory & Critical Care Medicine* **155**, 819-825.

Hamid, Q., Song, Y., Kotsimbos, T.C., Minshall, E., Bai, T.R., Hegele, R.G. & Hogg, J.C. (1997). Inflammation of small airways in asthma. *Journal of Allergy & Clinical Immunology* **100**, 44-51.

Hamid, Q.A. & Minshall, E.M. (2000). Molecular pathology of allergic disease: I: lower airway disease. *Journal of Allergy & Clinical Immunology* **105**, 20-36.

Hanna, L.M. & Scherer, P.W. (1986). Regional control of local airway heat and water vapor losses. *Journal of Applied Physiology* **61**, 624-632.

Hansson, L., Wollmer, P., Dahlback, M. & Karlsson, J.A. (1992). Regional sensitivity of human airways to capsaicin-induced cough. *American Review of Respiratory Disease* **145**, 1191-1195.

Harden, T.K. (1990). G protein-dependent regulation of phospholipase C by cell surface receptors. *American Review of Respiratory Disease* **141**, S119-S122

Harrison, N.K., Dawes, K.E., Kwon, O.J., Barnes, P.J., Laurent, G.J. & Chung, K.F. (1995). Effects of neuropeptides on human lung fibroblast proliferation and chemotaxis. *American Journal of Physiology* **268**, L278-L283

Hart, M.V., Rowles, J.R., Hohimer, A.R., Morton, M.J. & Hosenpud, J.D. (1984). Hemodynamics in the guinea pig after anesthetization with ketamine/xylazine. *American Journal of Veterinary Research* **45**, 2328-2330.

Hashimoto, M. (1994). Influence of cytokines on ICAM-1 expression and soluble ICAM-1 production by human endothelial cells. [Japanese] *Fukuoka Igaku Zasshi - Fukuoka Acta Medica* **85**, 178-186.

Hazbun, M.E., Hamilton, R., Holian, A. & Eschenbacher, W.L. (1993). Ozone-induced increases in substance P and 8-epi-prostaglandin F2 alpha in the airways of human subjects. *American Journal of Respiratory Cell & Molecular Biology* **9**, 568-572.

Heaney, L.G., Cross, L.J., Stanford, C.F. & Ennis, M. (1995). Substance P induces histamine release from human pulmonary mast cells. *Clinical & Experimental Allergy* **25**, 179-186.

Heard, B.E. & Hossain, S. (1973). Hyperplasia of bronchial muscle in asthma. *Journal of Pathology* **110**, 319-331.

Heilpern, S. & Rebuck, A.S. (1972). Effect of disodium cromoglycate (Intal) on sputum protein composition. *Thorax* **27**, 726-728.

Helke, C.J., Krause, J.E., Mantyh, P.W., Couture, R. & Bannon, M.J. (1990). Diversity in mammalian tachykinin peptidergic neurons: multiple peptides, receptors, and regulatory mechanisms. *The Federation of American Societies for Experimental Biology Journal* **4**, 1606-1615.

Hepler, J.R. & Gilman, A.G. (1992). G proteins. *Trends in Biochemical Sciences* **17**, 383-387.

Hirata, F., Lee, J.Y., Sakamoto, T., Nomura, A., Uchida, Y., Hirata, A. & Hasegawa, S. (1994). IL-1 beta regulates the expression of the Gi2 alpha gene via lipid mediators in guinea pig tracheal muscle. *Biochemical & Biophysical Research Communications* **203**, 1889-1896.

Hodge, L., Salome, C.M., Peat, J.K., Haby, M.M., Xuan, W. & Woolcock, A.J. (1996). Consumption of oily fish and childhood asthma risk. *Medical Journal of Australia* **164**, 137-140.

Hogg, J.C., James, A.L. & Pare, P.D. (1991). Evidence for inflammation in asthma. *American Review of Respiratory Disease* **143**, S39-S42

Hogg, J.C., Pare, P.D. & Moreno, R. (1987). The effect of submucosal edema on airways resistance. *American Review of Respiratory Disease* **135**, S54-S56.

Holgate, S.T. (1997). The cellular and mediator basis of asthma in relation to natural history. *Lancet* **350 Suppl 2:** SII5-SII9.

Holgate, S.T., Davies, D.E., Lackie, P.M., Wilson, S.J., Puddicombe, S.M. & Lordan, J.L. (2000). Epithelial-mesenchymal interactions in the pathogenesis of asthma. *Journal of Allergy & Clinical Immunology* **105**, 193-204.

Holtzman, M.J., Fabbri, L.M., O'Byrne, P.M., Gold, B.D., Aizawa, H., Walters, E.H., Alpert, S.E. & Nadel, J.A. (1983). Importance of airway inflammation for hyperresponsiveness induced by ozone. *American Review of Respiratory Disease* **127**, 686-690.

Holzer, P. (1988). Local effector functions of capsaicin-sensitive sensory nerve endings: involvement of tachykinins, calcitonin gene-related peptide and other neuropeptides. *Neuroscience* **24**, 739-768.

Honda, I., Kohrogi, H., Yamaguchi, T., Ando, M. & Araki, S. (1991). Enkephalinase inhibitor potentiates substance P- and capsaicin-induced bronchial smooth muscle contractions in humans. *American Review of Respiratory Disease* **143**, 1416-1418.

Horie, S., Gleich, G.J. & Kita, H. (1996). Cytokines directly induce degranulation and superoxide production from human eosinophils. *Journal of Allergy & Clinical Immunology* **98**, 371-381.

Hosenpud, J.D., Yung, N.N. & Morton, M.J. (1983). Left ventricular pressure-volume relations shift to the left after long-term loss of pericardial restraint. *Circulation* **68**, 155-163.

Hoshino, M., Nakamura, Y., Sim, J., Shimojo, J. & Isogai, S. (1998). Bronchial subepithelial fibrosis and expression of matrix metalloproteinase-9 in asthmatic airway inflammation. *Journal of Allergy & Clinical Immunology* **102**, 783-788.

Hotta, K., Emala, C.W. & Hirshman, C.A. (1999). TNF-alpha upregulates Gialpha and Gqalpha protein expression and function in human airway smooth muscle cells. *American Journal of Physiology* **276**, L405-L411.

Howarth, P.H., Bradding, P., Montefort, S., Peroni, D., Djukanovic, R., Carroll, M.P. & Holgate, S.T. (1994). Mucosal inflammation and asthma. *American Journal of Respiratory & Critical Care Medicine* **150**, S18-S22.

Howarth, P.H., Djukanovic, R., Wilson, J.W., Holgate, S.T., Springall, D.R. & Polak, J.M. (1991). Mucosal nerves in endobronchial biopsies in asthma and non-asthma. *International Archives of Allergy & Applied Immunology* **94**, 330-333.

Hua, X., Lundberg, J.M., Theodorsson-Norheim, E. & Brodin, E. (1984). Comparison of cardiovascular and bronchoconstrictor effects of substance P, substance K and other tachykinins. *Naunyn-Schmiedebergs Archives of Pharmacology* **328**, 196-201.

Hua, X.Y., Theodorsson-Norheim, E., Brodin, E., Lundberg, J.M. & Hokfelt, T. (1985). Multiple tachykinins (neurokinin A, neuropeptide K and substance P) in capsaicin-sensitive sensory neurons in the guinea-pig. *Regulatory Peptides* **13**, 1-19.

Huang, A.J., Manning, J.E., Bandak, T.M., Ratau, M.C., Hanser, K.R. & Silverstein, S.C. (1993). Endothelial cell cytosolic free calcium regulates neutrophil migration across monolayers of endothelial cells. *Journal of Cell Biology* **120**, 1371-1380.

Huang, J., Olivenstein, R., Taha, R., Hamid, Q. & Ludwig, M. (1999). Enhanced proteoglycan deposition in the airway wall of atopic asthmatics. *American Journal of Respiratory & Critical Care Medicine* **160**, 725-729.

Huber, H.L. & Koessler, K.K. (1922). The pathology of bronchial asthma. *Archives of Internal Medicine* **30**, 689-760.

Hudgin, R.L., Charleson, S.E., Zimmerman, M., Mumford, R. & Wood, P.L. (1981). Enkephalinase: selective peptide inhibitors. *Life Sciences* **29**, 2593-2601.

Hughes, A.R., Horstman, D.A., Takemura, H. & Putney, J.W.J. (1990). Inositol phosphate metabolism and signal transduction. *American Review of Respiratory Disease* **141**, S115-S118

Hughes, J.M., McKay, K.O., Johnson, P.R., Tragoulias, S., Black, J.L. & Armour, C.L. (1993). Neutrophil-induced human bronchial hyperresponsiveness in vitro--pharmacological modulation. *Clinical & Experimental Allergy* **23**, 251-256.

Hulsmann, A.R. & de Jongste, J.C. (1993). Studies of human airways in vitro: a review of the methodology. *Journal of Pharmacological & Toxicological Methods* **30**, 117-132.

Hulsmann, A.R. & De, J. (1996). Modulation of airway responsiveness by the airway epithelium in humans: putative mechanisms. *Clinical & Experimental Allergy* **26**, 1236-1242.

Humbert, M., Durham, S.R., Ying, S., Kimmitt, P., Barkans, J., Assoufi, B., Pfister, R., Menz, G., Robinson, D.S., Kay, A.B. & Corrigan, C.J. (1996a). IL-4 and IL-5 mRNA and protein in bronchial biopsies from patients with atopic and nonatopic asthma: evidence against "intrinsic" asthma being a distinct immunopathologic entity. *American Journal of Respiratory & Critical Care Medicine* **154**, 1497-1504.

Humbert, M., Grant, J.A., Taborda-Barata, L., Durham, S.R., Pfister, R., Menz, G., Barkans, J., Ying, S. & Kay, A.B. (1996b). High-affinity IgE receptor (FcepsilonRI)-bearing cells in bronchial biopsies from atopic and nonatopic asthma. *American Journal of Respiratory & Critical Care Medicine* **153**, 1931-1937.

Hunt, L.W., Gleich, G.J., Ohnishi, T., Weiler, D.A., Mansfield, E.S., Kita, H. & Sur, S. (1994). Endotoxin contamination causes neutrophilia following pulmonary allergen challenge. *American Journal of Respiratory & Critical Care Medicine* **149**, 1471-1475.

Ichinose, M., Miura, M., Yamauchi, H., Kageyama, N., Tomaki, M., Oyake, T., Ohuchi, Y., Hida, W., Miki, H., Tamura, G. & Shirato, K. (1996). A neurokinin 1-receptor antagonist improves exercise-induced airway narrowing in asthmatic patients. *American Journal of Respiratory & Critical Care Medicine* **153**, 936-941.

Ingenito, E.P., Pliss, L.B., Martins, M.A. & Ingram, R.H. (1991). Effects of capsaicin on mechanical, cellular, and mediator responses to antigen in sensitized guinea pigs. *American Review of Respiratory Disease* **143**, 572-577.

Inoue, H., Aizawa, H., Matsumoto, K., Shigyo, M., Takata, S., Hara, M. & Hara, N. (1997). Effect of beta 2-agonists on histamine-induced airway microvascular leakage in ozone-exposed guinea pigs. *American Journal of Respiratory & Critical Care Medicine* **156**, 723-727.

Ireland, S.J., Bailey, F., Cook, A., Hagan, R.M., Jordan, C.C. & Stephens-Smith, M.L. (1991). Receptors mediating tachykinin-induced contractile responses in guinea-pig trachea. *British Journal of Pharmacology* **103**, 1463-1469.

Iwama, T., Nagai, H., Suda, H., Tsuruoka, N. & Koda, A. (1992). Effect of murine recombinant interleukin-5 on the cell population in guinea-pig airways. *British Journal of Pharmacology* **105**, 19-22.

Jackowski, J., Chapman, G.A., Abraham, W.M. & Ahmed, T. (1993). Ovine tracheal muscle contraction in vitro: inhibition by calcium channel blockers gallopamil and verapamil. *Respiration* **60**, 27-31.

James, A. & Carroll, N. (1995). Theoretic effects of mucus gland discharge on airway resistance in asthma. *Chest* **107**, 110S-110S.

James, A.L., Hogg, J.C., Dunn, L.A. & Pare, P.D. (1988). The use of the internal perimeter to compare airway size and to calculate smooth muscle shortening. *American Review of Respiratory Disease* **138**, 136-139.

James, A.L., Pare, P.D. & Hogg, J.C. (1989). The mechanics of airway narrowing in asthma. *American Review of Respiratory Disease* **139**, 242-246.

Jancso, N., Jancso-Gabor, A. & Szolcsanyi, J. (1967). Direct evidence for neurogenic inflammation and its prevention by denervation and by pretreatment with capsaicin. *British Journal of Pharmacology* **31**, 138-151.

Jeffery, P.K. (1994). Comparative morphology of the airways in asthma and chronic obstructive pulmonary disease. *American Journal of Respiratory & Critical Care Medicine* **150**, S6-13.

Jeffery, P.K., Wardlaw, A.J., Nelson, F.C., Collins, J.V. & Kay, A.B. (1989). Bronchial biopsies in asthma. An ultrastructural, quantitative study and correlation with hyperreactivity. *American Review of Respiratory Disease* **140**, 1745-1753.

Jeng, A.Y., Ansell, J. & Erion, M.D. (1989). pH- and time-dependent inhibition of rat kidney neutral endoprotease 24.11 by thiorphan and phosphoramidon. *Life Sciences* **45**, 2109-2114.

Jiang, H., Rao, K., Halayko, A.J., Liu, X. & Stephens, N.L. (1992). Ragweed sensitization-induced increase of myosin light chain kinase content in canine airway smooth muscle. *American Journal of Respiratory Cell & Molecular Biology* **7**, 567-573.

Jiang, H. & Stephens, N.L. (1994). Calcium and smooth muscle contraction. *Molecular & Cellular Biochemistry* **135**, 1-9.

John, M., Au, B.T., Jose, P.J., Lim, S., Saunders, M., Barnes, P.J., Mitchell, J.A., Belvisi, M.G. & Chung, K.F. (1998). Expression and release of interleukin-8 by human airway smooth muscle cells: inhibition by Th-2 cytokines and corticosteroids. *American Journal of Respiratory Cell & Molecular Biology* **18**, 84-90.

Johnson, P.R., Ammit, A.J., Carlin, S.M., Armour, C.L., Caughey, G.H. & Black, J.L. (1997). Mast cell tryptase potentiates histamine-induced contraction in human sensitized bronchus. *European Respiratory Journal* **10**, 38-43.

Johnson, P.R., Black, J.L., Carlin, S., Ge, Q. & Underwood, P.A. (2000). The production of extracellular matrix proteins by human passively sensitized airway smooth-muscle cells in culture: the effect of beclomethasone. *American Journal of Respiratory & Critical Care Medicine* **162**, 2145-2151.

Johnson, S. & Knox, A. (1999). Autocrine production of matrix metalloproteinase-2 is required for human airway smooth muscle proliferation. *American Journal of Physiology* **277**, L1109-L1117

Johnston, S.L. (1995). Natural and experimental rhinovirus infections of the lower respiratory tract. *American Journal of Respiratory & Critical Care Medicine* **152**, S46-S52

Jones, S.V. (1993). Muscarinic receptor subtypes: modulation of ion channels. *Life Sciences* **52**, 457-464.

Jongejan, R.C., de Jongste, J.C., Van Strik, R., Raatgeep, H.R., Bonta, I.L. & Kerrebijn, K.F. (1988). Measurement of human small airway smooth muscle function in vitro. Comparison of bronchiolar strips and segments. *J Pharmacol Methods* **20**, 135-142.

Joos, G., Pauwels, R. & van der Straeten, M. (1987). Effect of inhaled substance P and neurokinin A on the airways of normal and asthmatic subjects. *Thorax* **42**, 779-783.

Joos, G., Pauwels, R. & van der Straeten, M. (1988). The effect of oxitropium bromide on neurokinin A-induced bronchoconstriction in asthmatic subjects. *Pulmonary Pharmacology* **1**, 41-45.

Joos, G.F., Germonpre, P.R., Kips, J.C., Peleman, R.A. & Pauwels, R.A. (1994). Sensory Neuropeptides and the Human Lower Airways - Present State and Future Directions. *Eur Resp J* **7**, 1161-1171.

Joos, G.F., Kips, J.C. & Pauwels, R.A. (1993). Direct and indirect bronchial responsiveness. *Respiratory Medicine* **87**, 31-36.

Joos, G.F. & Pauwels, R.A. (1990). Mechanisms involved in neurokinin-induced bronchoconstriction. *Archives Internationales de Pharmacodynamie et de Therapie 1990* **303**, 132-146.

Joos, G.F., Pauwels, R.A. & van der Straeten, M.E. (1988a). The mechanism of tachykinin-induced bronchoconstriction in the rat. *American Review of Respiratory Disease* **137**, 1038-1044.

Joos, G.F., Pauwels, R.A. & van der Straeten, M.E. (1989a). Effect of nedocromil sodium on the bronchoconstrictor effect of neurokinin A in asthmatics. *Drugs* **37**, 109-136.

Joos, G.F., Pauwels, R.A. & van der Straeten, M.E. (1989b). The effect of nedocromil sodium on the bronchoconstrictor effect of neurokinin A in subjects with asthma. *Journal of Allergy & Clinical Immunology* **83**, 663-668.

Joris, I., Majno, G., Corey, E.J. & Lewis, R.A. (1987). The mechanism of vascular leakage induced by leukotriene E4. Endothelial contraction. *American Journal of Pathology* **126**, 19-24.

Juniper, E.F., Frith, P.A., Dunnett, C., Cockcroft, D.W. & Hargreave, F.E. (1978). Reproducibility and comparison of responses to inhaled histamine and methacholine. *Thorax* **33**, 705-710.

Kaneko, T., Massion, P.R., Hara, M. & Nadel, J.A. (1996). Ragweed antigen causes interleukin-8 production in sensitized dog trachea. *American Journal of Respiratory & Critical Care Medicine* **153**, 136-140.

Kapanci, Y., Assimacopoulos, A., Irle, C., Zwahlen, A. & Gabbiani, G. (1974). "Contractile interstitial cells" in pulmonary alveolar septa: a possible regulator of ventilation-perfusion ratio? Ultrastructural, immunofluorescence, and in vitro studies. *Journal of Cell Biology* **60**, 375-392.

Karaki, H., Ozaki, H., Hori, M., Mitsui-Saito, M., Amano, K., Harada, K., Miyamoto, S., Nakazawa, H., Won, K.J. & Sato, K. (1997). Calcium movements, distribution, and functions in smooth muscle. *Pharmacol Rev* **49**, 157-230.

Karlsson, J.A. (1993). A Role for Capsaicin Sensitive, Tachykinin Containing Nerves in Chronic Coughing and Sneezing But Not in Asthma - A Hypothesis. *Thorax* **48**, 396-400.

Katsunuma, T., Roffel, A.F., Elzinga, C.R., Zaagsma, J., Barnes, P.J. & Mak, J.C. (1999). beta(2)-adrenoceptor agonist-induced upregulation of tachykinin NK(2) receptor expression and function in airway smooth muscle. *American Journal of Respiratory Cell & Molecular Biology* **21**, 409-417.

Kaziro, Y., Itoh, H., Kozasa, T., Nakafuku, M. & Satoh, T. (1991). Structure and function of signal-transducing GTP-binding proteins. *Annu Rev Biochem* **60**, 349-400.

Kelman, G.R. & Nunn, J.F. (1966). Nomograms for correction of blood Po2, Pco2, pH, and base excess for time and temperature. *Journal of Applied Physiology* **21**, 1484-1490.

Kenakin, T.P. & Beek, D. (1982). A quantitative analysis of histamine H2-receptor-mediated relaxation of rabbit trachea. *Journal of Pharmacology & Experimental Therapeutics* **220**, 353-357.

Kilbinger, H., Dietrich, C. & von Bardeleben, R.S. (1993). Functional relevance of presynaptic muscarinic autoreceptors. *Journal of Physiology, Paris* **87**, 77-81.

Kim, J.S., Rabe, K.F., Magnussen, H., Green, J.M. & White, S.R. (1995). Migration and proliferation of guinea pig and human airway epithelial cells in response to tachykinins. *American Journal of Physiology* **269**, L119-L126

Kimura, K., Inoue, H., Ichinose, M., Miura, M., Katsumata, U., Takahashi, T. & Takishima, T. (1992). Bradykinin causes airway hyperresponsiveness and enhances maximal airway narrowing. Role of microvascular leakage and airway edema. *American Review of Respiratory Disease* **146**, 1301-1305.

Kips, J.C., Joos, G.F., Peleman, R.A. & Pauwels, R.A. (1993). The effect of TNFalpha on the *in vitro* responsiveness of rat trachael rings. (Abstract). *European Respiratory Journal* **6**, 325S

Kips, J.C., Tavernier, J. & Pauwels, R.A. (1992). Tumor necrosis factor causes bronchial hyperresponsiveness in rats. *American Review of Respiratory Disease* **145**, 332-336.

Kips, J.C., Tavernier, J.H., Joos, G.F., Peleman, R.A. & Pauwels, R.A. (1993a). The potential role of tumour necrosis factor alpha in asthma. *Clinical & Experimental Allergy* **23**, 247-250.

Kirk, R.E. (1995). *Experimental Design: Procedures for the behavioral sciences.* Brooks/Cole Publishing Co. Pacific Grove,

Kirkpatrick, C.T. (1975a). Excitation and contraction in bovine tracheal smooth muscle. *Journal of Physiology* **244**, 263-281.

Kirkpatrick, C.T., Jenkinson, H.A. & Cameron, A.R. (1975b). Interaction between drugs and potassium-rich solutions in producing contraction in bovine tracheal smooth muscle: studies in normal and calcium-depleted tissues. *Clinical & Experimental Pharmacology & Physiology* **2**, 559-570.

Kirsch, C.M., Sigal, E., Djokic, T.D., Graf, P.D. & Nadel, J.A. (1988). An in vivo chemotaxis assay in the dog trachea: evidence for chemotactic activity of 8,15-diHETE. *Journal of Applied Physiology* **64**, 1792-1795.

Kirshenbaum, A.S., Goff, J.P., Dreskin, S.C., Irani, A.M., Schwartz, L.B. & Metcalfe, D.D. (1989). IL-3-dependent growth of basophil-like cells and mastlike cells from human bone marrow. *Journal of Immunology* **142**, 2424-2429.

Klein, R.C. & Salvaggio, J.E. (1966). Nonspecificity of the bronchoconstricting effect of histamine and acetyl-beta-methylcholine in patients with obstructive airway disease. *J Allergy* **37**, 158-168.

Kleinstiver, P.W. & Eyre, P. (1979). Evaluation ot the lung parenchyma strip preparation to measure bronchoactivity. *Journal of Pharmacological Methods* **2**, 175-185.

Knight, D.A., Adcock, J.A., Phillips, M.J. & Thompson, P.J. (1990). The effect of epithelium removal on human bronchial smooth muscle responsiveness to acetylcholine and histamine. *Pulmonary Pharmacology* **3**, 198-202.

Knight, D.A., Stewart, G.A., Lai, M.L. & Thompson, P.J. (1995). Epithelium-derived inhibitory prostaglandins modulate human bronchial smooth muscle responses to histamine. *European Journal of Pharmacology* **272**, 1-11.

Knight, D.A., Stewart, G.A. & Thompson, P.J. (1992). Histamine tachyphylaxis in human airway smooth muscle. The role of H2-receptors and the bronchial epithelium. *Am.Rev Respir Dis.* **146**, 137-140.

Knox, A.J. & Tattersfield, A.E. (1995). Airway smooth muscle relaxation. *Thorax* **50**, 894-901.

Knudson, C.B. & Knudson, W. (1993). Hyaluronan-binding proteins in development, tissue homeostasis, and disease. *The Federation of American Societies for Experimental Biology Journal* **7**, 1233-1241.

Koh, Y., Hybertson, B.M., Jepson, E.K. & Repine, J.E. (1996). Tumor necrosis factor induced acute lung leak in rats: less than with interleukin-1. *Inflammation* **20**, 461-469.

Kon, O.M. & Kay, A.B. (1999a). Anti-T cell strategies in asthma. *Inflammation Research* **48**, 516-523.

Kon, O.M. & Kay, A.B. (1999b). T cells and chronic asthma. *Int.Arch.Allergy Immunol* **118**, 133-135.

Kondepudi, A. & Johnson, A. (1993). Cytokines increase neutral endopeptidase activity in lung fibroblasts. *American Journal of Respiratory Cell & Molecular Biology* **8**, 43-49.

Kosaka, H., Sakaguchi, H., Sawai, Y., Kumura, E., Seiyama, A., Chen, S.S. & Shiga, T. (1994). Effect of interferon-gamma on nitric oxide hemoglobin production in endotoxin-treated rats and its synergism with interleukin 1 or tumor necrosis factor. *Life Sciences* **54**, 1523-1529.

Koshino, T., Arai, Y., Miyamoto, Y., Sano, Y., Itami, M., Teshima, S., Hirai, K., Takaishi, T., Ito, K. & Morita, Y. (1996). Airway basophil and mast cell density in patients with bronchial asthma: relationship to bronchial hyperresponsiveness. *Journal of Asthma* **33**, 89-95.

Koto, H., Mak, J.C., Haddad, E.B., Xu, W.B., Salmon, M., Barnes, P.J. & Chung, K.F. (1996). Mechanisms of impaired beta-adrenoceptor-induced airway relaxation by interleukin-1beta in vivo in the rat. *Journal of Clinical Investigation* **98**, 1780-1787.

Kubo, K., Kobayashi, T., Hayano, T., Koizumi, T., Honda, T., Sekiguchi, M. & Sakai, A. (1994). Effects of ONO-5046, a specific neutrophil elastase inhibitor, on endotoxin-induced lung injury in sheep. *Journal of Applied Physiology* **77**, 1333-1340.

Kuijpers, T.W., Hakkert, B.C., Hart, M.H. & Roos, D. (1992). Neutrophil migration across monolayers of cytokine-prestimulated endothelial cells: a role for platelet-activating factor and IL-8. *Journal of Cell Biology* **117**, 565-572.

Kuwano, K., Bosken, C.H., Pare, P.D., Bai, T.R., Wiggs, B.R. & Hogg, J.C. (1993). Small airways dimensions in asthma and in chronic obstructive pulmonary disease. *American Review of Respiratory Disease* **148**, 1220-1225.

Laitinen, A. (1985a). Autonomic innervation of the human respiratory tract as revealed by histochemical and ultrastructural methods. *European Journal of Respiratory Disease Suppl.* **140:** 1-42.

Laitinen, A., Partanen, M., Hervonen, A. & Laitinen, L.A. (1985b). Electron microscopic study on the innervation of the human lower respiratory tract:

evidence of adrenergic nerves. *European Journal of Respiratory Disease* **67**, 209-215.

Laitinen, A., Partanen, M., Hervonen, A., Pelto-Huikko, M. & Laitinen, L.A. (1985c). VIP like immunoreactive nerves in human respiratory tract. Light and electron microscopic study. *Histochemistry* **82**, 313-319.

Laitinen, L.A., Haahtela, T., Kava, T. & Laitinen, A. (1983). Non-specific bronchial reactivity and ultrastructure of the airway epithelium in patients with sarcoidosis and allergic alveolitis. *European Journal of Respiratory Disease Suppl.* **131:** 267-284.

Laitinen, L.A., Heino, M., Laitinen, A., Kava, T. & Haahtela, T. (1985). Damage of the airway epithelium and bronchial reactivity in patients with asthma. *American Review of Respiratory Disease* **131**, 599-606.

Laitinen, L.A. & Laitinen, A. (1987). Innervation of airway smooth muscle. *American Review of Respiratory Disease* **136**, S38-S42.

Laitinen, L.A., Laitinen, A. & Haahtela, T. (1992). A comparative study of the effects of an inhaled corticosteroid, budesonide, and a beta 2-agonist, terbutaline, on airway inflammation in newly diagnosed asthma: a randomized, double-blind, parallel-group controlled trial. *Journal of Allergy & Clinical Immunology* **90**, 32-42.

Laitinen, L.A., Laitinen, A., Salonen, R.O. & Widdicombe, J.G. (1987). Vascular actions of airway neuropeptides. *American Review of Respiratory Disease* **136**, S59-S64.

Lam, S., Leriche, J.C., Kijek, K. & Phillips, D. (1985). Effect of bronchial lavage volume on cellular and protein recovery. *Chest* **88**, 856-859.

Lambert, R.K. (1991). Role of bronchial basement membrane in airway collapse. *Journal of Applied Physiology* **71**, 666-673.

Lambert, R.K., Wiggs, B.R., Kuwano, K., Hogg, J.C. & Pare, P.D. (1993). Functional significance of increased airway smooth muscle in asthma and COPD. *Journal of Applied Physiology* **74**, 2771-2781.

Lamblin, C., Gosset, P., Tillie-Leblond, I., Saulnier, F., Marquette, C.H., Wallaert, B. & Tonnel, A.B. (1998). Bronchial neutrophilia in patients with noninfectious status asthmaticus. *American Journal of Respiratory & Critical Care Medicine* **157**, 394-402.

Lammers, J.W., Minette, P., McCusker, M.T., Chung, K.F. & Barnes, P.J. (1989). Capsaicin-induced bronchodilation in mild asthmatic subjects: possible role of nonadrenergic inhibitory system. *Journal of Applied Physiology* **67**, 856-861.

Lanes, S., Stevenson, J.S., Codias, E., Hernandez, A., Sielczak, M.W., Wanner, A. & Abraham, W.M. (1986). Indomethacin and FPL-57231 inhibit antigen-induced airway hyperresponsiveness in sheep. *Journal of Applied Physiology* **61**, 864-872.

Lange, N.A. & Forker, G.M. (1969). *Handbook of chemistry*. McGraw-Hill New York.

Lansing, M.W., Ahmed, A., Cortes, A., Sielczak, M.W., Wanner, A. & Abraham, W.M. (1993). Oxygen Radicals Contribute to Antigen-Induced Airway Hyperresponsiveness in Conscious Sheep. *American Review of Respiratory Disease* **147**, 321-326.

Laurent, T.C. & Fraser, J.R. (1992). Hyaluronan. *The Federation of American Societies for Experimental Biology Journal* **6**, 2397-2404.

Laurenzi, M.A., Persson, M.A., Dalsgaard, C.J. & Ringden, O. (1989). Stimulation of human B lymphocyte differentiation by the neuropeptides substance P and neurokinin A. *Scandinavian Journal of Immunology* **30**, 695-701.

Lazaar, A.L., Amrani, Y., Hsu, J., Panettieri, R.A., Fanslow, W.C., Albelda, S.M. & Pure, E. (1998). CD40-mediated signal transduction in human airway smooth muscle. *Journal of Immunology* **161**, 3120-3127.

Lazarus, S.C. (1987). The role of mast cell-derived mediators in airway function. *American Review of Respiratory Disease* **135**, S35-S38

Lefort, J., Bachelet, C.M., Leduc, D. & Vargaftig, B.B. (1996). Effect of antigen provocation of IL-5 transgenic mice on eosinophil mobilization and bronchial hyperresponsiveness. *Journal of Allergy & Clinical Immunology* **97**, 788-799.

Lemiere, C., Malo, J.L. & Gautrin, D. (1996). Nonsensitizing causes of occupational asthma. *Medical Clinics of North America* **80**, 749-774.

Lemjabbar, H., Gosset, P., Lamblin, C., Tillie, I., Hartmann, D., Wallaert, B., Tonnel, A.B. & Lafuma, C. (1999). Contribution of 92 kDa gelatinase/type IV collagenase in bronchial inflammation during status asthmaticus. *American Journal of Respiratory & Critical Care Medicine* **159**, 1298-1307.

Levine, J.D., Lam, D., Taiwo, Y.O., Donatoni, P. & Goetzl, E.J. (1986). Hyperalgesic properties of 15-lipoxygenase products of arachidonic acid. *Proceedings of the National Academy of Sciences of the United States of America* **83**, 5331-5334.

Levine, J.D., Lau, W., Kwiat, G. & Goetzl, E.J. (1984). Leukotriene B4 produces hyperalgesia that is dependent on polymorphonuclear leukocytes. *Science* **225**, 743-745.

Li, C.G. & Rand, M.J. (1991). Evidence that part of the NANC relaxant response of guinea-pig trachea to electrical field stimulation is mediated by nitric oxide. *British Journal of Pharmacology* **102**, 91-94.

Li, X. & Wilson, J.W. (1997). Increased vascularity of the bronchial mucosa in mild asthma. *American Journal of Respiratory & Critical Care Medicine* **156**, 229-233.

Liggett, S.B. (2000). The pharmacogenetics of beta2-adrenergic receptors: Relevance to asthma. *Journal of Allergy & Clinical Immunology* **105**, 487-492.

Lilly, C.M., Bai, T.R., Shore, S.A., Hall, A.E. & Drazen, J.M. (1995). Neuropeptide content of lungs from asthmatic and nonasthmatic patients. *American Journal of Respiratory & Critical Care Medicine* **151**, 548-553.

Lilly, C.M., Chapman, R.W., Sehring, S.J., Mauser, P.J., Egan, R.W. & Drazen, J.M. (1996). Effects of interleukin 5-induced pulmonary eosinophilia on airway reactivity in the guinea pig. *American Journal of Physiology* **270**, L368-L375

Lilly, C.M., Hall, A.E., Rodger, I.W., Kobzik, L., Haley, K.J. & Drazen, J.M. (1995). Substance P-induced histamine release in tracheally perfused guinea pig lungs. *Journal of Applied Physiology* **78**, 1234-1241.

Lilly, C.M., Kobzik, L., Hall, A.E. & Drazen, J.M. (1994). Effects of chronic airway inflammation on the activity and enzymatic inactivation of neuropeptides in guinea pig lungs. *Journal of Clinical Investigation* **93**, 2667-2674.

Lin, L.L., Lin, A.Y. & Knopf, J.L. (1992). Cytosolic phospholipase A2 is coupled to hormonally regulated release of arachidonic acid. *Proceedings of the National Academy of Sciences of the United States of America* **89**, 6147-6151.

List, S.J., Findlay, B.P., Forstner, G.G. & Forstner, J.F. (1978). Enhancement of the viscosity of mucin by serum albumin. *Biochem.J* **175**, 565-571.

Lotvall, J., Elwood, W., Tokuyama, K., Sakamoto, T., Barnes, P.J. & Chung, K.F. (1992). A thromboxane mimetic, U-46619, produces plasma exudation in airways of the guinea pig. *Journal of Applied Physiology* **72**, 2415-2419.

Lotvall, J.O., Lemen, R.J., Hui, K.P., Barnes, P.J. & Chung, K.F. (1990). Airflow obstruction after substance P aerosol: contribution of airway and pulmonary edema. *Journal of Applied Physiology* **69**, 1473-1478.

Lotvall, J.O., Tokuyama, K., Lofdahl, C.G., Ullman, A., Barnes, P.J. & Chung, K.F. (1991). Peptidase modulation of noncholinergic vagal bronchoconstriction and airway microvascular leakage [published errata appear in *Journal of Applied Physiology* 1991; 71(3): following Table of Contents and 1991;71(6): following Author Index]. *Journal of Applied Physiology* **70**, 2730-2735.

Lulich, K.M., Mitchell, H.W. & Sparrow, M.P. (1976). The cat lung strip as an in vitro preparation of peripheral airways: a comparison of beta-adrenoceptor agonists, autacoids and anaphylactic challenge on the lung strip and trachea. *British Journal of Pharmacology* **58**, 71-79.

Lundberg, J.M. (1995). Tachykinins, sensory nerves, and asthma - an overview. *Canadian Journal of Physiology & Pharmacology* **73**, 908-914.

Lundberg, J.M., Alving, K., Karlsson, J.A., Matran, R. & Nilsson, G. (1991). Sensory neuropeptide involvement in animal models of airway irritation and of allergen-evoked asthma. *American Review of Respiratory Disease* **143**, 1429-1430.

Lundberg, J.M., Brodin, E., Hua, X. & Saria, A. (1984). Vascular permeability changes and smooth muscle contraction in relation to capsaicin-sensitive substance P afferents in the guinea-pig. *Acta Physiologica Scandinavica* **120**, 217-227.

Lundberg, J.M., Brodin, E. & Saria, A. (1983). Effects and distribution of vagal capsaicin-sensitive substance P neurons with special reference to the trachea and lungs. *Acta Physiologica Scandinavica* **119**, 243-252.

Lundberg, J.M., Franco-Cereceda, A., Hua, X., Hokfelt, T. & Fischer, J.A. (1985). Co-existence of substance P and calcitonin gene-related peptide-like immunoreactivities in sensory nerves in relation to cardiovascular and bronchoconstrictor effects of capsaicin. *European Journal of Pharmacology* **108**, 315-319.

Lundberg, J.M., Hokfelt, T., Martling, C.R., Saria, A. & Cuello, C. (1984). Substance P-immunoreactive sensory nerves in the lower respiratory tract of various mammals including man. *Cell & Tissue Research* **235**, 251-261.

Lundberg, J.M., Lundblad, L., Martling, C.R., Saria, A., Stjarne, P. & Anggard, A. (1987). Coexistence of multiple peptides and classic transmitters in airway neurons: functional and pathophysiologic aspects. *American Review of Respiratory Disease* **136**, S16-S22

Lundberg, J.M., Martling, C.R. & Saria, A. (1983a). Substance P and capsaicin-induced contraction of human bronchi. *Acta Physiologica Scandinavica* **119**, 49-53.

Lundberg, J.M. & Saria, A. (1983b). Capsaicin-induced desensitization of airway mucosa to cigarette smoke, mechanical and chemical irritants. *Nature* **302**, 251-253.

MacGlashan, D.J., McKenzie-White, J., Chichester, K., Bochner, B.S., Davis, F.M., Schroeder, J.T. & Lichtenstein, L.M. (1998). In vitro regulation of FcepsilonRIalpha expression on human basophils by IgE antibody. *Blood* **91**, 1633-1643.

Machleidt, T., Kramer, B., Adam, D., Neumann, B., Schutze, S., Wiegmann, K. & Kronke, M. (1996). Function of the p55 tumor necrosis factor receptor "death domain" mediated by phosphatidylcholine-specific phospholipase C. *Journal of Experimental Medicine* **184**, 725-733.

Macklem, P.T. (1990). A hypothesis linking bronchial hyperreactivity and airway inflammation: implications for therapy. *Annals of Allergy* **64**, 113-116.

Maclagan, J. (1987). Presynaptic control of airway smooth muscle. *American Review of Respiratory Disease* **136**, S54-S57.

Maestrelli, P., di Stefano, A., Occari, P., Turato, G., Milani, G., Pivirotto, F., Mapp, C.E., Fabbri, L.M. & Saetta, M. (1995). Cytokines in the airway mucosa of subjects with asthma induced by toluene diisocyanate. *American Journal of Respiratory & Critical Care Medicine* **151**, 607-612.

Maggi, C.A. (1997). The effects of tachykinins on inflammatory and immune cells. *Regulatory Peptides* **70**, 75-90.

Maggi, C.A., Patacchini, R., Rovero, P. & Meli, A. (1989). The hamster isolated trachea: a new preparation for studying NK-2 receptors. *European Journal of Pharmacology* **166**, 435-440.

Magno, M. (1990). Comparative anatomy of the tracheobronchial circulation. *European Respiratory Journal Suppl.* **12:** 557s-562s.

Majno, G., Shea, S.M. & Leventhal, M. (1969). Endothelial contraction induced by histamine-type mediators: an electron microscopic study. *Journal of Cell Biology* **42**, 647-672.

Mak, J.C. & Barnes, P.J. (1990). Autoradiographic visualization of muscarinic receptor subtypes in human and guinea pig lung. *American Review of Respiratory Disease* **141**, 1559-1568.

Mak, J.C., Roffel, A.F., Katsunuma, T., Elzinga, C.R., Zaagsma, J. & Barnes, P.J. (2000). Up-regulation of airway smooth muscle histamine H(1) receptor mRNA, protein, and function by beta(2)-adrenoceptor activation. *Molecular Pharmacology* **57**, 857-864.

Manetti, R., Parronchi, P., Giudizi, M.G., Piccinni, M.P., Maggi, E., Trinchieri, G. & Romagnani, S. (1993). Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. *Journal of Experimental Medicine* **177**, 1199-1204.

Manning, D.C., Raja, S.N., Meyer, R.A. & Campbell, J.N. (1991). Pain and hyperalgesia after intradermal injection of bradykinin in humans. *Clinical Pharmacology & Therapeutics* **50**, 721-729.

Manning, P.J., Jones, G.L. & O'Byrne, P.M. (1987). Tachyphylaxis to inhaled histamine in asthmatic subjects. *Journal of Applied Physiology* **63**, 1572-1577.

Mark, K.S. & Miller, D.W. (1999). Increased permeability of primary cultured brain microvessel endothelial cell monolayers following TNF-alpha exposure. *Life Sciences* **64**, 1941-1953.

Marks, G.B., Yates, D.H., Sist, M., Ceyhan, B., De Campos, M., Scott, D.M. & Barnes, P.J. (1996). Respiratory sensation during bronchial challenge testing with methacholine, sodium metabisulphite, and adenosine monophosphate. *Thorax* **51**, 793-798.

Marthan, R., Armour, C.L., Johnson, P.R. & Black, J.L. (1987). The calcium channel agonist BAY K8644 enhances the responsiveness of human airway muscle to KCl and histamine but not to carbachol. *American Review of Respiratory Disease* **135**, 185-189.

Marthan, R., Crevel, H., Guenard, H. & Savineau, J.P. (1992). Responsiveness to histamine in human sensitized airway smooth muscle. *Respiration Physiology* **90**, 239-250.

Marthan, R., Roux, E. & Savineau, J.P. (1996). Human bronchial smooth muscle responsiveness after in vitro exposure to oxidizing pollutants. *Cell Biology & Toxicology* **12**, 245-249.

Martinez, F.D., Wright, A.L., Taussig, L.M., Holberg, C.J., Halonen, M. & Morgan, W.J. (1995). Asthma and wheezing in the first six years of life. The Group Health Medical Associates. *New England Journal of Medicine* **332**, 133-138.

Martling, C.R. (1987a). Sensory nerves containing tachykinins and CGRP in the lower airways. Functional implications for bronchoconstriction, vasodilatation and protein extravasation. *Acta Physiologica Scandinavica.* Suppl. **3**, 1-57.

Martling, C.R., Theodorsson-Norheim, E. & Lundberg, J.M. (1987b). Occurrence and effects of multiple tachykinins; substance P, neurokinin A and neuropeptide K in human lower airways. *Life Sciences* **40**, 1633-1643.

Matran, R., Naline, E., Advenier, C., Lockhart, A., Tricot, J.F. & Regoli, D. (1988). Role of extracellular calcium in the effects of substance P and neurokinin A on guinea pig trachea and human bronchus. *Fundamental and Clinical Pharmacology* **2**, 47-55.

Matsuba, K. & Thurlbeck, W.M. (1972). A morphometric study of bronchial and bronchiolar walls in children. *American Review of Respiratory Disease* **105**, 908-913.

Matsuda, H., Kannan, Y., Ushio, H., Kiso, Y., Kanemoto, T., Suzuki, H. & Kitamura, Y. (1991). Nerve growth factor induces development of connective tissue-type mast cells in vitro from murine bone marrow cells. *Journal of Experimental Medicine* **174**, 7-14.

Mauser, P.J., Pitman, A.M., Fernandez, X., Foran, S.K., Adams, G.K., Kreutner, W., Egan, R.W. & Chapman, R.W. (1995). Effects of an antibody to interleukin-5 in a monkey model of asthma. *American Journal of Respiratory & Critical Care Medicine* **152**, 467-472.

Mautino, G., Capony, F., Bousquet, J. & Vignola, A.M. (1999a). Balance in asthma between matrix metalloproteinases and their inhibitors. *Journal of Allergy & Clinical Immunology* **104**, 530-533.

Mautino, G., Henriquet, C., Jaffuel, D., Bousquet, J. & Capony, F. (1999b). Tissue inhibitor of metalloproteinase-1 levels in bronchoalveolar lavage fluid from asthmatic subjects. *American Journal of Respiratory & Critical Care Medicine* **160**, 324-330.

Mautino, G., Oliver, N., Chanez, P., Bousquet, J. & Capony, F. (1997). Increased release of matrix metalloproteinase-9 in bronchoalveolar lavage fluid and by alveolar macrophages of asthmatics. *American Journal of Respiratory & Critical Care Medicine* **17**, 583-591.

May, N.D.S. (1970). *The anatomy of the sheep: a dissection manual.* University of Queensland Press, St Lucia.

McDonald, D.M. (1988). Respiratory tract infections increase susceptibility to neurogenic inflammation in the rat trachea. *American Review of Respiratory Disease* **137**, 1432-1440.

McDonald, D.M. (1994). Endothelial gaps and permeability of venules in rat tracheas exposed to inflammatory stimuli. *American Journal of Physiology* **266**, L61-L83.

McDonald, D.M., Bowden, J.J., Baluk, P. & Bunnett, N.W. (1996). Neurogenic inflammation. A model for studying efferent actions of sensory nerves. *Advances in Experimental Medicine & Biology* **410**, 453-462.

McElroy, A.B., Clegg, S.P., Deal, M.J., Ewan, G.B., Hagan, R.M., Ireland, S.J., Jordan, C.C., Porter, B., Ross, B.C., Ward, P. & et, a. (1992). Highly potent and selective heptapeptide antagonists of the neurokinin NK-2 receptor. *Journal of Medicinal Chemistry* **35**, 2582-2591.

McEwan, J.R., Benjamin, N., Larkin, S., Fuller, R.W., Dollery, C.T. & MacIntyre, I. (1988). Vasodilatation by calcitonin gene-related peptide and by substance P: a comparison of their effects on resistance and capacitance vessels of human forearms. *Circulation* **77**, 1072-1080.

McFadden, E.R.J. & Lyons, H.A. (1968). Arterial-blood gas tension in asthma. *New England Journal of Medicine* **278**, 1027-1032.

McKay, K.O., Black, J.L. & Armour, C.L. (1991). The mechanism of action of endothelin in human lung. *British Journal of Pharmacology* **102**, 422-428.

McKay, K.O., Black, J.L. & Armour, C.L. (1992). Phosphoramidon potentiates the contractile response to endothelin-3, but not endothelin-1 in isolated airway tissue. *British Journal of Pharmacology* **105**, 929-932.

McKenzie, A.N., Culpepper, J.A., de Waal, M., Briere, F., Punnonen, J., Aversa, G., Sato, A., Dang, W., Cocks, B.G. & Menon, S. (1993). Interleukin 13, a T-cell-derived cytokine that regulates human monocyte and B-cell function. *Proceedings of the National Academy of Sciences of the United States of America* **90** , 3735-3739.

McMillian, M.K., Soltoff, S.P. & Talamo, B.R. (1987). Rapid desensitization of substance P- but not carbachol-induced increases in inositol trisphosphate and intracellular Ca++ in rat parotid acinar cells. *Biochemical and Biophysical Research Communications* **148**, 1017-1024.

Mead, J. & Whittenberger, J.L. (1953). Physical properties of human lungs measured during spontaneous respiration. *J Applied Physiology* **5**, 779-796.

Messer, J.W., Peters, G.A. & Benner, W.A. (1960). Causes of death and pathologic finding in 304 cases of bronchial asthma. *Diseases of the Chest* **38**, 612-624.

Michel, C.C. & Curry, F.E. (1999). Microvascular permeability. *Physiological Reviews* **79**, 703-761.

Michel, C.C. & Kendall, S. (1997). Differing effects of histamine and serotonin on microvascular permeability in anaesthetized rats. *ournal of Physiology (London)* **501**, 657-662.

Middleton, E.J. (1984). Airway smooth muscle, asthma, and calcium ions. *Journal of Allergy & Clinical Immunology* **73**, 643-650.

Mills, P.R., Davies, R.J. & Devalia, J.L. (1999). Airway epithelial cells, cytokines, and pollutants. *American Journal of Respiratory & Critical Care Medicine* **160**, S38-S43.

Minette, P.A. & Barnes, P.J. (1990). Muscarinic receptor subtypes in lung. Clinical implications. *American Review of Respiratory Disease* **141**, S162-S165.

Minette, P.A., Lammers, J.W., Dixon, C.M., McCusker, M.T. & Barnes, P.J. (1989). A muscarinic agonist inhibits reflex bronchoconstriction in normal but not in asthmatic subjects. *Journal of Applied Physiology* **67**, 2461-2465.

Ming, W.J., Bersani, L. & Mantovani, A. (1987). Tumor necrosis factor is chemotactic for monocytes and polymorphonuclear leukocytes. *Journal of Immunology* **138**, 1469-1474.

Mirbahar, K.B. & Eyre, P. (1982). Autacoid and autonomic reactivity of bovine and ovine bronchus: modifications by antigenic sensitization. *Archives Internationales de Pharmacodynamie et de Therapie* **258**, 60-70.

Mitchell, H.W. & Gray, P.R. (1996). Uncoupling in the wall of the cartilaginous bronchus of the pig produced by smooth muscle contraction. *Pulmonary Pharmacology* **9**, 29-34.

Mitchell, R.W., Koenig, S.M., Kelly, E., Stephens, N.L. & Leff, A.R. (1989). Ca2+-dependent facilitated shortening in isotonic contraction of trachealis muscle. *Journal of Applied Physiology* **66**, 632-637.

Mitchell, R.W., Rabe, K.F., Magnussen, H. & Leff, A.R. (1997). Passive sensitization of human airways induces myogenic contractile responses in vitro. *Journal of Applied Physiology* **83**, 1276-1281.

Mitzner, W., Wagner, E. & Brown, R.H. (1995). Is asthma a vascular disorder? *Chest* **107**, 97S-102S.

Miura, M., Ichinose, M., Kimura, K., Katsumata, U., Takahashi, T., Inoue, H. & Takishima, T. (1992). Dysfunction of nonadrenergic noncholinergic inhibitory system after antigen inhalation in actively sensitized cat airways. *American Review of Respiratory Disease* **145**, 70-74.

Miyakawa, T., Maeda, A., Yamazawa, T., Hirose, K., Kurosaki, T. & Iino, M. (1999). Encoding of Ca2+ signals by differential expression of IP3 receptor subtypes. *The EMBO Journal* **18**, 1303-1308.

Molfino, N.A. & Slutsky, A.S. (1994). Near-fatal asthma. *European Respiratory Journal* **7**, 981-990.

Molfino, N.A., Wright, S.C., Katz, I., Tarlo, S., Silverman, F., McClean, P.A., Szalai, J.P., Raizenne, M., Slutsky, A.S. & Zamel, N. (1991). Effect of low concentrations of ozone on inhaled allergen responses in asthmatic subjects. *Lancet* **338**, 199-203.

Molimard, M., Naline, E., Boichot, E., Devillier, P., Lagente, V., Begaud, B. & Advenier, C. (1998). In vitro-induced human airway hyperresponsiveness to bradykinin. *European Respiratory Journal* **12**, 1301-1306.

Monkawa, T., Miyawaki, A., Sugiyama, T., Yoneshima, H., Yamamoto-Hino, M., Furuichi, T., Saruta, T., Hasegawa, M. & Mikoshiba, K. (1995). Heterotetrameric complex formation of inositol 1,4,5-trisphosphate receptor subunits. *Journal of Biological Chemistry* **270**, 14700-14704.

Montefort, S., Herbert, C.A., Robinson, C. & Holgate, S.T. (1992). The bronchial epithelium as a target for inflammatory attack in asthma. *Clinical & Experimental Allergy* **22**, 511-520.

Montefort, S., Roche, W.R. & Holgate, S.T. (1993). Bronchial epithelial shedding in asthmatics and non-asthmatics. *Respiratory Medicine* **87**, 9-11.

Moreno, R.H., Hogg, J.C. & Pare, P.D. (1986). Mechanics of airway narrowing. *American Review of Respiratory Disease* **133**, 1171-1180.

Mosmann, T.R., Cherwinski, H., Bond, M.W., Giedlin, M.A. & Coffman, R.L. (1986). Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *Journal of Immunology* **136**, 2348-2357.

Munakata, M., Chen, H., Nasuhara, Y., Sato, A., Takahashi, T., Sato, R., Homma, Y. & Kawakami, Y. (1996). Interleukin-1 beta decreases sensitivity of guinea-pig airway to potassium chloride and isoproterenol by an epithelium-dependent mechanism [published erratum appears in *Respirology* 1996;1(2):152]. *Respirology* **1**, 61-67.

Murlas, C.G., Lang, Z., Williams, G.J. & Chodimella, V. (1992). Aerosolized neutral endopeptidase reverses ozone-induced airway hyperreactivity to substance P. *Journal of Applied Physiology* **72**, 1133-1141.

Murphy, T.M., Mitchell, R.W., Blake, J.S., Mack, M.M., Kelly, E.A., Munoz, N.M. & Leff, A.R. (1989). Expression of airway contractile properties and acetylcholinesterase activity in swine. *Journal of Applied Physiology* **67**, 174-180.

Mussap, C.J., Geraghty, D.P. & Burcher, E. (1993). Tachykinin receptors: a radioligand binding perspective. *Journal of Neurochemistry* **60**, 1987-2009.

Nadel, J.A. (1990). Decreased neutral endopeptidases: possible role in inflammatory diseases of airways. *Lung* **168**, 123-127.

Nadel, J.A. & Borson, D.B. (1991). Modulation of neurogenic inflammation by neutral endopeptidase. *American Review of Respiratory Disease* **143**, S33-S36.

Nagai, H., Kondo, M., Koda, A., Nakamura, S., Hashimoto, M., Yanagihara, Y. & Daikoku, M. (1992). Responses of isolated Japanese monkey tracheal muscle to allergic mediators. *International Archives of Allergy & Immunology* **98**, 70-75.

Nakamura-Craig, M. & Gill, B.K. (1991). Effect of neurokinin A, substance P and calcitonin gene related peptide in peripheral hyperalgesia in the rat paw. *Neuroscience Letters* **124**, 49-51.

Nakanishi, S., Nakajima, Y. & Yokota, Y. (1993). Signal transduction and ligand-binding domains of the tachykinin receptors. *Regulatory Peptides* **46**, 37-42.

Naline, E., Devillier, P., Drapeau, G., Toty, L., Bakdach, H., Regoli, D. & Advenier, C. (1989). Characterization of neurokinin effects and receptor selectivity in human isolated bronchi. *American Review of Respiratory Disease* **140**, 679-686.

Naline, E., Molimard, M., Regoli, D., Emonds-Alt, X., Bellamy, J.F. & Advenier, C. (1996). Evidence for functional tachykinin NK1 receptors on human isolated small bronchi. *American Journal of Physiology* **271**, L763-L767.

Newton, C.L., Mignery, G.A. & Sudhof, T.C. (1994). Co-expression in vertebrate tissues and cell lines of multiple inositol 1,4,5-trisphosphate (InsP3) receptors with distinct affinities for InsP3. *Journal of Biological Chemistry* **269**, 28613-28619.

Nieber, K., Baumgarten, C.R., Rathsack, R., Furkert, J., Oehme, P. & Kunkel, G. (1992). Substance P and beta-endorphin-like immunoreactivity in lavage fluids of subjects with and without allergic asthma. *Journal of Allergy & Clinical Immunology* **90**, 646-652.

Niemeier, R.W. & Bingham, E. (1972). An isolated perfused lung preparation for metabolic studies. *Life Sciences - Part* 2; **11**, 807-820.

Nilsen, E.M., Johansen, F.E., Jahnsen, F.L., Lundin, K.E., Scholz, T., Brandtzaeg, P. & Haraldsen, G. (1998). Cytokine profiles of cultured microvascular endothelial cells from the human intestine. *Gut* **42**, 635-642.

Nishizuka, Y. (1988). The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature* **334**, 661-665.

Nophar, Y., Kemper, O., Brakebusch, C., Englemann, H., Zwang, R., Aderka, D., Holtmann, H. & Wallach, D. (1990). Soluble forms of tumor necrosis factor receptors (TNF-Rs). The cDNA for the type I TNF-R, cloned using amino acid sequence data of its soluble form, encodes both the cell surface and a soluble form of the receptor. *The EMBO Journal* **9**, 3269-3278.

Noveral, J.P. & Grunstein, M.M. (1995). Tachykinin regulation of airway smooth muscle cell proliferation. *American Journal of Physiology* **269**, L339-L343.

O'Byrne, P.M., Dolovich, J. & Hargreave, F.E. (1987). Late asthmatic responses. *American Review of Respiratory Disease* **136**, 740-751.

O'Riordan, T.G., Zwang, J. & Smaldone, G.C. (1992). Mucociliary clearance in adult asthma. *American Review of Respiratory Disease* **146**, 598-603.

Oettgen, H.C. & Geha, R.S. (2001). IgE regulation and roles in asthma pathogenesis. *Journal of Allergy & Clinical Immunology* **107**, 429-440.

Ohashi, Y., Motojima, S., Fukuda, T. & Makino, S. (1992). Airway hyperresponsiveness, increased intracellular spaces of bronchial epithelium, and increased infiltration of eosinophils and lymphocytes in bronchial mucosa in asthma. *American Review of Respiratory Disease* **145**, 1469-1476.

Ohno, I., Tanno, Y., Yamauchi, K. & Takishima, T. (1990). Gene expression and production of tumour necrosis factor by a rat basophilic leukaemia cell line (RBL-2H3) with IgE receptor triggering. *Immunology* **70**, 88-93.

Okamoto, Y., Shirotori, K., Kudo, K., Ishikawa, K., Ito, E., Togawa, K. & Saito, I. (1993). Cytokine expression after the topical administration of substance P to human nasal mucosa. The role of substance P in nasal allergy. *Journal of Immunology* **151**, 4391-4398.

Okusawa, S., Gelfand, J.A., Ikejima, T., Connolly, R.J. & Dinarello, C.A. (1988). Interleukin 1 induces a shock-like state in rabbits. Synergism with tumor necrosis factor and the effect of cyclooxygenase inhibition. *Journal of Clinical Investigation* **81**, 1162-1172.

Ollerenshaw, S., Jarvis, D., Woolcock, A., Sullivan, C. & Scheibner, T. (1989). Absence of immunoreactive vasoactive intestinal polypeptide in tissue from the lungs of patients with asthma. *New England Journal of Medicine* **320**, 1244-1248.

Ollerenshaw, S.L., Jarvis, D., Sullivan, C.E. & Woolcock, A.J. (1991). Substance P immunoreactive nerves in airways from asthmatics and nonasthmatics. *European Respiratory Journal* **4**, 673-682.

Olson, L.E. & Wardle, R.L. (1992). Effect of a regional increase in alveolar pressure on pulmonary blood flow. *Journal of Applied Physiology* **73**, 1291-1296.

Orsida, B.E., Li, X., Hickey, B., Thien, F., Wilson, J.W. & Walters, E.H. (1999). Vascularity in asthmatic airways: relation to inhaled steroid dose. *Thorax* **54**, 289-295.

Owen, W.F.J., Rothenberg, M.E., Silberstein, D.S., Gasson, J.C., Stevens, R.L., Austen, K.F. & Soberman, R.J. (1987). Regulation of human eosinophil viability, density, and function by granulocyte/macrophage colony-stimulating factor in the presence of 3T3 fibroblasts. *Journal of Experimental Medicine* **166**, 129-141.

Paggiaro, P.L., Vagaggini, B., Dente, F.L., Bacci, E., Bancalari, L., Carrara, M., Di Franco, A., Giannini, D. & Giuntini, C. (1993). Bronchial hyperresponsiveness and toluene diisocyanate. Long-term change in sensitized asthmatic subjects. *Chest* **103**, 1123-1128.

Palmer, J.B. & Barnes, P.J. (1987). Neuropeptides and airway smooth muscle function. *American Review of Respiratory Disease* **136**, S50-S54.

Palmer, J.B., Cuss, F.M. & Barnes, P.J. (1986). VIP and PHM and their role in nonadrenergic inhibitory responses in isolated human airways. *Journal of Applied Physiology* **61**, 1322-1328.

Panettieri, R.A., Jr. (1998). Cellular and molecular mechanisms regulating airway smooth muscle proliferation and cell adhesion molecule expression. *American Journal of Respiratory & Critical Care Medicine* **158**, S133-S140.

Papka, R.E., Furness, J.B., Della, N.G., Murphy, R. & Costa, M. (1984). Time course of effect of capsaicin on ultrastructure and histochemistry of substance P-immunoreactive nerves associated with the cardiovascular system of the guinea-pig. *Neuroscience* **12** , 1277-1292.

Pare, P.D. & Bai, T.R. (1995). The consequences of chronic allergic inflammation. *Thorax* **50**, 328-332.

Pare, P.D. & Bai, T.R. (1996). Airway wall remodelling in chronic obstructive pulmonary disease. *European Respiratory Review* **6:39**, 259-263.

Parsons, G.H., Nichol, G.M., Barnes, P.J. & Chung, K.F. (1992). Peptide mediator effects on bronchial blood velocity and lung resistance in conscious sheep. *Journal of Applied Physiology* **72**, 1118-1122.

Patel, H.J., Barnes, P.J., Takahashi, T., Tadjkarimi, S., Yacoub, M.H. & Belvisi, M.G. (1995). Evidence for prejunctional muscarinic autoreceptors in human and guinea pig trachea. *American Journal of Respiratory & Critical Care Medicine* **152**, 872-878.

Pauwels, R.A., Brusselle, G.J. & Kips, J.C. (1997). Cytokine manipulation in animal models of asthma *American Journal of Respiratory & Critical Care Medicine* **156**, S78-S81.

Pavia, D., Bateman, J.R., Sheahan, N.F., Agnew, J.E. & Clarke, S.W. (1985). Tracheobronchial mucociliary clearance in asthma: impairment during remission. *Thorax* **40**, 171-175.

Pedersen, K.E., Meeker, S.N., Riccio, M.M. & Undem, B.J. (1998). Selective stimulation of jugular ganglion afferent neurons in guinea pig airways by hypertonic saline. *Journal of Applied Physiology* **84**, 499-506.

Pendino, K.J., Shuler, R.L., Laskin, J.D. & Laskin, D.L. (1994). Enhanced production of interleukin-1, tumor necrosis factor-alpha, and fibronectin by rat lung phagocytes following inhalation of a pulmonary irritant. *American Journal of Respiratory & Critical Care Medicine* **11**, 279-286.

Pendry, Y.D. (1993). Neuronal Control of Airways Smooth Muscle. *Pharmacology and Therapeutics* **57**, 171-202.

Pene, J., Rousset, F., Briere, F., Chretien, I., Wideman, J., Bonnefoy, J.Y. & De Vries, J.E. (1988). Interleukin 5 enhances interleukin 4-induced IgE production by normal human B cells. The role of soluble CD23 antigen. *European Journal of Immunology* **18**, 929-935.

Pennings, H.J., Kramer, K., Bast, A., Buurman, W.A. & Wouters, E.F. (1998). Tumour necrosis factor-alpha induces hyperreactivity in tracheal smooth muscle of the guinea-pig in vitro. *European Respiratory Journal* **12**, 45-49.

Persson, C.G. (1986). Role of plasma exudation in asthmatic airways. *Lancet* **2**, 1126-1129.

Persson, C.G. (1987). Leakage of macromolecules from the tracheobronchial microcirculation. *American Review of Respiratory Disease* **135**, S71-S75.

Persson, C.G. (1991). Plasma exudation in the airways: mechanisms and function. *European Respiratory Journal* **4**, 1268-1274.

Persson, C.G., Erjefalt, I., Gustafsson, B. & Luts, A. (1990). Subepithelial hydrostatic pressure may regulate plasma exudation across the mucosa. *International Archives of Allergy & Applied Immunology* **92**, 148-153.

Persson, C.G., Erjefalt, J.S., Andersson, M., Greiff, L. & Svensson, C. (1996). Extravasation, lamina propria flooding and lumenal entry of bulk plasma exudate in mucosal defence, inflammation and repair. *Pulmonary Pharmacology* **9**, 129-139.

Phan, S.H., Gharaee-Kermani, M., McGarry, B., Kunkel, S.L. & Wolber, F.W. (1992). Regulation of rat pulmonary artery endothelial cell transforming growth factor-beta production by IL-1 beta and tumor necrosis factor-alpha. *Journal of Immunology* **149**, 103-106.

Piedimonte, G., Hoffman, J.I.E., Husseini, W.K., Snider, R.M., Desai, M.C. & Nadel, J.A. (1993). NK(1) Receptors Mediate Neurogenic Inflammatory Increase in Blood Flow in Rat Airways. *Journal of Applied Physiology* **74**, 2462-2468.

Piguet, P.F., Grau, G.E. & Vassalli, P. (1990). Subcutaneous perfusion of tumor necrosis factor induces local proliferation of fibroblasts, capillaries, and epidermal cells, or massive tissue necrosis. *American Journal of Pathology* **136**, 103-110.

Pilewski, J.M. & Albelda, S.M. (1993). Adhesion molecules in the lung. An overview. *American Review of Respiratory Disease* **148**, S31-S37.

Pober, J.S. & Cotran, R.S. (1990). Cytokines and endothelial cell biology. *Physiological Reviews* **70**, 427-451.

Polito, A.J. & Proud, D. (1998). Epithelia cells as regulators of airway inflammation. *Journal of Allergy & Clinical Immunology* **102**, 714-718.

Polosa, R. (1995). Renaissance of the effector role of the mast cell in bronchial asthma. *Annali Italiani di Medicina Interna* **10**, 126-133.

Postma, D.S., Bleecker, E.R., Amelung, P.J., Holroyd, K.J., Xu, J., Panhuysen, C.I., Meyers, D.A. & Levitt, R.C. (1995). Genetic susceptibility to asthma--bronchial hyperresponsiveness coinherited with a major gene for atopy. *New England Journal of Medicine* **333**, 894-900.

Power, C., Sreenan, S., Hurson, B., Burke, C. & Poulter, L.W. (1993). Distribution of immunocompetent cells in the bronchial wall of clinically healthy subjects showing bronchial hyperresponsiveness. *Thorax* **48**, 1125-1129.

Qian, Y., Advenier, C., Naline, E., Bellamy, J.F. & Emonds-Alt, X. (1994). Effects of SR 48968 on the neuropeptide gamma-induced contraction of the human isolated bronchus. *Fundamental & Clinical Pharmacology* **8**, 71-75.

Rajah, R., Nachajon, R.V., Collins, M.H., Hakonarson, H., Grunstein, M.M. & Cohen, P. (1999). Elevated levels of the IGF-binding protein protease MMP-1 in asthmatic airway smooth muscle. *American Journal of Respiratory Cell & Molecular Biology* **20**, 199-208.

Rao, K., Jiang, H., Halayko, A.J., Pan, N., Kepron, W. & Stephens, N.L. (1991). Increased ATPase activity and myosin light chain kinase (MLCK) content in airway smooth muscle from sensitized dogs. *Advances in Experimental Medicine & Biology* **304**, 369-376.

Ray, D.W., Garland, A., Hernandez, C., Eappen, S., Alger, L. & Solway, J. (1991). Time course of bronchoconstriction induced by dry gas hyperpnea in guinea pigs. *Journal of Applied Physiology* **70**, 504-510.

Ray, D.W., Hernandez, C., Munoz, N., Leff, A.R. & Solway, J. (1988). Bronchoconstriction elicited by isocapnic hyperpnea in guinea pigs. *Journal of Applied Physiology* **65**, 934-939.

Ray, D.W., Ingenito, E.P., Strek, M., Schumacker, P.T. & Solway, J. (1989). Longitudinal distribution of canine respiratory heat and water exchanges. *Journal of Applied Physiology* **66**, 2788-2798.

Renzetti, L.M., Paciorek, P.M., Tannu, S.A., Rinaldi, N.C., Tocker, J.E., Wasserman, M.A. & Gater, P.R. (1996). Pharmacological evidence for tumor necrosis factor as a mediator of allergic inflammation in the airways. *Journal of Pharmacology and Experimental Therapeutics* **278**, 847-853.

Reynolds, A.M. & McEvoy, R.D. (1988). Prevention of abnormal pulmonary mechanics in the postmortem guinea pig lung. *Journal of Applied Physiology* **64**, 1322-1326.

Reynolds, A.M. & McEvoy, R.D. (1989). Tachykinins mediate hypocapnia-induced bronchoconstriction in guinea pigs. *Journal of Applied Physiology* **67**, 2454-2460.

Reynolds, A.M., Reynolds, P., Holmes, M. & Scicchitano, R. (1998). Tachykinin NK_2 receptors predominantly mediate tachykinin-induced contractions in ovine trachea. *European Journal of Pharmacology* **341**, 211-223.

Reynolds, A.M., Zadow, S.P., Scicchitano, R. & McEvoy, R.D. (1992). Airway hypocapnia increases microvascular leakage in the guinea pig trachea. *American Review of Respiratory Disease* **145**, 80-84.

Reynolds, P.N., Rice, A.J., Reynolds, A.M., Thornton, A.T., Holmes, M.D. & Scicchitano, R. (1997). Tachykinins contribute to the acute airways response to allergen in sheep actively sensitized to Ascaris suum. *Respirology* **2**, 193-200.

Rice, A.J., Reynolds, P.N., Reynolds, A.M., Holmes, M.D. & Scicchitano, R. (2001). Tachykinin-induced bronchoconstriction in sheep is NK-1 receptor mediated and exhibits tachyphylaxis. *Respirology* **6**, 113-123.

Richardson, J. & Beland, J. (1976). Nonadrenergic inhibitory nervous system in human airways. *Journal of Applied Physiology* **41**, 764-771.

Richardson, P.S. & Webber, S.E. (1987). The control of mucous secretion in the airways by peptidergic mechanisms. *American Review of Respiratory Disease* **136**, S72-S76.

Roberts, C.R. (1995). Is asthma a fibrotic disease? *Chest* **107**, 111S-117S.

Roberts, J.A., Raeburn, D., Rodger, I.W. & Thomson, N.C. (1984). Comparison of in vivo airway responsiveness and in vitro smooth muscle sensitivity to methacholine in man. *Thorax* **39**, 837-843.

Robinson, D.S., Hamid, Q., Ying, S., Tsicopoulos, A., Barkans, J., Bentley, A.M., Corrigan, C., Durham, S.R. & Kay, A.B. (1992). Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *New England Journal of Medicine* **326**, 298-304.

Robinson, D.S., Ying, S., Bentley, A.M., Meng, Q., North, J., Durham, S.R., Kay, A.B. & Hamid, Q. (1993). Relationships among numbers of bronchoalveolar lavage cells expressing messenger ribonucleic acid for cytokines, asthma symptoms, and airway methacholine responsiveness in atopic asthma. *Journal of Allergy & Clinical Immunology* **92**, 397-403.

Robinson, P., Okazawa, M., Bai, T. & Pare, P. (1992). In vivo loads on airway smooth muscle: the role of noncontractile airway structures. *Canadian Journal of Physiology & Pharmacology* **70**, 602-606.

Roche, W.R. (1998). Inflammatory and structural changes in the small airways in bronchial asthma. *American Journal of Respiratory & Critical Care Medicine* **157**, S191-S194.

Roche, W.R., Beasley, R., Williams, J.H. & Holgate, S.T. (1989). Subepithelial fibrosis in the bronchi of asthmatics. *Lancet* **1**, 520-524.

Rogers, D.F., Aursudkij, B. & Barnes, P.J. (1989). Effects of tachykinins on mucus secretion in human bronchi in vitro. *European Journal of Pharmacology* **174**, 283-286.

Rogers, D.F., Belvisi, M.G., Aursudkij, B., Evans, T.W. & Barnes, P.J. (1988). Effects and interactions of sensory neuropeptides on airway microvascular leakage in guinea-pigs. *British Journal of Pharmacology* **95**, 1109-1116.

Rogers, D.F., Boschetto, P. & Barnes, P.J. (1989). Plasma exudation. Correlation between Evans blue dye and radiolabeled albumin in guinea pig airways in vivo. *Journal of Pharmacological Methods* **21**, 309-315.

Rogers, D.F. & O'Connor, B.J. (1993). Airway hyperresponsiveness: relation to asthma and inflammation? *Thorax* **48**, 1095-1096.

Romagnani, S. (1992). Induction of TH1 and TH2 responses: a key role for the 'natural' immune response? *Immunology Today* **13**, 379-381.

Rona, R.J., Duran-Tauleria, E. & Chinn, S. (1997). Family size, atopic disorders in parents, asthma in children, and ethnicity. *Journal of Allergy & Clinical Immunology* **99**, 454-460.

Rossetti, M., Savineau, J.P., Crevel, H. & Marthan, R. (1995). Role of protein kinase C in nonsensitized and passively sensitized human isolated bronchial smooth muscle. *American Journal of Physiology* **268**, L966-L971.

Rossi, G.L. & Olivieri, D. (1997). Does the mast cell still have a key role in asthma? *Chest* **112**, 523-529.

Roth, M., Johnson, P., King, G., Tamm, M. & Black, J. (2000). Enhanced cell prolieration capacity of cultured bronchial human smooth muscle cells in asthma. (Abstract). *American Journal of Respiratory & Critical Care Medicine* **161**, A260.

Rothenberg, M.E., Owen, W.F.J., Silberstein, D.S., Woods, J., Soberman, R.J., Austen, K.F. & Stevens, R.L. (1988). Human eosinophils have prolonged survival, enhanced functional properties, and become hypodense when exposed to human interleukin 3. *Journal of Clinical Investigation* **81**, 1986-1992.

Roubin, R., Elsas, P.P., Fiers, W. & Dessein, A.J. (1987). Recombinant human tumour necrosis factor (rTNF)2 enhances leukotriene biosynthesis in neutrophils and eosinophils stimulated with the Ca2+ ionophore A23187. *Clincal and Experimental Immunology* **70**, 484-490.

Ruffin, R.E., Alpers, J.H., Crockett, A.J. & Hamilton, R. (1981). Repeated histamine inhalation tests in asthmatic patients. *Journal of Allergy & Clinical Immunology.* **67**, 285-289.

Saban, R., Dick, E.C., Fishleder, R.I. & Buckner, C.K. (1987). Enhancement by parainfluenza 3 infection of contractile responses to substance P and capsaicin in airway smooth muscle from the guinea pig. *American Review of Respiratory Disease* **136**, 586-591.

Saetta, M., di Stefano, A., Rosina, C., Thiene, G. & Fabbri, L.M. (1991). Quantitative structural analysis of peripheral airways and arteries in sudden fatal asthma. *American Review of Respiratory Disease* **143**, 138-143.

Said, S.I. (1991). VIP as a modulator of lung inflammation and airway constriction. *American Review of Respiratory Disease* **143**, S22-S24.

Saito, H., Hatake, K., Dvorak, A.M., Leiferman, K.M., Donnenberg, A.D., Arai, N., Ishizaka, K. & Ishizaka, T. (1988). Selective differentiation and proliferation of hematopoietic cells induced by recombinant human interleukins. *Proc.Natl.Acad.Sci U.S.A.* **85**, 2288-2292.

Sampson, P.M., Rochester, C.L., Freundlich, B. & Elias, J.A. (1992). Cytokine regulation of human lung fibroblast hyaluronan (hyaluronic acid) production. Evidence for cytokine-regulated hyaluronan (hyaluronic acid) degradation and

human lung fibroblast-derived hyaluronidase. *Journal of Clinical Investigation* **90**, 1492-1503.

Sanders, M.A. & LeVine, H. (1996). Desensitization of the neurokinin 1 receptor is mediated by the receptor carboxy-terminal region, but is not caused by receptor internalization. *Journal of Neurochemistry* **67**, 2362-2372.

Sanderson, M.J. & Sleigh, M.A. (1981). Serum proteins agglutinate cilia and modify ciliary coordination. *Pediatric Research* **15**, 219-228.

Sang, Q.X. (1998). Complex role of matrix metalloproteinases in angiogenesis. *Cell Research* **8**, 171-177.

Sanz, M.J., Weg, V.B., Bolanowski, M.A. & Nourshargh, S. (1995). IL-1 is a potent inducer of eosinophil accumulation in rat skin. Inhibition of response by a platelet-activating factor antagonist and an anti-human IL-8 antibody. *Journal of Immunology* **154**, 1364-1373.

Saria, A. & Lundberg, J.M. (1983). Evans blue fluorescence: quantitative and morphological evaluation of vascular permeability in animal tissues. *Journal of Neuroscience Methods* **8**, 41-49.

Schaffalitzky, D.M.O., Aggestrup, S. & Stentoft, P. (1986). Flushing and plasma substance P concentration during infusion of synthetic substance P in normal man. *Scandinavian Journal of Gastroenterology* **21**, 498-502.

Schellenberg, R.R. & Foster, A. (1984). In vitro responses of human asthmatic airway and pulmonary vascular smooth muscle. *International Archives of Allergy & Applied Immunology* **75**, 237-241.

Schmidt, J.A., Mizel, S.B., Cohen, D. & Green, I. (1982). Interleukin 1, a potential regulator of fibroblast proliferation. *Journal of Immunology* **128**, 2177-2182.

Schramm, C.M. & Grunstein, M.M. (1992). Assessment of signal transduction mechanisms regulating airway smooth muscle contractility. *American Journal of Physiology* **262**, L119-L139.

Schultheis, A.H., Bassett, D.J. & Fryer, A.D. (1994). Ozone-induced airway hyperresponsiveness and loss of neuronal M2 muscarinic receptor function. *Journal of Applied Physiology* **76**, 1088-1097.

Schwartz, L.B. (1992). Cellular inflammation in asthma: neutral proteases of mast cells. *American Review of Respiratory Disease* **145**, S18-S21.

Schwarze, J., Hamelmann, E., Bradley, K.L., Takeda, K. & Gelfand, E.W. (1997). Respiratory syncytial virus infection results in airway hyperresponsiveness and enhanced airway sensitization to allergen. *Journal of Clinical Investigation* **100**, 226-233.

Schwingshackl, A., Duszyk, M., Brown, N. & Moqbel, R. (1999). Human eosinophils release matrix metalloproteinase-9 on stimulation with TNF-alpha. *Journal of Allergy & Clinical Immunology* **104**, 983-989.

Scicchitano, R., Bienenstock, J. & Stanisz, A.M. (1988). In vivo immunomodulation by the neuropeptide substance P. *Immunology* **63**, 733-735.

Sears, M.R., Burrows, B., Flannery, E.M., Herbison, G.P., Hewitt, C.J. & Holdaway, M.D. (1991). Relation between airway responsiveness and serum IgE in children with asthma and in apparently normal children. *New England Journal of Medicine* **325**, 1067-1071.

Sears, M.R., Burrows, B., Flannery, E.M., Herbison, G.P. & Holdaway, M.D. (1993). Atopy in childhood. I. Gender and allergen related risks for development of hay fever and asthma. *Clinical & Experimental Allergy* **23**, 941-948.

Sears, M.R., Holdaway, M.D., Flannery, E.M., Herbison, G.P. & Silva, P.A. (1996). Parental and neonatal risk factors for atopy, airway hyper-responsiveness, and asthma. *Archives of Disease in Childhood* **75**, 392-398.

Seder, R.A., Paul, W.E., Davis, M.M. & Fazekas, d.S. (1992). The presence of interleukin 4 during in vitro priming determines the lymphokine-producing potential of CD4+ T cells from T cell receptor transgenic mice. *Journal of Experimental Medicine* **176**, 1091-1098.

Seeger, W., Stohr, G., Wolf, H.R. & Neuhof, H. (1985). Alteration of surfactant function due to protein leakage: special interaction with fibrin monomer. *Journal of Applied Physiology* **58**, 326-338.

Sekizawa, K., Caughey, G.H., Lazarus, S.C., Gold, W.M. & Nadel, J.A. (1989). Mast cell tryptase causes airway smooth muscle hyperresponsiveness in dogs. *Journal of Clinical Investigation* **83**, 175-179.

Sekizawa, K., Tamaoki, J., Graf, P.D., Basbaum, C.B., Borson, D.B. & Nadel, J.A. (1987a). Enkephalinase inhibitor potentiates mammalian tachykinin-induced contraction in ferret trachea. *Journal of Pharmacology & Experimental Therapeutics* **243**, 1211-1217.

Sekizawa, K., Tamaoki, J., Nadel, J.A. & Borson, D.B. (1987b). Enkephalinase inhibitor potentiates substance P- and electrically induced contraction in ferret trachea. *Journal of Applied Physiology* **63**, 1401-1405.

Shah, A., Church, M.K. & Holgate, S.T. (1995). Tumour necrosis factor alpha: a potential mediator of asthma. *Clinical & Experimental Allergy* **25**, 1038-1044.

Sheller, J.R. & Brigham, K.L. (1982). Bronchomotor responses of isolated sheep airways to electrical field stimulation. *Journal of Applied Physiology* **53**, 1088-1093.

Sheppard, D., Scypinski, L., Horn, J., Gordon, T. & Thompson, J.E. (1986). Granulocyte-mediated airway edema in guinea pigs. *Journal of Applied Physiology* **60**, 1213-1220.

Sheppard, D., Thompson, J.E., Scypinski, L., Dusser, D., Nadel, J.A. & Borson, D.B. (1988). Toluene diisocyanate increases airway responsiveness to substance P and decreases airway neutral endopeptidase. *Journal of Clinical Investigation* **81**, 1111-1115.

Sheppard, D., Wong, W.S., Uehara, C.F., Nadel, J.A. & Boushey, H.A. (1980). Lower threshold and greater bronchomotor responsiveness of asthmatic subjects to sulfur dioxide. *American Review of Respiratory Disease* **122**, 873-878.

Shore, S.A., Laporte, J., Hall, I.P., Hardy, E. & Panettieri, R.A.J. (1997). Effect of IL-1 beta on responses of cultured human airway smooth muscle cells to bronchodilator agonists. *American Journal of Respiratory Cell & Molecular Biology* **16**, 702-712.

Silverman, F. (1979). Asthma and respiratory irritants (ozone). *Environmental Health Perspectives* **29:** 131-136.

Siracusa, A., Vecchiarelli, A., Brugnami, G., Marabini, A., Felicioni, D. & Severini, C. (1992). Changes in interleukin-1 and tumor necrosis factor production by peripheral blood monocytes after specific bronchoprovocation test in occupational asthma. *American Review of Respiratory Disease* **146**, 408-412.

Skidgel, R.A., Engelbrecht, S., Johnson, A.R. & Erdos, E.G. (1984). Hydrolysis of substance p and neurotensin by converting enzyme and neutral endopeptidase. *Peptides* **5**, 769-776.

Smart, S.J. & Casale, T.B. (1994). TNF-alpha-induced transendothelial neutrophil migration is IL-8 dependent. *American Journal of Physiology* **266**, L238-L245.

Smith, C.H., Barker, J.N. & Lee, T.H. (1993). Adhesion molecules in allergic inflammation. *American Review of Respiratory Disease* **148**, S75-S78.

Smith, R.B. & Taylor, I.M. (1971). Observations on the intrinsic innervation of trachea, bronchi and pulmonary vessels in the sheep. *Acta Anatomica* **80**, 1-13.

Snider, R.M., Constantine, J.W., Lowe, J.A., Longo, K.P., Lebel, W.S., Woody, H.A., Drozda, S.E., Desai, M.C., Vinick, F.J. & Spencer, R.W. (1991). A potent nonpeptide antagonist of the substance P (NK1) receptor. *Science* **251**, 435-437.

Soler, M., Sielczak, M. & Abraham, W.M. (1991). Separation of late bronchial responses from airway hyperresponsiveness in allergic sheep. *Journal of Applied Physiology* **70**, 617-623.

Soler, M., Sielczak, M.W. & Abraham, W.M. (1989). A PAF antagonist blocks antigen-induced airway hyperresponsiveness and inflammation in sheep. *Journal of Applied Physiology* **67**, 406-413.

Solway, J. & Leff, A.R. (1991). Sensory neuropeptides and airway function. *Journal of Applied Physiology* **71**, 2077-2087.

Song, P., Milanese, M., Crimi, E., Rehder, K. & Brusasco, V. (1997). Allergen challenge of passively sensitized human bronchi alters M2 and beta2 receptor function. *American Journal of Respiratory & Critical Care Medicine* **155**, 1230-1234.

Sont, J.K., van Krieken, J.H., van Klink, H.C., Roldaan, A.C., Apap, C.R., Willems, L.N. & Sterk, P.J. (1997). Enhanced expression of neutral endopeptidase (NEP) in airway epithelium in biopsies from steroid- versus nonsteroid-treated patients

with atopic asthma. *American Journal of Respiratory Cell & Molecular Biology* **16**, 549-556.

Souhrada, J.F. & Dickey, D.W. (1976). Effect of antigen challenge on sensitized guinea pig trachea. *Respiration Physiology* **27**, 241-251.

Soussi-Gounni, A., Kontolemos, M. & Hamid, Q. (2001). Role of IL-9 in the pathophysiology of allergic diseases. *Journal of Allergy & Clinical Immunology* **107**, 575-582.

Spina, D. (1996). Airway sensory nerves: a burning issue in asthma? *Thorax* **51**, 335-337.

Spina, D. (1998a). Epithelium smooth muscle regulation and interactions. *American Journal of Respiratory & Critical Care Medicine* **158**, S141-S145.

Spina, D. (1998b). Modulation of sensory nerve function in the airways. *Pulmonary Pharmacology and Therapeutics* **11**, 319-330.

Spurzem, J.R. (1996). Function at the junction: dynamic interactions between lung cells and extracellular matrix. *Thorax* **51**, 956-958.

Standiford, T.J., Kunkel, S.L., Basha, M.A., Chensue, S.W., Lynch, J.P., Toews, G.B., Westwick, J. & Strieter, R.M. (1990). Interleukin-8 gene expression by a pulmonary epithelial cell line. A model for cytokine networks in the lung. *Journal of Clinical Investigation* **86**, 1945-1953.

Stanescu, D.C. & Frans, A. (1982). Bronchial asthma without increased airway reactivity. *European Journal of Respiratory Disease* **63**, 5-12.

Stephens, N.L. (1978). Physical properties of contractile systems. In *Methods in Pharmacology. Volume 3 Smooth Muscle* (Daniel, E.E. and Paton, D.M., eds.), Plenum Press, New York, pp. 265-280.

Sterk, P.J. (1993a). Bronchial hyperresponsiveness today. *Respiratory Medicine* **87**, 27-29.

Sterk, P.J. (1993b). Virus-induced airway hyperresponsiveness in man. *European Respiratory Journal* **6**, 894-902.

Stetler-Stevenson, W.G. (1999). Matrix metalloproteinases in angiogenesis: a moving target for therapeutic intervention. *Journal of Clinical Investigation* **103**, 1237-1241.

Stewart, A.G., Tomlinson, P.R., Fernandes, D.J., Wilson, J.W. & Harris, T. (1995). Tumor necrosis factor alpha modulates mitogenic responses of human cultured airway smooth muscle. *American Journal of Respiratory Cell & Molecular Biology* **12**, 110-119.

Stretton, D. (1991). Non-adrenergic, non-cholinergic neural control of the airways. *Clinical & Experimental Pharmacology & Physiology* **18**, 675-684.

Strunk, R.C. (1993). Death due to asthma. New insights into sudden unexpected deaths, but the focus remains on prevention. *American Review of Respiratory Disease* **148**, 550-552.

Sukkar, M.B., Hughes, J.M., Armour, C.L. & Johnson, P.R. (2001). Tumour necrosis factor-alpha potentiates contraction of human bronchus in vitro. *Respirology* **6**, 199-203.

Sunyer, J., Anto, J.M., Kogevinas, M., Barcelo, M.A., Soriano, J.B., Tobias, A., Muniozguren, N., Martinez-Moratalla, J., Payo, F. & Maldonado, J.A. (1997). Risk factors for asthma in young adults. Spanish Group of the European Community Respiratory Health Survey. *European Respiratory Journal* **10**, 2490-2494.

Sur, S., Crotty, T.B., Kephart, G.M., Hyma, B.A., Colby, T.V., Reed, C.E., Hunt, L.W. & Gleich, G.J. (1993). Sudden-onset fatal asthma. A distinct entity with few eosinophils and relatively more neutrophils in the airway submucosa? *American Review of Respiratory Disease* **148**, 713-719.

Svensson, C., Pipkorn, U., Alkner, U., Baumgarten, C.R. & Persson, C.G. (1992). Topical vasoconstrictor (oxymetazoline) does not affect histamine-induced mucosal exudation of plasma in human nasal airways. *Clinical & Experimental Allergy* **22**, 411-416.

Sweeney, H.L. (1998). Regulation and tuning of smooth muscle myosin. *American Journal of Respiratory & Critical Care Medicine* **158**, S95-S99.

Takahashi, T., Ichinose, M., Yamauchi, H., Miura, M., Nakajima, N., Ishikawa, J., Inoue, H., Takishima, T. & Shirato, K. (1993). Neuropeptide-Y Inhibits Neurogenic Inflammation in Guinea Pig Airways. *Journal of Applied Physiology* **75**, 103-107.

Takizawa, T. & Thurlbeck, W.M. (1971). Muscle and mucous gland size in the major bronchi of patients with chronic bronchitis, asthma, and asthmatic bronchitis. *American Review of Respiratory Disease* **104**, 331-336.

Tallarida, R.J., Raffa, R.B. & McGonigle, P. (1988). *Principles in general pharmacology*. Springer-Verlag New York, pp. 1-219.

Tamaoki, J., Yamawaki, I., Takeyama, K., Chiyotani, A., Yamauchi, F. & Konno, K. (1994). Interleukin-1 beta inhibits airway smooth muscle contraction via epithelium-dependent mechanism. *American Journal of Respiratory & Critical Care Medicine* **149**, 134-137.

Tanaka, D.T. & Grunstein, M.M. (1984). Mechanisms of substance P-induced contraction of rabbit airway smooth muscle. *Journal of Applied Physiology* **57**, 1551-1557.

Tanaka, D.T. & Grunstein, M.M. (1986). Effect of substance P on neurally mediated contraction of rabbit airway smooth muscle. *Journal of Applied Physiology* **60**, 458-463.

Tartaglia, L.A. & Goeddel, D.V. (1992). Two TNF receptors. *Immunology Today* **13**, 151-153.

Tee, R.D., Cullinan, P., Welch, J., Burge, P.S. & Newman-Taylor, A.J. (1998). Specific IgE to isocyanates: a useful diagnostic role in occupational asthma. *Journal of Allergy & Clinical Immunology* **101**, 709-715.

ten Berge, R.E., Santing, R.E., Hamstra, J.J., Roffel, A.F. & Zaagsma, J. (1995). Dysfunction of muscarinic M2 receptors after the early allergic reaction: possible contribution to bronchial hyperresponsiveness in allergic guinea-pigs. *British Journal of Pharmacology* **114**, 881-887.

ten Berge, R.E., Zaagsma, J. & Roffel, A.F. (1996). Muscarinic inhibitory autoreceptors in different generations of human airways. *American Journal of Respiratory & Critical Care Medicine* **154**, 43-49.

Terry, P.B., Menkes, H.A. & Traystman, R.J. (1987). Effects of maturation and aging on collateral ventilation in sheep. *Journal of Applied Physiology* **62**, 1028-1032.

Thomas, P.S., Yates, D.H. & Barnes, P.J. (1995). Tumor necrosis factor-alpha increases airway responsiveness and sputum neutrophilia in normal human subjects. *American Journal of Respiratory & Critical Care Medicine* **152**, 76-80.

Thompson-Snipes, L., Dhar, V., Bond, M.W., Mosmann, T.R., Moore, K.W. & Rennick, D.M. (1991). Interleukin 10: a novel stimulatory factor for mast cells and their progenitors. *Journal of Experimental Medicine* **173**, 507-510.

Thompson, J.E., Scypinski, L.A., Gordon, T. & Sheppard, D. (1987). Tachykinins mediate the acute increase in airway responsiveness caused by toluene diisocyanate in guinea pigs. *American Review of Respiratory Disease* **136**, 43-49.

Thomson, R.J., Bramley, A.M. & Schellenberg, R.R. (1996). Airway muscle stereology: implications for increased shortening in asthma. *American Journal of Respiratory & Critical Care Medicine* **154**, 749-757.

Tillie-Leblond, I., Pugin, J., Marquette, C.H., Lamblin, C., Saulnier, F., Brichet, A., Wallaert, B., Tonnel, A.B. & Gosset, P. (1999). Balance between proinflammatory cytokines and their inhibitors in bronchial lavage from patients with status asthmaticus. *American Journal of Respiratory & Critical Care Medicine* **159**, 487-494.

Tomaki, M., Ichinose, M., Miura, M., Hirayama, Y., Yamauchi, H., Nakajima, N. & Shirato, K. (1995). Elevated substance P content in induced sputum from patients with asthma and patients with chronic bronchitis. *American Journal of Respiratory & Critical Care Medicine* **151**, 613-617.

Tomioka, K., Garrido, R., Ahmed, A., Stevenson, J.S. & Abraham, W.M. (1989). YM461, a PAF antagonist, blocks antigen-induced late airway responses and airway hyperresponsiveness in allergic sheep. *European Journal of Pharmacology* **170**, 209-215.

Tomioka, K., Jackowski, J.T. & Abraham, W.M. (1991). Characterization of sulfidopeptide leukotriene responses in sheep tracheal smooth muscle in vitro. *Canadian Journal of Physiology & Pharmacology* **69**, 805-811.

Torphy, T.J. (1994). Beta-adrenoceptors, cAMP and airway smooth muscle relaxation: challenges to the dogma. *Trends in Pharmacological Sciences* **15**, 370-374.

Tsukagoshi, H., Haddad, E.B., Sun, J., Barnes, P.J. & Chung, K.F. (1995). Ozone-induced airway hyperresponsiveness: role of superoxide anions, NEP, and BK receptors. *Journal of Applied Physiology* **78**, 1015-1022.

Tsukagoshi, H., Sakamoto, T., Xu, W., Barnes, P.J. & Chung, K.F. (1994). Effect of interleukin-1 beta on airway hyperresponsiveness and inflammation in sensitized and nonsensitized Brown-Norway rats. *Journal of Allergy & Clinical Immunology* **93**, 464-469.

Tsukagoshi, H., Sun, J., Kwon, O., Barnes, P.J. & Chung, K.F. (1995a). Role of neutral endopeptidase in bronchial hyperresponsiveness to bradykinin induced by IL-1 beta. *Journal of Applied Physiology* **78**, 921-927.

Turner, N.C., Lamb, J., Worby, A. & Murray, K.J. (1994). Relaxation of guinea-pig trachea by cyclic AMP phosphodiesterase inhibitors and their enhancement by sodium nitroprusside. *British Journal of Pharmacology* **111**, 1047-1052.

Tyler, W.S. (1983). Comparative subgross anatomy of lungs. Pleuras, interlobular septa, and distal airways. *American Review of Respiratory Disease* **128**, S32-S36.

Uchida, Y., Nomura, A., Ohtsuka, M., Hasegawa, S., Goto, K., Kimura, S., Sugita, Y. & Uchiyama, Y. (1987). Neurokinin A as a potent bronchoconstrictor. *American Review of Respiratory Disease* **136**, 718-721.

Uddman, R. & Sundler, F. (1987). Neuropeptides in the airways: a review. *American Review of Respiratory Disease* **136**, S3-S8.

Van de Graaf, E.A., Out, T.A., Roos, C.M. & Jansen, H.M. (1991). Respiratory membrane permeability and bronchial hyperreactivity in patients with stable asthma. Effects of therapy with inhaled steroids. *American Review of Respiratory Disease* **143**, 362-368.

van Griensven, M., Stalp, M. & Seekamp, A. (1999). Ischemia-reperfusion directly increases pulmonary endothelial permeability in vitro. *Shock* **11**, 259-263.

van Haren, E.H., Lammers, J.W., Festen, J. & van Herwaarden, C.L. (1992). Bronchial vagal tone and responsiveness to histamine, exercise and bronchodilators in adult patients with cystic fibrosis. *European Respiratory Journal* **5**, 1083-1088.

van Herwerden, L., Harrap, S.B., Wong, Z.Y., Abramson, M.J., Kutin, J.J., Forbes, A.B., Raven, J., Lanigan, A. & Walters, E.H. (1995). Linkage of high-affinity IgE receptor gene with bronchial hyperreactivity, even in absence of atopy. *Lancet* **346**, 1262-1265.

van Oosterhout, A.J., Ladenius, A.R., Savelkoul, H.F., van Ark, I., Delsman, K.C. & Nijkamp, F.P. (1993). Effect of anti-IL-5 and IL-5 on airway hyperreactivity and

eosinophils in guinea pigs. *American Review of Respiratory Disease* **147**, 548-552.

van Oosterhout, A.J., van Ark, I., Folkerts, G., van der Linde, H.J., Savelkoul, H.F., Verheyen, A.K. & Nijkamp, F.P. (1995). Antibody to interleukin-5 inhibits virus-induced airway hyperresponsiveness to histamine in guinea pigs. *American Journal of Respiratory & Critical Care Medicine* **151**, 177-183.

Van Rossum, J.M. (1963). Cumulative concentration-response curves. II. Technique for the making of dose-response curves in isolated organs and the evaluation of drug parameters. *Archives Internationales de Pharmacodynamie et de Therapie* **143**, 299-330.

Vargaftig, B.B. (1997). Modifications of experimental bronchopulmonary hyperresponsiveness. *American Journal of Respiratory & Critical Care Medicine* **156**, S97-102.

Varpela, E., Laitinen, L.A., Keskinen, H. & Korhola, O. (1978). Asthma, allergy and bronchial hyper-reactivity to histamine in patients with bronchiectasis. *Clincal Allergy* **8**, 273-280.

Venter, J.C. & Fraser, C.M. (1990). Receptor mechanisms. Structure and molecular biology of transmitter receptors. *American Review of Respiratory Disease* **141**, S99-105.

Villanove, X., Marthan, R., Tunon, d.L., Johnson, P.R., Savineau, J.P., McKay, K.O., Alouan, L.A., Armour, C.L. & Black, J.L. (1993). Sensitization decreases relaxation in human isolated airways. *American Review of Respiratory Disease* **148**, 107-112.

Vincenc, K.S., Black, J.L., Yan, K., Armour, C.L., Donnelly, P.D. & Woolcock, A.J. (1983). Comparison of in vivo and in vitro responses to histamine in human airways. *American Review of Respiratory Disease* **128**, 875-879.

Von Essen, S.G., Rennard, S.I., O'Neill, D., Ertl, R.F., Robbins, R.A., Koyama, S. & Rubinstein, I. (1992). Bronchial epithelial cells release neutrophil chemotactic activity in response to tachykinins. *American Journal of Physiology* **263**, L226-L231.

Von Euler, U.S. & Gaddum, J.H. (1931). An unidentified depressor substance in certain tissue extracts. *Journal of Physiology (London)* **72**, 74-86.

Wagner, E.M., Kleeberger, S.R., Spannhake, E.W. & Adams, G.K. (1985). Increased in vitro airway responsiveness in sheep following repeated exposure to antigen in vivo. *Journal of Applied Physiology* **59**, 1874-1878.

Wagner, J.G. & Roth, R.A. (2000). Neutrophil migration mechanisms, with an emphasis on the pulmonary vasculature. *Pharmacological Reviews* **52**, 349-374.

Wallenstein, S., Zucker, C.L. & Fleiss, J.L. (1980). Some statistical methods useful in circulation research. *Circulation Research* **47**, 1-9.

Walsh, D.A., Salmon, M., Featherstone, R., Wharton, J., Church, M.K. & Polak, J.M. (1994). Differences in the distribution and characteristics of tachykinin NK1 binding sites between human and guinea pig lung. *British Journal of Pharmacology* **113**, 1407-1415.

Wang, S.Z. & Forsyth, K.D. (2000). The interaction of neutrophils with respiratory epithelial cells in viral infection. *Respirology* **5**, 1-10.

Wanner, A. (1989). Circulation of the airway mucosa. *Journal of Applied Physiology* **67**, 917-925.

Wanner, A. & Abraham, W.M. (1982). Experimental models of asthma. *Lung* **160**, 231-243.

Wanner, A., Mezey, R.J., Reinhart, M.E. & Eyre, P. (1979). Antigen-induced bronchospasm in conscious sheep. *Journal of Applied Physiology* **47**, 917-922.

Wanner, A., Salathe, M. & O'Riordan, T.G. (1996). Mucociliary clearance in the airways. *American Journal of Respiratory & Critical Care Medicine* **154**, 1868-1902.

Ward, J.K., Belvisi, M.G., Fox, A.J., Miura, M., Tadjkarimi, S., Yacoub, M.H. & Barnes, P.J. (1993). Modulation of Cholinergic Neural Bronchoconstriction by Endogenous Nitric Oxide and Vasoactive Intestinal Peptide in Human Airways Invitro. *Journal of Clinical Investigation* **92** , 736-742.

Warner, E.A., Krell, R.D. & Buckner, C.K. (1990). Pharmacologic studies on the differential influence of inhibitors of neutral endopeptidase on nonadrenergic, noncholinergic contractile responses of the guinea pig isolated hilar bronchus to transmural electrical stimulation and exogenously applied tachykinins. *Journal of Pharmacology & Experimental Therapeutics* **254**, 824-830.

Wasserman, S.I. (1994). Mast cells and airway inflammation in asthma. *American Journal of Respiratory & Critical Care Medicine* **150**, S39-S41.

Watson, N., Bodtke, K., Coleman, R.A., Dent, G., Morton, B.E., Ruhlmann, E., Magnussen, H. & Rabe, K.F. (1997). Role of IgE in hyperresponsiveness induced by passive sensitization of human airways. *American Journal of Respiratory & Critical Care Medicine* **155**, 839-844.

Watson, N., Maclagan, J. & Barnes, P.J. (1993). Endogenous tachykinins facilitate transmission through parasympathetic ganglia in guinea-pig trachea. *British Journal of Pharmacology* **109**, 751-759.

Watson, N., Magnussen, H. & Rabe, K.F. (1995). Pharmacological characterization of the muscarinic receptor subtype mediating contraction of human peripheral airways. *Journal of Pharmacology and Experimental Therapeutics* **274**, 1293-1297.

Webb, B.L., Lindsay, M.A., Barnes, P.J. & Giembycz, M.A. (1997). Protein kinase C isoenzymes in airway smooth muscle. *The Biochemical Journal* **324**, 167-175.

Weiss, S.T., Tager, I.B., Speizer, F.E. & Rosner, B. (1980). Persistent wheeze. Its relation to respiratory illness, cigarette smoking, and level of pulmonary function in a population sample of children. *American Review of Respiratory Disease* **122**, 697-707.

Welkowitz, J., Ewen, R.B. & Cohen, J. (1982). *Introductory statistics for the behavioral sciences.* Academic Press New York, pp. 247-251.

Wesselius, L.J., Smirnov, I.M., O'Brien-Ladner, A.R. & Nelson, M.E. (1995). Synergism of intratracheally administered tumor necrosis factor with interleukin-1 in the induction of lung edema in rats. *Journal of Laboratory & Clinical Medicine* **125**, 618-625.

Wheeler, A.P., Jesmok, G. & Brigham, K.L. (1990). Tumor necrosis factor's effects on lung mechanics, gas exchange, and airway reactivity in sheep. *Journal of Applied Physiology* **68**, 2542-2549.

Widdicombe, J. (1993). The airway vasculature. *Experimental Physiology* **78**, 433-452.

Widdicombe, J. & Davies, D. (1983). Control of breathing. In *Respiratory Physiology* (Hardy, R.N., Hobsley, M. and Saunders, K.B., eds.), Edward Arnold, London, pp. 90-102.

Wiedermann, C.J., Auer, B., Sitte, B., Reinisch, N., Schratzberger, P. & Kahler, C.M. (1996). Induction of endothelial cell differentiation into capillary-like structures by substance P. *European Journal of Pharmacology* **298**, 335-338.

Wiedermann, F.J., Kahler, C.M., Reinisch, N. & Wiedermann, C.J. (1993). Induction of normal human eosinophil migration in vitro by substance P. *Acta Haematologica* **89**, 213-215.

Wiggs, B.R., Bosken, C., Pare, P.D., James, A. & Hogg, J.C. (1992). A model of airway narrowing in asthma and in chronic obstructive pulmonary disease. *American Review of Respiratory Disease* **145**, 1251-1258.

Wiggs, B.R., Hrousis, C.A., Drazen, J.M. & Kamm, R.D. (1994). The implications of airway wall buckling in asthmatic airways. *American Journal of Respiratory & Critical Care Medicine* **149**, A585.

Wiggs, B.R., Hrousis, C.A., Drazen, J.M. & Kamm, R.D. (1997). On the mechanism of mucosal folding in normal and asthmatic airways. *Journal of Applied Physiology* **83**, 1814-1821.

Wills-Karp, M. & Gilmour, M.I. (1993a). Increased Cholinergic Antagonism Underlies Impaired beta- Adrenergic Response in Ovalbumin-Sensitized Guinea Pigs. *Journal of Applied Physiology* **74**, 2729-2735.

Wills-Karp, M., Uchida, Y., Lee, J.Y., Jinot, J., Hirata, A. & Hirata, F. (1993b). Organ culture with proinflammatory cytokines reproduces impairment of the beta-adrenoceptor-mediated relaxation in tracheas of a guinea pig antigen model. *American Journal of Respiratory Cell & Molecular Biology* **8**, 153-159.

Wilson, J. (2000). The bronchial microcirculation in asthma. *Clinical & Experimental Allergy* **30**, 51-53.

Wojcikiewicz, R.J. (1995). Type I, II, and III inositol 1,4,5-trisphosphate receptors are unequally susceptible to down-regulation and are expressed in markedly different proportions in different cell types. *Journal of Biological Chemistry* **270**, 11678-11683.

Wojcikiewicz, R.J. & Luo, S.G. (1998). Differences among type I, II, and III inositol-1,4,5-trisphosphate receptors in ligand-binding affinity influence the sensitivity of calcium stores to inositol-1,4,5-trisphosphate. *Molecular Pharmacology* **53**, 656-662.

Wood, P.R. & Seow, H.F. (1996). T cell cytokines and disease prevention. *Veterinary Immunology and Immunopathology* **54**, 33-44.

Woolcock, A.J. & Read, J. (1966). Lung volumes in exacerbations of asthma. *American Journal of Medicine* **41**, 259-273.

Woolcock, A.J. & Read, J. (1968). The static elastic properties of the lungs in asthma. *American Review of Respiratory Disease* **98**, 788-794.

Woolcock, A.J., Salome, C.M. & Yan, K. (1984). The shape of the dose-response curve to histamine in asthmatic and normal subjects. *American Review of Respiratory Disease* **130**, 71-75.

Woolf, C.J., Safieh-Garabedian, B., Ma, Q.P., Crilly, P. & Winter, J. (1994). Nerve growth factor contributes to the generation of inflammatory sensory hypersensitivity. *Neuroscience* **62**, 327-331.

Wozniak, A., McLennan, G., Betts, W.H., Murphy, G.A. & Scicchitano, R. (1989). Activation of human neutrophils by substance P: effect on FMLP-stimulated oxidative and arachidonic acid metabolism and on antibody-dependent cell-mediated cytotoxicity. *Immunology* **68**, 359-364.

Xiu, Q., Fujimura, M., Nomura, M., Saito, M., Matsuda, T., Akao, N., Kondo, K. & Matsushima, K. (1995). Bronchial hyperresponsiveness and airway neutrophil accumulation induced by interleukin-8 and the effect of the thromboxane A2 antagonist S-1452 in guinea-pigs. *Clinical & Experimental Allergy* **25**, 51-59.

Yager, D., Butler, J.P., Bastacky, J., Israel, E., Smith, G. & Drazen, J.M. (1989). Amplification of airway constriction due to liquid filling of airway interstices. *Journal of Applied Physiology* **66**, 2873-2884.

Yamawaki, I., Tamaoki, J., Takeda, Y. & Nagai, A. (1997). Inhaled cromoglycate reduces airway neurogenic inflammation via tachykinin antagonism. *Research communications in molecular pathology and pharmacology* **98**, 265-272.

Yanaga, F., Abe, M., Koga, T. & Hirata, M. (1992). Signal transduction by tumor necrosis factor alpha is mediated through a guanine nucleotide-binding protein in osteoblast-like cell line, MC3T3-E1. *Journal of Biological Chemistry* **267**, 5114-5121.

Yap, D.Y., Liebkemann, W.D., Solway, J. & Gaver, D.P. (1994). Influences of parenchymal tethering on the reopening of closed pulmonary airways. *Journal of Applied Physiology* **76**, 2095-2105.

Yau, W.M. & Youther, M.L. (1982). Direct evidence for a release of acetylcholine from the myenteric plexus of guinea pig small intestine by substance P. *European Journal of Pharmacology* **81**, 665-668.

Yeo, C.J., Jaffe, B.M. & Zinner, M.J. (1984). The effects of intravenous substance P infusion on hemodynamics and regional blood flow in conscious dogs. *Surgery* **95**, 175-182.

Yi, E.S. & Ulich, T.R. (1992). Endotoxin, interleukin-1, and tumor necrosis factor cause neutrophil-dependent microvascular leakage in postcapillary venules. *American Journal of Pathology* **140**, 659-663.

Ying, S., Humbert, M., Barkans, J., Corrigan, C.J., Pfister, R., Menz, G., Larche, M., Robinson, D.S., Durham, S.R. & Kay, A.B. (1997). Expression of IL-4 and IL-5 mRNA and protein product by CD4+ and CD8+ T cells, eosinophils, and mast cells in bronchial biopsies obtained from atopic and nonatopic (intrinsic) asthmatics. *Journal of Immunology* **158** , 3539-3544.

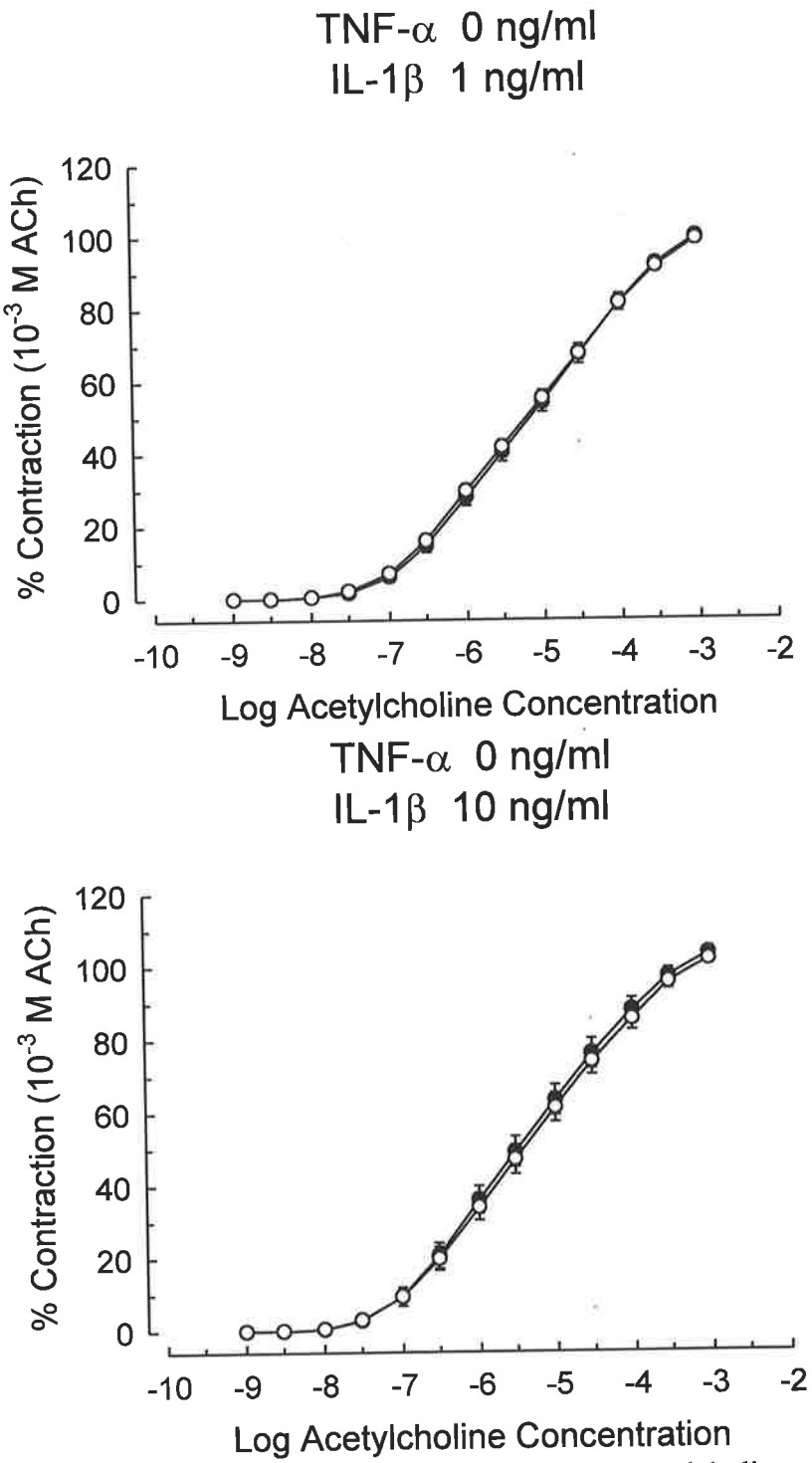
Young, B. & Heath, J.W. (2000). *Wheater's Functional Histology 4E CD-ROM.* Churchill Livingstone London,

Zaagsma, J., van Amsterdam, R.G., Brouwer, F., van der Heijden, P.J., van der Schaar, M.W., Verwey, W.M. & Veenstra, V. (1987). Adrenergic control of airway function. *American Review of Respiratory Disease* **136**, S45-S50.

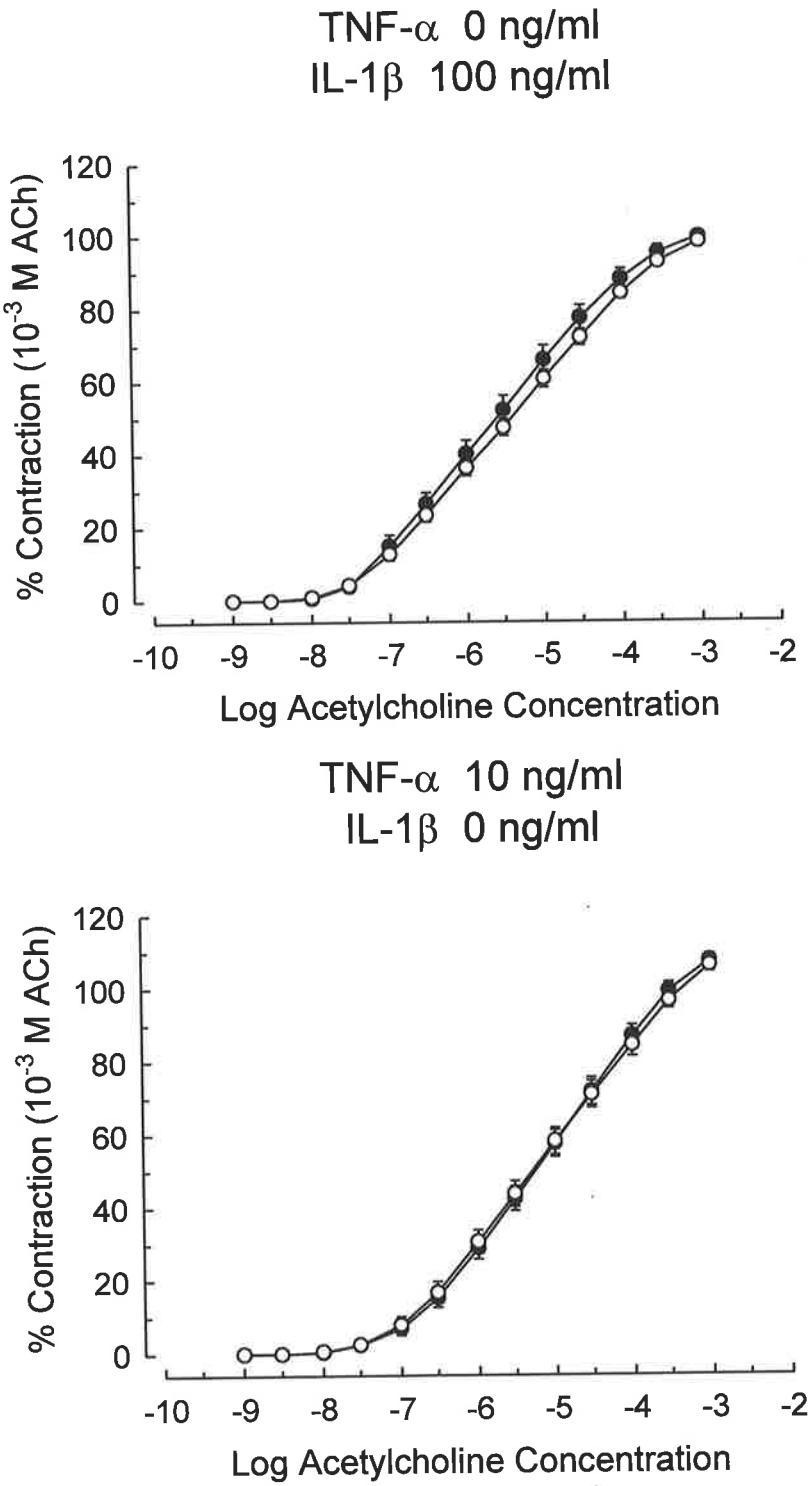
Zeng, X.P. & Burcher, E. (1994). Use of selective antagonists for further characterization of tachykinin NK-2, NK-1 and possible "septide-selective" receptors in guinea pig bronchus. *Journal of Pharmacology & Experimental Therapeutics* **270**, 1295-1300.

Zimmermann, D.R. & Ruoslahti, E. (1989). Multiple domains of the large fibroblast proteoglycan, versican. *The EMBO Journal* **8**, 2975-2981.

Appendix A Enlarged cytokine incubation graphs



Mean cumulative concentration response curve for acetylcholine on tracheal smooth muscle strips in control (○) and cytokine pretreated smooth muscle (●) (note different IL-1β and TNFα). Mean responses are from 9 animals, expressed as a percentage of the reference contraction to 10^{-3} M ACh. Vertical bars indicate SEM. * Denotes a statistically significant difference between control and cytokine treated tissues ($P < 0.05$, paired *t*-test).



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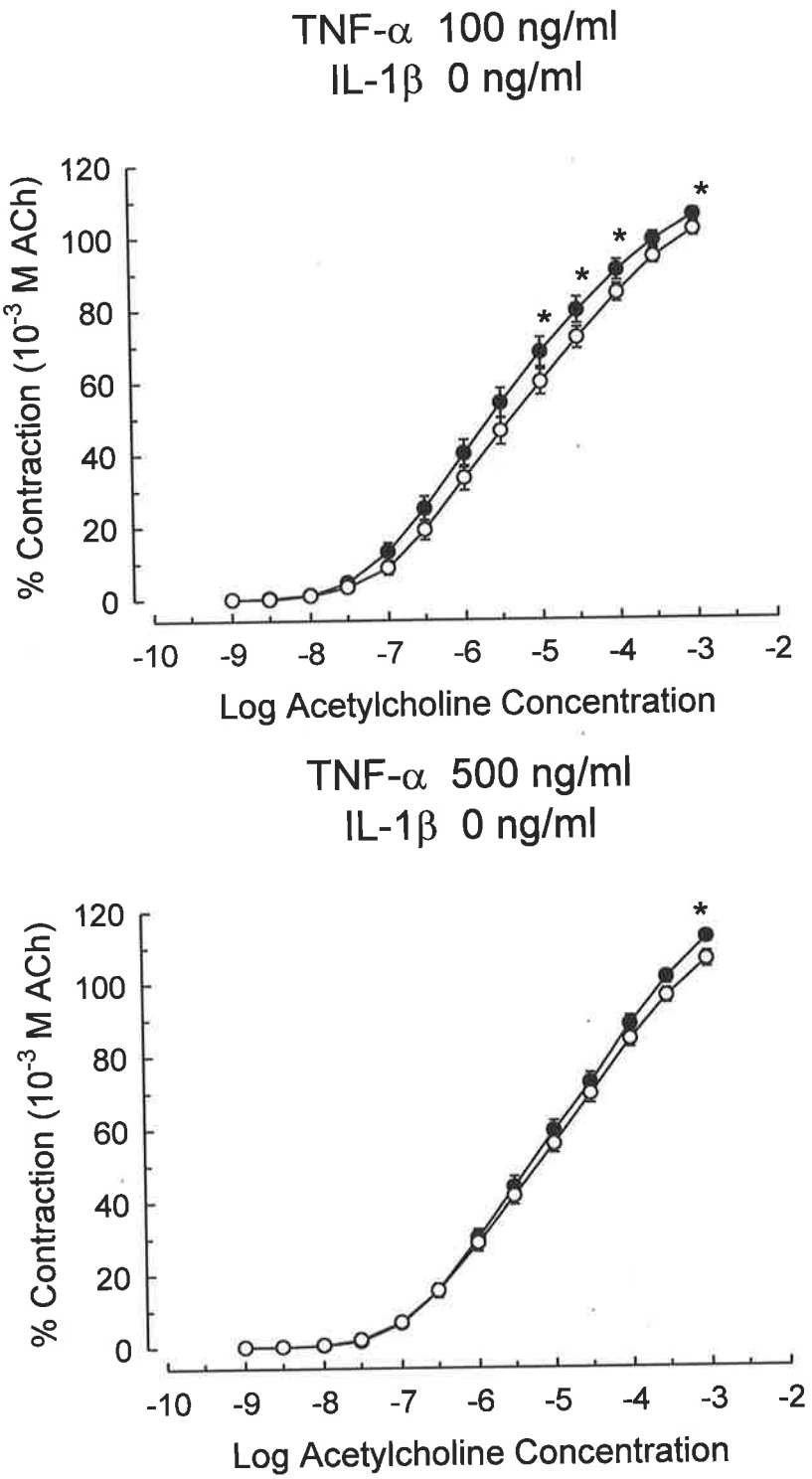

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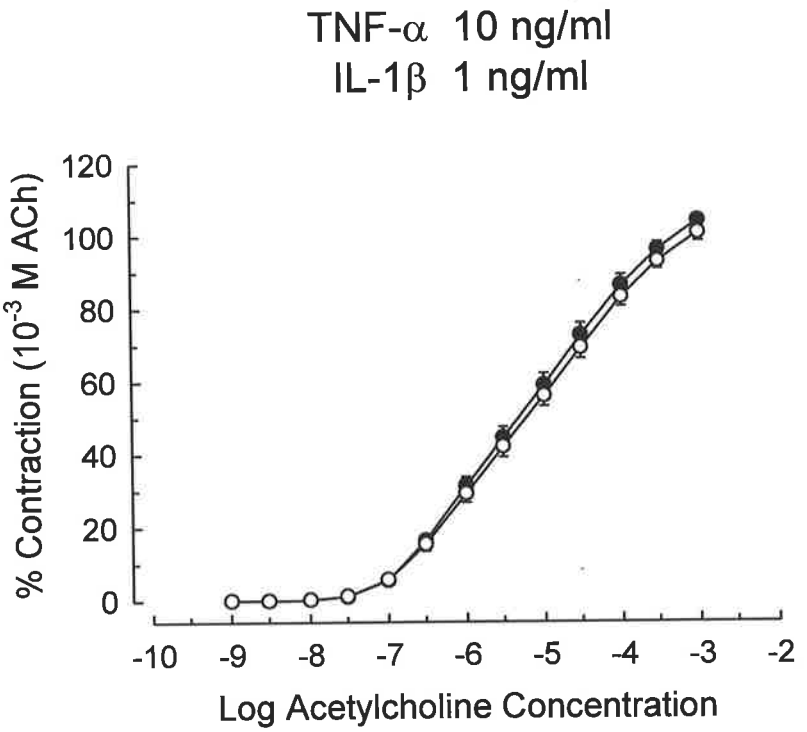

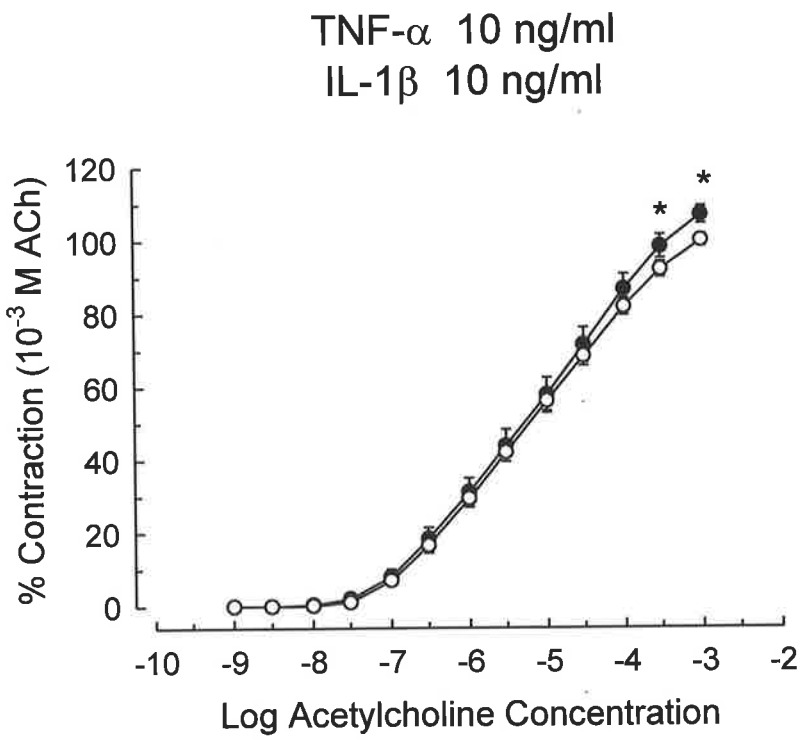

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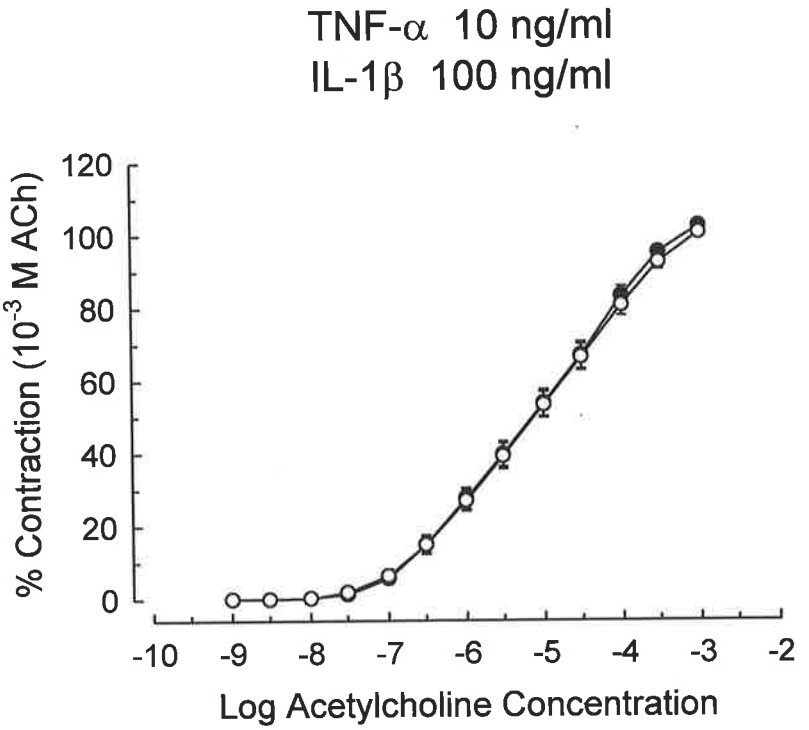

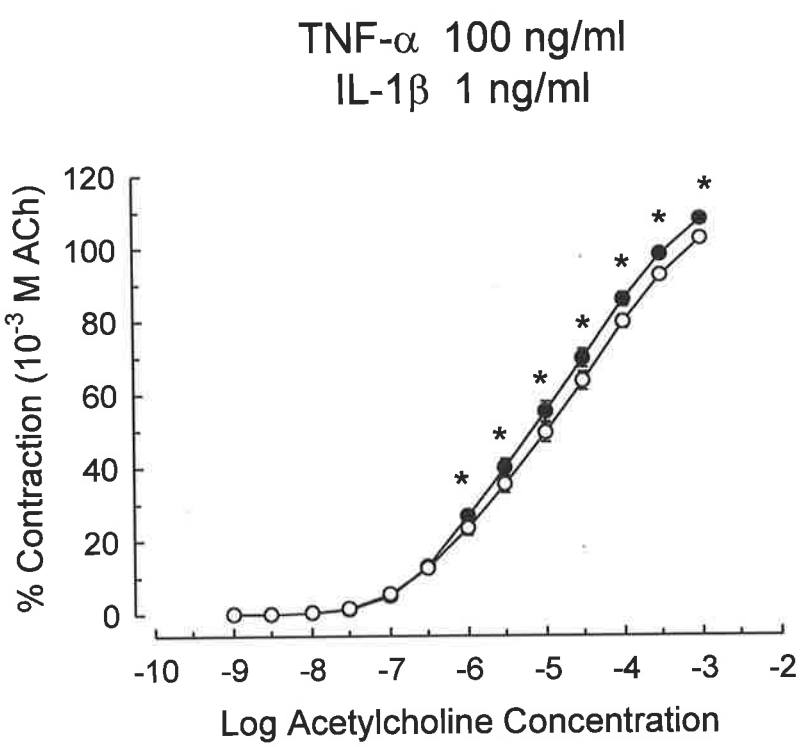

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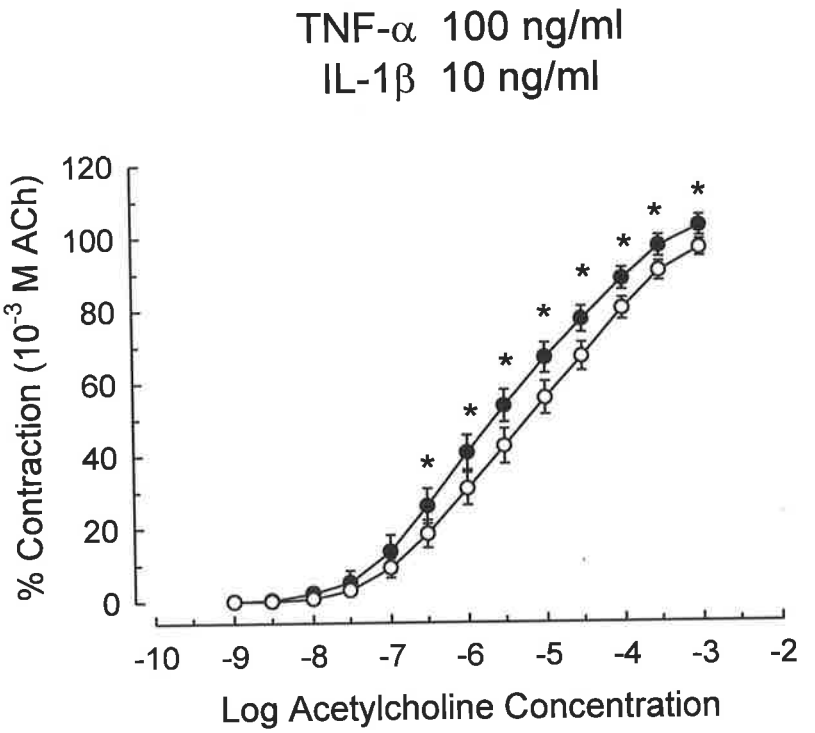

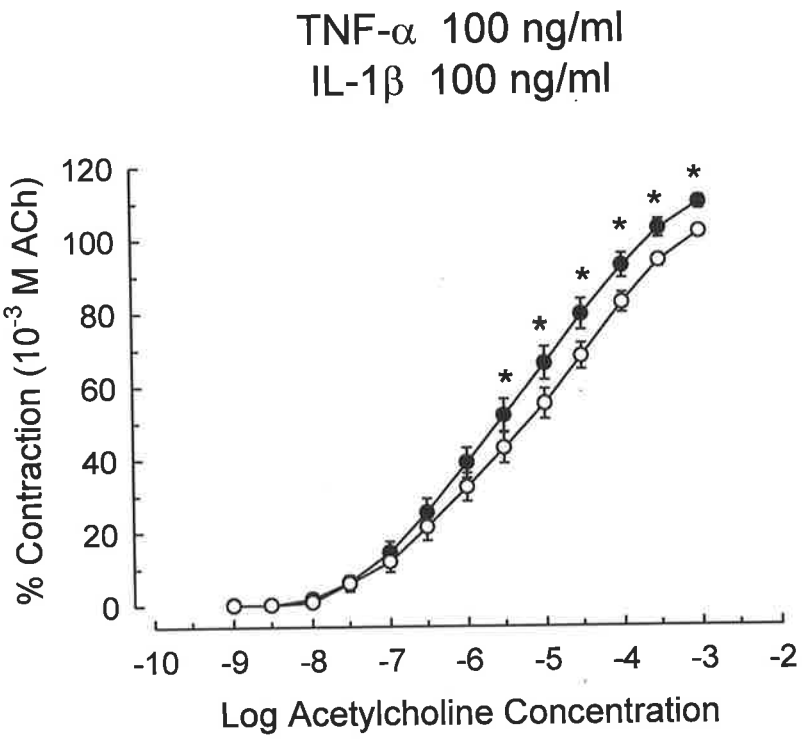

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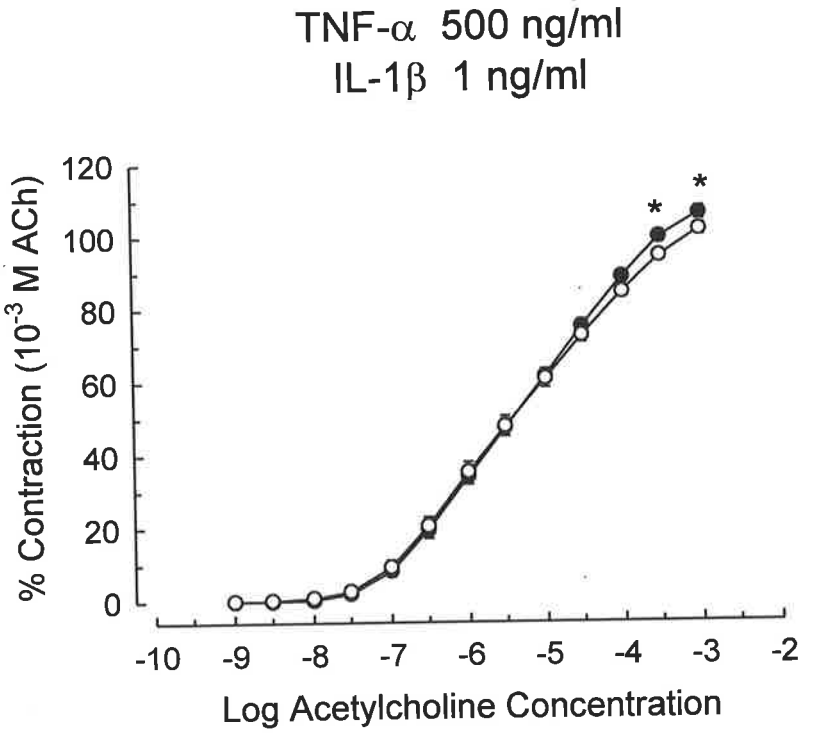

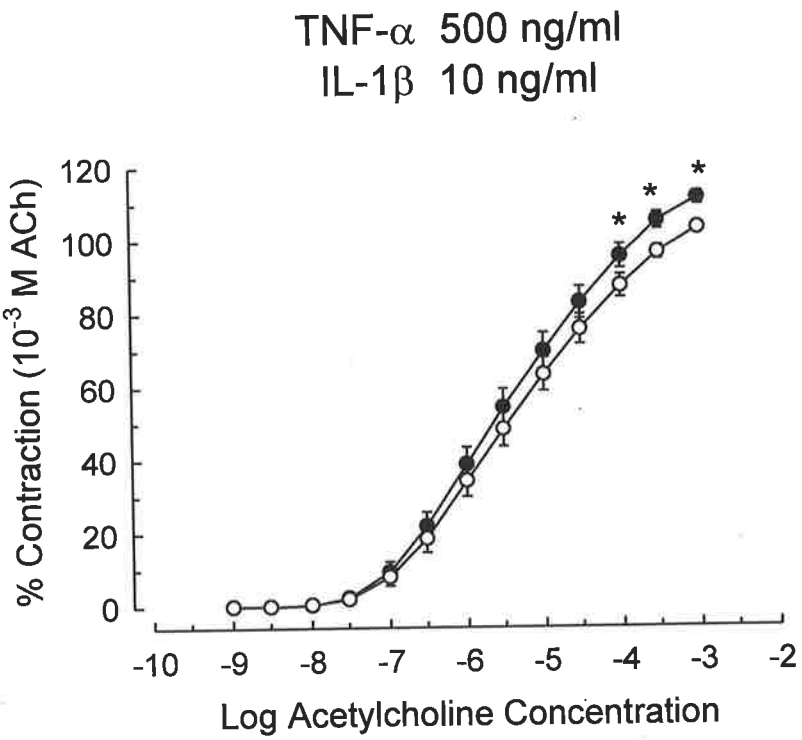

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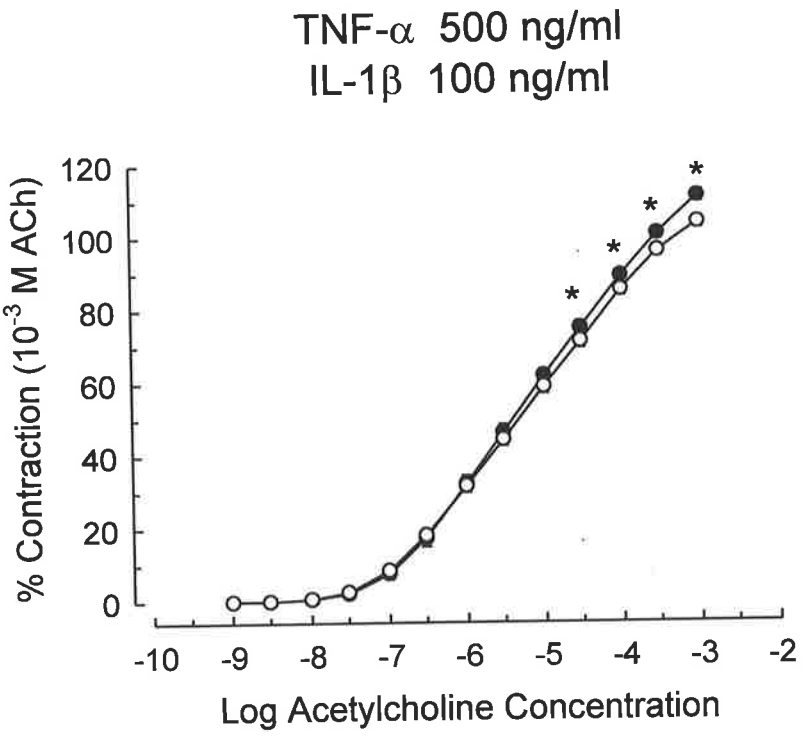

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Appendix B Publications relevant to this thesis

Reynolds, P.N., Rice, A.J., Reynolds, A.M., Thornton, A.T., Holmes, M.D., and Scicchitano, R., (2007) Tachykinins contribute to the acute airways response to allergen in sheep actively sensitized to *Ascaris suum.*
Respirology, v. 2 (3), pp. 193-200.

Reynolds, A.M., Reynolds, P., Holmes, M., and Scicchitano, R., (1998) Tachykinin NK2 receptors predominantly mediate tachykinin-induced contractions in ovine trachea.
European Journal of Pharmacology, v. 341 (2-3), pp. 211-223.

Reynolds, A.M., Holmes, M., and Scicchitano, R.D., (2000) Cytokines enhance airway smooth muscle contractility in response to acetylcholine and neurokinin A. *Respirology, v. 5 (2), pp. 153-160.*

Rice, A.J., Reynolds, P.N., Reynolds, A.M., Holmes, M.D., and Scicchitano, R., (2001) Tachykinin-induced bronchoconstriction in sheep is NK-1 receptor mediated and exhibits tachyphylaxis.
Respirology, v. 6 (2), pp. 113-123.

Reynolds, A.M., Holmes, M.D., and Scicchitano, R., (2002) Interleukin-1β and tumour necrosis factor-α increase microvascular leakage in the guinea pig trachea. *Respirology, v. 7 (1), pp. 23-28.*