THE ACTIONS OF 5-HYDROXYTRYPTAMINE ON THE MARMOSET VASCULATURE

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Thesis submitted for the degree of

Doctor of Philosophy

in the

Department of Clinical and Experimental Pharmacology

The University of Adelaide

(Faculty of Medicine)

CONTENTS

ABSTRACT

The principle aim of the current studies was to characterise the responses to 5-Hydroxytryptamine (5-HT) occurring in the aorta of a primate species, the common marmoset. Further aims were to determine whether the 5-HT-induced response of the aorta is representative of the serotonergic responses of other vessels from the marmoset, in particular the coronary artery. Finally, studies were extended to vascular disease states following a high lipid diet and aortic balloon-catheterisation.

In the marmoset aorta, responses to 5-HT differed greatly from those described in the aorta of commonly used rodent species. In the marmoset aorta with intact endothelium and other agents absent, 5-HT is inactive. However, when the tone is increased with a secondary contractile agent both contractile and relaxant responses become apparent. It appears that three 5-HT-induced responses are present in the marmoset aorta. At low concentrations (0.001 - 0.1μM), 5-HT produced an endothelium-dependent relaxant response. At high concentrations (0.1 - 10μM), 5-HT induces a strong relaxant response which is unaffected by endothelial integrity and is likely to be mediated by a 5-HT_7 receptor. Under conditions of reduced relaxation, a 5-HT_1-like receptor mediates a weak contractile response which exhibits synergism with the thromboxane (Tx) -mimetic U44069 but not noradrenaline (NA). The amplification response was greatest when endothelial function was impaired and the lack of a synergistic interaction of 5-HT with NA was not due to ß-receptor activation on the part of NA. Importantly, the interaction between U44069 and 5-HT was also present in marmoset carotid, mesenteric and coronary arteries and the 5-HT_1-like receptor also plays a role in the contractile effect in the coronary artery.

In response to dietary lipid loading, marmosets exhibited a large variation in responsiveness of plasma lipid levels, such that there were "hyper-responding" and "hypo-

responding" marmosets. Hypercholesterolaemia for 10 months did not result in the development of atheromatous lesions. The subset of animals that were hyper-responsive to dietary lipid had reduced aortic contractile sensitivity to sumatriptan and aortic basal NO production was reduced by comparison with the hypo-responders. In addition, hypo-responders exhibited an enhanced relaxant response to the endothelium-independent component of 5-HT-induced relaxation (possibly 5-HT_7-mediated) in both the aorta and carotid arteries.

In aortae subjected to balloon angioplasty, endothelium-dependent relaxation was reduced. Serotonergic responses in angioplastied arteries were characterised by increased 5-HT_1-like-mediated contraction and reduced relaxation. These responses exemplify the pathophysiological significance of serotonergic responses in vascular disease; ie. when endothelial function is impaired Tx A_2 and 5-HT will interact and the vasoconstrictive effects of 5-HT will predominate, impeding flow in the damaged artery.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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PUBLICATIONS IN SUPPORT OF THIS THESIS

DYER SM, DE LA LANDE IS, FREWIN DB, HEAD RJ (1998). 5-hydroxytryptamine-induced contraction of the marmoset isolated aorta is mediated by a 5-HT_1-like receptor. *Clinical and Experimental Pharmacology and Physiology* **25**: 246-251.

DYER SM, BEXIS S, MANO MT, DE LA LANDE IS, FREWIN DB, HEAD RJ (1994). Interactions between 5-hydroxytryptamine, noradrenaline and the thromboxane-A_2 mimetic U44069 in the marmoset isolated aorta. *Clinical and Experimental Pharmacology and Physiology* **21**: 201-206.

PRESENTATIONS TO LEARNED SOCIETIES

DYER SM, DE LA LANDE IS, FREWIN DB, HEAD RJ (1994). Characterisation of 5-hydroxytryptamine-induced, smooth muscle mediated relaxation in the marmoset aorta. Abstract number 156. Third IUPHAR Satellite Meeting on Serotonin, Chicago, U.S.A. 1994.

HEAD RJ, DE LA LANDE IS, BEXIS S, DYER SM, MANO M (1993). Action of 5-hydroxytryptamine (5-HT) on the marmoset aorta. *Proceedings of the Western Pharmacology Society* 36: 353. Joint Meeting of the Western Pharmacological Society and the Australasian Society of Clinical and Experimental Pharmacologists, Lake Tahoe, Nevada, USA, 1993

ACKNOWLEDGEMENTS

I am indebted to my supervisors, Professors Derek Frewin and Richard Head for their advice, encouragement and guidance throughout my candidature.

I also owe particular thanks to Professor Ivan de la Lande for his continual inspiration and advice throughout the course of my studies. I would like to thank Dr Anne Tonkin for her understanding and assistance in reviewing this thesis during its preparation.

Many thanks also to the following for their contributions: Dr Marilyn Henderson for contributions to histological studies; Sotiria Bexis and Mark Mano for organ bath experiments, Mark Mano for conducting the second dietary atherosclerosis study and Cherie Keatch for performing dietary cholesterol measurements. I am also grateful to Gordon Crabb for advice on IT matters. I am grateful for the assistance of many staff members of the CSIRO Division of Human Nutrition and the Department of Clinical and Experimental Pharmacology, The University of Adelaide for their assistance.

I will always remember the friendship and encouragement of Nicole Kerry, Sarah Jeffreson, Scott Smid, Alice Owen, Sotiria Bexis, Cath Danz and Rod Irvine who provided an enjoyable working environment.

I would like to thank my family and friends for their support during the many years of study leading to the production of this thesis and in particular Anthony Pope for his unceasing support and understanding during the thesis preparation.

This thesis is dedicated to the memory of my mother, Gwenneth Irene Dyer.

ABBREVIATIONS

ACh	acetylcholine
cyclic AMP	adenosine 3':5'-cyclic monophosphate
5-CT	5-carboxamidotryptamine
DAG	diacylglycerol
DRC	dose-response curve
EDHF	endothelium-derived hyperpolarising factor
EDRF	endothelium-derived relaxing factor
EGTA	ethyleneglycol-bis-(ß-aminoethyl ether) N,N,N',N'-tetraacetic acid
ET	endothelin-1
HDL	high density lipoproteins
5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine, serotonin
IBMX	3-isobutyl-1-methylxanthine
IDL	intermediate density lipoproteins
IP_3	inositol-1,4,5-triphosphate
KPSS	potassium physiological salt solution
LDL	low density lipoproteins
L-NMMA	N^G-monomethyl-L-arginine
NOLA	Nω-nitro-L-arginine
5-MeOT	5-methoxytryptamine
METH	methoxamine
NA	noradrenaline
NO	nitric oxide
NOS	nitric oxide synthase

8-OH-DPAT	8-hydroxy-2-(di-n-propylamino)tetralin
PCTA	percutaneous transluminal angioplasty
PE	phenylephrine
PSS	physiological salt solution
SNP	sodium nitro-prusside
Tx	thromboxane
U44	U44069
VLDL	very low density lipoproteins
VSMC	vascular smooth muscle cell

CHAPTER 1

INTRODUCTION

1.1 Epidemiology of cardiovascular disease

Cardiovascular disease is the leading cause of death in most societies, accounting for almost 50% of all deaths. Whilst the mortality attributable to cardiovascular disease in industrialized countries has declined over the past 30 years, its incidence has increased in developing countries. During the period 1965-1990, male mortality due to cardiovascular disease has fallen by approximately 50% in Australia, the U.S.A., Canada and France but has risen by between 40 and 80% in Bulgaria, Poland, Hungary and Czechoslovakia (Wielgosz, 1993). Thus, the prevalence of cardiovascular disease on a global scale, and accordingly the cost of medical care (an estimated $3.5 billion in Australia in 1989-1990 (National Heart Foundation of Australia, 1996)) is still a great burden on society.

In 1994, 43.3% of all deaths in Australia were attributable to cardiovascular diseases and of these approximately half (55.7%) were due to myocardial infarction and a quarter (23.4%) to cerebrovascular accident (National Heart Foundation of Australia, 1996). In addition, the high prevalence of peripheral vascular disease which does not contribute as significantly to mortality but commonly causes loss of lower limb function must not be overlooked (Balkau *et al.*, 1994). The pathology of the vasculature in coronary artery disease, cerebrovascular disease and peripheral arterial disease is usually characterised by atherosclerosis, a common form of arteriosclerosis involving the accumulation of lipid deposits within the arterial intima. Thus hyperlipidaemia, smoking, hypertension, diabetes, obesity, age, male gender and a sedentary lifestyle which are risk factors for

atherosclerosis, are all risk factors for these cardiovascular diseases.

1.2 Vasospasm

Vasospasm is the pathological contraction of the smooth muscle cells of an artery which excessively reduces the luminal diameter, impairing flow and even occluding the vessel. This produces local ischaemia and if sustained may result in permanent pathological damage to the surrounding tissue. Atherosclerotic vessels are predisposed to vasospasm (Shimokawa *et al.,* 1983; Houston & Vanhoutte, 1989; Ganz *et al.,* 1991; Heistad & Armstrong, 1992). Thus, vasospasm may be the initiating factor in many sudden cardiovascular incidents. Vasospasm occurring in arteries of the coronary circulation is believed to be an important factor in Prinzmetal (variant) and unstable angina (Kaski *et al.,* 1986) and may be a precipitating event in myocardial infarction (Maseri *et al.,* 1978; Conti, 1983; Willerson, 1995). Vasospasm of the cerebral circulation occurs following subarachnoid haemorrhage (Ohta *et al.,* 1983; Vorkapic *et al.,* 1991) and is also likely to be one of the causative factors of cerebrovascular accident. In the periphery, spasm of the digital arteries is part of the aetiology of Raynaud's phenomenon (Cohen & Coffman, 1984; Van Zwieten *et al.,* 1990; Coffman, 1991).

The true aetiology of vasospasm is unknown, but it is commonly believed that it may be initiated by platelet aggregation occurring at sites of vascular damage (eg. atherosclerotic plaques) (Rubenstein *et al.,* 1981; Vanhoutte & Houston, 1985; Willerson *et al.,* 1990). As part of the normal haemostatic process, exposure of platelets to collagen and thrombin at a site of vessel injury causes adherence of the platelets to the vessel wall and platelet activation - shape change, aggregation, secretion of granule contents and the release of endogenous mediators. Agents are released from the platelet which contribute to

coagulation and the formation of a haemostatic plug. Vasoactive agents released by platelets will cause local vasoconstriction, thereby reducing local blood flow at the injury site. Some of these compounds will then also act as a stimulus to platelet activation, potentiating the haemostatic response. Thus, local blood loss is minimised whilst vessel repair takes place. However, in certain disease states (eg. cardiovascular disease, hyperlipidaemia, increased age) platelets are hyperresponsive to platelet activators and platelet aggregation may occur pathologically (Carvalho *et al.,* 1974; Tremoli *et al.,* 1984; De Cree *et al.,* 1985; De Cree *et al.,* 1988; Buhler *et al.,* 1990). In addition, the release of nucleotides and the generation of thromboxane A_2 (Tx A_2) from aggregating platelets from hyperlipidaemic patients has been reported to be increased in disease states (Carvalho *et al.,* 1974; Tremoli *et al.,* 1984). Furthermore, diseased vessels can respond to platelet released vasoactive agonists in an aberrant manner, resulting in prolonged or excessive constriction (Kaski *et al.,* 1986; Shimokawa & Vanhoutte, 1989; Kaul *et al.,* 1991). Thus, combined with the mechanical obstruction of a platelet thrombus, a pathological haemostatic response can completely occlude an artery and result in local tissue ischaemia and end-organ damage (Conti, 1983; Bush *et al.,* 1984).

The two principal vasoconstrictors released by aggregating platelets are serotonin (5-hydroxytryptamine, 5-HT) and Tx A_2 and as such these are major candidates as potential mediators of vasospasm.

1.3 Thromboxane A_2

Thromboxane A_2 is an eicosanoid that is generated de novo from arachidonic acid subsequent to its liberation from membrane phospholipids by phospholipases. It is formed in platelets, macrophages, kidney, spleen and lung tissue (Moncada & Vane, 1979).

Cyclooxygenase catalyses the formation of the unstable cyclic endoperoxides (Prostaglandins G_2 and H_2) from arachidonic acid. The endoperoxide PGH_2 is then metabolised by thromboxane synthetase into Tx A_2 which undergoes nonenzymatic degradation into the stable endproduct Tx B_2. Tx B_2 is physiologically inactive, is eliminated from the body by urinary excretion and its levels in urine are commonly used as a measure of Tx A_2 synthesis and release (Patrono *et al.,* 1992). Average basal plasma concentrations of Tx B_2 are < 2 pg/ml but following platelet activation and initiation of Tx A_2 production and release this can rise to 270 ng/ml in serum (Patrono *et al.,* 1986). The major physiological effects of Tx A_2 are the contraction of vascular and bronchial smooth muscle and platelet aggregation (Moncada & Vane, 1979). The endoperoxides can also activate Tx receptors and thus possess the same spectrum of physiological activities as Tx A_2, as well as many other prostanoids due to their conversion into the prostaglandins (Salzman, 1977; Moncada & Vane, 1979; Gresele *et al.,* 1991). The Tx/endoperoxide receptor is G-protein coupled and initiates hydrolysis of phosphatidyl inositol and thereby liberation of Ca^{2+} from intracellular stores (Gresele *et al.,* 1991; Hirata *et al.,* 1991; Baldassare *et al.,* 1993).

The endoperoxides and Tx A_2 are extremely labile (half-lives of approximately 5 min. and 30 sec. at 37°C respectively); hence studies of their biological activity are generally achieved by utilising stable chemical analogues of PGH_2. Such analogues have been shown to possess the same physiological effects as the endogenous agents, but are not substrates for the endoperoxide converting enzymes and generally have a greater potency than the natural compounds. Platelet aggregation and contraction of many isolated smooth muscle preparations, including rabbit and rat aorta, guinea-pig lung and dog saphenous vein have been demonstrated with these synthetic analogues (Malmsten, 1976; Best *et al.,* 1979; Coleman *et al.,* 1981). Two of the commercially available and commonly used Tx

A_2 mimetics are U44069 (9,11-dideoxy-9α,11α-epoxymethano-prostaglandin $F_{2\alpha}$) and U46619 (9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$) (Bundy, 1975).

1.4 5-Hydroxytryptamine

Serotonin, or 5-hydroxytryptamine (5-HT), is a biogenic amine whose precursor is the essential amino acid L-tryptophan. The conversion of tryptophan to 5-HT occurs in many tissues including mast cells, the pineal gland, central and peripheral neurons (where it acts as a neurotransmitter), renal tissue, and in the greatest quantities in the enterochromaffin cells of the gastrointestinal mucosa. The 5-HT produced in the enterochromaffin cells is released in response to a variety of stimuli to increase gastrointestinal motility and secretion as an aid to digestion. Some of the 5-HT enters the portal circulation where the majority of it is destroyed by hepatic monoamine oxidases. Thus, in the liver and also the pulmonary epithelium, a large proportion of 5-HT is metabolised by oxidative deamination into 5-hydroxyindoleacetic acid (5-HIAA) which is eliminated from the body by urinary excretion and can serve as a marker of body 5-HT production. Other minor metabolic pathways include *N*-acetylation followed by *O*-methylation to melatonin in the pineal gland, reductive deamination, sulphation and glucuronidation.

The fraction of 5-HT remaining in the circulation does not circulate freely, but is accumulated by the blood platelets as they pass through the intestinal circulation via a high-affinity active carrier-mediated transport process (Da Prada & Picotti, 1979; Marazziti *et al.,* 1989). In the platelets, 5-HT is stored within the dense granules, where it is available for release along with other platelet-derived vasoactive agents upon platelet aggregation. Thus, plasma 5-HT concentrations are extremely low, with the levels reported in the literature ranging from <0.9 nM to 120 nM (Da Prada & Picotti, 1979; Hindberg &

Naesh, 1992; Coffman & Cohen, 1994; Martin, 1994). Plasma sampling and processing results in varying degrees of platelet activation and 5-HT release, and thus the disparate and possibly erroneously high reported plasma 5-HT concentrations. In contrast, 5-HT levels in whole blood can be as high as 1.7 μM (Badcock *et al.,* 1987) and platelet aggregation can produce serum concentrations of up to 900 nM (Engbaek & Voldby, 1982; Chauveau *et al.,* 1991). Thus, at local vascular sites of platelet aggregation, concentrations of 5-HT can increase to many times their basal level.

The nature of the local response of a vessel to released 5-HT is determined by a variety of factors and experimental conditions, including vessel calibre, the presence or absence of disease states, the level of pre-existing tone, the 5-HT concentration and the types of 5-HT receptors present in the vessel.

1.4.1 5-Hydroxytryptamine Receptors

Over recent years, research into 5-HT receptor subtypes has expanded greatly, such that a plethora of new and putative 5-HT receptors have been discovered. The initial 'D' and 'M' classification introduced by Gaddum and Picarelli in 1957 was revised more than a decade ago by Bradley *et al.* (1986) to the scheme that is used in an expanded form today. The current 5-HT receptor classification system recognises 7 principal 5-HT receptor types (Hoyer *et al.,* 1994; Martin & Humphrey, 1994; Saxena, 1995) which are related as depicted in *Figure 1.1.* Some receptor subtype specific agonists and antagonists are listed in *Table 1.1.* Current guidelines for full characterization of new receptors state that structural, operational and transductional criteria must be met before classification is considered definitive (Hoyer *et al.,* 1994; Martin & Humphrey, 1994). Recombinant receptors for which the operational and transductional characteristics remain unclearly defined are represented by lower case letters. In addition, there are certain 'orphan'

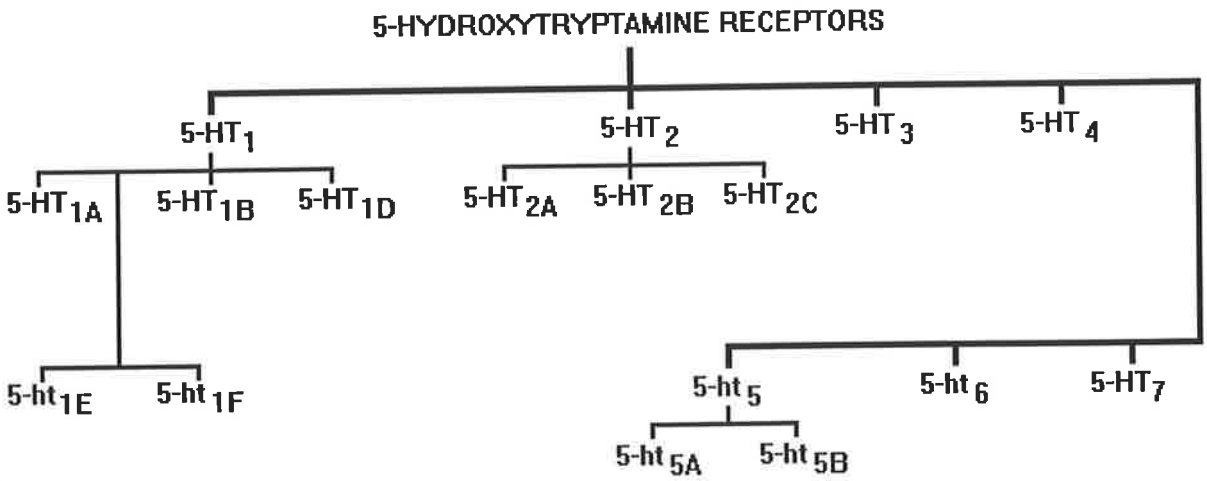

Figure 1.1 The subclassification of 5-Hydroxytryptamine receptors. Recombinant receptors are indicated by lower case letters.

Table 1.1 Some agonists and antagonists at 5-HT receptors.

RECEPTOR	AGONISTS	ANTAGONISTS
5-HT_1	5-carboxamidotryptamine 8-OH-DPAT (1A) sumatriptan (1B/1D)	methiothepin methysergide GR127935 (ID)
5-HT_2	α-Methyl-5-HT	ketanserin (2A) LY53857 methiothepin methysergide
5-HT_3	2-methyl-5-HT	tropisetron MDL7222 ondansetron
5-HT_4	5-methoxytryptamine	tropisetron (weak)
5-ht_5	5-carboxamidotryptamine	methiothepin methysergide
5-ht_6	5-methoxytryptamine	methiothepin
5-HT_7	5-carboxamidotryptamine 5-methoxytryptamine	methiothepin LY53857 methysergide

receptors (most operationally identified) which do not definitively belong to any of the known 5-HT receptor subtypes on the basis of the characterisation information available.

The 5-HT_1 class of receptors consists of 5 formally recognised subtypes, 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-ht_{1E} and 5-ht_{1F}. Members of this class of receptors are linked via G-proteins to the inhibition of adenylate cyclase and their genes lack introns (Martin & Humphrey, 1994). Operationally, they generally exhibit a high nanomolar affinity for 5-HT, an equivalent or higher affinity for the agonist 5-carboxamidotryptamine (5-CT) and are antagonised by methiothepin. This class of receptors are generally located neuronally and predominantly in the central nervous system; however, functional 5-HT_1-like receptors are now known to mediate contraction in certain vascular smooth muscle preparations (Hamel & Bouchard, 1991a; Perren *et al.,* 1991; Sahin-Erdemli *et al.,* 1991; Cushing *et al.,* 1994). The term 5-HT_1-like has been applied to these receptors since they appear to belong to the 5-HT_1 class but do not fit the characteristics of any identified subtype precisely (these receptors comprise some of the 'orphan' atypical 5-HT receptors). 8-Hydroxy-dipropylaminotetralin (8-OH-DPAT) is a selective agonist for the 5-HT_{1A} receptor which is widely distributed in the CNS and is principally implicated in emotional and behavioural responses (Arvidsson *et al.,* 1981; Middlemiss & Fozard, 1983). The 5-HT_{1B} receptor, which functions as a central autoinhibitory receptor, has recently been reclassified (Hoyer *et al.,* 1996). It now contains the previously classified 5-HT_{1B} receptor found only in rodents, plus its non-rodent species homologue encoded by the 5-$HT_{1Dß}$ gene and previously included in the receptor subtype classified as 5-HT_{1D} by Hoyer *et al.* (1994). These two receptors have a high sequence homology and similar distribution of expression within the CNS, but quite different operational characteristics (Voigt *et al.,* 1991; Jin *et al.,* 1992; Hoyer *et al.,* 1994; Hartig et al, 1996). The 5-HT_{1D} receptor has consequently been reclassified (Hartig *et al.*, 1996). It now includes only the receptor

encoded by the 5-$HT_{1D\alpha}$ gene and expressed in relatively small levels in rodent and non-rodent species, which was previously included in the receptor subtype classified as 5-HT_{1D} by Hoyer *et al.* (1994). It has quite a different structure but very similar ligand binding profile to the non-rodent 5-HT_{1B} gene (Weinshank *et al.,* 1992; Hoyer *et al.,* 1994). This reclassified 5-HT_{1B} and 5-HT_{1D} terminology (Hartig *et al.*, 1996) is used throughout the remainder of this thesis. The anti-migraine drug sumatriptan is an agonist at 5-HT_{1B} (non-rodent) and 5-HT_{1D} receptor subtypes and produces contraction of cerebral arteries *in vitro* (Connor *et al.,* 1989a; Peroutka & McCarthy, 1989; Hamel *et al.,* 1993; Deckert *et al.,* 1994). Only recently have potent antagonists with selectivity for the 5-HT_{1B} (non-rodent) and 5-HT_{1D} receptor subtypes (including GR 127935) been developed (Skingle *et al.,* 1995; Pauwels, 1997). The only antagonists able to distinguish these subtypes are ketanserin and ritanserin, which have little affinity for the 5-HT_{1B} (non-rodent) receptor subtype but appreciable affinity for the 5-HT_{1D} receptor, as well as 5-HT_{2A} receptors, plus 5-HT_{2C}, 5-ht_6 and 5-HT_7 receptors in the case of ritanserin (Hoyer *et al.,* 1994; Pauwels, 1997). The recombinant 5-ht_{1e} and 5-ht_{1f} receptors both exhibit lower affinities for 5-CT and methiothepin than other receptors of the 5-HT_1 class, but nevertheless their high sequence homology to the other receptors, high affinity for 5-HT and negative linkage to adenylate cyclase justifies their inclusion in the 5-HT_1 class (Amlaiky *et al.,* 1992; McAllister *et al.,* 1992; Adham *et al.,* 1993; Gudermann *et al.,* 1993). The 5-HT_1-like receptors mentioned previously resemble the 5-HT_{1B} (non-rodent) and 5-HT_{1D} receptors pharmacologically, including a high affinity for sumatriptan and GR 127935, but have not been classified definitively as yet because of a lack of structural information, limited evidence of inhibitory coupling to adenylate cyclase and some discrepancies in their pharmacology (Hoyer *et al.,* 1994). These 5-HT_1-like receptors display a lower than expected affinity for metergoline and a higher than expected affinity for 8-OH-DPAT. They are found mainly in particular blood vessels (especially cranial vessels, coronary

arteries and saphenous veins) where they mediate constrictor responses, as well as in other peripheral amooth muscle and neuronal tissues.

The 5-HT_2 class contains the 5-HT_{2A} subtype which corresponds to the 'D' class of receptors according to Gaddum and Picarelli's nomenclature (1957). This receptor is the classical 5-HT_2 receptor that is found on the surface membrane of platelets, mediating 5-HT-induced platelet aggregation, causes contraction of vascular and other smooth muscle and also appears to play a role in psychiatric disorders (Pandey *et al.,* 1995). Ketanserin is a 5-HT_{2A} antagonist, selective over the 5-HT_{2B} and 5-HT_{2C} subtypes, but also showing affinity for 5-HT_{1D} and α_1-adrenergic receptors. The 5-HT_{2B} receptor (also previously known as 5-HT_{2F}) has so far been identified mainly in peripheral tissues and mediates contraction of the rat stomach fundus and also some vascular serotonergic responses (Kursar *et al.,* 1992; Wainscott *et al.,* 1993; Choi *et al.,* 1994; Ellis *et al.,* 1995; Watts *et al.,* 1995). The 5-HT_{2C} (previously known as 5-HT_{1C}) subtype is localised in the central nervous system and is present in particularly high concentrations in the choroid plexus where it may mediate the regulation of cerebrospinal fluid production, although its functional role is still uncertain (Pandey *et al.,* 1995; Watson & Brown, 1995). The 5-HT_2 class of receptors is linked transductionally via a G-protein to phospholipase C and thereby increases phosphatidyl inositol turnover and internal calcium release. The genes for these receptors contain introns and exons and the receptors have a relatively low (micromolar) affinity for 5-HT but a high affinity for α-methyl-5-HT. Antagonists of 5-HT_2 receptors include ketanserin (5-HT_{2A}), ritanserin, methiothepin, methysergide, LY53857 and spiperone (5-HT_{2A}).

The 5-HT_3 receptor is equivalent to Gaddum and Picarelli's 'M' class and is a ligand-gated cation channel which, upon activation, is permeable to Na^+ and K^+ ions and thereby leads to membrane depolarization. Structurally, the 5-HT_3 receptor lacks the 7

hydrophobic transmembrane domains which are characteristic of the G-protein linked receptors (Richardson & Engel, 1986; Maricq *et al.,* 1991). High affinity antagonists for these receptors include the agents MDL7222, ondansetron and tropisetron (ICS 205-930), whereas 2-methyl-5-HT acts as an agonist at the 5-HT_3 receptor, with a potency similar to that of 5-HT itself (Richardson *et al.,* 1985). The 5-HT_3 receptor is located on central and peripheral neurons where it mediates many functions including neurotransmitter release from peripheral postganglionic neurones, nociception, a variety of cardiovascular effects, centrally controlled nausea and vomiting and many behavioural effects (Richardson & Engel, 1986; Hoyer *et al.,* 1994).

The 5-HT_4 class comprises another group of G-protein linked 5-HT receptors which are positively linked to cyclic AMP formation (Dumuis *et al.,* 1988; Gerald *et al.,* 1995). This class includes short and long splice variants, 5-ht_{4S} and 5-ht_{4L} (Gerald *et al.,* 1995). At these receptors tropisetron acts as an antagonist at concentrations greater than its 5-HT_3 antagonistic effects and 5-methoxytryptamine acts as a potent agonist (Bockaert *et al.,* 1992). This receptor subtype is responsible for positive inotropic effects, tachycardia, adrenal steroid release and many of the gastro-intestinal and peristaltic effects of 5-HT (Kaumann *et al.,* 1990; Clarke & Bockaert, 1993).

The classes 5-ht_5 and 5-ht_6 are recombinant receptors which have been cloned in recent years from central nervous system tissues and for which there is currently a lack of selective ligands. Receptor classification for these groups is still only tentative since operational and transductional data are limited. These receptors show high affinity for 5-CT and methiothepin but have low sequence homology with all other classes of 5-HT receptors. The 5-ht_5 group consists of 5-ht_{5A} and 5-ht_{5B} subtypes and the transduction system linked to this receptor is unknown but the 5-ht_{5A} receptor may be negatively coupled to adenylate cyclase (Plassat *et al.,* 1992; Erlander *et al.,* 1993; Matthes *et al.,*

1993; Carson *et al.,* 1996). The 5-ht_6 receptor is positively coupled to adenylate cyclase and the relative binding affinity for 5-CT as compared with 5-HT is less than 1 (reported as 0.2 and 0.09 (Monsma *et al.,* 1993; Kohen *et al.,* 1996)) whereas its affinity at the 5-ht_5 receptor is greater than 1 (reportedly 5.7 - 19.0 (Plassat *et al.,* 1992; Erlander *et al.,* 1993; Matthes *et al.,* 1993).

Cloning of the 5-HT_7 receptor was reported by many different researchers in 1993 and it has low homology with other 5-HT receptor subtypes (Bard *et al.,* 1993; Lovenberg *et al.,* 1993; Meyerhof *et al.,* 1993; Monsma *et al.,* 1993; Plassat *et al.,* 1993; Ruat *et al.,* 1993a; Ruat *et al.,* 1993b; Shen *et al.,* 1993). Splice variants also exist for this receptor (Heidmann *et al.,* 1997; Stam *et al.,* 1997). Transductionally, the 5-HT_7 receptor is positively linked to adenylate cyclase. 5-HT_7 mRNA has been located in many central tissues and also the human coronary artery. Functionally, the 5-HT_7 receptor appears to play a role in mediating smooth muscle relaxation (Carter *et al.,* 1995; Cushing *et al.,* 1996; Leung *et al.,* 1996). The 5-HT_7 receptor has a high affinity for 5-CT (affinity relative to 5-HT 2.5 - 10.8; Lovenberg *et al.,* 1993; Tsou *et al.,* 1994)), methiothepin and LY53857, which was originally developed as a 5-HT_2 antagonist (Cushing *et al.,* 1996).

1.4.2 In Vivo Vascular Responses to 5-HT

Functional responses of the vasculature to 5-HT vary greatly and may manifest as either vasodilatation or vasoconstriction or a combination of the two. The species concerned, the anatomical origin of the vascular bed studied and the level of pre-existing tone are amongst the factors that influence the response observed.

In healthy animals *in vivo*, infusion of 5-HT tends to cause an overall decrease in resistance, or a vasodilator effect in many vascular beds from a variety of species. In dogs,

infusion of 5-HT causes an overall decrease in mean arterial pressure and total peripheral resistance which becomes a vasopressor effect at high concentrations (Meschig *et al.,* 1985; Cambridge *et al.,* 1995). A bolus 5-HT dose has a transient hypotensive effect in pigs (Fukai *et al.,* 1995). Infusion of 5-HT into the iliac artery decreases total hindlimb resistance in healthy monkeys (Heistad *et al.,* 1984) and when administered into the forearm of healthy human volunteers it has a similar vasodilator effect (Blauw *et al.,* 1988; Bruning *et al.,* 1993). However, as the 5-HT concentrations are increased, a vasoconstrictor response can be seen which is antagonised by the 5-HT_{2A} antagonist ketanserin (Blauw *et al.,* 1988). Similarly, intracoronary infusions of 5-HT in healthy humans (Golino *et al.,* 1991), dogs (Woodman, 1990; Cappelli-Bigazzi *et al.,* 1991; Cambridge *et al.,* 1995) and cats (Lamping *et al.,* 1989) generally cause an increase in coronary blood flow which becomes a constrictor response at high concentrations (McFadden *et al.,* 1991). The vasorelaxant response in dogs was not reduced by the administration of an inhibitor of NO formation (Cambridge *et al.,* 1991).

In contrast, other vascular beds respond to infused 5-HT with a vasoconstrictor response. In the pulmonary vascular bed, a bolus injection of 5-HT induces an increase in pulmonary arterial pressure in dogs (Janssen, 1985) and a mixture of vasoconstrictor and vasodilator responses in the cat when the concentration and pre-existing tone are varied (Neely *et al.,* 1993). The renal vascular bed of the dog responds with an initial vasoconstriction and secondary vasodilatation after injection of 5-HT into the renal artery (Takahashi *et al.,* 1992). The vasoconstrictor response appears to be due to 5-HT_{1}-like receptors similar to the 5-HT_{1B} (non-rodent) and 5-HT_{1D} receptors (Cambridge *et al.,* 1995). Infusion of 5-HT causes an overall reduction of total carotid blood flow in cats, pigs and dogs (Saxena & Verdouw, 1983; Cambridge *et al.,* 1995).

In yet other vascular beds or experimental situations, 5-HT may produce no significant

change in blood flow. This has been observed in cerebral vessels supplying primate grey matter (Eidelman *et al.,* 1978). Since the response of a whole vascular bed is the sum of the responses of the arteries, veins, conducting and resistance vessels, a clearer indication of the response of the individual vessels is given by isolated preparations.

1.4.3 In Vitro Vascular Responses to 5-HT

The response to 5-HT in isolated healthy vessels is still very variable, but there is a general tendency for venules and large arteries to constrict and for arterioles to dilate in response to 5-HT. However, the same vessels may produce different responses under varying conditions. In what have probably been the most commonly used *in vitro* vascular preparations, the rabbit and the rat aorta, 5-HT causes a concentration-dependent contraction which is mediated by a 5-HT_2 receptor (Cohen *et al.,* 1981; Feniuk *et al.,* 1985; Leff & Martin, 1986; Roth *et al.,* 1986; Tagawa *et al.,* 1993; Ogawa *et al.,* 1995).

In cerebral arteries and arterioles from the human (Hamel & Bouchard, 1991a; Hatake *et al.,* 1992), primate (Heistad *et al.,* 1987a; Connor *et al.,* 1989a), dog (Connor *et al.,* 1989a; Brian & Kennedy, 1993), cow (Hamel *et al.,* 1993), sheep (Drummond & Wadsworth, 1994), rat (Edvinsson *et al.,* 1983; Deckert & Angus, 1992), rabbit (Deckert *et al.,* 1994) and cat (Edvinsson *et al.,* 1983) 5-HT also induces vasoconstriction. This response appears to be mediated by a 5-HT_1-like receptor, similar to the 5-HT_{1B} (non-rodent) and 5-HT_{1D} receptor in particular because of the potent agonist actions of 5-CT and sumatriptan, antagonism by methiothepin and the lack of effect of ketanserin (Hamel & Bouchard, 1991a; Ebersole *et al.,* 1993; Deckert *et al.,* 1994). In human pial arterioles the 5-HT_{1B} (non-rodent) and 5-HT_{1D} receptor antagonist metergoline also inhibited the 5-HT induced contraction (Hamel & Bouchard, 1991a; Hamel & Bouchard, 1991b). In addition, 5-HT agonists have been demonstrated to inhibit cyclic AMP formation in bovine

cerebral vascular smooth muscle cells (Ebersole *et al.,* 1993). The mRNA for the 5-$HT_{1D\beta}$ receptor has been isolated from human and bovine cerebral arteries again supporting a role for this receptor (Hamel *et al.,* 1993). In the basilar artery, however, there appears to be some contribution of 5-HT_2 receptors to the contraction (Chang & Owman, 1989; Connor *et al.,* 1989a).

Other vessels which exhibit contractile responses to 5-HT include human mammary arteries (Yang *et al.,* 1989; Conti *et al.,* 1990) and saphenous veins (Yang *et al.,* 1989; Docherty, 1994), hand and mesenteric arteries and veins (Arneklo-Nobin *et al.,* 1985; Kaumann *et al.,* 1993), canine (Feniuk *et al.,* 1985; Humphrey *et al.,* 1988; Sumner *et al.,* 1992) and rabbit saphenous veins (Martin & MacLennan, 1990), rabbit femoral (MacLennan & Martin, 1992), guinea-pig iliac (Sahin-Erdemli *et al.,* 1991) and rat pulmonary (Ogawa *et al.,* 1995) arteries. The contractile response to 5-HT in the dog saphenous vein has been studied extensively and is mediated by a 5-HT_1-like receptor. The pharmacological profile of this receptor matches that of the 5-HT_{1B} (non-rodent) and 5-HT_{1D} receptor closely except for the low potency of metergoline as an antagonist (Humphrey *et al.,* 1988; Perren *et al.,* 1991; Sumner *et al.,* 1992). Inhibition of cyclic AMP formation by 5-HT agonists and expression of the 5-HT_{1B} (non-rodent) and 5-HT_{1D} receptor has been demonstrated in this tissue (Sumner & Humphrey, 1990; Cushing *et al.,* 1994).

A contractile response to 5-HT is also observed in human (Kaumann & Frenken, 1985; Toda & Okamura, 1990), primate (Toda & Okamura, 1990), canine (Toda & Okamura, 1990), ovine (Drummond & Wadsworth, 1994), rat (Nyborg, 1991; Tschudi *et al.,* 1991; Tschudi & Luscher, 1995), rabbit (Yokoyama *et al.,* 1983) and porcine (Cushing & Cohen, 1993) coronary arteries. Contraction to 5-HT in coronary arteries is likely to be due to a mixture of 5-HT_1-like and 5-HT_2 receptors, with the proportions varying between species

(Connor *et al.,* 1989b; Toda & Okamura, 1990; Cushing & Cohen, 1992a; Foy *et al.,* 1992; Parsons *et al.,* 1992; Bax *et al.,* 1993; Kaumann *et al.,* 1994).

In vessels pre-contracted with a separate constrictor agent, 5-HT elicits relaxation in isolated human and cat cerebral arteries (Edvinsson *et al.,* 1983). Endothelium-dependent relaxation due to 5-HT has been demonstrated in rat jugular vein (Bodelsson *et al.,* 1993; Ellis *et al.,* 1995) and porcine coronary (Cocks & Angus, 1983; Richard *et al.,* 1990), renal and mesenteric (Cocks & Angus, 1983) arteries. The endothelium-dependent 5-HT-induced relaxation in the pig coronary artery was initially discovered when the contractile effects of 5-HT in this preparation were blocked with ketanserin (Cocks & Angus, 1983). This endothelium-dependent relaxation appears to be mediated by a 5-HT_1-like receptor, similar to the 5-HT_{1B} (non-rodent) and 5-HT_{1D} receptor, since 5-CT and sumatriptan act as agonists and methiothepin and metergoline as antagonists of the response (Schoeffter & Hoyer, 1990). In contrast, endothelium-dependent relaxation in the rat jugular vein is mediated by a 5-HT_{2B} receptor (Ellis *et al.,* 1995). Importantly, expression of 5-$HT_{1D\beta}$ and 5-HT_{2B} receptor mRNA has been demonstrated in endothelial cells (Schoeffter *et al.,* 1995).

5-HT-induced vasodilatation via both endothelium-dependent (Sumner, 1991) and -independent (Sumner *et al.,* 1989) mechanisms has been demonstrated in the porcine vena cava, the rabbit (Martin *et al.,* 1987) and guinea-pig (Gupta, 1992) jugular vein and the canine coronary artery (Cohen *et al.,* 1983; Houston & Vanhoutte, 1988; Cushing & Cohen, 1992b). Direct vascular smooth muscle-mediated relaxation has also been reported in the sheep pulmonary vein (Eyre, 1975; Cocks & Arnold, 1992), cat saphenous vein (Feniuk *et al.,* 1983) and the arterioles of skeletal muscle (De Clerck *et al.,* 1984). Previous research conducted in this laboratory (CSIRO Division of Human Nutrition) has demonstrated endothelium-independent relaxation due to 5-HT in the primate aorta (Head

et al., 1990). The 5-HT receptor mediating smooth muscle relaxation of the canine coronary artery and porcine vena cava has properties similar to a 5-HT_1-like receptor in as much as 5-CT acts as a potent agonist and methiothepin and methysergide as antagonists of the response whilst ketanserin is without effect (Houston & Vanhoutte, 1988; Sumner *et al.,* 1989; Cushing & Cohen, 1992b). In these preparations, however, the 5-HT_{1B} (non-rodent) and 5-HT_{1D} receptor agonist sumatriptan is ineffective as a relaxant. Moreover, in the porcine vena cava 5-HT has been shown to be linked to stimulation of cyclic AMP formation - an effect contrary to the classification of this receptor as of the 5-HT_1 type (Trevethick *et al.,* 1984; Trevethick *et al.,* 1986; Sumner *et al.,* 1989). Thus, this 5-HT receptor was originally classed as an orphan receptor, but now is more like the 5-HT_7 subtype (Eglen *et al.,* 1997). In contrast, direct relaxation of the sheep pulmonary vein is reported to be mediated by a 5-HT_4 receptor (Cocks & Arnold, 1992; Zhang *et al.,* 1995).

One of the reasons for the large variation in responses to 5-HT observed between different vessels and species is obviously a disparate distribution of the different 5-HT receptor subtypes. Whilst characterisation of the receptor subtypes involved in some of the above mentioned responses has been achieved, in many cases this is not so. In many vessels, eg. porcine and canine coronary arteries, different responses to 5-HT are observed depending on the level of pre-existing tone and the endothelial integrity. In addition, in some preparations normally silent responses can be unmasked in the presence of low concentrations of other agonists or of antagonists of other 5-HT receptors (Cocks & Angus, 1983; Sahin-Erdemli *et al.,* 1991; Choppin & O'Connor, 1995). Thus, the presence of more than one 5-HT receptor subtype in the same vessel is common and this greatly complicates the full characterisation of 5-HT effects in many arteries. The utilisation of agents in vascular studies which are more selective with regard to 5-HT receptor populations will assist in understanding the vast variations observed in vascular responses

to 5-HT. In addition, utilising primates in studies on vascular 5-HT effects, where possible, will provide more information of relevance to human vascular function.

1.4.4 General Vascular Effects of 5-HT

5-HT, in addition to the above mentioned direct vascular effects, can exert actions on the vasculature via indirect means. 5-HT has been shown to greatly amplify the vasoconstrictor effects of other vasoactive agents both *in vivo* and *in vitro* (de la Lande, 1990; Van Nueten & Janssens, 1990; Yildiz & Purdy, 1998). This effect has been demonstrated with a wide variety of tissues and agonists, including adrenergic agonists in the rabbit ear artery (de la Lande *et al.,* 1966; Meehan *et al.,* 1987; Meehan *et al.,* 1988; de la Lande, 1992) and aorta (Stupecky *et al.,* 1986; Christ *et al.,* 1990). Synergy between potassium and 5-HT occurs in the rabbit aorta and the dog mesenteric artery and vein (Stupecky *et al.,* 1986; Shimamoto *et al.,* 1994). In the rat aorta (Yang *et al.,* 1992) and human mammary and coronary arteries (Yang *et al.,* 1990), 5-HT potentiates contraction to endothelin. Importantly, in considering the possible role of vasospasm in myocardial infarction, synergy between 5-HT and U46619 has been demonstrated in the human coronary artery (Cocks *et al.,* 1993). Similarly, whilst 5-HT is actually only a weak direct platelet activator, it has synergistic actions with other agonists of platelet activation. This potentiation in the platelet is clearly mediated by a 5-HT_{2A} receptor, as demonstrated by antagonism with agents such as ketanserin (De Clerck *et al.,* 1984).

5-HT can act as a substrate for uptake 1 in sympathetic neurons and as such can act as an indirect sympathomimetic (Feniuk & Humphrey, 1989). In addition, 5-HT can exert vascular relaxant effects via the pre-synaptic inhibition of adrenergic neurotransmission in peripheral sympathetic nerves, an action mediated by 5-HT_1 receptors (Mylecharane &

Phillips, 1989; Mylecharane, 1990). Some of the vascular effects of 5-HT are in fact not elicited via interaction with 5-HT receptors but through its affinity for adrenergic receptors (Apperley *et al.,* 1976; Feniuk & Humphrey, 1989; Fernandez *et al.,* 1995). Tachyphylaxis to the vasoconstrictor actions of 5-HT has also been observed (Salomone *et al.,* 1997).

5-HT has a direct mitogenic effect on vascular smooth muscle cells and also enhances the mitogenic effects of other growth factors (Fanburg & Lee, 1997). Endothelial cells are also subject to the mitogenic effects of 5-HT (Pakala *et al.,* 1994).

1.5 Vascular Responses to Aggregating Platelets

In vivo, vascular responses to aggregating platelets are in fact the sum of the effects of the many vasoactive mediators (including 5-HT and Tx A_2) released by the platelets. Thus, studies on the vascular effects of aggregating platelets provide an important link between studies on isolated agents and the true clinical situation. In humans, the formation of an intracoronary thrombus during percutaneous transluminal coronary angioplasty results in a vasoconstrictor response at a site distal to the thrombus (Zeiher *et al.,* 1991). In isolated human coronary arteries, incubation with platelets induced a contractile response which appeared to be mediated by both 5-HT and Tx A_2 (Bax *et al.,* 1994).

In vitro it has been shown that exposure of endothelium-denuded rat caudal arteries to platelets (and hence activation by exposed collagen) causes a contraction which is mediated mainly by 5-HT (De Clerck & Van Nueten, 1982). In coronary arteries from healthy pigs, aggregating platelets produced a contraction in vessels which were at resting tone, and endothelium-dependent relaxation in vessels with pre-imposed tone (Shimokawa

et al., 1987; Shimokawa & Vanhoutte, 1989; Park *et al.,* 1995). This relaxation was demonstrated to be due to both 5-HT and adenine nucleotides (Shimokawa *et al.,* 1987). Endothelium-intact, pre-contracted dog coronary arteries also relax in response to aggregating platelets, a response in part mediated by 5-HT as demonstrated by inhibition with methysergide (Cohen *et al.,* 1983). The products from aggregated platelets, when injected into the iliac artery, cause vasodilatation in the primate hindlimb (Kawamura *et al.,* 1993).

Intra-arterial infusion of the platelet activator collagen produces platelet aggregation and the release of mediators *in situ.* Using this approach, researchers have demonstrated decreases in vascular resistance in the primate hindlimb and increases in vascular resistance in the feline hindlimb *in vivo* (Kaul *et al.,* 1991; Loots & De Clerck, 1993). The primate model exhibited a contraction to infused collagen in large arteries, which was antagonised by a Tx receptor antagonist or an inhibitor of prostaglandin production, indomethacin, but was resistant to blockade by ketanserin (Kaul *et al.,* 1991). In sharp contrast, the vasoconstriction in the feline model was unaffected by a Tx antagonist or indomethacin, but was antagonised by ketanserin (Loots & De Clerck, 1993). Thus, whilst both 5-HT and Tx appear to play roles in the vascular response to aggregating platelets, the overall picture is far from clear.

1.6 Vascular Morphology

Blood vessels generally consist of three structural layers, the *tunica intima*, *tunica media* and the *tunica adventitia.* The intima consists of a single, uninterrupted layer of endothelial cells lining the lumen of the vessel, with a subendothelial connective tissue layer. In arteries the internal elastic lamina separates the intima from the media, which

consists of concentric layers of smooth muscle cells, plus elastin and collagen fibres in varying proportions. The substantial part of the media of medium to large muscular arteries comprises smooth muscle cells, whilst the media of large elastic arteries also contains several concentric elastic lamellae. An external elastic lamina separates the media from the adventitia in large vessels. The adventitia consists of loose connective tissue, with fibroblasts, adipose tissue, elastin and collagen fibres, vasa vasorum and nerve fibres.

1.6.1 The Vascular Endothelium

The vascular endothelium plays an active role in modulating the contractile tone of the underlying smooth muscle and also the activation level of the circulating platelets. In 1980, Furchgott and Zawadzki revealed that the endothelium mediates the vasodilatory actions of acetylcholine via the release of a potent relaxing factor . It is now widely accepted that this endothelium-derived relaxing factor is the free radical nitric oxide (NO) (Feelisch *et al.,* 1994). NO production occurs in the cerebellum, macrophages and vascular endothelial cells during the conversion of L-arginine to L-citrulline by nitric oxide synthase (NOS). Thus synthetic analogues of L-arginine such as Nω-nitro-L-arginine (NOLA) or N^G-monomethyl-L-arginine (L-NMMA) inhibit NOS and hence the formation of NO (Rees *et al.,* 1990b). NO production by the endothelium can be stimulated by a wide variety of vasoactive agents including acetylcholine, bradykinin, histamine, the calcium ionophore A23187 and 5-HT. Endotoxin and cytokines can induce the expression of the NO synthase enzyme in vascular smooth muscle cells, an effect which can be prevented by glucocorticoids (Radomski *et al.,* 1990; Rees *et al.,* 1990a; Moritoki *et al.,* 1992).

NO causes a decrease in the active tone of the vasculature by the direct activation of soluble guanylate cyclase in the vascular smooth muscle cells and the consequent elevation

of intracellular cyclic GMP levels. Two relatively non-specific inhibitors of the actions of NO which are commonly used *in vitro* are haemoglobin and methylene blue. Recently, a highly specific inhibitor, a quinoxaline derivative (ODQ) has been introduced (Ohara *et al.,* 1993). The ferrous heme group in haemoglobin sequesters and binds extracellular NO in much the same way as guanylate cyclase, whilst methylene blue and ODQ are believed to directly inhibit the enzyme (Martin *et al.,* 1985; Ohara *et al.,* 1993).

In addition to agonist-stimulated NO production, there is a continuous low basal level of NO release from the endothelium. Thus, acute or chronic administration of inhibitors of NO formation *in vivo* results in a hypertensive response (Rees *et al.,* 1989; Rees *et al.,* 1990b; Kobayashi *et al.,* 1991) and transgenic mice lacking the endothelial NOS gene are hypertensive (Huang *et al.,* 1995). Similarly, inhibition of NO production *in vitro* results in an increase in vascular tone in some preparations and enhances the vascular response to contractile agonists (Rees *et al.,* 1990b; Ignarro *et al.,* 1991; Yang *et al.,* 1991). Thus, an intact endothelial cell layer greatly inhibits the vascular contraction produced by aggregating platelets *in vitro* (Luscher & Vanhoutte, 1986). Experimentally-induced endothelial damage *in vivo* causes platelet aggregation and hence vasoconstriction which can be blocked by 5-HT or Tx receptor antagonists (Golino *et al.,* 1989).

Extensive research into the role of the endothelium since Furchgott and Zawadzki's discovery has resulted in the recognition of a range of other endothelium-derived relaxing and contracting factors including endothelium-derived hyperpolarising factor, endothelin and angiotensin II. The continuous basal release of endothelium-derived relaxing factors by a functional endothelial layer serves not only to maintain vascular tone but also to inhibit platelet activation and the adhesion of blood-borne cells to the vessel wall (Luscher & Noll, 1994).

1.7 Vascular Disease States

1.7.1 Atherosclerosis and Plasma Lipids

Atherosclerosis is a common vascular disease characterised by the accumulation of lipid deposits within the intima of large and medium-sized arteries, and often occurs in association with increased circulating plasma lipids (hyperlipidaemia). The majority of dietary lipid is consumed in the form of triglycerides (triacylglycerols) and absorbed as non-esterified fatty acids and monoacylglycerols. In the circulation, lipids are transported in association with apoproteins within lipoprotein complexes. The densities of these lipoprotein complexes varies with the proportions of lipid and protein they contain, and as such can be separated by differential ultracentrifugation. The major lipoprotein classes, in order of increasing density and therefore decreasing ratio of lipid to protein, are chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL) (Thompson, 1990). However, these are far from discrete classes and represent a general division of a continuum of lipoprotein densities.

Chylomicrons are formed by the intestinal mucosa during digestion and consist predominantly of the absorbed and reconstituted triglycerides. They also contain some newly absorbed cholesterol, which is transported from the intestine to the liver and adipose tissue. The hepatically synthesised VLDL, however, contain the majority of the plasma triglycerides and in man most of the plasma cholesterol is associated with LDL. Progressive peripheral metabolism of VLDL (which transport triglycerides and cholesterol esters synthesised in the liver) by lipoprotein lipases results in the formation of intermediate density lipoproteins (IDL) and LDL as the apoproteins and triglycerides are degraded or deposited in the tissues. The IDL are then either removed intact from the circulation, or are further degraded by hepatic and/or circulating lipoprotein lipase to give

LDL which consist of approximately 60 % cholesterol, the majority of it in the esterified form (Goldstein & Brown, 1977). The primary function of LDL is to transport cholesterol to the peripheral tissues, where they are absorbed into cells via both a specific receptor-mediated and a non-receptor-mediated absorptive endocytotic mechanism. HDL are involved in the transport of excess cholesterol from the peripheral tissues to the liver for catabolism and excretion (reverse cholesterol transport).

The plasma lipid and lipoprotein profile of the rabbit (as in the rat) varies greatly from that of the human. In the human, more than half of the IDL are converted to LDL and thus the LDL class constitutes greater than 50 % of all of the circulating lipoproteins, containing approximately 70 % of circulating cholesterol (Goldstein & Brown, 1977; Gurr & Harwood, 1991). However, in many other species (including the rabbit and the rat) the majority of the IDL are absorbed by the liver, hence the predominant plasma lipoprotein class is often HDL in non-primate species (Gurr & Harwood, 1991). The plasma lipoprotein profile of the common marmoset is more similar to that of humans than most other species, both in terms of composition and concentration (Chapman *et al.,* 1979; Crook *et al.,* 1990). A difference still remains in that in the marmoset approximately 50% of circulating cholesterol is associated with HDL (Chapman *et al.,* 1979; Schouten *et al.,* 1986). In the marmoset, serum concentrations of LDL and HDL cholesterol have been reported to be within the ranges 170-280 and 338-408 mg/dL (Chapman *et al.,* 1979) as compared with approximately 100-145 and 35-85 in the human, respectively (Creager *et al.,* 1992; Institute of Medical and Veterinary Science, 1989; Goldstein & Brown, 1977). Thus, observations of the pharmacological vascular effects of hypercholesterolaemia and atherosclerosis in a non-human primate such as the marmoset are obviously of greater physiological relevance than observations in species which are phylogenetically less similar to humans.

Hyperlipidaemia, in particular hypercholesterolaemia, is a major risk factor for atherosclerosis (reviewed in (Thompson, 1990)). Specifically, LDL has been shown to exhibit a direct relationship, and HDL an inverse relationship, with the risk of coronary heart disease (Kannel *et al.,* 1979). Thus the total cholesterol to HDL cholesterol ratio is a strong indicator of risk (Castelli, 1984). The correlation between circulating lipid levels and atherosclerosis led to the development of the Lipid Hypothesis of atherosclerosis. This theory essentially states that hyperlipidaemia results in the deposition of circulating lipids into the arterial wall, an event which is the major initiating factor in the pathology of atherosclerosis (Ross & Harker, 1976; Goldstein & Brown, 1977; Steinberg, 1987). Alternative explanations of the aetiology of atherosclerosis include the Response-to-Injury hypothesis (see *1.7.3*), Benditt's hypothesis and Duguid's hypothesis (reviewed by (Steinberg & Olefsky, 1987)).

1.7.2 Atherosclerosis and Vascular Morphology

The development and progression of atherosclerosis is gradual and without discrete phases, but is typically characterised by three general types of atherosclerotic lesion; the fatty streak, the atherosclerotic (fibrous) plaque and the complicated lesion (reviewed by Ross (1986)). A fatty dot or streak consists of a local accumulation of lipid deposits, predominantly cholesteryl esters, within the intima. Circulating monocytes adhere to the endothelial cells and invade the arterial intima to form macrophages which accumulate lipid, forming foam cells. Lipid droplets are also found extracellularly along the internal elastic lamina and in smooth muscle cells which have migrated from the media into the subendothelial space. Fatty streaks consisting of foam cells exist in suckling rabbits but disappear following weaning without further progression (Jokinen *et al.,* 1985). In humans these lesions occur from early childhood onwards and are not associated with clinical

symptoms. However, progressively, the fatty dots may enlarge and join to form fatty streaks. This process continues over many decades, eventually leading to lipid deposits which cover increasingly larger areas of the intima.

The atherosclerotic plaque appears as an elevation above the surrounding intima which protrudes into the arterial lumen and thus decreases blood flow. This lesion consists of a basal, lipid-rich deposit containing many foam cells and extracellular lipid deposits. This lipid pool is covered by a fibrous cap of connective tissue, which morphologically is of an opaque, white appearance. The relative proportions of the lipid-pool and the fibrous layer vary greatly. The plaque also contains an abundance of smooth muscle cells and connective tissue matrix. An advanced plaque may become vascularised by the vasa vasorum from the adventitia. In rabbits and humans, atherosclerosis is associated with morphological alterations of endothelial cells (Tokunaga *et al.,* 1989; Tashiro *et al.,* 1994).

In a complicated lesion, the atherosclerotic plaque is associated with secondary changes such as calcification or ulceration. Ulceration may result in exposure of the subendothelial connective tissue to circulating platelets, hence platelet activation and the formation of a thrombus may occur.

1.7.3 The Response-to-Injury Hypothesis

The response-to-injury hypothesis is a theory developed to explain the pathogenesis of atherosclerosis and is the result of a combination of theories proposed by many researchers, but was formally presented by Ross in 1973. The basis of this theory is that endothelial "injury", which may take the form of chemical (eg. oxidised LDL) or mechanical insults, leads to endothelial dysfunction. This is the initiating event in a

cascade including platelet aggregation, the release of platelet-derived growth factors and cellular proliferation, which leads to the intimal hyperplasia and vascular lesions characteristic of atherosclerosis (Ross & Glomset, 1976).

In accord with this theory, the common risk factors for atherosclerosis, eg. hypertension (Linder *et al.,* 1990; Panza *et al.,* 1990; Dyer *et al.,* 1993), hypercholesterolaemia (Shimokawa & Vanhoutte, 1989; Rossitch *et al.,* 1991; Creager *et al.,* 1992), increasing age (Tschudi *et al.,* 1991) family history and male gender (Vita *et al.,* 1990) and diabetes (Oyama *et al.,* 1986; Mayhan *et al.,* 1991) are associated with impaired endothelial function. *In vitro* exposure of arteries to oxidised LDL also inhibits endothelium-dependent relaxation (Tanner *et al.,* 1991a; Galle *et al.,* 1994). Hence the atherosclerotic risk factors fit into this model as potential and importantly often chronic sources of endothelial injury. It is also important to note that the Lipid Hypothesis (*1.7.1*) and the Response-to-Injury Hypothesis are not necessarily mutually exclusive and can be combined to form the Unified Hypothesis to explain the pathogenesis of atherosclerosis (Steinberg, 1987).

1.7.4 Balloon Angioplasty

Percutaneous transluminal angioplasty (PCTA), or the dilatation of a stenotic artery by the use of an intra-luminal balloon catheter, was first performed clinically by Andreas Grüntzig in 1977 . It is now a commonly used non-surgical method for the treatment of arteries obstructed by atherosclerotic plaques. The success of this treatment, however, is hampered by the occurrence of restenosis in approximately 30% of cases (Rowe, 1989; Cowley, 1992).

Balloon embolectomy catheters are commonly used experimentally to induce an initial

vessel wall injury which subsequently leads to intimal hyperplasia and thus provides an animal model of vascular disease (Clowes *et al.,* 1983; Shimokawa *et al.,* 1989; Gonschior *et al.,* 1995). Passage of the catheter with the balloon inflated along an artery causes mechanical endothelial denudation and some medial damage (Enna & Karbon, 1987). The endothelial layer then regenerates and intimal hyperplasia occurs, giving a lesion which shows strong similarities to the morphology of the vascular lesions of atherosclerosis, however without the deposition of lipids. The intimal hyperplasia that occurs is primarily due to the migration and multiplication of vascular smooth muscle cells and the accumulation of extracellular matrix synthesised by these cells (Austin *et al.,* 1985; Nikkari & Clowes, 1994; Hadoke *et al.,* 1995). Migration occurs within days and proliferation halts within weeks of injury, thus neointimal growth over an extended period is due to accumulation of connective tissue components (Clowes *et al.,* 1983; Clowes & Reidy, 1991; Hadoke *et al.,* 1995; Geary *et al.,* 1996). The VSMC proliferation and migration may be due to the release of growth factors from platelets adhering to the damaged vessel wall, haemodynamic factors, the loss of endothelium-derived inhibitory factors, and/or the release of mitogens from damaged medial smooth muscle cells (; Liu *et al.,* 1989; Dzau *et al.,* 1991; Casscells, 1992). Platelet-derived growth factor, transforming growth factor-ß, angiotensin II, basic fibroblast growth factor and heparin have all been implicated (Libby & Tanaka, 1997). Endothelial regrowth begins immediately after injury, regenerating from undamaged cells at the boundary of injury and gradually covering the denuded area. In rats growth halts at approximately 6-12 weeks after intervention, and in rabbits 2 weeks following angioplasty, thus in extensively damaged vessels the central portion remains denuded of endothelium (Reidy *et al.,* 1983; Clowes *et al.,* 1983). The regenerated endothelial cells and myointimal smooth muscle cells are also morphologically different from native cells (Shimokawa *et al.,* 1987; Hadoke *et al.,* 1995).

Intimal hyperplasia induced experimentally by the use of balloon catheters in animals differs from restenosis occurring following clinical angioplasty in human arteries. In patients, PTCA is performed on arteries which exhibit severe atherosclerotic stenosis. Thus, the severity of the insult and the resultant lesion in animals is often increased experimentally by the combination of angioplasty with an atherosclerotic diet (Shimokawa *et al.,* 1983; Hadoke *et al.,* 1995).

1.8 Vascular Responses in Cardiovascular Disease States

With the onset of cardiovascular disease, the normal responses of the vasculature to vasoactive agents become altered in both humans and animals. In particular, inhibition of vascular endothelial function has been shown to be associated with many of the risk factors for cardiovascular diseases, as discussed above (see *1.7.3*). However, conflicting findings have also been reported. Endothelium-dependent relaxation has been reported to be reduced, enhanced or unchanged in healthy smokers (Rangemark & Wennmalm, 1992; Celermajer *et al.,* 1993; Jacobs *et al.,* 1993; Zeiher *et al.,* 1995). *In vitro* incubation of segments of rabbit carotid artery with cholesterol has also been shown to enhance endothelium-dependent relaxation (Bialecki & Tulenko, 1993). Interestingly, cholesterol feeding may induce vascular non-endothelial NOS production (Verbeuren *et al.,* 1993).

The vascular contraction occurring in response to many vasoconstrictors becomes enhanced in association with atherosclerotic risk factors, most likely due to the removal of the inhibiting influence of basal endothelium-derived relaxing factors (Woodman, 1995; Weisser *et al.,* 1991).

The response of diseased arteries to aggregating platelets is of particular interest and

pathophysiological relevance. The contraction of large arteries to infused collagen is enhanced and the vasodilation in response to injection of the products of platelet aggregation is reduced in the hindlimb of atherosclerotic primates (Kaul *et al.,* 1991; Kawamura *et al.,* 1993). In the coronary arteries of hypercholesterolaemic pigs without atheroma, the endothelium-dependent relaxation of pre-contracted vessels in response to aggregating platelets is reduced and the contraction of quiescent vessels to the same is enhanced (Shimokawa & Vanhoutte, 1989). In the spontaneously hypertensive rat, the contractile response of aortic rings to aggregating platelets is significantly increased (Luscher & Vanhoutte, 1986). Thus, in general, diseased arteries tend to exhibit reduced relaxant responses and enhanced contractile responses to many agents (including platelet-released mediators) by comparison with healthy arteries.

1.8.1 Vascular Responses to 5-HT in Cardiovascular Disease States

This general statement with regard to the effects of cardiovascular disease states on vascular responses translates well to the specific case of 5-HT. Endothelial removal or inhibition of NO production in dogs increases the 5-HT-induced contraction in coronary arteries and inhibits 5-HT induced relaxation in coronary arterioles *in vivo* (Lamping *et al.,* 1985; Cappelli-Bigazzi *et al.,* 1991; De Fily & Chilian, 1991). The relaxation of porcine coronary arterioles (Kuo *et al.,* 1992) and of pre-contracted canine (Cocks & Angus, 1983) and porcine (Cocks & Angus, 1983; Cohen *et al.,* 1983) coronary arteries to 5-HT *in vitro* is also dependent upon intact endothelial function. Likewise, incubation of human coronary arteries with methylene blue or an inhibitor of NO formation *in vitro* increases the contractile effect of 5-HT (Berkenboom *et al.,* 1989; Richard *et al.,* 1990). Thus, any disease state which affects endothelial function can be expected to affect 5-HT responses in either a specific or non-specific manner.

Contraction mediated by the 5-HT_{1D} agonist sumatriptan has been shown to be greater in human mammary arteries from human hypertensive patients than from normotensives (Yildiz *et al.,* 1996). In pial arterioles from normotensive rat the vasodilator response to 5-HT is reversed to vasoconstriction in vessels from hypertensive rats (Mayhan *et al.*, 1987). Hypertension in rats is also associated with an increased contractile response of mesenteric resistance arteries to 5-HT (Tesfamariam & Halpern, 1988). In diabetic rabbits, the contractile effect of 5-HT in pulmonary artery rings was enhanced by comparison with normal rabbits (el Kashef, 1996).

Alterations in vascular responsiveness to 5-HT can be observed in the presence of atherosclerotic risk factors alone *before the appearance of pathological lesions.* In rat coronary arteries 5-HT-induced contraction increases with age (Nyborg, 1991; Tschudi & Luscher, 1995). This enhancement was shown to occur even in the absence of age-associated inhibition of endothelium-dependent relaxation (Tschudi & Luscher, 1995). In genetic and DOCA rat models of hypertension, contractions to 5-HT are greater than in control rats (Webb, 1984; Clozel *et al.,* 1990; Watts, 1998). Hypercholesterolaemia has been shown to reduce maximal endothelium-dependent relaxations to 5-HT in porcine coronary arteries (Shimokawa & Vanhoutte, 1989) and to enhance aortic 5-HT-induced vasoconstriction *in vivo* (measured using ultrasonography) and *in vitro* in rabbits (Chin *et al.,* 1990; Galle *et al.,* 1991). Incubation of porcine coronary arteries with oxidised LDL *in vitro* inhibits endothelium-dependent relaxations to both 5-HT and aggregating platelets, but not to the endothelium-dependent relaxant A23187 (a calcium ionophore) (Tanner *et al.,* 1991a). Capelli-Bigazzi *et al.* (1990) demonstrated that canine coronary arteries removed from dogs which had been subjected to an acute hypertensive episode exhibited a four-fold increase in 5-HT-induced contraction. This effect may have been dependent upon the release of vasoactive factors from leucocytes and platelets which adhere to the

damaged endothelial layer following the hypertensive phase rather than upon inhibition of endothelium-derived relaxing factors (Cappelli-Bigazzi *et al.,* 1990). In contrast to the above, the maximal contraction to 5-HT has been shown to decrease with increasing age in the isolated human basilar artery (Hatake *et al.,* 1992). Hypercholesterolaemia had no effect on the 5-HT-induced constriction of large arteries or upon hindlimb resistance in monkeys (Heistad *et al.,* 1984). In Watanabe heritable hyperlipidaemic rabbits, coronary artery contraction to 5-HT was shown only to be enhanced once atherosclerotic lesions occurred in the vasculature (Yokoyama *et al.,* 1983).

In vessels from atherosclerotic humans or animals, the alterations in 5-HT responses are marked. In the human coronary vascular bed *in vivo*, the 5-HT-induced vasodilatation observed in healthy vessels is reversed to vasoconstriction in patients with coronary artery atherosclerosis or variant angina (Golino *et al.,* 1991; McFadden *et al.,* 1991). Isolated human atherosclerotic coronary arteries exhibit increased contraction to 5-HT by comparison with vessels free of coronary artery disease (CAD) (Kalsner & Richards, 1984). Since 5-HT_1-like receptors play a role in mediating coronary 5-HT-induced contraction, CAD is a serious contra-indication for the use of the anti-migraine drug sumatriptan. There have also been reports of chest pain associated with the use of sumatriptan in patients without evidence of CAD (Ottervanger *et al.,* 1993; Barnard, 1992).

In monkeys, hindlimb vasodilatation reverses to vasoconstriction and the vasoconstriction of large arteries is enhanced with the occurrence of atherosclerosis (Heistad *et al.,* 1984; Heistad *et al.,* 1987b). The contraction of large cerebral arteries and coronary arteries to 5-HT *in vivo* is increased in atherosclerotic monkeys (Heistad *et al.,* 1987a; Lamping *et al.,* 1994). The blood flow in retinal and coronary vascular beds in normal monkeys is unaffected by 5-HT, but in atherosclerotic monkeys 5-HT reduces flow (Faraci *et al.,*

1991; Lamping *et al.,* 1994). The contraction of isolated mesenteric arteries from atherosclerotic monkeys to 5-HT is increased (Toda *et al.,* 1988). Endothelium-dependent relaxation to 5-HT is reduced in atherosclerotic porcine coronary arterioles (Kuo *et al.,* 1991; Kuo *et al.,* 1992) and aortic (Tagawa *et al.,* 1993; Miwa *et al.,* 1994) and coronary artery (Yokoyama *et al.,* 1983) contraction to 5-HT is increased in Watanabe heritable hyperlipidaemic rabbits. In contrast, Simonsen *et al.* fed a high cholesterol diet to New Zealand white rabbits which induced atherosclerotic lesions in the aorta but did not affect the responses of the cerebral, femoral or mesenteric small arteries to 5-HT (there was no evidence of atherosclerotic changes in these small vessels) and only slightly increased the responsiveness of the aorta to 5-HT at low doses (Simonsen *et al.,* 1991a).

Vessels with intimal hyperplasia induced by endothelial-denudation using the balloon catheterisation method show similar functional alterations. Following balloon catheterisation, vasoconstriction to 5-HT is increased and endothelium-dependent relaxation to 5-HT and aggregating platelets is reduced in porcine coronary arteries although relaxation to other endothelium-dependent vasodilators remains unaltered (Shimokawa *et al.,* 1987; Park *et al.,* 1995).

Whilst there are alterations in vascular responsiveness to many vasoactive agents in cardiovascular disease states, there is some evidence that changes in serotonergic effects may occur earlier than for other substances. During the development of atherosclerosis in cholesterol-fed rabbits, an increase in the sensitivity of the aorta to the contractile effects of 5-HT occurred before contractions to noradrenaline or potassium were enhanced and prior to inhibition of endothelium-dependent relaxation to acetylcholine (Galle *et al.,* 1991). Also, in a rabbit model of atherosclerosis where intimal lesions are produced by the placement of a Silastic collar around the carotid artery, relaxations to ACh and contractions to 5-HT are altered earlier than contraction to phenylephrine (Dusting *et al.,*

1990).

In a study on atherosclerotic primates, contractions of mesenteric arteries to 5-HT were enhanced whilst contractions to electrical stimulation, noradrenaline and angiotensin II were unaffected. In porcine coronary arteries which have been subjected to balloon catheterisation, endothelium-dependent relaxations to 5-HT and aggregating platelets are impaired 4 weeks after intervention, but relaxation responses to ADP and bradykinin are not suppressed until much later and then to a lesser degree, whilst A23187 responses are not inhibited at all (Shimokawa *et al.,* 1989). Similarly, vascular responses to 5-HT in hypertensive animals are enhanced to a greater degree than responses to many other agonists (Webb, 1984).

Nevertheless, the evidence supporting this theory is not unequivocal. Heistad *et al.* demonstrated an increase in vascular contraction to noradrenaline in small vessels from hypercholesterolaemic primates whereas there was no change in the sensitivity to 5-HT (Heistad *et al.,* 1984). Noradrenaline but not 5-HT sensitivity was also increased in small cerebral arteries from atherosclerotic rabbits in the study by Simonsen *et al.* (1991a).

In summary, serotonergic vascular responses are altered in disease states, generally exhibiting increased contractile responses and decreased relaxant responses. These changes have been observed to occur, (in some models), in the presence of atherosclerotic risk factors alone and in the absence of morphological alterations. In fact, the changes in serotonergic resposes may occur earlier than changes in the response to other vasoactive agents. However, there are some studies contradicting these trends and further investigations utilising 5-HT receptor specific agents in a primate model would be useful.

1.9 Summary and Aims

The vasospasm of atherosclerotic arteries may be a principal causative factor in a variety of cardiovascular incidents. The aetiology of vasospasm is unknown but it may be initiated by platelet aggregation occurring at sites of vascular damage, which results in the release of a variety of vasoactive agents including Tx A_2 and 5-HT. The ensuing response of the vasculature to 5-HT is widely variable and may depend upon many factors including the anatomical and species origin of the vessel, the level of pre-existing tone, the presence or absence of disease states and the types of 5-HT receptors present.

Much of the research conducted into vascular responses to 5-HT to date does not attempt to include characterisation of the receptor subtype(s) mediating the 5-HT effects concerned. Over the past few years the number of 5-HT receptors discovered has rapidly increased such that only quite recent research tends to relate to the current 5-HT receptor classification system. At present, there are 7 classes of 5-HT receptors ($5\text{-}HT_1$ to $5\text{-}HT_7$) with $5\text{-}ht_5$ and $5\text{-}ht_6$ being putative recombinant 5-HT receptors. In addition, past research which includes detailed studies of the receptor subtypes mediating the 5-HT effects have often been in species (eg. pig, dog) which may be of questionable relevance to the human. Moreover, when detailed investigations of the effects of disease states on 5-HT responses have been conducted generally little attention, if any, has been paid to the specific 5-HT receptor subtypes involved.

The overall aims of this study were to characterise the 5-HT receptors mediating the vascular responses to 5-HT in a primate, the common cotton-eared marmoset monkey, and to determine the changes in these responses that occur in the presence of a vascular disease state, specifically diet-induced atherosclerosis and balloon catheter-induced intimal hyperplasia. The main part of these studies utilizes the aorta since the large size of this

vessel enables many parallel experiments to be conducted in a single vessel from any given animal. Early in these studies (utilising arteries available from control animals in a separate dietary investigation) it became apparent that the response of the marmoset aorta to 5-HT was much more complex than that reported in the rabbit aorta. Thus it became necessary to establish in detail the nature of and the 5-HT receptor subtype involvement in the 5-HT response. The study was then expanded to other vessels and the coronary artery in particular since this vessel plays a key role in the mortality due to cardiovascular disease. More specifically, the aims of the study were:

1) To determine the 5-HT receptor subtypes responsible for the 5-HT-induced responses of the marmoset aorta.

2) To determine whether the 5-HT-induced response of the marmoset aorta is parallelled by the serotonergic responses of other marmoset vessels, particularly the coronary arteries.

3) To determine the effects of vascular disease states on the responsiveness of the marmoset vasculature to 5-HT.

CHAPTER 2

GENERAL METHODS

2.1 Animals

Adult common cotton-eared marmoset monkeys (*Callithrix jacchus jacchus*) of both sexes, weighing approximately 280 g, were obtained from the Commonwealth Scientific and Industrial Research Organisation (CSIRO), Division of Human Nutrition, Glenthorne laboratories (O'Halloran Hill, South Australia) and the Queen Elizabeth Hospital (Woodville, South Australia) breeding colonies (McIntosh & Looker, 1982). Since animals of a consistent age or sex were not available for all of the experiments described in this thesis, groups of marmosets of a similar age and sex were generally used in any one study and statistical comparisons confined to these groupings. The animals were housed in stainless steel cages under conditions of an artificial 12/12 hour light/dark cycle in an ambient temperature of 24-26^{o}C. Diet (except where stated otherwise) consisted of a standard colony pellet diet custom made by Milling Industries Ltd. (Dulwich, South Australia) (refer Appendix I). This was supplemented with 3 varieties of fruit daily (approximately 10g pieces) and a multivitamin supplement ("Pentavite", Roche) on bread twice weekly. During later studies, vitamin D_3 levels in every batch of the pellet diet were monitored and a dietary supplement of this was adjusted accordingly to achieve an intake of 400 µg/kg/day. Water and pellets were administered *ad libitum*.

Adult male hooded Wistar and Wistar Kyoto (WKY) rats, weighing approximately 300 g, were obtained from the CSIRO, Division of Human Nutrition, Glenthorne breeding colony and were housed in wire-bottomed cages under conditions of an artificial 12/12 hour

light/dark cycle and an ambient temperature of 22-24°C. These animals received water and standard colony rat chow (Milling Industries Ltd.) *ad libitum.*

All of the experiments described in this thesis were approved by the CSIRO Animal Care and Ethics committee prior to the commencement of the studies. Tissues from many animals were also used in studies other than those described in this thesis.

2.1.1 Tissue Collection

The marmosets were killed under alphaxalone/alphadolone anaesthesia ("Saffan"; 24 mg/kg i.m.). The animals were anaesthetised at the colony location and then transported to the CSIRO, Division of Human Nutrition, Kintore Ave, Sth. Aust. (with the permission of the Australian Quarantine and Inspection Service) where they were sacrificed within 1-1.5 hours of commencement of anaesthesia. The animals were killed by exsanguination from the abdominal aorta after which the vessels required were removed and placed in physiological salt solution (PSS) gassed with 95% O_2/5% CO_2. The PSS was composed of (mmol/l): NaCl 113; KCl 4.8; KH_2PO_4 1.2; $MgSO_4.7H_2O$ 1.2; $NaHCO_3$ 25; $CaCl_2$ 2.5; glucose 11.2 and ascorbic acid 0.57 in order to inhibit *in vitro* oxidation of unstable pharmacological agents.

2.1.2 Blood Sampling

Blood samples were taken from the femoral vein of restrained, conscious marmosets which had been fasted overnight for the purpose of analysing their plasma lipids (*2.4*). Up to 1.5 ml of blood was collected into heparin (approximately 12.5 U/ml) using a 23 or 25 G

needle and placed on ice. Plasma was obtained by centrifuging the whole blood at 1,000 g for 20 minutes at 4°C. The plasma was stored at 4°C and analysed within 36 hours of collection or stored at -80°C for analysis at a later date. Immediately prior to analysis, frozen plasma samples were centrifuged at 3,000 rpm for 5 min at room temperature to remove fibrin from the sample.

2.2 Organ Bath Preparations

2.2.1 Aortic Ring Preparation

Within a period of 45 min after removal of the aorta, it was cut into 6-10 rings of 2-3 mm length which were suspended horizontally between two stainless steel stirrups inserted into the vessel lumen, in an organ bath containing 15 ml PSS maintained at 37°C and bubbled with 95% O_2/5% CO_2. One stirrup was anchored in the organ bath and the other connected to the force transducer. Preliminary experiments were conducted to investigate the relationship between preload and contraction to KCl at a concentration (80 mM) which induced maximum active force of the marmoset aortic rings. With a preload of 3, 4 or 5g the aortic rings contracted with an active force of 1.98 ± 0.48, 2.25 ± 0.51 or 2.71 ± 0.65 g respectively (n = *3*). Since these values were not significantly different, all aortic rings were placed under a pre-load of 4g to enable comparisons with previous work carried out in this laboratory.

When required, the endothelial layer was removed by gentle rubbing of the luminal surface with the tip of a pair of forceps or by rubbing between the fingertips, with or without inversion of the vessel segment. After mounting, the tissues were equilibrated for at least 45 min prior to eliciting responses. Isometric contractions were measured using FT03 force transducers (Grass Medical Instruments, Quincy, MA, USA). These were

coupled to a JRAK WR3701 amplifier (JRAK Biosignals, Windsor, Vic., Australia) and recorded on a Linearcorder (Graphtec, Yokohama, Japan); or to a Grass 79D Polygraph.

Following equilibration under tension, all artery segments were contracted with K^+ (20 mM in early experiments, 40 mM or KPSS (120 mM) in later experiments) to confirm tissue viability. KPSS was equivalent to PSS (*2.1.1*) with NaCl replaced by KCl on an equimolar basis. This was followed by a cumulative dose-response curve (DRC) to a contractile agonist. A minimum of 30 min equilibration time was allowed between successive DRCs. All agonists were applied in a cumulative manner. Following wash out and re-equilibration, a DRC to a 5-HT agonist under one of a variety of conditions was obtained. In experiments where the arteries were contracted prior to the 5-HT DRC, the contractile agent was applied at its EC_{50} as determined in the previous DRC (in the presence of antagonist when appropriate). If precontractile tone was not at a stable level (± approximately 0.08g) for several minutes following addition of the contractile agent, then the artery ring was excluded from the experiment. Since the responses to 5-HT extended over a wide range of concentrations (0.001 - 10.0 μM), 5-HT was cumulated at ten fold increments of concentration. Antagonists were added 30 minutes prior to the contractile agent DRC and also 30 min prior to the 5-HT DRC. At the completion of experiments investigating the role of endothelial function, the response to an endothelium-dependent relaxing agent (usually 1.0 μM A23187) was tested in order to assess the integrity of the endothelial layer. Endothelium-intact preparations were required to exhibit greater than 60%, and "endothelium-denuded" preparations less than 30% relaxation to A23187 (1 μM) for inclusion in final data. In all other experiments where endothelial integrity is not specifically referred to, no attempt was made to remove the endothelial layer.

2.2.2 Carotid Artery Preparations

The right common carotid artery was removed from the animals and placed into PSS at ambient temperature (approximately 21°C). Within a period of 1 hour, the artery was cut into 2 segments of 2-3 mm length and suspended between two stainless steel stirrups under tension in an organ bath containing 15 ml PSS maintained at 37°C and bubbled with 95% O_2/5% CO_2. On the basis of preload-active force relationships, the carotid arteries were suspended under 3g basal tension. The recording equipment and protocol details for the carotid artery segments were as described above for the thoracic aortic rings (*2.1.1*).

2.2.3 Mesenteric Artery Preparations

The superior mesenteric artery was removed from the animals and placed into PSS at ambient temperature (approximately 21°C). Within a period of 1 hour, the artery was cut into 2 segments of 2-3 mm length and suspended between two stainless steel stirrups under 3g tension in an organ bath containing 15 ml PSS maintained at 37°C and bubbled with 95% O_2/5% CO_2. On the basis of the preload-active force relationship, the superior mesenteric artery segments were mounted under 3g tension. The recording equipment and protocol details for this preparation were as described above for the thoracic aortic rings (*2.1.1*).

2.2.4 Coronary Artery Preparations

The heart was removed from the animals and placed into ice-cold Ca^{2+}-free PSS continuously gassed with 95% O_2/5% CO_2. Within 3 hours, segments of the large coronary arteries, ie. the left anterior descending and circumflex coronary arteries, were dissected from the heart, with the aid of a dissecting microscope.

In early experiments the Ca^{2+}-free dissection solution contained 1 mM EGTA to ensure a calcium free environment to keep the lumen patent and thus enable wires to be passed through the vessel. This treatment did not significantly affect the pEC_{50} of contraction of coronary artery segments to U44069 (*Figure 2.1*). Following dissection, the vessels were mounted either immediately, or after storage overnight at 4°C in an air-tight receptacle containing 50 ml oxygenated PSS. The pEC_{50} for U44069-induced contraction of coronary arteries was not significantly affected by overnight refrigeration (*Figure 2.1*). (Cold storage of arteries prior to determination of pharmacological responses is commonly practised by other researchers and has not been shown to significantly affect vascular responsiveness (personal communication, I.S. de la Lande)). Two vessel segments were mounted on supports in a dual small vessel Mulvany/Halpern myograph (Mulvany & Halpern, 1976) model 400A (J.P. Trading, Aarhus, Denmark) connected to a Rikadenki recorder (Japan) for measurement of isometric contractions. Briefly, two wires of 40 μM diameter were threaded through the lumen of the artery segments. One wire was attatched to a support connected to an isometric force transducer and the other to a moveable support controlled by a manual micrometer. Throughout this process the vessels were immersed in chilled, oxygenated Ca^{2+}-free PSS. When the mounting procedure was complete, the organ chambers were gradually warmed to 37°C.

The arteries were equilibrated under zero tension in 5 ml standard PSS maintained at 37.0°C and bubbled with 95% O_2/5% CO_2 for at least 10 minutes. The vessel length (a) was measured using a calibrated eyepiece graticule mounted in a dissecting microscope. A passive wall tension-vessel diameter curve for each vessel was then obtained by incrementally increasing the tension on the vessel segments (in approximately 4-8 steps) at 1 minute intervals until the internal circumference was slightly greater than that which the vessel would have had if relaxed and exposed to a transmural pressure of 100 mmHg

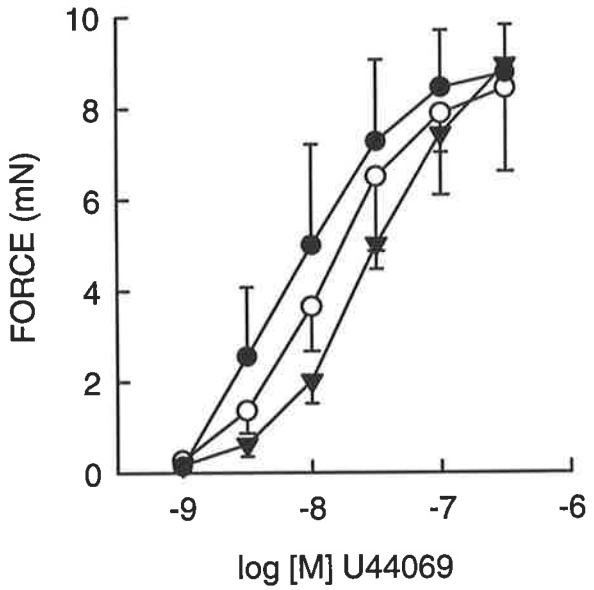

Figure 2.1 Dose response curves of marmoset coronary arteries to U44069 in the absence (o) or presence of EGTA (1mM) in the dissection medium (●) or overnight refrigeration without EGTA (▼); n = 3-6.

(L_{100}) (Mulvany & Halpern, 1977). The internal circumference (L) was calculated from the distance between the supporting wires (x; as determined by utilising the micrometer mounted on one of the supports in the myograph) and the diameter (d) of the two supporting wires; $L = (\pi+2)d + 2x$. The internal diameter of the vessel is then $D = L/\pi$. The wall tension (T) is determined by the force (F, as measured by the transducer in the myograph) and the wall length (2a); $T = F/2a$. Thus, the effective transmural pressure is determined by Laplace's equation, $p = 2\pi T/L$. These calculations were made using a BASIC "normalization" program as described in the myograph manual (Mulvany, 1992). The tension on every vessel was then set at the same point on the passive tension-diameter curve, such that the internal circumference of all vessels was equivalent to 90% of the internal circumference at L_{100}. The arteries were then equilibrated for a minimum of 30 minutes under tension before commencing the experimental protocol.

After the equilibration period the artery segments were contracted with potassium ($\geq$ 40mM) to confirm tissue viability. This was followed by a cumulative DRC to an agonist. At the completion of most experiments a DRC to an endothelium-dependent relaxing agent was obtained in order to assess the integrity of the endothelial layer. Where endothelial integrity is not specifically referred to, no attempt was made to remove the endothelial layer.

2.3 Cyclic AMP Formation

Measurement of cyclic AMP levels was based on the method described by Sumner *et al.* (Sumner *et al.,* 1989). Artery segments were placed in 5 ml PSS containing the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, 100 μM) at 37°C,

bubbled with 95% O_2/5% CO_2 for 30 or 60 min. The vessels then were incubated with the agonists and antagonists of interest, after which they were rapidly frozen in liquid nitrogen.

Subsequently, the tissues were homogenised in 400 µl absolute ethanol using a motorised glass/glass tissue grinder. The homogenate was centrifuged at 12,500 g (max) for 15 min at 4°C and 300 µl of the supernatant extracted and dried under a gentle stream of nitrogen at ambient temperature. The protein pellets were frozen for later determination of protein content by an automated Lowry protein assay (see *2.4*). The dried supernatant extracts were reconstituted and appropriately diluted in assay buffer (0.05 M acetate with sodium azide). The cyclic AMP content of each vessel segment was then determined using a cyclic AMP [^{125}I]scintillation proximity assay kit (Amersham International). All samples were assayed in duplicate. Briefly, each sample was acetylated by the addition of 0.05 volumes of acetic anhydride/triethylamine 1:2. To 100 µl of sample was added 100 µl of adenosine 3',5'-cyclic phosphoric acid 2'-0-succinyl-3-[^{125}I]iodotyrosine methyl ester, 100 µl of rabbit anti-succinyl cAMP serum and 100 µl donkey anti-rabbit IgG coupled to scintillation proximity reagent fluomicrospheres. After mixing on an orbital shaker (200 rpm for 15-20 hours at ambient temperature), CPM were measured in a Packard ß liquid scintillation counter for 2 minutes. Results were expressed as pmol cyclic AMP per mg tissue protein.

2.4 Protein Measurement

The protein concentration of tissue homogenates was determined by the method of Lowry *et al.* (1951) adapted for use on a Cobas-Bio centrifugal analyzer (F. Hoffman-La Roche, Basel, Switzerland) (Clifton *et al.,* 1988). The precipitated protein pellets were

reconstituted by incubation in 1M NaOH for 2 hours at 60°C. The standard samples (final concentration range 0.125 - 1 mg/ml) were prepared from bovine serum albumin and were treated in an identical manner. To 20 µl of sample was added 250 µl of 2% $NaCO_3$ in 0.1 M NaOH/2% NaK tartrate/1% $CuSO_4$ (100:1:1) and allowed to incubate for 60s. To this mixture 50% Folin-Ciocalteu reagent was added and incubated for a further 60s before the absorbance at 750 nm was read. All samples were assayed in duplicate and diluted appropriately in deionised H_2O to fall within the linear range of the assay (0.125 - 0.8 mg/ml). On all assay days an inter-assay coefficient of variation of <1.5% for a potassium dichromate precision assay on the Cobas-Bio centrifugal analyzer was required for assay acceptance.

2.5 Plasma Lipids

2.5.1 Plasma Cholesterol

Total plasma cholesterol concentrations were determined using an enzymatic colorimetric assay kit (Ultimate 7 CHOL, Roche Diagnostics, F. Hoffman-La Roche, based on the method of Allain *et al.* (1974)) conducted on the Cobas-Bio centrifugal analyzer (F. Hoffman La Roche, Basel, Switzerland). Briefly, 10 µl of plasma sample was incubated with 1 ml of reagent solution for 15 min at 25 °C. The reagent solution contained phosphate buffer (pH 7.2, 150 mM), cholesterol esterase (≥1.7 µkat) and cholesterol oxidase (≥1.0 µkat) which catalysed the hydrolysis and subsequent oxidation of the cholesterol esters within the sample to free cholesterol and then cholest-4-en-3-one and hydrogen peroxide. The formation of hydrogen peroxide was quantitated by the method of Trinder (1969). In the presence of 4-aminoantipyrine (0.4 mM), sodium phenolate (7.8 mM) and peroxidase (≥20 µkat) the hydrogen peroxide forms a quinoneimine derivative,

the absorbance of which is determined photometrically at 500 nm. Plasma cholesterol is stable for at least 7 days at room temperature. All samples were assayed in duplicate and quality control samples were included in each assay (Ciba Corning, Australian Diagnostics). On all assay days an inter-assay coefficient of variation of <1.5% for a potassium dichromate precision assay on the Cobas-Bio centrifugal analyzer was required for assay acceptance.

Plasma HDL cholesterol levels were measured similarly, but in plasma from which other lipoproteins had been precipitated with polyethylene glycol (PEG). PEG (20% w/v, pH 10 plus glycine 1.5% w/v) was incubated with plasma (1:1) for 10 minutes at room temperature. The supernatant was isolated by centrifugation at 2800 g for 10 min. The LDL/VLDL plasma cholesterol fraction was determined as the total plasma cholesterol measured less the HDL cholesterol component.

2.5.2 Plasma Triglycerides

Plasma triglyceride levels were determined using an enzymatic colorimetric assay kit (Ultimate 5 TRIG, Roche Diagnostics, F. Hoffman-La Roche) based on the methods of Fossati and Prencipe (1982) and McGowan *et al.* (1983)) on the Cobas-Bio centrifugal analyzer. Briefly, 10 µl of plasma sample was incubated with 1 ml of reagent solution for 20 min at 25 ^{o}C. The reagent solution contained phosphate buffer (pH 7.5, 42mM), lipoprotein lipase (≥50 µkat), adenosine triphosphate (1 mM) and glycerol kinase (≥13 µkat) which catalysed the hydrolysis and subsequent phosphorylation of the triglycerides within the sample to glycerol and free fatty acids and then glycerol 3-phosphate. This serves as a substrate for glycerol phosphate oxidase (≥25 µkat), thus generating hydrogen peroxide. The formation of hydrogen peroxide was quantitated by the method of Trinder

(1969). In the presence of 4-aminoantipyrine (0.5 mM), 4-chlorophenol (6 mM) and peroxidase (≥5 μkat) the hydrogen peroxide forms a quinoneimine derivative, the absorbance of which is determined photometrically at 500 nm. Plasma triglycerides are stable for 3 days at 4 °C (Muhlfellner *et al.,* 1972). All samples were assayed in duplicate and quality control samples were included in each assay (Ciba Corning, Australian Diagnostics). On all assay days an inter-assay coefficient of variation of <1.5% for a potassium dichromate precision assay on the Cobas-Bio centrifugal analyzer was required for assay acceptance.

2.6 Diet Analysis

2.6.1 Total fat

The water content of unpelletted or crushed diets was determined by loss of mass after drying for 24 hours under vacuum at 40 °C. The fat content of marmoset diets was determined in duplicate samples according to the method of Daugherty and Lento (1983) as follows. 5 g samples of diet expected to contain < 10% fat, and 3 g samples of diet expected to contain > 10% fat were incubated with 32 ml (adjusted for water content of diet sample) of 1% clarase in 0.5 M sodium acetate for 1 hour at 45 - 50 °C. The incubation mix was then transferred to a Waring blender and mixed for 2 min with 80 ml methanol and 40 ml chloroform. A further 40 ml of chloroform was added and mixed for 30 sec and then a further 40 ml of water added and mixed for 30 sec. The mixture was then centrifuged at 4000 rpm for 15 min to separate the layers. 20 ml of the chloroform layer was then removed and evaporated in a fumehood and the fat residue dried at 100 °C for 15 min. After cooling to room temperature in a dessicator, the mass of extracted fat residue was determined and the percentage fat content of the diet calculated.

2.6.2 Cholesterol

The cholesterol content of the diets was determined by gas chromatographic analysis of another portion of the chloroform extraction fraction of the diet (*2.6.1*) as follows. These analyses were kindly conducted by Mrs. Cherie Keatch. The internal standard 5α-cholestane was added to samples prior to the extraction procedure described above to give a concentration of 10 μg/ml (*2.6.1*). One ml of the chloroform sample or 65 μl of 5α-cholestane/cholesterol standards in ethyl acetate were evaporated to dryness under a stream of nitrogen. The samples/standards were then dissolved and hydrolysed by the addition of 5 ml of 0.5M KOH in 95% ethanol and incubation for 1 hour at 70°C. The cholesterol was then extracted by the addition of 4 ml water and 4 ml hexane. The mixture was shaken for 30 seconds and the aqueous layer removed. The extraction was then repeated with 2 ml hexane. The hexane solvent was then evaporated to dryness under a stream of nitrogen. The methyl esters were prepared by heating in 50 μl ethylacetate and 50 μl of trifluoroacetic anhydride for 30 min at 50°C. The solution was then cooled and dried under nitrogen, and reconstituted in 50 μl of hexane for injection into a gas chromatograph.

The gas chromatograph utilised was a DANI 6500 with split/splitless injection system (split ratio 1:20) set at a temperature of 250°C and a vitreous silica capillary column (25cm x 0.25mm, 1mm film thickness). The carrier gas was hydrogen, with an inlet pressure of 1.2 bar. The retention times were approximately 5.5 min for 5α-cholestane and 7.4 min for cholesterol.

2.7 Histology

2.7.1 Fixation

Arteries utilised in *in vitro* organ bath studies were fixed by immersion in 1.25% glutaraldehyde (Appendix II).

2.7.2 Processing

The majority of the processing and all of the sectioning of samples was kindly conducted by Dr. Marilyn Henderson of the Centre for Electron Microscopy and Microstructure Analysis (CEMMSA, The University of Adelaide).

Following fixation, tissues were dissected into small (1-3mm) segments for dehydrating (using an automated Lynx processor) and embedding as follows. Sections were washed in phosphate buffered saline (PBS) plus 4% sucrose for 2 x 30 min. Then they were post-fixed in 1% OsO_4 in PBS for 1-2 hours on a rotator. Dehydration consisted of washes in 70% ethanol (3 x 20 min), 90 % ethanol (3 x 20 min), 95% ethanol (3 x 20 min) and 100% ethanol (3 x 20 min, 1 x 1 hour). This was followed by 8 hours overnight in 50% ethanol / 50% resin, then 100% resin (3 x 8 hours at 4°C). Tissues were then embedded in resin by polymerizing at 70°C. Tissues were sectioned as 1 μm thick on an automated microtome with a glass knife.

2.7.3 Staining

Sections were stained using a method based on that by Humphrey and Pittman (1974). Details of the formulae for the stains are given in Appendix II. Sections were transferred to a drop of distilled water on a glass slide and dried on a hotplate at 80-100°C. Then a

drop of methylene blue solution was applied on the hotplate for 2 min. This was rinsed with distilled water and dried. A drop of working solution of basic fuscin was then applied for 30 sec. followed by rinsing and drying. The slides were then mounted in Ghee, covered with a glass cover slip and set at room temperature.

2.8 Data Analysis

In pharmacological experiments the response to an agonist represents the increase or decrease in tension which was maintained at a steady state level. Force of contraction is expressed in absolute (g or mN) terms, ie. not standardised per mg tissue weight, since active tension was demonstrated not to directly correlate with aortic tissue weight ($r = -0.17 \pm 0.39$, $n = 3$). The magnitudes of relaxant responses cited in the test and figure legends and expressed as a % of the pre-contractile vessel tone; all graphical data illustrating relaxant responses express vessel tome as a % of the pre-contractile tone. The molar concentration of an agonist producing 50% of maximal effect (EC_{50}) was calculated utilizing the DResponse program (courtesy of G.A.Crabb, The University of Adelaide) or GraphPad PRISM program (Graphpad Software Inc) and expressed as the negative logarithm to base 10 (pEC_{50}). All results are presented as mean ± standard error of the mean (s.e.m.) and *n* refers to the number of animals.

Data were initially tested for homogeneity of variance with a Bartlett's test. The data were then analysed using parametric or non-parametric tests as appropriate, ie. Student's *t*-tests (with or without Welch's correction) or Mann-Whitney U-test, or one-way ANOVAs (parametric or Kruskal Wallis), utilizing a compiled pharmacological statistics program (Pharcal v 7.1p G.A. Crabb, The University of Adelaide) or GraphPad PRISM program (Graphpad Software Inc). Responses in arteries exposed to a pre-contractile agent at the pre-determined EC_{50} were required to exhibit >20% maximal response to this

concentration for inclusion in final data. These responses were expressed as a percentage of the tone imposed by the pre-contractile agent. EC_{50}s were analysed statistically using the log (pEC_{50}) values. Statistical significance was assumed at the $P<0.05$ level.

CHAPTER 3

GENERAL REACTIVITY OF THE MARMOSET VASCULATURE

3.1 Introduction

The aorta of rodent species (rat, rabbit, guinea-pig) is arguably historically the most commonly used artery in which laboratory research on vascular reactivity is conducted. However, extrapolation of findings between species or even different vascular beds is virtually impossible (see *1.4.2, 1.4.3*). It has been well established that the action of 5-HT on the aorta of the rabbit and rat is predominantly vasoconstrictor and is mediated by the 5-HT_{2A} receptor (Apperley *et al.,* 1976; Cohen *et al.,* 1981; Feniuk *et al.,* 1985; Leff & Martin, 1986; Tagawa *et al.,* 1993; Ogawa *et al.,* 1995). However, the action of 5-HT on the aorta of other species, in particular the primate (including human), has received little attention. Preliminary studies in this author's laboratory have indicated that the response of a primate aorta to 5-HT differs greatly from that of rodent species (Head *et al.,* 1990). Thus, the current study is an expansion of this previous work and aimed to characterise the responsiveness of the aorta from a primate, the marmoset monkey (*Callithrix jacchus jacchus*) to a variety of vasoactive agents, especially 5-HT, in order to provide a baseline for further studies on this vessel.

Whilst the large size of the aorta minimises experimental variation by allowing many experimental replications to be conducted, its pattern of responsiveness is unlikely to mimic that of smaller resistance vessels (see *1.4.3*). It is possible, however, that it may serve as a suitable indicator of the responsiveness of other large conducting vessels within the same species. Therefore, the current study also aimed to determine whether or not the activity of the aorta mimics the pattern of responsiveness of other vessels from the

marmoset, specifically the coronary, carotid and superior mesenteric arteries.

Since the work of Furchgott and Zawadzki in 1980, researchers have become increasingly aware of the role of the endothelium in influencing vascular responsiveness to many agents (see *1.6.1*). Endothelial production of NO, prostacyclin and/or endothelium-derived hyperpolarising factor (EDHF) mediates the vasodilatory actions of many agents and the basal release of these endothelium-derived relaxing agents can also produce physiological antagonism of the actions of vasoconstrictors. Thus, a further aim of the present study was to determine the influence and involvement of endogenous prostanoids or NO in the response of the marmoset aorta to 5-HT.

3.2 Methods

3.2.1 Animals

Adult marmoset monkeys were obtained and tissues collected as described previously (*2.1*).

3.2.2 Vascular Preparations

The aorta, the coronary, carotid and superior mesenteric arteries were prepared and mounted as described previously (*2.2*).

The organ bath protocols followed that detailed in *2.2.1*. In aortic ring preparations, following equilibration and contraction to K^+, contractile agonists tested were noradrenaline (NA, 0.003 - 33 μM), phenylephrine (PE, 0.01 - 33 μM), methoxamine (METH, 0.1 - 333 μM), endothelin-1 (ET, 0.001 - 0.1 μM), the Tx A_2 mimetic U44069

(0.001 - 0.3 μM), histamine (in the absence and presence of cimetidine (100 μM), 1.0 μM - 1 mM), prostaglandin $F_{2\alpha}$ (0.0001 - 1.0 μM), platelet activating factor (0.001 - 1.0 μM) and arginine vasopressin (0.01 - 1.0 μM). 5-HT was applied at basal tone or following pre-contraction with NA or U44069 at the EC_{50}. 5-HT dose response curves (DRCs) were also tested in the presence of the cyclo-oxygenase inhibitor indomethacin (10 μM, NA pre-contraction), NOLA (100 μM, basal tone and NA pre-contraction), dexamethasone (100 nM, NA pre-contraction), the combination of indomethacin (10 μM) and NOLA (100 μM, U44069 pre-contraction), the combination of NOLA (100 μM) and the 5-HT_2 antagonist LY53857 (0.1 μM, basal tone) and rubbed preparations (basal tone, NA and U44069 pre-contraction). Finally, preparations were tested for relaxation to the calcium ionophore A23187 (0.01 - 1.0 μM), or bradykinin (0.001 - 1.0 μM), acetylcholine (0.001 - 10 μM), or substance P (1.0 μM) followed by A23187 (1.0 μM).

In carotid and mesenteric arteries, a DRC to NA (0.007 - 80 μM) or U44069 (0.003 - 0.24 μM) was constructed, followed by a DRC to 5-HT in the presence of NA or U44069 at the EC_{50} and application of A23187 (1.0 μM) after pre-contraction with NA.

In coronary arteries, the agonists NA (0.01 - 100 μM), METH (0.1 μM - 1 mM), U44069 (0.001 - 1.0 μM) and arginine vasopressin (1.0 μM) were tested. A DRC to 5-HT at basal tone or in the presence of U44069 (at the EC_{50}) was constructed, followed by DRCs to bradykinin (0.001 - 1.0 μM), acetylcholine (0.001 - 10 μM) or histamine (0.033 - 33 μM) after pre-contraction with U44069.

3.2.3 Data Analysis

Data analysis was conducted as described previously (*2.8*). All results are presented as mean ± s.e.m. The effects of antagonists on the EC_{50}s and E_{max}s of agonist DRCs and on responses to individual concentrations of 5-HT were assessed using Student's *t*-tests. The

effects of endothelium removal and incubation with NOLA on U44 DRCs, NA DRCs and responses to 5-HT (at each concentration) were compared using an ANOVA.

3.3 Results

3.3.1 Aorta

Contractile responses. The marmoset aorta exhibited concentration-dependent contraction to NA, PE, METH, ET and the Tx A_2 mimetic U44069. The EC_{50} and E_{max} values are in *Table 3.1.* The adrenergic agonists NA (α_1, α_2 and ß), PE (α_1) and METH (α_1) had relative potencies (as calculated from the EC_{50}s and compared with NA) of 1, 0.85 and 10.4 respectively and E_{max}s of a similar magnitude to that observed for exposure to K^+. Histamine also acted as a contractile agent, but the contractions were poorly maintained, both in the absence and presence of cimetidine (100 µM) (*n = 6,4* respectively; data not shown). The marmoset aorta exhibited little or no contractile response to prostaglandin $F_{2\alpha}$ (*n = 3*), platelet activating factor (*n = 2*) or arginine vasopressin (*n = 1*).

Table 3.1 EC_{50} and E_{max} values for contractile agonists in marmoset isolated aortic rings. $^{\#}P < 0.05$ vs. NA.

AGONIST (*n*)	pEC_{50}	E_{max} (g)
Noradrenaline (*7*)	6.0 ± 0.3	2.03 ± 0.37
Phenylephrine (*7*)	6.0 ± 0.1	1.82 ± 0.32
Methoxamine (*6*)	4.9 ± 0.2$^{\#}$	1.84 ± 0.31
U44069 (*7*)	7.8 ± 0.1$^{\#}$	1.05 ± 0.29
Endothelin-1 (*4*)	7.7 ± 0.2$^{\#}$	1.09 ± 0.22

Relaxant responses. In the presence of NA at its EC_{50} the marmoset aorta exhibited strong relaxation to A23187 (1.0 μM, 69.8 ± 3.7%; *n = 7*) but weaker relaxation to bradykinin in the same artery segments (1.0 μM, 38.1 ± 5.3%; *n = 7*). Little or no relaxant response was observed to acetylcholine (10.0 μM, 5.3 ± 2.0% vs. A23187 1.0 μM, 67.3 ± 5.8%; *n = 10*) or substance P (1.0 μM 5.8 ± 5.8% vs. A23187 1.0 μM, 89.4 ± 3.2%; *n = 2*).

Responses to 5-HT. Responses to 5-HT were tested in preparations at basal tone and contracted with NA and U44069 (depicted in *Figure 3.1*). At basal tone (endothelium intact, other agents not present), 5-HT 0.001 - 10 μM was either without effect or caused slight contraction. In NA-contracted arteries, 5-HT (0.001 - 0.1 μM) produced either slight contraction or relaxation, but in higher concentrations (1.0 - 10 μM) consistently produced relaxation, sometimes preceded by a transient contraction (see *Figures 3.2a, 3.4*). The latter response was consistently observed in vessels at various levels of pre-imposed tone (NA at its EC_{30}, EC_{55} or EC_{80}, *n* = 5-10; data not shown). Note:- Subsequent analysis of the response to 0.1 μM 5-HT in the NA-contracted artery from male animals revealed a modest but significant negative correlation between relaxation and age (r=0.71, *n* = 49; P<0.0001; *Figure 3.3*). This correlation, however, only appears to be strong in animals aged above approximately 70 months.

In contrast, when the tone was elevated by U44069, the effect of 5-HT was predominantly excitatory (*Figure 3.1, 3.2b*). The threshold for contraction was between 0.001 and 0.01 μM and the maximum was in the vicinity of 0.1 - 1.0 μM. The response to 1.0 μM 5-HT declined after reaching its peak value and only relaxation was seen when the concentration was increased to 10 μM. DRCs to 5-HT did not significantly alter with sequential repetition. This applied when either NA or U44069 was the pre-contractile agent (*Figure 3.2 a,b*).

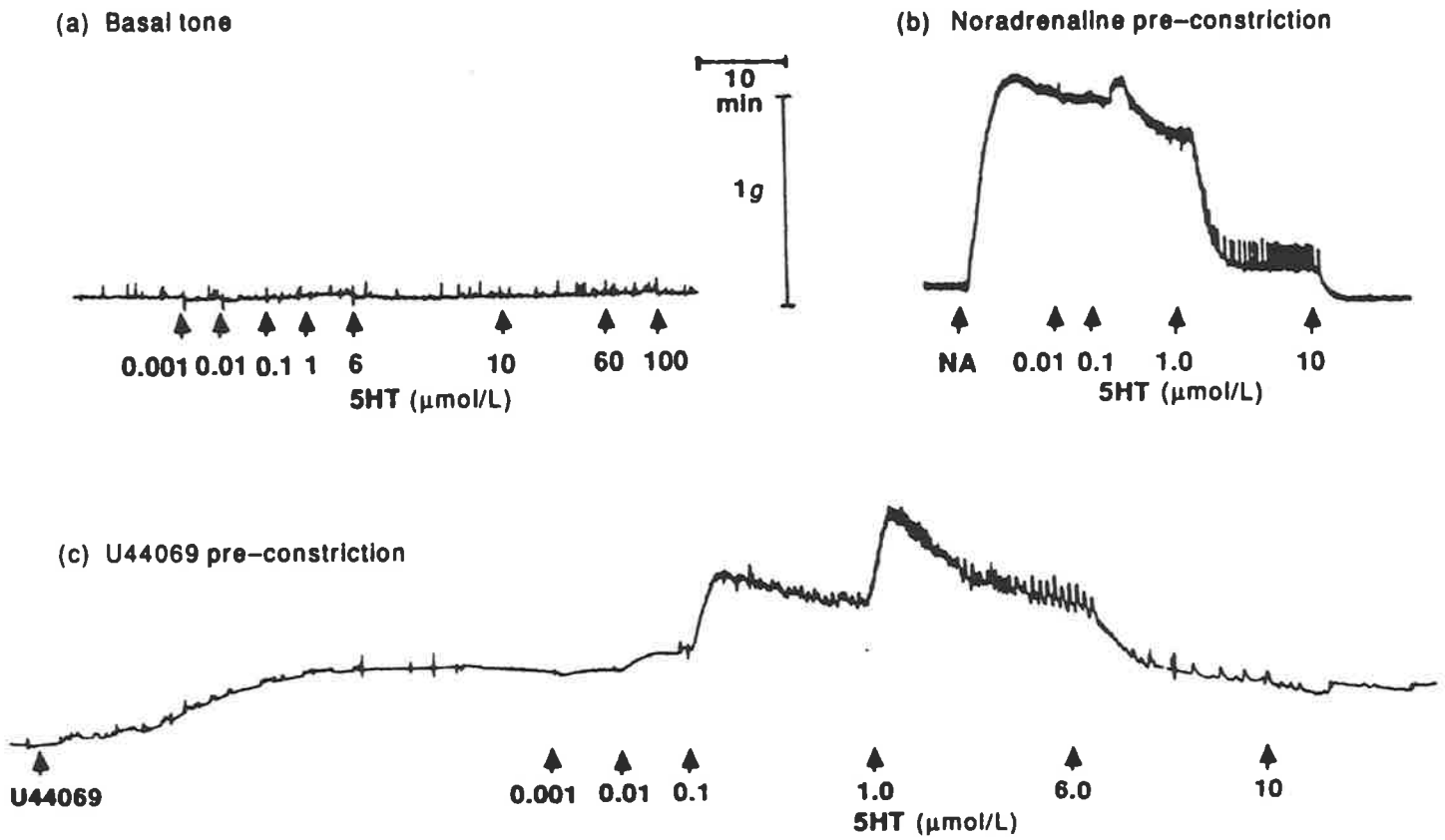

Figure 3.1 Typical responses of the marmoset aorta to 5-HT at (a) basal tone and in the presence of EC_{50} concentrations of (b) noradrenaline or (c) U44069.

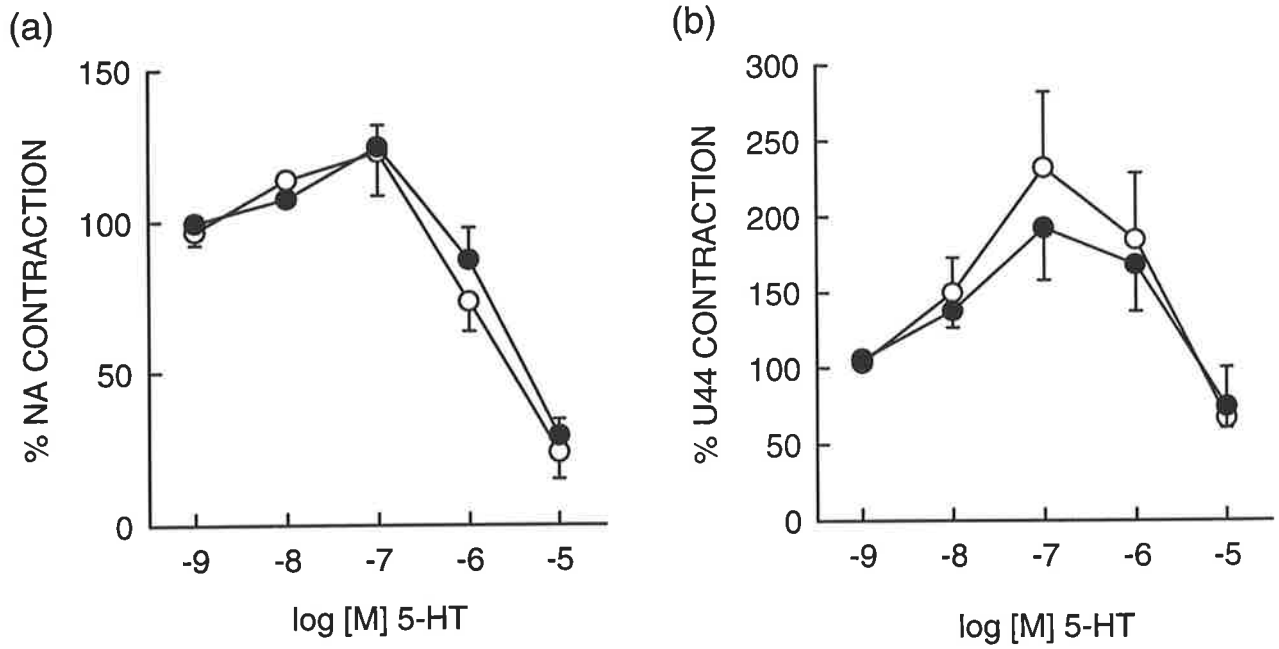

Figure 3.2 Sequential repetition of 5-HT dose response curves in aortic rings pre-contracted with (a) noradrenaline or (b) U44069 at the EC_{50}; n = 5.
○ initial DRC; ● second DRC. NOTE: differing Y-axis scales.

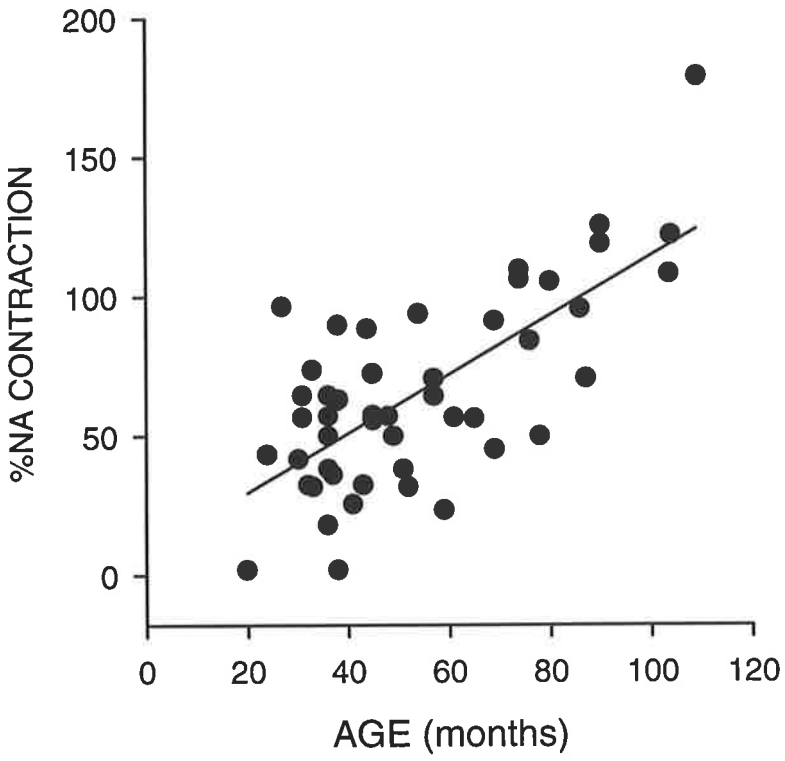

Figure 3.3 Response to 0.1μM 5-HT in aortic rings pre-contracted with NA versus animal age; n = 49; r = 0.71.

The cyclo-oxygenase inhibitor indomethacin (10 μM) was without effect on the relaxation to 5-HT in NA-contracted arteries (*Figure 3.4*). The NA DRC was also unaffected by indomethacin (*Table 3.2*). In NA-contracted preparations, endothelium-denuded arteries exhibited significantly reduced relaxation to A23187 (0.1 μM; 4.5 ± 3.1 % vs. 61.1 ± 8.4 % relaxation in rubbed vs. unrubbed arteries) and significantly increased sensitivity to NA (*Table 3.2*), but an unaltered response to 5-HT (*Figure 3.5*). The inhibitor of NO synthase, NOLA (100 μM), abolished the relaxant response and unmasked a contractile response to low concentrations of 5-HT; however, the strong component of relaxation at 10 μM was unaffected (*Figure 3.5*). NOLA also decreased the EC_{50} for the DRC to NA (*Table 3.2*). The response to 5-HT (0.001 - 10 μM) in NA pre-contracted marmoset aortic rings was not affected by incubation with dexamethasone (100 nM) throughout the standard protocol (*n = 3*, data not shown).

Table 3.2 EC_{50} and E_{max} values for noradrenaline (NA) or U44069 (U44) dose response curves in marmoset isolated aortic rings, in the absence and presence of indomethacin (INDO, 10 μM), endothelium removal (ENDO), or N-nitro-L-arginine (NOLA, 100 μM). $^{\#}P < 0.05$ vs. control; $^{*}P < 0.05$ vs. U44±ENDO.

	CONTROL		WITH TREATMENT	
AGONIST (*n*)	pEC_{50}	E_{max}(g)	pEC_{50}	E_{max}(g)
NA ± INDO (*6*)	6.5±0.1	3.43±0.23	6.6±0.2	3.29±0.28
NA ± ENDO (*5*)	6.1±0.1	2.82±0.28	6.5±0.1$^{\#}$	3.30±0.35
NA ± NOLA (*5*)	6.1±0.1	2.82±0.28	6.9±0.1$^{\#}$	3.94±0.48
U44±ENDO(*6-8*)	7.9±0.2	1.07±0.30	8.3±0.1$^{\#}$	1.99±0.17$^{\#}$
U44±NOLA&INDO (*5-6*)	7.9±0.2	1.07±0.30	8.1±0.0	3.05±0.13$^{\#*}$

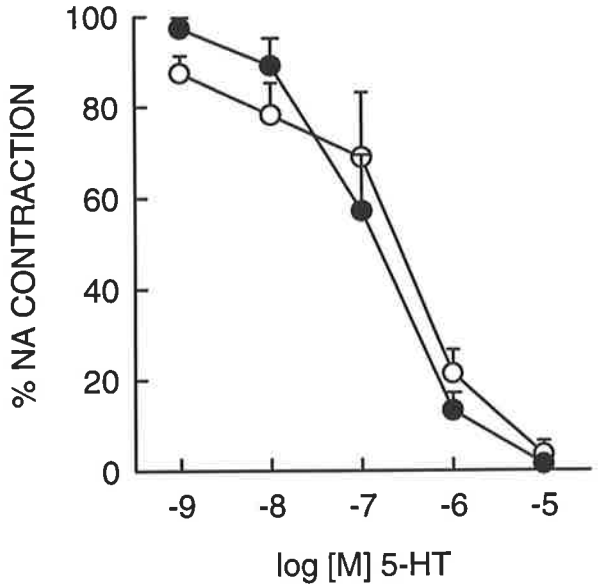

Figure 3.4 5-HT dose response curves in aortic rings pre-contracted with noradrenaline at the EC_{50} and in the absence (o) or presence (●) of indomethacin (10μM); n = 6.

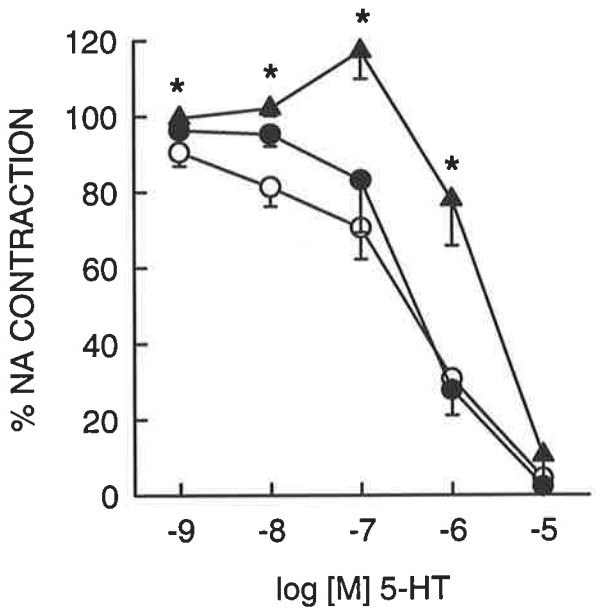

Figure 3.5 5-HT dose response curves in aortic rings pre-contracted with noradrenaline at the EC_{50} and under control conditions (o), or endothelium denuded (●), or in the presence of NOLA (100 μM, ▲); n = 5.
* $P < 0.05$, NOLA vs. control. NOTE: For graphical purposes, control data was pooled. Statistical analysis was confined to experimental groupings.

In an experiment utilising aortae from female marmosets the contractile action to 5-HT in U44069 pre-contracted preparations was weak, but removal of the endothelium (relaxation to 1.0 μM A23187 0 % vs. 88.2 ± 9.8 % in rubbed vs. unrubbed preparations) or incubation with NOLA (100 μM) plus indomethacin (10 μM) unmasked strong contractile actions which were of a comparable magnitude (*Figure 3.6*). Inhibition of endothelial function by both mechanical and chemical means significantly increased the E_{max} for the DRCs to U44069 and endothelial removal decreased the EC_{50} (*Table 3.2*). The E_{max} was increased by a significantly greater amount by NOLA+INDO than by mechanical endothelium removal alone.

At basal tone, vessels with intact endothelium were unresponsive to 5-HT. Consistent, although weak, contractile responses to 5-HT were elicited in endothelium-denuded arteries, as well as in arteries exposed to NOLA (100 μM; *Figure 3.7*). The combination of LY 53857 (0.1 μM) and NOLA (100 μM) further increased the contractile response to 5-HT. Thus in response to 1.0μM 5-HT, the contraction increased from 112 ± 29 mg tension (NOLA alone) to 364 ± 52 mg tension ($P<0.01$; *t*-test, $n = 5$; *Figure 3.7*). Characteristic of the response was that it was well sustained at concentrations of 5-HT to 1.0 μM, but it displayed pronounced fade from a peak value at higher concentrations.

3.3.2 Common Carotid Artery

The common carotid artery with intact endothelium exhibited a profile of responses to NA, U44069, 5-HT and A23187 which was similar to those of the aorta. NA and U44069 elicited concentration-dependent contractions in segments of common carotid artery, with pEC_{50}s of 6.7 ± 0.3 ($n = 6$) and 7.7 ± 0.1 ($n = 5$) and E_{max}s of 1.52 ± 0.13 and 1.03 ± 0.10 g respectively (*Figure 3.8 a,b*).

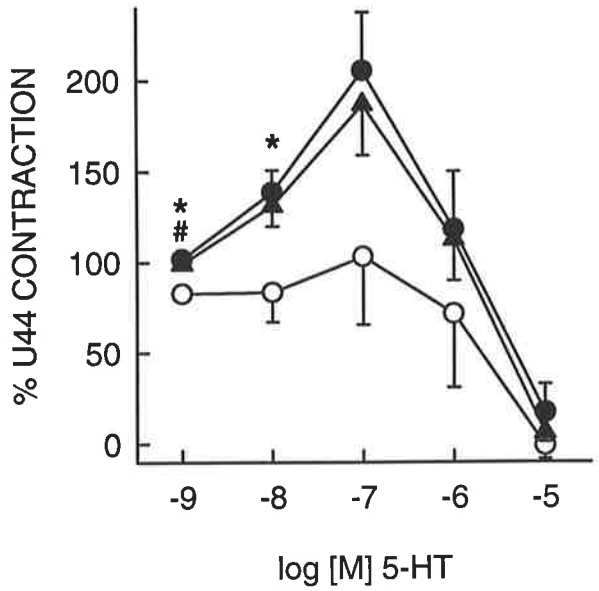

Figure 3.6 5-HT dose response curves in aortic rings pre-contracted with U44069 at the EC_{50} and under control conditions (○), or endothelium denuded (●), or in the presence of NOLA (100 μM) plus indomethacin (10 μM; ▲); n = 5-8; * $P < 0.05$, denuded vs. control, $^{\#}P < 0.05$, NOLA vs. control.

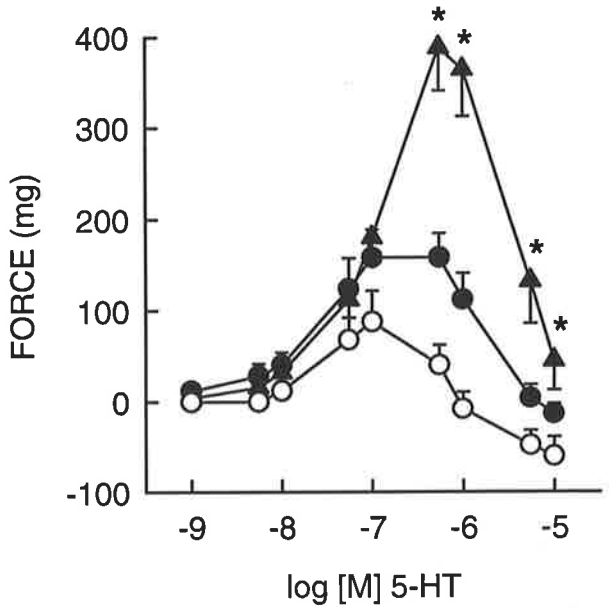

Figure 3.7 5-HT dose response curves in aortic rings at basal tone and which were endothelium denuded (○), or incubated with NOLA (100μM, ●), or incubated with NOLA (100 μM) plus LY53857 (0.1μM, ▲), 5-HT induced nil contraction in control arteries; n = 5. *$P < 0.05$ denuded vs. NOLA plus LY53857.

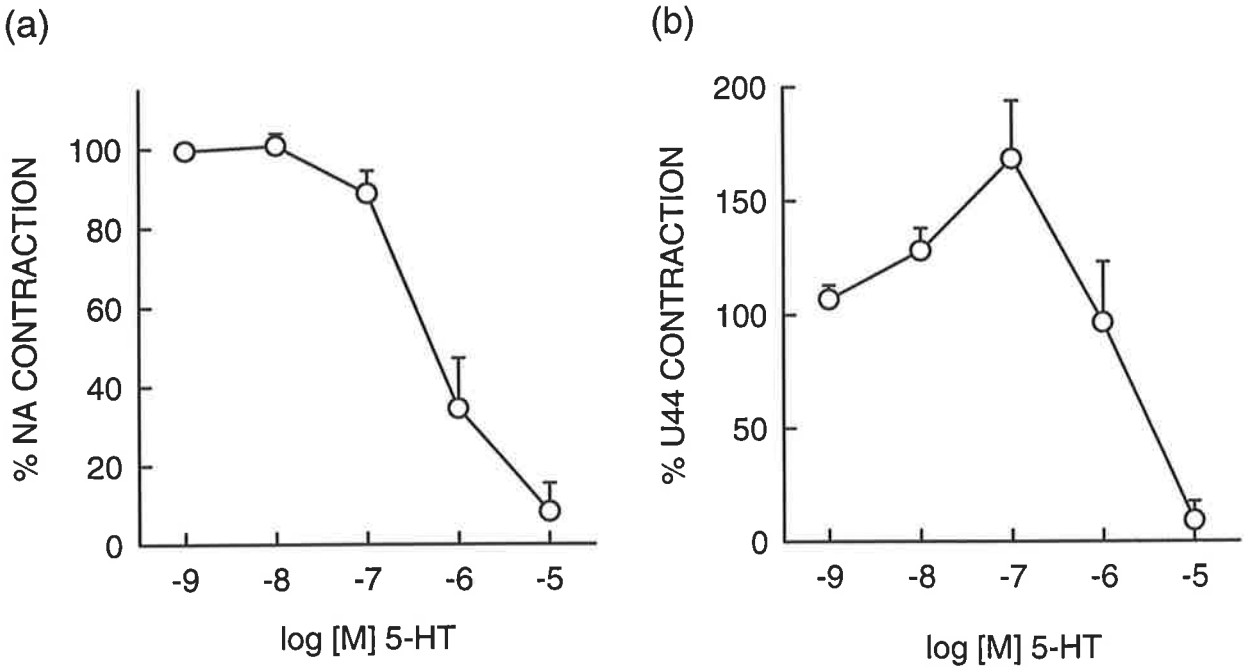

Figure 3.8 5-HT dose response curves in carotid arteries pre-contracted with (a) noradrenaline (n = 6) or (b) U44069 (n = 5) at the EC_{50}. Relaxation to A23187 (1 μM) in these vessels was (a) 72.3 ± 2.6 % and (b) 81.4 ± 5.2 %.

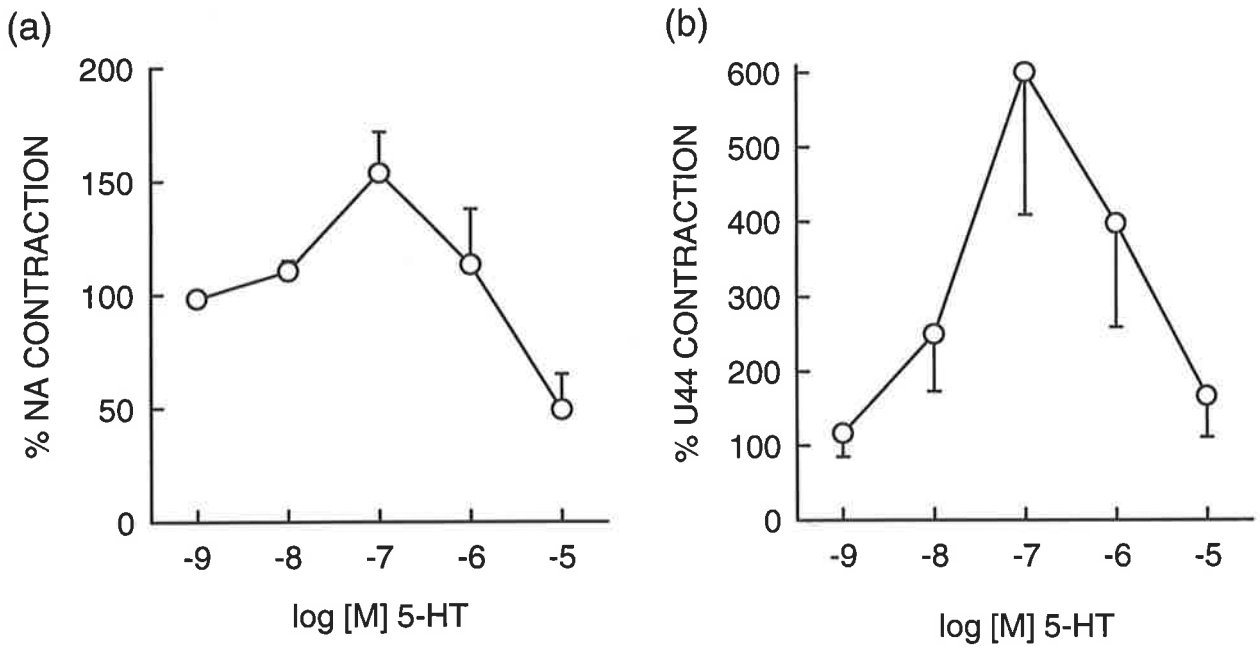

Figure 3.9 5-HT dose response curves in mesenteric arteries pre-contracted with (a) noradrenaline (n = 6) or (b) U44069 (n = 3) at the EC_{50}. Relaxation to A23187 (1 μM) in these vessels was (a) 92.7 ± 2.5 % and (b) 76.4 ± 20.6 %.

3.3.3 Superior Mesenteric Artery

Ring preparations of superior mesenteric artery also exhibited a profile of responses to NA, U44069, 5-HT and A23187 which was similar to those of the aorta and common carotid artery. NA and U44069 elicited concentration-dependent contractions in segments of superior mesenteric artery with pEC_{50}s of 5.5 ± 0.2 and 7.4 ± 0.1 and E_{max}s of 1.06 ± 0.13 and 0.47 ± 0.20 g respectively (*Figure 3.9 a,b*).

3.3.4 Coronary artery

Contractile responses. Segments of marmoset coronary artery exhibited strong concentration-dependent contractions to U44069 (as depicted in *Figure 2.1*) with a pEC_{50} of 7.8 ± 0.1 (n = 14) and an E_{max} of 8.01 ± 0.54 mN (n = 21). However, NA elicited little or no contractile effect (< 10 % of U44069 E_{max}; n = 7). Methoxamine exhibited weak and variable contractile responses (n = 6). Arginine vasopressin (1 μM) had a weak and transient contractile effect, amounting to 10.5 ± 4.1 % of the U44069 E_{max} (n = 3). In view of these findings, only U44069 was used as a contractile agent in further studies.

Relaxant responses. Coronary arteries contracted with U44069 at its EC_{50} were relaxed by BK (maximum relaxation at 1.0 μM was 72.0 ± 8.3%, pEC_{50} = 8.0 ± 0.2, n = 7), acetylcholine (maximum relaxation at 10 μM was 60.1 ± 15.2%, pEC_{50} = 7.1 ± 0.5, n = 4) and histamine (maximum relaxation at 33 μM was 79.8 ± 0.8%, pEC_{50} = 5.9 ± 0.1, n = 2).

5-HT responses. At basal tone, 5-HT in the range 0.001 - 1.0 μM produced a weak dose-dependent contraction in coronary arteries (*Figure 3.10*). Although the pEC_{50} for contraction was 7.1 ± 0.2, the E_{max} was only 9 % of the E_{max} for U44069. In artery segments pre-contracted with U44069 at its EC_{50}, the contraction to 5-HT (0.001 - 0.1

μM) was greater but at higher concentrations (1.0 - 10 μM) 5-HT produced a decline in tension, but not to a level less than the initial U44069 contraction (*Figure 3.10*).

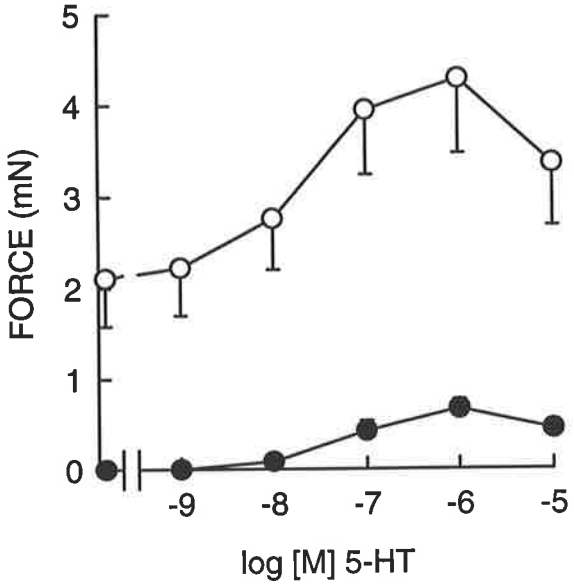

Figure 3.10 5-HT dose response curves in coronary arteries at basal tone (●), or pre-contracted with U44069 at the EC_{50} (o); n = 6-8. Relaxation to bradykinin (1 μM) in these vessels was 72.0 ± 8.3 %. NOTE: First data point indicates level of pre-contraction.

3.4 Discussion

In marmoset aortic ring preparations adrenergic agonists behaved as full tissue agonists. The ratio of potencies of the adrenergic agonists noradrenaline (NA), phenylephrine (PE) and methoxamine (METH) in the marmoset aorta were comparable with those reported in binding studies on α_{1A} adrenergic receptors (1.0, 0.9 and 4.5 for NA, PE and METH respectively (Schwinn *et al.,* 1990)), indicating that this receptor most likely mediates the contractile action of adrenergic agonists in this vessel.

In vessels where the endothelial function was inhibited either mechanically or chemically the E_{max} for contraction to U44069 was significantly increased. However, this was not the case with NA (*Table 3.2*). In endothelium intact vessels, the U44069-induced maximal contractile force in the aorta was less than that induced by the α-agonists (although not significantly). Thus endothelium-derived relaxing factors inhibit U44069-induced, but not NA-induced, contraction. The combination of NOLA and indomethacin increased the E_{max} for U44069 contraction to a greater extent than endothelium removal alone. Thus, endothelium-independent prostanoids may also suppress U44069-induced contraction. It may be that there is less receptor reserve for Tx receptors than α-adrenergic receptors in this tissue, such that U44069-induced contraction is unable to overcome the inhibitory effects of EDRFs in endothelium-intact vessels. This situation has important physiological implications. *In vivo*, vessel segments with intact endothelial function will have an endogenously limited ability to contract to Tx A_2 released from aggregating platelets, thus preserving patency in undamaged sections of arteries during vessel repair. *In vitro*, physiological inhibition of contractile responses by endothelium-derived NO has been demonstrated in many isolated arteries (Topouzis *et al.,* 1991; Woodman, 1995; Schwarzacher *et al.,* 1996).

The failure to observe an excitatory action of 5-HT in the marmoset isolated aorta under basal conditions (ie. at basal tone, with endothelium-intact and other agents not present) may be due to masking by either a simultaneous inhibitory action of 5-HT (as observed in the presence of NA) and/or basally released EDRFs. A strong contractile effect of 5-HT was observed when the tone of the isolated preparation was elevated by U44069. This effect of U44069 is most likely to be due to the well-documented amplifying interaction whereby 5-HT and a second contractile agent enhance each other's excitatory actions (de la Lande *et al.,* 1966; de la Lande, 1989; Tanner *et al.,* 1991b; Cocks *et al.,* 1993). In particular, $PGF_{2\alpha}$ and another Tx A_2 agonist, U46619, have been shown to unmask contractile effects of 5-HT in the guinea-pig ileal artery (Sahin-Erdemli *et al.,* 1991) and to enhance 5-HT's contractile effects in the rabbit femoral artery (MacLennan & Martin, 1992). Unmasking of excitatory effects of 5-HT (in the range 0.0001 - 1.0 μM) by precontraction with a Tx A_2-mimetic has also been demonstrated in the rabbit isolated mesenteric artery (Choppin & O'Connor, 1995). However, the failure of 5-HT to elicit strong contraction when the tone was elevated by NA is unexpected, particularly since mutual amplification has been demonstrated between 5-HT and NA in other vascular preparations (Van Nueten, 1987; de la Lande, 1990). The discrepancy between the interactions of 5-HT with NA and U44069 is addressed further in a later chapter (*Chapter 6*).

The role of the endothelium. The endothelium appears to play a role in inhibiting the contractile activity of 5-HT at low concentrations (0.001- 0.1 μM), as demonstrated by the effects of endothelium removal (*Figure 3.6, 3.7*). The fact that this effect was mimicked to the same or a greater extent by NOLA, but not indomethacin (*Figure 3.4*) indicates that the EDRF involved in this effect of the endothelium is NO, not a prostanoid. It is difficult to determine whether this effect is purely due to a masking effect of basally

released NO, or an endothelium-dependent relaxant action of 5-HT itself, particularly since no significant effect of endothelium removal was detected in the NA contracted artery (*Figure 3.3*). The lack of effect of endothelium removal, however, may be due to the fact that endothelium removal (although significant) was not complete in these arteries. Nevertheless, blockade of relaxation and unmasking of 5-HT's contractile activity by exposure to NOLA was evident in the NA contracted preparation. Thus it appears that there is a component of relaxation which is endothelium dependent, but which is confined to low concentrations of 5-HT. It is noteworthy that this is the same concentration range at which the contractile response to 5-HT occurs. In isolated vessels precontracted with a second agent, 5-HT has been shown to cause endothelium-dependent relaxation in a variety of vessels including the jugular vein of the rat, rabbit and guinea-pig (Martin *et al.,* 1987; Gupta, 1992; Bodelsson *et al.,* 1993; Ellis *et al.,* 1995). However, it is clear that the effect of endothelium-derived NO in the marmoset aorta is only significant at low concentrations of 5-HT and that the major relaxant response to 5-HT at higher concentrations (> 1.0 μM) is independent of endothelium or NO (*Figures 3.5, 3.6*).

The apparent enhancement of the contractile activity of 5-HT in the presence of the 5-HT receptor antagonist LY53857 (*Figure 3.7*) also suggests that 5-HT's excitatory activity is not only masked by basal NO production, but also by an inhibitory action of 5-HT itself. The endothelium-independent relaxant effect of 5-HT observed at higher concentrations in the marmoset aorta is investigated in Chapter 5.

In view of the evidence that endothelium-derived NO influences the response to low concentrations of 5-HT in the marmoset aorta, the variability in response to 5-HT at these low concentrations observed in control data with different experimental groups throughout this thesis may be due to innate differences in endothelial function between animal groups of different age and sex (due to limited animal availability it was not possible to use

animals of a constant age and sex in different experiments). The negative correlation between increasing age and relaxation to 0.1 μM 5-HT in the NA pre-contracted artery would tend to support this possibility. Other researchers have demonstrated influences of age and sex upon endothelium-dependent responses (Mantelli *et al.,* 1995; Taddei *et al.,* 1995; Gerhard *et al.,* 1996; Vita *et al.,* 1990; Hayashi *et al.,* 1992). However, these factors were minimised in the current studies by using as controls vessel rings from the same animal wherever possible.

Others have reported activation of the inducible form of NO synthase in the vascular smooth muscle of aortic rings in *in vitro* preparations, probably due to the presence of endotoxin in the bathing solution (Rees *et al.,* 1990a; Moritoki *et al.,* 1992). Expression of this inducible NO synthase in vascular tissue can be prevented by glucocorticoids (Radomski *et al.,* 1990; Rees *et al.,* 1990a; Moritoki *et al.,* 1992). Since dexamethasone had no effect on the response to 5-HT in the marmoset aortic ring preparations, it is concluded that inducible NOS has not been activated under the current experimental conditions and is not responsible for the apparent involvement of NO in this response.

An amplification interaction between 5-HT and U44069 was demonstrated in all vessels tested, ie. the aorta, carotid and mesenteric arteries, and the coronary artery. Qualitatively, the responses to 5-HT in the marmoset aorta are very similar to those observed in the carotid artery. However, the balance between the contractile and relaxant effects of 5-HT appears to favour contraction in the mesenteric artery since relaxation to 10 μM 5-HT in the presence of NA was reduced in this artery compared with the carotid artery or aorta. Importantly, the amplification interaction occurring between 5-HT and U44069 in the marmoset artery has also been demonstrated in the human coronary artery (Cocks *et al.,* 1993).

There was no evidence for a strong relaxant response to 5-HT in the coronary artery. Since NA alone did not contract the coronary artery preparation, pre-contraction with this agent to establish conditions which were optimal for relaxation in the aorta was not possible. Canine and porcine large coronary arteries exhibit endothelium-dependent relaxation to 5-HT, when 5-HT_{2A} receptor mediated vasoconstriction is blocked with ketanserin (Cocks & Angus, 1983; Richard *et al.,* 1990; Schoeffter & Hoyer, 1990). Endothelium-dependent relaxation to 5-HT has not, however, been demonstrated in the human isolated large coronary artery (in the presence of ketanserin to inhibit 5-HT_{2A}-mediated contractions), despite intact endothelium-dependent relaxation in response to other agents (Berkenboom *et al.,* 1989; Toda & Okamura, 1989). Whilst the current study did not utilize ketanserin to assist in unmasking any relaxant responses present in the coronary artery, relaxant responses were observed in other vessels from the marmoset in the absence of this agent. Thus 5-HT induced relaxation is not apparent in the marmoset coronary artery under the conditions tested (endothelium-intact, other agents not present). This again highlights the difficulties associated with extrapolating results from different vascular beds and from non-primate species to the human.

In conclusion, the present study has shown that 5-HT exerts complex actions on the marmoset aorta which may be inhibitory or excitatory depending on the presence and nature of a second contractile agent and the integrity of the endothelium. Although the endothelium (by generating NO) plays a role in affecting the responsiveness of the marmoset aorta to 5-HT at low concentrations (0.001 - 1.0 μM), the major components of 5-HT-induced contraction and relaxation appear to be endothelium-independent. In summary, the multiplicity of actions of 5-HT in the marmoset aorta distinguishes this vessel from the aortae of common laboratory animals (rat and rabbit), where the main response to 5-HT is endothelium-independent contraction which does not require the

presence of a second agent for its manifestation.

CHAPTER 4

EXCITATORY ACTIONS OF 5-HYDROXYTRYPTAMINE IN THE MARMOSET VASCULATURE

4.1 Introduction

The responsiveness of the marmoset isolated aorta to 5-hydroxytryptamine (5-HT) was demonstrated in the previous chapter to be quite different to that of aortae from rodent species. In the latter, the action of 5-HT is contractile and is mediated by 5-HT_{2A} receptors (Apperley *et al.,* 1976; Cohen *et al.,* 1981; Leff & Martin, 1986; Ogawa *et al.,* 1995). In contrast, at resting tone the marmoset aorta is unresponsive to 5-HT, but elevation of the tone of the preparation by the Tx A_2 mimetic U44069 or inhibition of endothelial NO production reveals a contractile response to 5-HT. The aim of the present study was primarily to determine the receptor subtypes involved in this contractile action of 5-HT in the marmoset aorta and to obtain some insight into whether the same receptors mediate contraction in the marmoset coronary artery.

4.2 Methods

4.2.1 Animals

Adult marmoset monkeys were obtained and tissues collected as described previously (*2.1*). NOTE: Male animals were used throughout except in one instance where only a group of female animals were available. In this group contractile responses to 5-HT in the

U44069 precontracted vessels were relatively weak (Figure 4.2 in Results), but whether this was the reason for the variation was not investigated further.

4.2.2 Vascular Preparations

The aorta and coronary artery was prepared and mounted as described previously (*2.2*).

The organ bath protocols followed the outline detailed in *2.2.1*. Investigations were conducted in aortic rings in which the conditions for the contractile activity of 5-HT were optimal (as established in Chapter 3), ie. either (i) contracted with U44069 or (ii) at basal tone but incubated with NOLA (100 μM) plus LY 53857 (0.1 μM). The effects of four 5-HT receptor antagonists were examined, namely methysergide (1.0 μM, 5-HT_1 and 5-HT_2), ketanserin (1.0 μM, 5-HT_{2A}), LY 53857 (0.1 μM, 5-HT_2) and GR 127935 (0.01 μM, 5-HT_{1B} (non-rodent) and 5-HT_{1D} receptor and 5-HT_1-like) on 5-HT's contractile activity were studied using procedure (i). The effects of the antagonists tropisetron (at 8.0 μM an antagonist of both 5-HT_3 and 5-HT_4 receptors (Bockaert *et al.,* 1992)) and GR 127935 were studied using procedure (ii). A comparison of the relative potencies of the 5-HT agonists 5-HT, 5-CT, sumatriptan and 8-OH-DPAT was conducted using procedure (ii). An investigation of the agonist effects of methysergide utilised procedure (i). In addition, identical experiments to those carried out with 5-HT as the agonist in Figure 3.5 were conducted with sumatriptan as the agonist, ie. at basal tone with endothelium intact, absent, in the presence of NOLA (100 μM) and in the presence of NOLA (100 μM) plus LY 53857 (0.1 μM). The effect of GR 127935 (0.01 μM) on contraction to sumatriptan was assessed using procedure (ii).

NOTE: Exceptions to the standard protocol outlined in *2.2.1* were the series of experiments on the effect of the 5-HT receptor antagonists ketanserin and methysergide

where two 5-HT DRCs were constructed, the first in the absence and the second in the presence of the antagonist (the two curves did not differ significantly in the absence of antagonists, see *Figure 3.1b*). The effect of ketanserin was assessed by comparing the second DRCs from separate artery rings, one of which was not treated with the antagonist (parallel control). The effect of methysergide was assessed by comparing the two DRCs conducted in the same artery ring (sequential control). However, this method proved extremely time consuming and was abandoned in favour of procedures (i) and (ii).

In coronary artery preparations, studies on the effect of GR 127935 (0.01 μM) on the response to 5-HT in U44069 precontracted preparations were conducted utilising a protocol identical to procedure (i). The actions of the 5-HT receptor agonist 5-CT and sumatriptan were studied in coronary arteries contracted with U44069 at the EC_{50}.

4.2.3 Cyclic AMP Formation

For investigation of the effects of 5-HT on cyclic AMP formation, aortic rings were incubated in IBMX (100 μM) for 60 min. Subsequently, the rings were incubated with 5-HT (0.1 μM), forskolin (5 μM) or vehicle (ethanol) for 2 min before being rapidly frozen in liquid nitrogen. 5-HT was used in a concentration of 0.1 μM which was between the EC_{50} and EC_{100} for contraction. Either the $5\text{-}HT_2$ antagonist LY 53857 (0.1 μM) or saline were added 30 min prior to addition of 5-HT. The cyclic AMP content was then determined as previously described (*2.3*).

4.2.4 Data Analysis

Data analysis was conducted as previously described (*2.8*). The effects of antagonists were assessed using a Student's *t*-test or Mann-Whitney U-test. Apparent EC_{50}s for 5-HT

receptor agonists were estimated from the maximum sustained contractile response, utilising the DResponse program (courtesy G.A. Crabb, The University of Adelaide). Cyclic AMP data was analysed using an ANOVA with a Newman-Keuls post-hoc test.

4.3 Results

4.3.1 Aortic Rings Precontracted with U44069

In preparations contracted with U44069, 5-HT elicited contractile responses in the concentration range 0.001 - 1.0 μM and relaxation at higher concentrations. Experiments conducted under these conditions were confined to the effects of the 5-HT receptor antagonists ketanserin (1.0 μM, 5-HT_{2A}), LY 53857 (0.1 μM, 5-HT_2), methysergide (1.0 μM, 5-HT_1 and 5-HT_2) and GR 127935 (0.01 μM, 5-HT_{1B} (non-rodent) and 5-HT_{1D} receptor and 5-HT_1-like). Ketanserin (1.0 μM; data not shown) and LY53857 (0.1μM; *Table 4.1*) were without effect on the level of contraction to U44069. However, their effects on the contractile action of 5-HT differed widely in that a) ketanserin was without effect (*Figure 4.1*) whilst b) LY 53857 markedly enhanced the contractile response to 5-HT (0.1 μM; *Figure 4.2*). In contrast, GR 127935 (0.01 μM) eliminated 5-HT's contractile activity and unmasked relaxant responses in U44069 contracted preparations (*Figure 4.3*). GR 127935 itself did not elicit contraction, although it tended to increase the response to U44069, manifested by a decrease in the EC_{50} and an increase in the E_{max} of the initial U44069 DRC (*Table 4.1*). The effect of methysergide was less straightforward because it contracted the U44069 pre-contracted artery (pEC_{50} = 7.60 ± 0.07), with a maximum in the vicinity of 1.0 μM (*Figure 4.4*). In the presence of 1.0 μM methysergide, 5-HT did not elicit any further contraction and its relaxant activity was greatly diminished (*Figure 4.4*).

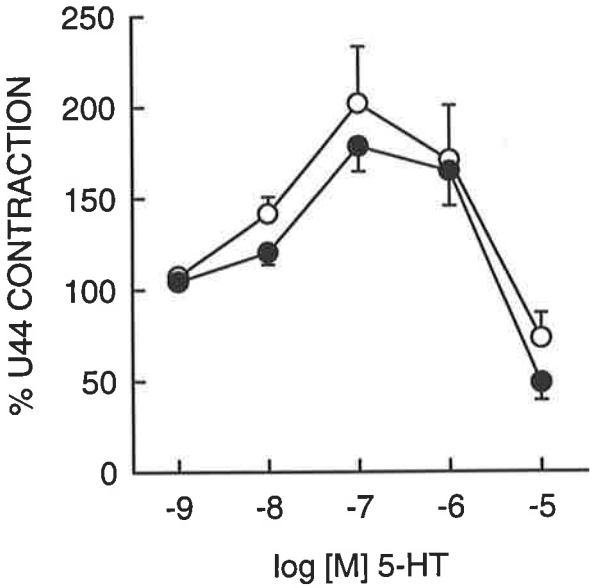

Figure 4.1 5-HT dose response curves in aortic rings pre-contracted with U44069 at the EC_{50} and in the absence (o) or presence (●) of ketanserin (1.0 μM); n = 5.

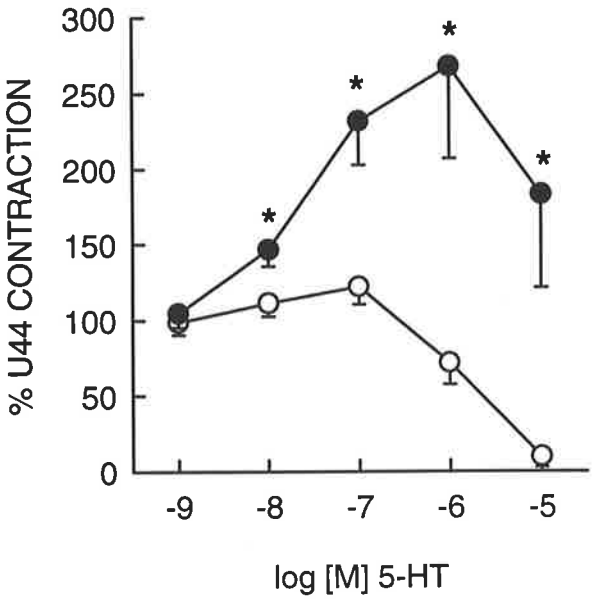

Figure 4.2 5-HT dose response curves in aortic rings pre-contracted with U44069 at the EC_{50} and in the absence (o) or presence (●) of LY 53857 (0.1 μM); n = 8. *P < 0.05.

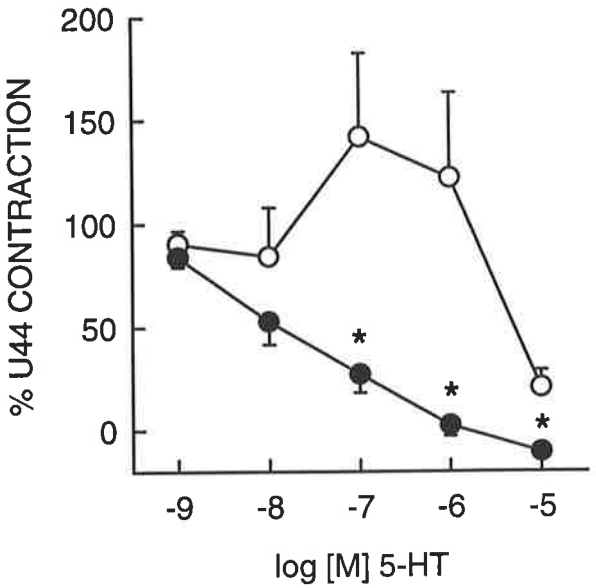

Figure 4.3 5-HT dose response curves in aortic rings pre-contracted with U44069 at the EC_{50} and in the absence (o) or presence (●) of GR 127935 (0.01 μM); n = 6. *P < 0.05.

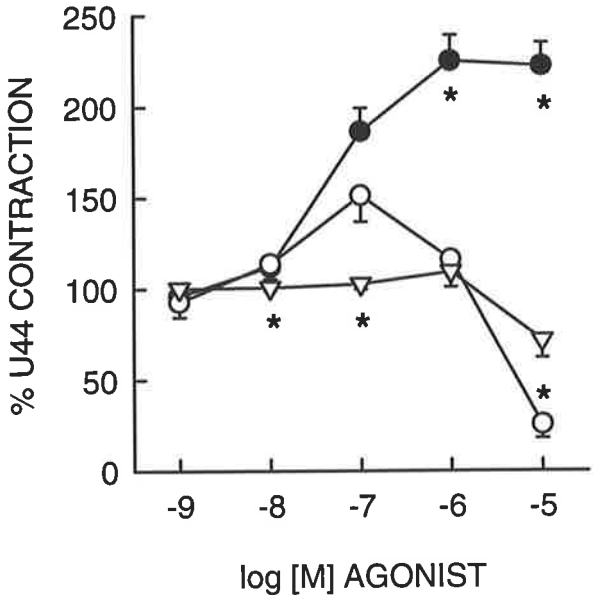

Figure 4.4 Dose response curves of aortic rings pre-contracted with U44069 at the EC_{50} to 5-hydroxytryptamine (o), or methysergide (●), or 5-hydroxytryptamine in the presence of methysergide (▽); n = 5-8. *P < 0.05, vs. 5-HT alone. NOTE: For graphical purposes, control data was pooled. Statistical analysis was confined to experimental groupings.

Table 4.1 EC_{50} and E_{max} values for U44069 (U44) dose response curves in aortic rings, in the absence and presence of LY 53857 (LY, 0.1 μM; n = 8) or GR 127935 (GR, 0.01 μM; n = 7). $^{\#}P < 0.05$ vs. control.

	CONTROL		WITH ANTAGONIST	
	pEC_{50}	E_{max}(g)	pEC_{50}	E_{max}(g)
U44 ± LY	7.9 ± 0.1	1.78 ± 0.28	7.8 ± 0.1	1.46 ± 0.31
U44 ± GR	7.9 ± 0.1	1.34 ± 0.15	8.1 ± 0.1$^{\#}$	1.86 ± 0.16$^{\#}$

4.3.2 Aortic Rings at Basal Tone

In the presence of NOLA (100 μM) and LY 53857 (0.1 μM), the effect of 5-HT was compared with that of the 5-HT receptor agonists 5-CT (5-HT_1), sumatriptan (5-HT_{1B} (non-rodent) and 5-HT_{1D}) and 8-OH-DPAT (5-HT_{1A}). Each of these agents elicited contraction, with pEC_{50} values of 8.46 ± 0.04, 7.47 ± 0.06, 6.65 ± 0.09 and 5.75 ± 0.10 for 5-CT (n=6), 5-HT (n=5), sumatriptan (n=6) and 8-OH-DPAT (n=6), respectively (*Figure 4.5*). Thresholds for contraction were 0.001-0.01, 0.01-0.1, 0.01-0.1 and 0.1-1.0 μM for 5-CT, 5-HT, sumatriptan and 8-OH-DPAT, respectively. The rank order of relative potencies derived from the EC_{50} values is summarised in *Table 4.2*.

Table 4.2 Contractile potencies of 5-hydroxytryptamine agonists relative to 5-HT, calculated from the apparent EC_{50} values. [a]Data from Humphrey *et al.* (1988) and Perren *et al.* (1991) .

TISSUE	5-CT	5-HT	Suma	8-OH-DPAT
Marmoset aorta	9.9	1.0	0.14	<0.018
Dog saphenous vein[a]	3.3	1.0	0.22	0.015

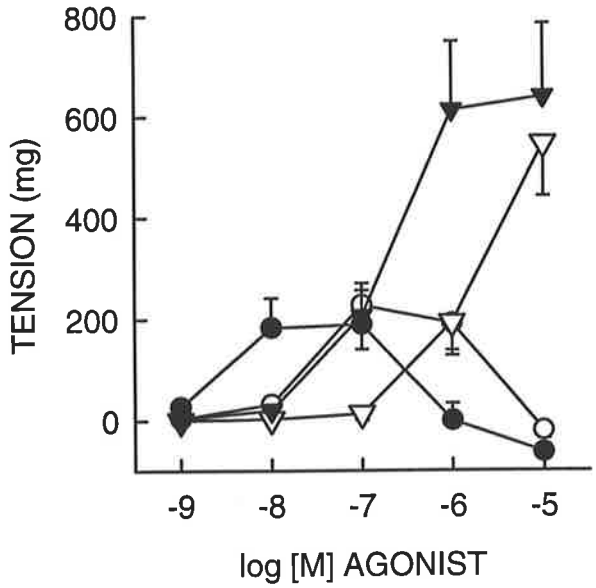

Figure 4.5 Dose response curves of aortic rings to 5-HT (○), 5-CT (●), sumatriptan (▼), or 8-OH-DPAT (▽) at basal tone and in the presence of NOLA (100 μM) plus LY 53857 (0.1 μM); n = 5-6.

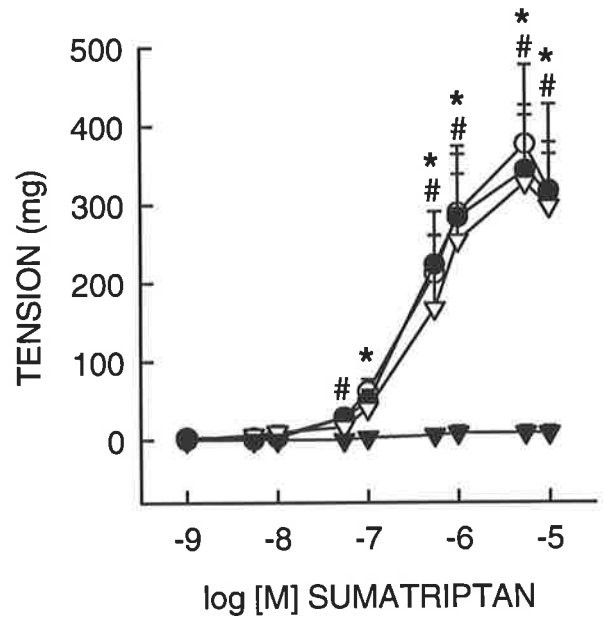

Figure 4.6 Sumatriptan dose response curves in aortic rings (▼), or aortic rings which were endothelium denuded (▽), or incubated with NOLA (100 μM, ○), or incubated with NOLA (100 μM) plus LY 53857 (0.1 μM, ●); n = 6-8. # $P < 0.05$ control vs. NOLA plus LY 53857; * $P < 0.05$ control vs. NOLA alone

The effects of the endothelium, LY53857, GR127935 and threshold concentrations of U44069 on contraction to sumatriptan were also investigated. Arteries at basal tone were unresponsive to sumatriptan, but contractile activity was observed in arteries in which endothelial function was reduced (*Figure 4.6*). Endothelial denudation was manifest by a decrease in relaxation to A23187 (1.0 μM) from 91.5 ± 6.1% to 8.7 ± 4.4% (n = 6). Contraction to sumatriptan at concentrations $\geq$0.1μM were significantly enhanced by incubation with NOLA (100 μM; *Figure 4.6*). LY 53857 (0.1 μM) did not further affect the response to sumatriptan in arteries exposed to NOLA (100 μM; *Figure 4.6*). In the presence of NOLA (100 μM) and LY 53857 (0.1 μM), the contractile response to sumatriptan was completely abolished by GR 127935 (0.01 μM, n = 3; data not shown).

The effect of tropisetron (8.0 μM, 5-HT_3 and 5-HT_4) on 5-HT's contractile action was examined in the arteries at basal tone with NOLA (100μM) and LY 53857 (0.1μM) present; it was without a statistically significant effect (n = 7; data not shown).

4.3.3 Coronary Arteries Precontracted with U44069

In coronary arteries contracted with U44069, GR 127935 (0.01 μM) abolished the contractile response to 5-HT and unmasked relaxant activity (*Figure 4.7*). Neither the EC_{50} nor the E_{max} of the DRC to U44069 were affected by GR 127935 (pEC_{50}s were 7.78 ± 0.06 vs. 7.78 ± 0.19 and E_{max}s were 7.91 ± 1.05mN vs. 6.61 ± 1.52mN in the absence and presence of GR 127935, respectively). 5-CT and sumatriptan both acted as contractile agonists in coronary arteries precontracted with U44069 (*Figure 4.8*). The pEC_{50} values for contraction were 8.32 ± 0.12, 7.64 ± 0.06 and 7.12 ± 0.09 for 5-CT, 5-HT and sumatriptan, respectively.

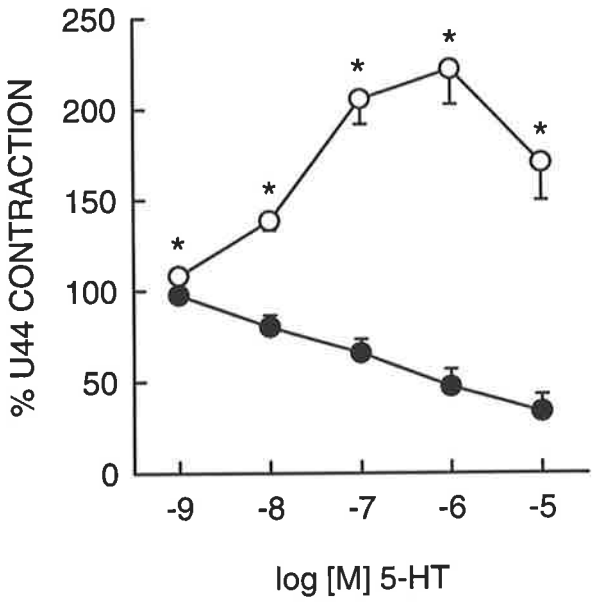

Figure 4.7 5-HT dose response curves of coronary arteries in the absence (○) or presence (●) of GR 127935 (0.01 μM); n = 7. *P < 0.05.

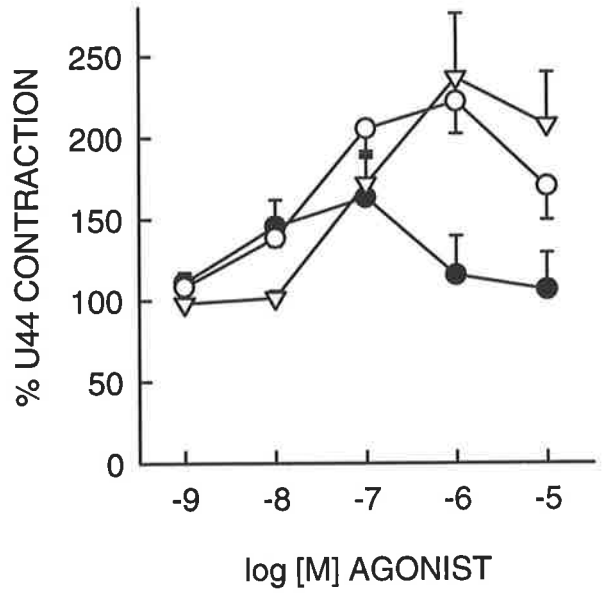

Figure 4.8 Dose response curves of coronary arteries to 5-HT (○), 5-CT (●) or sumatriptan (▽) in the presence of U44069 at the EC_{50}; n = 7.

4.3.4 Cyclic AMP

The basal level of cyclic AMP in rings of aorta incubated with the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) for 60 minutes was 2.39 ± 0.43 mg^{-1} protein. Neither 5-HT (0.1 μM) nor sumatriptan (1.0 μM) had any effect on basal cyclic AMP accumulation (n = 5; data not shown). 5-HT reduced cyclic AMP formation slightly, but not significantly, when the latter had been increased by forskolin (5.0 μM; *Figure 4.9*). In the presence of LY53857 (0.1 μM) inhibition was more pronounced; in the absence of 5-HT, LY 53857 was without effect (*Figure 4.9*).

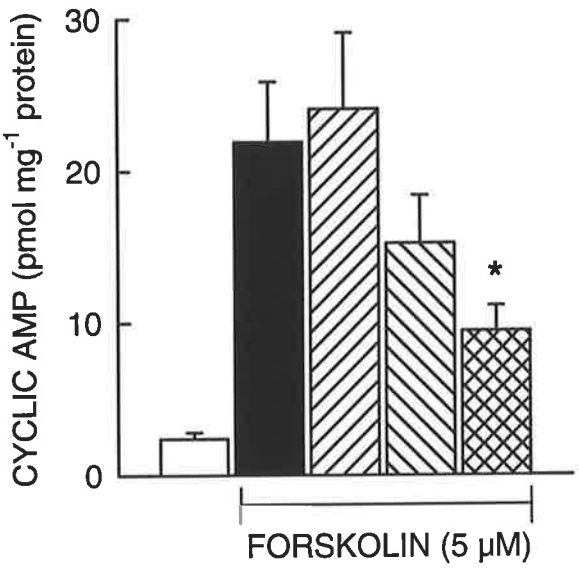

Figure 4.9 Cyclic AMP levels (pmol mg^{-1} protein) in aortic rings incubated with 3-isobutyl-1-methyl-xanthine (100 µM) for 60 min and (a) vehicle ( ), (b) forskolin (5 µM;), (c) forskolin plus LY 53857 (0.1 µM;), (d) forskolin plus 5-hydroxytryptamine (5-HT, 0.1 µM;), or (e) forskolin plus LY 53857 and 5-HT ( ); n = 4-5. $^{*}P < 0.05$ vs. forskolin alone.

4.4 Discussion

The effects of 5-HT receptor agonists and antagonists in the marmoset aorta point strongly to a 5-HT_1-like receptor as the mediator of 5-HT's contractile action. The effects of antagonists accord with the various operational criteria for a 5-HT_1-like receptor as defined by Hoyer *et al.* (1994) as follows (i) insensitivity of 5-HT's action to ketanserin in a concentration adequate for 5-HT_{2A} receptor blockade and to tropisetron in a concentration adequate for blockade of both 5-HT_3 and 5-HT_4 receptors (this study), (ii) a contractile response to methysergide, which conforms with evidence that this agent behaves as a partial agonist at the 5-HT_1-like receptor in other vessels including the rabbit ear artery (de la Lande *et al.,* 1966; Martin & MacLennan, 1990; Gupta, 1992) and (iii) inhibition of contractile responses to 5-HT and to sumatriptan by GR 127935 in a low nanomolar concentration. GR 127935 has been demonstrated to be a potent and selective antagonist of the 5-HT_{1B} (non-rodent) and 5-HT_{1D}-like receptor in dog saphenous vein (Skingle *et al.,* 1995; De Vries *et al.,* 1997).

Another operational criterion for characterising the 5-HT_1-like receptor is agonist potencies in the order 5-CT ≥ 5-HT > sumatriptan > 8-OH-DPAT. In arteries exposed to NOLA and LY 53857 the marmoset aorta exhibits the same rank order when the apparent EC_{50}s are used as a guide to potency (see comparison with agonist potencies in the dog saphenous vein, the prototype tissue for the 5-HT_1-like receptor, *Table 4.2*). The rank order differs only with respect to the relative positions of 5-HT and sumatriptan (which become equipotent) when contractile threshold concentrations are used as the guide. The latter measurement is probably more reliable since the apparent EC_{50} assumes that the observed maximum is the true maximum. This assumption may not be valid for 5-HT and 5-CT since these agonists became relaxant instead of contractile at high concentrations and their peak contractile effects were considerably smaller than those of sumatriptan and 8-

OH-DPAT (which were relatively well sustained over the range of concentrations tested and did not exhibit relaxant effects). However, the equipotent ranking of 5-HT and sumatriptan (based on threshold contractile potencies) does not weaken the evidence for a 5-HT_1-like receptor since the comparative summary in Table 6 of Hoyer *et al.* (1994) indicates a wide variation in relative potencies and includes another vessel (rabbit renal artery) in which sumatriptan and 5-HT are equipotent. Furthermore, the measured apparent EC_{50} of sumatriptan (pEC_{50} = 6.65) is within the range of the pEC_{50}s quoted by Hoyer *et al.* (1994) for activation of 5-HT_1-like receptors in other vessels, namely 5.8 - 8.2. A proviso is that the potency of 8-OH-DPAT, although low, may be overestimated since the response observed at 10 μM may not have attained its maximum, but again this has no effect upon the rank of 8-OH-DPAT in the relative potencies.

A property common to all receptors in the 5-HT_1 class is negative coupling to adenylate cyclase. Thus, further insight into the 5-HT receptors mediating contraction was sought by examining the effects of 5-HT on cyclic AMP formation. 5-HT was used in a concentration of 0.1 μM which was optimal for contraction but usually threshold for relaxation in the pharmacological studies. In the present study, inhibition of forskolin-stimulated cyclic AMP formation is indicative of negative coupling to adenylate cyclase, as has been similarly demonstrated in the dog saphenous vein (Sumner & Humphrey, 1990). The finding that the inhibitory effect of 5-HT on cyclic AMP accumulation in the marmoset aorta was increased in the presence of LY53857, an agent which facilitated the contractile effect, points to an association between these effects.

In some arteries the 5-HT_1-like receptor mediates an amplifying interaction between 5-HT and a second contractile agent. This type of interaction has been documented in the rabbit ear artery (de la Lande, 1992) and presumably occurs in other arteries where the presence of a second contractile agent is required before 5-HT_1-like receptor activation is

manifested, eg. rabbit iliac, femoral and mesenteric arteries (MacLennan & Martin, 1992; Choppin & O'Connor, 1995; Yildiz & Tuncer, 1995), guinea-pig iliac artery (Sahin-Erdemli *et al.,* 1991). In this respect the amplifying interaction between 5-HT and U44069 as well as methysergide and U44069 in the marmoset aorta can be viewed as additional evidence for a 5-HT_1-like receptor.

Hoyer *et al.* (1994) point out that the properties of the 5-HT_1-like receptor distinguish it from the various 5-HT_1 binding sites. However, the high potencies of 5-CT, sumatriptan and GR 127935 exhibited for the marmoset aorta (this study) and the dog saphenous vein (Perren *et al.,* 1991; Skingle *et al.,* 1995) indicate similarities to the 5-HT_{1B} (non-rodent) and 5-HT_{1D} receptor and it is noteworthy that expression of 5-$HT_{1D\beta}$ receptor mRNA has been demonstrated in the dog saphenous vein (Cushing *et al.,* 1994) as well as human aortic smooth muscle (Ullmer *et al.,* 1995).

Although the present study has focussed on the contractile component of 5-HT's action, the results provide some additional information on the nature of the relaxant activity. The extent of this activity was highlighted in the U44069 contracted arteries where blockade of contractile responses to 5-HT by GR127935 unmasked relaxation responses extending down to the lowest concentration of 5-HT tested (0.001 μmol/L). The ability of NOLA to facilitate the appearance of the contractile activity in arteries at basal tone (Figure 3.5) suggested that some of the relaxation may be mediated by endothelium-derived nitric oxide (EDNO). However the difficulty with this interpretation is that NOLA produced comparable increases in the contractile effect of sumatriptan, although this agonist appears to be devoid of relaxant properties. (The lack of further effect of LY53857 above that of NOLA provides support for the lack of relaxant effect of sumatriptan. However, investigation of the effects of sumatriptan in the presence of GR127935 and a pre-contractile agent is required to confirm a lack of effect on an endothelium-dependent

relaxant receptor at low concentrations.) This implies that inhibition of basal generation of EDNO is largely responsible for the effect of NOLA on the response to sumatriptan and presumably also on the response to 5-HT.

In the marmoset coronary artery, the ability of the 5-HT_{1B} (non-rodent) and 5-HT_{1D} receptor antagonist GR 127935 to block the contractile effect of 5-HT provides some evidence that a 5-HT_1-like receptor may also mediate contraction in this vessel, as do the high agonist potencies of 5-CT and sumatriptan. However, the lack of information regarding other antagonists means that the possibility that other receptor subtypes may play a role cannot be ruled out and classification as a 5-HT_1-like receptor is indicated but not conclusive. The 5-HT_1-like receptor has been implicated in the contractile action of dog coronary arteries (Parsons *et al.,* 1992; Cushing *et al.,* 1994) and in the human coronary artery both 5-HT_2 and 5-HT_1-like receptors contribute to 5-HT-induced contraction (Connor *et al.,* 1989b; Bax *et al.,* 1993; Kaumann *et al.,* 1994), whereas only 5-HT_2 receptors mediate contraction in rat coronary arteries (Nyborg, 1991). 5-$HT_{1D\beta}$ mRNA is expressed in porcine coronary arteries (Ullmer *et al.,* 1995). The involvement of 5-HT_1-like receptors to 5-HT induced contraction in human coronary arteries is also apparent *in vivo* (MacIntyre *et al.,* 1993), thus it is likely that this receptor also mediates 5-HT-induced contraction in the primate aorta. Blockade of contraction by GR 127935 revealed the presence of a relaxant response to 5-HT in the coronary artery, but the nature of the receptors mediating this response were not investigated.

In summary, the marmoset aorta joins the growing number of vessels in which the contractile effect of 5-HT is mediated by a 5-HT_1-like receptor. In many vessels the 5-HT_{2A} receptor also contributes (Connor *et al.,* 1989b; Connor *et al.,* 1989a; MacLennan & Martin, 1992; Noguchi *et al.,* 1993; Kaumann *et al.,* 1994), but insensitivity to ketanserin argues against a significant contribution from it in the marmoset aorta. The present

findings represent the first evidence for the presence of a functional 5-HT_1-like receptor in the aorta of any primate species, including human.

CHAPTER 5

INHIBITORY ACTIONS OF 5-HYDROXYTRYPTAMINE IN THE MARMOSET VASCULATURE

5.1 Introduction

The previous two chapters have demonstrated that the responsiveness of the marmoset isolated aorta to 5-HT is quite different from that of rodent species. In arteries contracted with U44069 or in arteries at basal tone with endothelial function impaired, a contraction mediated by a 5-HT_1-like receptor is predominant. When the tone of the preparation is elevated with a second contractile agent, 5-HT exhibits an inhibitory response, the strongest component of which (at concentrations above 1.0 μM) is endothelium-independent (*Chapter 3*). The aim of the present study was to determine the 5-HT receptor subtype mediating this low-affinity relaxant action in the marmoset aorta and to obtain some insight as to whether or not this is the same receptor as that mediating relaxation in the marmoset common carotid artery.

5.2 Methods

5.2.1 Animals

Adult marmoset monkeys were obtained and tissues collected as described previously (*2.1*).

5.2.2 Vascular Preparations

The aorta and carotid artery were prepared and mounted as described previously (*2.2*).

The organ bath protocols followed the outline detailed in *2.2.1*. Investigations were conducted in aortic rings precontracted with NA (the conditions under which relaxation was optimal). The 5-HT receptor agonists utilised were 5-CT (5-HT_1), 5-methoxytryptamine (5-MeOT, 5-HT_4), 8-OH-DPAT (5-HT_{1A}) and sumatriptan (5-HT_{1B} (non-rodent) and 5-HT_{1D}). The effects of the 5-HT receptor antagonists methysergide (5-HT_1 and 5-HT_2), LY 53857 (5-HT_2) and tropisetron (at 10 μM an antagonist at 5-HT_3 and 5-HT_4 (Bockaert *et al.*, 1992)) on responses to 5-HT were investigated. The use of ketanserin in the concentration range commonly used to assess 5-HT receptor activity was vitiated by its α-adrenergic antagonistic effects. The effects of the phosphodiesterase inhibitor IBMX (10 μM) on 5-HT responses were also investigated. Subsequent to the dose response curve (DRC) to 5-HT in some control arteries, the actions of forskolin were studied by the addition of NA at the EC_{50} followed by forskolin (10 μM). Estimations of the potency of 5-HT and 5-CT were also conducted in the presence of NOLA (100 μM) plus GR 127935 (0.1 μM).

NOTES: *i*) A single exception to the standard protocol outlined in *2.2.1* was the series of experiments investigating the effect of the 5-HT_2 antagonist LY 53857, which was conducted by Ms Sotiria Bexis. These arteries were contracted with 0.6 μM NA and a (control) cumulative 5-HT DRC (0.01 - 10 μM) was constructed. Following wash out, the same artery was again contracted with NA (0.6 μM), then LY 53857 (0.1 μM) added and allowed to incubate for 5 min before construction of a second 5-HT DRC. The effect of LY 53857 was assessed by comparing the second and first DRCs from the same artery ring (sequential control). *ii*) The experiment investigating the relative potencies of 5-CT

and 5-HT in arteries incubated with NOLA and GR 127935 were conducted by Mr Mark Mano. *iii*) GR 127935 was not available when the initial experiments were conducted.

In carotid artery preparations, the effects of the 5-HT receptor agonists 5-CT and sumatriptan were compared with that of 5-HT in arteries contracted with NA at the EC_{50}.

5.2.3 Cyclic AMP Formation

Further insight into the 5-HT receptors mediating relaxation was sought by examining the effects of 5-HT on cyclic AMP formation. Measurement of cyclic AMP levels was based on the method described by Sumner *et al.* (Sumner *et al.*, 1989) and as described previously (*2.3*). Rings of thoracic aorta, 2-3 mm in length (6 per animal) were placed in 5 ml PSS containing the phosphodiesterase inhibitor 3-isobutyl-1-methyl-xanthine (IBMX, 100μM) at 37°C and bubbled with 95% O_2, 5% CO_2 for 30 or 60 min. The rings were then incubated with either the antagonist or saline for 30 min, after which they were exposed to saline or 5-HT (100nM or 10μM) for 2 min before being rapidly frozen in liquid nitrogen. The antagonists utilised were LY 53857 (0.1μM) and methiothepin (1.0μM). In addition the left anterior descending and left circumflex coronary arteries were incubated with IBMX (100μM) for 60 min, followed by incubation with saline or 5-HT (10μM) for 2 min and snap freezing in liquid nitrogen.

5.2.4 Data Analysis

Data analysis was conducted as previously described (*2.8*). The EC50s for the 5-HT agonists were determined assuming a maximum relaxation of 100%, using GraphPad Inplot v.4.05 (GraphPad Software Inc). The effects of antagonists on organ bath responses were assessed with Student's *t*-tests (paired for LY 53857). Cyclic AMP data was

analysed utilising ANOVAs with a Tukey's post-hoc test.

5.3 Results

5.3.1 Aortic Relaxation

Arteries which were contracted with NA relaxed in response to 5-HT. 5-CT, 8-OH-DPAT and 5-MeOT also exerted relaxant effects when tested in concentrations up to 10 μM (*Figure 5.1*). Sumatriptan was inactive as a relaxant agent in the same concentration range (n=4; data not shown). The pEC_{50} values (with s.e.mean) were 5-CT 7.6 (0.1); 5-HT 6.1 (0.1); 5-MeOT 5.5 (0.2) and 8-OH-DPAT 4.9 (0.2). The potencies relative to 5-HT (= 1.0) are given in *Table 5.1*. Endothelium removal was without effect on the pEC_{50} for 5-CT (relaxation to A23187 was 84.4 ± 9.6% vs. 0% in endothelium-intact vs. -denuded preparations, n = 4), but significantly reduced relaxation to a single concentration of 5-CT only (10 nM, data not shown). In preparations incubated with NOLA (100 μM) and GR 127935 (0.1 μM) the high relative potency of 5-CT compared to that of 5-HT was maintained (pEC_{50}s 5-CT 9.1 ± 0.1, 5-HT 7.3 ± 0.2; n = 4; 5-CT relative potency = 59).

Table 5.1 Relaxant potencies of 5-hydroxytryptamine agonists relative to 5-HT, calculated from the apparent EC_{50}s.

TISSUE	5-CT	5-HT	5-MeOT	8-OH-DPAT	SUMA
Marmoset aorta	29	1.0	0.21	0.05	inactive
Dog coronary[a]	6.3	1.0	0.01	inactive	inactive
Pig vena cava[b]	50	1.0	N.A.	<0.02	inactive

N.A. = data not available.
[a]Data calculated from Figure 3, Cushing and Cohen (1992) .
[b]Data from Sumner *et al.* (1989) .

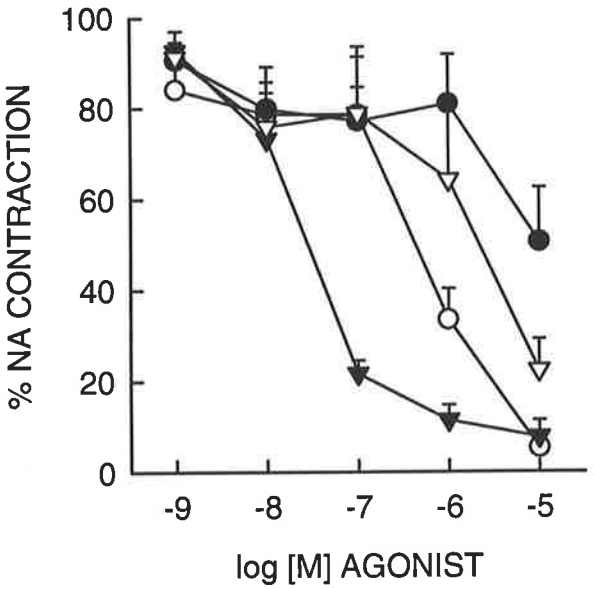

Figure 5.1 Dose response curves of aortic rings pre-contracted with NA at its EC_{50} to 5-HT (o), 5-CT (▼), 5-methoxytryptamine (▽), or 8-OH-DPAT (●); n = 5-6.

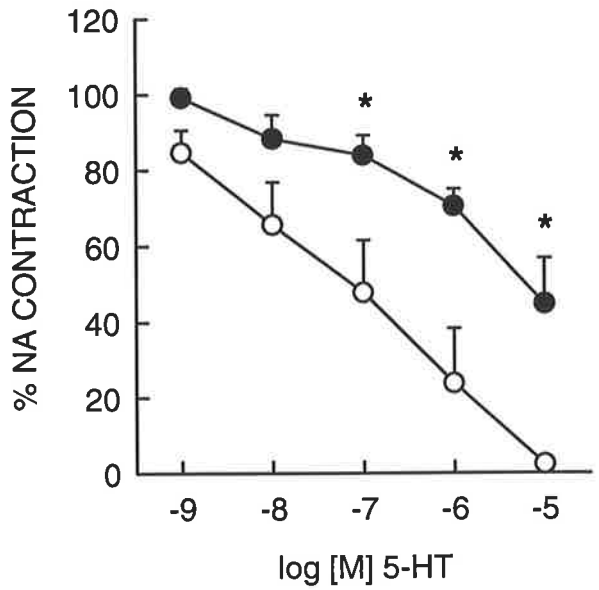

Figure 5.2 5-HT dose response curves of aortic rings precontracted with NA at the EC_{50} and in the absence (o), or presence (●) of methysergide (1.0 μM); n = 5. *P < 0.05.

Methysergide (1.0 μM), an antagonist at multiple 5-HT receptors, caused marked blockade of relaxant activity (*Figure 5.2*) without affecting the EC_{50} or E_{max} of the NA DRC (*Table 5.2*). LY 53857 (0.1 μM, 5-HT_2) significantly reduced relaxation to 5-HT (*Figure 5.3*). LY 53857 (0.1 μM) itself did not elicit a response in NA contracted arteries. Tropisetron (an antagonist of 5-HT_3 and 5-HT_4 receptors at 10 μM) was without significant effect on 5-HT relaxant activity (*Figure 5.4*) or the NA DRC (*Table 5.2*). The phosphodiesterase inhibitor IBMX (10 μM) slightly, but not significantly shifted the curve for relaxation to 5-HT to the left (*Figure 5.5*) and was without effect on the response to NA (*Table 5.2*). The stimulator of cyclic AMP production forskolin (10.0 μM) completely relaxed preparations contracted with NA at the EC_{50} (relaxation was 97.7 ± 3.6% of pre-contraction, n = 4).

Table 5.2 EC_{50}s and E_{max}s for noradrenaline (NA) dose response curves in marmoset aortic rings, in the absence and presence of methysergide (METHYS, 1.0 μM; n = 5), tropisetron (ICS, 10 μM; n = 3) or 3-isobutyl-1-methyl-xanthine (IBMX, 10 μM; n = 6).

	CONTROL		WITH ANTAGONIST	
	pEC_{50}	E_{max}(g)	pEC_{50}	E_{max}(g)
NA ± METHYS	6.08 ± 0.15	2.68 ± 0.34	6.20 ± 0.11	2.40 ± 0.34
NA ± ICS	6.91 ± 0.06	3.43 ± 0.55	7.08 ± 0.04	3.32 ± 0.52
NA ± IBMX	6.33 ± 0.22	2.50 ± 0.35	6.67 ± 0.26	3.81 ± 0.57

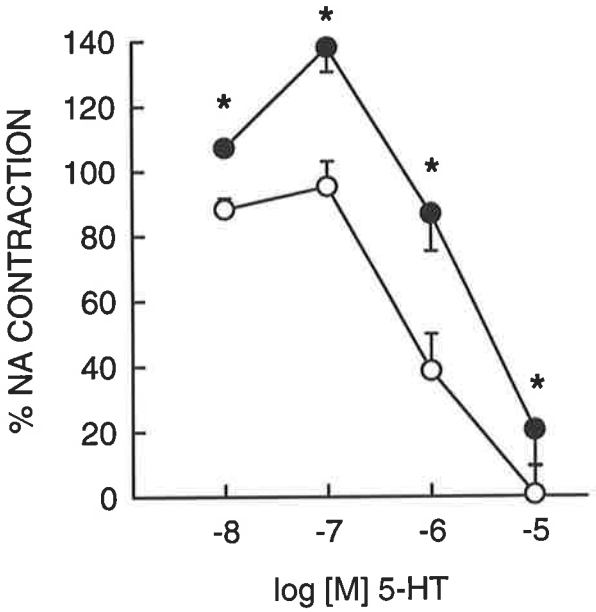

Figure 5.3 5-HT dose response curves of aortic rings pre-contracted with NA at the EC_{50} and in the absence (o) or presence (●) of LY 53857 (0.1 μM); n = 5. *P < 0.05.

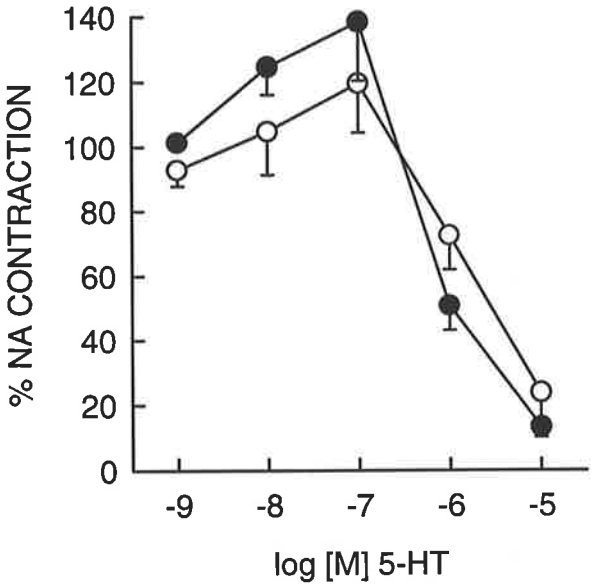

Figure 5.4 5-HT dose response curves of aortic rings pre-contracted with NA at the EC_{50} and in the absence (o) or presence (●) of tropisetron (0.1 μM); n = 5.

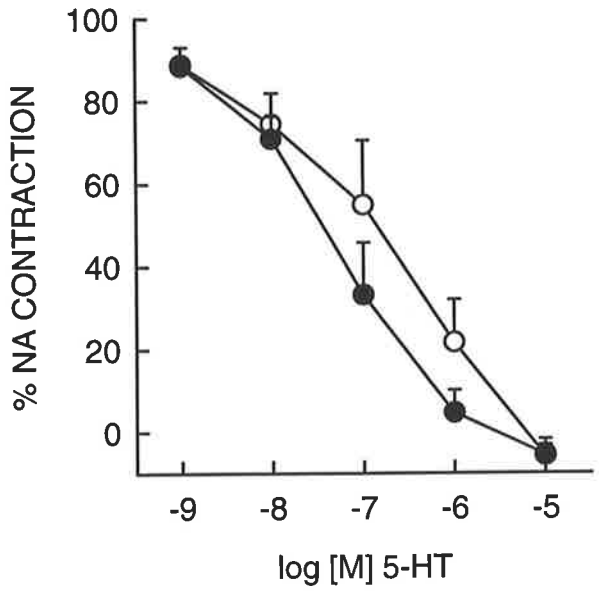

Figure 5.5 5-HT dose response curves of aortic rings pre-contracted with NA at the EC_{50} and in the absence (o) or presence (●) of IBMX (10 μM); n = 6.

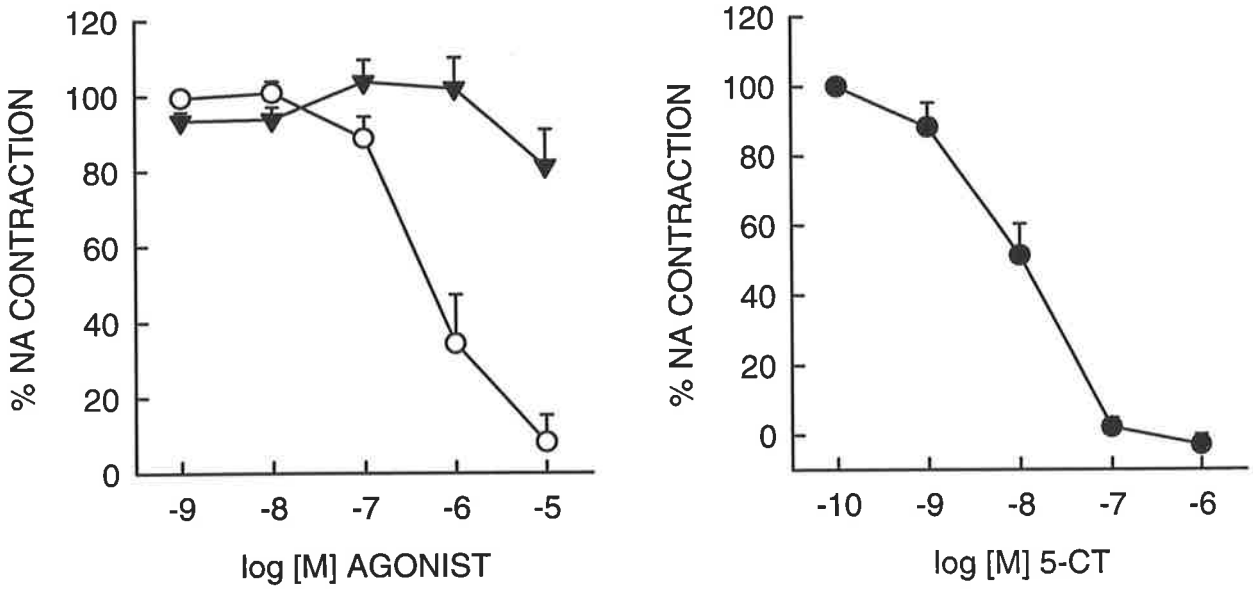

Figure 5.6 Dose response curves of carotid arteries pre-contracted with NA at its EC_{50} to (a) 5-HT (o) or sumatriptan (▼), n = 6; or (b) 5-CT in the presence of GR 127935 (0.01 μM; ●), n = 5.

5.3.2 Carotid Relaxation

In carotid arteries contracted with NA, 5-HT induced relaxation with pEC_{50} = 6.3 ± 0.2 while sumatriptan was inactive in concentrations of 0.001 - 1.0 μM, exerting a small relaxant effect only at 10 μM (*Figure 5.6a*). In contrast, 5-CT produced potent relaxation in the presence of 0.01 μM GR127935 (pEC_{50} = 8.1 ± 0.2; *Figure 5.6b*).

5.3.3 Cyclic AMP Formation

The basal levels of cyclic AMP accumulation in rings of aorta incubated with the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) for 30 and 60 minutes were 1.89 ± 0.27 and 3.52 ± 0.98 pmol mg^{-1} protein, respectively. In the lower concentration (0.1μM), 5-HT was without effect on cyclic AMP formation. In the higher concentration (10μM) which was maximal for relaxation, 5-HT increased cyclic AMP formation approximately 2 and 7 fold (in different experiments; *Figures 5.7a,b*). The increase did not occur when methiothepin (1.0μM; *Figure 5.7a*) or LY53857 (0.1μM; *Figure 5.7b*) were present.

The level of cyclic AMP accumulation in coronary arteries was not affected by 10μM 5-HT. Cyclic AMP levels were 5.18 ± 1.80 pmol mg^{-1} protein in control vessels (n = 4) and 3.61 ± 0.96 pmol mg^{-1} protein in vessels incubated with 10μM 5-HT (n = 5).

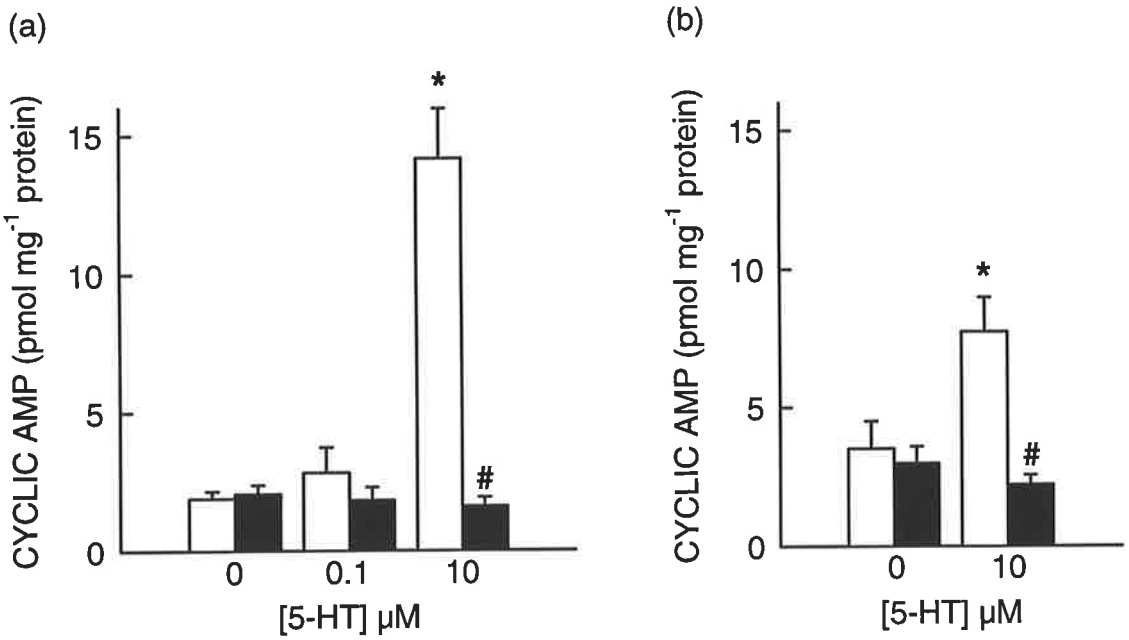

Figure 5.7 Cyclic AMP levels (pmol mg^{-1} protein) in aortic rings incubated with IBMX (100 µM) for (a) 30 min or (b) 60 min. Arteries were exposed to saline or 5-HT (0.1 µM or 10 µM) in the absence (☐) or presence (■) of (a) methiothepin (1.0 µM; n = 5) or (b) LY 53857 (0.1 µM; n = 4-5). #P < 0.05 vs. 10 µM 5-HT alone. *P < 0.05 vs. saline control.

5.4 Discussion

5-HT receptor agonists and antagonists were selected in the expectation that they would indicate whether the receptor mediating endothelium-independent relaxation fell within one of the four most extensively characterised receptor groups, ie. 5-HT_1, 5-HT_2, 5-HT_3 and 5-HT_4. The 5-HT_3 and 5-HT_4 subtypes can be excluded on the basis that tropisetron, in a concentration where it blocks the 5-HT_4 receptor as well as the 5-HT_3 receptor (Bockaert *et al.,* 1992), was without effect on relaxation. Blockade of relaxation by LY 53857 (which was developed as a potent 5-HT_2 receptor antagonist (Cohen *et al.,* 1985)) would appear to favour a 5-HT_2 receptor, as does the inhibitory action of methysergide, which blocks this receptor and also the 5-HT_1 receptors. However, a strong argument against the 5-HT_{2A} receptor is the lack of effect of ketanserin on relaxation responses to 5-HT in the U44069-contracted artery (*Figure 4.1*).

The relative effects of the 5-HT agonists provides a further insight into the nature of the receptor mediating relaxation. Although the high potency of 5-CT is a characteristic of the 5-HT_1-like group of receptors, the 5-HT_{1B} (non-rodent) and 5-HT_{1D} receptor subtypes seem excluded by the lack of activity of sumatriptan and the association of relaxation with increased rather than decreased cyclic AMP levels. Similarly, the low potency of 8-OH-DPAT argues against the 5-HT_{1A} receptor subtype. On the other hand, the order of agonist potencies is similar to those for endothelium-independent relaxation in the dog coronary artery (Houston & Vanhoutte, 1988; Cushing & Cohen, 1992b) and the pig vena cava (Trevethick *et al.,* 1984; Sumner *et al.,* 1989) (*Table 5.1*). In case the order of potencies in the marmoset aorta was unduly influenced by the involvement of endothelium-derived NO and by excitatory effects mediated by the $_{5\text{-HT1B}}$ (non-rodent) and 5-HT_{1D} receptor-like receptor (*Chapter 4*), the relative potencies of 5-CT and 5-HT were also estimated in the presence of NOLA and the $_{5\text{-HT1B}}$ (non-rodent) and 5-HT_{1D}

receptor antagonist GR 127935. Although the arteries were sensitised to both agonists, the high potency of 5-CT relative to 5-HT was maintained, indicating the difference in potencies is a genuine reflection of their relative relaxant actions on vascular smooth muscle. This result again highlights the minor role the endothelium plays in relaxant responses to 5-HT in this preparation, since blockade of any endothelium-dependent component of consequence would be expected to decrease the efficacy of 5-HT. Instead, the apparent effect was an increase in efficacy, presumably due to blockade of interaction with the 5-HT_1-like contractile receptor. Endothelium removal alone had no effect on 5-CT-induced relaxation. Also, in a later chapter (see *Table 7.2*) NOLA (100μM) was without affect on the relaxant response to 5-CT in arteries precontracted with phenylephrine and incubated with GR127935 (10nM). Thus there is no endothelium-dependent component to the 5-CT-induced relaxation. Unfortunately limited availability of animals precluded assessment of relative potencies of the other agonists under these conditions. Ideally, all agonist/antagonist experiments should be conducted in the presence of NOLA (or endothelium removal) plus GR127935 to prevent any interaction at other receptors. Under these conditions, a K_D for antagonism by LY53857 could be determined.

The 5-HT-induced responses of the dog coronary artery bear further similarities to the marmoset aorta in that both arteries exhibit endothelium-dependent relaxation at low concentrations, endothelium-independent relaxation at high concentrations which is blocked by LY53857 but not ketanserin and contraction in endothelium-denuded preparations which is mediated by a 5-HT_1-like receptor (Houston & Vanhoutte, 1988; Cushing & Cohen, 1992b; Cushing *et al.,* 1994; Cushing *et al.,* 1996). In the marmoset aorta maximal relaxation was endothelium-independent and was associated with increased cyclic AMP formation which was blocked by methiothepin and LY53857. Similarly, in

pig vena cava 5-HT stimulates cyclic AMP formation and this effect is prevented by methiothepin (Trevethick *et al.,* 1984; Sumner *et al.,* 1989). These comparisons suggest that the same receptor type may mediate endothelium-independent relaxation in the three preparations.

Evidence has accumulated that the receptor mediating relaxation in the dog coronary artery and pig vena cava is the functional equivalent of the cloned 5-ht_7 receptor (Cushing *et al.,* 1996; Terrón, 1996; Eglen *et al.,* 1997). The 5-HT_7 receptor, like the 5-ht_6 receptor, is positively linked to adenylate cyclase. However the former, but not the latter possesses an affinity for 5-CT which is greater than that for 5-HT (Bard *et al.,* 1993; Lovenberg *et al.,* 1993; Meyerhof *et al.,* 1993; Plassat *et al.,* 1993; Ruat *et al.,* 1993b; Shen *et al.,* 1993; Monsma *et al.,* 1993; Kohen *et al.,* 1996). Furthermore, the 5-HT_7 receptor has a relatively high affinity for methysergide and methiothepin and a low affinity for ketanserin (Bard *et al.,* 1993; Plassat *et al.,* 1993; Shen *et al.,* 1993). The relative binding affinities of the agonists at the 5-HT_7 receptor also accord well with the relative potencies in the marmoset aorta (Bard *et al.,* 1993; Plassat *et al.,* 1993; Ruat *et al.,* 1993b; Shen *et al.,* 1993). The characteristics of 5-HT-mediated relaxation in the marmoset aorta as discussed above suggest strongly that the relaxation is mediated by the 5-HT_7 receptor. Expression of the 5-HT_7 receptor has been demonstrated in human aortic smooth muscle, strongly indicating similarities between human and marmoset aortic serotonergic responses (Ullmer *et al.,* 1995).

This receptor mediates the vasodilator response to 5-HT observed in the canine coronary vascular bed *in vivo* (Cambridge *et al.,* 1995) and mRNA was found in the human coronary artery (Bard *et al.,* 1993). The 5-HT_7 receptor is also the likely mediator of endothelium-independent relaxation in the rabbit jugular and femoral vein (Martin *et al.,* 1987; Martin & Wilson, 1995), the sheep pulmonary vein (Zhang *et al.,* 1995), the

Cynomolgus monkey jugular vein (Leung *et al.*, 1996) and relaxation in the guinea pig ileum and cat saphenous vein (Feniuk *et al.*, 1983). Functional 5-HT_7 receptors are also expressed in human uterine artery smooth muscle cells (Schoeffter *et al.*, 1996).

The 5-HT receptor mediating relaxation in the marmoset carotid artery exhibits the high potency of 5-CT as an agonist and a lack of relaxant effect of sumatriptan, which is reminiscent of the effect in the marmoset aorta and is suggestive of the presence of the 5-HT_7 receptor. However, there is a need for additional data regarding the actions of 5-HT receptor agonists and antagonists before the 5-HT relaxant receptor in the marmoset carotid artery can be conclusively characterised.

Relaxation to 5-HT was demonstrated in marmoset coronary arteries incubated with GR127935 in the previous chapter (*Figure 4.7*). This relaxation extended over four orders of magnitude from 0.001 - 10μM 5-HT. No attempt has been made to operationally characterise the nature of the receptor(s) mediating this response. Cyclic AMP formation was not stimulated by 10μM 5-HT at 10μM in the coronary arteries. Further investigations should focus upon establishing the endothelium-dependence of the relaxation and then the nature of the receptor mediating it.

In summary, low-affinity, endothelium-independent relaxation to 5-HT ($\geq$1.0μM) in the marmoset aorta is most likely mediated by the 5-HT_7 receptor.

CHAPTER 6

ANALYSIS OF SEROTONERGIC AMPLIFYING INTERACTIONS IN THE MARMOSET AORTA

6.1 Introduction

Amplifying interactions between 5-HT and either Tx agonists or NA are well documented in other vessels (see *1.4.4*). Thus, the absence of an amplifying interaction between 5-HT and NA in the marmoset aorta seemed paradoxical (see *Chapter 3, 3.3.1*). Similarly, the 5-HT_1-like partial agonist methysergide enhanced U44069-induced but not NA-induced contraction (*4.3.1, Table 5.2*). In the present study the response to sumatriptan in the presence of U44069 and NA were investigated. In addition, responses to 5-HT in the presence of other contractile agents (endothelin (ET), K^+) were explored to assess whether these conformed to the pattern exhibited by U44069 or NA. The possibility that ß-receptor activity on the part of NA had contributed to masking the appearance of amplification with 5-HT was also examined.

6.2 Methods

6.2.1 Animals

Adult marmoset monkeys were obtained and tissues collected as described previously (*2.1*).

6.2.2 Aortic Ring Preparations

The aorta was prepared and aortic rings mounted as described previously (*2.2*).

The organ bath protocols followed the outline detailed in *2.2.1.* The response to sumatriptan in the presence of a threshold concentration (9 ± 2nM) of U44069 and in the presence of NA at the EC_{50} was determined. The response to 5-HT (0.001 - 10μM) in the presence of noradrenaline, U44069 or methoxamine was determined. DRCs to 5-HT in the presence of NA plus the adrenergic ß-receptor antagonist propranolol (1.0μM) were determined. Preliminary studies indicated that tachyphylaxis occurred in response to endothelin-1 (ET). Thus, full dose response curves (DRCs) to ET were not constructed. To investigate interactions of 5-HT with ET, arteries were precontracted with ET at 10 - 20nM, a pre-determined EC_{50} for ET (see *Table 3.1*). Similarly, KCl was applied at 20 - 40mM for precontraction prior to a cumulative 5-HT DRC. The possibility of an amplifying interaction between NA and U44069 was also investigated. Cumulative DRCs to 5-HT in the presence of KCl at a sub-threshold concentration (10 mM) were also constructed. In addition, DRCs to NA in the presence of U44069 at 1nM (an approximate threshold concentration) were constructed.

6.2.3 Cyclic AMP Formation

For investigation of possible effects of NA on cyclic AMP formation, aortic rings were incubated in IBMX (100μM) for 60 min. Subsequently, the rings were incubated with saline, 5-HT (10μM) or noradrenaline (2μM or 20μM) for 2 min before being rapidly frozen in liquid nitrogen. The concentrations of NA used were close to the EC_{50} and EC_{100} previously determined for contraction to NA in this system.

6.2.4 Data Analysis

Data analysis was conducted as previously described (*2.8*). Cyclic AMP data was analysed in $\log_{10}$ form using an ANOVA.

6.3 Results

6.3.1 Organ Bath Preparations

Sumatriptan. Sumatriptan produced a concentration-dependent contractile response in arteries which were endothelium intact and contracted with a threshold dose of U44069 (*Figure 6.1a*). In endothelium-intact NA-contracted arteries, sumatriptan also produced a concentration-dependent contractile response (*Figure 6.1b*).

Endothelin. In the presence of ET, 5-HT exhibited relaxant responses which were indistinguishable from those of 5-HT in the NA-contracted vessel (*Figure 6.2*). However, in endothelium-denuded arteries the response to 5-HT in concentrations to 1.0μM was reversed to contraction, although relaxation remained pronounced at the highest concentration (10μM; *Figure 6.2*). The level of precontraction to ET was 0.55 ± 0.18 vs. 0.90 ± 0.19g in unrubbed vs. rubbed preparations ($P>0.05$).

Potassium. 5-HT to 1.0 μM was without significant effect when the artery was pre-contracted with K^+ rather than NA or U44069 (*Figure 6.3*). 10μM 5-HT had a modest relaxant effect. In the presence of a subthreshold concentration of KCl (10mM) 5-HT gave little or no contractile response (<5% KCl max; data not shown).

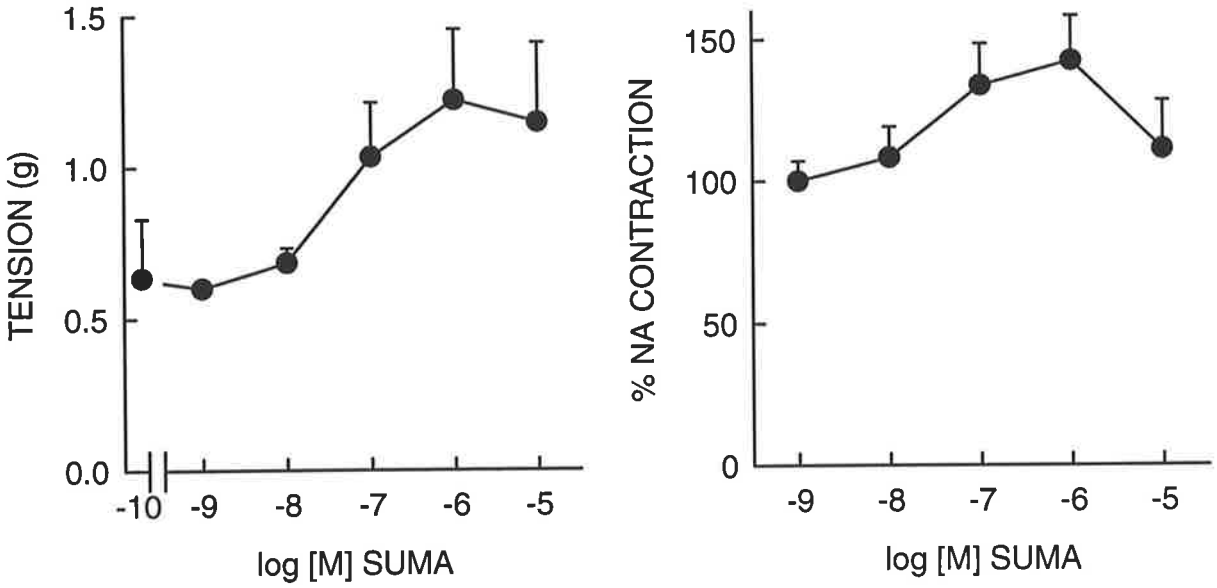

Figure 6.1 Sumatriptan dose response curves in aortic rings (a) pre-contracted with a threshold concentration of U44069 or (b) NA at the EC_{50}; n = 6.
NOTE: In (a) First data point indicates level of pre-contraction.

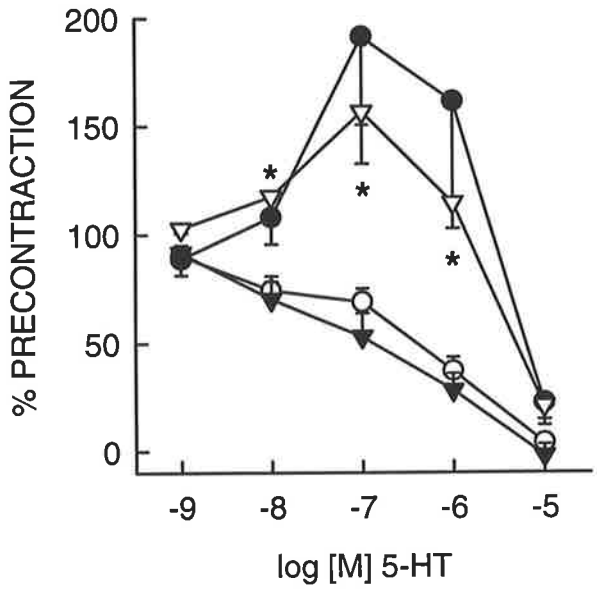

Figure 6.2 5-HT dose response curves of aortic rings pre-contracted with noradrenaline (○), U44069 ( ), ET + endothelium (▼) or or ET - endothelium (▽); n = 5-8. *P < 0.05 ET + endothelium vs. ET - endothelium.

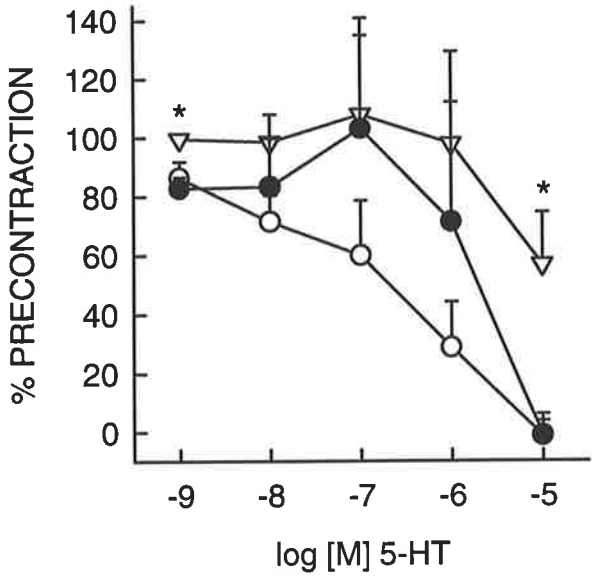

Figure 6.3 5-HT dose response curves of aortic rings pre-contracted with NA (○), U44069 (●), or KCl (▽); n = 5. *P < 0.05 K^+ vs. NA and U44069.

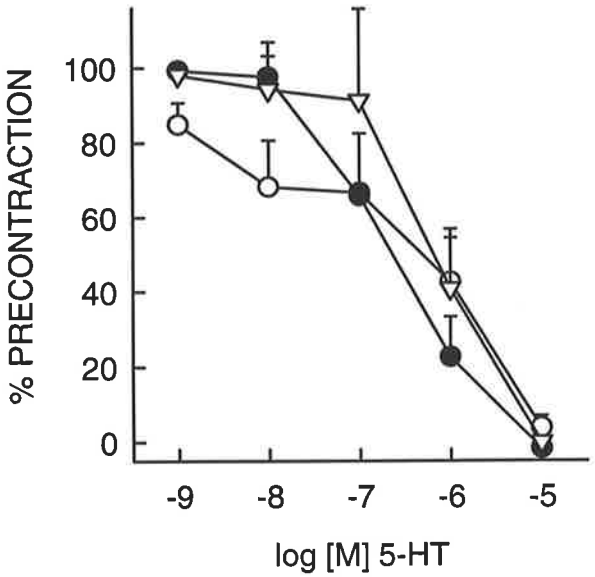

Figure 6.4 5-HT dose response curves of aortic rings pre-contracted with noradrenaline (○), noradrenaline plus propranolol (1.0μM; ●), or methoxamine (▽); n = 5-6.

Influence of ß-receptors. Propranolol did not affect the response to NA (*Table 6.1*), nor did it influence the nature of the interaction between 5-HT and NA (*Figure 6.4*). The relaxant response to 5-HT did not differ when NA was replaced with the specific α_1-agonist methoxamine (1.0 μM; *Figure 6.4*).

Interaction between NA and U44069. The response to NA was not affected by the presence of 1nM U44069 (*Table 6.1*).

Table 6.1 EC_{50} and E_{max} values for NA DRCs in aortic rings, in the absence and presence of propranolol (1.0μM) or U44069 (1nM); (NA, noradrenaline; PROP, propranolol; U44, U44069).

	CONTROL		+ 2nd AGENT	
AGONIST (*n*)	pEC_{50}	E_{max}(g)	pEC_{50}	E_{max}(g)
NA ± PROP (*6*)	6.3 ± 0.1	2.14 ± 0.33	6.4 ± 0.2	2.40 ± 0.25
NA ± U44 (*8*)	6.6 ± 0.1	3.63 ± 0.44	6.9 ± 0.3	4.17 ± 0.34

6.3.2 Cyclic AMP Formation

The basal level of cyclic AMP accumulation in aortic rings incubated with IBMX for 60 min was 3.5 ± 0.1 pmol/mg protein. 5-HT (10μM) significantly increased cyclic AMP formation. Noradrenaline in a low (0.2mM) or a high concentration (2mM) had no significant effect on cyclic AMP accumulation (*Figure 6.5*).

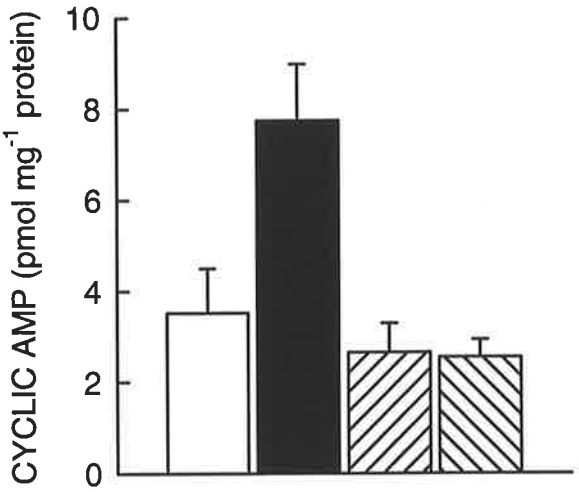

Figure 6.5 Cyclic AMP levels (pmol mg^{-1} protein) in aortic rings incubated with IBMX (100 μM) for 60 min and (a) vehicle (▭), (b) 5-HT (10 μM; ■), (c) noradrenaline (0.2 mM; ▨), or (d) noradrenaline (2 mM; ▨); n = 4-5.

6.4 Discussion

The presence of an amplifying interaction between sumatriptan and both U44069 and NA is in contrast to the effects of 5-HT. This indicates that the masking of 5-HT's contractile effect in the presence of NA is a specific interaction between NA and a non-5-HT_1-like component of 5-HT's action. The lack of effect of the ß-antagonist propranolol and the lack of difference between responses to 5-HT in the presence of NA (a non-specific adrenergic agonist) and methoxamine (α_1-agonist) indicate that ß-receptor involvement is not responsible for masking amplification interactions. Accordingly, noradrenaline had no significant adenylate cyclase stimulatory properties in the current study.

A mechanism for the synergistic contractile interaction between U44069 and 5-HT is not apparent, however *Figure 3.6* indicates that amplification is greatest when endothelium-function is reduced. This is of important pathophysiological relevance when considering the role of these vasoactive agents in vasospasm occurring in conditions where endothelial function is impaired. Similarly, in ET contracted, endothelium-intact preparations, relaxation to 5-HT predominated. However, under conditions of reduced endothelial function, a synergistic contractile interaction occurred. Synergistic interactions between 5-HT_1-like receptor mediated contraction and a secondary agonist has also been demonstrated with prostaglandin $F_{2\alpha}$, U46619 and methoxamine in a variety of vessels, including the human coronary artery (Sahin-Erdemli *et al.,* 1991; de la Lande, 1992; Cocks *et al.,* 1993; Choppin & O'Connor, 1995). In contrast, U46619 had no effect on contractile responses to sumatriptan in the dog coronary artery or saphenous vein (Kemp & Cocks, 1995).

Synergy between activated G_i- and G_q-coupled receptors in vascular smooth muscle is widespread, although the molecular basis for these amplification interactions is still

unknown (Martin *et al.,* 1998; Selbie & Hill, 1998). In the rabbit basilar artery, where 5-HT-induced contraction is also mediated by a 5-$HT_{1\text{-like}}$ receptor, 5-HT appears to cause DAG release independently of IP_3 generation (Clark & Garland, 1991). If this occurs in the marmoset aorta, synergism between the second messenger systems of U44069 and 5-HT at the level of DAG may occur. In the rabbit mesenteric artery, where 5-HT_1-like receptors also mediate contraction, 5-HT appears to increase the Ca^{2+} sensitivity of the contractile apparatus (Seager *et al.,* 1994). This would therefore increase the contractile response to U44069. $_{5\text{-HT1B}}$ (non-rodent) and 5-HT_{1D} receptors expressed in a murine cell line can induce IP_3 generation in addition to decreasing cyclic AMP accumulation (Zgombick *et al.,* 1993). If IP_3 generation is also present in marmoset aortic vascular smooth muscle cells, then this would provide another potential site for synergistic contractile interactions with other agonists. The reason for the discrepancy between the 5-HT interactions with U44069 and NA in the marmoset preparation, however, remains unknown. Further understanding of this phenomenon awaits determination of the precise mechanisms involved in mediating the contractions to U44069 and NA in this tissue.

5-HT contraction does not occur to any significant extent in arteries precontracted with K^+. However, amplification may occur in arteries with reduced endothelial function. Relaxation at 10μM 5-HT is reduced in K^+-contracted arteries. At high concentrations of 5-HT, relaxation presumably occurs via a 5-HT_7 receptor, activation of cyclic AMP and thereby inhibition of L-type calcium channels as well as regulation of the activity of the sarcolemmal Ca^{2+} pump and the sensitivity of the contractile apparatus (Bolton, 1986; Hathaway *et al.,* 1991; Hosey *et al.,* 1992; Xiong & Sperelakis, 1995; Ozaki *et al.,* 1996). Activation of the 5-HT receptor may cause inhibition of current flow through a different subtype of L-type Ca^{2+} channel to that activated by K^+-induced depolarization. Cyclic GMP and NO inhibit Ca^{2+} entry through channels activated by NA but not those activated

by K^+ depolarization in rat aorta (Godfraind, 1986). In fact, the inhibitory effects of cyclic AMP in vascular smooth muscle may be mediated by cyclic GMP-dependent protein kinase, rather than cyclic AMP-dependent protein kinase (Jiang *et al.,* 1992; Lincoln *et al.,* 1990; Lincoln & Cornwell, 1991). Thus, in marmoset aortic smooth muscle cells, cyclic AMP may activate cyclic GMP-dependent protein kinase and thereby inhibit calcium channels which are activated by NA but not K^+.

Many more experiments could be conducted to elucidate further the nature of serotonergic interactions in the marmoset aorta. Mutual amplification interactions may be more simply investigated by constructing DRCs to a secondary agonist in the presence of a threshold concentration of 5-HT, thus enabling EC_{50} and E_{max} calculations and comparisons. In addition, contractile interactions would be better investigated in the presence of LY53857 and NOLA, or by the use of sumatriptan, in order to minimise relaxant effects. Interpretation of results can be complicated by the possible presence of a 5-HT receptor-mediated endothelium-dependent relaxant response. Measurement of IP_3 levels in aortic vascular smooth muscle would also greatly extend the current studies.

In summary, 5-HT-induced contraction (mediated by the 5-HT_1-like receptor) exhibits a synergistic contractile interaction with U44069 and ET, but not NA. Synergistic contractile interactions between 5-HT and a secondary agonist in the marmoset aorta are normally masked by EDRF production. The lack of a synergistic interaction of 5-HT with NA is not due to the involvement of ß-adrenoceptors.

CHAPTER 7

EFFECTS OF AN ATHEROGENIC DIET ON SEROTONERGIC RESPONSES OF THE MARMOSET VASCULATURE

7.1 Introduction

In the previous chapters of this thesis it was shown that the aorta isolated from healthy marmoset monkeys exhibits responses to 5-HT which differ from those reported in the aorta of non-primate species. The differences comprise contraction, mediated by a 5-HT_1-like receptor, relaxation possibly mediated by a 5-HT_7 receptor and an amplification interaction which occurs between 5-HT and the Tx A_2 mimetic U44069, but not NA.

Many previous studies have investigated the effects of vascular disease, including atherosclerosis and its risk factors (eg. hypercholesterolaemia) on vascular responses to 5-HT (see *1.8*) in animal models, however few have provided information on the effects of 5-HT receptor subtype specific agonists. The subsequent chapters in this thesis address the issue of how 5-HT specific responses in the marmoset vasculature are affected by vascular disease states.

For many researchers, the first animal model used in atherosclerosis research, ie. the rabbit (Castelli, 1984), is still the laboratory animal of choice for investigations into the atherogenic effects of a lipid-rich diet. In contrast, whilst the rat is commonly used for vascular reactivity research, this animal is relatively resistant to the development of atherosclerosis and thus is not a convenient animal model for studies on this condition. In

the rat, it is usually necessary to combine hypercholesterolaemia with a second experimental intervention to produce atherosclerotic lesions (Gill *et al.,* 1989). Although induction of atherosclerotic lesions in the rabbit is much simpler, it should be noted that this animal is a herbivore and naturally occurring arterial lesions in it do not normally contain lipid. Thus, experimental dietary intervention usually produces increases in serum lipid levels far in excess of that seen in humans. In addition, the plasma lipid and lipoprotein profile of the rabbit (as in the rat) varies greatly from that of the human. Thus, studies on the vascular effects of pharmacological agents under conditions of hypercholesterolaemia and atherosclerosis in a non-human primate are obviously of greater physiological relevance than observations in species which are phylogenetically less similar to humans. In addition, the common cotton-eared marmoset has been demonstrated to be susceptible to diet-induced atherosclerosis at the CSIRO Division of Human Nutrition and in other laboratories (McIntosh *et al.,* 1988; Crook *et al.,* 1990).

The aim of the current study was therefore to determine the effects of hypercholesterolaemia (and atherosclerosis) on the vascular responses to 5-HT in the aorta, common carotid and large coronary arteries of the marmoset.

7.2 Methods

Introductory Note

This chapter describes the results from two separate dietary experiments, as indicated by "atherogenic dietary experiment #1" vs. "atherogenic dietary experiment #2".

Atherogenic Dietary Experiment #1

7.2.1 Animals

Marmosets were sourced and housed as described previously (*2.1*). Sixteen adult male animals aged approximately 4-6 years were used since both sex and age have been shown to influence plasma lipid levels (McIntosh *et al.,* 1984; Kaplan *et al.,* 1991). The marmosets were periodically administered supplementary vitamin D_3 i.m. injections by the veterinarian responsible for the animal house, in view of the requirement of this species for large amounts of this vitamin (Takahashi *et al.,* 1985). The animals were randomly assigned to one of two groups and fed either a control or an atherogenic experimental diet for 10 months. Before commencement of the diet and after 1, 2, 3, 5, 7 and 9 months the animals were fasted overnight and blood samples were taken for analysis of plasma lipoprotein and triglyceride levels, as described previously (*2.1.2, 2.5*). The 10 month samples were also analysed by the Institute of Medical and Veterinary Science (Adelaide, South Australia).

At the completion of the experimental feeding period, the animals were fasted overnight, anaesthetised with Saffan, killed by exsanguination from the abdominal aorta (providing a final blood sample) and the aorta, right common carotid artery and left anterior descending coronary artery were collected for organ bath studies (*2.1*). The left common carotid artery and abdominal aorta (distal to the needle puncture site) to iliac arteries were removed, rinsed in phosphate buffered saline and transferred to 1.25% glutaraldehyde for histological analysis (as described in *2.7*).

7.2.2 Diets

The animals were fed a prepared diet for 10 months. The base diet was the standard colony diet supplied by Milling Industries in the non-pelleted form and was stored at 4°C. Details of the constitution and nutrient levels of this diet are given in Appendix I. This diet was pelleted following supplementation with 7% (w/w) sheep kidney fat (Coldi Meat Foods Pty Ltd, Beverley, Sth. Aust.) and 0.3% (w/w) cholesterol (Ajax) to give the atherogenic diet, or pelleted without supplementation for the control diet. Experimental diets were stored at -20°C for up to 90 days. The control diets contained 7% fat and 0.02% cholesterol and the final atherogenic diets were prepared to contain 14% fat and 0.3% cholesterol. The fat and cholesterol contents of the diets were analysed retrospectively as described previously (*2.6*).

7.2.3 Vascular Preparations

Six aortic rings, two common carotid artery segments and two coronary artery segments were dissected from each animal, prepared and mounted for organ bath studies as described previously (*2.2*).

The organ bath protocols followed the outline detailed in *2.2.1*. The six aortic rings were treated as follows:-

In two aortic rings contracted with NA, DRCs to the following agents were constructed:

- ring 1 (i) 5-HT (ii) A23187
- ring 2 (i) 5-HT (ii) sodium nitro-prusside (SNP)

In two aortic rings contracted with U44069, DRCs to the following agents were constructed:

- ring 3 (i) 5-HT (ii) A23187

- ring 4 (i) 5-HT (ii) SNP

In the final two aortic rings, a DRC to NA was followed by DRCs to:

(i) 5-HT at basal tone (ii) A23187

The two carotid artery segments were treated the same as aortic rings 1 and 3 in the aortic ring protocol.

In the coronary artery segments, DRCs to 5-HT and bradykinin (BK) were constructed with U44069 pre-contraction.

Atherogenic Dietary Experiment #2

A second trial of the atherogenic dietary study was conducted with the same experimental outline. During the course of the first dietary experiment it became apparent that the response of the marmoset's plasma lipids to increased dietary fat and cholesterol was greatly variable. Thus, animals in the second study were screened with an initial 2 months feeding of the atherogenic diet to enable selection of approximately equal numbers of animals who exhibited either a strong or a weak plasma lipid response. The timetabling of the animal treatment, diet preparation, blood sampling and plasma lipoprotein analyses were conducted by Mr M.T. Mano. Differences in the protocols were as follows.

7.2.4 Animals, Experiment #2

Adult male animals of 2-3 years of age were used. Seven animals were fed the control diet and 15 the atherogenic diet, for a period of 10 months. Blood sampling and plasma

analysis took place before the diet commenced and at every month during feeding. At the completion of the experimental feeding period, the animals were killed under anaesthesia by exsanguination from the femoral artery. The abdominal aorta was removed, rinsed in phosphate buffered saline and fixed in 1.25% glutaraldehyde prior to Sudan IV staining for lipid deposits (Holman *et al.*, 1958).

7.2.5 Diet, Experiment #2

The animals were fed a purely synthetic diet prepared to contain 4% fat and 0.02% cholesterol in the control diet and 12% fat and 0.4% cholesterol in the atherogenic diet. The details of the constitution of these diets are given in Appendix I.4.

7.2.6 Vascular Preparations, Experiment #2

Six aortic rings, two carotid artery segments and two coronary artery segments from each animal were prepared and mounted as described previously (2.2).

The protocol was designed to establish the influence of the diets on (i) the contractile effects of 5-HT and sumatriptan; (ii) relaxant effects of 5-HT and 5-CT, the latter in the presence of GR127935 (10nM) to block contraction mediated by 5-HT_1-like receptors and (iii) endothelium-dependent and independent relaxation to NO donors, using A23187 and SNP, respectively. Phenylephrine (PE) was used as a supplementary pre-contractile agent because in trial experiments it exhibited maintenance of tone without fade of contraction more consistently than NA in the hyperlipidaemic animal groups. Since the protocols included the initial effect of KPSS and DRCs to NA, PE or U44069, the results also indicated possible effects of the diets on the activities of these contractile agents.

The organ bath protocols followed the outline detailed in *2.2.1*. The six aortic rings were treated as follows:-

- ring 1 NA precontraction (i) 5-HT (ii) A23187 (iii) SNP
- ring 2 U44069 precontraction (i) 5-HT (ii) A23187 (NA) (iii) SNP (NA)
- ring 3 NA precontraction (i) 5-CT + GR127935 (ii) BK (iii) SNP
- ring 4 PE precontraction (i) 5-CT + GR127935 (ii) A23187
  (iii) 5-CT + GR127935 + NOLA
- ring 5 NA precontraction (i) sumatriptan (ii) BK (iii) SNP
- ring 6 PE precontraction (i) sumatriptan (iii) A23187
  (iii) sumatriptan + NOLA

The two carotid artery preparations, were treated as follows:

- ring 1 NA precontraction (i) 5-CT + GR127935 (ii) A23187 (iii) SNP
- ring 2 U44069 initial DRC, then NA precontraction (i) 5-HT (ii) A23187 (iii) SNP

The two coronary artery segments were precontracted with U44069, without an initial U44069 DRC. DRCs to either 5-CT or sumatriptan were conducted.

7.2.7 Data Analysis

Data analyses were conducted as previously described (*2.8*). Comparisons of vascular responses between control, hypo-responder and hyper-responder groups were conducted using a one way ANOVA.

7.3 Results

Atherogenic Dietary Experiment #1

The animals tolerated the diet reasonably well after an initial period of a few weeks during which they were fed a gradually increasing proportion of supplemented diet. At the time of collection of blood samples, some joint stiffness was noticed in a few of the animals.

7.3.1 Diets

Analysis revealed that the base (pre-pelleted) diet contained 6.9 ± 0.2% fat and 0.021 ± 0.001% cholesterol (n = 5) in the first batch and 6.1 ± 0.0% fat (n = 2) in the second (and final) batch. Samples of prepared final diet, analysed throughout the feeding period contained 6.5 ± 0.1% fat in the control diet (n = 4) and 13.9 ± 0.2% fat in the atherogenic diet (n = 5). The cholesterol content of the final diets was 0.02% and 0.31% in the control and atherogenic diets respectively.

7.3.2 Plasma Lipids

Total plasma cholesterol concentrations did not significantly differ between control and atherogenic animals before commencement of the dietary feeding protocol (controls 6.1 ± 0.3 mM; atherogenic 5.1 ± 0.4 mM). The response of total plasma cholesterol to dietary cholesterol and fat loading was highly variable between individual animals and was also inconsistent over time (*Figure 7.1*). In general, mean total cholesterol, HDL and LDL+VLDL fractions rose over time in animals fed an atherogenic diet, while triglyceride levels remained unaffected (*Figure 7.2*). Plasma cholesterol concentrations in the final (10 month) sample were significantly lower than in samples taken from conscious animals at 7 or 9 months (P<0.0001, paired t-tests, n=16). Total cholesterol levels for 10 month

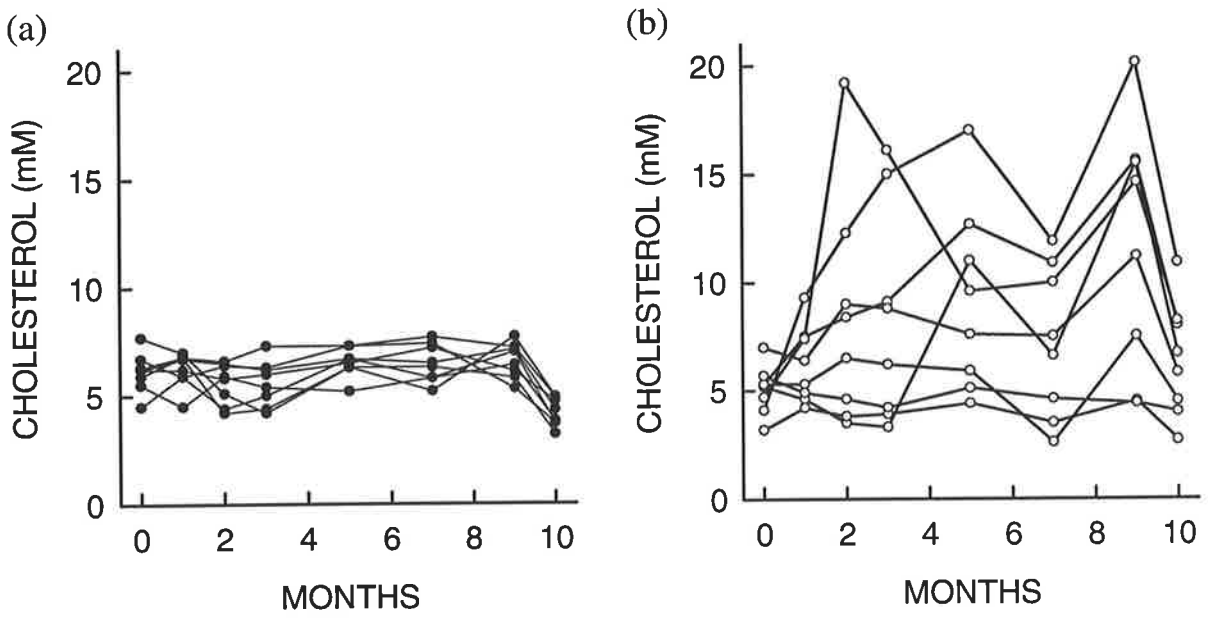

Figure 7.1 Total plasma cholesterol levels in individual marmosets maintained on (a) a control or (b) atherogenic diet #1 for 10 months.

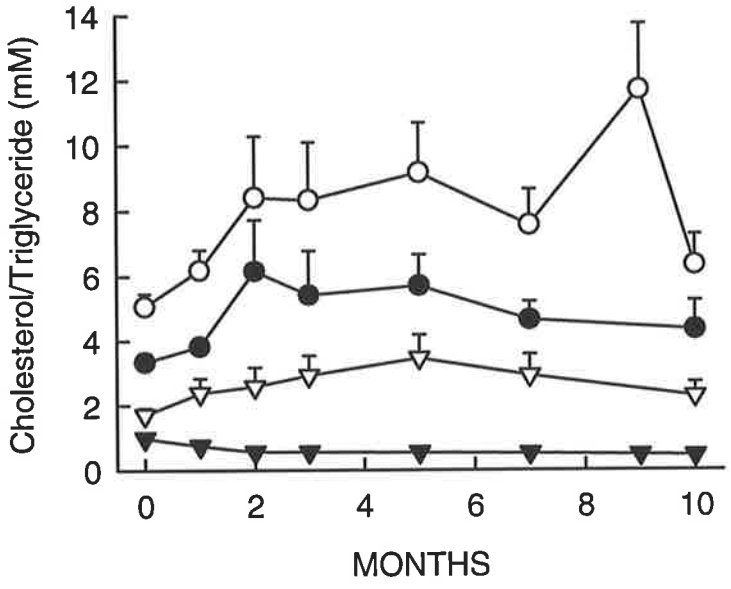

Figure 7.2 Plasma lipid levels in marmosets maintained on atherogenic diet #1 for 10 months. Total plasma cholesterol (○); HDL cholesterol (▽); LDL + VLDL cholesterol (●); triglyceride (▼); $n = 8$.

samples as measured by the IMVS were similar but 0.29 ± 0.03 mM lower than levels obtained by this author.

7.3.3 Histology and Vascular Responses

At a macroscopic level, some aortae in both control and treated groups appeared obviously stiffened (calcified) and fragile upon dissection. Histological analysis of vessels from the animals revealed atherosclerotic changes in some of the animals maintained on the atherogenic diet (including foam cells and early plaque development), but also extensive degeneration of the media in animals from both treatment groups, characterised by lack of structural elastic tissue (collagen) and also calcification.

The aortic ring preparations did not consistently exhibit a satisfactory amplitude of response to vasoactive agents and the sensitivity of the preparations was highly variable. In the light of the microscopy findings it was determined that the animals were likely to be exhibiting signs of Vitamin D toxicity. Thus, details of the organ bath preparation results are deemed meaningless and are not presented here; neither is there any further discussion of these data.

Atherogenic Dietary Experiment #2

7.3.4 Plasma Lipids, Experiment #2

Results from animals in the atherogenic dietary group are arbitrarily divided into "hypo-responder" and "hyper-responder" categories due to the extreme variability in responsiveness of plasma cholesterol levels to dietary lipid loading, as demonstrated in the previous dietary experiment (#1; *Figure 7.1*). Hyper-responders were defined as those animals whose total plasma cholesterol levels exceeded 8 mM at more than 5 measured time points, the remainder of animals being classified as hypo-responders. Over the 10 months of feeding total plasma cholesterol levels of hyper-responding animals rose following a lag period of approximately 2 months, whereas hypo-responders had no response to dietary lipid loading until after 6 months, and even this response was relatively blunted (*Figure 7.3*). Total plasma cholesterol levels across all treatment groups were significantly lower at 10 months (in anaesthetised animals) than at 9 months ($P<0.001$, paired t-test, $n=20$; there was no difference between levels obtained at 8 and 9 months, paired t-test).

7.3.5 Morphology, Experiment #2

The abdominal aorta from all marmosets showed no visible signs of gross atherosclerotic lesions on the intimal surfaces and Sudan IV staining for lipid deposits revealed a few fatty dots, staining <5% of the intimal surface area in all vessels.

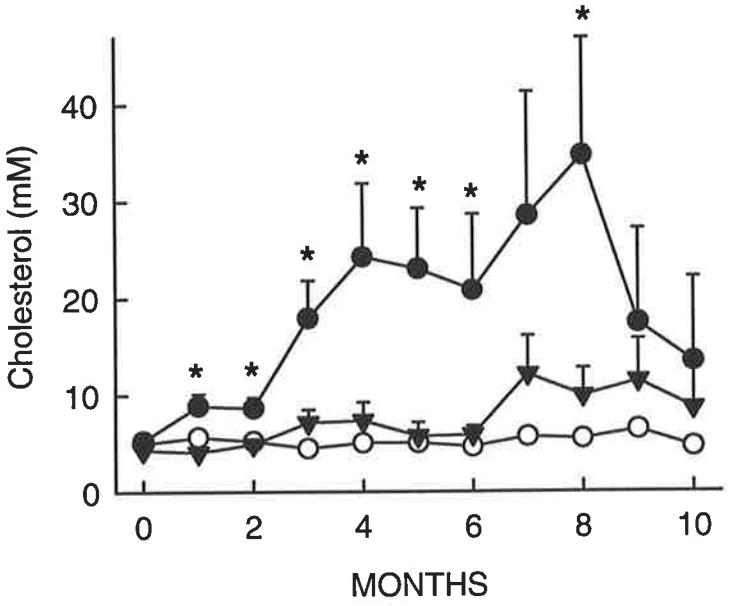

Figure 7.3 Total plasma cholesterol levels in marmosets maintained on control (white symbols; n = 7) or atherogenic (#2, black symbols) diets for 10 months. Hyper-responding animals (● ; n = 5) hypo-responding animals (▼ ; n = 10). *P<0.05 vs. control.

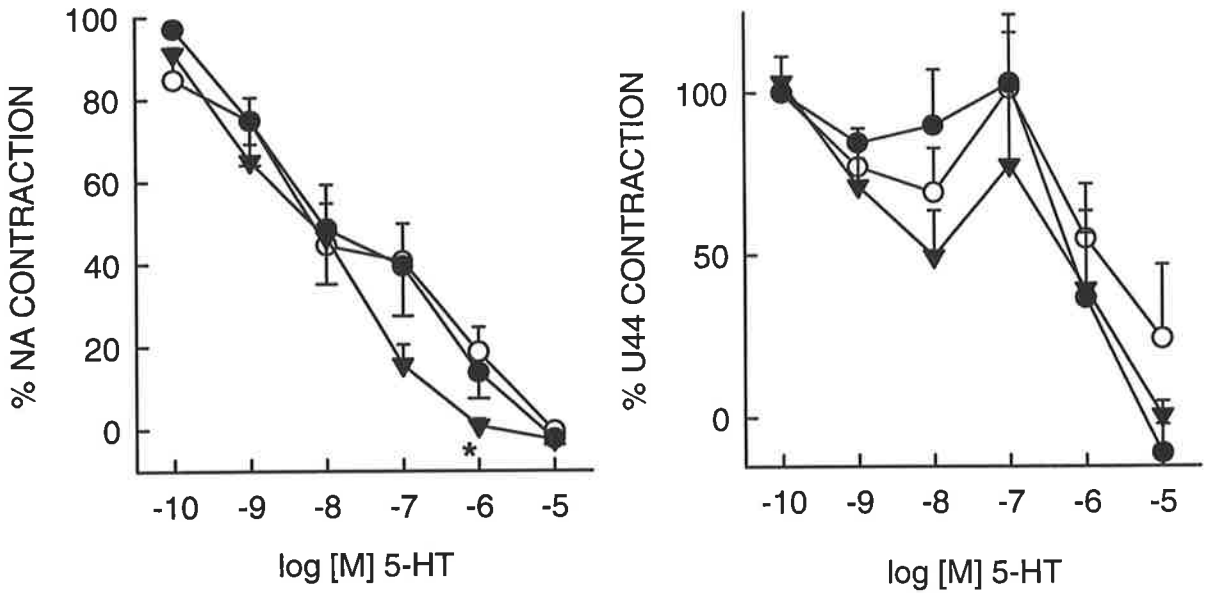

Figure 7.4 5-HT dose response curves of aortic rings from control (○), hyper-responding (●) or hypo-responding (▼) marmosets pre-contracted with (a) NA or (b) U44069 at the EC_{50}. *P<0.05 vs. control.

7.3.6 Vascular Preparations, Experiment #2

Aortic rings and carotid artery segments from one animal in the control group exhibited maximal contraction to NA, PE and U44069 which was consistently <30% of the relative mean E_{max} for this group; thus, vascular results from this animal were excluded.

Aortic rings. There were no significant differences in contractile responses to KPSS, NA, PE or U44069 between control, hyper-responder and hypo-responder groups (*Table 7.1*).

In NA contracted arteries, relaxation to 1.0μM 5-HT was significantly greater in hypo-responding animals (n=9) than in control animals (n=6, *Figure 7.4a*; P=0.051 for 0.1μM 5-HT). Relaxation to 5-CT (in the presence of GR127935, 10nM) did not significantly differ between any of the marmoset groups, in PE contracted vessels both in the absence and presence of NOLA (100 μM; *Figure 7.5b; Table 7.2*). However, in NA pre-contracted aortae the pEC_{50} significantly differed between hypo- and hyper-responders (*Figure 7.5a, Table 7.2*). The mean pEC_{50} for 5-CT did not significantly differ between vessels pre-contracted with NA as compared with PE; however, the variance of the pEC_{50}s was significantly greater in the PE than in the NA contracted vessels in the hypo-responders (P<0.05).

In U44069 contracted arteries, the response to 5-HT was variable and (unexpectedly) exhibited very little of the contractile activity seen in most of the experimental groups documented in earlier chapters. The response did not significantly differ between control, hypo-responding and hyper-responding groups (*Figure 7.4b*). A fade of contraction to sumatriptan at the high dose of 10μM was observed in these experiments with PE contracted arteries. The net effect was a significantly greater final response to 10μM sumatriptan in hyper-responding than in hypo-responding vessels (n = 5, *Figure 7.6a*). In NA contracted arteries, responses to 10μM sumatriptan did not significantly differ between

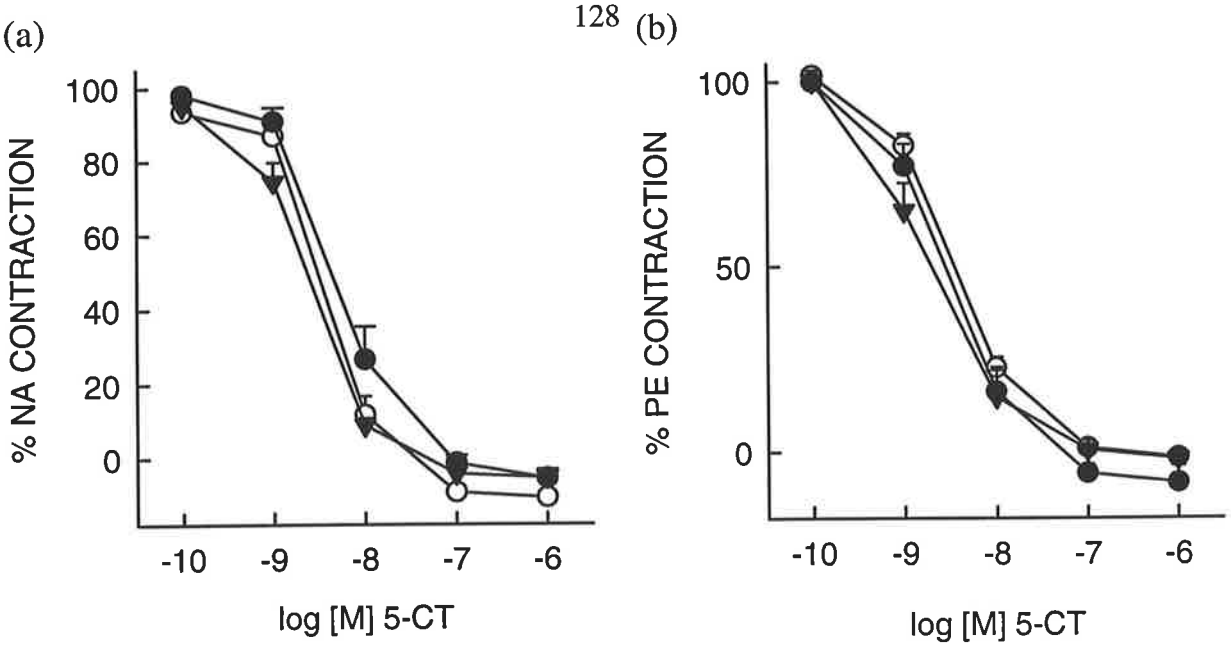

Figure 7.5 5-CT dose response curves of aortic rings from control (○), hyper-responding (●) or hypo-responding (▼) marmosets in the presence of GR127935 (10nM) and (a) NA or (b) PE plus NOLA (100μM).

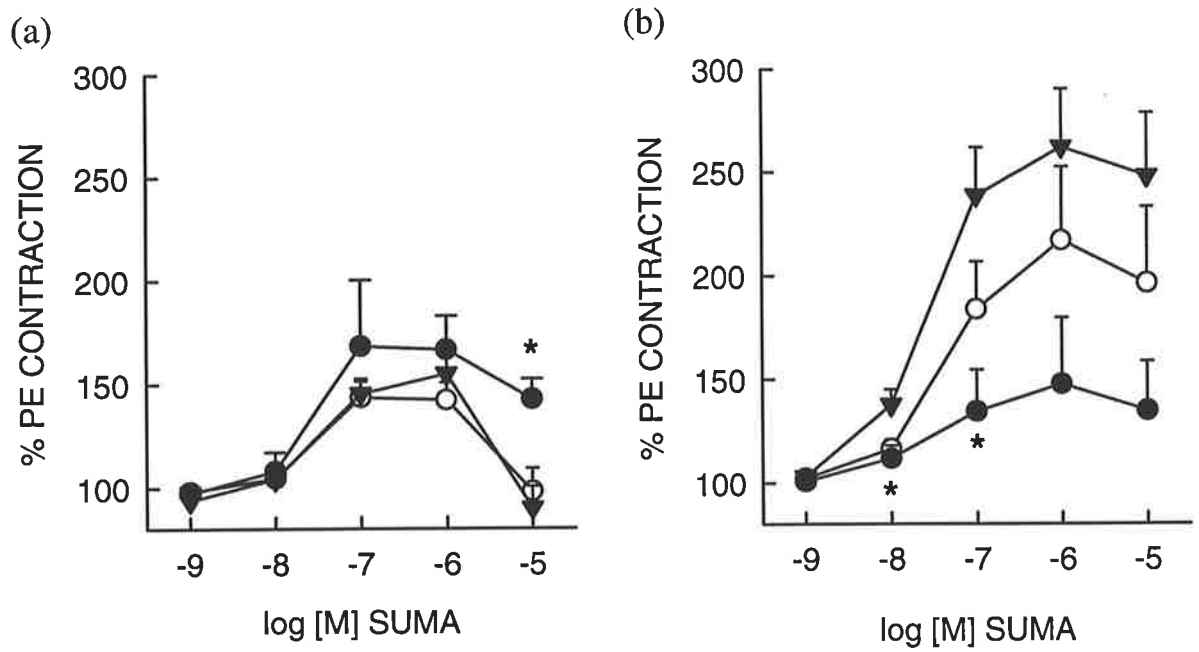

Figure 7.6 Sumatriptan dose response curves of aortic rings from control (○), hyper-responding (●) or hypo-responding (▼) marmosets pre-contracted with PE at the EC_{50} and in the absence (a) or presence (b) of NOLA (100μM). *P<0.05 hyper- vs. hypo-responders.

	CONTROLS (*n=6-7*)		HYPER-RESPONDERS (*n=5*)		HYPO-RESPONDERS (*n=10*)	
AGONIST	pEC_{50}	E_{max}(g)	pEC_{50}	E_{max}(g)	pEC_{50}	E_{max}(g)
KPSS	N/A	2.53 ± 0.14	N/A	2.48 ± 0.27	N/A	2.57 ± 0.11
NA	6.1 ± 0.1	2.41 ± 0.36	6.1 ± 0.1	2.20 ± 0.37	6.1 ± 0.1	2.60 ± 0.10
PE	6.0 ± 0.1	2.10 ± 0.25	6.1 ± 0.1	2.14 ± 0.30	6.0 ± 0.1	2.43 ± 0.16
U44069	7.8 ± 0.1	1.21 ± 0.31	7.9 ± 0.1	0.84 ± 0.19	7.8 ± 0.1	1.20 ± 0.82

Table 7.1 EC_{50} and E_{max} values for dose response curves to various contractile agonists in marmoset aortic rings isolated from animals maintained on control or atherogenic diets for 10 months. NA, noradrenaline; PE, phenylephrine; N/A, not applicable.

RELAXANT AGENT	**CONTROLS** (*n*)	**HYPER-RESPONDERS**	**HYPO-RESPONDERS**
5-CT + GR (NA)	8.5 ± 0.1 (*6*)	8.3 ± 0.1 (*5*)	8.6 ± 0.1 (*10*)$^{\#}$
5-CT + GR (PE)	8.5 ± 0.1 (*5*)	8.5 ± 0.1 (*5*)	8.8 ± 0.2 (*10*)
5-CT + GR + NOLA (PE)	8.5 ± 0.0 (*6*)	8.5 ± 0.1 (*5*)	8.7 ± 0.1 (*9*)
BK (NA)	8.7 ± 0.2 (*5*)	8.2 ± 0.1 (*3*)	8.7 ± 0.1 (*8*)
A23187 (NA)	8.6 ± 0.0 (*6*)	8.5 ± 0.1 (*5*)	8.6 ± 0.0 (*10*)
A23187 (PE)	8.5 ± 0.0 (*6*)	8.5 ± 0.1 (*5*)	8.6 ± 0.1 (*10*)
SNP (NA)	7.9 ± 0.2 (*6*)	8.2 ± 0.1 (*5*)	8.2 ± 0.1 (*10*)

Table 7.2 pEC_{50} values for dose response curves to various relaxant agonists in marmoset aortic rings isolated from animals maintained on control or atherogenic diets for 10 months. 5-CT, 5-carboxamidotryptamine; GR, GR127935 (10nM); BK, bradykinin; SNP, sodium nitroprusside. Chemicals in brackets indicate the pre-contractile agents; NA, noradrenaline; PE, phenylephrine; NOLA, N-nitro-L-arginine. $^{\#}P<0.05$ vs. hyper-responders.

groups (n = 3; data not shown). In the presence of NOLA (100μM) the contractile response to sumatriptan was significantly greater than in the absence of NOLA, for the hypo-responding and control animal groups (control: sumatriptan 10μM ± NOLA, $P<0.05$; hypo-responders: sumatriptan 10nM-10μM ± NOLA, $P<0.05$). In hyper-responding marmosets, the response to 0.01 μM and 0.1 μM sumatriptan was significantly less than in hypo-responding animals in the presence of NOLA (*Figure 7.6b*).

Responses to the endothelium-dependent relaxing agent A23187 were not significantly different between marmoset groups, with either NA or PE as the pre-contractile agent (*Figure 7.7a, Table 7.2*). The pEC_{50} for relaxation to BK was not significantly different betweeen groups (P= 0.07). Relaxation to the 0.3nM concentration only was significantly less in hyper-responding marmosets than in control or hypo-responding groups (*Figure 7.7b, Table 7.2*). Relaxation to SNP did not differ between groups (*Table 7.2*).

Carotid arteries. Contraction to KPSS in hyper-responding animals maintained on the atherogenic diet was significantly greater than in the control animals (*Figure 7.8*; *Table 7.3*). Responses to NA and U44069 did not differ between groups (*Table 7.3*). In NA-contracted arteries, relaxation to 5-CT in the presence of GR127935 (10nM) was greater in hypo-responding than control animals, without a statsitically significant shift in the EC_{50} (*Figure 7.9a, Table 7.4*). Responses to sumatriptan in carotid arteries contracted with NA did not differ significantly between groups (*Figure 7.9b*). The pEC_{50} for relaxation to A23187 was significantly greater in hyper- than hypo-responding animals, without an effect on the maximum relaxation (*Figure 7.10, Table 7.4*).

Coronary arteries. Responses to 5-CT (0.001 μM - 10 μM; n = 5-7) and sumatriptan (0.001 μM - 10 μM; n = 2-10) in coronary arteries contracted with U44069 did not differ significantly between animal groups (data not shown).

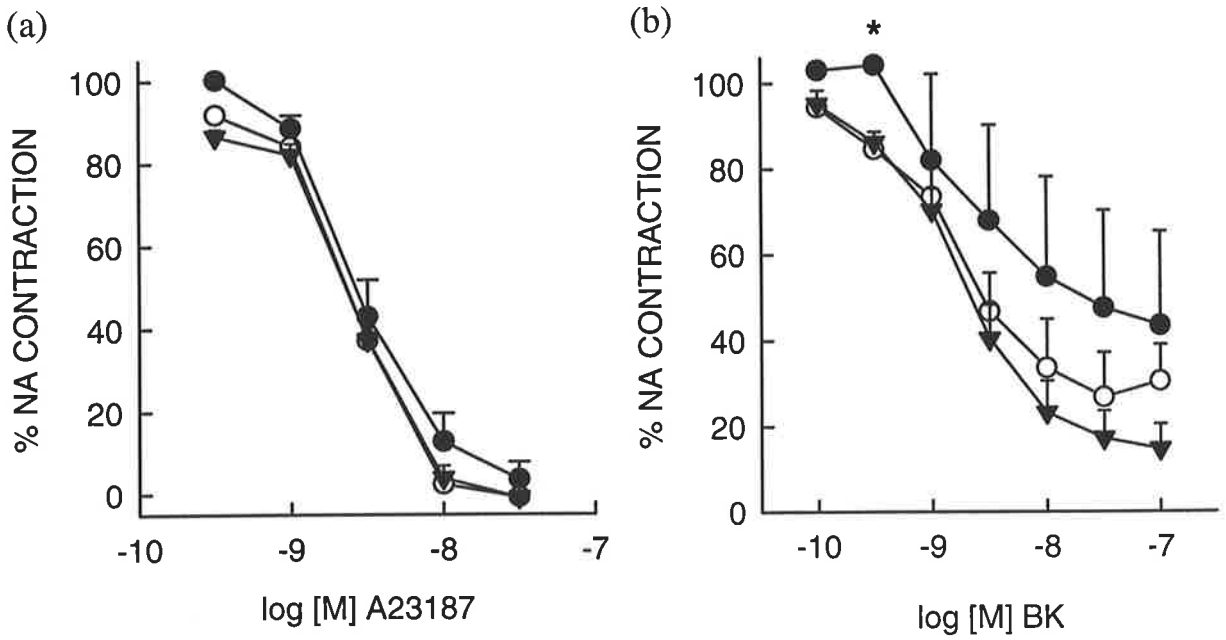

Figure 7.7 Dose response curves of aortic rings from control (○), hyper-responding (●) or hypo-responding (▼) marmosets to (a) A23187 or (b) bradykinin (BK), both pre-contracted with NA at the EC_{50}. *P<0.05 vs. control and hypo-responders.

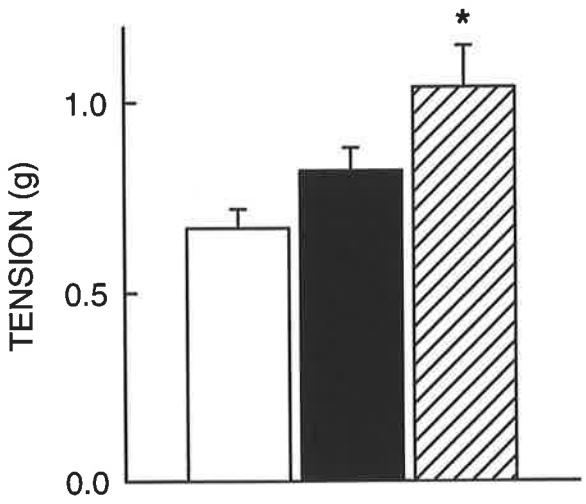

Figure 7.8 Response of carotid arteries from control (▭), hypo-responders (▬), or hyper-responders (▨) to KPSS. *P < 0.05 vs. control.

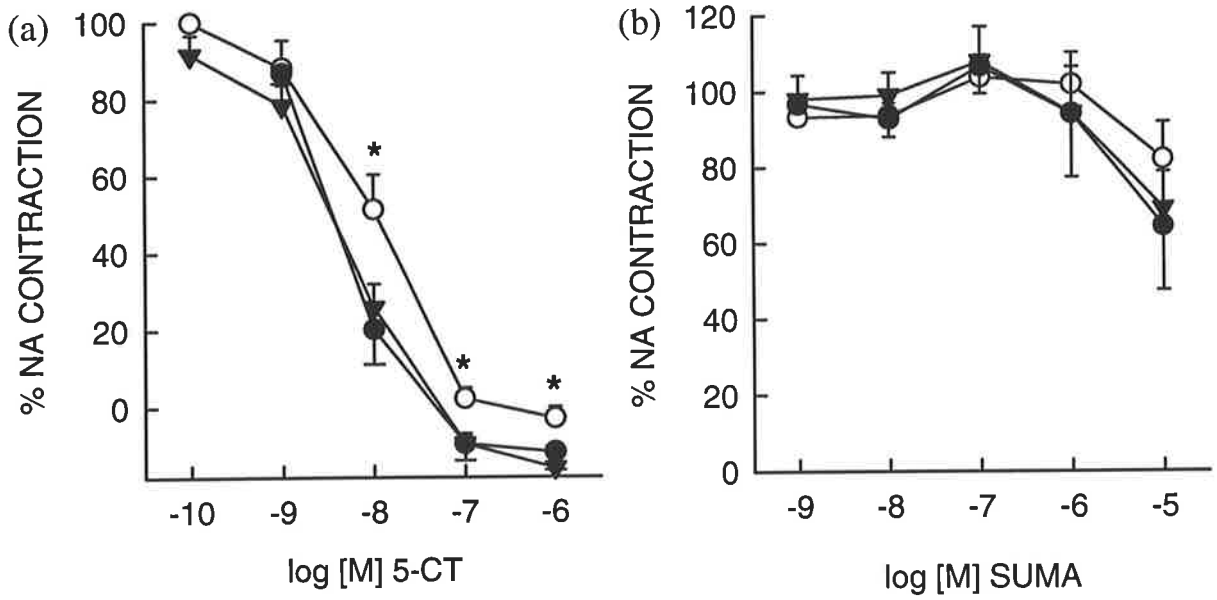

Figure 7.9 Dose response curves of carotid artery rings from control (○), hyper-responding (●) or hypo-responding (▼) marmosets to (a) 5-CT in the presence of GR127935 (10nM) or (b) sumatriptan, both pre-contracted with NA at the EC_{50}. *P<0.05 vs. hypo-responders.

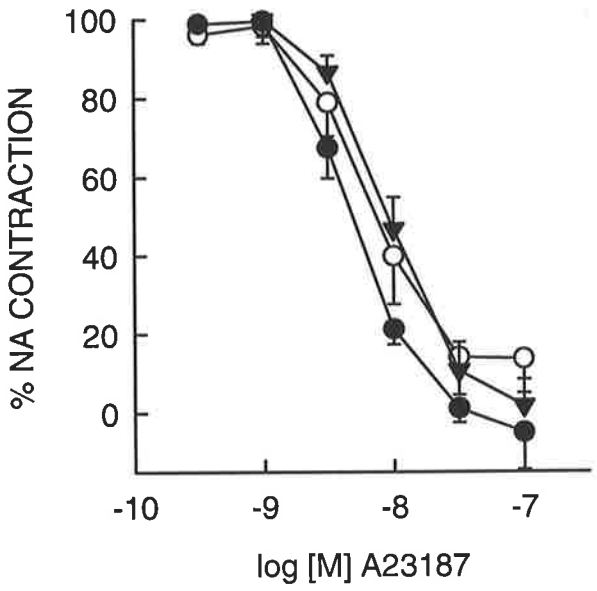

Figure 7.10 Dose response curves of carotid arteries from control (○), hyper-responding (●) or hypo-responding (▼) marmosets to A23187 in the presence of NA at the EC_{50}.

Table 7.3 EC_{50} and E_{max} values for dose response curves to various contractile agonists in marmoset carotid artery rings isolated from animals maintained on control or atherogenic diets for 10 months. NA, noradrenaline. *P<0.05 vs. controls.

	CONTROLS (*n=5-7*)		**HYPER-RESPONDERS** (*n=4-5*)		**HYPO-RESPONDERS** (*n=8-9*)	
AGONIST	pEC_{50}	E_{max}(g)	pEC_{50}	E_{max}(g)	pEC_{50}	E_{max}(g)
KPSS	N/A	0.67 ± 0.05	N/A	1.04 ± 0.11*	N/A	0.82 ± 0.06
NA	6.1 ± 0.2	0.64 ± 0.09	5.9 ± 0.1	0.91 ± 0.24	5.9 ± 0.1	0.66 ± 0.08
U44069	8.1 ± 0.1	0.68 ± 0.19	7.6 ± 0.2	0.30 ± 0.10	7.8 ± 0.0	0.41 ± 0.07

Table 7.4 pEC_{50} values for dose response curves to various relaxant agonists in marmoset carotid artery rings isolated from animals maintained on control or atherogenic diets for 10 months. 5-CT, 5-carboxamidotryptamine; GR, GR127935 (10nM). Chemicals in brackets indicate the pre-contractile agents; NA, noradrenaline. #P<0.05 vs. hyper-responders.

RELAXANT AGENT	**CONTROLS** (*n*)	**HYPER-RESPONDERS**	**HYPO-RESPONDERS**
5-CT + GR (NA)	8.1 ± 0.2 (*5*)	8.3 ± 0.1 (*4*)	8.3 ± 0.1 (*9*)
A23187 (NA)	8.1 ± 0.1 (*6*)	8.3 ± 0.1 (*5*)	8.0 ± 0.1 (*9*)#

7.4 Discussion

7.4.1 Plasma Lipids

The response of circulating plasma lipid levels to dietary cholesterol and fat supplementation was highly variable between individual marmosets. This large degree of variation is typical of outbred species such as the human and non-human primates due to the large contribution of genetic factors to the dietary cholesterol response (; Clarkson *et al.,* 1976). Segregation of responsiveness of plasma lipid levels to dietary lipid supplementation into hyper- and hypo-responders has previously been documented in non-human primates as well as lower species (Clarkson *et al.,* 1976; McGill *et al.,* 1988; Battacharyya *et al.,* 1989; Crook *et al.,* 1990). These differences may be due to variations in cholesterol absorption, metabolism and/or excretion (Clarkson *et al.,* 1976; Battacharyya *et al.,* 1989; Turley *et al.,* 1997).

Final plasma total cholesterol levels measured at 10 months were significantly less than levels measured at 9 months. Quality control samples were included in all assay runs, thus assay error is unlikely to be the cause. Final (10 month) samples were taken from anaesthetised animals, whereas samples at all other time points were taken from conscious animals. This may be an effect of the anaesthetic, since at least one general anaesthetic (propofol) has been shown to have significant plasma lipid lowering effects in humans (Myles *et al.,* 1995).

Histological staining of the abdominal aorta from marmosets fed the atherogenic diet revealed minimal deposition of lipid, despite elevated circulating plasma cholesterol levels. The development of atherosclerotic lesions has been demonstrated in marmosets in some previous studies (Dreizen *et al.,* 1973; McIntosh *et al.,* 1988; Crook *et al.,* 1990), but not in others (Abbey, 1990). Dietary supplementation with 12% fat (but not cholesterol) for

90 weeks in 1 year old animals produced only minimal hypercholesterolaemia and atheroma (McIntosh *et al.,* 1987). Similarly, a diet supplemented with 0.2% cholesterol and 10% fat for 30 weeks, in 1.5-2 year old marmosets did not result in atheroma development (Abbey, 1990). In contrast, a study utilising 0.32% cholesterol and 12% fat for 42 weeks in 2-3 year old animals produced moderate lesions (Crook *et al.,* 1990). Further, 0.5% cholesterol and 10% fat in 5-9 year old animals for 11 months produced extensive lesions (McIntosh *et al.,* 1988). These studies involved varying levels of dietary cholesterol supplementation and the hypercholesterolaemic response may be proportional to the dietary cholesterol concentration given (McIntosh *et al.,* 1984). In addition, 4 year old marmosets have significantly higher total plasma cholesterol levels than 2 year old animals maintained on a normal diet and are more prone to the lipid-raising effects of vitamin C deficiency (McIntosh *et al.,* 1984). Thus, susceptibility to atheroma development following hypercholesterolaemia may rise with increasing age. The marmosets utilised in the current study were fed 0.4% cholesterol and were aged 2-3 years and thus their young age may have provided some protection against atheroma development. Another factor which has a strong influence upon susceptibility to atherosclerosis is dietary anti-oxidant content (gershoff, 1993; Mahfouz *et al.,* 1997). Analysis of the anti-oxidant levels in the different diets, however, was beyond the scope of this study. In summary, the animals in this study exhibited a nil, low level (hypo-responders) and high level (hyper-responders) of hypercholesterolaemia with no atheroma development.

7.4.2 Vascular Reactivity

Aortic Rings. The absence of differences in contractile responses of control, hypo-responding or hyper-responding marmoset aortae to K^+, NA, PE or U44069 implies that smooth muscle contractility was not affected by the dietary treatment. Similarly, relaxation to sodium-nitroprusside did not differ between the groups, implying that the sensitivity of the smooth muscle to nitric oxide (NO) had not been affected.

The actions of 5-HT on the aorta did differ in some respects between treatment groups. First, however, it should be noted that in the control aorta there was a lack of the usual contractile response and instead the presence of a relaxant effect at low concentrations of 5-HT in the U44069 contracted artery. There was also a stronger relaxant effect of 5-HT in the range 0.001 - 0.1μM in the NA contracted arteries (*Figure 7.4a*) than that observed in the earlier studies (eg. *Figure 3.2*). This is the concentration range where there is endothelium-dependent relaxation (see *Chapter 3*). This apparently greater effect of the endothelium may reflect the younger age of the animals (2-3 years) used in the dietary studies since there is evidence of an age relationship of this response (see *Figure 3.3*). Endothelium-dependent effects are stronger in vessels from younger animals or patients (Tschudi *et al.,* 1991; Mantelli *et al.,* 1995; Taddei *et al.,* 1995; Gerhard *et al.,* 1996). These responses to the low concentrations of 5-HT did not significantly differ between treatment groups, with either NA or U44069 pre-contraction.

At the higher concentrations of 0.1 and 1.0μM 5-HT, the hypo-responding marmosets exhibited enhanced relaxation in NA contracted aortae. Since relaxation to the endothelium-dependent relaxing agents A23187 or BK, however, was unchanged, the enhanced relaxation to 5-HT is unlikely to be endothelium-mediated. In view of the evidence presented earlier for the presence of an endothelium-independent receptor

resembling the 5-HT_7 subtype, it is reasonable to suggest that expression of this receptor is increased in the hyporesponders. In accord with this suggestion is the observation that the EC_{50} for relaxation to 5-CT in hypo-responders is significantly lower than the EC_{50} in hyper-responders. The difference cannot be attributed to an influence of the contractile 5-HT_1-like receptor since an antagonist of the latter receptor, GR127935, was present. Against this suggestion is the fact that the EC_{50}s for 5-CT-induced relaxation did not differ between groups when vessels were contracted with PE instead of NA.

Another difference between the groups was in the response to sumatriptan. Sumatriptan contracted the arteries in the absence of NOLA, the only difference being greater maintenance of contraction to 10μM sumatriptan in hyper-responders relative to hypo-responders. NOLA enhanced contraction in aortae from hypo-responders and controls but not in hyper-responders. Therefore, it is suggested that underlying basal NO production there is a greater sensitivity of hypo-responder than hyper-responder aortae to sumatriptan. NO-production may also mask underlying differences in sensitivity to other contractile agonists. The effect of production of NO on the response to sumatriptan was greatest in the order hypo- > control > hyper-responders. Hyper-responding aortae produce relatively less basal NO and have a reduced sensitivity to sumatriptan compared to hypo-responders. In addition, NO could be responsible for the fade in contraction to sumatriptan observed at supramaximal concentrations.

The relatively reduced basal NO production in hyper-responders was reflected in decreased relaxation to 0.3nM bradykinin only (however a reduced relaxant response in this group may have been detected with a greater *n*). This reduction was not reflected in the response to A23187 although both responses were endothelium-dependent. This difference could be related to the implied impaired NO generating capacity of the hyper-responders. Since there is evidence in other tissues that BK generates endothelium-derived

hyperpolarizing factor (Drummond & Cocks, 1996; Kemp & Cocks, 1997), NOLA would only suppress the NO contribution to relaxation.

Carotid Arteries. The responsiveness of the carotid arteries differed from that of the aorta. The carotid arteries from hyper-responders exhibited greater contraction to potassium, but not NA or U44069. Enhanced responses to vasoconstrictors in association with hypercholesterolaemia prior to atherosclerotic changes has been widely documented (Heistad *et al.,* 1984; Chin *et al.,* 1990; Galle *et al.,* 1991; Woodman, 1995). This may occur due to reduced inhibition of contraction by basal NO production. However, Godfraind (1986) has found that endothelium removal did not affect contraction to potassium. Alternatively, this could indicate a hyper-responsiveness to calcium influx in the carotid arteries, although this would be expected to be reflected in responses to all contractile agonists. The sensitivity of the contractile apparatus in vascular smooth muscle to calcium is altered in some pathological states (Uehata *et al.,* 1997; Savineau & Marthan, 1997). *In vitro* exposure to cholesterol has been shown to sensitize canine coronary arteries to Ca^{2+} and to increase the contractile response to K^+ (Yokoyama & Henry, 1979). There is presumably not the same magnitude of difference in responses between control and hypo-responding animals since vessels from this group were exposed to a far more moderate increase in plasma cholesterol.

Relaxation to 5-CT was greater in hypo-responders than control animals. This is in accord with the greater relaxation to high concentrations of 5-HT in the aorta, again suggesting that expression of the receptor resembling the 5-HT_7 subtype is increased in the hypo-responders. However, in the carotid artery the hyper-responders show the same trend in increased relaxation (although in this case it did not reach statistical significance).

Unexpectedly, hyper-responder carotid arteries displayed *greater* relaxation to the

endothelium-dependent relaxing factor A23187 than hypo-responders (although not controls). The majority of studies have demonstrated reduced endothelium-dependent relaxation in the presence of hypercholesterolaemia, in a wide range of species and vessels (Jayakody *et al.,* 1985; Vita *et al.,* 1990; Simonsen *et al.,* 1991b; Creager *et al.,* 1992; O'Rourke & Docherty, 1998). However, cholesterol feeding has been shown to induce vascular non-endothelial NO production (Verbeuren *et al.,* 1993). Galle *et al.* (1991) suggest that the effect of hypercholesterolaemia in the absence of atherosclerosis differs from that of hypercholesterolaemia maintained for a longer period of time (until the occurrence of atherosclerotic lesions). In Galle's rabbit model, the degree of inhibition of endothelium-dependent relaxation was dependent on the level of development of atherosclerosis (not present with hypercholesterolaemia alone) and many of the aforementioned studies do not include histological assessment. A study utilizing rabbits fed a high cholesterol diet found that endothelium-dependent relaxation was inhibited in both atherosclerotic and hypercholesterolaemic aortae, despite increased production of NO (Minor *et al.,* 1990). Bialecki and Tulenko (1993) reported that endothelium-dependent relaxation in rabbit carotid arteries was enhanced by *in vitro* exposure to cholesterol, due to an increase in EDRF half life and/or release (Bialecki & Tulenko, 1993). In most tissues this can be reconciled with decreased endothelium-dependent relaxation due to a simultaneous (and presumably greater) increase in superoxide anion production (Ohara *et al.,* 1993). However, it is possible that in the carotid arteries from cholesterol fed animals in the current study NO production may be in excess of superoxide anions.

7.4.3 Summary

Dietary cholesterol and fat supplementation for 10 months produced hypercholesterolaemia without atherosclerosis. Marmosets exhibited either a marked increase (hyper-responders)

or small increase (hypo-responders) in plasma lipid levels in response to dietary fat and cholesterol supplementation. No differences were observed in aortic responses to K^+, NA, PE, U44069, or the relaxing agents A23187 and SNP between groups. Neither were there any differences in coronary responses or carotid responses to NA or U44069. Within the marmosets whose dietary response to lipid-loading is reduced (hypo-responders), an mildly increased capacity for 5-HT_7-mediated relaxation was present in aortae and carotid arteries. This may provide an underlying vascular protective difference in this subset of animals. In addition, contractile sensitivity to sumatriptan was reduced and indications were that basal NO production was less in hyper-responder than hypo-responder aortae, in the absence of atheromatous changes. In carotid arteries, endothelium-dependent relaxation in hyper-responders was enhanced rather than impaired.

CHAPTER 8

EFFECTS OF BALLOON ANGIOPLASTY ON SEROTONERGIC RESPONSES OF THE MARMOSET AORTA

8.1 Introduction

The previous chapter described the effects of hypercholesterolaemia on the responses to 5-HT in the marmoset vasculature. In that study, dietary intervention was used with the aim of inducing vascular disease. However, prolonged hypercholesterolaemia for 10 months produced no obvious atheroma. In other experimental animal models utilising balloon catheters, intimal hyperplasia is limited and does not produce the extensive restenosis seen clinically without the additional insult of dietary intervention. The extent of hyperplasia is also dependent upon the degree of medial damage induced by the angioplasty (Fingerle *et al.,* 1990; Gonschior *et al.,* 1995). The intimal proliferation observed following balloon angioplasty is predominantly due to the migration and multiplication of vascular smooth muscle cells and accumulation of extracellular matrix synthesised by these myointimal cells (see *1.7.4*). According to other animal models of balloon injury, at the 6-week post-surgery stage, vascular smooth muscle cell (VSMC) growth should have ceased and endothelial regrowth should be almost complete (Clowes & Reidy, 1991; Bauters & Isner, 1997).

Thus, the study described in the current chapter utilised mechanical arterial damage (balloon angioplasty) to induce a non-lipid form of arteriosclerosis as a model of vascular disease. Specifically, the aim was to determine what influence this model of vascular disease has on 5-HT receptor-specific responses in the marmoset aorta at an early (3

weeks) and later stage (6 weeks) of intimal hyperplasia.

8.2 Methods

8.2.1 Animals

Adult marmoset monkeys were sourced and housed as described previously (*2.1*). Due to limited availability of marmosets, the controls (aged 34 ± 1 months) used were those described in the preceding experiment (*Chapter 7*). There was an age difference between the marmosets subjected to balloon angioplasty (53 ± 3 months) and the controls. At these ages there was no clear correlation with the level of responses to 5-HT (see *Figure 3.3*). Nevertheless, in case the correlation seen in *Figure 3.3* applied rigorously to all ages, the data on 5-HT obtained in the current experiments was age corrected for a single dose (0.1 μM 5-HT) of one experiment (NA pre-contracted aortae). The trend in results observed in this experiment was sustained when the response was age corrected.

8.2.2 Surgery (Angioplasty)

The descending aorta was denuded of endothelium utilising Fogarty arterial embolectomy catheters (2F; Baxter Health Care, Santa Ana, CA, USA) utilising a procedure based on that described by Davies *et al.* (1994). The marmosets were administered a single oral dose of prophylactic antibiotic (amoxycillin/clavulanic acid, "Clavulox", Pfizer Animal Health, Australia; 12.5 mg/kg), fasted overnight, and anaesthetised with Saffan (20mg/kg i.m.). The iliac artery was exposed and a saline-filled embolectomy catheter inserted and passed along the length of the aorta. Subsequently, the balloon was inflated to generate slight resistance and passed along the length of the aorta. The procedure was repeated twice more to achieve complete denudation. (In preliminary experiments utilising Wistar

rats, histological analysis revealed that this procedure was effective in removing the intimal layer from the aorta.) The groin wound was closed and the animals monitored until recovery from anaesthesia. Catheters were sterilised with 1% benzalkonium chloride and rinsed with sterile water between uses (one catheter was used for 3-4 animals). For five days following surgery the marmosets were administered the antibiotic "Amoxil" (amoxycillin, SmithKline Beecham, U.K.; 10 mg/kg) in the drinking water. Seven animals were sacrificed 3 weeks following and seven animals 6-7 weeks following surgery. In a rat model of balloon carotid angioplasty these time periods would correspond to hyperplastic stages before and after the cessation of smooth muscle cell growth (Clowes & Reidy, 1991).

8.2.3 Aortic Ring Preparations

The thoracic aorta was prepared and 8 aortic rings mounted as described previously (*2.2*).

The organ bath protocols followed the outline detailed in *2.2.1* and were based on that for the atherogenic experiment, as described in *7.2.6*. Aortic rings 1-4 were duplicates of arteries 1&2 described in *7.2.6*. Aortic rings 5-8 were treated as follows:

- ring 5 PE precontraction (i) 5-CT + GR127935 (ii) A23187 (iii) SNP
- ring 6 PE precontraction (i) 5-CT + GR127935 (ii) A23187
  (iii) 5-CT + GR127935 + NOLA
- ring 7 PE precontraction (i) sumatriptan (ii) A23187 (iii) SNP
- ring 8 PE precontraction (i) sumatriptan (ii) A23187 (iii) sumatriptan + NOLA

In aortic rings 6&8 part (iii) was conducted in 6-week post-surgery animals only.

8.2.4 Data Analysis

Data analyses were conducted as previously described (*2.8*). Comparisons of vascular responses between control, 3-week and 6-week post-surgery groups were conducted using one way ANOVAs.

8.3 Results

Maximum contractile force generated in response to K^+, NA, PE, and U44069 did not significantly differ between control and angioplasty animals (*Table 8.1*). The pEC_{50} for contraction to NA, but not PE, was significantly greater in angioplasty animals than control animals for both time periods post-surgery (*Figure 8.1a; Table 8.1*). Three-weeks post-surgery the pEC_{50} for contraction to U44069 was also significantly enhanced. However, this returned to normal 6 weeks after surgery (*Figure 8.1b, Table 8.1*).

In U44069 contracted arteries, contraction to 10 and 100nM 5-HT was enhanced in angioplasty animals (*Figure 8.2b*). Sumatriptan-induced contraction was significantly greater in arteries from either group of angioplasty animals than in control arteries (*Figure 8.3a*). Following incubation with NOLA (100μM) 6-week post-surgery animals exhibited enhanced contraction to 10nM sumatriptan (*Figure 8.3b*). In these experiments, NOLA did not affect basal tone.

Relaxation in response to 0.1μM 5-HT in NA contracted preparations was reduced in marmosets subjected to angioplasty by comparison with controls (*Figure 8.2a*). Based on the correlation between age and 0.1μM 5-HT response in *Figure 3.2*, the age-corrected untreated response to 0.1μM 5-HT for the groups would be control, 55.9 ± 5.3%; 3-weeks, 32.9 ± 2.3%; and 6-weeks, 31.2 ± 5.2% relaxation. This compares to actual responses in

	CONTROLS ($n=6$)		3-WEEKS ($n=7$)		6-WEEKS ($n=7$)	
AGONIST	pEC_{50}	E_{max}(g)	pEC_{50}	E_{max}(g)	pEC_{50}	E_{max}(g)
KPSS	N/A	2.50 ± 0.16	N/A	1.79 ± 0.19	N/A	1.97 ± 0.36
NA	6.1 ± 0.1	2.41 ± 0.36	6.8 ± 0.2*	2.64 ± 0.36	6.8 ± 0.1*	2.55 ± 0.38
PE	6.0 ± 0.1	2.10 ± 0.25	6.4 ± 0.2	2.14 ± 0.22	6.5 ± 0.1	2.15 ± 0.33
U44069	7.8 ± 0.1	1.21 ± 0.31	8.3 ± 0.2*	1.20 ± 0.25	8.0 ± 0.1	1.47 ± 0.31

Table 8.1 EC_{50} and E_{max} values for dose response curves to various contractile agonists in marmoset aortic rings isolated from control marmosets or marmosets 3 or 6 weeks after angioplasty. NA, noradrenaline; PE, phenylephrine; N/A, not applicable. *$P<0.05$ vs. control.

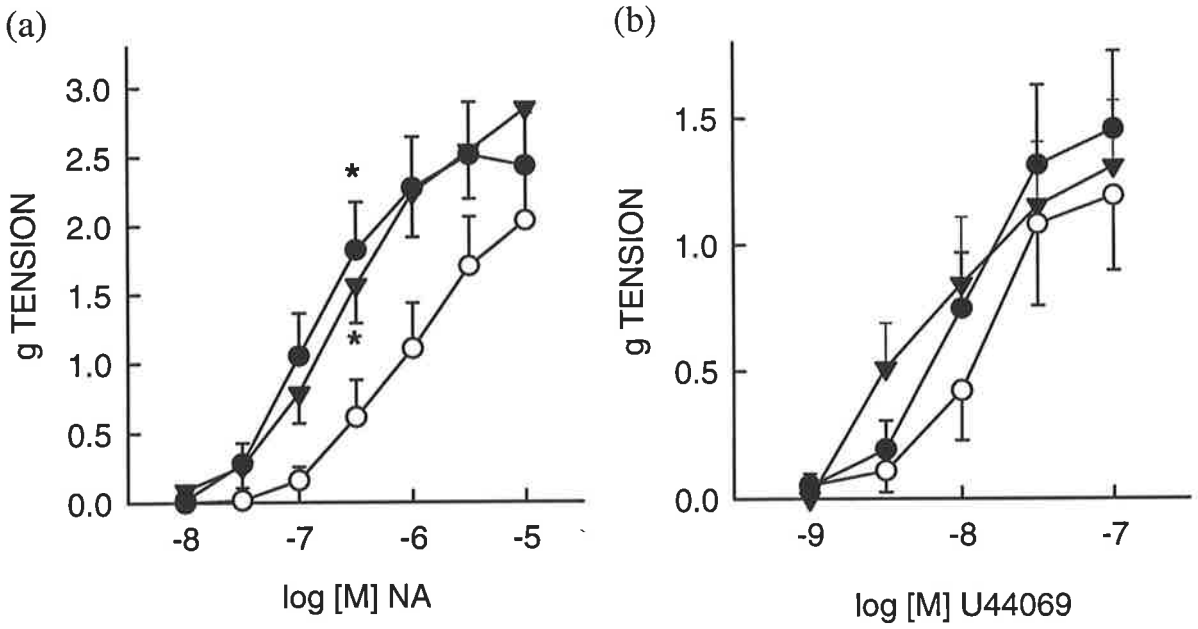

Figure 8.1 Dose response curves of aortic rings from control (○), 3-week (▼) or 6-week (●) post-angioplasty marmosets to (a) NA or (b) U44069. *P<0.05 vs. control.

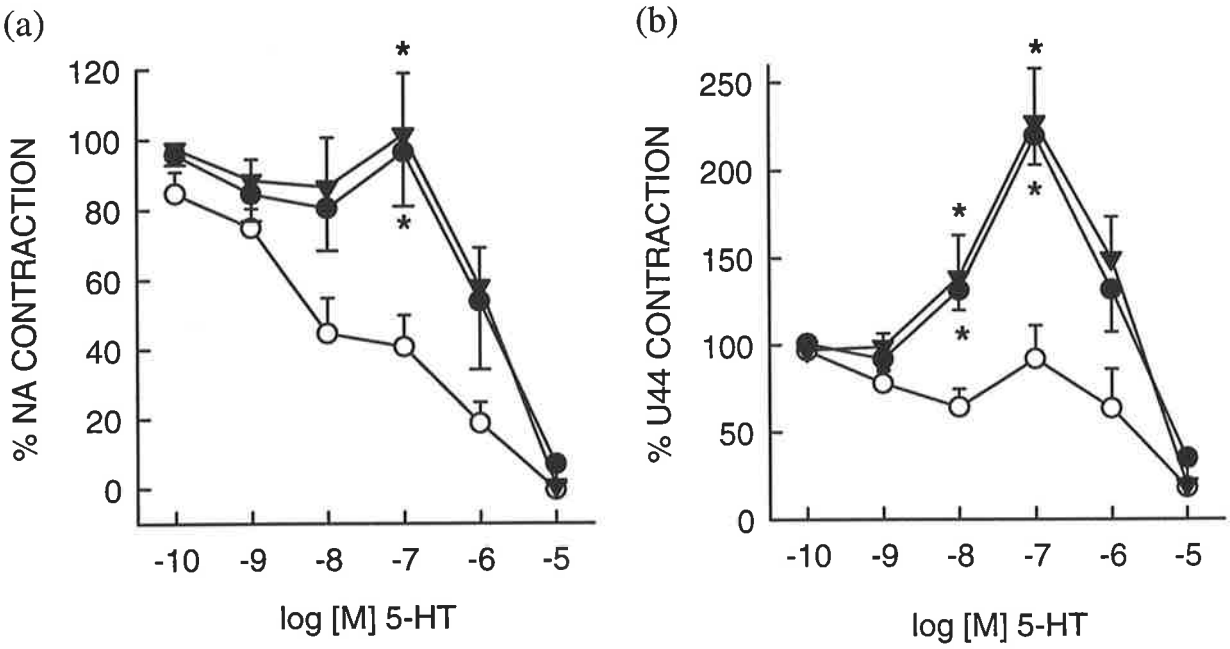

Figure 8.2 5-HT dose response curves of aortic rings from control (○), 3-week (▼) or 6-week (●) post-angioplasty marmosets pre-contracted with (a) NA or (b) U44069 at the EC_{50}. *P<0.05 vs. control.

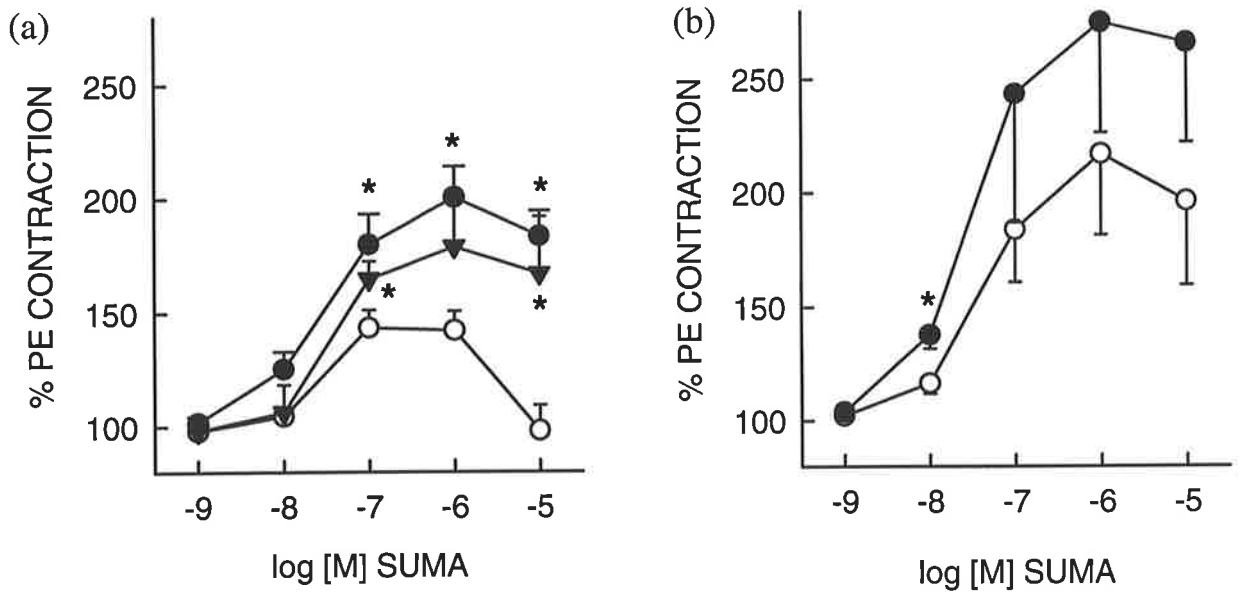

Figure 8.3 Sumatriptan dose response curves of aortic rings from control (○), 3-week (▼) or 6-week (●) post-angioplasty marmosets, pre-contracted with PE at the EC_{50} and in the absence (a) or presence (b) of NOLA (100μM). *P<0.05 vs. controls.

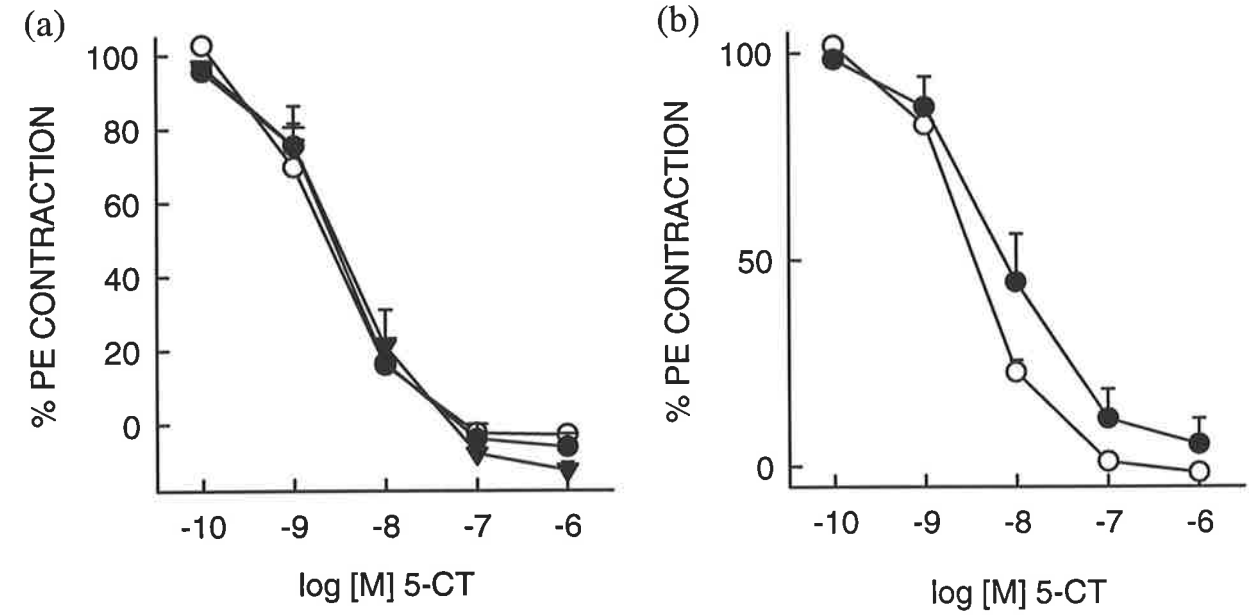

Figure 8.4 5-CT dose response curves of aortic rings from control (○), 3-week (▼) or 6-week (●) post-angioplasty marmosets, in the presence of GR127935 (10nM) and PE at the EC_{50} and the absence (a) or presence (b) of NOLA (100μM).

treated groups of control, 59.3 ± 9.1%; 3-weeks, -1.4 ± 17.4%; and 6-weeks, 3.6 ± 15.4 % relaxation. Thus, treated groups exhibited approximately 30% less relaxation than predicted by their age and this difference was significant by *t*-test for the 3-week post-angioplasty group ($P<0.05$, $n = 7$). In PE-contracted arteries, relaxation to 5-CT in the presence of GR127935 was unaltered by angioplasty (*Figure 8.4, Table 8.2*). In the presence of NOLA (100μM) the pEC_{50} for relaxation to 5-CT was reduced in 6-week animals (*Figure 8.4b, Table 8.2*). NOLA itself raised basal tone by 0.13 ± 0.04g and 0.17 ± 0.06g in control and 6-week post-angioplasty aortae respectively.

The pEC50 for responses to the endothelium-dependent relaxing agent A23187 was significantly less in both 3-week and 6-week post-surgery animals than in control animals without an effect on the maximum relaxation achieved (*Figure 8.5a, Table 8.2*). Relaxation to SNP did not differ between treatment groups (*Figure 8.5b, Table 8.2*).

RELAXANT AGENT	**CONTROLS** (*n=6*)	**3-WEEKS** (*n=7*)	**6-WEEKS** (*n=7*)
5-CT + GR (PE)	8.5 ± 0.1	8.4 ± 0.2	8.5 ± 0.1
5-CT + GR + NOLA (PE)	8.5 ± 0.0	N/A	8.0 ± 0.1*
A23187 (NA)	8.6 ± 0.0	8.0 ± 0.2*	7.9 ± 0.1*
A23187 (PE)	8.5 ± 0.0	7.9 ± 0.1*	7.9 ± 0.1*
SNP (NA)	7.9 ± 0.2	8.2 ± 0.1	8.0 ± 0.1

Table 8.2 pEC_{50} values for dose response curves to various relaxant agonists in aortic rings isolated from control marmosets or marmosets 3 or 6 weeks after angioplasty. 5-CT, 5-carboxamidotryptamine; GR, GR127935 (10nM); SNP, sodium nitroprusside. Chemicals in brackets indicate the pre-contractile agents; NA, noradrenaline; PE, phenylephrine; N/A, not applicable. $^{\#}P<0.05$ vs. controls.

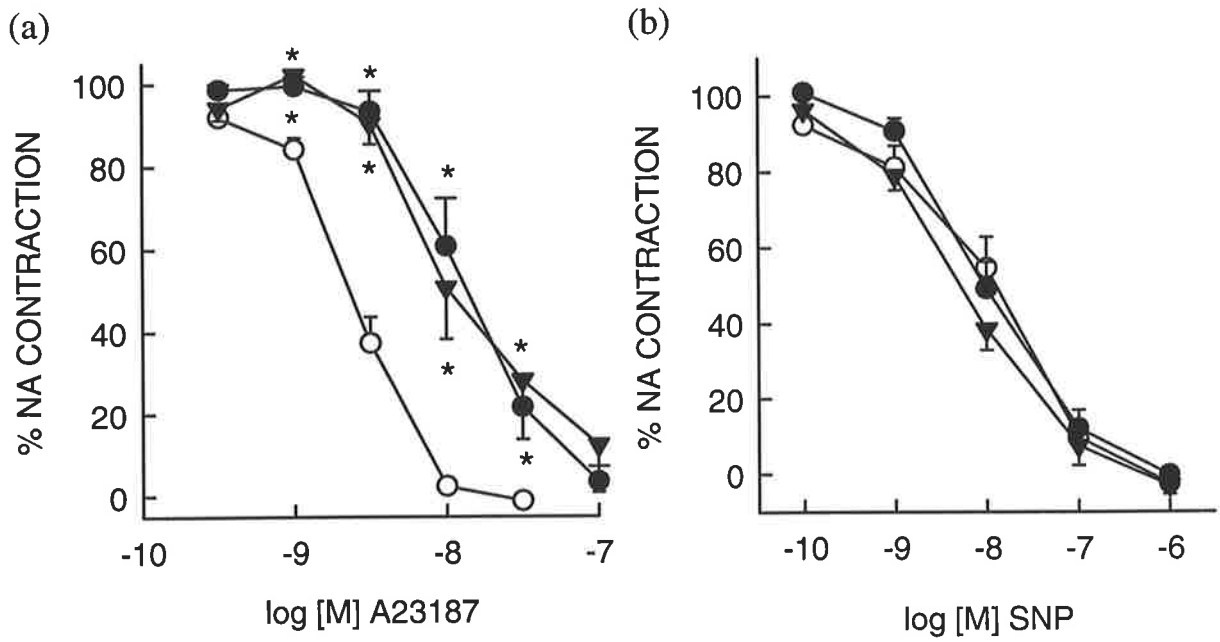

Figure 8.5 Dose response curves of aortic rings from control (o), 3-week (▼) or 6-week (●) post-angioplasty marmosets to (a) A23187 or (b) sodium nitroprusside (SNP), both pre-contracted with NA at the EC_{50}. *P<0.05 vs. controls.

8.4 Discussion

Following balloon angioplasty in marmoset monkeys, the maximal contractile strength of the vessels was not significantly altered. Thus, any medial damage occurring during surgery had been functionally repaired within 3 weeks. In addition, there were no differences in the relaxant response to sodium nitroprusside between groups, suggesting that the sensitivity of the smooth muscle response to NO was unimpaired. Hence, the observed increase in sensitivity to NA, 5-HT and sumatriptan in ballooned arteries could be explained by impaired endothelial function leading to a reduction in basal NO-production, in accord with the evidence of reduced endothelial function demonstrated by the decline in relaxation to A23187. Increased sensitivity to NA and 5-HT following angioplasty has also been documented in porcine coronary arteries (Park *et al.,* 1995). However, in the current study sensitivity to U44069 returned to control levels 6 weeks following surgery. An implication is that responses to NA and 5-HT may be more sensitive to dysfunctional endothelium than U44069. Likewise, Tx receptor-mediated contraction may more readily indicate some recovery of the endothelial function, being more easily suppressed by EDRFs. This is in accord with the effects of endothelium removal on the E_{max} for contraction as determined in *Chapter 3*. The effects of *in vitro* inhibition of endothelial function suggested that U44069-induced contraction may be more sensitive to NO-inhibitory effects than NA. Hadoke *et al* (1995) have suggested that "the regrown endothelium is at first defective, but later resumes its normal function". Differential sensitivity during recovery has been noted by Shimokawa *et al* (1987) and Park *et al* (1995), who saw no change in the responsiveness of porcine coronary arteries to prostaglandin $F_{2\alpha}$ 4 weeks after angioplasty, despite altered responsiveness to other agents.

A limitation in the present study is that it was not possible to age match the control and treated groups. Retrospective analysis of data from earlier studies on NA-contracted

arteries revealed that the extent of relaxation to 0.1 μM 5-HT is crudely correlated with age (*Figure 3.3*). Many studies have indicated that increasing age reduces endothelium-dependent responses in animals and humans (Tschudi *et al.,* 1991; Mantelli *et al.,* 1995; Gerhard *et al.,* 1996; Sirtori & Vega, 1997). However, relaxation to 0.1μM 5-HT (a concentration at which the endothelium influence the response) in the angioplasty treated groups was reduced by a greater amount than that predicted by the age difference alone. Thus, it is likely that the treatment is the major cause of the differences observed.

Responses to 5-HT in ballooned arteries were characterised by reduced relaxation and enhanced contraction. The question arises as to whether the decrease in relaxation is genuine or simply due to masking by the increased contractile component of the response. Since relaxations mediated by 5-CT (in the presence of GR127935) did not vary between treatment groups, it is unlikely that differences in responses are at the relaxant (possibly 5-HT_7) receptor level. In contrast, contractions to sumatriptan in the presence of PE were enhanced. As discussed in earlier chapters, such differences may be due to either reduced basal NO levels or reduced 5-HT receptor-mediated endothelium-dependent inhibitory effects. Either mechanism could explain the enhanced contractions and reduced relaxations produced by 5-HT in aortae from angioplasty animals.

When NO production was inhibited by incubation with NOLA, a slight but statistically significant difference in the pEC_{50}s for 5-CT between control and 6-week post-angioplasty animals was observed. The pEC_{50}s for the control group did not differ in the absence and presence of NOLA; ie., NO had an influence upon this response in the angioplasty animals, but not controls. This exemplifies the complex nature of the interactions between vasoactive factors occurring in this tissue. In the absence of any influence of NO, angioplasty animals exhibited a lesser sensitivity to relaxation mediated by 5-CT than controls. However, in the presence of NO, its greater contribution to relaxation concealed

the underlying difference in relaxation between the two groups. It is not possible to fully explain this. However, it may be that in angioplasty animals there is an additive effect of basal NO with the 5-HT_7 mediated response in these arteries. Balloon angioplasty has been demonstrated to induce NOS activity in the vessel wall of rat carotid arteries 24 hours after injury (Joly *et al.,* 1992). Thus, whilst receptor-mediated EDRF release is reduced, basal NO may be increased. The theory of enhanced basal NO production in angioplasty treated arteries, however, contradicts the trend of increased sensitivity to NA and the effects on 5-HT-induced responses in angioplasty animals, in particular, the increase in sumatriptan-induced contractions (*Figure 8.3a*), as discussed above. It is also unlikely that basal endothelium-derived NO production is increased, since receptor-mediated endothelium-dependent NO release was reduced (*Figure 8.5a*). The reason for the discrepancy in the effects of NOLA on basal tone in rings 6 and 8 is also unknown.

The reduced relaxation in response to A23187 which was observed indicates impaired endothelial function. Thus, if the endothelium has in fact fully regenerated, its functional properties have not been fully retained.

In summary, the current study demonstrated that balloon angioplasty of the aorta increased the sensitivity to NA. Sensitivity to U44069 was increased at 3-weeks but not at 6-weeks following surgery. Thus, NA-mediated contraction is more susceptible to the effects of endothelial damage than U44069-mediated contraction. In addition, angioplasty enhanced the contractile response to 5-HT and sumatriptan, thus reducing relaxation to 5-HT in NA-contracted arteries. The differences observed are most likely due to reduced endothelial function, as demonstrated by a decline in relaxation to A23187.

CHAPTER 9

GENERAL DISCUSSION

Vasospasm is the excessive constriction of an artery which reduces local blood flow and may be an initiating factor in many pathophysiological cardiovascular incidents. 5-hydroxytryptamine and Tx A_2, as platelet released vasoactive factors, are potential mediators of vasospasm. Thus, studies of the vascular effects of these agents are a key component in advancing the understanding of the pathophysiology of cardiovascular disease, a major cause of morbidity and mortality in today's society.

Functional responses of the vasculature to 5-HT are however quite diverse and manifest as either vasodilatation or vasoconstriction or a combination of the two. Responses vary between vessels from different species and vascular beds and also depend upon the resting tone and endothelial integrity of the vessel studied. Thus, extrapolations of findings between species can be unreliable and studies in primate species such as those presented in this thesis are particularly relevant to the clinical situation (Pregenzer *et al.,* 1997).

In Chapter 3 of this thesis the responses of the marmoset aorta to a variety of vasoactive agents were determined. This vessel was demonstrated to possess strong α_1-adrenergic contractile responses and also contraction in response to the Tx A_2-mimetic U44069. There was greater functional antagonism by NO of U44069-mediated contraction than NA-induced contraction. This could occur if there was less receptor reserve for Tx receptors than α_1-receptors in this tissue. An important physiological implication for this would be the preservation of vessel patency in undamaged arterial segments, which produce NO, in areas adjacent to sites of platelet aggregation, where Tx A_2 is released.

The marmoset aorta exhibited responses to 5-HT which differ greatly from those observed in the aorta of commonly used rodent species, where 5-HT_{2A} receptors mediate a strong contractile response. In the marmoset aorta with intact endothelium and other agents absent, 5-HT was inactive. However, when the tone was increased with a contractile agent both contractile and relaxant responses to 5-HT became apparent. It appears that three 5-HT-induced responses are present in the marmoset aorta. At low concentrations (0.001 - 0.1 μM) 5-HT produces both an endothelium-dependent relaxant response and a contractile response and at higher concentrations (0.1 - 10μM) there is endothelium-independent relaxation.

At low concentrations, the relaxant response is endothelium-dependent and can therefore be influenced by factors that affect endothelial function, such as age (Docherty, 1990; Mantelli *et al.,* 1995; Taddei *et al.,* 1995; Gerhard *et al.,* 1996), sex (Hayashi *et al.,* 1992) and the presence or absence of disease states (see *1.7.3*). This relaxant response was most apparent in younger animals, such as those used in the studies described in Chapter 7. Specifically, this could account for the negative correlation of relaxation to 0.1μM 5-HT with age. Whilst no attempt was made to determine the nature of the receptor subtype mediating the endothelium-dependent relaxant response to 5-HT, some information is available. Relaxation at low concentrations of 0.001 - 0.1μM 5-HT occurred in the presence of the 5-HT_{1B} (non-rodent) and 5-HT_{1D} (and 5-HT_1-like) receptor-selective antagonist GR127935 (*Figure 4.3*). In addition, relaxation to the 5-HT_1-agonist 5-CT was unaffected by incubation with the NO synthase inhibitor NOLA (*Table 7.2*), indicating that 5-CT is not an agonist of the receptor responsible for endothelium-dependent relaxation. Thus, it is unlikely that the receptor is of the 5-HT_1 group. Endothelium-dependent relaxation mediated by a 5-HT_1-like receptor has been demonstrated in porcine coronary artery (Schoeffter & Hoyer, 1990) whereas endothelium dependent relaxation of the rat

jugular vein is mediated by a 5-HT_{2B} receptor (Ellis *et al.*, 1995). Expression of both 5-$HT_{1D\beta}$ and 5-HT_{2B} receptor types has been demonstrated in human aortic endothelial cells (Ullmer *et al.*, 1995). Utilising a variety of receptor specific agonists and antagonists in a group of young animals will provide further information on the receptor subtype involved in the marmoset aortic response. These investigations should focus on endothelium-dependent relaxation in arteries incubated with GR127935 to block the contractile interaction. It will be necessary to determine if LY53857 antagonises the endothelium-dependent relaxation. The existing evidence suggests that this is likely considering the enhancement of contractile responses by LY53857 which occurs within the low range of 5-HT concentrations (*Figure 5.3*).

At high concentrations (0.1 - 10μM), 5-HT induced a strong endothelium-independent relaxant response. The endothelial-independence was described in Chapter 3 and confirmed in Chapter 7 by the lack of effect of the NO synthase inhibitor NOLA on 5-CT mediated relaxation. In Chapter 5 it was demonstrated that this relaxation was antagonised by methysergide and LY53857, but not tropisetron. Ketanserin was without effect on relaxation in the U44069-contracted artery (Chapter 4). 5-CT was a potent agonist whilst sumatriptan was inactive with respect to relaxation. The relaxation was also associated with stimulation of cyclic AMP production. On the basis of this it was concluded that the endothelium-independent relaxation is most likely to be mediated by a 5-HT_7 receptor. The receptor identity could be further tested by expanding the experiments conducted in Chapter 5 in arteries incubated with NOLA (or denuded of endothelium) plus the 5-HT_1-like receptor antagonist GR127935, by utilizing other agonists in addition to 5-CT and 5-HT. Under these conditions a K_D for antagonism by LY53857 could also be determined. Confirmation of the presence of 5-HT_7 receptor mRNA in the marmoset aorta by the use of Northern blotting or reverse transcription-

polymerase chain reaction would provide proof that this receptor is actively transcribed in this tissue.

Evidence was presented in Chapter 3 that under conditions of reduced relaxation, eg. incubation with NOLA and LY53857, 5-HT produces a contractile response in the aorta. In Chapter 4 it was shown that a 5-HT_1-like receptor mediates this response, since a) both 5-CT and sumatriptan acted as agonists, b) the specific 5-HT_1-like antagonist GR 127935 was an antagonist of the response, c) methysergide acted as a partial agonist, d) ketanserin, LY53857 and tropisetron had no antagonistic activity and e) the response was associated with the inhibition of forskolin-stimulated cyclic AMP accumulation. This receptor is also responsible for an amplification interaction occurring between 5-HT and U44069 or endothelin but not NA or K^+ (Chapter 6). It was shown that ß-receptor activity on the part of NA was not responsible for masking a contractile interaction with 5-HT under these conditions. The amplification response was greatest when endothelial function was reduced (Chapters 3 & 6). Thus, the synergistic interaction existing between U44069 and 5-HT_1-like responses is of potential pathophysiological importance as a mechanism for vasospasm occurring in arteries where endothelial function is impaired.

In coronary artery disease and in the presence of the risk factors increased age, hypertension and hyperlipidaemia, platelets become hyperreactive (Carvalho *et al.,* 1974; Tremoli *et al.,* 1984; De Cree *et al.,* 1985; Koutouzov *et al.,* 1989; Akopov, 1992). LDL has been reported to amplify 5-HT-induced platelet aggregation (Fetkovska *et al.,* 1989) and 5-HT uptake may be reduced in hypertension so that perivascular 5-HT concentrations may be raised (Fetkovska *et al.,* 1990). Thus, platelets may aggregate and release vasoactive factors like 5-HT and Tx A_2 readily under the pathological conditions prevailing in atherosclerosis. Since endothelial function is reduced in atherosclerosis (Berkenboom *et al.,* 1987; Chester *et al.,* 1990; Sellke *et al.,* 1990; Ganz *et al.,* 1991;

Luscher *et al.*, 1993), these factors may cause a pathological level of constriction and amplify each other's vasoconstrictive response, as demonstrated in this thesis.

In Chapter 3 comparisons were made between responses to 5-HT in the aorta, carotid, mesenteric and coronary arteries. The marmoset common carotid artery exhibited contractile and relaxant responses similar to those of the aorta but the nature of the receptors responsible remains to be examined in detail. The presence of 5-CT mediated and sumatriptan insensitive relaxation, however, was suggestive of a relaxant receptor similar to that present in the aorta (Chapter 7). In the mesenteric artery there was evidence of relaxation and contraction but with stronger contractile responses than in either the aorta or the carotid artery. Again the nature of the receptors responsible has not been examined. The coronary artery differed from these vessels in that NA was inactive and the contractile effects of 5-HT predominated. In Chapter 4 it was demonstrated that the contractile response in the coronary artery was blocked by GR 127935 and mimicked by 5-CT and sumatriptan suggesting that a 5-HT_1-like receptor was responsible for this response. In the presence of GR 127935 a relaxant response was evident but the nature of this response was not further investigated. Significantly, the amplification interaction between U44069 and 5-HT was also present in carotid, mesenteric and coronary arteries, indicating the importance of this interaction across many vascular beds. Further investigations should focus on characterising the receptors involved in mediating 5-HT responses in these vascular beds, in particular determining the endothelium-dependence and nature of the relaxation observed in the coronary artery.

Importantly, it was demonstrated in Chapter 7 that marmosets exhibited a large variation in responsiveness of plasma lipid levels to dietary fat and cholesterol loading. Presumably this is because they are an outbred species. In a group of young marmosets, hypercholesterolaemia for 10 months was not associated with the development of

atherosclerotic lesions. Responses to the contractile agents K^+, NA, PE or U44069 and the endothelium-dependent and -independent relaxing agents A23187 and sodium nitroprusside respectively did not differ between aortae from animals in different treatment groups. Neither were there any differences in responses in the coronary artery or to NA or U44069 in the carotid artery. However, in the subset of animals who were hyper-responsive to dietary cholesterol, contractile sensitivity to sumatriptan was reduced and indications were that basal NO production was less than in hypo-responders, as revealed in the presence of NOLA. It has been reported that nitric oxide may have many anti-atherogenic effects (Cooke & Tsao, 1994) and that hypercholesterolaemia is generally associated with a reduced capacity to release EDRF (Henry *et al.,* 1995). However, this may not be the case in hypo-responding animals. Surprisingly, endothelium-dependent relaxation in the carotid arteries from hyper-responders was greater than in hypo-responders. Interestingly, the hyporesponders exhibited a slightly enhanced relaxant response to the high concentration, endothelium-independent component of 5-HT-induced relaxation in the aorta (by comparison with hyper-responders) and also to 5-CT in the carotid artery (by comparison with controls). These relaxant effects may provide an important vascular protective mechanism, whose absence in hyper-responding animals place the latter at a higher risk of cardiovascular disease. Importantly, these differences were observed in the presence of hypercholesterolaemia but the absence of atherosclerotic lesions. Expansions of these studies should focus on quantification of 5-HT_7 receptor expression in hyper- and hypo-responding animals.

In Chapter 8 the potential for a pathophysiological synergistic interaction between U44069 and 5-HT_1-like responses in arteries where endothelial function is impaired was demonstrated by the greater contractile interactions and reduced relaxant effects of 5-HT in animals several weeks after balloon vascular injury. In aortae subjected to balloon

angioplasty, contraction to NA and U44069 was enhanced, this effect persisting to 6-weeks (after surgery) for NA but not U44069 and indicating a lesser sensitivity to the effects of endothelium damage in the case of U44069. Importantly, the contractile effects of sumatriptan were increased and the relaxant effects of 5-CT reduced in angioplastied animals, as revealed in the presence of NOLA. These effects are most likely to be due to reduced endothelial function, as indicated by decreased relaxation to A231287 both 3- and 6-weeks following surgery. Thus, in arteries with reduced endothelial function, 5-HT will produce greater contractile effects and amplification responses will be enhanced, providing a potential for pathological vasoconstriction.

This interaction between 5-HT and Tx A_2 is a potential cause of vasospasm occurring in human atherosclerotic arteries. The discovery of the 5-HT_7 receptor and the increasing evidence of its widespread vascular relaxant activities provide an important new site for pharmacological intervention. New potent agonists of this receptor may provide specific vascular protective effects which may assist in negating the vasoconstrictor effects of 5-HT and other agonists in situations of reduced endothelial function.

In summary, the marmoset aorta presents a system whereby relaxant effects of 5-HT, mediated both by the endothelium and by an endothelium-independent (possibly 5-HT_7-receptor) mechanism occur in opposition to a 5-HT_1-like contractile effect of 5-HT in the vasculature. Under conditions of intact endothelial function, the relaxant effects of 5-HT predominate. However, when endothelial function is impaired, 5-HT's contractile action and amplification of Tx A_2-mediated contraction predominates. This could be of major importance in the pathophysiology of atherosclerosis and its complications and provides a model likely to be physiologically very similar to the human.

APPENDIX I

MARMOSET DIETS

I.1 Standard Colony Diet

Diet Constituent	*Quantity (%w/w)*
Wheat	38.9
Field peas	13.9
Oats	8.0
Meatmeal	8.5
Rice pollard	8.0
Fishmeal	5.0
Soyabean meal	6.0
Cottonseed meal	4.0
Sunflower oil	1.9
Calcite (calcium source)	1.5
Wafex (binder)	1.2
Tallow	1.0
Kynophos (phosphorus source)	1.0
Vitamin/mineral premix (see Appendix I.2)	0.4
Salt	0.2
Choline chloride	0.2
L-lysine	0.17
Ascorbyl PM (heat stable form of vitamin C)	0.01
DF 750 (flavouring)	0.1
Wheat pollard	0.02

I.2 Vitamin/mineral premix

Premix Constituent	*Quantity (%w/w)*	*Activity (mg/kg)*
BHT	2.5	
Cal-Dl-Pantothanate	1.125	20
Cobalt sulphate	0.125	1.0
Copper sulphate	1.5	1.0
Calcium iodate	0.125	1.0
D meal (pollard carrier)	69.625	
Ferrous sulphate	8.375	100
Folic acid	0.125	5.0
Hetrazeen (vitamin K_3)	0.75	4.5
Manganous oxide	2.0	50
Niacin	1.5	60
Pyrodoxine HCl	1.25	20
Riboflavin	0.75	30
Biotin	0.375	0.3
Thiamine HCl	1.625	65
Vitamin B_{12}	0.25	0.01
Vitamin A	1.375	8.3
Vitamin E	4.5	90
Vitamin D_3	0.625	0.312
Zinc oxide	2.25	63

PLEASE NOTE:

1 mcg retinol = 3.3 IU vitamin A
1 mcg cholecalciferol = 40.0 IU vitamin D_3
1 mg α tocopherol = 1.1 IU vitamin E

I.3 Standard Diet Nutrient Levels

Nutrient	*Quantity (%)*
DE (pigs)	13.7 mJ/kg
Crude protein	21.1
Crude fat	7.2
Fat added	2.9
Crude fibre	4.2
Ash	7.1
Moisture	13.6
Calcium	1.86
Phosphorus - total	1.28
- available	0.96
Sodium	0.22

I.4 Atherogenic Experimental Diet #2

Constituent	*Control (%)*	*Atherogenic (%)*
Casein	20	20
Poultry meal	7.9	7.9
Corn starch	33.4	30
Sugar	16	11
Wheat bran	10	10
Fat	4	12
Cholesterol	0.02	0.4
Minerals	5.9	5.9
Vitamins	0.2	0.2
Flavouring	0.15	0.15
Gelatine	2.5	2.5

APPENDIX II

CHEMICALS AND SOLUTIONS

II.1 Chemical Sources

Standard laboratory chemicals were obtained from Ajax Chemicals, Sydney, Australia. Except for the following, all other chemicals were obtained from Sigma Chemical Company, MO, U.S.A. Clarase was obtained from Solvay Enzymes. Saffan (Pitman-Moore), containing 0.9% alphaxalone and 0.3% alphadolone acetate, was purchased from Lyppard S.A. Pty Ltd, Sth. Aust., Australia. Heparin sodium was obtained from CSL, Melbourne, Australia. Clavulox (amoxycillin/clavulanic acid) was obtained from Pfizer Animal Health, Australia and Amoxil from Smith Kline Beecham, U.K. Methysergide maleate was purchased from Research Biochemicals Inc., Natwick, MA, USA and Substance P from Auspep, Australia. LY 53857 maleate was a gift from Eli Lilly & Co., Indianapolis, Indiana, USA. 5-carboxamidotryptamine maleate, GR 127935 and sumatriptan succinate (GR 43175C) were gifts from Glaxo Group Research Ltd, Greenford, Middlesex, UK, and tropisetron (ICS 205-930) hydrochloride was a gift from Sandoz Australia Pty Ltd, Sydney, Aust. Forskolin and indomethacin were dissolved in absolute ethanol; all other agents were dissolved in saline with ascorbic acid (0.01%) when appropriate.

II.2 Histology Solutions

Phosphate Buffered Saline (PBS) 1 LITRE

Dipotassium hydrogen tetroxide	6.71 g
Potassium dihydrogen tetroxide	1.57 g
Sodium chloride	8.5 g
Sodium azide	0.1 g

Adjust to pH 7.4

Fixative

Glutaraldehyde	1.25%
Paraformaldehyde	4%
Sucrose	4%

Paraformaldehyde dissolved in PBS buffer at 60-65°C, then sucrose added. Solution cooled, then EM grade glutaraldehyde added and made up to volume with PBS.

Stains

Methylene Blue

Methylene blue	0.13 g
Azure II	0.02 g
Glycerol	10 ml
Methanol	10 ml
0.15M phosphate buffer, pH 6.0	30 ml
Distilled water	50 ml

Basic Fuschin

Solution A

Basic fuschin	0.125 g
Glycerol	5 ml
Distilled water	50 ml

Solution B

Sodium tetraborate	0.125 g
Distilled water	50 ml

Working solution - make up immediately before use

Mix equal volumes of solutions A and B

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