5-HT-Receptor Mediated Responses In Pulmonary Arteries: Changes With Developmental Age And Pulmonary Hypertension.

by

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Abstract

(1) 5-HT receptor mediated responses were investigated in perinatal (foetal, 0-24 hours, 4 day and 7 day old), and adult rabbit isolated pulmonary conduit (PCAs) and resistance (PRAs) arteries and in human PRAs.

(2) In all rabbit vessels 5-HT and α-methyl-5HT, which is selective for 5-HT_{2A} receptors, (1nM-100μM) were equipotent in causing contractions at each age. In the perinatal PRAs, sumatriptan, a selective 5-$HT_{1B/1D}$ agonist, and 5-CT (5-HT_1 and 5-HT_7 receptor agonist) produced negligible contractions, but in adult PRAs, 5-CT and sumatriptan were potent agonists with pEC_{50}s of 6.05 ± 0.3 and 5.7 ± 0.2 respectively.

(3) The nitric oxide, synthase inhibitor N^{ω}-nitro-L-arginine methylester (L-NAME;100μM) markedly increased the maximum contraction to 5-HT in the 0-24h, 4 day and 7 day PRAs and in all perinatal PCAs. L-NAME increased 5-HT potency in the 4-, 7-day old and adult rabbit PRA and the 0-24 hour PCAs.

(4) In all perinatal vessels, contractions to 5-HT, with L-NAME present, were antagonized by ketanserin (30nM and 0.1μM), which is selective for 5-HT_{2A} receptors, but not by GR55562 (1μM; 5-$HT_{1B/1D}$ antagonist). A small ketanserin (30nM) resistant, GR55562-sensitive component was observed in 0-24 hour PRAs but not PCAs.

(5) In adult PCAs, 5-HT-evoked contractions were competitively antagonized by low concentrations of ketanserin (10nM and 0.1μM) giving a pA_2 value of 8.9, but not by GR55562 (0.1-3μM).

(6) In adult PRAs, both ketanserin (30nM and 0.1μM) and GR55562 (1μM) inhibited 5-HT-evoked contractions. The selective 5-HT_{1B} receptor antagonist SB-224289 (200nM) and 5-HT_{1D} antagonist BRL-15572 (500nM) antagonized 5-CT-evoked contractions in these vessels.

These data suggest the presence of 5-HT_{2A}, 5-HT_{1B} and/or 5-HT_{1D} receptors in adult rabbit PRAs.

(7) Vasodilator responses to 5-CT were observed in pre-contracted PRAs from 4-and 7-day old rabbits but not in the foetus, 0-24h old or rabbit vessels nor the PCAs at all perinatal ages. At 4 days the vasodilator response was inhibited both by L-NAME (100μM) and GR55562 (1μM). At 7 days the response was only partly blocked by L-NAME and resistant to GR55562. The L-NAME resistant component was antagonized by the 5-HT_7 receptor antagonist spiperone (1μM).

(8) In precontracted adult PCAs, 5-HT and 5-CT induced relaxations which were inhibited by the 5-HT_7 receptor antagonists spiperone (1μM), pimozide (30nM) and risperidone (3nM) but not affected by endothelium removal.

(9) Collectively the results suggest that 5-HT_{2A} receptors predominantly mediate 5-HT-induced contractions in perinatal rabbit PCAs and PRAs and adult rabbit PCAs. The 5-$HT_{1B/1D}$ receptor also contributes in adult rabbit PRAs. The 5-$HT_{1B/1D}$ receptor mediates NO-dependent vasodilation in PRAs from 4 day rabbits whilst 5-HT_7 receptors mediate NO-independent vasodilation by 7 days.

(10) In human PRAs, the contractile effects of 5-HT (>0.1μM) were partially antagonized by ketanserin (0.1μM), whilst GR55562 (1μM) inhibited 5-HT-induced contractions at all concentrations of 5-HT (estimated $pK_B = 7.7 \pm 0.2$). Sumatriptan evoked contractions that were resistant to blockade by BRL-15572 (500nM) and ketanserin (0.1μM) but antagonized by SB-224289 (200nM; estimated $pK_B = 8.4 \pm 0.1$) and blocked by GR55562 (1μM). These data suggest that 5-HT-induced contraction is mediated via the 5-HT_{1B} and 5-HT_{2A} receptors in human PRAs.

(11) The influence of vascular tone induced by endothelin-1 (ET-1), neuropeptide Y (NPY), KCl 4-aminopyridine (inactivator of Kv channels, 4-AP) or the calcium ionophore A23187 on contractile responses to 5-CT was studied in PRAs from control rats and those exposed to chronic hypoxia (CH rats). The influence of L-NAME was also studied.

(12) In control rat smPAs, only high concentrations of 5-CT (> 1μM) induced vasoconstriction. Tone induced by NPY, 4-AP and A23187 had no effect on responses to 5-CT whilst responses to 5-CT were increased by ET-1- and KCl-induced tone. In the presence of ET-1- or KCl-induced vascular tone, L-NAME enhanced 5-CT-induced contraction further, increasing responses 5- and 2-fold (ET-1) and 6- and 2-fold (KCl).

(13) Responses to 5-CT were maximally enhanced in CH rat smPAs and increasing tone pharmacologically had no further effect on responses to 5-CT.

(14) The results suggest that inhibition of NOS combined with KCL- or ET-1-induced vascular tone potentiates responses to 5-CT in rat PRAs in a synergistic fashion and mimics the effects of chronic hypoxic exposure.

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Declaration

This thesis is entirely my own composition and the experimental work detailed within was undertaken wholly by myself except for the RT-PCR study described in chapter 4 which was undertaken by Dr. Robert Heeley, Dept. Neuroscience and Biomedical Systems, IBLS, University of Glasgow.

Signed [redacted].

Some of the results in this thesis have been published, details of which are given below.

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Glossary of acronyms and abbreviations

5-HTT	5-hydroxytryptamine transporter
AC	adenylate cyclase
bPAs	extralobar branch pulmonary arteries
cAMP	cyclic 3'5' adenosine monophosphate
CCRCs	cumulative concentration response curves
cGMP	cyclic 3'5' guanosine monophosphate
CHPHT	chronic hypoxic pulmonary hypertensive
EDHF	endothelium derived hyperpolarizing factor
eNOS	endothelial nitric oxide synthase
HPV	hypoxic pulmonary vasoconstriction
i.d.	internal diameter
IP_3	inositol trisphosphate
MAO	monoamine oxidase
MAPK	mitogen activated protein kinase
MLC	myosin light chain
MLCK	myosin light chain kinase
NO	nitric oxide
PAH	pulmonary arterial hypertension
PCAs	pulmonary conduit arteries
PDE	phosphodiesterase
PGI_2	prostacyclin
PHT	pulmonary hypertension
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PNECs	pulmonary neuroendocrine cells
PPHN	persistent pulmonary hypertension of the newborn
PRAs	pulmonary resistance arteries
PTX	pertussis toxin
PVR	pulmonary vascular resistance
PVSMC	pulmonary vascular smooth muscle cell
RVH	right ventricular hypertrophy
smPAs	small muscular pulmonary arteries
SMPP-1M	smooth muscle myosin phosphatase

Glossary II- Drugs used in this thesis

Drug	Description/Use in this thesis
NP-Y	Neuropeptide-Y; used to induce threshold contractions in smPAs
4-AP	4-Aminopyridine; voltage operated K+ channel blocker used to induce threshold contractions in smPAs
5-CT	5-carboxamidotryptamine; 5-HT_1 and 5-HT_7 receptor agonist used to investigated 5-HT receptor mediated vasoconstriction (5-HT_1) and vasorelaxation (5-HT_7) in pulmonary arteries
5-HT	5-hydroxytryptamine; used to investigate 5-HT receptor mediated responses
8-OHDPAT	8-hydroxy-2- (di-n-propylamino) tetralin; 5-HT_{1A} agonist used to investigate 5-HT receptor mediated contractions in pulmonary arteries.
α-me-5-HT	α-methyl-5-hydroxytryptamine; 5-HT_2 receptor agonist used to investigate 5-HT receptor mediated contractions in pulmonary arteries.
ACh	acetylcholine; used to investigate endothelium-dependent relaxation in precontracted pulmonary arteries
BW 723C86	1-[5-(2-thenyloxy)-1H-indol-3-yl]propan-2-amine; 5-HT_{2B} receptor agonist used to investigate 5-HT receptor mediated relaxation in precontracted pulmonary arteries
ET-1	endothelin-1; potent vasoconstrictor peptide used to precontract pulmonary arteries for subsequent vasodilator studies
L-NAME	N^{ω}-nitro-L-arginine methylester; endothelial nitric oxide synthase blocker used to inhibit basal and stimulated NO release

NA	noradrenaline; adrenoceptor agonist used to precontract pulmonary arteries for subsequent vasodilator studies
PE	phenylephrine; α-adrenoceptor agonist used to precontract pulmonary arteries for subsequent vasodilator studies
SNP	sodium nitroprusside; used to investigate endothelium-independent relaxation in precontracted pulmonary arteries
sumatriptan	5-HT1B/1D receptor agonist; used to investigate 5-HT1B/1D receptor mediated responses in pulmonary arteries

Table of 5-HT receptor agonists used in this thesis

	5-HT Receptor						
	1A	1B	1D	2A	2B	7	
Agonists							
8-OH-DPAT	*						(Hoyer *et al.*, 1994)
Sumatriptan		*	*				(Humphrey *et al.*, 1994)
5-CT	*	*	*			*	(Hoyer *et al.*, 1994)
BW723C86					*		(Kennet *et al.*, 1996)
α-me-5-HT				*			(Hoyer *et al.*, 1994)

Table 1 5-HT receptor-selective agonists used in this thesis.

Glossary III-Antagonist affinity values at vascular 5-HT receptors

Antagonist	Reported antagonist affinity for vascular 5-HT receptor subtypes				
	5-HT_{1B}	5-HT_{1D}	5-HT_{2A}	5-HT_{2B}	5-HT_7
5-HT_{1B}					
SB-224289	**9.0**[a]	6.2[a]	5.8[b]	—	—
5-HT_{1D}					
BRL-15572	6.1[a]	**7.9**[a]	—	—	—
5-$HT_{1B/1D}$					
GR55562	**7.9**[e]	**6.3**[e]	5.6	—	—
5-HT_{2A}					
ketanserin	5.3[f]	7.1[f]	**9.3**	5.4	<6.0
methiothepin	8.1	7.7	**9.0**	7.6[c]	9.7[d]
5-HT_7					
spiperone	4.4	4.8	9.1	5.5[c]	**7.3**[d]
pimozide	—	—	—	—	**8.2**[g]
risperidone	—	—	—	—	**6.9**[g]

Affinity values for 5-HT receptor antagonists used in this thesis. Particular receptor subtype for which each antagonist was used in this thesis is emphasised in **bold type**. All data from Hoyer *et al.*, 1994 unless otherwise stated.

[a] Verheggen *et al.*, 1998; [b] Roberts *et al.*, 1997; [c] Hoyer *et al.*, 1989; [d] Leung *et al.*, 1996; [e] Conner *et al.*, 1995; [f] Bard *et al.*, 1996; [g] Roth *et al.*, 1994..

Chapter 1

Introduction and Literature Review

Introduction

The pulmonary circulation can no longer be simply regarded as a passive conduit circuit, involved in gas exchange of the continual circulation of blood around the body. Extensive research investigating structural and functional aspects of this unique circulation has highlighted complex systems involved in the regulation of blood flow in the lung as well being involved in several non-respiratory functions. In particular, a vast amount of evidence exists for the importance of the endothelium, with its ability to release vasoactive agents, in understanding the mechanisms for the local regulation of pulmonary (as well as systemic) vascular tone in health and disease states.

The research presented in this thesis has focused on the vascular reactivity of pulmonary arteries, both conductance and resistance vessels, and in particular has focused on the role of 5-hydroxytryptamine (5-HT, serotonin), a potent bioamine known to have complex vascular effects. Until recently, the majority of *in vitro* investigations on the pulmonary circulation have focused on the large calibre conductance vessels. From indirect evidence, it is thought however, that the small pulmonary resistance arteries play an important role in controlling pulmonary vascular tone (Leach *et al.*, 1989)) and are the major contributors to increased pulmonary vascular resistance in the pulmonary hypertensive state (Singhal, *et al.*, 1973). Of the relatively few studies that have investigated these vessels, most have reported a number of important differences between the response of capacitance (1-2 mm internal diameter) and small muscular resistance vessels (100-300 μm internal diameter) (Leach, *et al.*, 1992; McCulloch, *et al.*, 1996). For example, previous studies recently demonstrated heterogeneity within the pulmonary

arterial circulation with regard to endothelin (ET) receptors (MacLean *et al.*, 1994b; McCulloch *et al.*, 1996). Coupled with the observation that the vascular smooth muscle cells of the pulmonary resistance arteries are phenotypically distinct from the larger vessels (Frid *et al.*, 1997), it is important to make a separate characterisation of the vascular reactivity of pulmonary arteries of different sizes.

The physiology and pharmacology of 5-HT and its putative role in the hypertensive pulmonary circulation is reviewed in this introduction in sections 1.4 and 1.5 respectively. The responsiveness of the pulmonary vasculature was investigated mainly under two different states; a) during the transitional development from foetal to neonatal life and into adulthood and b) in the pulmonary hypertensive state due to chronic hypoxia. It is important to first gain an understanding of the normal pulmonary circulation.

1.1 The Pulmonary Circulation

The characteristics of the normal, adult pulmonary circulation are uniquely those of a low pressure, highly compliant, vascular bed which has low vascular resistance. This circuit can accommodate the total cardiac output from the right ventricle to the gas exchanging surface at less than 20% of systemic vascular pressure. The pulmonary circulation is thought to be under a state of very little or no resting vascular tone (Fishman, 1985) as vasodilators have little or no effect on pulmonary vascular pressures. The high compliability of this system, along with capillary recruitment, ensures low pressure maintenance even in situations of increased cardiac output and elevated flow rate during exercise. Lung perfusion pressure therefore remains constantly low.

A vast body of evidence from extensive studies of the pulmonary circulation of many species has revealed this special circulation as being more than simply a passive conduit for blood flow to and from the alveoli and has provided much information on its various aspects.

1.1.1 Functions of the Pulmonary Circulation

As mentioned above, the entire cardiac output flows through the pulmonary circulation, essential for gas exchange to occur. A vast, 'sheet' network of pulmonary capillaries in the walls of the alveoli receives deoxygenated blood from the pulmonary arterial system, to allow gas exchange to occur. Once oxygenated, the blood returns to the left atrium of the heart via the pulmonary venous system. Apart from the primary role of the pulmonary circulation in gas exchange, the pulmonary vascular bed is also a major site of many non-respiratory functions including key metabolic processes. The large surface area of the pulmonary microvasculature is involved in the biological processing of several vasoactive substances (Vane, 1969). Angiotensin converting enzyme is present on the luminal surface of the pulmonary capillary endothelial cells and catalyses the conversion of Angiotensin I to angiotensin II as well as inactivating bradykinin (Johnson & Erdos, 1977; Said, 1982). Several other mediators such as ET, noradrenaline (NA) and 5-HT are removed from the circulation on passing through the pulmonary vasculature (DeNucci, *et al.*, 1988; Bakhle, 1970; Said, 1982).

1.1.2 Structure of the pulmonary arteries

Upon leaving the right ventricle, the main pulmonary artery trunk rapidly divides into two branches, the left branch extending to

the hilum of the left lung and dividing into a further two branches, one passing to each lobe. The right pulmonary artery divides into two branches, the larger extending to the middle and lower lung lobes while the smaller branch extends to the upper lobe of the right lung. Upon entering the lung the pulmonary arteries form a rapidly branching structure, daughter branches generally form at the distal end of the parent vessel. Some side arteries, however, may have side branches coming off at right angles to the parent branch. The majority of pulmonary arteries follow the branching pattern of the airways. In man, 17 orders of pulmonary arterial vessels have been estimated (Singhal, *et al.*, 1973). From the main trunk down to an internal diameter of ~1 mm, pulmonary arteries are defined as elastic pulmonary arteries and retain the structure of typical conducting arteries. They consist of a thin intima, thick adventitia and a media consisting of several elastic laminae interposed with a small amount of smooth muscle. The number of elastic fibrils gradually decreases with continued branching in the human pulmonary arterial tree, concomitant with an increased proportion of medial smooth muscle as the arteries diminish in size. the thickness of this smooth muscle also increases relative to diameter (Daly & Hebb, 1966). The arteries accompanying the terminal bronchioles (100μm-1mm internal diameter) are predominantly muscular and contain four to six smooth muscle cell layers, obliquely arranged and bound by distinct internal and external elastic laminae (Brenner, *et al.*, 1935; Heath & Edwards, 1958). At respiratory bronchiole level, the smooth muscle cell layer reduces abruptly with more distal branches being only either partially muscular or non muscular (Meynick & Reid, 1983). The pulmonary arterial vessels less than 100μm internal diameter in the human lung consist of a single elastic lamina, with very sparse

smooth muscle (Heath & Edwards, 1958); this is the region of the pulmonary circulation most affected in pulmonary hypertension and is discussed further in section 1.3. of this introduction.

Considerable variation exists both within and between species (Kay, 1983) in the structure of pulmonary vessels. The structure of the pulmonary arteries is generally similar in man, monkey and ferret, whilst most other animals exhibit more muscular pulmonary arteries (Kay, 1983). In rabbit lungs, the pulmonary arteries have a consistently thick muscle coat (Daly & Hebb, 1966). In some animals, muscular pulmonary arteries can extend to vessels with an internal diameter less than 100μm, while in man, vessels below 100μm are normally non-muscular.

1.1.3 Pulmonary Pressures

The pulmonary circulation is a low pressure system and in humans, the pulmonary systolic, diastolic and mean arterial pressures average approximately 23, 8 and 13 mmHg respectively. This low pressure state is associated with the structural features of the pulmonary arteries which have comparatively larger luminal diameters than systemic arteries of similar size. The pulmonary vascular resistance (PVR) is approximately one-tenth of total (systemic) peripheral resistance (Fishman, 1985) as the mean pulmonary arterial-to-venous pressure difference is only about one-tenth that in the systemic circulation. In contrast to the systemic circulation, where the arterioles represent ~70% of resistance to blood flow, a more even distribution of PVR through the lung is indicated. Pressure declines gradually to ~10mmHg in the pulmonary capillaries and to a mean of about 5mmHg in the venous bed. Some evidence from morphometric studies in human lung, however,

suggests that small muscular pulmonary arteries and arterioles are the major sites of PVR (Singhal, 1973), especially in PHT.

1.1.4 Regulation of low pulmonary vascular tone

The systemic circulation is under the predominant control of neural and humoral mechanisms. Contrasting this, the pulmonary circulation is under the control of both active and passive factors (Daly & Hebb, 1966). Active factors, including humoral factors, autonomic nerves and respiratory gases, alter pulmonary vascular tone and resistance by either contracting or relaxing pulmonary arterial smooth muscle. Passive factors, such as changes in airway and interstitial pressure or changes in cardiac output and left atrial pressure, gravitational force and vascular recruitment or obstruction all influence pulmonary vascular pressure independently of changes in vascular tone. The investigations in this thesis concentrate on active factors.

1.1.4.1 Neural Mechanisms

The autonomic nervous system innervates the pulmonary vasculature primarily via the pulmonary plexus (Mitchell, 1956; Downing & Lee, 1980) and may modify the pulmonary circulation under physiological conditions. It may also be involved in the pathophysiology of pulmonary vascular diseases. Stimulation of sympathetic (adrenergic) nerve fibres in the pulmonary vasculature generally mediates a vasoconstrictor response, whereas stimulation of parasympathetic (cholinergic) nerve fibres mediates vasodilation (Downing & Lee, 1980; Barnes & Lui, 1995).

The type and degree of innervation of the pulmonary vasculature varies markedly, dependent both on the species under

investigation and on the location and size of the pulmonary vessel (Downing & Lee, 1980; Barnes & Lui, 1995). Generally, extralobar and large muscular pulmonary arteries appear to be more densely innervated compared to smaller pulmonary arteries and arterioles which generally receive a very sparse innervation. Exceptions to these general observations include both the rabbit and man, which both display extensive, dense adrenergic innervation, extending to arteries <70μm and 60 μm outer diameter, respectively (Cech & Dolezel, 1967; McLean, 1986). Adrenergic innervation in the intralobar pulmonary arteries of the rat, in contrast, are absent (McLean, *et al.*, 1985). The pulmonary circulation in most species receives comparatively little cholinergic innervation when compared to adrenergic innervation, but notable exceptions include the rabbit pulmonary arteries, where extensive innervation with acetylcholine esterase-positive nerve fibres, extending down to vessels <100 μm is observed (Barnes & Lui, 1995). Pulmonary arteries contain heterogeneous populations of both adrenergic and cholinergic receptors with variable regional distribution in terms of receptor subtype. The overall response, either vasoconstriction or vasodilation, in the pulmonary circulation will therefore be determined by this as well as the degree of pre-existing pulmonary vascular tone (Hyman & Kadowitz, 1988; 1989). The contribution of the autonomic nervous system to basal pulmonary vascular tone is uncertain, although some evidence suggests that sympathetic nerves may contribute, at least in some species, as α-adrenergic antagonists reduce PVR in anaesthetised cats and conscious dogs (Barer, 1966; Murray, *et al.*, 1986).

1.1.4.2 Humoral factors

Several circulating mediators and hormones are known to affect pulmonary vascular tone either by causing vasoconstriction or vasodilation. The effects of these mediators are via stimulation of multiple receptor types and vary significantly with species, age and pre-existing tone (Barnes & Lui, 1995). In general, angiotensin II, thromboxane, neuropeptide Y and several prostaglandins (PGs) are pulmonary vasoconstrictors, whereas atrial natriuretic peptide and PGs E_1 and I_2 (prostacyclin) are pulmonary vasodilators. In man, for example, PGI_2 causes a dose-dependent decrease in PVR when infused into healthy volunteers (Szczeklik ,*et al.*, 1980) and it is now used in the treatment of PHT (Hoeper *et al.*, 2000). The effects of some mediators such as bradykinin, histamine, ETs and substance P are dependent on pre-existing vascular tone, generally causing contraction when pulmonary vascular tone is low, but relaxation ensues when vascular tone is high. One such mediator is 5-HT and its role in the pulmonary circulation is introduced in sections 1.4 and 1.5.

1.1.4.3 Regulation by the Endothelium

Despite the large volume of research conducted over many years, the factors involved in maintaining low pulmonary vascular tone still remain, to some extent, ambiguous. Increasingly, however, the endothelium derived factors are thought to play an important role in affecting the tone of vascular smooth muscle.

Endothelial cells can influence vascular smooth muscle tone in several ways. The vascular endothelial cells of the lung play an important role in regulating levels of biologically active factors participating in their uptake and metabolism from the blood for example, 5-HT (Said, 1983). Endothelial cells can also synthesise

and release a variety of paracrine substances with vasoactive properties. Among these are the potent peptide vasoconstrictor ET-1 (Yanagisawa, *et al.*, 1988) as well as endothelium derived relaxing factor-nitric oxide (NO). Prostacyclin (PGI_2), produced from arachidonic acid by the cyclooxygenase pathway has been identified as a potent endothelium-derived vasodilator (Moncada and Vane., 1979). In the rat pulmonary artery, ACh-induced relaxation was reported to be partially inhibited by meclofenamate, a cyclooxygenase inhibitor, suggesting that cyclooxygenase products modulate ACh-induced relaxation of these vessels (Yaghi, *et al.*, 1997).

Endothelium derived hyperpolarizing factor (EDHF) is another relaxing factor derived from the endothelium (Nagao and Vanhoutte, 1993) and is distinct from NO. EDHF causes the opening of K^+ channels, without the involvement of cyclic 3'5' -adenosine monophosphate (cAMP) or cyclic 3'5' guanosine monophosphate cGMP, leading to smooth muscle hyperpolarization (Garland, *et al.*, 1995) and has been reported in rat pulmonary arteries (Chen *et al.*, 1988). Recently, in rat isolated pulmonary arteries, Karamsetty *et al.* (2000) demonstrated that NO-independent relaxation was completely abolished by charybdotoxin plus apamin, suggesting that EDHF contributes to endothelium-dependent, NO-independent relaxation in these vessels.

1.1.4.4 Regulation by second messengers

Cyclic nucleotides are important in the regulation of pulmonary vascular tone. A wide variety of vasoactive agents exert their effects by regulating the intracellular concentration of cyclic 3'5' -adenosine monophosphate (cAMP) and cyclic 3'5' guanosine monophosphate cGMP (Bentley & Beavo, 1992) and these second

messengers can, in turn, regulate pulmonary vascular tone. Exogenous cGMP and cAMP are potent pulmonary vasodilators (Hayes, et al., 1992; McMahon, et al., 1992; 1993). NO-evoked pulmonary vasodilation involves cGMP as the key second messenger, and cAMP plays an important central role in mediating the pulmonary vasodilator response to several direct acting vasodilators including PGI_2, β-adrenoceptor and 5-HT_7 receptor agonists. Cyclic nucleotide levels may also play a critical role in the responsiveness of pulmonary arteries to activation of G-protein coupled receptors as decreased cGMP levels potentiate Gi-coupled responses in human and bovine pulmonary arteries (MacLean, et al., 1993; 1994) and this phenomenon has been investigated in the studies of chapter 6 of this thesis. This may be pathophysiologically important in pulmonary hypertension where decreased levels of cGMP and cAMP are observed in pulmonary arteries from the chronic hypoxic pulmonary hypertensive rat which also exhibit enhanced vasoconstriction to 5-HT (MacLean, et al., 1996).

Cyclic nucleotide inactivation occurs via phosphodiesterase (PDE) enzyme-mediated hydrolysis to the corresponding 5' nucleotide and PDEs represent the only known means whereby cells can inactivate cyclic nucleotides. There are currently seven PDE families which are derived from at least 15 genes in the mammalian genome (Conti *et al*, 1995). Four of the PDE families (1, 3, 4 and 5) are known to play a significant role in the regulation of vascular tone and may be important in the regulation of vascular responses to injury (Beavo 1995; Conti, *et al.*, 1995; Polson & Strada, 1996). PDEs 1, 3, 4 and 5 have been identified in the pulmonary vasculature (Rabe *et al.*, 1994; Polson & Strada, 1996; Dent, *et al.*,1994) and inhibitors of PDEs 3, 4 and 5 relax preconstricted human pulmonary

arteries (Rabe, et al., 1994). Recent research has focused on the role that PDEs may play in regulating pulmonary vasoreactivity in the hypertensive pulmonary vasculature (MacLean, *et al.*, 1997; Wagner, et al., 1997) including persistent pulmonary hypertension of the newborn (Hanson, *et al.*,1998).

1.1.4.5 Regulation by respiratory gases

Pulmonary vascular tone can be greatly influenced by the relative composition of the respiratory gas with both hypoxia and hypercapnia causing pulmonary vasoconstriction (Fishman, 1961). Uniquely, pulmonary arteries are known to contract in response to acute hypoxia and this phenomenon of hypoxic pulmonary vasoconstriction (HPV) was first described by Von Euler and Liljestrand (1947). HPV causes greatest constriction in small muscular pulmonary arteries (Fishman, 1976). In contrast, the systemic circulation exhibits a vasodilator response to hypoxia. This pulmonary vasoconstrictor reflex to acute hypoxia is of physiological importance in both foetal and adult life. In the foetus, HPV, as a result of the relatively hypoxic environs *in utero*, aids in diverting blood away from the foetus' non functioning lungs. (see section 1.2.1). In the adult, HPV aids in ventilation perfusion matching, redirecting circulating blood towards better ventilated regions of the lung and away from less well vented alveoli, thus maximising arterial oxygenation.

Despite many decades of investigation, the mechanism(s) responsible for HPV remain controversial although the mechanism is believed to reside in the vascular smooth muscle cells as HPV can be demonstrated in pulmonary arterial rings devoid of endothelium as well as in single pulmonary arterial smooth muscle cells (Yaun, *et*

al., 1990; Madden, *et al.*, 1992). Recent evidence suggests one mechanism may be hypoxia-induced inhibition of potassium current in the smooth muscle cells of pulmonary resistance arteries. This would lead to membrane depolarisation and calcium entry through voltage gated calcium channels (Smirnov *et al.*, 1994; Weir & Archer, 1995). The channels initiating HPV are thought to be 4-aminopyridine sensitive delayed rectifier channels. In CHPHT rat pulmonary artery smooth muscle cells, Osipenko (1998) recently demonstrated that increased 4-AP sensitivity reflects a switch in the major K^+ current determining resting potential from the non-inactivating K^+ current to the delayed rectifier K+ current. Hypoxic inhibition of K^+ current is specific for pulmonary smooth muscle cells and may play a key intermediary step in HPV.

Several studies have implicated an essential role for either Ca^{2+} influx (Cornfield *et al.*, 1994) or Ca^{2+} release from intracellular stores. In canine small pulmonary arteries, for example, ryanodine (inhibitor of intracellular Ca^{2+} release) plus caffeine can inhibit sustained hypoxia-mediated vasoconstriction by ~80%, suggesting that HPV in this preparation is mainly mediated by Ca^{2+} release (Jabr, *et al*; 1997). It is apparent therefore that HPV is associated with a rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$).

HPV in pulmonary arteries typically consists of two superimposed phases (e.g. Robertson *et al.*, 1995), the first being transient in nature. The second is a more slowly developing, but sustained, endothelium-dependent constriction. During phase 2 $[Ca^{2+}]_i$ does not rise in parallel with tension. It has been proposed that the rise in tension during phase 2 is produced via sensitization of the myofilaments to Ca^{2+} (Robertson *et al.*, 1995). Ca^{2+}-sensitization refers to the ability of agonists to enhance smooth

muscle force development at a given level of $[Ca^{2+}]_i$ (see section 1.1.4.7.2). Recently interest has focused upon the role of RhoA, and its effector Rho-associated kinase in agonist-induced Ca^{2+} sensitization. Robertson *et al* (2000) recently demonstrated that Rho-associated kinase caused a concentration-dependent inhibition of acute sustained HPV in rat pulmonary arteries. This is consistent with the activation of RhoA-associated kinases, and presumably Ca^{2+} sensitization, being involved in the mechanism underlying the generation of sustained HPV.

The presence of a functional vascular endothelium has been shown to enhance or facilitate HPV (Ward and Robertson, 1995; Karamsetty, *et al.*, 1996) The influence of the vascular endothelium on HPV, however, has still to be satisfactorily explained.

Acute hypoxic episodes produce an HPV which lasts for the duration of the stimulus. However, HPV appears to be malign following chronic hypoxic exposure and is sustained even upon return to normoxia (see 1.3).

1.1.4.6 Regulation by ion channels

A variety of ion channels have been characterized in the plasma membrane of VSMCs and their roles in controlling vascular tone have been extensively studied.

1.1.4.6.1. Potassium ($K^{\pm}$) channels

In the pulmonary vasculature, K^+ channels play a major role in the regulation of pulmonary vascular tone by governing membrane potential (Nelson *et al.*, 1990). Closure of K^+ channels results in a build up of positive charge on the inside of the membrane causing depolarisation of the pulmonary arterial (PA) smooth muscle cells (PASMC). Depolarisation results in opening of voltage-activated Ca^{2+}

channels and thus increases the cytoplasmic free Ca^{2+} concentration $[Ca^{2+}]_i$ (Nelson, *et al.*,1990, Yuan, 1995). In contrast, K^+ channel activation hyperpolarises PASMCs and inhibits the evoked rise in $[Ca^{2+}]_i$ (Archer, *et al.*, 1994, Yuan, et al., 1996). The increase in $[Ca^{2+}]_i$ in PASMC is a major trigger for pulmonary vasoconstriction and an important stimulus for vascular smooth muscle cell proliferation, leading to pulmonary vascular remodelling (see section 1.3.1). K^+ channels in vascular smooth muscle cells are also potential targets of vasoactive neurotransmitters or therapeutic agents (e.g., nitric oxide) (Archer, *et al.*, 1994; Yaun, *et al.*, 1996). Altered K^+-channel function/activity has been implicated in the pathogenesis of several cardiovascular diseases including primary pulmonary hypertension (Yuan, *et al.*, 1996). Three types of K+ channel have been identified in PASMC:

(i) Voltage-gated K^+ (K_V) channels regulate resting membrane potential and hence pulmonary vascular tone, especially in the pulmonary resistance arteries (Yuan, 1995; Archer, *et al.*, 1996). In the rat isolated perfused lung, inhibition of K_V channels by 4-aminopyridine results in depolarisation of the PVSMCs, increased $[Ca^{2+}]_i$ and increased pulmonary arterial pressure and PVR (Hasunuma, *et al.*, 1991). Recently the molecular identity of the K_V channels regulating $[Ca^{2+}]i$ in rat PASMCs has also been elucidated (Yuan, *et al.*, 1998). Evidence exists for a distinct, non-delayed rectifier, K_V channel active at resting membrane potential, and therefore important in the modulation of tone) in rabbit pulmonary artery smooth muscle (Evans, *et al.*, 1996).

ii. Ca^{2+}-dependent K^+ (K_{Ca}) channels are a second major class of channels located in pulmonary vascular smooth muscle (Albarwani, *et al.*, 1994; Archer, *et al.*, 1994; Gelband & Hume, 1992; Peng, *et al.*,

1996). Under resting conditions K_{Ca} channels are largely inactive and inhibition of these channels with charybdotoxin has been reported to have negligible effect on resting membrane potential, $[Ca^{2+}]_i$, or PA pressure (Peng, *et al.*, 1996). These channels are activated by membrane depolarisation and increases in $[Ca^{2+}]_i$. During PASMC contraction, therefore, these channels tend to repolarise the membrane and limit vasoconstriction. In contrast to the K_V channels, the major determinants in controlling resting membrane potential, K_{Ca} channels are thought to comprise a negative feedback pathway in regulating active tension (Brayden, *et al.*, 1992, Peng, *et al.*, 1996) and are primarily located in large, conduit pulmonary arteries (Peng, *et al.*, 1996).

iii. Voltage-independent ATP-sensitive K^+ (K_{ATP}) channels (Clapp & Gurney, 1992) are thought to play more of a modulatory role in regulating pulmonary vascular tone in times of metabolic stress. Under normal conditions the K_{ATP} channel blocker glibenclamide has little effect on pulmonary vascular tone (Clapp & Gurney, 1992).

1.1.4.6.2. Calcium (Ca^{2+}) Channels

The role of Ca^{2+} channels in the control of pulmonary vascular tone is a regulatory one as the activation threshold of the L-type Ca^{2+} channel is more depolarised than the resting membrane potential of the PVSMCs. L-type Ca^{2+} channel blockers such as nifedipine do not alter resting tone in isolated PA rings or lungs (Young, *et al.*, 1983) but do reduce artificially-induced pulmonary vasoconstriction (McMurtry *et al.*, 1976). There is some evidence for the presence of small conductance T-type Ca^{2+} channels in the chronic hypoxic pulmonary vasculature which are generally more active at more negative membrane potentials than L-type channels.

T-type channel blockers have been shown to partially dilate agonist-constricted chronic hypoxic rat lungs (Maramutsu, *et al.*, 1997).

1.1.4.6.3. Chloride (Cl^-) channels

Under physiological conditions, the most abundant intra- and extracellular anion is chloride. Activation of Cl^- channels results in a movement of negative Cl^- ions out of the cell translating into an inward current and producing membrane depolarization (Hille, 1992). Modulation of Cl^- channels could, therefore, play an important role in the control of pulmonary vascular tone. The majority of reports concerning PVSMCs have identified the calcium-activated chloride channels (Cl_{Ca}) (Clapp, *et al.*, 1996;, Salter, *et al.*, 1995, Wang & Large, 1993, Wang & Kotlikoff, 1996) which are activated by increases in $[Ca^{2+}]_i$. These channels may therefore be important in maintaining agonist-induced responses (Yaun, 1997) rather than determining resting pulmonary vascular tone. Blockers of Cl_{Ca} channels have been reported to have no effect on basal pulmonary vascular tone (Yaun, 1997).

Agonist-induced pulmonary arterial constriction is frequently associated with an initial but transient increase in $[Ca^{2+}]_i$ via release from intracellular stores to trigger contraction (Somlyo & Somlyo, 1994). This is accompanied by a sustained $[Ca^{2+}]_i$ plateau due to influx of Ca^{2+} through sarcolemmal voltage-gated Ca^{2+} channels leading to sustained contraction (Nelson, *et al.*, 1990). Elevated $[Ca^{2+}]_i$ activates Cl_{Ca} channels and elicits inward Cl^- current. The resulting Cl^- currents would cause membrane depolarisation and subsequent opening of voltage-activated Ca^{2+} channels, leading to an additional increase in $[Ca^{2+}]_i$. Ca^{2+}-induced activation of Cl_{Ca} channels would therefore explain why vasoconstrictor agonists such

as ET-1, 5-HT and PE, in addition to increasing $[Ca^{2+}]_i$, also cause membrane depolarisation and sustained vasoconstriction in pulmonary arteries (Yuan,1997; Salter, *et al.*, 1995).

Many vasoconstrictors, are known to induce membrane depolarisation and Leblanc and Leung (1995) reported that Ca^{2+} activated Cl^- current is an important contributor to increase Ca^{2+} and vascular tone by inducing membrane depolarisation. Membrane depolarisation makes an important contribution to the triggering and maintenance of arterial contractions (Somlyo & Somlyo, 1994) and the activation of Cl_{Ca} channels (Salter, *et al.*, 1995; Wang & Kotlikoff, 1996), as well as inhibition of delayed rectifier K^+ channels, could play a crucial role in depolarising PVSMCs when $[Ca^{2+}]_i$ is elevated by vasoconstrictor agonists. Agonist-induced release from inositol trisphosphate (IP_3)-sensitive stores has been reported to activate Cl_{Ca} channels in PVSMCs (Salter *et al.*, 1995; Yuan, 1997).

1.1.4.7 Importance of $[Ca^{2+}]_i$ and Ca^{2+} sensitivity

It is communally agreed that an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is a determinant for vascular smooth muscle contraction in response to various contractile stimuli including agonists which bind to G-protein coupled receptors and mobilise Ca^{2+} (Somlyo and Somlyo, 1994). Recent studies have shown that most of these agonists are also able to modulate contraction by altering Ca^{2+} myofilament sensitivity or through Ca^{2+} -independent pathways (Horowitz, *et al.*, 1996; Weber, *et al.*, 1999).

1.1.4.7.1. Calcium mobilisation

Changes in intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ are important in vascular smooth muscle contraction and relaxation and the involvement and importance of ion channels in these changes have been highlighted in section 1.1.4.6. $[Ca^{2+}]_i$ regulation involves a complex interplay between Ca^{2+} entry and extrusion across the plasma membrane, and Ca^{2+} release and re-uptake from intracellular stores. Ca^{2+} influx is an important pathway for increased $[Ca^{2+}]_i$ and contractile stimuli often increase $[Ca^{2+}]_i$ via Ca^{2+} entry from the extracellular milieu through voltage-gated or receptor-operated Ca^{2+} channels or Ca^{2+} release from the sarcoplasmic reticulum through Ca^{2+} release channels (inositol 1,4,5-trisphosphate receptors or ryanodine receptors).

The major regulatory mechanism of vascular smooth muscle contraction and relaxation is phosphorylation/dephosphorylation of a 20k dalton protein myosin light chain (MLC) (Somlyo and Somlyo, 1994). MLC is phosphorylated by myosin light chain kinase (MLCK), an enzyme regulated by Ca^{2+} through calmodulin. A rise in $[Ca^{2+}]_i$, therefore, produces VSM contraction by activation of MLCK which phosphorylates one of the constituents of myosin, allowing myosin to interact with actin and subsequently initiating contraction.

Conversely, relaxation of VSM results from a decrease in cytosolic Ca^{2+} concentration due to extrusion of Ca^{2+} from the cell by the sarcolemmal Ca^{2+}-ATPase and the Na^{+}-Ca^{2+} exchanger or uptake by the sarcoplasmic reticulum membrane Ca^{2+}-ATPase. This results in dephosphorylation of MLC by a heterotrimeric smooth muscle myosin phosphatase (SMPP-1M) (Hartshore, *et al.*, 1998).

1.1.4.7.2. Ca^{2+} sensitisation and Ca^{2+}-independent contraction of vascular smooth muscle

It is now well established that MLC phosphorylation and contraction can be induced independently of change in $[Ca^{2+}]_i$. Recent studies using permeabilized arteries have shown that Ca^{2+} mobilising agonists are able to modulate contraction by altering contractile myofilament Ca^{2+} sensitivity (Horowitz, *et al.*, 1996). Endothelin receptor activation can induce constriction of arterial smooth muscle not only by raising the intracellular Ca^{2+} concentration (Highsmith *et al.*, 1992; Pang *et al.*, 1989; Enoki *et al.*, 1995), but by increasing the sensitivity of the contractile apparatus to Ca2+ (Nishimura *et al.*, 1992; Ohanian *et al.*, 1997). For example, in rat permeabilized pulmonary artery rings, ET-1 was found to induce a sustained and reversible constriction at a fixed $[Ca^{2+}]_i$. Under these conditions, ET-1-induced constriction could only result from an increase in the sensitivity of the contractile apparatus to Ca^{2+} (Evans, *et al.*, 1999).

Agonist-induced Ca^{2+} sensitization of vascular smooth muscle contractile myofilaments to is thought to occur through inhibition of MLCP via different mechanisms involving the independent participation of several kinases:

i) Phosphorylation of the 130 kDa subunit of SMPP-1M and inhibition of the phophatase may occur via activation of the small GTPase RhoA and subsequent Rho-associated kinase (Kimura, *et al.*, 1996). RhoA dependent Ca^{2+} sensitization constitutes a major component of the sustained rise in tension induced by vasoconstrictors in various vascular beds including the pulmonary vasculature (Gong, *et al.*, 1997). In human omental arteries, the Rho-kinase inhibitor Y-27632 strongly inhibited agonist-induced contractions without affecting $[Ca^{2+}]_i$, suggesting the involvement of the Rho-kinase associated pathway in agonist-induced Ca^{2+}

sensitization of these vessels (Martinez, *et al.*, 2000). Recently, Hirata *et al.* (1992) reported that G protein-mediated Ca^{2+} sensitization in permeabilized vascular smooth muscle cells was inhibited by pretreatment with C3 exotoxin, a selective inhibitor of rho p21, a small G protein associated with Rho kinase. This phenomenon has now been confirmed in several other investigations, suggesting that activation of rho p21-Rho kinase is involved in Ca^{2+} sensitization presumably due to modification of the phosphorylation state of myosin light chain (Fujita, *et al.*,1995; Noda, *et al.*, 1995).

ii) The involvement of tyrosine kinase in Ca^{2+} sensitization has also been reported in permeabilized arteries. Tyrosine kinases have been implicated in agonist induced Ca^{2+} sensitization in human small omental arteries (Martinez, *et al.*, 2000). Ohania *et al.* (1997) suggested that ET-1 may induce an increase in myofilament Ca^{2+} sensitivity in rat mesenteric artery smooth muscle by activating a tyrosine kinase and subsequent protein tyrosine phosphorylation. In the rat pulmonary artery, however, the observed increase in Ca^{2+} sensitivity was mediated by a mechanism independent of the TK pathway (Evans, *et al.*, 1999). this may be of some importance, as the identification of a distinct Ca^{2+} sensitizing mechanism associated with the development of pulmonary hypertension (see section 1.3) may lead to the development of more selective and effective therapies for this disorder.

iii) Myofilament Ca^{2+} sensitization can occur through a protein kinase C-dependent pathway as PKC may also inhibit the dephosphorylation of myosin light chain by an inhibitory action on SMPP-1M (Akopov, *et al.*, 1998). In the rabbit mesenteric artery, 5-$HT_{1B/1D}$ receptor-induced contraction occurs via myofilament Ca^{2+}

sensitization, which can be blocked by PKC inhibitors (Parsons, *et al.*, 1996).

Ca^{2+} sensitization via the above pathways is therefore interpreted as SMPP-1M inhibition leading to an increase in the MLCK: SMPP-1M activity ratio, light chain phosphorylation and contraction without a change in $[Ca^{2+}]_i$.

Phosphorylation of other proteins, such as caldesmon by MAP-kinase or calponin by PKC has also been suggested to play a role in Ca^{2+} sensitization (Horowitz, et al., 1996). Recently, 5-$HT_{1B/1D}$ receptor mediated contraction in the rabbit renal artery was reported to be blocked by inhibitors of MAP-kinase (Hinton, *et al.*, 2000).

Cross-talk between the different kinase pathways in the regulation of Ca^{2+} sensitization has been suggested as a key signalling event of Ca^{2+} sensitization of the contractile apparatus during agonist-induced contraction of vascular smooth muscle (Sasaki, *et al.*, 1998).

Of relevance to the pulmonary circulation, calcium sensitization has also been implicated in the development of HPV (Robertson *et al.*, 1995; see section 1.1.4.5)

1.2 The perinatal pulmonary circulation

1.2.1 The Foetal pulmonary circulation

The foetal pulmonary circulation is characterised by high resistance and low blood flow, receiving only less than 10% of the foetal cardiac output. High PVR causes the majority of the foetal right ventricular output (~55-65% of total cardiac output) to be diverted through a patent ductus arteriosus to the descending thoracic aorta and enhances perfusion of the foetal organ of gas exchange, the placenta, thereby diverting the lungs (Rudolph 1979).

Pulmonary blood flow, although low *in utero*, remains essential for providing substrates, nutrients and hormones necessary for the normal growth and functional maturation of the lung before birth. Blood returning to the heart is shunted through the foramen ovale to the left atrium and ventricle. As the lung is in parallel with the placenta, the maintenance of high PVR results in this preferential distribution of blood flow to the comparatively low-resistance placenta. Much of the current understanding of the foetal pulmonary circulation has arisen from investigation in the foetal lamb. PVR in this model was shown to be high at ~6 mmHg $ml^{-1}min^{-1}$ at 0.4 gestation with a progressive 20 fold fall by term (Rudolph, 1977). This fall in PVR is a result of increased cross sectional area of the pulmonary vascular bed, due to decreased resting vascular vasoconstriction, increased vessel diameter and formation of new vessels. When the lung is growing rapidly, during the latter half of gestation, the number of arterioles in the foetal lamb right lung increases nearly 50-fold from ~100 thousand to 4.8 million (Levin, *et al.*, 1976). At term mean pulmonary arterial pressure is ~50mmHg.

Several mechanisms are thought to play a role in maintaining high foetal basal PVR, but are not understood completely. These include the low oxygen tension of the blood perfusing the lungs. Hypoxia is a potent pulmonary vasoconstrictor (see section 1.1.4.5), so the persistent hypoxaemia that exists produces persistent active pulmonary vasoconstriction. The pulmonary vascular endothelium is known to release a number of vasoactive agents which cause either vasoconstriction or vasodilation (see section 1.1.4.3); the maintenance of high resting tone may therefore reflect a balance between these mediators. For example, low basal production of vasodilator products (such as PgI_2 and NO), combined with increased

production of vasoconstrictors including ET-1 (Ivy *et al.*, 1994) or leukotrienes (Soifer *et al.*, 1985, Cassin *et al.*, 1988). Recently, *in vitro* studies in ovine foetal pulmonary arteries have suggested that perinatal pulmonary arteries may exhibit a potent myogenic response, contributing to the high PVR in the foetal pulmonary circulation (Belik 1994; 1995). More recent, *in vivo* studies, have confirmed this (Storme *et al.*, 1999).

It is possible that locally released 5-HT from foetal pulmonary neuroendocrine cells (PNECs) may also contribute to the maintenance of high PVR. Human foetal PNECs have been shown to be immunoreactive for 5-HT from early in gestation (Cutz *et al.*,1995) and in rabbit cultured foetal PNECs, exposure of these cells to hypoxia resulted in decreased intracellular content of 5-HT accompanied by increased exocytosis of dense core vesicles. The amount of 5-HT release correlated with the degree of hypoxia (Cutz *et al.*, 1993). Assuming this released 5-HT is able to reach pulmonary arterial smooth muscle, contribution to vasoconstriction and hence high PVR is possible in the relatively hypoxic environment of the foetal pulmonary vasculature.

1.2.2 Transition of the pulmonary circulation at birth

At birth, pulmonary arterial blood flow increases 8 to 10-fold (Cassin, *et al.*, 1964; Dawes & Mott, 1962) and pulmonary arterial pressure rapidly declines by about 50% within the first 24 hours of extra-uterine life (Emmanouilides, *et al.*, 1964). These key events are essential for the normal transition of gas exchange from placenta to the lungs to occur and are dependent on a dramatic decrease in the PVR within the first few hours of birth. The increased blood flow at birth raises left atrial pressure above right atrial pressure, forcing the

closure of the foramen ovale. As PVR falls below systemic resistance, flow through the ductus arteriosus reverses. Over the first few hours after birth, the ductus closes.

The fall in PVR occurs as a result of a rapid sequence of integrated morphological (Haworth & Hislop, 1981; Hall & Haworth, 1986) and functional (Zellers & Vanhoutte, 1991; Lui, *et al.*, 1992) changes. Within minutes of the initial vasodilator response, increased pulmonary blood flow distends the vasculature, resulting in a "structural reorganization" of the vascular wall that includes flattening of the endothelium and thinning of smooth muscle cells and matrix.

In extensive studies, Haworth and colleagues have examined pulmonary arterial structural adaptation in the perinatal piglet observing immediate changes at birth. For example, at birth, in peripheral pulmonary arteries, thick interdigitating endothelial cells and brick-like immature smooth muscle cells show an immediate increase in surface/volume ratio and 'spread' within the wall to increase lumen diameter, lower resistance and halve right ventricular work by 2 weeks of age (Hall & Haworth, 1986a,b; Haworth, *et al.*, 1987; Greenwald, *et al.*, 1985). There is no reduction in the amount of vascular smooth muscle. This rapid remodelling is probably facillitated by the relative lack of fixed collagen (Haworth, *et al.*, 1987; Allen & Haworth, 1988). Changes in PVSMC shape are associated with a transient depolymerization of contractile and cytoskeletal filaments. The different SMC phenotypes identified in the vessel wall show rapid postnatal changes in the types of filament proteins and contractile-associated proteins (Haworth, 1995). Growth and development of the lung was observed to continue until

an adult pattern was reached by the age of 6 months (Haworth & Hislop, 1981).

The mechanisms responsible in the normally successful cardiopulmonary transition from foetal to neonatal life are incompletely understood and involve multiple factors. Birth-related stimuli, such as ventilation, increased blood oxygen content, and endothelium-derived vasoactive substances such as prostacyclin (PGI_2) (Leffler, *et al.*, 1984) and endothelin-1 (ET-1) (Ziegler, *et al.*, 1995) have all been implicated. In particular, growing evidence suggests that NO contributes to the transitional processes at birth. Physical stimuli, including, ventilation, increased shear stress, and increased oxygen, cause pulmonary vasodilation in part by increasing production of NO (Abman, *et al.*, 1992; Cornfield , *et al.*, 1992; McQueston, *et al.*, 1993; Moore *et al.*, 1992). Pretreatment with the NOS inhibitor, N^{ω}-nitro-L-arginine, attenuates pulmonary blood flow after delivery by 83% in near-term fetal lambs (Grifiths, *et al.*, 1987). These findings suggest that a significant part of the rise in pulmonary blood flow at birth may be related directly to the acute release of NO. Each of the birth-related stimuli can stimulate NO release independently, followed by vasodilation through cGMP kinase-mediated stimulation of Ca^{2+}-activated K^{+} channels (Saqueston, *et al.*, 1999). As endothelial NOS (eNOS) is present early in gestation, NO may also be involved in angiogenesis during lung development (Halbower, *et al.*, 1994). This hypothesis was strengthened when Xue *et al.* (1996) detected increasing endothelial cells imunnopositive for eNOS progressing from 15 days of gestation to at least 7 days postnatally in perinatal rat lung.

The potent vasoconstrictor, ET-1 has been implicated in playing an important role in the postnatal adaptation of the

pulmonary circulation (Zielger, *et al.*, 1995) as circulating levels are markedly high in the foetal and transitional pulmonary circulations (Endo, *et al.*, 1996; Malamitsi Puchner, *et al.*, 1993). Docherty & MacLean (1998) recently investigated contractile responses to ET-1 in isolated, perinatal rabbit PRAs. They observed a rapid alteration in ET-receptor-mediated contraction in these vessels with a marked hypersensitivity to ET_B receptor stimulation in newborn rabbits. Complex changes in the functional properties and interaction of both the endothelial and smooth muscle cells of pulmonary arteries are therefore thought to occur during postnatal development. Levy *et al.*, (1995) for example, demonstrated that at birth a functional endothelium enhanced contractile responses to $PGF_{2\alpha}$ and KCl in the newborn pig, but this was reversed at 10 days postnatal age. These changes may, therefore, also be reflected in perinatal pulmonary circulatory responses to other known vasoactive substances such as 5-HT.

Other vasodilators, including adrenomedullin (Rudolph, 1998) and PGI_2, may also modulate changes in pulmonary vascular tone at birth. PGI_2 infusion *in vivo* into foetal lung produces a potent vasodilation (Cassin, *et al.*, 1981), however, PGI_2 production declines rapidly immediately after birth (Leffler, *et al.*, 1984). Inhibition of prostaglandin synthesis only modestly attenuates the fall in PVR at birth in near term foetal lambs and does not alter postnatal adaptation. Collectively, the results suggest that PGI_2 may contribute to pulmonary vascular changes during the transitional period at birth, but has minimal involvement in the postnatal adaptation.

1.3 Pulmonary hypertension

Pulmonary hypertension (PHT) is defined as being present when mean pulmonary arterial pressure exceeds 25 mmHg (Fishman, 1985). Regardless of aetiology, the clinical importance of PHT is related to high resistance to right ventricular outflow, low cardiac output, diminished arterial oxygenation and right ventricular pressure overload, right heart failure and death.

PHT can arise as a primary phenomenon (idiopathic PHT) where the mechanism and cause are unknown. Primary PHT is a rare syndrome and is regarded as a disease of young adults, predominantly women aged between 30-40 years, with an average survival rate of 2-3 years (Rich, 1988). More commonly, PHT usually occurs secondary to other disease states and many mechanisms appear to either cause or contribute to the pathogenesis of PHT (Rich, et *al.*,1987). The principal causes of PHT fall into four main categories; (1) passive increases in pulmonary arterial pressure secondary to elevated left atrial pressure and left ventricular dysfunction. (2) veno-occlusive disorders. (3) conditions that cause an increased blood flow through the pulmonary vasculature beyond the ability of the pulmonary circulation to compensate; e.g. persistent pulmonary hypertension of the newborn (PPHN) caused by patent ductus arteriosus (see section 1.3.2) and ventricular or atrial septal defects. (3) Vaso-occlusive type resulting in a diminished effective cross sectional area of the pulmonary vascular bed by obstructive e.g. thrombosis, chronic obstructive lung disease (COLD); obliterative, eg. emphysema or advanced fibrosis; and vasoconstrictive means which is usually associated with hypoxia. Despite the different aetiologies of PHT, the condition is characterised by a relentless increase in PVR.

The high compliance of the major pulmonary blood vessels, and the tonic state of the peripheral precapillary resistance vessels both ensure the maintenance of low pulmonary arterial pressure in normal subjects, however, the structure of this highly compliant circuit may alter markedly with disease. The earliest changes include the muscularisation of the terminal part of the pulmonary arterial tree and narrowing of the peripheral vessels. Thickening of the major vessels also occurs (see section 1.3.1). Pulmonary arterial vasoconstriction elevates pulmonary arterial pressure, increasing the pressure on the right-hand side of the heart and causing right ventricular dysfunction. Pulmonary venous constriction can result in pulmonary oedema due to increased pulmonary capillary pressure. PHT, therefore, arises due to the combination of both vasoconstriction and anatomical restriction.

The underlying mechanism(s) in the development of PHT have not been fully elucidated with multifactorial influences on both vascular remodelling (see section 1.3.1) and reactivity. Not surprisingly, altered endothelium function is thought to play an important role in altering pulmonary vascular tone in PHT. The pulmonary clearance of circulating catecholamines such as noradrenaline is abolished in patients with both primary and secondary PHT (Sole, *et al.*, 1979). Impaired pulmonary vasodilation to stimulated NO release has been observed *in vivo* in patients with both primary (Conraads, *et al.*, 1993) and secondary PHT (Celermajer, *et al.*, 1993). Recently, Giaid & Saleh (1995) observed a decreased expression in eNOS from the lungs of patients with severe PHT. Tuder and co-workers (1999) observed a marked reduction in PGI_2 synthase expression in lungs of severe PHT patients. Collectively, these results indicate an impairment of endothelium-dependent

vasodilator responses in PHT. In the normal pulmonary circulation, low pulmonary vascular tone is controlled by a balance between vasodilator agents such as NO and PGI_2 and vasoconstrictors such as 5-HT and ET-1 (Fishman, 1998; MacLean, 1999) (see section 1.1.4.3). Alterations in this balance, in favour of the vasoconstrictors, through loss or reduction of protective NO and or PGI_2,, or indeed increased levels of endogenous vasoconstrictors such as ET-1 (Stewart, *et al.*, 1991) will predispose a patient to pulmonary vasoconstriction. Elevated levels of the endogenous vasoconstrictors angiotensin II and thromboxane A_2 may also be present in PHT, contributing to the increased vascular tone (Cargill & Lipworth, 1995; Christman, 1998). Substantial evidence indicates that 5-HT (see section 1.4.5.4) and ET-1 may also regulate growth and proliferation of vascular cell types. For example, ET-1 stimulates DNA synthesis and cell proliferation of cultured pulmonary vascular smooth muscle cells (Janakidevi, *et al.*, 1992). Coupled with the loss of NO, known to inhibit vascular smooth muscle mitogenesis and proliferation (Garg & Hassid, 1989), altered pulmonary endothelial function could facilitate the process of pulmonary vascular remodelling and accelerate the development of PHT.

Several other important factors could also be responsible for the increased vascular tone characteristic in PHT. These include depolarisation by inactivation of K^+ channels, and decreased levels of cGMP and cAMP (due to increased phosphodiesterase activity); both have been observed in the chronic hypoxic rat model of PHT (Osipenko, *et al.*, 1998; MacLean, *et al.*, 1996; 1997). Recently, Hanson and co-workers (1998) demonstrated a marked increase in PDE activity in the foetal lamb model of persistent pulmonary

hypertension of the newborn, suggesting that decreased cGMP contributed to altered pulmonary vasoreactivity in perinatal PHT.

Most standard vasodilators have been unsuccessful in the treatment of PHT due to lack of selectivity. Pulmonary vascular remodelling (see section 1.3.1) renders the pulmonary circulation relatively resistant to vasodilator therapy, and standard vasodilators exert a greater effect on the systemic circulation. This results in intolerable side-effects such as systemic hypotension. Inhaled NO, offers an attractive, pulmonary circulation selective, therapy in the treatment of PHT (Adnot, *et al.*, 1992). Recent studies have demonstrated that inhaled NO causes marked improvement in oxygenation in many newborn infants with PPHN (see section 1.3.2). Kinsella *et al.* (1992) reported rapid improvement in oxygenation in neonates with severe PPHN with the use of doses of 20 ppm NO for 4 hours, after which the dose was decreased to 6 ppm for the duration of treatment; this strategy resulted in sustained improvement in oxygenation. Inhaled NO therapy may also be useful in other PHT states including acquired heart disease (Girard, *et al.*, 1992) and PHT secondary to congenital heart disease (Berner, *et al.*, 1993). Oxygen is also an effective pulmonary vasodilator in several forms of PHT such as secondary to congenital heart disease (Bush, *et al.*, 1985) and bronchopulmonary dysplysia (Palmisano, *et al.*, 1990) and is the only selective vasodilator generally available. Increasing evidence suggests that 5-HT plays an important role in the aetiology of PHT (see section 1.5.3). The role of 5-HT in the pulmonary circulation and its putative involvement in PHT is introduced in section 1.5.2. 5-HT antagonists, therefore, present a possible therapeutic intervention. Recent studies, however have shown ketanserin, a 5-HT_{2A} receptor antagonist (see section 1.4.3.2), to be ineffective or of limited use in

the treatment of PHT secondary to platelet storage pool disease (Herve, *et al.*, 1995) or chronic obstructive pulmonary disease (Domenighetti, *et al.*, 1997), due to systemic hypotension. Targeting of a more pulmonary vasculature-selective 5-HT receptor subtype may be of more benefit.

1.3.1 Pulmonary vascular remodelling

Figure 1.1 illustrates the progressive muscularisation of the pulmonary arteries in the pulmonary hypertensive lung. In PHT of all aetiologies, the main changes involved in pulmonary vascular remodelling are smooth muscle hypertrophy, intimal proliferation and thickening of the adventitia of the pulmonary resistance vessels (Reid, 1990). As mentioned in section 1.3, the earliest changes that occur in the development of PHT are a progressive muscularisation of the, originally non-muscular, terminal portion of the pulmonary arterial tree (Heath, *et al.*, 1987) and is due to hyperplasia of the PVSMCs which extend distally in a layer to the original internal elastic lamina (Heath, *et al*, 1987), effectively forming an inner layer of longitudinal smooth muscle. Medial extension also occurs peripherally along the pulmonary arterial tree to the elastic arteries. Although both primary and secondary PHT share common pathobiologies, during the latter stages of pulmonary vascular remodelling, the patterns of smooth muscle cell proliferation and migration can be different. In primary PHT, distinct plexiform lesions occur and occlude the pulmonary vascular lumen, smooth muscle cell migration is also widespread (Heath, 1992). In contrast migration is limited and plexiform lesions are rare in hypoxic PHT.

Although the study of vascular remodelling has focused extensively on the pulmonary arterial medial layer, all three vascular

layers of the pulmonary arteries, the intima, media and adventitia, all undergo varying patterns of thickening in PHT dependent on the size of the pulmonary artery (Peacock, *et al.*, 1993).

The processes involved in remodelling are modification of cell growth, cell migration and extracellular matrix production and though not completely understood, several factors are thought to influence pulmonary vascular remodelling. Endothelial cells respond to shear stress arising from altered blood flow and as a result may secrete mediators, such as vascular endothelial growth factor, causing vascular SMC growth (Cool, *et al.*, 1999). Altered endothelial cells may also lead to angiogenesis, resulting in hypertrophy and hyperplasia (Lee, *et al.*, 1998). Vascular injury may elicit vascular remodelling via inflammatory mediators. Wright *et al.* (1998) recently observed increased levels of transforming growth factor-β, and interleukins 1 and 6, resulting in production of inflammatory leukotrienes, in patients with primary PHT. Increased production of endogenous vascular elastase in PVSMCs may result in cell migration from tunica media into the intima where they proliferate and secrete proteoglycans and matrixproteins (Rabinovitch, 1999). Once PHT has developed, the associated vascular structural changes renders the pulmonary circulation relatively resistant to standard vasodilator therapy (section 1.3).

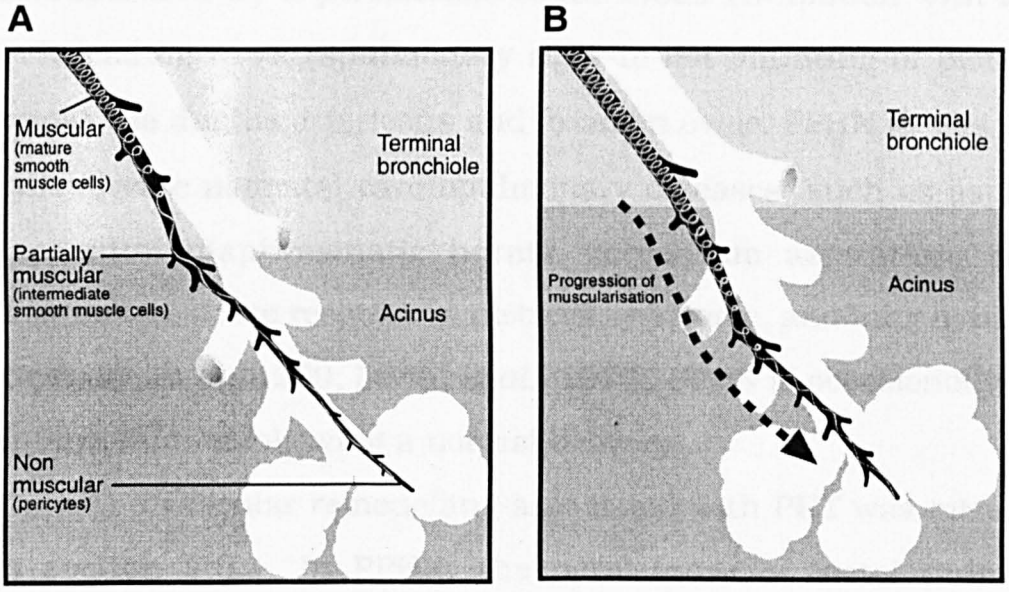

Figure 1.1
Diagrammatic representation of the pulmonary arteries within the lung. (A) In the normal lung there is an uneven distribution of smooth muscle phenotypes and numerous non-muscular precapillary vessels. (B) In the pulmonary hypertensive lung there is progression of muscularization into the non-muscular terminal portion of the arterial tree. This is due to hyperplasia and redistribution of smooth muscle cell phenotypes (see text for details).
From: *MacLean et al., (2000) British Journal of Pharmacology 131, 161-168.*

1.3.2 Persistent pulmonary hypertension of the newborn (PPHN)

The pulmonary circulation of some infants fails to adapt to life outside the uterus, which leads to severe respiratory distress and hypoxemia, referred to as persistent pulmonary hypertension of the newborn (PPHN). PPHN contributes significantly to morbidity and mortality in both term and preterm neonates (Hageman, 1984) and is characterised by a persistence of the foetal circulation with a high PVR leading to extrapulmonary right-to-left shunting of blood flow across the ductus arteriosus and foramen ovale. PPHN is associated with diverse neonatal cardiopulmonary diseases, such as asphyxia, congenital diaphragmatic hernia, meconium aspiration, sepsis, pneumonia, acute respiratory distress syndrome, and lung hypoplasia (Gersony, *et al.*, 1969; Levin, *et al.*, 1970). PPHN is occasionally found in term infants following a normal delivery.

The vascular remodelling associated with PHT was introduced in section 1.3.1. In PPHN, the persistence of foetal pulmonary vasculature morphology is often evident.

Because PPHN represents the failure of postnatal adaptation of the lung circulation at birth, understanding basic mechanisms of normal functional and structural development of the pulmonary circulation *in utero* and mechanisms that contribute to pulmonary vasodilation at birth (see section 1.2.2) may provide insights into the syndrome of PPHN and its treatment.

1.4 5-HT (serotonin)

1.4.1 Discovery

In 1948, Rapport and colleagues isolated a substance released from platelets in clotting blood that was subsequently shown to be a powerful vasoconstrictor and was originally termed serotonin (Rapport, *et al*, 1948). Rapport quickly went on to identify the active component of this substance as 5-hydroxytryptamine (5-HT), a bioactive indolealkylamine.

1.4.2 5-HT distribution, biosynthesis and degradation

5-HT occurs at its highest concentrations in three different locations throughout the body. In mammals, the main source of 5-HT present in the body, approximately 90% of the overall total, is found in the enterochromaffin cells of the gastrointestinal tract; after being extracted from strips of gastrointestinal tract it was originally given the name enteramine (Erspamer & Asero, 1952; Stacey, 1962a). 5-HT is also present in high concentrations in blood platelets which possess mechanisms for its uptake, storage and release (see later in this subsection).

5-HT also acts as a neurotransmitter in the central nervous system, is selectively contained in neurones with cell bodies mainly located in the midline area of the brainstem and terminals diffusely distributed throughout the central nervous system.

Hamlin and co-workers (1951) originally described the synthesis of 5-HT and although considerable amounts of 5HT are found in normal diet, the high levels of 5HT in enterochromaffin cells does not arise as a consequence of 5-HT uptake from the intestine but by "in-situ" synthesis from the essential amino acid tryptophan (Douglas, 1980).

The amino-acid precursor for 5-HT biosynthesis is tryptophan and is converted to 5-hydroxytryptophan in chromaffin cells (and neurones) through the action of the mixed functional oxidase enzyme, tryptophan hydroxylase requiring molecular oxygen and a reduced pteridine cofactor for activity. The resulting 5-hydroxytryptophan then undergoes carboxylation by L-aromatic acid decarboxylase to 5-hydroxytryptamine. Platelets do not directly synthesise 5-HT, but possess a high affinity uptake system with relative specificity for 5-HT and avidly take up and store the bioamine as they pass through the intestinal circulation (Coppen, *et al.*, 1978; Pletscher, 1978). Basal release of enteric 5-HT can be augmented by mechanical stretching and vagal stimulation (Forsberg & Miller, 1982). Na^+-dependent active transport of 5-HT across platelet surface membranes leads to uptake into very dense core, delta granules across a H^+-translocating ATPase-dependent electrochemical gradient. It has been estimated that 10^{12} platelets circulating in a 70kg adult contain in total approximately 500μg 5-HT (Fozard, 1981). Plasma also contains some free 5-HT. This fraction has been estimated to account for 15-120nM (Crawford, 1965; Engback & Voldby, 1982). Low circulating free 5-HT levels are achieved by a combination of the avid uptake and storage by platelets and by the effective uptake and subsequent metabolism in the pulmonary vascular endothelium.

Breakdown of 5-HT primarily occurs as a two-step process through oxidative deamination, resulting in the formation of an aldehyde via the action of monoamine oxidase (MAO). Two isoforms of this enzyme exist, MAO-A and MAO-B, and have been cloned (Shih, 1991). MAO-B is the predominant form in platelets. The

aldehyde produced is subsequently oxidised to 5-hydroxyindoleacetic acid (5HIAA) which is excreted in the urine.

1.4.3 5-HT receptor classification

The first studies on 5HT receptors concerned their pharmacological characteristics on guinea pig ileum and led Gaddum & Picarelli (1957) to propose the existence of 2 receptor types; originally described as the musculotrophic 'D' receptor and neurotrophic 'M'. These 'criteria' were based on their sensitivity to morphine (M) and dibenzyline (phenoxybenzamine) (D). Subsequent studies by Peroutka & Snyder (1979) gave rise to the 5-HT_1, 5-HT_2 classification of receptors based on radioligand binding studies on rat brain cortex. Bradley *et al.* (1986) in a pragmatic approach proposed the reconciliation of these two nomenclatures and suggested that 5-HT receptors could be classified into three groups designated '5-HT_1-like', 5-HT_2 and $5HT_3$.

For nearly ten years the classification of Bradley was widely accepted as it provided a logical framework in terms of operational characteristics for classifying 5-HT receptors. Recent advances in molecular biology have served to confirm, extend and establish the distinct identity of the 5-HT receptors. On this basis, the state of knowledge on 5-HT receptors was re-addressed by Hoyer *et al* in 1994. The three criteria used to classify receptors are operational (i.e. functional pharmacological and radioligand binding data), transductional (receptor-effector coupling events) and structural (gene & receptor structural sequences for their nucleotide & amino acid components, respectively) (Hoyer and Martin, 1996); none of the three have precedence. The classification of 5-HT receptors described in this chapter is based on an international view sanctioned by the

Receptor Nomenclature Committee of IUPHAR (see Hoyer *et al*, 1994) coupled with the alignment of the nomenclature with the human genome (Hoyer and Martin, 1996).

Figure 1.2 shows the different classes of 5-HT receptor which are currently classified into at least seven classes of receptor. They comprise the $5HT_1$ receptors, with subtypes $5\text{-}HT_{1A\text{-}F}$, $5\text{-}HT_2$ receptors with subtypes $5\text{-}HT_{2A\text{-}C}$, $5\text{-}HT_3$ as well as the $5\text{-}HT_4$, $5\text{-}HT_5$, $5\text{-}ht_6$ and $5\text{-}HT_7$ receptors. With the exception of the $5\text{-}HT_3$ receptor, which comprises a ligand-gated ion channel, all other 5-HT receptors belong to a 'superfamily' that are coupled with guanine nucleotide binding (G)-proteins and contain seven transmembrane-spanning hydrophobic regions, an extracellular N terminus and a cytosolic C terminus.

It is beyond the scope of this chapter to fully review each subtype of 5-HT receptor belonging to the seven classes. This review will concentrate on the 5-HT receptors identified on vascular smooth muscle and endothelial cells, focusing on those receptors recently implicated in mediating vascular smooth muscle function, i.e. the $5\text{-}HT_{1B}$, $5\text{-}HT_{1D}$, $5\text{-}HT_{2A}$, $5\text{-}HT_{2B}$ and $5\text{-}HT_7$ receptors.

1.4.3.1 $5\text{-}HT_{1B}$ and $5\text{-}HT_{1D}$ receptors

$5\text{-}HT_1$ receptors were first identified as a high-affinity site for 5-HT in radioligand-binding studies on brain homogenates using [^{3}H]-5-HT (Peroutka & Snyder, 1979). Later the subtypes $5\text{-}HT_{1A}$, $5\text{-}HT_{1B}$, $5\text{-}HT_{1C}$, $5\text{-}HT_{1D}$, $5\text{-}HT_{1E}$ and $5\text{-}HT_{1F}$ were identified, which can all be selectively labelled. All these receptors have also been cloned and shown to share a high degree of homology (>60%

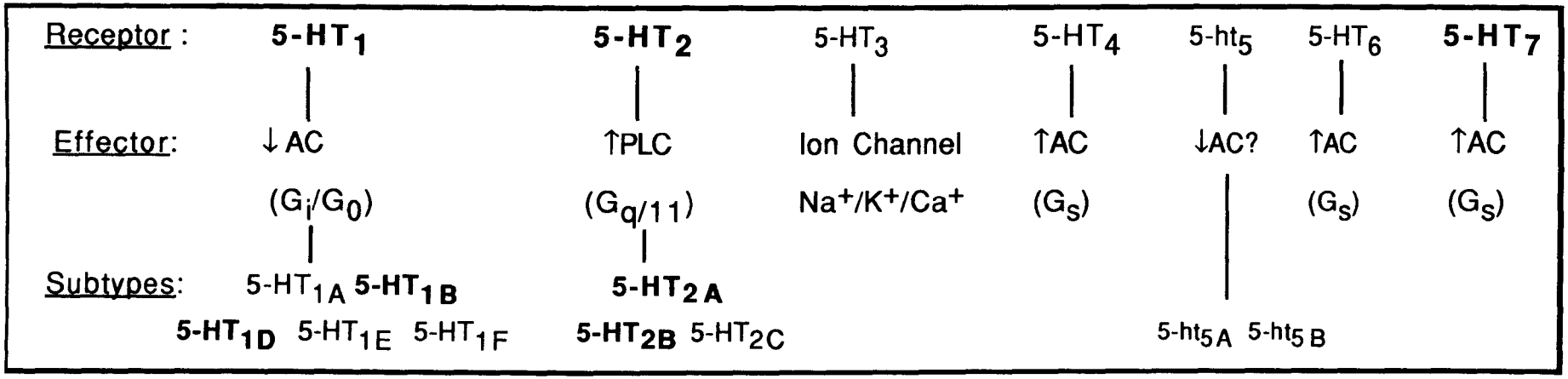

Figure 1.2 Current classification of 5-hydroxytryptamine receptors. 5-HT receptors currently divided into 7 classes based upon operational, transductional and molecular data. Receptor subtypes mediating vascular responses shown in **bold type.** AC, adenylate cyclase; PLC,phospholipase C; G_s,stimulatory G protein; G_i/G_o, inhibitory G protein; $G_{q/11}$, Gq GTP binding protein.

in transmembrane domains). They also have a high affinity for 5-HT and share a common transduction system in being negatively coupled to adenylate cyclase via regulatory G-proteins (Hoyer, *et al.*, 1994; see 1.4.3.1.i).

Selective receptor agonists are available for 5-HT_{1A-D} receptors and an agonist potency order of 5-CT > 5-HT > sumatriptan ≥ α-me-5-HT > 8-OH-DPAT satisfies one of the criteria for identifying a 5-HT_1 receptor mediated response (Hoyer, *et al.*, 1994). The originally named the 5-HT_{1C} receptor (Pazos *et al.*, 1984) has now been renamed 5-HT_{2C} (Humphrey *et al.*, 1993) based on characterisation criteria. It mediates activation of protein kinase C via increased phosphoinositide metabolism is entirely consistent with operational and structural data of the 5-HT_2 receptor class.

Two closely related human genes that encode for G protein-coupled receptors have been cloned and share very similar pharmacological profiles including high affinity for the vasoconstrictor compound sumatriptan (Weinshank, *et al.*, 1992). These receptors have been named the 5-HT_{1B} and 5-HT_{1D} receptors. Until recently, with their very similar pharmacology, these receptors were termed the 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors respectively but their nomenclature has recently been re-aligned (Hartig, *et al.*, 1996) and compounds that are able to distinguish between them are beginning to be produced. Until recently, no truly selective antagonist existed which was able to distinguish between these two receptors and pharmacological classification relied upon agonist potency orders and resistance to antagonists with known selectivity at other 5-HT receptor subtypes.

Involvement of either of these receptors, without clear distinction between the two, was often given the appellation 5-

$HT_{1B/1D}$ receptor. Sumatriptan, used as a selective agonist for 5-$HT_{B/1D}$ receptors, was initially identified by its ability to constrict dog isolated saphenous vein (Humphrey, *et al.*, 1988). It has subsequently been shown to produce potent contractile responses in several vascular preparations including cerebral (Janssen, *et al.*, 1993), coronary (Kaumann, *et al.*, 1994) and pulmonary arteries (MacLean, *et al.*, 1996; Cortijo, *et al.*, 1997) in man. Used extensively throughout this thesis as a 5-$HT_{1B/1D}$ receptor agonist, sumatriptan has an affinity value (pK_i) at cloned rabbit 5-HT_{1B} receptors of ~6.8 (Bard, *et al.*, 1996). The following antagonists were used in this thesis: (i) GR55562 is a selective antagonist at the 5-HT_{1B} and 5-HT_{1D} receptors and has a pA_2 value of 7.9 against sumatriptan-induced contractions of the canine and monkey isolated basilar artery (Conner, *et al.*, 1995) and has been shown to be a potent antagonist against the 5-$HT_{1B/1D}$ receptor in human isolated large pulmonary arteries (MacLean, *et al.*, 1996). (ii) Recently, two compounds have been shown to be selective for the 5-HT_{1B} and 5-HT_{1D} receptors. SB-224289 a 5-HT_{1B} receptor selective antagonist, has affinity values of 8.0 and 6.2 at 5-HT_{1B} and 5-HT_{1D} receptors respectively (Roberts, *et al.*, 1997). BRL-15572, a 5-HT_{1D} receptor-selective antagonist has antagonist affinity values of 6.1 and 7.9 at 5-HT_{1B} and 5-HT_{1D} receptors respectively (Price, *et al.*, 1997; Schlicker, *et al.*, 1997). These compounds were used in the studies in chapter 4 of this thesis.

With regard to the possible pathophysiology associated with vascular 5-HT_{1B} and 5-HT_{1D} receptors, it is thought that the 5-HT_{1B} receptor mediates vasoconstriction in human cerebral vessels (Verheggen, *et al.*, 1998) which is part of the basis of migraine treatment with sumatriptan and other similar triptan derivatives.

Vascular 5-HT_{1B} receptors may also be important in mediating pulmonary vasoconstriction both in the normal as well as the pulmonary hypertensive state (see section 1.5.3).

1.4.3.1.1 5-$HT_{1B/1D}$ receptor signal transduction/effector mechanisms

Activation of 5-HT_{1B} and 5-HT_{1D} receptors results in inhibition of adenylate cyclase (Brancheck *et al.*, 1991; Hartig *et al.*, 1996) and in a number of vascular preparations, activation of these receptors has been shown to be negatively coupled to the production of cAMP. In the canine saphenous vein for example, sumatriptan decreased the stimulated accumulation of cAMP (Sumner & Humphrey, 1990).Recently, the rabbit recombinant saphenous vein 5-HT_{1B} receptor stably transfected in C6-glial cells displayed significant reduction in cAMP when exposed to various 5-$HT_{1B/1D}$ receptor selective agonists including sumatriptan (Wurch, *et al.*, 1997).

The 5-HT_{1B} receptor is located on human chromosome 6q13. In cells stably expressing human 5-HT_{1B} receptors, stimulation of these receptors blocks forskolin-stimulated adenylate cyclase and cAMP accumulation (Veldman & Bienkowki, 1992; Hamblin, *et al.*, 1992) and these receptor mediated effects are blocked by pertussis toxin pretreatment, indicating the involvement of $G_{i/o}$ proteins (Dickenson & Hill, 1995). The 5-HT_{1D} receptor has 63% structural homology with the 5-HT_{1B} receptor, is located on human gene 1p36.3-p34.3 and has been shown to be negatively coupled to adenylate cyclase (Weinshank, *et al.*, 1992).

Although decreased cyclic AMP levels provide a useful indicator for 5-$HT_{1B/1D}$ receptor function, it is unclear, however, if such a

reduction , following 5-$HT_{1B/1D}$ receptor stimulation, is responsible *per se* for vascular smooth muscle contraction.

In the bovine isolated pulmonary artery, contraction to the 5-$HT_{1B/1D}$ receptor selective agonist sumatriptan correlated well with the concomitant decrease in basal cyclic AMP (Sweeney, *et al.*, 1995). Sumatriptan alone caused no significant contraction in those vessels and had no effect on intracellular cyclic AMP concentration. In the presence of agonist-induced tone, however, sumatriptan evoked a potent contraction in the bovine pulmonary arteries, concomitant with a decrease in basal cyclic AMP. Furthermore, administration of forskolin, a direct activator of adenylate cyclase (Koch, H., 1982), potentiated the contractile response to sumatriptan and allowed sumatriptan to induce a further decrease in cyclic AMP. Collectively, the results suggest that the ability of sumatriptan to decrease cyclic AMP is positively correlated to its ability to induce vasoconstriction in bovine pulmonary arteries (Sweeney, *et al.*, 1995).

In contrast, Sumner & Humphrey (1990) reported that decreased levels of cyclic AMP, in response to 5-$HT_{1B/1D}$ receptor activation, were only observed if adenylate cyclase was prestimulated with forskolin in canine saphenous vein. Furthermore, an involvement of extracellular Ca^{2+} in 5-$HT_{1B/1D}$ receptor-mediated vasoconstriction was carried out by Sumner *et al.* (1992), who found that sumatriptan-induced contraction in the dog saphenous vein, was blocked by removal of extracellular Ca^{2+} and markedly attenuated by the L-type Ca^{2+} channel antagonist verapamil. This suggests an important role for Ca^{2+} influx through voltage operated Ca^{2+} channels in 5-$HT_{1B/1D}$ receptor-mediated vasoconstriction. Sumatriptan also inhibited agonist-stimulated cAMP in these vessels (Sumner *et al.*, 1992). Evidence exists against a causal relationship

between 5-$HT_{1B/1D}$ receptor-induced decrease in cyclic AMP and ensuing smooth muscle contraction (Movahedi & Purdy, 1997; Randall, *et al.*, 1996). 5-HT alone, through activation of 5-$HT_{1B/1D}$ receptors, was reported to significantly reduce basal levels of cyclic AMP without causing any significant contraction in the rabbit femoral artery (Randall, *et al.*, 1996). In the same study, in pre-contracted vessels, 5-HT also failed to reduce modest increases of cellular cyclic AMP but caused a potent contractile response.

The rabbit ear artery possesses a significant population of 5-$HT_{1B/1D}$ receptors which induce a potent vasoconstriction in the presence of agonist-induced tone and mediate inhibition of adenylate cyclase through a pertussis toxin (PTX)-sensitive G-protein (Movahedi & Purdy, 1997). In that study, PTX did not significantly alter the 5-$HT_{1B/1D}$ receptor-induced contraction but did mediate uncoupling of the receptor to adenylate cyclase and cyclic AMP formation. The authors concluded that the 5-$HT_{1B/1D}$ receptor-mediated inhibition of adenylate cyclase, and therefore decreased cyclic AMP, was not per se responsible for the ensuing contraction. The contraction, however, was completely dependent on the availability of extracellular Ca^{2+} (Movahedi & Purdy, 1997), suggesting an alternative second messenger pathway associated with calcium influx. In a recent study using rabbit mesenteric arteries, Hinton and colleagues (1999) reported that 5-HT decreased cyclic AMP following forskolin stimulation, and without the requirement to raise external potassium, a pre-requisite for 5-$HT_{1B/1D}$ receptor-mediated contraction in this preparation. The observations argue against any major role for cyclic AMP in contraction (Hinton, *et al.*, 1999). Taken together, the findings suggest that contractile responses to 5-HT are mediated via a cAMP-independent pathway and evidence

for the link between decreased cellular cAMP and smooth muscle contraction is, therefore, not substantive. Vascular smooth muscle contraction via 5-$HT_{1B/1D}$ receptor stimulation may, therefore, involve other signalling pathways operating either in addition to, or instead of decreased cyclic AMP.

The precise role of Ca^{2+} in 5-$HT_{1B/1D}$ receptor-induced contractions may vary between different vessels. Vascular 5-$HT_{1B/1D}$ receptors have been shown to mediate extracellular Ca^{2+} mobilization in cultured bovine basilar artery VSMCs (Ebersole, *et al.*, 1993). In rabbit isolated ear artery, 5-$HT_{1B/1D}$ receptor-mediated contraction following precontraction with phenylephrine was attenuated by the voltage-operated Ca^{2+} channel blocker nifedipine and completely abolished by removal of extracellular Ca^{2+} (Movahedi & Purdy, 1997). In contrast, in rabbit saphenous vein, 5-$HT_{1B/1D}$ receptor-induced contractions were insensitive to nifedipine (Razzaque *et al.*, 1995).

Agonist-induced elevation in intracellular Ca^{2+} may also result from release of Ca^{2+} from intracellular stores. 5-$HT_{1B/1D}$ receptor stimulation is known to induce transient increases in intracellular Ca^{2+} in the absence of extracellular Ca^{2+} in VSMCs from bovine basilar artery (Ebersole, *et al.*, 1993). In contrast, in rabbit isolated mesenteric artery, the Ca^{2+} -ATPase inhibitor thapsigargin failed to inhibit 5-$HT_{1B/1D}$ receptor-induced contractions, providing evidence that Ca^{2+} release from intracellular stores did not contribute to the contractions (Hill, *et al.*, 2000).

In addition to requiring extracellular Ca^{2+}, several studies, mainly in the rabbit mesenteric artery, have demonstrated that 5-HT can stimulate contraction in permeabilized cells in the presence of a fixed concentration of Ca^{2+} (Parsons, *et al.*, 1996; Seager *et al.*, 1994;

Hinton, *et al.*, 1999). This suggests that 5-$HT_{1B/1D}$ receptor-mediated contraction also involves sensitization of the contractile myofilaments to Ca^{2+}. The myofilament sensitization evoked with 5-HT was reported to be blocked by inhibitors of either phosholipase A_2 (PLA_2) (Parsons, *et al.*, 1996) or protein kinase C (PKC) (Seager, *et al.*, 1994; Parsons, *et al.*, 1996) and mimicked by arachidonic acid. In the same artery, 5-HT_{1B} receptor-mediated activation is associated with the activation of the enzyme phospholipase D (PLD) (Hinton, *et al.*, 1999) which may be downstream of PKC activity and without the concurrent activation of phospholipase C (PLC) (Seager, *et al.*, 1994). this provides evidence, albeit indirect, to suggest that PLD could represent a component of the link between 5-HT_{1B} receptor-activation and vascular smooth muscle contraction.

Recently, Hinton *et al* (2000) reported that inhibitors of phosphatidylinositol 3-kinase and activated mitogen-activated protein kinase (MAPK) significantly reduced the 5-$HT_{1B/1D}$ receptor mediated contractile response in the rabbit renal artery. This indicates that MAPK may also play an important role in the contractile pathway resulting from 5-$HT_{1B/1D}$ receptor activation.

It is clear that the second messenger system linking 5-$HT_{1B/1D}$ receptor activation and vascular smooth muscle contraction is not clearly defined and that these receptors mediate the ensuing contraction in a complex way. This may involve the interaction of several intracellular signalling pathways.

1.4.3.2 5-HT_{2A} receptors

Peroutka & Snyder (1979) showed that 5-HT_2 sites could be labelled with [^{3}H] LSD and [^{3}H] spiperone but not [^{3}H] 5-HT; this led to the proposed existence of 5-HT_1 and 5-HT_2 sites. Three 5-HT_2

receptor subtypes are currently recognised (5-HT_{2A}, 5-HT_{2B} & 5-HT_{2C}) and each has been cloned and shown to be a G-protein linked single protein molecule of similar size and close homology. All three subtypes mediate their effects through activation of phosphoinositide metabolism (see section 1.4.4.2).

Originally described as the 5-HT_2 receptor, the 5-HT_{2A} receptor is located on human chromosome 13q14-q21. Widely distributed in peripheral tissues, this receptor mediates contraction of many urinary, uterine, gastrointestinal and vascular smooth muscle tissues. (Hoyer, *et al.*, 1994). A number of compounds have high affinity for the 5-HT_{2A} receptor but few are truely selective, especially with regard to the other members of the 5-HT_2 receptor class. The agonist α-methyl-5-HT, often used as an agonist probe for the 5-HT_{2A} receptor, is also a potent agonist at the 5-HT_{2B} and 5-HT_{2C} receptors. Nevertheless, an agonist potency order of 5-HT ≥ α-me-5-HT >> 8-hydroxy-2-(di-n-propylamino)-tetralin (8-OH-DPAT) > 5-carboxamidotryptamine (5-CT) still provides useful supporting evidence for mediation via the 5-HT_{2A} receptor. One of the most useful antagonists is ketanserin, first described by Leysen and colleagues (1982) having a high binding affinity for 5-HT_2 receptors (pK_i = 8.9), this compound demonstrates ~70 -100 fold selectivity over 5-HT_{2B} and 5-HT_{2C} receptors and is used extensively in the studies of this thesis. The contractile response to 5-HT is mediated by the 5-HT_{2A} receptor in a wide variety of arterial and venous preparations especially conduit vessels (Saxena & Villalon, 1990) and ketanserin has provided a strong basis in the identification of this receptor. In the rabbit isolated aorta, for example, Leff & Martin (1986) showed this compound to have a pA_2 against 5-HT-induced contraction of 8.6. This affinity value is typical for ketanserin showing a range of

8.1 - 10.4 in a variety of isolated conduit vascular preparations from different species including the rat basilar and rat caudal arteries (Chang & Owman, 1989; Leff & Martin, 1986) and the calf coronary artery, displaying subnanomolar antagonist affinity (pK_B = 9.5) (Frenken & Kaumann, 1984).

Several studies have shown that ketanserin is an antagonist at 5-$HT_{1B/1D}$ receptors at μM concentrations. In cloned rabbit and human 5-HT_{1B} and 5-HT_{1D} receptors, Bard, *et al* (1996) demonstrated that ketanserin was able to discriminate between these two 5-HT receptor subtypes showing high affinity (pK_i = 7.66) for the 5-HT_{1D} subtype and a 20-fold selectivity over the 5-HT_{1B} subtype. In rabbit isolated coronary arteries, high concentrations of ketanserin (1-10μM) were shown to antagonise sumatriptan-induced contractions (pA_2 ~6.5) (Ellwood and Curtis, 1997). The possibility that ketanserin may antagonise 5-$HT_{1B/1D}$ receptor-mediated responses must therefore be taken into consideration when interpreting the results of pharmacological studies. Evidence indicates that the vasoconstrictor effect of 5-HT in human systemic arteries is normally mediated mainly through the 5-HT_{2A} receptor (Arneklo-Nobin, *et al.*, 1985).

1.4.3.3 5-HT_{2B} receptors

The 5-HT_{2B} receptor is located on human chromosome 2q36.3-2q37.1 . First cloned from rat and mouse stomach fundus (Foguet *et al.*, 1992; Kursar, *et al.*, 1992), it is the most recent member of the 5-HT_2 receptor class, named because of its close structural homology with this family. Cloned human rat and mouse 5-HT_{2B} receptors are coupled to $G_{q/11}$ G-proteins and signal transduction mechanisms include, primarily, an increased phosphoinositide hydrolysis (Kursar,

et al., 1992). Cox and co-workers (1995) showed that stimulation of this receptor also results in the activation of protein kinase C in rat stomach fundus.

5-HT_{2B} receptors display a similar pharmacology to that of the 5-HT_{2A} and 5-HT_{2C} receptor in terms of the agonists 5-HT and α-me-5-HT having high affinity for this receptor (Wainscott, *et al.*, 1993), with the affinity for 5-HT at this receptor (~8.36) being higher than at any other functional 5-HT receptor described (Leff, *et al.*, 1987). 5-CT displays a 7-fold lower affinity than 5-HT at this receptor. Receptor binding affinities for ketanserin, however, at this receptor are comparatively poor (pK_i = 5.4) compared to the 5-HT_{2A} receptor (Wainscott, *et al.*, 1993). Recently, SB 204741 has been reported as a selective 5-HT_{2B} receptor antagonist (pK_i = 7.8) with ~100-fold selectivity over the 5-HT_{2A} and 5-HT_{2C} sites. One of the most selective agonists, BW 723C86, has been reported to have 100 fold and 10 fold selectivity (pK_i = 7.9) over the rat 5-HT_{2A} and 5-HT_{2C} receptors respectively (Kennet, *et al.*, 1996) and this compound has recently been shown to induce a potent, endothelium-dependent vasorelaxation in isolated porcine pulmonary arteries (Glusa,1992; 2000). 5-HT_{2B} receptor mediated, endothelium-dependent, relaxation has also been reported in other isolated vascular preparations including the rat and rabbit jugular vein (Ellis, *et al.*, 1995; Leff, *et al.*, 1987). In the rabbit jugular vein, for example, BW 723C86 was shown to be a potent agonist (pEC_{50} = 7.5) in mediating endothelium-dependent relaxation (Martin, *et al.*, 1993). The observed relaxation is thought to occur indirectly via endothelium-dependent nitric oxide release (Baxter, *et al.*, 1995; Ishida, *et al.*, 1998). Ullmer and colleagues (1996) observed calcium release from ryanodine-sensitive intracellular stores in human pulmonary artery

endothelial cells upon activation of the 5-HT_{2B} receptor. This is consistent with the NO-induced vessel relaxation. Strong evidence for an endothelial location for this receptor has also been provided using molecular studies as 5-HT_{2B} receptor mRNA is present in cultured endothelial cells from human umbilical vein, coronary artery and aorta (Ullmer, *et al.*, 1995). 5-HT_{2B} receptors are also present on smooth muscle and have been shown to mediate contraction of longitudinal muscle in human small intestine (Borman & Burleigh, 1995). The presence of 5-HT_{2B} receptor mRNA in the cerebral vasculature (Ullmer, *et al.*, 1995) suggests that activation of this receptor may play a role in migraine headache. In many forms of hypertension the vasoconstrictor response to 5-HT is markedly increased (Collis & Vanhoutte, 1977; Thompson & Webb, 1987; Turla & Webb, 1989) and in some forms of hypertension, 5-HT antagonists such as ketanserin are shown to be ineffective. Recently Watts and co-workers (1996) demonstrated a marked 5-HT_{2B} receptor mediated vasoconstriction and increased mRNA for this receptor in mesenteric arteries from deoxycorticosterone acetate-salt hypertensive rats compared to controls (5-HT_{2A} receptor mediation). 5-HT_{2B} receptors may therefore be implicated in hypertension.

<u>1.4.3.3.1 5-HT_2 receptor signal transduction mechanisms- Activation of phospholipase C (PLC) /protein kinase C (PKC)</u>

The 5-HT_2 class of receptor, including the 5-HT_{2A} and 5-HT_{2B} receptors are thought to be linked to the phosphinositol hydrolysis signal transduction system coupled to the α subunit of the Gq GTP binding protein (Hoyer, *et al.*, 1994). PLC catalyses the formation of inositol phosphate (IP_3) and diacylglycerol (DAG). This occurs through the hydrolysis of phosphotidyl 1 inositol (4,5) biphosphate

(PIP_2). Doyle and co-workers (1986) demonstrated that 5-HT_{2A} agonists activate PLC in rat culture aortic smooth muscle cells. IP_3 then acts via specific receptors to release intracellularly stored Ca^{2+}. This increase results in activation of the enzyme myosin light-chain kinase (MLCK), which leads to the phosphorylation of myosin light-chain protein, triggering contractile events. DAG may in turn activate PKC and ultimately cause the opening of voltage -dependent Ca^{2+} channels and the elevation of cytosolic Ca^{2+}. Recently, in the isolated blood-perfused dog lung, Barman and co-workers (1997) demonstrated that PKC activation and subsequent activation of voltage-dependent Ca^{2+} channels was involved in 5-HT_{2A} receptor mediated pulmonary vasoconstriction.

1.4.3.4 5-HT_7 Receptors

The 5-HT_7 receptor is the most recently identified member belonging to the family of G-protein coupled 5-HT receptors, has been cloned from several species including man and is located on the human chromosome 10q23.3-q24.4. Multiple splice variants of this receptor have been discovered (Heidmann, *et al.*, 1997) but are not associated with markedly different functional capabilities.

A rank order of agonist potency for this receptor has been suggested; 5-CT > 5-HT > 8-OH-DPAT > sumatriptan, with affinities for 5-HT and 5-CT being 8.1- 9.4 and 9.0 - 9.7 respectively in human recombinant receptors expressed in mammalian cells (Bard, *et al.*, 1993; Jasper, *et al.*, 1996). Indeed, this receptor was originally classed as "5-HT_1-like" due to high affinity for 5-CT. Several antipsychotic drugs and some 5-HT receptor antagonists have been found to bind to 5-HT_7 receptors with high affinity. These include spiperone, risperidone and pimozide with affinity values (pK_i) of 8, 8.86 and

respectively (Roth, *et al.*, 1994). In chapter 3 of this thesis, risperidone, pimozide and spiperone were used as 5-HT_7 antagonists and in chapter 5, spiperone was used. The 5-HT_7 receptor has been identified in the vascular smooth muscle of several species using pharmacological and molecular analysis and this receptor subtype mediates 5-HT-induced vasorelaxation. The presence of 5-HT_7 receptor mRNA has been demonstrated in canine and human coronary artery (Bard, *et al.*, 1993) and in various rat and porcine blood vessels including the porcine pulmonary artery (Ullmer, *et al.*, 1995). Functional studies show that endothelium-independent, 5-HT-induced vasorelaxation can be attributed to this receptor. In the endothelium-denuded, pre-constricted canine coronary artery, for example, 5-CT produced concentration-dependent relaxations with high potency which were significantly antagonised by risperidone, spiperone and pimozide with pKB values of 9.4, 7.14 and 8.27 respectively, strongly indicative of a 5-HT_7 receptor mediated response (Terron, *et al.*, 1996). Similar observations have also been made in monkey isolated jugular vein (Leung, *et al.*, 1996) and dog cerebral arteries (Terron, *et al.*, 1999). In the rabbit, the 5-HT_7 receptor is thought to mediate 5-HT-induced relaxation in the femoral and jugular veins (Martin & Wilson, 1995; Leff, *et al.*, 1987).

Collectively, these data show that the 5-HT_7 receptor plays a role in mediating vascular smooth muscle relaxation. Interestingly, the predominant response to 5-HT in the cerebral arterial bed is vasoconstriction thought to result from stimulation of predominantly 5-HT_{2A} and 5-HT_{1B} receptors (Deckert, *et al.*, 1994; Hamel, *et al.*, 1993). This is also true of the coronary artery (Ellwood & Curtis, 1997). A physiological role for the 5-HT_7 receptor has been suggested in modulating the vasoconstrictor effects of 5-HT_{2A} and 5-

HT_{1B} receptor activation (Eglen, *et al.*, 1997). Two important vascular beds where this receptor appears to be located are the cerebral (Terron, *et al.*, 1999) and coronary (Terron, *et al.*, 1996) vasculature. This may suggest a pathophysiological involvement in angina and migraine headache respectively.

Schoeffter and co-workers (1996) demonstrated that 5-CT and 5-HT enhanced forskolin-stimulated cAMP accumulation in human cultured uterine artery vascular smooth muscle cells and several studies have conclusively shown that the 5-HT_7 receptor is positively coupled to adenylate cyclase in various tissue preparations (Eglen, *et al.*, 1997; Kitazawa, *et al.*, 1998), a mechanism of action that is consistent with direct smooth muscle relaxation due to inhibition of myosin light chain kinase. For example, 5-HT_7 receptor stimulation in the porcine myometrium results in inhibition of contractility of this tissue, coupled with increased cAMP synthesis (Kitazawa, *et al.*, 1998). No evidence exists for coupling of the receptor to other intracellular signalling pathways.

1.4.5 5-HT and the cardiovascular system

The cardiovascular actions of 5-HT are complex due to the action of this bioamine at a multiplicity of 5-HT receptors. The end response depends on a number of factors including species, the target vascular bed and the concentration employed.

1.4.5.1 Haemodynamic effects

Intravenous infusion of 5-HT into conscious, anaesthetised rats causes a triphasic effect on blood pressure, comprising a rapid and transient vasodilation, followed by a moderate increase in blood pressure and a long lasting decrease in blood pressure (Kalkman, *et*

al., 1984; Saxena, *et al.*, 1985). The initial transient depressor response has since been demonstrated to be mediated via activation of the 5-HT_3 receptor on afferent cardiac vagal nerve endings which produce a marked bradycardia and decreased cardiac output (see section 1.4.8.2). The magnitude of the pressor response is dependent upon species, being poor in rabbits (Wright, *et al.*, 1989) and cats (Saxena, *et al.*, 1985), whereas a prominent pressor response ensues in the dog (Scneider & Yonkman, 1954). This pressor response is thought to be mediated via vascular 5-HT_{2A} receptor activation as ketanserin, the selective 5-HT receptor antagonist blocks the response (Kalkman, *et al.*, 1984). The long lasting decrease in blood pressure is not altered by selective antagonists for 5-HT_2 and 5-HT_3 receptors and was originally classed as "5-HT_1-like" due to the high potency of 5-CT in mediating this response, coupled with blockade by methiothepin (Saxena, *et al.*, 1985). Recent results from *in vivo* studies, however, suggest the hypotensive effects of i.v. 5-HT in the rat involve activation of receptors closely similar to the cloned 5-HT_7 subtype (DeVries, *et al.*, 1997; Terron, 1997).

1.4.5.2 Cardiac actions

5-HT can elicit a variety of responses by the heart resulting from the activation of receptor subtypes, stimulation or inhibition of autonomic activity, or dominance of reflex responses to 5-HT. 5-HT action on the vagus nerve endings produces the Bezold-Jarish reflex mediated via the vagus nerve, resulting in extreme bradycardia, and involving 5-HT_3 receptor activation (cardiac sensory receptors). This has been demonstrated in the rabbit (Wright, *et al.*, 1989) as well as other species.

1.4.5.3 Vascular actions

Due to the focus of this thesis being on the pulmonary circulation, the actions of 5-HT on the pulmonary vasculature in particular are discussed in section 1.5.2.

It is clear that 5-HT mediates its vascular responses via action at a number of 5-HT receptor subtypes (see section 1.4.3). The response of a particular vessel to 5-HT will ultimately depend, therefore, on which 5-HT receptors are located on the particular vessel, and which subtype predominates. 5-HT, therefore, regulates vasoconstriction and vasorelaxation in a complex way and involving interaction of several 5-HT receptor subtypes. This is schematically summarised in figure 1.3. In systemic vascular tissue preparations, the vasoconstrictor actions of 5-HT have been attributed to mediation via either, predominantly, the 5-HT_{2A} (see section 1.4.3.2) or, to a lesser extent, the 5-$HT_{1B/1D}$ (see section 1.4.3.1) receptors located on vascular smooth muscle. In contrast, endothelium-located 5-HT_{2B} (see section 1.4.3.3) and vascular smooth muscle 5-HT_7 receptors (see section 1.4.3.4) are thought to mediate the vasodilator responses to 5-HT and therefore may modulate the vasoconstrictor actions resulting from 5-HT_{2A} or 5- $HT_{1B/1D}$ receptor activation. As mRNA for the 5-HT_{1B} receptor is expressed in abundance in several artery endothelial cells (Ullmer *et al.*, 1995), a functional role in mediating NO-dependent vascular relaxation can not be ruled out.

Several studies have reported that, at high concentrations (>1μM), 5-HT can mediate an indirect vasoconstrictor effect via activation of α-adrenoceptors in some rabbit large blood vessels including the femoral artery (Grandaw & Purdy, 1996), ear artery (Black *et al.*, 1981) and aorta (Purdy *et al.*, 1987). In the rabbit isolated femoral artery, for example, 5-HT elicits a biphasic

concentration response curve. The first phase being sensitive to antagonism by ketanserin and the second phase blocked by the selective α_1-adrenoceptor antagonist benextramine (Grandaw & Purdy, 1996). The involvement of an α_1-adrenoceptor in the contractile response to 5-HT in the rat pulmonary conduit artery has also been reported (Ogawa *et al.*, 1995).

It has also been reported that high concentrations of 5-HT can have indirect sympathomimetic actions by releasing noradrenaline from perivascular sympathetic nerve endings (McGrath, 1977; Saxena & Villalon, 1990).

With respect to the ability of 5-HT to interfere with sympathetic transmission, 5-HT inhibits, via pre-junctional 5-HT_1 receptors, the contractile responses to adrenergic nerve stimulation in several blood vessels including canine (Humphrey *et al.*, 1988) and human (Molderings *et al.*, 1990) saphenous veins and the rat vena cava (Molderings et al., 1987).

1.4.5.4 Morphological effects on the vasculature

Substantial evidence indicates that 5-HT may regulate growth and proliferation of vascular cell types. 5-HT stimulates vascular smooth muscle cell proliferation cultured from porcine, rat and bovine aorta (Nemecek,*et al.*,1986). Several groups have demonstrated that 5-HT increases DNA synthesis in cultured pulmonary vascular smooth muscle cells from different regions of the pulmonary vascular tree (Lee, *et al.*, 1991; Pitt, *et al.*, 1994).

The mechanism responsible for the mitogenic effects of 5-HT varies with cell type and species. Several reports suggest mediation via the 5-HT_{2A} receptor, as ketanserin (a selective 5-HT_{2A} receptor antagonist) inhibits 5-HT mediated proliferation in rat cultured

pulmonary artery smooth muscle cells (Pitt, *et al.*, 1994). Other reports suggest the mitogenic action is initiated by energy-dependent transport into the cell as known selective inhibitors of 5-HT transport inhibit 5-HT-induced proliferation of bovine and rat pulmonary artery SMCs (Lee, *et al.*, 1991; Eddahibi, *et al.*, 1999). The signal transduction mechanisms involved in the mitogenic effects of 5-HT post-transport still remains uncertain. Recently, superoxide anion formation has been implicated in transport-associated 5-HT-induced mitogenesis (Lee, *et al.*, 1998). The mitogenic effects mediated by cell surface 5-HT_{2A} receptors have been shown to occur via the phosphatidylcholine-specific phospholipase C/mitogen activating protein kinase path in rat cultured mesangial cells (Goppelt-Struebe & Stroebel, 1998). The pathway in pulmonary vascular smooth muscle cells has yet to be confirmed.

It has been proposed that growth and migration of endothelial cells are prerequisites for vascular remodelling. Pakala *et al.* (1994) demonstrated that 5-HT dose-dependently causes a mitogenic effect on cultured vascular endothelial cells. Collectively these results suggest that 5-HT can contribute to remodelling of vascular tissues.

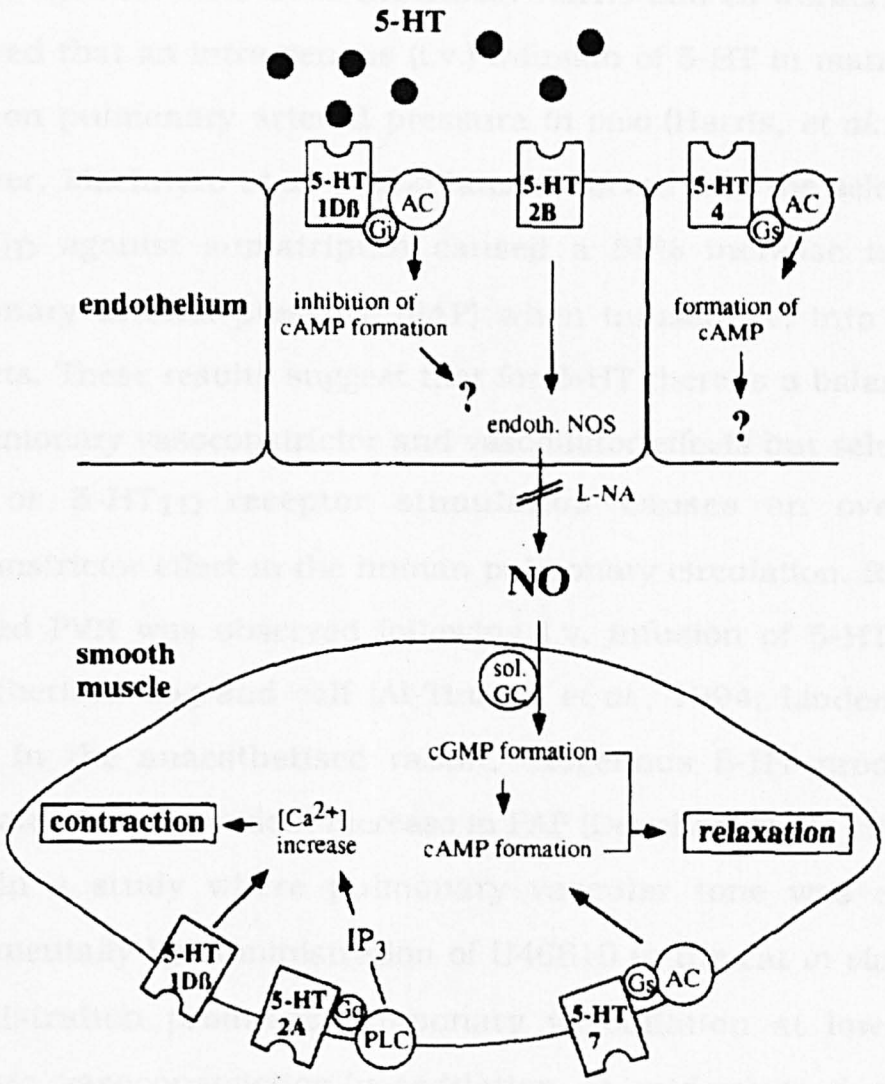

Figure 1.3 Schematic model of vascular 5-HT (serotonin) receptors. (see text for details).
From: *Ullmer et al., (1995) FEBS Letters 370, 215-221.*

1.5 5-HT and the pulmonary circulation

1.5.1 Pulmonary actions of 5-HT

1.5.1.1 *In vivo* studies

In the pulmonary circulation, varying responses *in vivo* to 5-HT and its agonists have been described. Harris and co-workers (1960) observed that an intra-venous (i.v.) infusion of 5-HT in man had no effect on pulmonary arterial pressure *in vivo* (Harris, *et al.*, 1960). However, MacIntyre *et al.* (1992) later observed that the selective 5-$HT_{1B/1D}$ agonist sumatriptan caused a 58% increase in mean pulmonary arterial pressure (PAP) when infused i.v. into human subjects. These results suggest that for 5-HT there is a balancing of its pulmonary vasoconstrictor and vasodilator effects but selective 5-HT_{1B} or 5-HT_{1D} receptor stimulation causes an overriding vasoconstrictor effect in the human pulmonary circulation. Recently, elevated PVR was observed following i.v. infusion of 5-HT in the anaesthetised dog and calf (Al-Tinawi, *et al.*, 1994; Linden, *et al.*, 1996) In the anaesthetised rabbit, exogenous 5-HT produced a moderate, dose-dependent increase in PAP (Deuchar, *et al.*, 1997).

In a study where pulmonary vascular tone was elevated experimentally by administration of U46619 in the cat *in vivo*, 5-HT administration produced pulmonary vasodilation at low doses, biphasic vasoconstriction/vasodilation at mid-range doses and predominantly vasoconstriction at high doses of 5-HT, suggesting the presence of more than one 5-HT receptor subtype (Neely, *et al.*, 1993)

1.5.1.2 In vitro studies

5-HT is a potent vasoconstrictor of animal and human pulmonary arteries *in vitro* (Barnes & Lui, 1994). The use of selective 5-HT receptor agonists and antagonists has aided the progressive

identity of the 5-HT receptor subtypes that mediate vascular effects. In isolated rat pulmonary arteries, functional vasoconstrictor responses to 5-HT were inhibited by the selective 5-HT_{2A} receptor antagonist ketanserin, thus indicating 5-HT_{2A} receptor activation (MacLean, *et al.*, 1996a). Until recently, it was assumed that this receptor mediates pulmonary arterial contraction as this has also been shown in isolated canine and bovine pulmonary arteries (Chand & Altura, 1980; MacLean, *et al.*, 1994). However, evidence presented in this thesis indicates that the 5-HT_{1B} receptor is predominant in mediating 5-HT-induced vasoconstriction in human pulmonary arteries. In isolated human large pulmonary arteries, 5-CT was shown to be more potent than 5-HT, which was equipotent to sumatriptan. The selective 5-HT_{1B} and 5-HT_{1D} antagonist GR55562 inhibited responses to both 5-HT and sumatriptan with a pK_B value of 8.88 against sumatriptan (MacLean, *et al.*, 1996b), indicating 5-HT_{1B} (or 5-HT_{1D}) receptor activation. These results were later confirmed by Cortijo *et al.*, (1997) who observed effective antagonism of sumatriptan-induced contraction with GR127935, an antagonist at 5-HT_{1B} and 5-HT_{1D} receptors (Conner, *et al.*, 1989). Collectively, these observations may explain why ketanserin has demonstrated a limited use in the treatment of PHT. The observations also collectively emphasise the importance of making comparative studies in human tissue where possible.

Evidence for a role of vasodilator 5-HT receptors has also been reported in pulmonary vessels *in vitro*. For example, Glusa and co-workers (1993) showed that 5-HT evoked endothelium-dependent relaxation of preconstricted porcine pulmonary arteries. Recently, further investigation by the same group, provided strong evidence that the 5-HT_{2B} receptor mediates endothelium-dependent relaxation

in the same preparation (Glusa & Pertz, 2000). In ovine preconstricted pulmonary vein, Zhang, *et al.* (1995) demonstrated an endothelium-independent relaxation to 5-HT which was positively coupled to adenylate cyclase and stimulated cyclic AMP formation. These observations are consistent with 5-HT mediating its action either via the recently described 5-HT_7 receptor (see section 1.4.3.4) or via the 5-HT_4 receptor, also positively coupled to adenylate cyclase (Hoyer, *et al.*, 1994).

In contrast, in the isolated pulmonary arteries of some species, experimentally elevated vascular tone results in augmented 5-HT-receptor-induced contractions. In bovine pulmonary arteries for example, precontraction with U46619, uncovers a significant population of 5-HT_1 receptors that were previously silent (MacLean, *et al.*, 1994).

Molecular analysis of 5-HT receptor mRNA expression has revealed the presence of mRNA for the 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2B} and 5-HT_7 receptor in pig pulmonary arteries and in cultured human pulmonary artery smooth muscle cells (Ullmer, *et al.*, 1995).

All the aforementioned studies indicate apparent species- and preparation- related differences in the functional effects of 5-HT-receptor mediated responses on the pulmonary vasculature with a number of concomitant variables determining the overall response, as well as distinct differences compared to the systemic circulation.

As previously mentioned in section 1.4.5.4, 5-HT is also a potent mitogenic and proliferative agent, and has been shown to be a potent stimulus for DNA synthesis and proliferation of bovine, and rat pulmonary vascular smooth muscle cells in culture (Lee, *et al.*, 1991; Pitt, *et al.*, 1994).

1.5.2 Physiological role for 5-HT in the pulmonary circulation

The pulmonary vascular bed of the lungs under normal conditions, acting as a secondary filter downstream of the liver, does not encounter excessive levels of 5-HT and a possible physiological role for 5-HT in the pulmonary circulation is unclear. However, the noted pulmonary vascular effects of 5-HT alluded to above (section 1.5.1) suggest the influence of this bioamine in the physiological regulation of pulmonary vascular tone seems plausible. Due to the variation in 5-HT mediated effects from various studies which have been reported, the definitive physiological role is difficult to determine. The vasodilator action of 5-HT could be implicated in the maintenance of low pulmonary vascular tone through interaction with the vascular endothelium, or through direct action on vascular smooth muscle. The pulmonary circulation appears to be a primary site of 5-HT clearance and metabolism, probably limiting the vasopressor effects of circulating 5-HT.

1.5.3 Pulmonary hypertension

Several studies, both in the clinical and experimental settings have suggested a role for 5-HT in the aetiology of PHT and Figure 1.4 schematically summarises this "5-HT Hypothesis of pulmonary hypertension". Due to the avid ability of the platelets to store large amounts of 5-HT (see section 1.4.2) and the fact that lungs are located downstream of the liver, and therefore act as a secondary filter, the pulmonary vasculature is not subject to excessive amounts of 5-HT under normal conditions. Changes in local, pulmonary, availability of 5-HT may be associated with the development of PHT although no link between plasma levels of free 5-HT and PHT have been conclusively proven. Local to the pulmonary circulation, 5-HT is

released from pulmonary neuroendocrine cells and neuroepithelial bodies distributed throughout the airways. Airway hypoxia and hypercapnia causes secretion of large amounts of 5-HT from these cells and may contribute to secondary PHT (Gosney, et al., 1989; Johnson & Gieorgieff, 1989). 5-HT levels have been reported to be increased in several clinical settings. Hervé *et al.* (1995) reported that plasma 5-HT levels are increased in primary PHT, even following normalisation of pulmonary arterial pressures post lung transplantation, ruling out PHT as the cause of the platelet dysfunction. PHT is often associated with diseases which exhibit elevated 5-HT levels due to altered platelet 5-HT storage including platelet storage pool disease (Herve, et al., 1990) and systemic sclerosis (Klimiuk, *et al.*, 1989). McGoon and Vanhoutte (1984) demonstrated that aggregating platelets can release 5-HT, inducing a vasoconstrictor response in canine isolated pulmonary arteries and platelet aggregation often occurs in primary (Nakonechnicov, *et al.*, 1996) and secondary pulmonary hypertension (Chouat, *et al.*, 1996). For example, Sibbald and co-workers (1980) observed a correlation between elevated plasma 5-HT levels and pulmonary hypertension in patients with adult respiratory distress syndrome due to sepsis. A long association between anorexigenic drugs and PHT has been observed with the possibility that drugs such as fenfluramine trigger PHT by both inducing or augmenting impaired platelet 5-HT storage (Eddahibi, et al., 1998). The fawn hooded rat, as an experimental model of platelet storage pool disease, can develop very severe PHT after modest hypoxic exposure (Ashmore, *et al.*, 1991) and demonstrates increased vasoconstrictor responses to 5-HT (Ashmore, *et al.*, 1991) along with reduced 5-HT removal by lung tissue (Hilliker, *et al.*, 1983). Pulmonary arteries from patients with primary PHT who

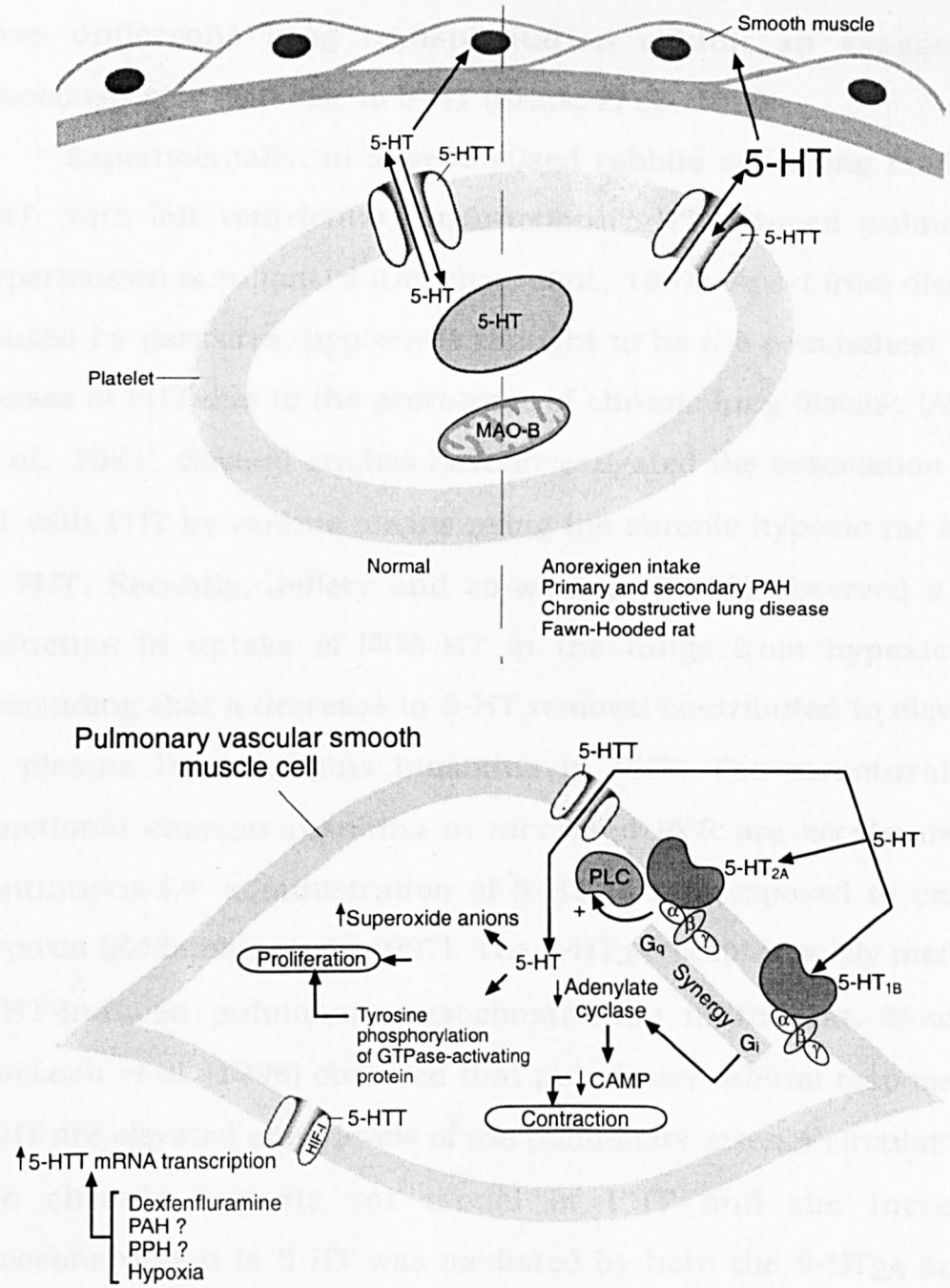

Figure 1.4
The 5-HT Hypothesis of pulmonary arterial hypertension. (A) Platelet handling of 5-HT (see text for details). (B) 5-HT and human pulmonary vascular smooth muscle cells (see text for details).
From: *MacLean et al., (2000) British Journal of Pharmacology 131, 161-168.*

have undergone lung transplantation exhibit an exaggerated vasoconstrictor response to 5-HT (Brink, *et al.*, 1988).

Experimentally, in anaesthetised rabbits exhibiting moderate PHT, with left ventricular dysfunction, 5-HT-induced pulmonary hypertension is enhanced (Deuchar, *et al.*, 1997). Apart from diseases caused by parasites, hypoxia is thought to be the commonest of all causes of PHT due to the prevalence of chronic lung disease (Alpert, *et al.*, 1981). Several studies have investigated the association of 5-HT with PHT by various means using the chronic hypoxic rat model of PHT. Recently, Jeffery and co-workers (2000) observed a 50% reduction in uptake of [^{3}H]5-HT in the lungs from hypoxic rats concluding that a decrease in 5-HT removal contributed to elevation in plasma levels of this bioamine in PHT. The structural and functional changes resulting in increased PVR are accelerated by continuous i.v. administration of 5-HT to rats exposed to chronic hypoxia (Eddahibi, *et al.*, 1997). The 5-HT_{2A} receptor solely mediates 5-HT-induced pulmonary vasoconstriction in the rat. However, MacLean *et al.* (1996) observed that pulmonary arterial responses to 5-HT are elevated at all levels of the pulmonary arterial circulation in the chronic hypoxic rat model of PHT and the increased vasoconstriction to 5-HT was mediated by both the 5-HT_{2A} and 5-HT_{1D} or 5-HT_{1B} receptor. Heeley *et al.* (1998) recently showed that the mRNA for the 5-HT_{1B} receptor is increased in the pulmonary arteries from these rats. In human isolated large pulmonary arteries 5-HT mediates its vasoconstrictor action via stimulation of the 5-$HT_{1B/1D}$ receptor (MacLean, *et al.*, 1996b; Cortijo, *et al.*, 1997). The evidence accumulated from the human and rat studies, therefore, implicates the importance of the 5-HT_{1B} receptor in mediating pulmonary vasoconstriction and may make a significant contribution

to increased pulmonary arterial vasoconstriction in PHT. This is in contrast to the systemic circulation, at least in humans, where the 5-HT_{2A} receptor is thought to mediate the vasoconstrictor actions of 5-HT (Arneklo-Nobin, *et al.*, 1985).

Further observations also implicate the importance of the 5-HT_{1B} receptor in mediating increased pulmonary arterial vasoconstriction in PHT. 5-HT_1 receptor mediated vasoconstriction is often only observed in the presence of increased vascular tone (Movahedi *et al.*, 1997), a phenomenon known as pharmacological synergism. This is thought to occur as a result of amplification of signal transduction mechanisms due to crosstalk between two G-protein coupled receptors on the same cell, ultimately producing a greater than additive effect (Martin, *et al.*, 1996). Systemic arteries are under a degree of tonic sympathetic tone *in vivo* and such synergy may, therefore, normally operate *in vivo*. In contrast, pulmonary arteries are fully dilated in the normal lung and such synergy may operate only in conditions when pulmonary vessels are subjected to increased vascular tone. The observations that 5-$HT_{1B/1D}$ receptor-induced contractions are uncovered in bovine pulmonary arteries in the presence of agonist-induced tone (MacLean, *et al.*, 1994), coupled with the fact that in the chronic hypoxic rat model of PHT, pulmonary arteries exhibit elevated vascular tone, strongly suggest that these synergistic effects become pathophysiologically important in PHT. Pharmacological synergy may therefore contribute to the increased 5-HT_{1B} receptor mediated vasoconstrictor response in PHT (MacLean, *et al.*, 1999).

The reported ability of 5-HT to stimulate hypertrophy and proliferation of pulmonary vascular smooth muscle cells suggests that it may also play a role in the concomitant vascular remodelling

in PHT. The mechanism responsible for this action of 5-HT is thought to be either via a cell surface 5-HT receptor (Pitt, *et al.*, 1994) or via the active transporter (5-HTT) located on pulmonary vascular smooth muscle cells (Eddahibi, *et al.*, 1999) or a combination of both. This has yet to be resolved. Several observations suggest, however, that 5-HT mediates the development of PHT through the activity of the 5-HTT. Pharmacological inhibition of 5-HT uptake results in the prevention of 5-HT-induced mitogenesis in cultured pulmonary artery SMCs suggesting the necessity of 5-HT internalisation by the 5-HTT. Recently, Eddahibi and co-workers (2000) observed a marked attenuation of chronic hypoxia-induced PHT development and vascular remodelling in mice that were deficient in the 5-HTT gene compared to mice with the gene. Coupled with the observations that exposure of pulmonary artery SMCs to hypoxia increases the expression and activity of the 5-HTT (Eddahibi *et al.*, 1999; Lee, *et al.*, 1991), these results provide strong evidence for the importance of the 5-HTT in 5-HT-evoked vascular remodelling in hypoxia-induced pulmonary hypertension.

Collectively, these findings and the marked effects of 5-HT on the pulmonary circulation (see section 1.5.1) suggests that 5-HT may contribute to the elevated pulmonary vascular resistance and pulmonary vascular remodelling, characteristic of all forms of PHT.

The potential effectiveness of various 5-HT receptor antagonists in the treatment of PHT in man depend not only on their efficacy in the pulmonary circulation, but also on having a desirable influence on the systemic circulation. Considering the collective evidence suggesting a role for the 5-HT_{1B} receptor, antagonists at this receptor subtype may therefore be of therapeutic benefit it the treatment of PAH.

1.6 Project Aims

The studies in this thesis focused primarily on the pulmonary vasculature of the perinatal rabbit. In the perinatal pulmonary circulation the pulmonary vasculature undergoes very marked changes in terms of structure and function in order to adapt to extrauterine life. In the normal lung, the pulmonary circulation is characterised by low pressure with low vascular resistance compared to the systemic circulation (see section 1.1.3). *In utero*, the foetal pulmonary circulation is under high pressure and resistance (see section 1.2.1) both of which markedly fall when the lungs start their main functional role in life at birth (section 1.2.2). It is of interest to study the pulmonary vasculature at this important stage of life where the marked structural and functional changes are modulated by or indeed affect the response to known pulmonary vasoactive substances. The effect of this transition from foetal to early neonatal life was therefore studied in the perinatal rabbit allowing the investigation of the "active" regulation of the pulmonary circulation due to changes in O_2. Similar studies in the adult rabbit would provide a basis for comparison.

The factor common to all the investigations in this thesis is 5-HT. The involvement of 5-HT has been implicated in pulmonary hypertension of various aetiologies (section 1.5.3). The effects of 5-HT on the pulmonary resistance vasculature in man is uncertain, therefore identification of the 5-HT receptors mediating these responses is of interest.

With particular relevance to the perinatal pulmonary circulation, 5-HT has also been linked to PHT associated with hypoxia in the newborn infant and 5-HT turnover has also been

shown to be increased in children with PHT secondary to congenital heart disease (section 1.5.3). Little is currently known about the perinatal pulmonary vascular actions of 5-HT. Hence it is of interest to examine the 5-HT-receptor mediated responses of pulmonary arteries in the perinatal period as well as in adulthood. The chronic hypoxic rat is a well studied model of hypoxic PHT. Rats exposed to chronic hypoxia display significant PHT, developing similar morphological alterations in the pulmonary vasculature that are exhibited in human PHT (Hislop & Reid, 1976; Rabonitch, *et al.*, 1979). In this model, important alterations in the responsiveness of the pulmonary arteries to 5-HT occur (section 1.5.3) in terms of magnitude and sensitivity of the contractile response as well as in 5-HT receptor subtype(s) involved. These alterations occur in an environment of altered pulmonary vascular tone, cyclic nucleotide levels and 5-HT receptor expression. Thus, it is of interest to examine the 5-HT-receptor mediated responses in rat pulmonary vessels under conditions which mimic these altered factors.

Chapters 3 and 5 show the results of the *in vitro* studies of pulmonary arteries in the adult and perinatal rabbit respectively. Chapter 4 focused on the in vitro examination of human small muscular pulmonary arteries, whilst the examination of pulmonary resistance vessels from chronic hypoxic pulmonary hypertensive and control rats are presented in chapter 6.

Chapter 2

Materials And Methods

2.1 Animal models used in the studies

2.1.1 Adult rabbits

Male New Zealand White rabbits weighing ~2.5 kg were purchased from Harlan UK Ltd., a credited commercial supplier of laboratory animals. These were held in the Central Research Facility, Biological Services, University of Glasgow. The rabbits were killed with an overdose of sodium pentobarbitone (200 $mgkg^{-1}$) through the left ear marginal vein. Lungs were rapidly removed into ice cold Krebs solution. The pulmonary arteries were dissected out as described in section 2.3.4.1

2.1.2 Foetal and neonatal rabbits

Pregnant female New Zealand White rabbits were obtained from Harlan UK Ltd. These rabbits were at 23 days gestation with their first litter (normal gestation to litter down date is 31 days) and were housed in the Central Research Facility, Biological Services, University of Glasgow. As previously described in this thesis (1.2.2), the pulmonary circulation undergoes rapid structural and functional changes at birth. The rabbit pups were studied either 2 day preterm foetal, 0-24 hours, 4 days or 7 days following birth. All pups were sacrificed with an overdose of sodium pentobarbitone (200 $mgkg^{-1}$). For foetal pups, the mother rabbit was overdosed and a caesarean section performed. The foetal rabbits were then rapidly overdosed and decapitated to ensure the foetus did not take a first breath. The lungs were rapidly removed to ice cold (4°C) Krebs solution for subsequent dissection of the pulmonary arteries. The dissection of these vessels is described in section 2.3.4.2.

2.1.3 Human pulmonary tissue

Due to limited availability, only crucial, key experimental protocols were applied to human tissue. Macroscopically normal human lung tissue was obtained from patients (both male and female) undergoing surgery for bronchial carcinoma at the Western Infirmary, Glasgow and the Royal infirmary, Glasgow. Verbal information available from the medical staff indicated that these patients did not have any documented evidence of any other chronic pulmonary pathology. Information concerning individual patient age, clinical histories and variable treatments was not, however, made available. Acknowledgement must be made about the difficulties associated with using human tissue classified as 'normal'. Patient variables such as age and specific patient medication may affect vascular responses. In addition, bronchial carcinoma is known to induce chronic inflammation, which may alter pulmonary vascular responses. These points must be taken into account when interpreting the data. The animal tissue studies in this thesis were performed with their relation to the situation in man in mind. Thus it is beneficial to compare the responses in animal models with those in similar preparations obtained from human tissue when available. Lung tissue specimens were placed in cooled (4^{o}C), fresh Krebs solution on site and transferred rapidly to the laboratory and studied within 12 hours postoperatively.

2.1.4 Chronic hypoxic rat model of pulmonary hypertension (CHRPHT)

2.1.4.1 Introduction to the model

Several experimental techniques can be employed to induce experimental pulmonary hypertension including the rabbit coronary ligated model of left ventricular dysfunction and monocrotaline

induced pulmonary hypertension. The chronic hypoxic rat model of PHT is a well studied model of hypoxia induced PHT (Hislop & Reid, 1976; Rabonivitch, *et al.*, 1979). It is well established that rats exposed to a chronic, hypobaric hypoxic insult are known to exhibit well defined PHT with similar development of the morphological changes that are observed in the human pulmonary vascular bed in pulmonary hypertension (Hislop & Reid, 1976; Rabonitch, *et al.*, 1979; MacLean *et al.*, 1996). This model therefore, provides highly reproducible means for examining the evolution of the structural and functional changes associated with PHT in the pulmonary vascular bed.

Male Wistar rats 28 to 30 days old (at the start of the experiment) were placed in a specially designed perspex hypobaric chamber obtained from the Royal Hallamshire Hospital Sheffield. This chamber was depressurized very slowly at a rate of 30 mbar per hour, over 2 days, to 550 mbar. The oxygen concentration equivalent at this pressure is equivalent to about 10%. The temperature of the chamber was maintained at 21-22^{o}C, and the chamber was ventilated with room air at approximately 45 $lmin^{-1}$ to prevent accumulation of CO_2, NH_3, and water vapour. Rats were maintained in these hypoxic/hypobaric conditions for 14 days. The chamber was recompressed briefly every 3 days to clean the cages and add fresh food and water. Age-matched normoxic control animals were held in room air.

All animals received humane care in compliance with the Home Office (UK) "Guidance on the operation of the Animals (Scientific Procedures) Act" published by Her Majesty's Stationery Office.

2.1.4.2 Assessment of pulmonary hypertension

2.1.4.2.i Measurement of right ventricular hypertrophy (RVH)

Right ventricular wall thickening occurs with the development of both clinical and experimentally-induced forms of PHT. The degree of right ventricular hypertrophy is a well established index of the degree and development of pulmonary hypertension (Hunter *et al.*, 1974). CHRPHT rats from the hypobaric chamber and age-matched normoxic controls were overdosed with sodium pentobarbitone (100 $mgkg^{-1}$) intraperitonealy. The thoracic cage was carefully opened and the heart and lungs removed to ice-cold Krebs solution. Heart atria and associated large calibre blood vessels were dissected from the heart ventricular mass. The ventricular mass was then dissected very carefully in order to separate the free wall of the right ventricle from the left ventricle plus septum as fully described by Fulton *et al.* (1952). Once dissected, the ventricles were washed with fresh Krebs and blotted dry on tissue paper. The wet weights were then measured on a Mettler AT261 balance. The ratio of RV weight to left ventricular plus septum weight was used as an index of RVH. This index is a commonly used and reliable index of the degree of pulmonary hypertension present in experimental animals (Herget *et al.*,1978; Wanstall *et al.*, 1991; Sheedy *et al.*, 1996).

2.2 Functional *in vitro* pharmacological studies of isolated pulmonary arteries using tissue baths.

2.2.1 Tissue bath set up

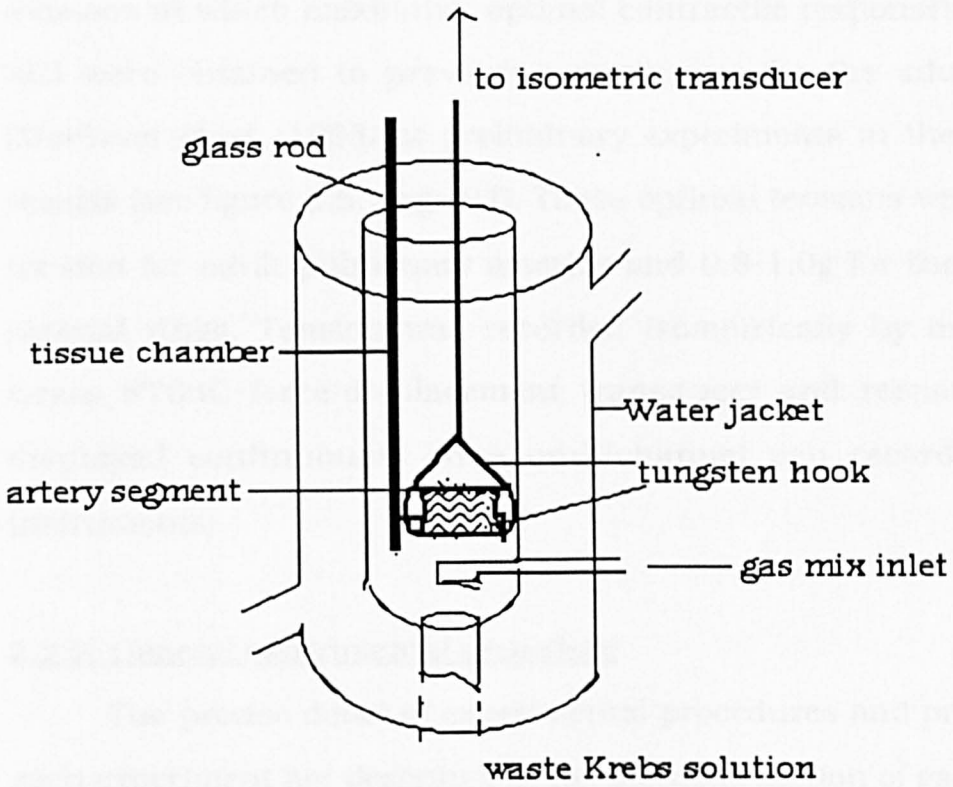

Figure 2.1 Tissue bath arrangement for investigating *in vitro* pharmacological functional properties of large (conduit) pulmonary arteries.

Figure 2.1 shows a typical tissue bath arrangement for the investigation of large pulmonary arteries. Extralobar and intralobar pulmonary arteries from adult and perinatal rabbits (internal diameters for each age group are described in the relevant results chapters of this thesis) were dissected free of surrounding parenchymal lung tissue (fully described in sections 2.3.4.1 and 2,3.4.2; except for the size and location of the vessels used, the technique for dissection was identical) and prepared as rings ~7 mm in length. The rings were placed over hooks made of tungsten wire and inserted over a second tungsten wire hook attached to a glass

holding rod and suspended in water-jacketed (37°C) tissue baths containing 5 ml of Krebs' solution. Preparations were gassed continuously with gas mixtures appropriate for adult and perinatal pulmonary arteries (see section 2.3.7.). Vessels were set up under tensions at which maximum, optimal contractile responses to 50mM KCl were obtained in previous experiments for the adult vessels (MacLean *et al.*, 1993) or preliminary experiments in the perinatal vessels (see figure 2.5, page 97). These optimal tensions were 1.5g wt tension for adult pulmonary arteries and 0.8-1.0g for the perinatal arterial rings. Tension was recorded isometrically by means of a Grass FT03C force-displacement transducer and responses were displayed continuously on a multichannel pen recorder (Gould Instruments).

2.2.2. General experimental procedure

The precise detail of experimental procedures and protocols for each experiment are described in the methods section of each chapter of experimental results. Each vessel investigated however was subjected to a general, core experimental procedure listed below:

(A) Each vessel, once mounted in the tissue bath and tensioned to an appropriate tension, was allowed to equilibrate for 45 - 60 minutes in Krebs solution bubbled with an appropriate gas mixture (see section 2.3.7) and maintained at 37°C. No drug was in contact with the vessel prior to the equilibration period.

(B) KCl at 50mM final bath concentration was used to stimulate the vessels to assess their viability. This concentration of KCl was chosen as that which has previously been reported as giving maximum, optimal contractile responses to KCl in rabbit pulmonary arteries (MacLean *et al.*, 1993). Once the contractile response attained a plateau, the vessels were washed 6-8 times with fresh

Krebs solution and left to return to baseline tension. This procedure with KCl was repeated in order to obtain a reference contractile response. 50mM KCl was used as a reference contraction in all adult and perinatal vessels examined in this thesis.

(C) The vessels were allowed another 30 - 40 minutes equilibration before proceeding with the addition of any drugs.

(D) The assessment of endothelial integrity is described in individual results chapters but invariably involved contraction with a submaximal concentration of a known vasoconstrictor followed by the addition of a known endothelium-dependent vasodilator such as acetylcholine once the contractile response had plateaued. Vessels were considered as having a functional endothelium if the endothelium-dependent vasodilator evoked a $\geq$ 70% reversal of the precontraction. No more than 10% of the vessels in any particular study had to be discarded when using this criteria.

(E) Where cumulative concentration response curves (CCRCs) were constructed to required agonists, the concentration range was such that each agonist achieved a maximum contractile response.

(F) For agonists which were known either to bind irreversibly to their receptor(s) or to produce marked desensitisation in certain tissues, only one CCRC could be constructed in each tissue. Separate vessels had to be used, therefore, to conduct experiments using inhibitors or antagonists.

Inhibitor and antagonist incubation periods are clearly stated in each results chapter.

2.3 Functional *in vitro* pharmacological studies of isolated pulmonary arteries using wire myography.

2.3.1 Background to wire myography

A technique initially described by Bevan and Osher (1972) for

the investigation of small blood vessels with internal diameters as small as 100μm was further developed in 1976 by Mulvany and Halpern who first described the use of the newly developed wire myograph. Until then, *in vitro* pharmacological examination of the functional properties of blood vessels was restricted to large diameter vessels. With the development of the new myographic technique, the *in vitro* pharmacology of blood vessels with internal diameters between 100-400 μm, i.e. those thought to make a major contribution to peripheral vascular resistance (Bohlen, 1986), could now be investigated. Initially wire myography naturally focused on the investigation of systemic resistance vessels, the technique can however, be applied to small veins (Jensen *et al.*, 1987), bronchi (Chopra *et al.*, 1994) and importantly in this thesis, pulmonary resistance vessels (Leach *et al.*, 1992).

2.3.2 Wire myograph equipment

Throughout the period of study, Mulvany/Halpern small vessel wire myographs (models 410 or 610M); Danish Myo-technology Ltd., Aarhus, Denmark) were used. These consist of 4 individual stainless steel organ chamber baths capable of having one vessel mounted in each bath. Vessels are mounted (see small vessel mounting procedure in section 2.3.5) by means of small, 40μm thick stainless steel wires which can be attached to metal vessel-supporting heads shown in figure 2.2. Each myograph chamber contains two vessel support heads, one is attached to a sensitive isometric force displacement transducer. The other support head is attached to an adjustable metal support arm which can be manipulated by a micrometer. This micrometer enables the sensitive adjustment of the distance between the two support heads to be carried out, allowing tension to be applied to the vessel located between the support heads.

Fine control of the myograph chamber temperature (to ± 0.1°C) is achieved by electronically controlled, internal heating units. Digital readout of force and temperature is displayed on the separate myograph control base-unit. This base-unit allows for output connections to a 4-channel, flatbed Linseis chart recorder (model no. 6514LB), from which a hard copy of force measurement from each myograph transducer can be obtained.

2.3.3 Myograph calibration

The myography equipment was calibrated on a regular basis every 4 weeks using a force calibration device consisting of a calibration 'pan' balance and standard 2g weight. The calibration balance consists of a horizontal metal arm containing a holding pan at each end with a thin pivotal vertical transducer arm located central to the horizontal arm. A 40μm stainless steel wire is securely attached to the force transducer-connected mounting head of the myograph chamber. The transducer arm is positioned carefully just behind the wire. When a given load W (e.g 2g weight) is placed on the appropriate holding pan, the force transducer is subjected to a force (F) equivalent to:

$F = W \times g \times \text{arm ratio}$

where, $g = 9.81\ mNg^{-1}$ and arm ratio = 0.5

Hence for calibration purposes using a 2g weight

$F = 2 \times 9.81 \times 0.5 = 9.81mN$

For calibration, the magnitude of deflection displayed on the pen recorder was equivalent to 1g weight or 9.81mN.

This provided a calibration factor (a), defined as the value of the force (mN) for a given deflection, and used both for calculating force as described in the normalisation procedure described in section 2.3.5. and for calculating the force as mg weight tension for a given

deflection. The absolute magnitude of functional responses in each experiment could then be calculated.

Each calibration resulted in negligible variation being observed in the readings.

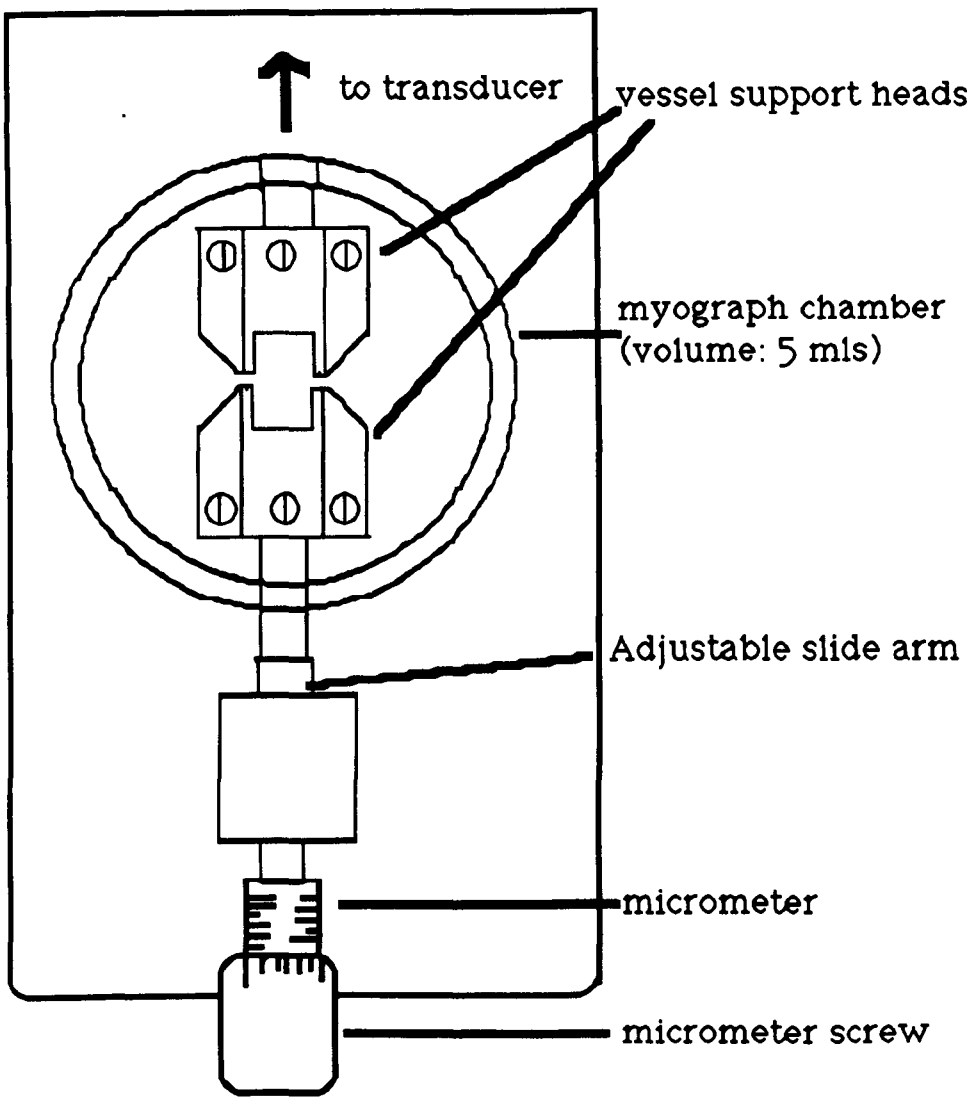

Figure 2.2 Illustration of Mulvany/Halpern wire myograph model 610M. Isometric tension is measure by an integral force transducer.

2.3.4 Small muscular pulmonary artery dissection procedure

2.3.4.1 Adult rabbit

Adult rabbit lung was obtained as described in section 2.1.1 and the right lung cut free, pinned to a dissecting dish with the parietal surface lying in an inferior position. Periodically, the tissue was washed with fresh, ice-cold Krebs solution during the dissection to prevent tissue drying. An incision was made along the superficial section of the airway, cutting in the direction from the large proximal airway, along the bronchial tree and through to the distal bronchiole. Using a dissection microscope (Zeiss),the pulmonary artery which branched parallel to the bronchus was identified. Bronchiole tissue was carefully dissected free to expose the pulmonary artery beneath. After gentle dissection away of the lung tissue lying lateral to the pulmonary artery, the distal portion of the artery (intralobar pulmonary artery **(B)** in figure 2.3), of size ~150-200μm internal diameter, was removed. These isolated arteries were placed in a vial of ice-cold Krebs solution, ready for mounting on the myograph.

2.3.4.2 Foetal and neonatal rabbit

A similar dissection technique employed for the dissection of adult small muscular pulmonary arteries was used to dissect out vessels from foetal and neonatal rabbit lungs. Slight modifications, however, were made as the lung lobe and associated pulmonary vascular tree are smaller than in the adult, The right lung was processed as for the adult lobe and using a dissecting microscope, an incision made along the superficial aspect of the airway. The first

branch of the main intrapulmonary artery (illustrated in figure 2.3 as **(A)**) was located beneath the bronchus parallel to it and lateral lung tissue associated with it was again carefully disssected free. Vessels smaller than this first branch pulmonary artery (~280-320μm internal diameter) could not be used due to their extreme fragility and subsequent significant damage upon dissection. Once dissected free, the isolated first branch intralobar pulmonary arteries were placed in ice cold Krebs in a glass vial in preparation for mounting.

2.3.4.3 CHPHT and control rats

A similar dissection technique employed for the dissection of adult rabbit small muscular pulmonary arteries was used to dissect out vessels from rat lungs. Slight modifications, however, were made as the lung lobe and associated pulmonary vascular tree are smaller than in the adult rabbit. The right lung was processed and vessels dissected out as for the adult rabbit lobe. Rat small intralobar pulmonary arteries (resistance arteries (PRAs) of internal diameter ~150-250 μm were used (vessels size **(B)** in figure 2.3.

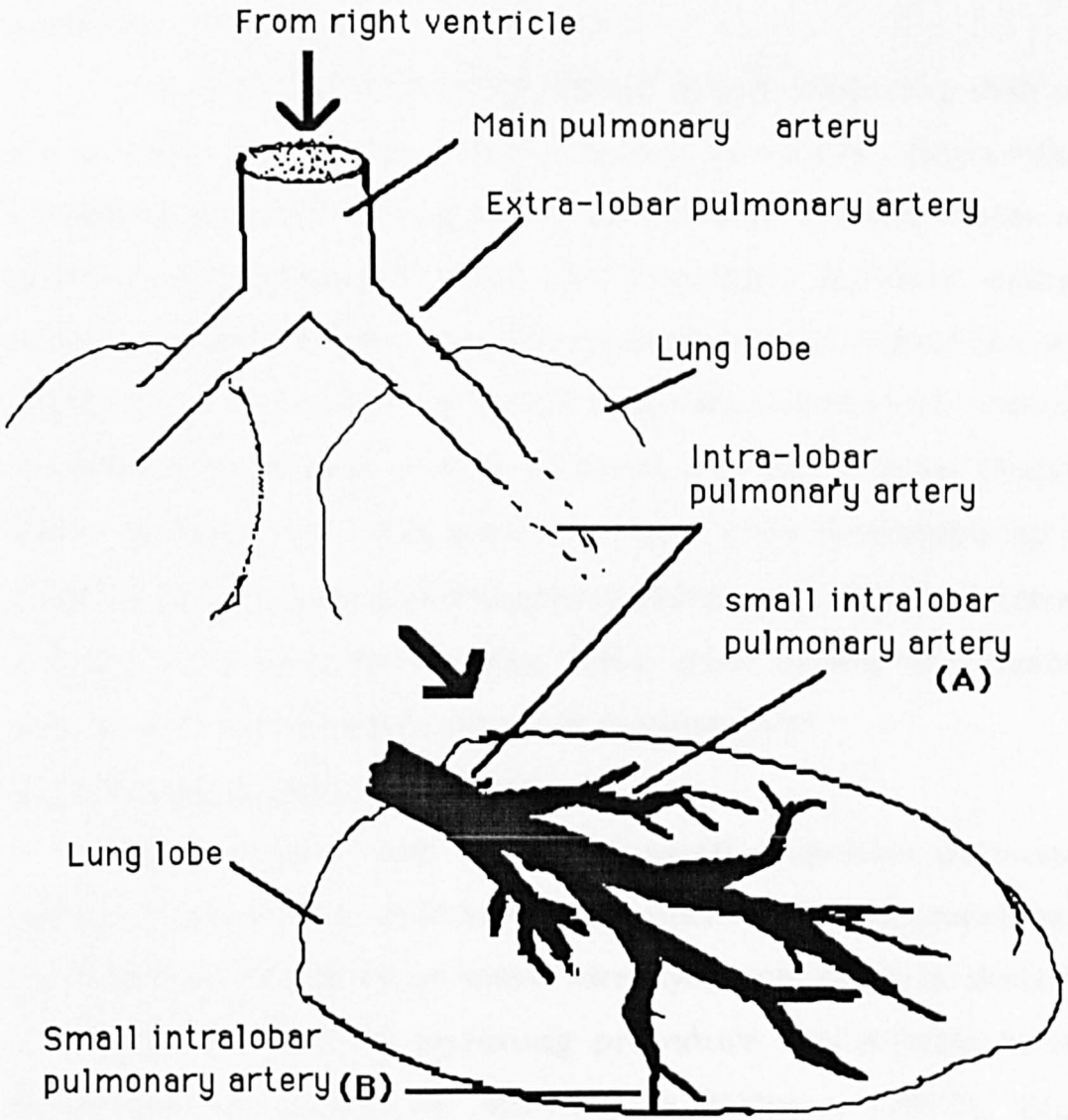

Figure 2.3 Diagram of dissection of pulmonary arteries. See text for details.

2.3.4.3 Human pulmonary arteries

Human lung samples obtained during the course of the study varied greatly in sample size and also in location of where in the lung samples were removed from during surgery. This variation was unavoidable due to the surgical requirements of the individuals from whom the samples were obtained. Great care was exercised however during the postoperative dissection of these samples in order to obtain pulmonary arteries of an appropriately similar size from each

individual lung sample.

The tissue samples were pinned into a dissecting dish and periodically washed with Krebs during dissection. Segments of intralobar large pulmonary artery were located in the samples and progressively dissected along the branching pathway using a dissecting microscope. The subsequently smaller branches were followed by dissecting away lateral lung tissue until small muscular pulmonary resistance arteries of about 200 μm internal diameter were uncovered and dissected out. These were identifiable by the close proximity of accompanying bronchioles. Once dissected free and cleaned of adherent surrounding tissue, these vessels were removed into ice cold Krebs for subsequent wire myography.

2.3.5 Vessel mounting procedure

Irrespective of the source of small muscular pulmonary arteries, these vessels underwent an identical mounting procedure on the Mulvany/Halpern small vessel wire myograph; which is described in section 2.3.2. This mounting procedure was similar to that described previously by Mulvany & Halpern (1977). Slight modifications to the procedure were employed as Mulvany & Halpern used small systemic arteries.

The mounting procedure for the pulmonary arteries is shown in figure 2.4. Small muscular pulmonary arteries isolated previously were placed in a pert dish containing cold Krebs solution. Using a pair of very fine forceps, a 40μm stainless steel wire was slowly inserted into and passed through the lumen of the vessel whilst simultaneously holding onto the end of the vessel with a second pair of fine forceps.

A) This was transferred to the wire myograph chamber containing Krebs solution and carefully secured between the vessel mounting heads by advancing the head attached to the micrometer until the

two heads touched and held the wire such that the vessel was positioned in the space between the mounting head jaws.

B) The wire was securely fixed at each end to the left mounting head by wrapping the ends clockwise around the top and bottom screws which were then tightened.

C) The mounting heads were then separated and any excess arterial segment located outside the jaw space was carefully cut away, this was necessary because any excess segment beyond the jaw would affect normalisation (described in section 2.3.6.). The distance between the jaws on each mounting head is 2mm, thus defining the vessel length.

D) Another 40μm wire was carefully inserted and passed through the vessel lumen.

E) The movable mounting head was again advanced until it touched the other mounting head and the second wire fixed and secured around the mounting head screws on the right vessel mounting head.

F) The mounting heads were separated slightly and the vessel allowed an appropriate equilibration period before an appropriate tension could be applied to the pulmonary artery. The Krebs was gassed with an appropriate mixture of gas (see section 2.3.7) once the vessels were mounted. The heating unit was engaged in order to heat to and maintain the Krebs solution at 37^{o}C.

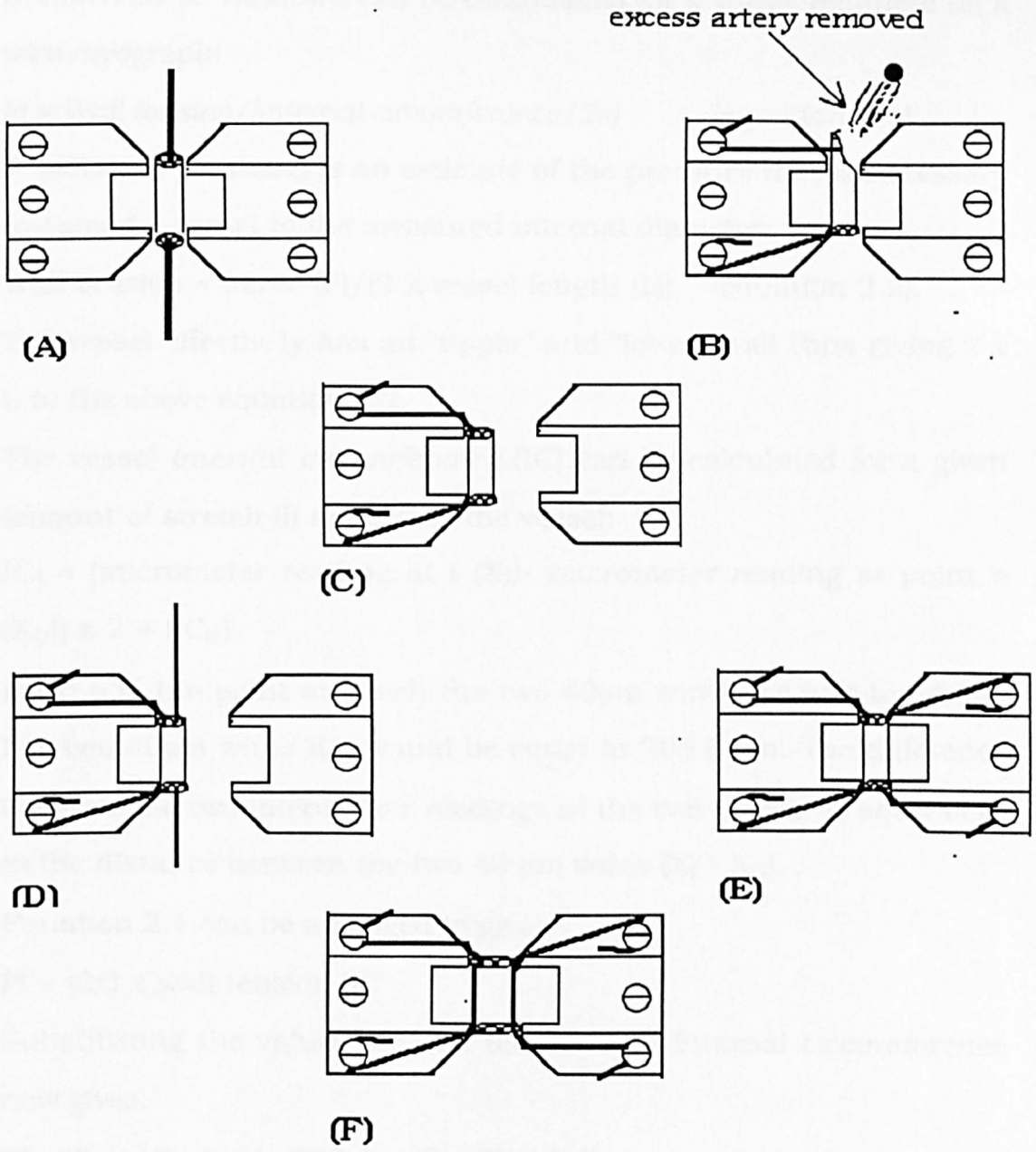

Figure 2.4 Procedure for mounting isolated pulmonary resistance arteries. Points **(A)** - **(F)** explained in body of preceding text.

2.3.6 Vessel normalisation procedure

In 1977, Mulvany & Halpern originally described a normalisation procedure for small systemic vessels. These vessels were stretched to a specified resting transmural pressure. Modifying the Laplace equation (equation 2.1), linking the wall tension in a cylinder with its radius and pressure within it, the relationship

between these variables can be established for a vessel mounted on a wire myograph:

Pi = Wall tension/(internal circumference/2π) (equation 2.1)

Pi (effective pressure) is an estimate of the pressure that is necessary to extend a vessel to the measured internal diameter.

Wall tension = Force (F)/(2 x vessel length (L)). (equation 2.2).

The vessel effectively has an "upper" and "lower" wall thus giving 2 x L in the above equation 2.2.

The vessel *internal circumference* (IC) can be calculated for a given amount of stretch (i) applied to the vessel:

IC_i = (micrometer reading at i (X_i)- micrometer reading at point o (X_o)) x 2 + (IC_o)

Point o is the point at which the two 40μm wires are just touching. For two 40μm wires IC_o would be equal to 205.6 μm. The difference between the two micrometer readings at the two points is equivalent to the distance between the two 40 μm wires (X_i - X_o).

Equation 2.1 can be arranged to give:

Pi = (2π) x wall tension/IC

Substituting the values for wall tension and internal circumference now gives:

$Pi = (2\pi) \times F / 2 \times L (205.6 + (2 \times (Xi - Xo))$

This relationship can be used to stretch a particular vessel to mimic a required transmural pressure equivalent.

2.3.6.1 Tension applied to pulmonary arteries

The majority of small systemic vessels are normalised to defined distending pressures (Mulvany & Halpern,1977). This procedure sets the arteries at a passive tension equivalent to 90% of their internal circumference (0.9 L_{100}) if they had been relaxed and perfused with a pressure of 100mmHg. Under these conditions the

equivalent pressure of the systemic vessel is in the range 60 - 70mmHg. The pulmonary circulation is a low pressure and low resistance circulation and therefore more appropriate conditions need to be applied which are more suitable for these pulmonary arteries. *In vivo*, pulmonary arteries would normally experience an equivalent transmural pressure of ~15 mmHg (Fishman, 1976). The isolated small muscular pulmonary arteries were therefore set at this equivalent transmural pressure of ~15 mmHg.

Note

In the chronic hypoxic rat model of pulmonary hypertension, tension on the pulmonary vessels was set to an equivalent transmural pressure of around 33 mmHg again to mimic the *in vivo* transmural pressures that these pulmonary hypertensive arteries would experience and this setting was previously used in earlier studies (MacLean, et al., 1996; MacLean and McCulloch, 1998).

2.3.7 Gas mixtures

The majority of *in vitro* pharmacological studies investigating isolated blood vessels use a "bubbling" gas mixture of 95% O_2, which is equivalent to a PO_2 of > 500 mmHg. In these studies, a gas mixture mimicking physiological conditions was chosen as the pulmonary vasculature is markedly sensitive to both O_2 and CO_2 concentrations. The percentage gas mixtures used for bubbling the Krebs solution surrounding the isolated pulmonary arteries consisted of: 16% O_2, 5% CO_2 and balance N_2. Bubbling the Krebs solutions in this way gave final bath gas tensions of pO_2 100-120 mmHg and pCO_2 35 mmHg at pH 7.4 (measurements taken by oxygen electrode and blood gas analyser), which mimic *in vivo* pO_2 values. Moreover, large, conduit pulmonary arteries are well supplied by the vasa-

vasorum despite receiving mixed arterio-venous blood. Hence the vessels are exposed to O_2 tensions of ~120mmHg. PRAs have walls < 1.5 μm thick, and, hence, tissue diffusional problems are not encountered with active bubbling. This gas mixture was used when studying the majority of vessels from the adult and neonatal rabbits, the human lung tissue samples and from the lungs of the CHRPHT and normoxic control rats, thus minimising variables between the different groups.

The gas mixtures used to bubble arteries isolated from the 2 day preterm foetal rabbits were 3% O_2, 5% CO_2, balance N_2. The measured gas tensions surrounding these vessels were pO_2 ~18-23 mmHg, CO_2 ~35-40 mmHg. This mimicked the hypoxic conditions experienced by the foetus *in utero*. This is a more appropriate O_2 level than that used in the other vessels as a major factor initiating the structural and functional changes in the pulmonary circulation at birth is the loss of the hypoxic pulmonary vasoconstrictor effect on first inhalation by the neonate from comparatively hyperoxic atmospheric air.

2.3.8 General experimental procedure

The precise detail of experimental procedures for each experiment are described in the methods section of each chapter of experimental results.

Each vessel undergoing wire myography however was subjected to a general, core experimental procedure listed below.

(A) Each vessel, once mounted in the myograph and tensioned to an appropriate equivalent transmural pressure was allowed to equilibrate for 45 - 60 minutes in Krebs solution bubbled with an appropriate gas mixture and maintained at 37°C. No drug was in contact with the vessel prior to the equilibration period.

(B) KCl at 50mM final bath concentration, giving optimum, maximal contractions to KCl, was used to stimulate the vessels to assess their viability. Once the contractile response attained a plateau, the vessels were washed 6-8 times with fresh Krebs solution and left to return to baseline tension. This procedure with KCl was repeated in order to obtain a reference contractile response.

(C) The vessels were allowed another 30 - 40 minutes equilibration before proceeding with the addition of any drugs.

(D) The assessment of endothelial integrity is described in individual results chapters but invariably involved contraction with a submaximal concentration of a known vasoconstrictor followed by the addition of a known endothelium-dependent vasodilator such as acetylcholine once the contractile response had plateaued. Vessels were considered as having a functional endothelium if the endothelium-dependent vasodilator evoked a $\geq$ 70% reversal of the precontraction. No more than 10% of the vessels in any particular study had to be discarded when using this criteria.

(E) Where cumulative concentration response curves (CCRCs) were constructed to required agonists, the concentration range was such that each agonist achieved a maximum contractile response.

(F) For agonists which were known to either bind irreversibly to their receptor(s) or to produce desensitisation in certain tissues, only one CCRC could be constructed in each tissue. Separate vessels had to be used, therefore, to conduct experiments using inhibitors or antagonists.

Inhibitor and antagonist incubation periods are clearly stated in each results chapter.

2.4 Data Analysis

2.4.1 Interpretation of results

For the *in vitro* pharmacological measurements of isometric tension using both the organ bath and the wire myograph, data for preparations subject to identical protocols were grouped and expressed as the mean value ± the standard error of the mean (s.e.m.) In contractile studies, functional data are expressed as a percentage of an agonists own maximum response in each preparation (% own maximum) for a particular agonist. This provides an indication of, as well as any changes in, the sensitivity to the agonist of a particular vessel. In order to standardise agonist responses, data are also expressed as a percentage of a reference contraction to a second exposure to 50mM KCl (% 50mM KCl). This also provides an indication of, as well as any changes in, the maximum contractile response of an agonist in a particular vessel. Some results are expressed as an absolute contractile response (mN tension or mg weight tension). For responses in relaxation studies, data are expressed as a percentage of the response relative to agonist-induced pre-contraction in each vessel.

2.4.2 Agonist potency

For contractile responses, as a measurement of agonist potency in pulmonary arterial preparations pEC_{50} values are given; pEC_{50} being the -log of the concentration of an agonist that evokes 50% of the maximum possible contractile effect of that particular agonist. For the comparison of potency at low concentrations of agonists, values for the pEC_{10} and pEC_{25} were also calculated being the -log of the concentration of an agonist that evokes 10 % or 25% respectively of the maximum possible effect of that particular agonist. For vasodilator responses, as a measurement of agonist potency in pulmonary arterial preparations pIC_{50} values are given; pIC_{50} being the -log of the concentration of an agonist that evokes

50% of the maximum possible vasodilator effect of that particular agonist. These *p*EC and *p*IC values were calculated by computer interpolation from individual CCRCs (% agonists own maximum response) for each vessel. *p*EC and *p*IC values presented in the tables throughout this thesis are drawn from individual response curves expressed as % own maximum response for a particular agonist.

2.4.3 Determination of antagonist potency

To appraise the effects of antagonists, pK_B values were calculated according to the methods described by Arunlakshana and Schild (1959), giving an estimation of antagonist affinity. In preliminary experiments, 5-HT caused significant desensitization in the small pulmonary arteries investigated using wire myography in the studies. This effect violates classical Schild conditions, and in order to avoid this pK_B values were determined by constructing CCRCs to agonists obtained in separate arterial segments either in the presence or absence of a particular antagonist. Owing to either the large number of study groups, large number of antagonist-agonist combinations employed or to limited availability of tissue and equipment, allocation of only one or two arterial segments from each tissue to the determination of each estimate of antagonist affinity. Therefore pK_B values were determined instead of pA_2 values, which requires analysis of the effects of at least three concentrations of antagonist using a Schild plot calculation. Where this was possible, this was clearly stated in the appropriate results chapter(s) in this thesis.

For a particular antagonist, the pK_B value is the - log of the dissociation equilibrium constant (K_B) for that antagonist. This constant is the molar concentration of ligand needed to occupy 50% of the receptor pool.

For the experiments, an estimate of the pK_B value was calculated using the following equation:

pK_B = log (DR-1)-log[B]; where DR is the ratio of the EC_{50} value in the presence of antagonist to the EC_{50} value in the absence of antagonist for a particular agonist (Choppin and O'Conner, 1995).

Several important assumptions have to be made and taken into account when calculating pK_B values using this direct equation. The equation assumes that the antagonist behaves in a competitive manner by interacting with the agonist for unoccupied receptors in a simple, reversible manner and a maximum response to the agonist is achieved in the concentration range studied.

2.4.4 Statistical analysis methods

Comparisons between two groups of data were made using Students *t*-test. Unless otherwise stated, the nature of the experiments were such that an un-paired t-test was used. For comparisons between three or more groups of data, one way analysis of variance (ANOVA) followed by an appropriate ad hoc post test was the statistical method used to analyse significant differences between groups. $P< 0.05$ was considered to be statistically significant. The statistical software package "InStat P203" for MacIntosh computers was used.

2.5 Solutions

All solutions, unless stated otherwise, were stored at 4^oC and made up fresh each day.

2.5.1 *In vitro* pharmacological studies

Modified Krebs-Heinslet solution was composed of the following: NaCl 118.4mM, $NaHCO_3$ 25 mM, KCl 4.7mM, KH_2PO_4

1.2mM, $MgSO_4$ 0.6mM, $CaCl_2$ 2.5mM, glucose 11.0mM.

2.6 Drugs and chemical reagents

The following is an alphabetical list of the drugs and chemical reagents used in the experiments presented in this thesis:

Drug/Reagent	Supplier
4-Aminopyridine	Sigma
5-Carboxamidotryptamine (5-CT)	Semat
5-Hydroxytryptamine creatine sulphate (5-HT)	Sigma
8 OH-DPAT (8-hydroxy-2- (di-n-propylamino) tetralin)	Semat
α-methyl-5-hydroxytryptamine	Sigma
A23187 (calcimycin)	Sigma
Acetylcholine	Sigma-Aldrich
BRL-15572 (1-(3-cholrophenyl)-4-[3,3-diphenyl(2-(S,R) hydroxy-propanyl) piperazine] hydrochloride	Smith Kline-Beecham
BW 723C86 (1-[5-(2-thenyloxy)-1H-indol-3-yl]propan-2-amine)	Sigma
Endothelin-1 (ET-1)	Thistle Peptides
GR55562 (3-[3-(dimethylamino) propyl]-4-hydroxy-N-[4(4pyridinyl)-phenyl] benzamide	
Ketanserin Tartrate	Roche
Methiothepin	Semat

Neuropeptide Y (NP-Y)	Tocris-Cookson
N^{ω}-nitro-L-arginine methylester (L-NAME)	Sigma
Phenylephrine	Sigma
Pimozide	Semat
Risperidone	Semat
SB-224289 (2,3,6,7-tetrahydro-1'-methyl-5-[2'methyl-4'(5-methyl-1,2,4-oxadiazol-3-yl) (biphenyl-4-carbonyl]furo [2,3,f] indol-3-spiro-4'-piperidine hydrochloride	Smith Kline-Beecham
Spiperone	Semat
Sumatriptan (GR 43175)	Glaxo-Wellcome

Stock solutions of SB-224289 and BRL15572 were prepared in 1.0% DMSO. All other drugs and all subsequent dilutions were prepared in distilled water.

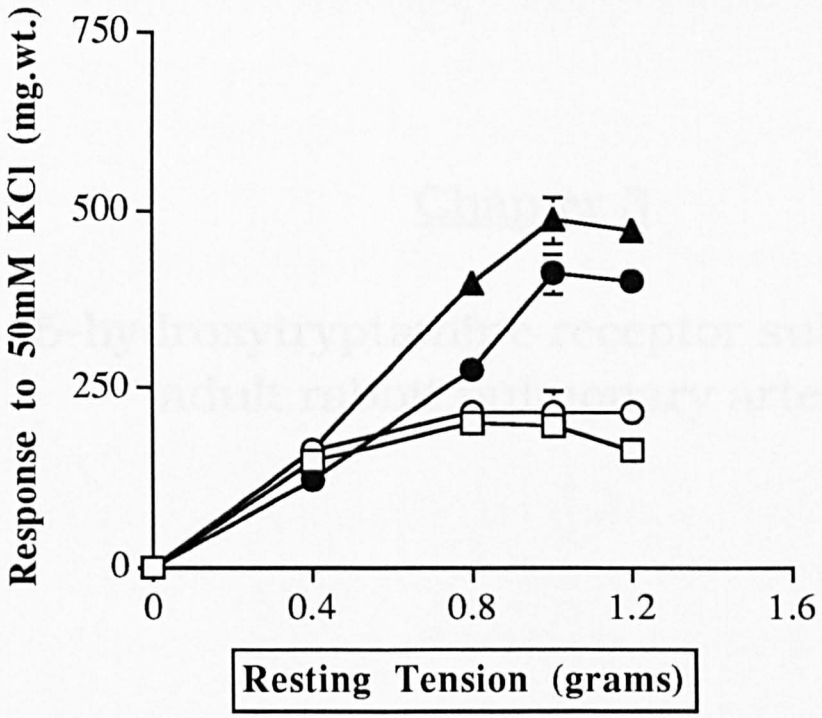

Figure 2.5 Preliminary experiments to show how the resting tension applied to Foetal (□), 0-24 hour (○), 4 day old (●) and 7 day old (▲) rabbit branch pulmonary arteries affects the size of the response to 50mM KCl. Data are expressed in mg.wt. Values are given as mean ± s.e.m. (n = 5 for each age group).

Chapter 3

5-hydroxytryptamine receptor subtypes in adult rabbit pulmonary arteries

3.1 Introduction

The structural changes which take place in the pulmonary arteries due to pulmonary hypertension have been intensively studied and several factors have been implicated in this condition, such as the potent vasoconstrictor peptide endothelin-1 (Kiowski, *et al.*, 1995). Recently, several important clinical and experimental studies have suggested a role for 5-hydroxytryptamine (5-HT) in the aetiology of pulmonary hypertension. 5-HT is released by activated platelets and pulmonary neuroendocrine cells and has been implicated in PHT in the presence of pulmonary thromboemboli (Comroe, *et al.*, 1953).

Elevated plasma levels of 5-HT due to platelet release have been reported in primary PHT (Herve *et al*; 1995) and platelet release of 5-HT is thought to be involved in PHT secondary to cardiac surgery (Johnson & Giorgieff, 1989). Isolated pulmonary arteries from PHT patients undergoing transplantation exhibit exaggerated vasoconstrictor responses to 5-HT (Brink *et al*, 1988). In the rat monocrotoline-induced PHT model, responses to 5-HT are significantly potentiated (Wanstall & O'Donnell, 1990).

Pulmonary neuroendocrine cells have been implicated as the source of increased 5-HT production, resulting in acute PHT postoperatively in children with heart defects (Breuer *et al.*, 1996; Schindler *et al.*, 1995). 5-HT has also been linked to PHT associated with hypoxia in the newborn infant (Johnson & Georgieff; 1989). Hence, the 5-HT receptors mediating pulmonary vasoconstriction in the perinatal period are of interest and the studies in chapter 5, extensively examined and discussed the role of 5-HT receptor mediated responses in the pulmonary vasculature of the perinatal rabbit.

5-HT interacts with a multiplicity of receptors causing either

vasoconstriction or vasodilation depending on receptor subtype, species, pulmonary vascular location and degree of underlying vascular tone (Hoyer *et al.*, 1994). For example, 5-HT causes vasoconstriction in isolated bovine intrapulmonary arteries via a 5-$HT_{1D/1B}$ receptor under conditions of raised vascular tone (MacLean *et al*, 1994) but induces endothelium-dependent relaxation in isolated porcine pulmonary arteries via the '5-HT_{1C}' receptor (Glusa & Richter, 1993). The 5-HT_{1C} receptor has subsequently been renamed the 5-HT_{2C} receptor (Hoyer *et al.*, 1994).

In human large pulmonary arteries, 5-HT-mediated vasoconstriction is via stimulation of 5-$HT_{1D/1B}$ receptors (MacLean *et al*, 1996b) and in chapter 4 the importance of the 5-HT_{1B} receptor in mediating 5-HT-receptor-induced vasoconstriction in the pulmonary small muscular resistance arteries in man is discussed. In other vascular beds, 5-HT can induce endothelium-dependent vasodilation via the 5-HT_{2C} receptor (Bodelson *et al.*, 1993), the 5-HT_{2B} receptor (Glusa & Roos, 1996; Ellis *et al.*, 1995) and the 5-$HT_{1D/1B}$ receptor (Gupta, 1992). There is also evidence that 5-HT can mediate vasodilation directly through 5-HT_4 and 5-HT_7 receptors located on vascular smooth muscle (Cocks & Arnold, 1992; Leung *et al.*, 1996; Terron, 1996). Hence, in this study, 5-HT receptor mediated responses in isolated rabbit pulmonary arteries were studied, both in PCAs and PRAs in adult rabbits. This gives a more complete picture of 5-HT receptor mediated responses along the pulmonary vascular tree as the vascular smooth muscle cells from these two regions are phenotypically distinct (Frid *et al.*, 1997). The investigations in the adult rabbit would also provide a basis for comparison with the studies in chapter 5 in the perinatal rabbit

isolated pulmonary arteries. The selective agonists and antagonists used in this study are described in chapter 1, section 1.4.3.

3.2 Methods

Rabbit large pulmonary arteries

New Zealand White adult rabbits were studied. They were killed by sodium pentobarbitone overdose (200mgkg^{-1}) and the lungs removed and placed in cold Krebs. First pulmonary artery extralobar branches were carefully dissected out as described in section 2.3.4.1. Arterial rings were mounted between two stainless steel hooks for isometric tension recording in 5 ml tissue baths and bathed in Krebs-buffer solution (pH 7.4) (composition (mM): NaCl 118.4, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 0.6, $CaCl_2$ 2.5, glucose 11.0, and EDTA 23.0) at 37°C Vessels were bubbled with 16%O_2/5%CO_2 balance N_2 to give values similar to those found *in vivo* given that these vessels are well supplied by the vasa vasorum. Initial tension was set to 1.5g, being that which consistently gives optimum contraction to a reference 50mM KCl challenge in these vessels (MacLean *et al.*, 1993). The endothelium was removed from some PCAs by carefully, but thoroughly, rubbing a pair of blunt forceps around the luminal surface of the vessel whilst placed on tissue soaked in Krebs solution.

Rabbit pulmonary resistance arteries

Adult male New Zealand White rabbits were overdosed with sodium pentobarbitone and the lungs removed and placed in ice-cold Krebs. Under a dissecting microscope, intralobar small pulmonary resistance arteries (~250 - 300μm internal diameter) from the right lung were carefully dissected out and mounted as ring preparations

in isometric wire myographs as described in section 2.3.5 and bathed in Krebs solution at 37°C. The vessels were bubbled with 16%O_2/5%CO_2 balance N_2 to give values similar to those found *in vivo*. Using the normalisation process explained in section 2.3.6., tension was then applied to all vessels to give a transmural pressure equivalent of approximately 15 mmHg, similar to *in vivo* pressures of pulmonary arterioles.

Experimental protocol

Large pulmonary arteries

Vessels were allowed to equilibrate for 45-60 minutes prior to the addition of any drugs. The response to 50mM KCl was determined twice; once the contraction to KCl had reached a plateau, the vessels were washed out at least 8 times with fresh Krebs solution and allowed to equilibrate for a further 45 minutes. The presence of a functional endothelium was assessed with acetylcholine (ACh; 1μM) in vessels precontracted with 0.1μM phenylephrine. The vessels were then subjected to one of the following experiments: In order to evaluate the 5-HT receptor(s) involved in the pulmonary vasoconstrictor activity in the large pulmonary arteries, CCRCs were constructed to the 5-HT agonists 5-carboxamidotryptamine (5-CT, 1nM-0.1mM; 5-HT_1 receptor agonist), α-methyl-5-HT (5-HT_2-selective agonist, 1nM-0.1mM) 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT, 5-HT_{1A} agonist) and the 5-$HT_{1B/1D}$ receptor agonist, sumatriptan. The antagonists used were ketanserin (selective for 5-HT_{2A} receptors), methiothepin (non-selective antagonist at 5-HT_1 and 5-HT_{2A} receptors and GR55562 (3-[3-(dimethylamino)propyl]-4-hydroxy-N-[4(4pyridinyl)phenyl] benzamide) (selective 5-$HT_{1B/1D}$

antagonist). In additional experiments, some antagonist studies were performed in the presence of 0.1mM N^{ω}-nitro-L-arginine methylester (L-NAME). This concentration of L-NAME was chosen to ensure complete inhibition of endothelial NOS activity. 100μM L-NAME completely inhibits endothelial cytosol cyclic GMP formation, a measure of NOS activity, in rat aorta (Rees *et al.*, 1990). A 10-fold lower concentration of L-NAME completely abolished ACh-induced relaxation of rat isolated aorta in the same study (Rees *et al.*, 1990). Moreover, Steeds *et al* (1997) recently reported that 100μM L-NAME abolished ACh-induced relaxation as well as basal-released NO in rat isolated PRAs.

A second protocol examined the 5-HT receptor(s) mediating vasodilation in the adult rabbit large pulmonary arteries and these experiments were conducted in the presence of 0.1μM ketanserin to exclude any 5-HT_{2A}-receptor mediated effects. Vessels were contracted with phenylephrine (PE; 0.1μM) as it reliably induces a maintained contraction in these arterial segments (MacLean *et al.*, 1993). Vessels were pre-contracted to give a contraction approximately half the maximal contractile response to this PE as preliminary studies indicated that contraction above this level actually decreased the sensitivity of vasodilators including 5-CT. I studied vasodilation in responses to 5-CT, 5-HT, sumatriptan and BW 723C86 (selective 5-HT_{2B} agonist). For antagonist studies, CCRCs to 5-CT was constructed in each vessel either in the presence or absence of one of the following antagonists; GR55562 (1μM), spiperone (1μM,5-HT_7 receptor antagonist), pimozide (30nM, 5-HT_7 receptor antagonist) or risperidone (3nM, 5-HT_7 receptor antagonist). Antagonists were allowed a 45 minute equilibrium period prior to

constructing the CCRCs to 5-CT. All CCRCs were in the concentration range 0.1nM-0.1mM.

Pulmonary resistance arteries

Following a 45 minute equilibration period, the response to 50mM KCl was determined, followed by wash-out and further equilibration. 50mM is the concentration at which KCl produces an maximum contractile response in adult rabbit PRAs (Docherty, 1997). This concentration was subsequently used to constrict vessels at the beginning of each protocol for all functional studies carried out in this thesis. The magnitude of the response to the second application of 50mM KCl was used as a reference contraction to compare subsequent vasoconstrictor responses to other agents, in the same preparation. In preliminary experiments, some PRAs were preconstricted with 1μM 5-HT followed by CCRC to sodium nitroprusside (SNP; 1nM-0.1mM) in order to assess the ability of the adult PRAs to relax to endothelium-independent vasodilator agents. The vessels were subjected to one of the following experiments.

Cumulative concentration-response curves (CCRCs) to 5-HT (1nM-0.1mM) were constructed either in the presence or absence of 100μM L-NAME.

In order to evaluate the 5-HT receptor(s) involved in the pulmonary vasoconstrictor activity in the PRAs, CCRCs were also constructed to the 5-HT agonists 5-carboxamidotryptamine (1nM-0.1mM), α-methyl-5-HT (1nM-0.1mM) and sumatriptan (1nM-0.1mM). Previous experience dictates that attempts at mechanical removal of the vascular endothelium in these arteries damages the thin and fragile underlying smooth muscle. Antagonist experiments

were, therefore, also carried out in the presence of 100μM L-NAME to prevent any inhibitory role of NO release on constrictor responses as the initial findings described in this study showed that NO from the endothelium markedly affected the sensitivity, as indicated by the pEC_{50} value, to 5-HT-induced vasoconstriction in these PRAs. CCRCs to 5-HT are not reproducible in each tissue as desensitisation often occurs with a second CCRC, therefore, only one CCRC to 5-HT was constructed either in the absence or presence of an antagonist in each vessel. The antagonists used were ketanserin (selective for 5-HT_{2A} receptors) and GR55562 (3-[3-(dimethylamino)propyl]-4-hydroxy-N-[4-(4-pyridinyl)phenyl] benzamide) (selective 5-HT1B/1D antagonist). CCRCs to 5-CT were also constructed in the presence of SB224289 (selective 5-HT_{1B} receptor antagonist; 200nM) and BRL15572 (selective 5-HT_{1D} antagonist; 500nM). All antagonists studies were also carried out in the presence of L-NAME (100μM). All antagonist concentrations which were studied, were chosen due to their affinity values in other *in vitro* vascular preparations. In all antagonist studies, antagonists were allowed a 45 minute equilibration period before the construction of any CCRCs.

Data analysis

Contractile responses are expressed as a percentage of the contraction to 50mM KCl determined at the start of the experiment in each preparation. The results are shown as the mean ± s.e.mean. Dilator responses are expressed as the % reduction of PE-induced tone. pEC_{50} and pIC_{50} values were calculated according to methods stated in chapter 2. Statistical comparison of the means of groups of data were made by Student's unpaired t-test or one way analysis of variance where appropriate. $P < 0.05$ was considered statistically

significant. n = number of vessels/number of animals. In antagonist studies in the large pulmonary arteries, the antagonist potencies of ketanserin against 5-HT were calculated according to Arunlakshana and Schild (1959) using EC_{50} values obtained in the presence and absence of ketanserin to give pA_2 values. The slope of the Schild regression was also determined. In the other antagonist studies, in particular for PRAs, estimated pK_B values calculated assuming the antagonists behaves in a competitive manner and a maximum response to the agonist is achieved in the concentration range studied. Apparent pK_B values were calculated for a single stated concentration of antagonist according to the methods described in chapter 2.

3.3 Results

Rabbit Large (conduit) pulmonary arteries (PCAs)

The absolute magnitude of the contractile response to 50mM KCl in these vessels was 2275 ± 175 mg wt tension (n > 15).

Assessment of endothelial integrity with ACh

ACh (1μM) caused a relaxation of phenylephrine-precontracted PCAs of 64 ± 5%(n = 24). In endothelium-denuded vessels, ACh either had no effect or caused a slight contraction.

Response to 5-HT receptor agonists

Figure 3.1 demonstrates CCRCs to 5-HT, α–methyl 5-HT, 5-CT, 8-OH-DPAT and sumatriptan in rabbit PCAs. All agonists caused concentration-dependent contractions in these vessels. The following rank order of potency was obtained, based on the *p*EC_{50} values for each agonist as summarised in table 3.1. 5-HT = αmethyl5-HT > 5-CT

> 8-OH-DPAT > sumatriptan. The maximum contractile response to α–methyl-5-HT was significantly greater than 5-HT ($P<0.05$). Maximum contractile responses to 5-CT and 8-OH-DPAT were not significantly different to 5HT. The maximum contractile response to sumatriptan was not reached within the concentration range studied. The potency of the 5-HT_2 receptor agonist α–methyl-5-HT strongly indicated the presence of vasoconstrictor 5-HT_2 receptors in this tissue.

Agonist	Maximum (% KCl)	pEC_{50}	n
5-HT	114 ± 8	7.01 ± 0.07	36/36
5-CT	112 ± 10	5.91 ± 0.08***	12/12
8-OH-DPAT	95 ± 14	5.32 ± 0.15***	11/11
α-me5-HT	146 ± 10*	6.88 ± 0.08	12/12
sumatriptan	71 ± 21$^{\alpha}$	nc	12/12

Table3.1 Characteristics of contractile response to 5-HT agonists in adult rabbit large pulmonary arteries. Statistical comparisons were made by ANOVA, agonist vs. 5-HT, *$P<0.05$, ***$P<0.001$. Data are expressed as arithmetic mean ± s.e.mean. Maximum represents maximum contractile response expressed as percentage response to reference 50mM KCl contraction. n/n, number of ring preparations/number of animals.
Note α represents contractile response at maximum sumatriptan concentration; nc, not calculated.

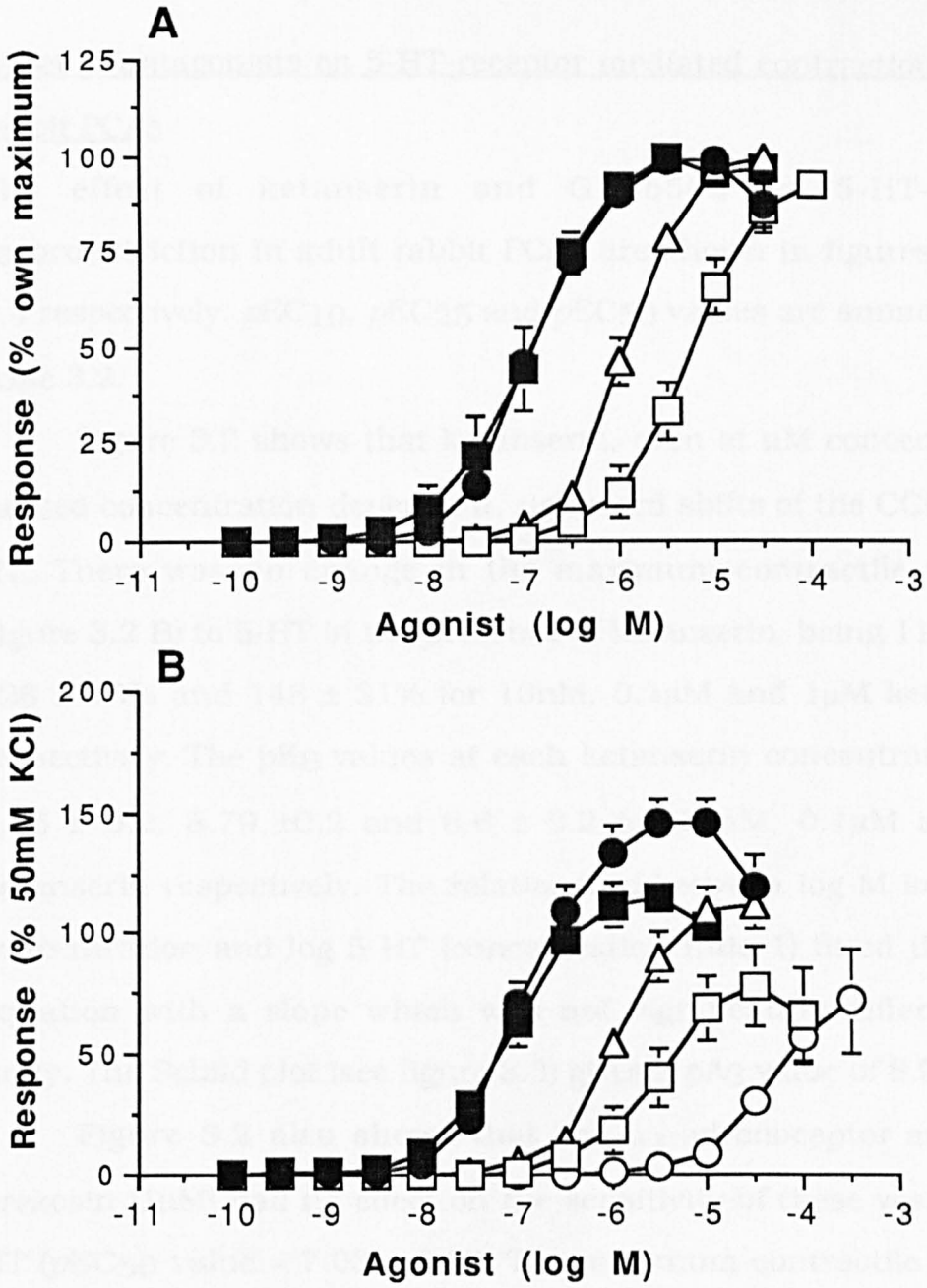

Figure 3.1 Responses to 5-HT receptor agonists in PCAs from adult rabbits. CCRCs to 5-HT (□, n/n = 12/12), α-methyl 5-HT (●, n/n = 12/12), 5-CT (Δ, n/n = 12/12), 8-OH-DPAT (□, n/n = 11/11) and sumatriptan (O, n/n = 12/12). A. Data are expressed as a percentage of their own maximum. B Data are expressed as a percentage reference contraction to 50mM. Each point represents mean ± s.e.mean.

Effect of antagonists on 5-HT-receptor mediated contraction of adult rabbit PCAs

The effect of ketanserin and GR55562 on 5-HT-induced vasoconstriction in adult rabbit PCAs are shown in figures 3.2 and 3.4 respectively. pEC_{10}, pEC_{25} and pEC_{50} values are summarised in table 3.2.

Figure 3.2 shows that ketanserin, even at nM concentrations, caused concentration-dependent, rightward shifts of the CCRCs to 5-HT. There was no change in the maximum contractile response (figure 3.2 B) to 5-HT in the presence of ketanserin, being 116 ± 23%, 126 ± 16% and 148 ± 31% for 10nM, 0.1μM and 1μM ketanserin, respectively. The pK_B values at each ketanserin concentration were 8.95 ± 0.2, 8.79 ±0.2 and 8.6 ± 0.2 for 10nM, 0.1μM and 1μM ketanserin respectively. The relationship between log M ketanserin concentration and log 5-HT (concentration ratio-1) fitted the Schild equation with a slope which was not significantly different from unity. The Schild plot (see figure 3.3) gives a pA_2 value of 8.9.

Figure 3.2 also shows that the α_1-adrenoceptor antagonist prazosin (1μM) had no effect on the sensitivity of these vessels to 5-HT (pEC_{50} value = 7.03 ± 0.2)). The maximum contractile response to 5-HT in the presence of prazosin was 121 ± 20% of the reference contraction to 50mM KCl. This value was not significantly different to the control 5-HT maximum contraction.

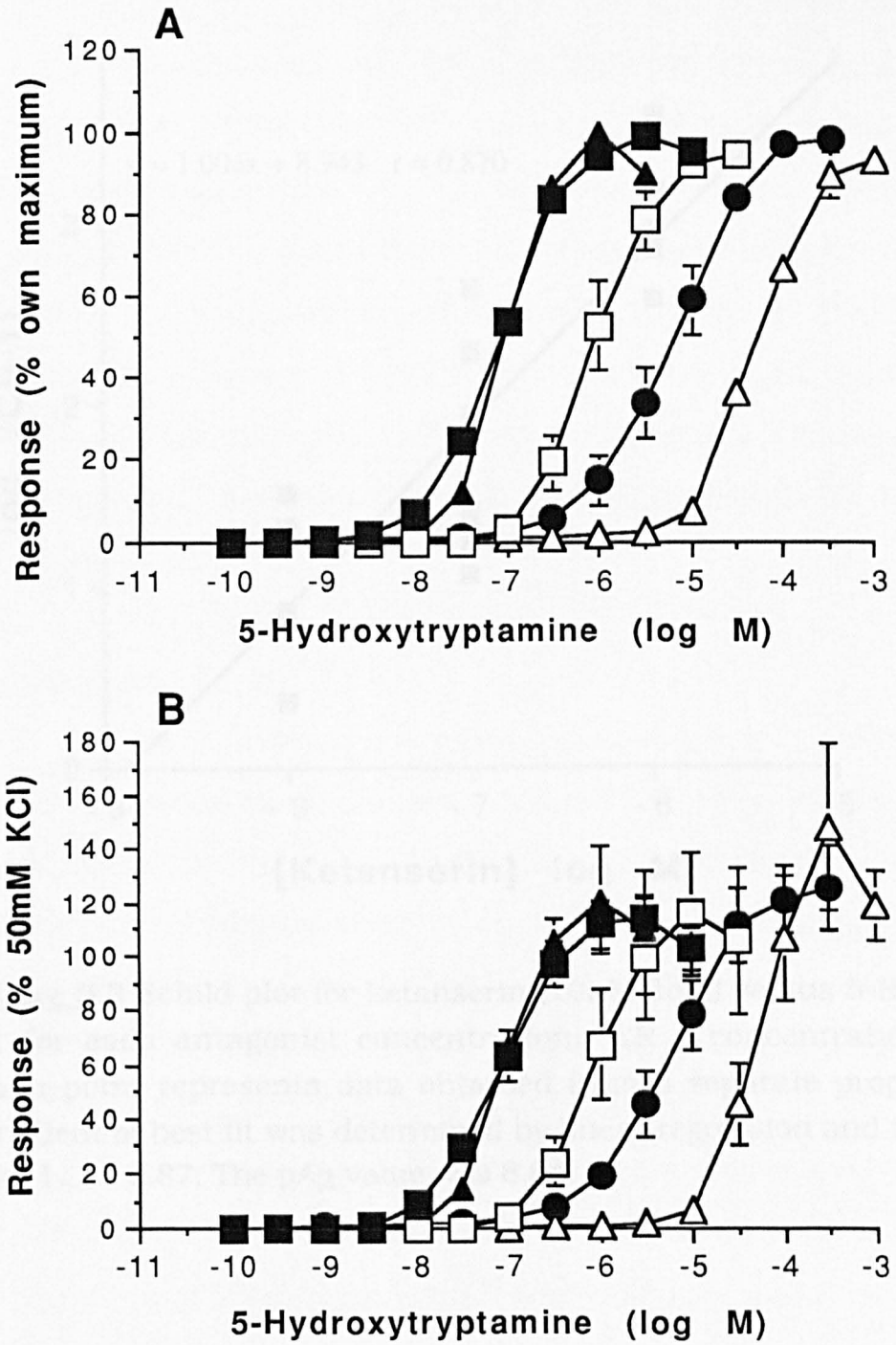

Figure 3.2 Effect of ketanserin and prazosin on 5-HT-induced contraction in PCAs from adult rabbits. CCRCs to 5-HT (■, n = 24/11), and in the presence of 10nM (□, n= 7/7), 0.1μM (●, n=11/11) and 1.0μM (△, n= 6/6) ketanserin; in the presence of 1.0μM prazosin (▲, n = 5/5). **A**. Data are expressed as a percentage of their own maximum. **B** Data are expressed as a percentage of the reference contraction to 50mM KCl. Each point represents mean ± s.e.mean.

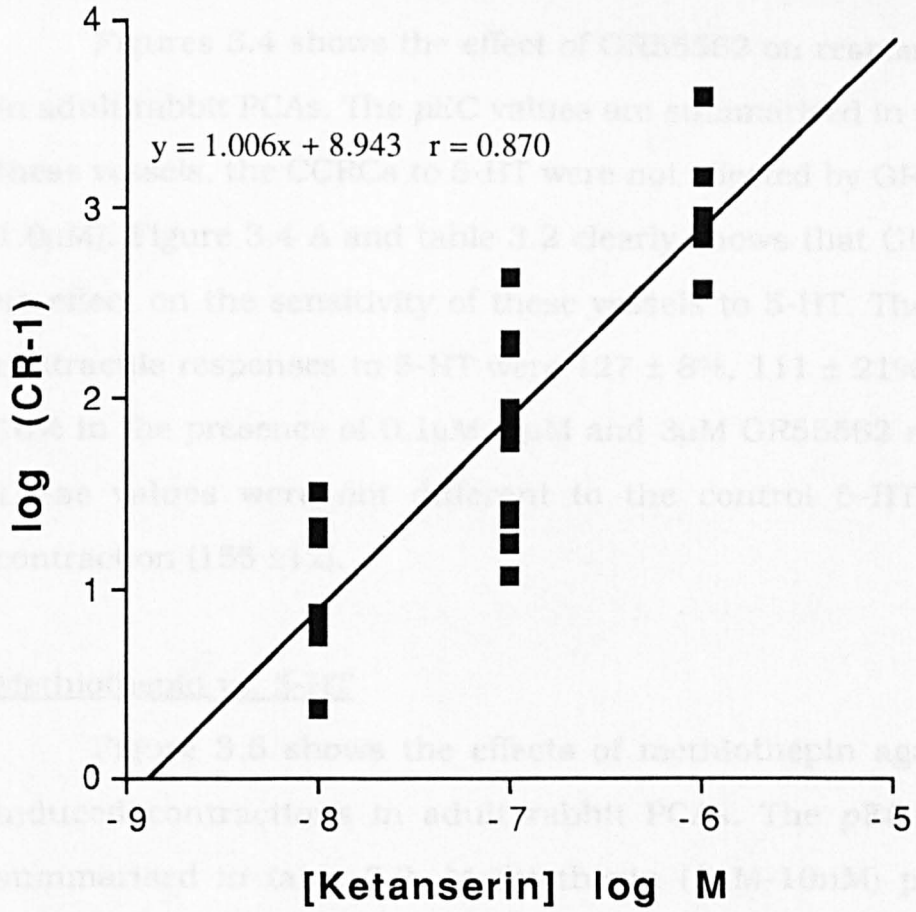

Figure 3.3 Schild plot for ketanserin (10nM-1μM) versus 5-HT (n= 6-11 for each antagonist concentration). CR = concentration ratio. Each point represents data obtained from a separate preparation. Gradient of best fit was determined by linear regression and the slope was 1., r= 0.87. The pA_2 value was 8.94.

GR55562 vs 5-HT

Figures 3.4 shows the effect of GR55562 on responses to 5-HT in adult rabbit PCAs. The *p*EC values are summarised in table 3.2. In these vessels, the CCRCs to 5-HT were not affected by GR55562 (0.1-1.0μM). Figure 3.4 A and table 3.2 clearly shows that GR55562 had no effect on the sensitivity of these vessels to 5-HT. The maximum contractile responses to 5-HT were 127 ± 8%, 111 ± 21% and 129 ± 10% in the presence of 0.1μM, 1μM and 3μM GR55562 respectively. These values were not different to the control 5-HT maximum contraction (155 ±12).

Methiothepin vs. 5-HT

Figure 3.5 shows the effects of methiothepin against 5-HT-induced contractions in adult rabbit PCAs. The *p*EC values are summarised in table 3.2. Methiothepin (1nM-10nM) produced a significant rightward shift of the concentration response curve. Methiothepin at 10nM did not significantly further decrease the sensitivity to 5-HT than 3nM methiothepin. A decrease in the maximum contractile effect of 5-HT was observed at methiothepin concentrations of 3nM (maximum contractile response = 77± 10%, n = 6/6; $P<0.05$ cf. control (maximum contractile response = 123 ± 8, n = 10/10); and 10nM (maximum contractile response = 37 ± 16, n = 6/6; $P<0.01$ cf. control).

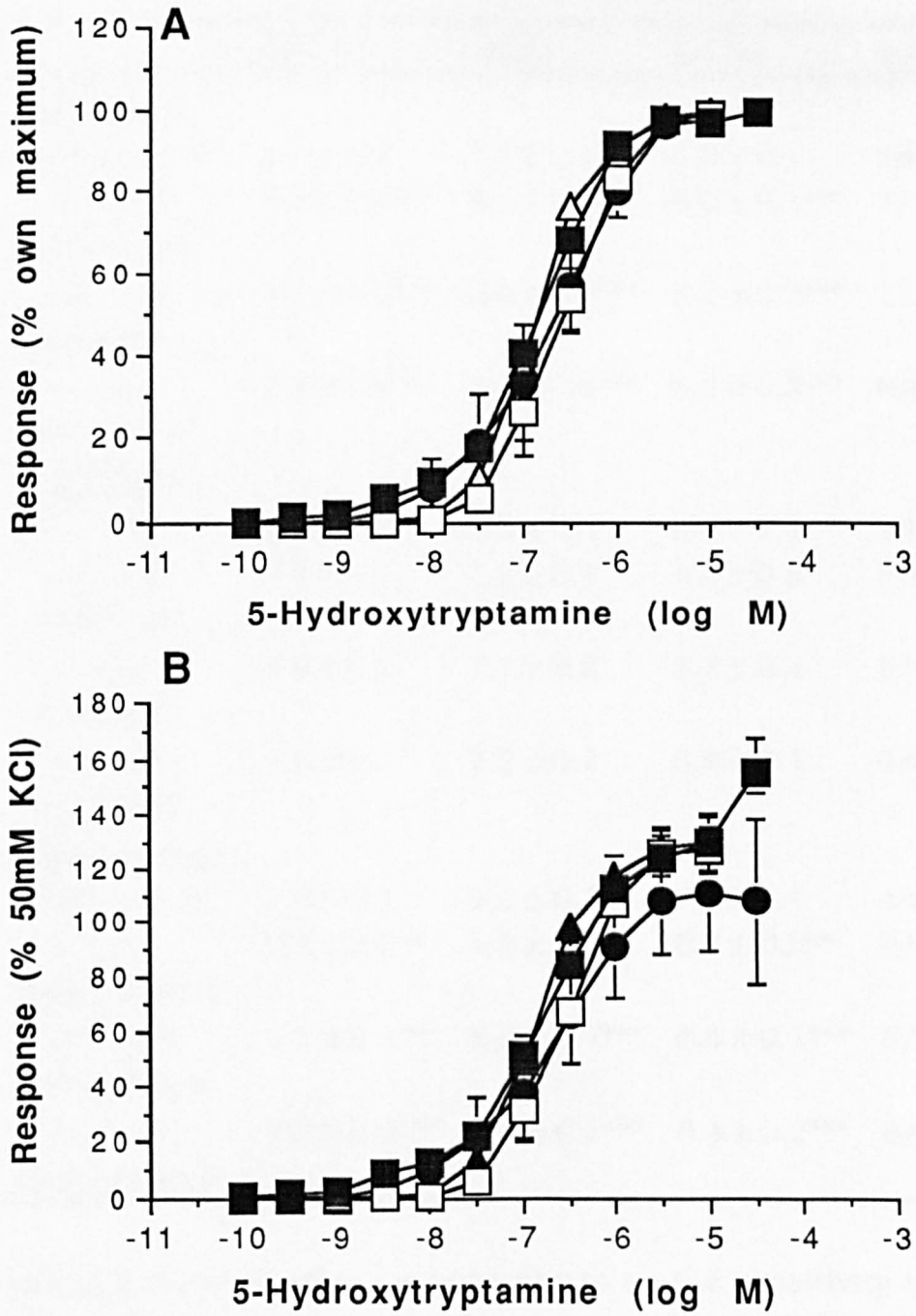

Figure 3.4 Effect of GR55562 on 5-HT-induced contraction in PCAs from adult rabbits. CCRCs to 5-HT (■, n = 18/6), and in the presence of 0.1μM (□, n= 6/6), 1μM (●, n= 6/6) and 3μM (△, n= 6/6) GR55562. **A**. Data are expressed as a percentage of their own maximum. **B** Data are expressed as a percentage of the reference contraction to 50mM. Each point represents mean ± s.e.mean.

	pIC_{10}	pIC_{25}	pIC_{50}	n/n
Ketanserin				
5-HT control	7.7 ± 0.1	7.3 ± 0.1	7.0 ± 0.1	24/11
+10 nM ketanserin	6.6 ± 0.1***	6.3 ± 0.1***	6.0 ± 0.1***	7/7
+0.1 μM ketanserin	6.2 ± 0.2***	5.6 ± 0.2***	5.2 ± 0.2***	11/11
+1.0 μM ketanserin	5.2 ±0.2***	4.9 ± 0.2***	4.4 ± 0.2***	6/6
GR55562				
5-HT control	7.9 ± 0.2	7.3 ± 0.1	6.8 ± 0.1	18/6
+ 0.1 μM GR55562	7.3 ± 0.1	7.0 ± 0.1	6.6 ± 0.2	6/6
+1.0 μM GR55562	7.6 ± 0.2	7.1 ± 0.2	6.7 ± 0.2	6/6
+3.0 μM GR55562	7.6 ±0.1	7.2 ± 0.1	6.8 ± 0.1	6/6
methiothepin				
5-HT control	7.9 ± 0.1	7.6 ± 0.1	7.2 ± 0.1	18/6
+1.0 nM methiothepin	7.3 ± 0.2**	7.0 ± 0.2**	6.7 ± 0.2**	6/6
+3.0 nM methiothepin	7.2 ± 0.1***	6.8 ± 0.1***	6.4 ± 0.1***	6/6
+ 10 nM methiothepin	7.0 ± 0.2***	6.8 ± 0.1***	6.4 ± 0.2***	6/6

Table 3.2 Effect of selective antagonists on the sensitivity to 5-HT in large pulmonary arteries from the adult rabbit. Statistical comparisons were made by Students' paired t-test: antagonist vs. control **P<0.01, ***P<0.001. Values are expressed as arithmetic mean ± s.e.mean., 5-HT, 5-hydroxytryptamine; n/n, number of ring preparations/ number of animals.

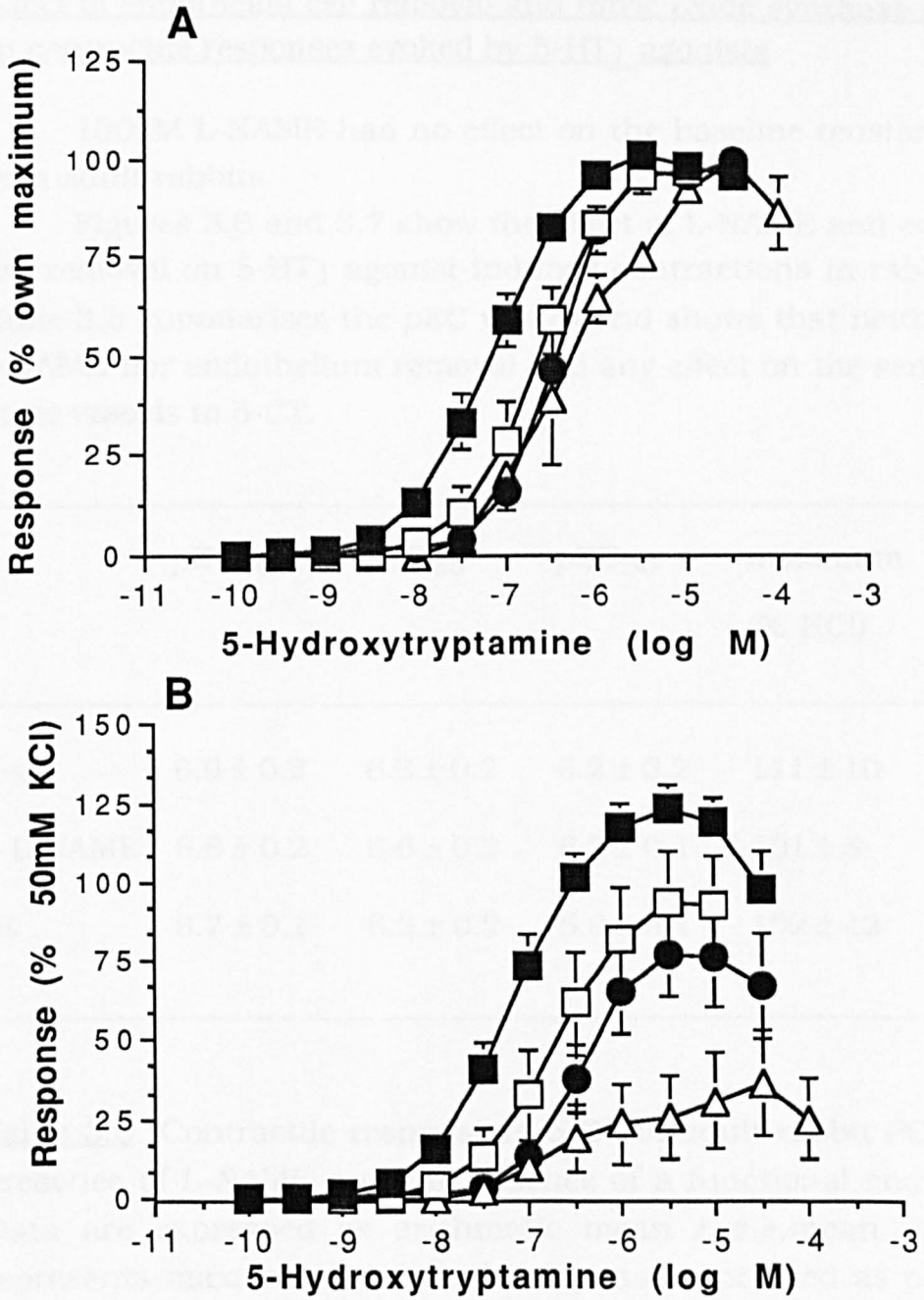

Figure 3.5 Effect of methiothepin on 5-HT-induced contraction in PCAs from adult rabbits. CCRCs to 5-HT (■, n = 18/6), and in the presence of 1nM(□ n= 6/6), 3nM (●,n= 6/6) and 10nM (△, n= 6/6) methiothepin. **A**. Data are expressed as a percentage of their own maximum. **B** Data are expressed as a percentage of the reference contraction to 50mM KCl. Each point represents mean ± s.e.mean.

Effect of endothelial cell removal and nitric oxide synthase inhibition on contractile responses evoked by 5-HT_1 agonists

100μM L-NAME had no effect on the baseline tension in PCAs from adult rabbits.

Figures 3.6 and 3.7 show the effect of L-NAME and endothelial cell removal on 5-HT_1 agonist-induced contractions in rabbit PCAs. Table 3.3 summarises the *p*EC values and shows that neither100μM L-NAME nor endothelium removal had any effect on the sensitivity of these vessels to 5-CT.

	*p*EC10	*p*EC25	*p*EC50	maximum (% KCl)	n
5-CT	6.9 ± 0.2	6.6 ± 0.2	6.2 ± 0.2	111 ± 10	6/6
+ L-NAME	6.8 ± 0.2	6.6 ± 0.2	6.3 ± 0.1	101 ± 8	6/6
-E	6.7 ± 0.1	6.3 ± 0.2	5.9 ± 0.1	102 ± 12	6/6

Table 3.3 Contractile response to 5-CT in adult rabbit PCAs in the presence of L-NAME and the absence of a functional endothelium. Data are expressed as arithmetic mean ± s.e.mean. Maximum represents maximum contractile response expressed as percentage response to reference 50mM KCl contraction. n/n, number of ring preparations/number of animals. -E: no endothelium

100μM L-NAME had no effect on the sumatriptan-induced contraction in these vessels (figure 3.7)

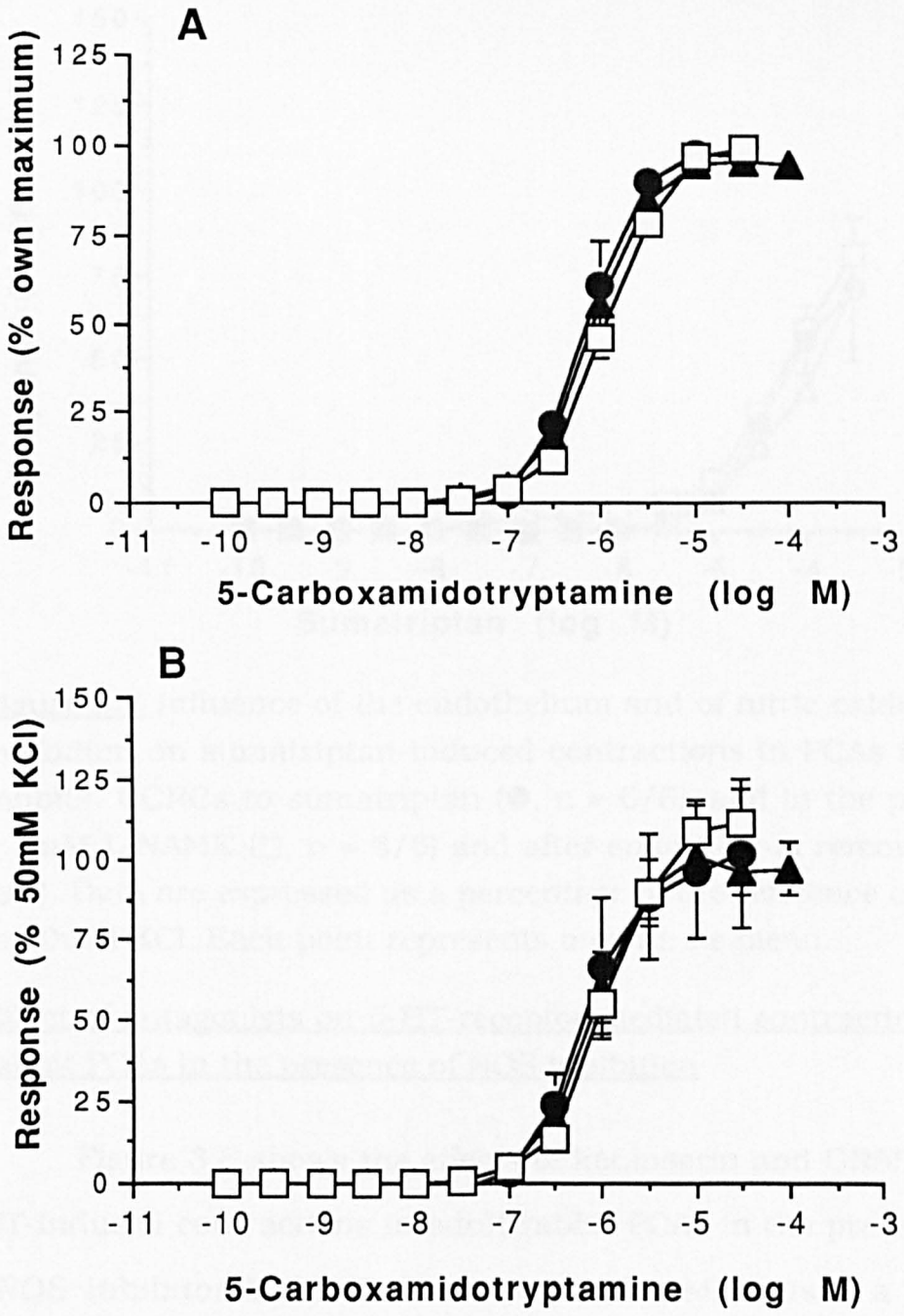

Figure 3.6 Influence of the endothelium and of nitric oxide synthase inhibition on 5-CT induced contractions in PCAs from adult rabbits. CCRCs to 5-CT (□, n = 6/6), and in the presence of 0.1mM L-NAME (▲, n = 6/6) and without endothelium present (●, n = 6/6). **A** Data are expressed as a percentage of their own maximum. **B** Data are expressed as a percentage of the reference contraction to 50mM KCl. Each point represents mean ± s.e.mean.

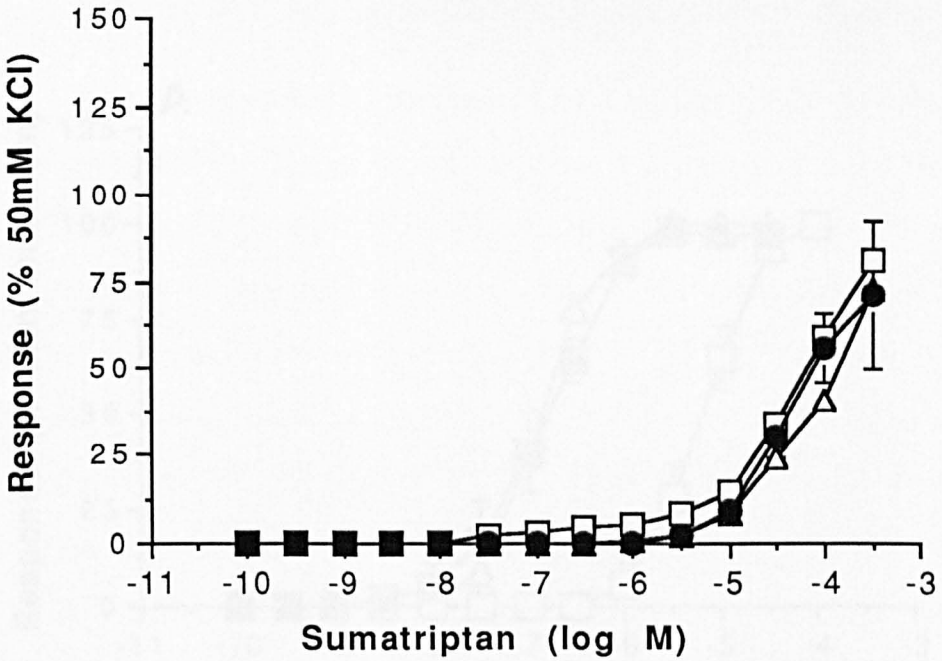

Figure 3.7 Influence of the endothelium and of nitric oxide synthase inhibition on sumatriptan-induced contractions in PCAs from adult rabbits. CCRCs to sumatriptan (●, n = 6/6), and in the presence of 0.1mM L-NAME (□, n = 6/6) and after endothelium removal (△, n = 6/6). Data are expressed as a percentage of the reference contraction to 50mM KCl. Each point represents mean ± s.e.mean.

Effect of antagonists on 5-HT-receptor mediated contraction of adult rabbit PCAs in the presence of NOS inhibition

Figure 3.8 shows the effects of ketanserin and GR55562 on 5-HT-induced contractions in adult rabbit PCAs in the presence of the eNOS inhibitor L-NAME. Ketanserin (0.1μM) caused a rightward displacement of the CCRC to 5-HT, reducing the pEC_{50} value from 6.8 ± 0.1 to 5.15 ± 0.17 ($P<0.001$,n= 6/6), without significantly affecting the maximum contractile response (126 ± 16%; cf control maximum contractile response = 105 ± 7). The estimated pK_B for ketanserin was 8.64 ± 0.20. GR55562 (3μM) did not significantly affect the sensitivity to 5-HT (pEC_{50} = 6.8 ± 0.1; figure 3.8A) nor the maximum response (119 ± 9%; figure 3.8B).

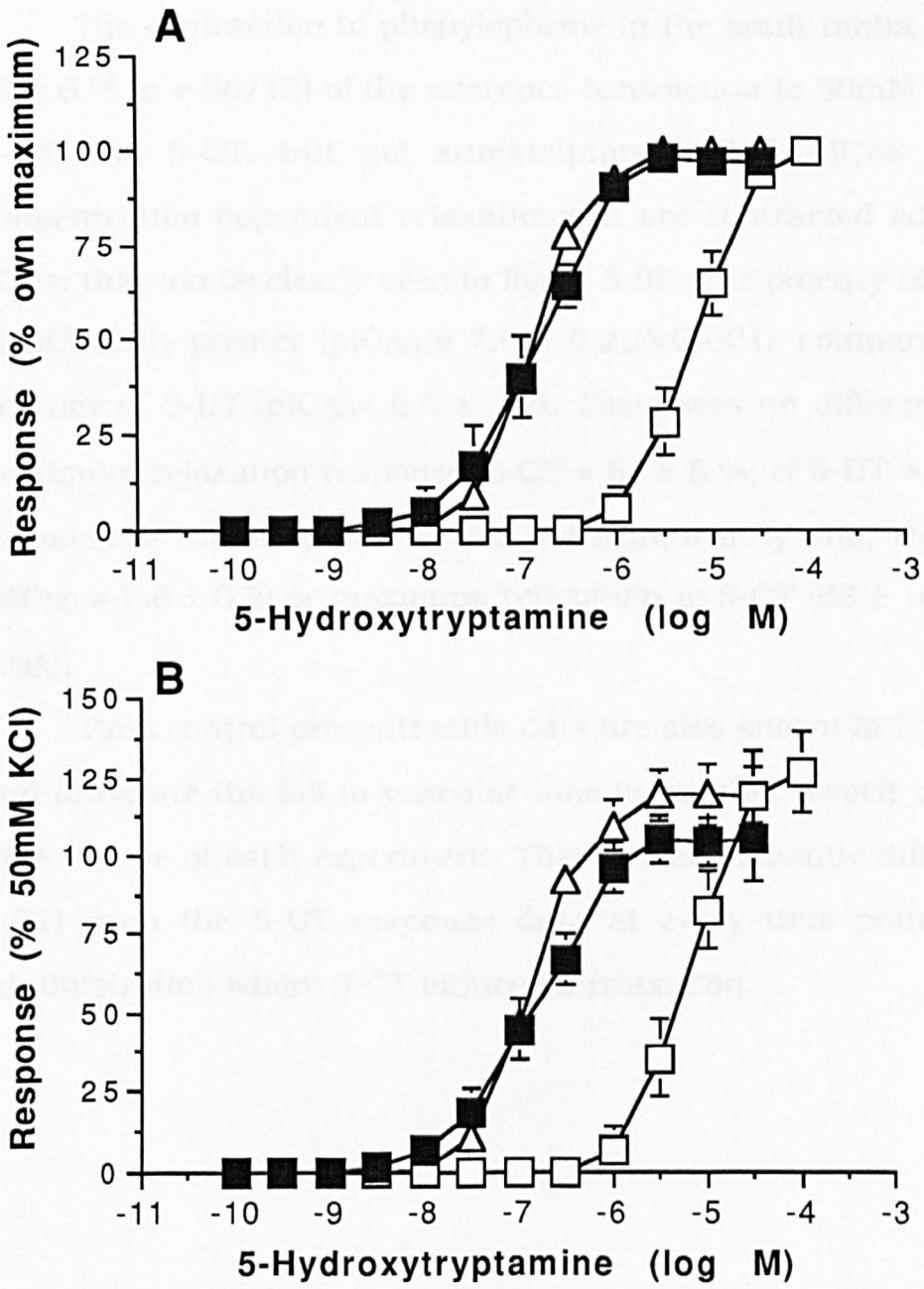

Figure 3.8 Effect of selective antagonists on 5-HT-induced contraction in PCAs from adult rabbits. CCRCs to 5-HT (■, n = 6/6), and in the presence of 1μM(□, n= 6/6) ketanserin, and 1μM (△, n= 6/6) GR55562.**A**. Data are expressed as a percentage of their own maximum. **B** Data are expressed as a percentage of the reference contraction to 50mM KCl. Each point represents mean ± s.e.mean. All CCRCs in the presence of 0.1mM L-NAME.

5-HT receptor mediated vasodilation in adult rabbit PCAs

The contraction to phenylephrine in the adult rabbit PCAs was 52 ± 6 % (n = 36/12) of the reference contraction to 50mM KCl. Both 5-HT and 5-CT, but not sumatriptan or BW723C86 produced concentration dependent relaxations in pre-contracted adult rabbit PCAs, this can be clearly seen in figure 3.9B. The potency of 5-CT was significantly greater (pIC_{50} = 7.6 ± 0.2;P<0.001) compared to the potency of 5-HT (pIC_{50}= 6.5 ± 0.2). There was no difference in the maximum relaxation response (5-CT = 64 ± 8 %; cf 5-HT = 65 ± 4%). Removal of the endothelium did not significantly alter the potency (pIC_{50} = 7.6 ± 0.2) or maximum relaxation to 5-CT (68 ± 10%; figure 3.9A).

Time control precontractile data are also shown in figures 3.9A and illustrate the fall in vascular tone in parallel vessels during the time course of each experiment. This was significantly different (P < 0.01) from the 5-CT response data at every time point of 5-CT administration where 5-CT induced a relaxation.

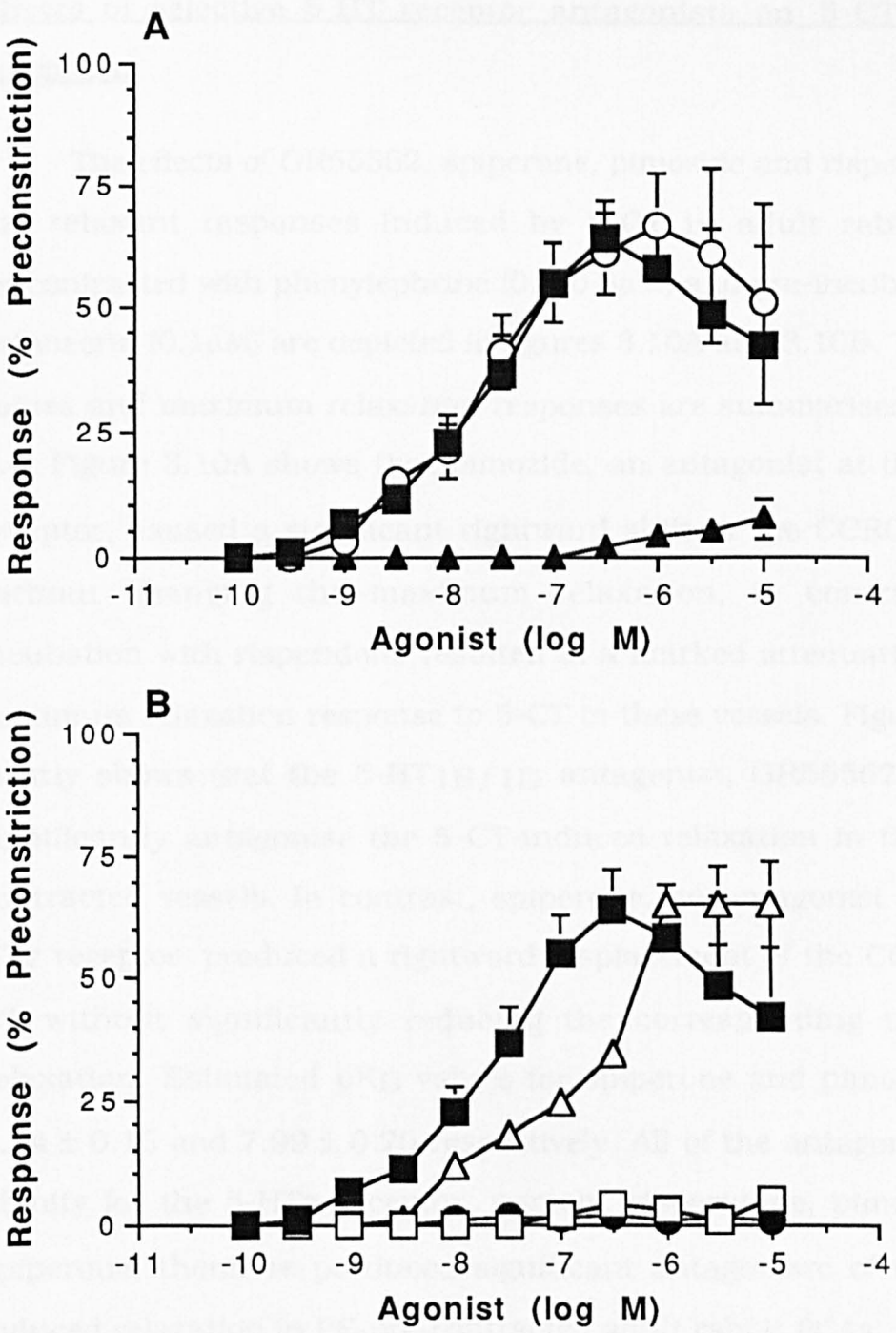

Figure 3.9 5-HT agonist-induced vasodilation of adult rabbit PCAs. **A.** CCRCs to 5-CT in the absence (○, n = 6/6) and presence 5-CT (■, n = 12/12) of a functional endothelium. Time control pre-contractile data are also shown (▲, n = 12/12). **B.** CCRCs to 5-HT (△, n = 6/6), 5-CT (■, n = 12/12), BW723C86 (□, n = 5/5) and sumatriptan (●, n = 5/5). CCRCs expressed as percentage of PE-induced tone. Each point represents arithmetic mean ± s.e.mean. All CCRCs are in the presence of 0.1μM ketanserin.

Effects of selective 5-HT receptor antagonists on 5-CT-induced relaxation

The effects of GR55562, spiperone, pimozide and risperidone on the relaxant responses induced by 5-CT in adult rabbit PCAs precontracted with phenylephrine (0.1-0.3μM) and pre-incubated with ketanserin (0.1μM) are depicted in figures 3.10A and 3.10B. The pIC_{50} values and maximum relaxation responses are summarised in table 3.4. Figure 3.10A shows that pimozide, an antagonist at the 5-HT7 receptor, caused a significant rightward shift in the CCRC to 5-CT without changing the maximum relaxation, in contrast, pre-incubation with risperidone resulted in a marked attenuation of the maximum relaxation response to 5-CT in these vessels. Figure 3.10B clearly shows that the 5-$HT_{1B/1D}$ antagonist, GR55562, did not significantly antagonise the 5-CT-induced relaxation in these pre-contracted vessels. In contrast, spiperone, an antagonist at the 5-HT7 receptor, produced a rightward displacement of the CCRC to 5-CT without significantly reducing the corresponding maximum relaxation. Estimated pK_B values for spiperone and pimozide were 6.84 ± 0.15 and 7.99 ± 0.20 respectively. All of the antagonists with affinity for the 5-HT7 receptor, namely, risperidone, pimozide and spiperone, therefore produced significant antagonism of the 5-CT-induced relaxation in PE-pre-contracted adult rabbit PCAs.

Time control precontractile data are also shown in figure 3.10 and illustrate the fall in vascular tone in parallel vessels during the time course of each experiment. This was significantly different ($P < 0.01$) from the 5-CT response data at every time point of 5-CT administration where 5-CT induced a relaxation.

	pIC_{50}	maximum relaxation (%)	(n)
5-CT	7.6 ± 0.2	64 ± 8	12/12
5-CT - endothelium	7.6 ± 0.2	68 ±10	6/6
5-CT + pimozide (30nM)	7.0 ± 0.2**	62 ± 8	6/6
5-CT + GR55562 (1μM)	7.4 ± 0.2	69 ± 12	6/6
5-CT + risperidone (3nM)	nc	13 ± 8***	6/6
5-CT + spiperone (1μM)	6.7 ± 0.2***	66 ±7	6/6

Table 3.4 Effect of selective antagonists on the relaxation response to 5-CT in adult rabbit PCAs in the presence of 0.1μM ketanserin. Data are expressed as arithmetic mean ± s.e.mean. Statistical comparisons were made by ANOVA: antagonist vs. control **P<0.01, ***P<0.001. Maximum represents maximum relaxation response expressed as percentage PE-induced contraction. n/n, number of ring preparations/number of animals.

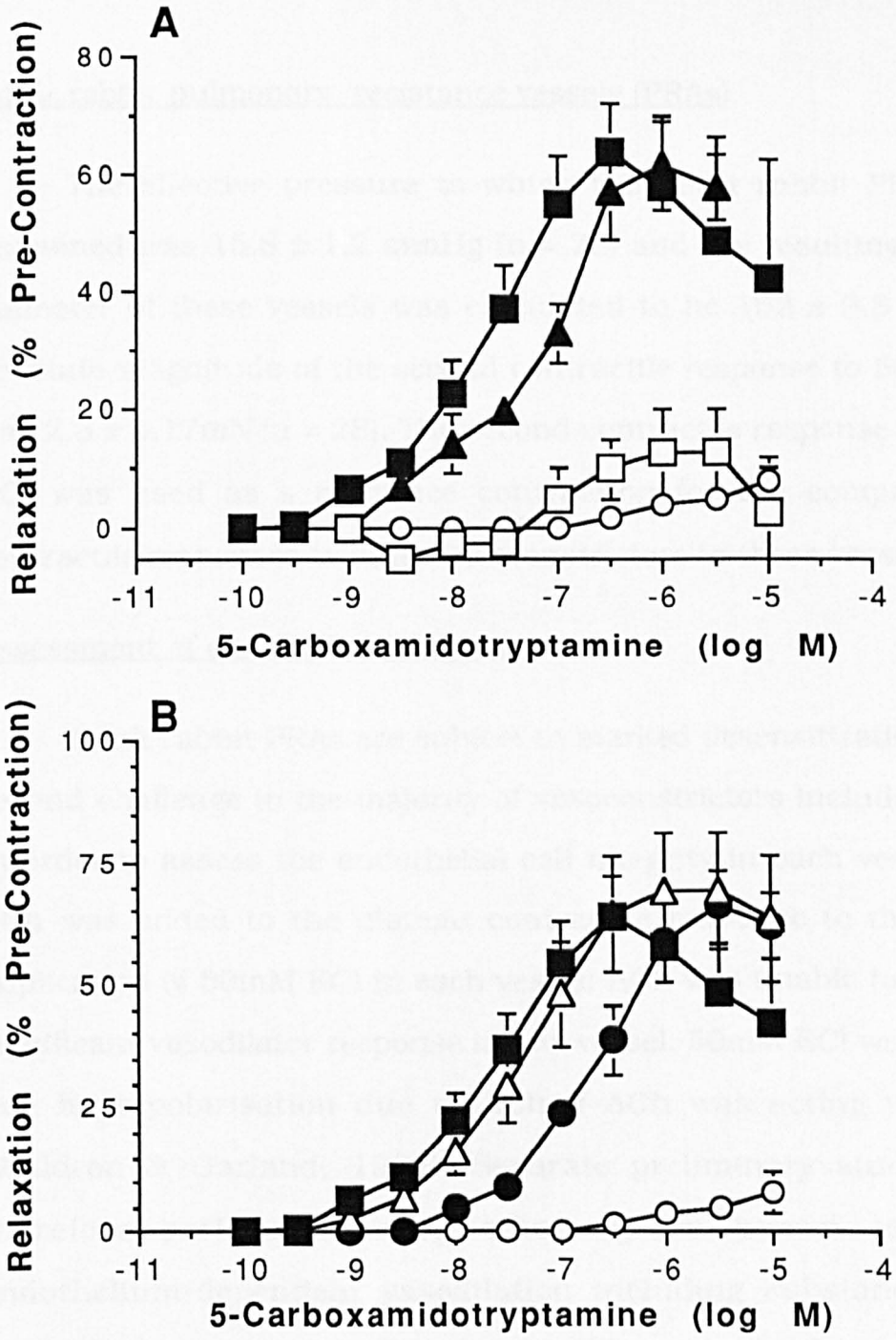

Figure 3.10 Effect of selective 5-HT antagonists on 5-CT-induced vasodilation in adult rabbit PCAs precontracted with phenylephrine. 5-CT CCRCs (■, n = 7/7), and in the presence of **A)** 30nM pimozide (▲, n = 6/6) or risperidone (□, n= 6/6). **B)** 1μM GR55562 (△, n = 5/5), or 1μM spiperone (●, n = 6/6); All CCRCs are in the presence of 0.1μM ketanserin. Time control pre-contractile data are also shown (○, n = 12/12). Data are expressed as % reversal of PE preconstriction. Each point represents mean ± s.e.mean.

Adult rabbit pulmonary resistance vessels (PRAs)

The effective pressure to which the adult rabbit PRAs were tensioned was 15.8 ± 1.2 mmHg (n = 28) and the resulting internal diameter of these vessels was calculated to be 162 ± 9.8 μm. The absolute magnitude of the second contractile response to 50mM KCl was 2.3 ± 0.17mN (n = 28). The second contractile response to 50mM KCl was used as a reference contraction for the comparison of contractile responses to other vasoconstrictors in these vessels.

Assessment of endothelial integrity

Adult rabbit PRAs are subject to marked desensitisation to any second challenge to the majority of vasoconstrictors including 5-HT. In order to assess the endothelial cell integrity in each vessel, 1μM ACh was added to the plateau contractile response to the second application of 50mM KCl in each vessel; ACh was unable to induce a significant vasodilator response in any vessel. 50mM KCl would block any hyperpolarisation due to ACh if ACh was acting via EDHF (Waldron & Garland, 1994). Separate preliminary studies were therefore performed using other agents known to induce endothelium-dependent vasodilation including substance P and A23187. These agents, including 1μM ACh, were added at 1μM once a contractile response to a single challenge of 1μM 5-HT had plateaued. None of the agents used caused any significant relaxation of the 5-HT pre-contracted vessels. The reason for this apparent general lack of relaxation to endothelium-dependent vasodilators in these vessels is unclear. However it is unlikely that damage to the endothelium accounted for this. Although the presence of an intact

endothelium was not confirmed histologically, similar sized PRAs, studied in this thesis, and mounted in the myograph exhibit endothelium-dependent relaxation (see chapters 4 and 5 of this thesis). It was concluded that adult rabbit PRAs were unresponsive to agents known to induce endothelium-dependent relaxation in agreement with previous studies in adult rabbit PRAs (Docherty & MacLean, 1997).

Effect of NOS inhibition

The effect of 100μM L-NAME alone on baseline tension of the PRAs was assessed. This nitric oxide synthase inhibitor evoked variable responses. Of the vessels examined 40% showed an increase in baseline tone of 23 ± 11 % of the reference contraction to 50mM KCl.

Effect of endothelium-independent vasodilator agent, sodium nitroprusside

1μM 5-HT induced a precontraction of the magnitude 48 ± 6.3 % of response to 50mM KCl. Figure 3.11i shows that SNP produced a concentration dependent relaxation in the precontracted adult rabbit PRAs. The maximum relaxation response was 63.0 ± 7.0 % and the pIC_{50} was 7.2 ±0.2 (n/n = 6/6).

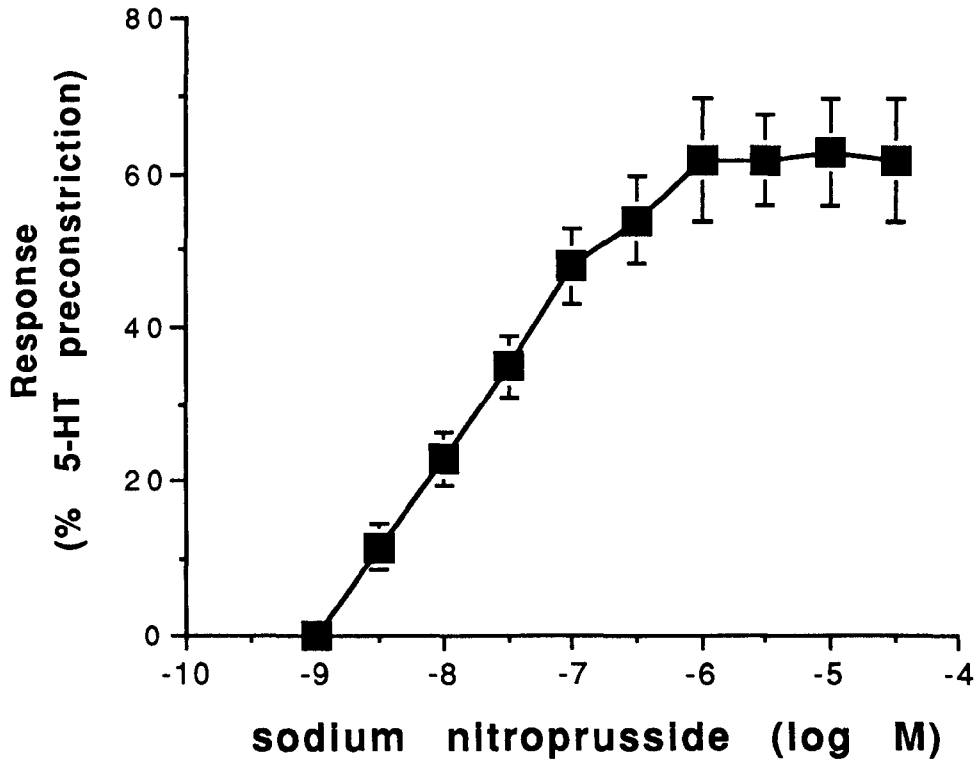

Figure 3.11i Effect of SNP on 5-HT-precontracted adult rabbit PRAs. Data are expressed as % 1μM 5-HT-induced tone. CCRC to SNP (■, n = 6/6). Each point represents mean ± s.e.mean. n/n, number vessels/number of animals.

Effect of 5-HT receptor agonists

The effect of the 5-HT receptor agonists 5-HT, 5-CT, α-methyl-5-HT and sumatriptan on PRAs from adult rabbits is shown in figure 3.11 and the *p*EC50 values are summarised in table 3.5. 5-HT elicited concentration-dependent contractions in the adult rabbit PRAs. The selective 5-HT_{2A} receptor agonist α-methyl 5-HT elicited concentration-dependent contractions in these vessels with a significantly smaller maximum contractile response compared to 5-HT. 5-CT evoked a concentration-dependent contractile response and the CCRC to 5-CT was biphasic in nature (see figure 3.11 B. The *p*EC50 value for 5-CT was significantly different from 5-HT. The maximum contractile response to 5-CT was also significantly smaller ($P<0.05$) than that of 5-HT (see table 3,5. Sumatriptan also elicited a concentration dependent contraction in the adult rabbit PRAs with a significantly lower potency compared to 5-HT (figure 3.11 A; table 3.5). The maximum contractile response was also significantly lower than that to 5-HT. Both 5-CT and sumatriptan were equipotent in these vessels.

Effect of L-NAME on 5-HT agonist-induced responses

The response of adult rabbit PRAs to 5-HT-receptor agonists in the presence and absence of 0.1mM L-NAME are shown in figures 3.11 and 3.15. *p*EC50 values and maximum contractile responses are shown in table 3.5. L-NAME significantly increased the sensitivity to 5-HT and to 5-CT in the adult PRAs. In comparison, L-NAME did not significantly increase the sensitivity to α-methyl-5-HT or to sumatriptan in these vessels. The magnitude of the maximum contractile response to all the agonists was not significantly changed in the presence of L-NAME.

	pEC_{50}	$E_{max.}$(%)	(n)
5-HT	6.08 ± 0.12	69 ± 9	(8/8)
+L-NAME	6.75 ± 0.13**	75 ±8	(8/8)
α-methyl-5-HT	6.51 ± 0.2	42 ± 3†	(6/6)
+L-NAME	6.63 ± 0.22	43 ± 2	(6/6)
5-CT	5.32 ± 0.2††	37 ± 6†	(6/6)
+L-NAME	6.05 ± 0.3*	56 ± 7	(6/6)
sumatriptan	5.19 ± 0.15††	37 ± 8†	(6/6)
+L-NAME	5.7 ± 0.2	39 ± 6	(6/6)

Table 3.5 Characteristics of 5-HT agonist-induced vasoconstriction in adult rabbit PRAs in the presence and absence of nitric oxide synthase inhibitor L-NAME (0.1mM).Statistical comparisons were made by Students unpaired t test, control agonist vs. control 5-HT, †$P<0.05$, ††$P<0.01$; control vs L-NAME, *$P<0.05$, **$P<0.01$. Data are expressed as arithmetic mean ± s.e.mean. $E_{max.}$ represents maximum contractile response expressed as percentage response to reference 50mM KCl contraction. n/n, number of ring preparations/number of animals. L-NAME, L-N^{ω}-nitro-L-arginine methylester.

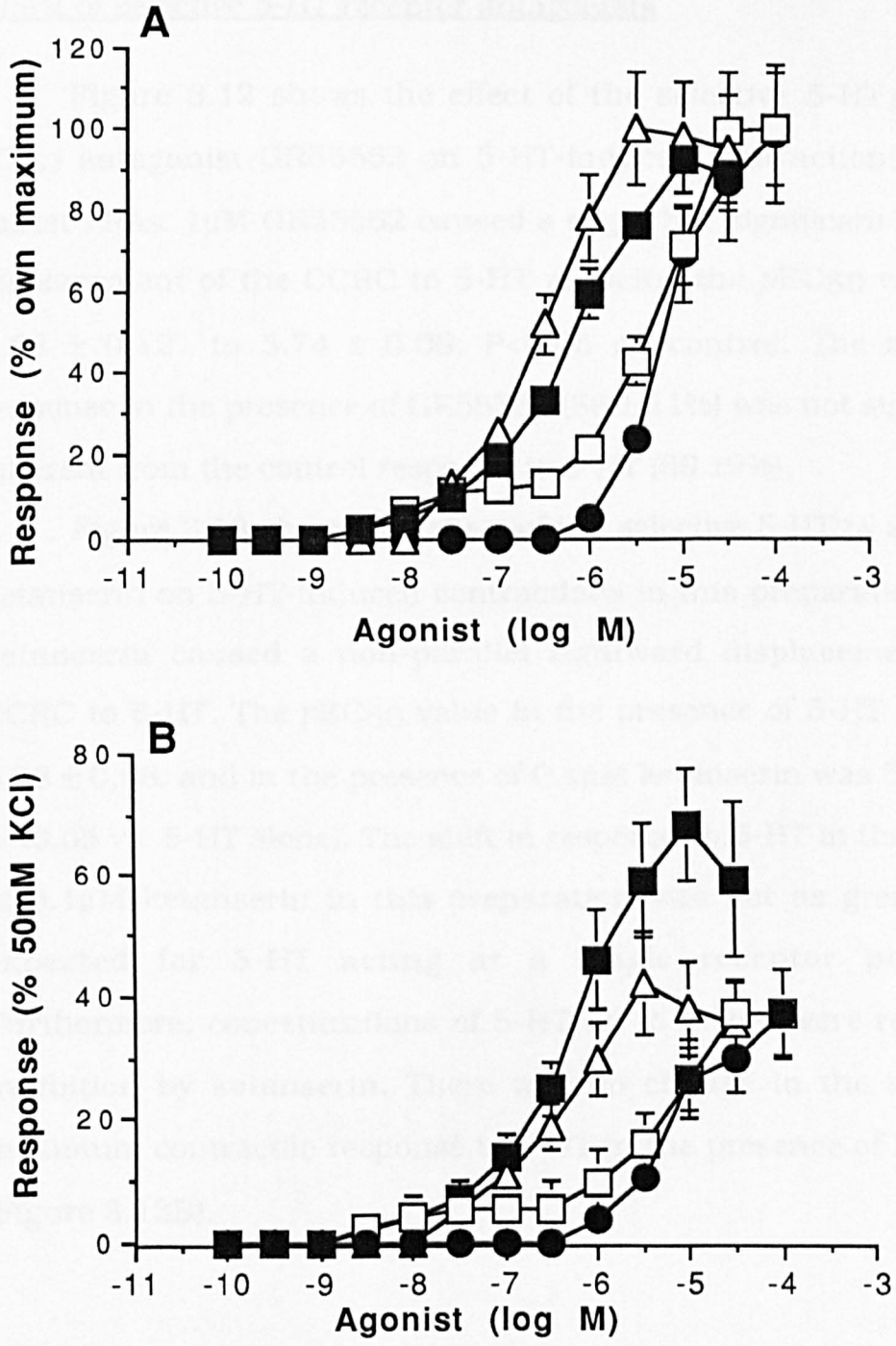

Figure 3.11 Responses to 5-HT receptor agonists in adult rabbit PRAs. CCRCs to 5-HT (■, n = 8/8), α-methyl 5-HT (△, n = 6/6), 5-CT (□, n= 6/6) and sumatriptan (●, n= 6/6). **A**. Data are expressed as a percentage of their own maximum. **B** Data are expressed as a percentage of the reference contraction to 50mM KCl. Each point represents mean ± s.e.mean.

Effect of selective 5-HT receptor antagonists

Figure 3.12 shows the effect of the selective 5-HT_{1B} and 5-HT_{1D} antagonist GR55562 on 5-HT-induced contractions in adult rabbit PRAs. 1μM GR55562 caused a slight but significant rightward displacement of the CCRC to 5-HT reducing the *p*EC_{50} value from 6.08 ± 0.12 to 5.74 ± 0.08; $P<0.05$ cf. control. The maximum response in the presence of GR55562 (86 ± 11%) was not significantly different from the control response to 5-HT (69 ±9%).

Figure 3.13 shows the effect of the selective 5-HT_{2A} antagonist ketanserin on 5-HT-induced contractions in this preparation. 0.1μM ketanserin caused a non-parallel rightward displacement of the CCRC to 5-HT. The *p*EC_{50} value in the presence of 5-HT alone was 6.08 ± 0.08, and in the presence of 0.1μM ketanserin was 5.21 ± 0.1. ($P<0.05$ vs. 5-HT alone). The shift in response to 5-HT in the presence of 0.1μM ketanserin in this preparation was not as great as that expected for 5-HT acting at a single receptor population. Furthermore, concentrations of 5-HT up to 0.1μM were resistant to inhibition by ketanserin. There was no change in the size of the maximum contractile response to 5-HT in the presence of ketanserin (Figure 3.13B).

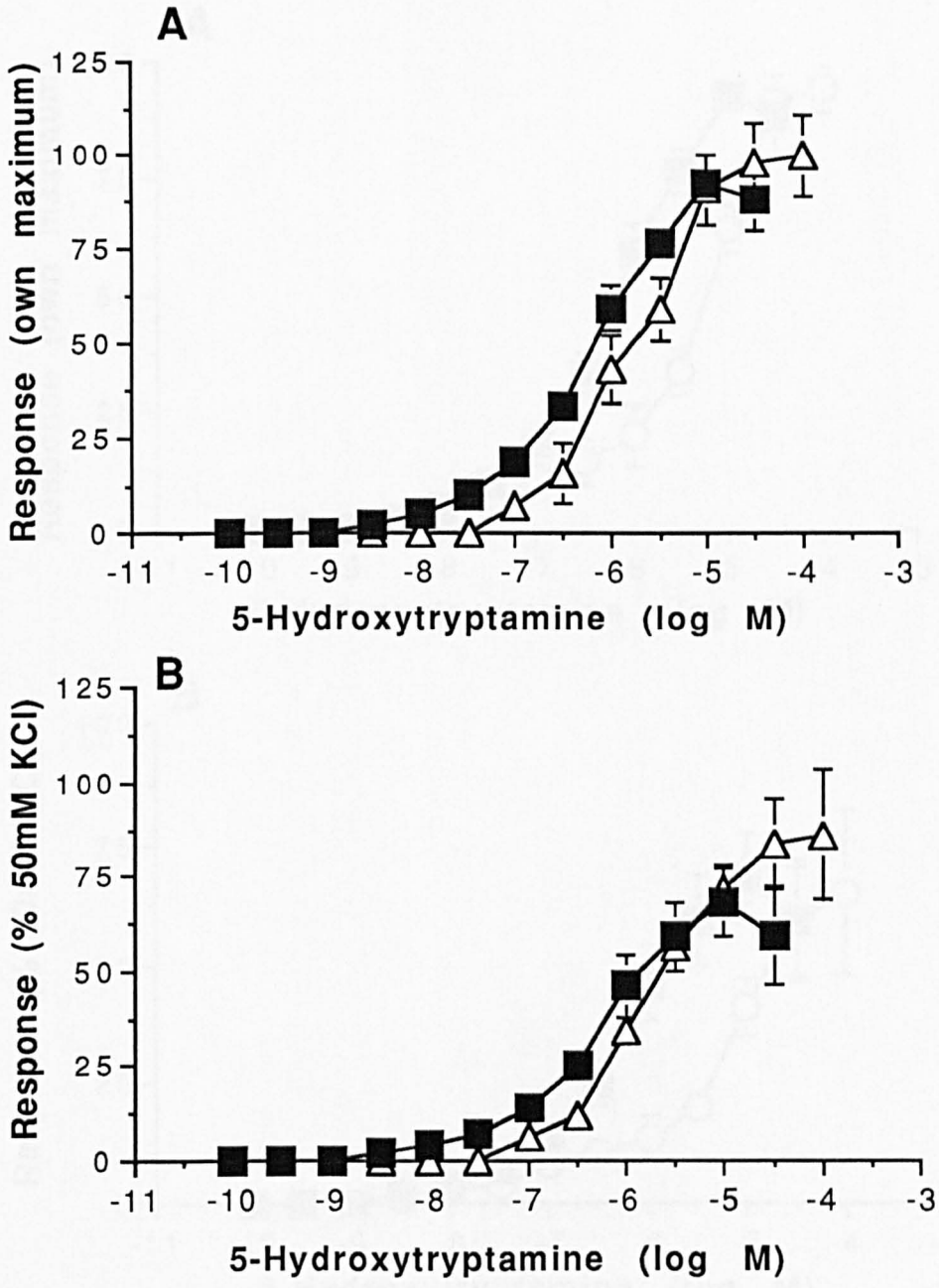

Figure 3.12 Effect of GR55562 (1μM) on responses to 5-HT in adult rabbit PRAs. CCRCs to 5-HT (■, n = 8/8), and in the presence of GR55562 (△, n= 6/6). **A**. Data are expressed as a percentage of their own maximum. **B** Data are expressed as a percentage of the reference contraction to 50mM KCl. Each point represents mean ± s.e.mean.

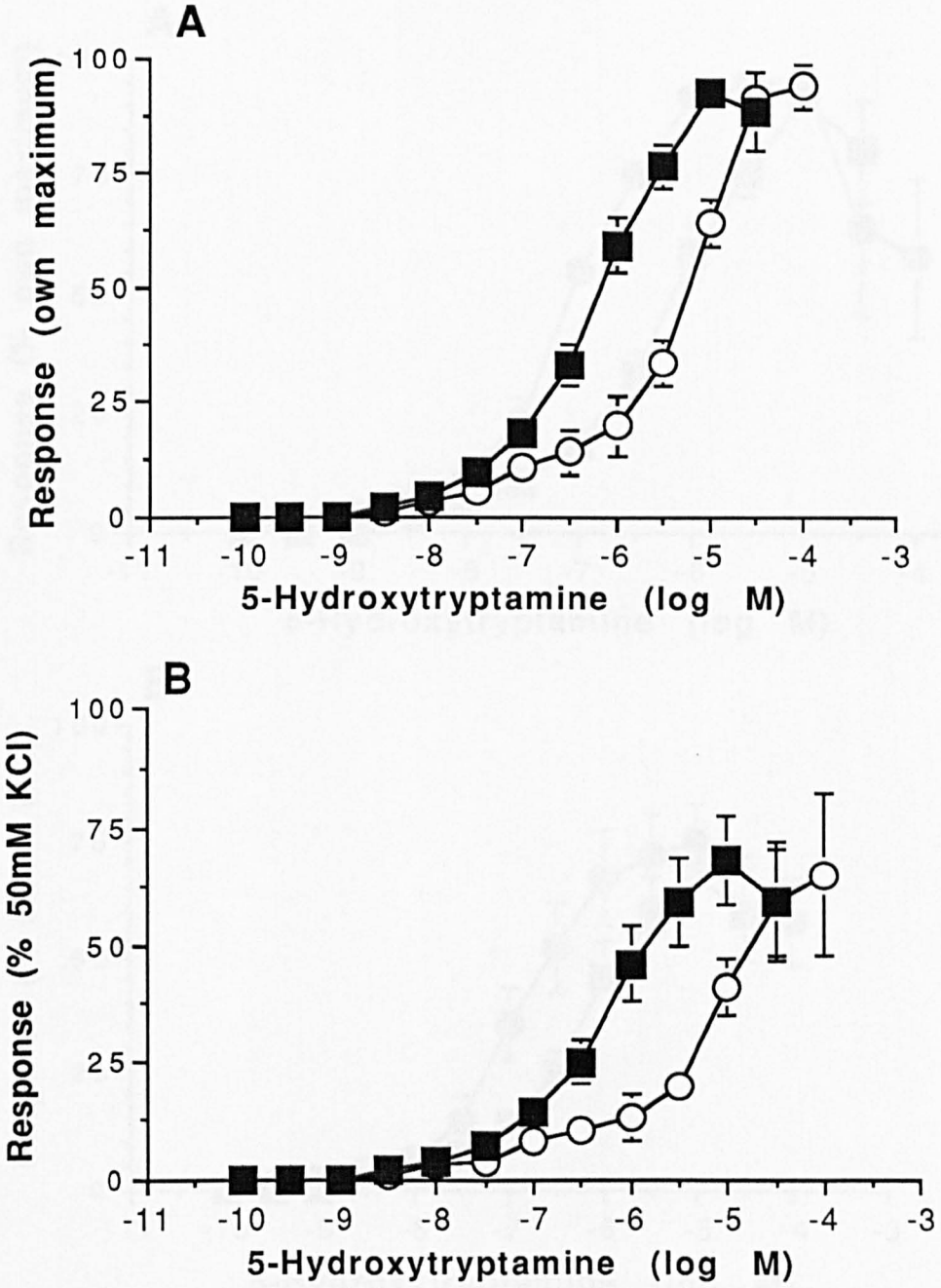

Figure 3.13 Effect of ketanserin (0.1μM) on responses to 5-HT in adult rabbit PRAs. CCRCs to 5-HT (■, n = 8/8), and in the presence of ketanserin (O, n= 6/6). **A**. Data are expressed as a percentage of their own maximum. **B** Data are expressed as a percentage of the reference contraction to 50mM KCl. Each point represents mean ± s.e.mean.

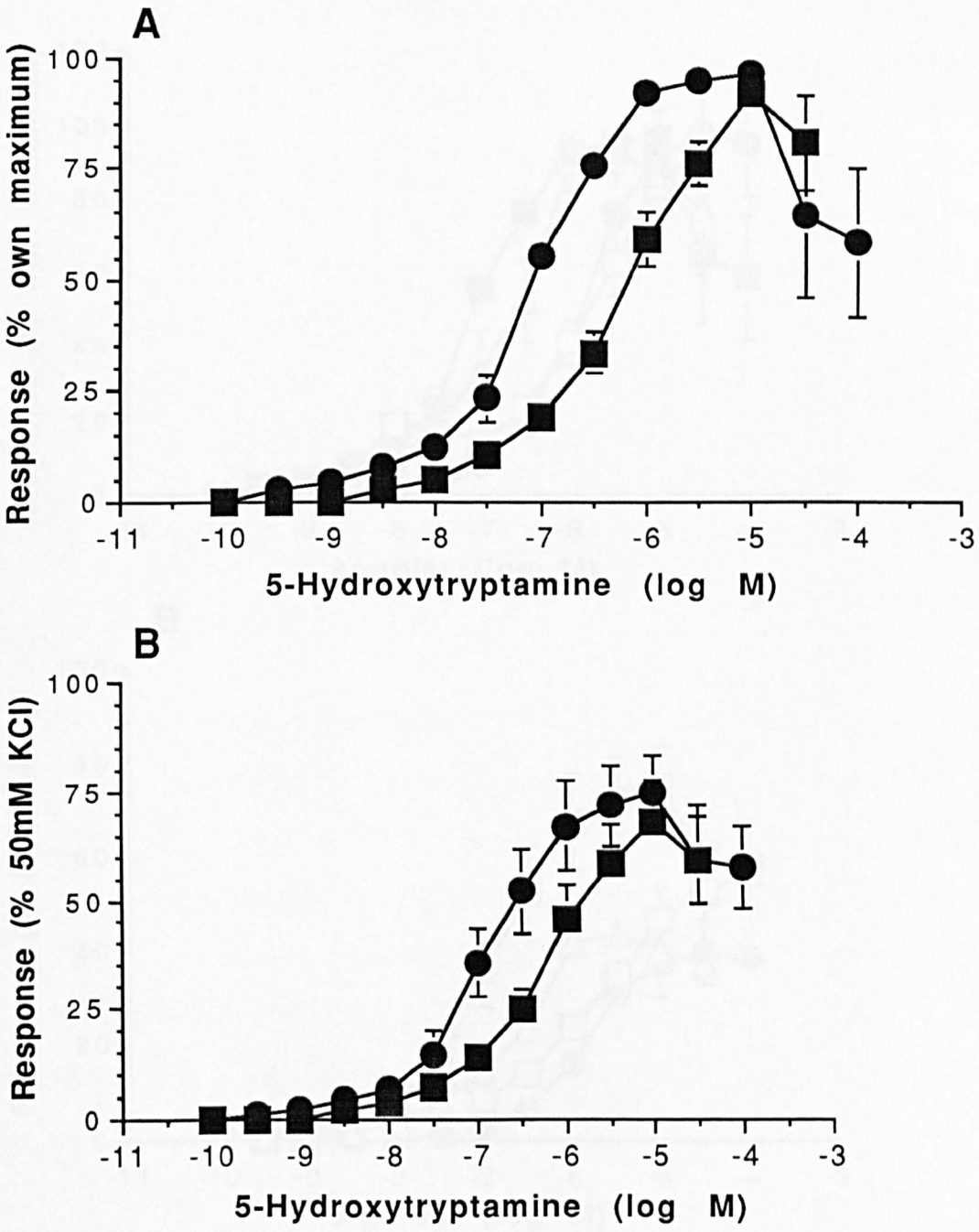

Figure 3.14 Effect of eNOS inhibition on responses to 5-HT in adult rabbit PRAs. CCRCs to 5-HT (■, n = 8/8), and in the presence of 0.1mM L-NAME (●, n= 8/8). **A**. Data are expressed as a percentage of their own maximum. **B** Data are expressed as a percentage of the reference contraction to 50mM KCl. Each point represents mean ± s.e.mean.

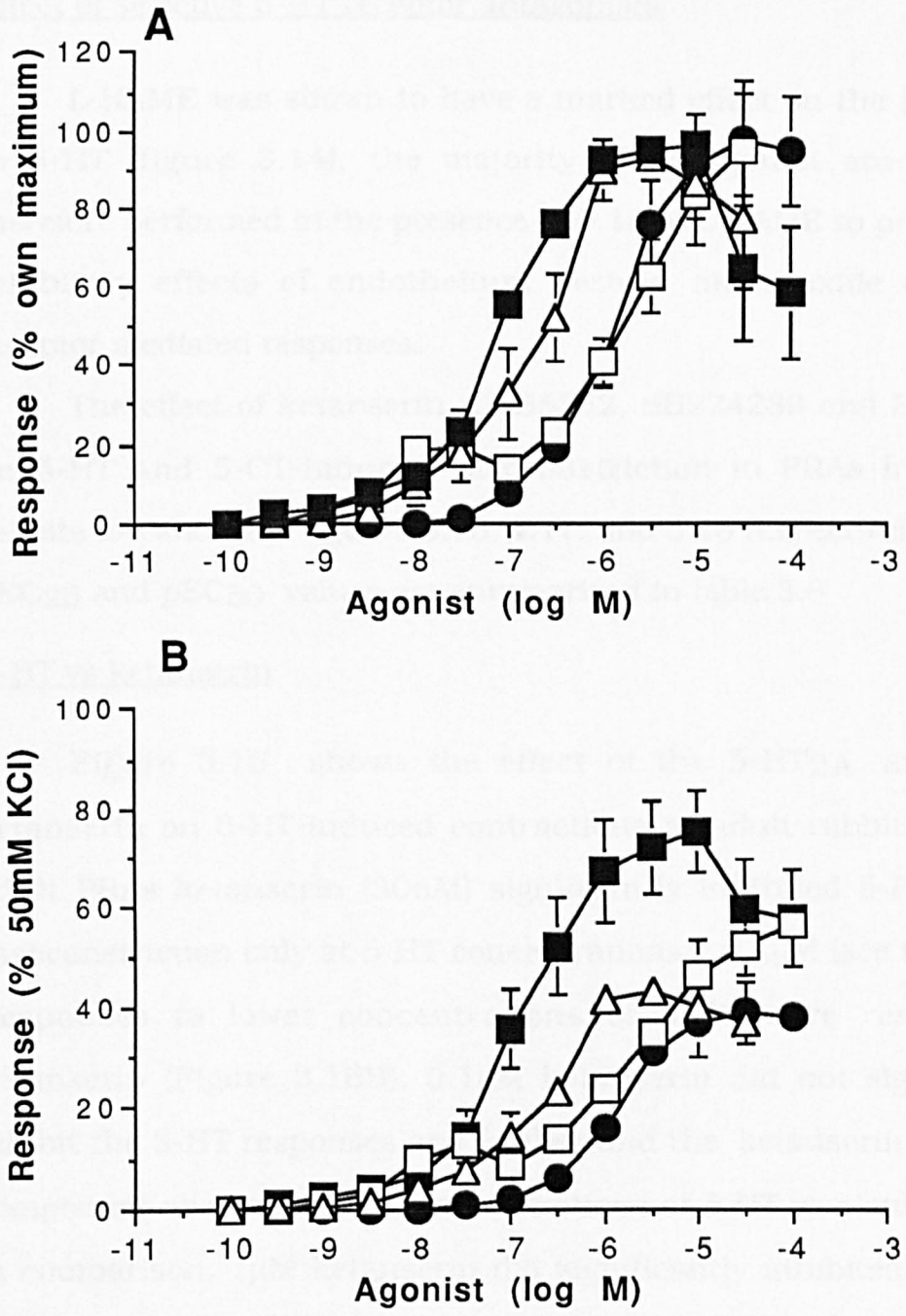

Figure 3.15 Effect of eNOS inhibition on responses to 5-HT receptor agonists in adult rabbit PRAs. CCRCs to 5-HT (■, n = 8/8), α-methyl 5-HT (△, n = 6/6), 5-CT (□, n= 6/6) and sumatriptan (●, n= 6/6). **A.** Data are expressed as a percentage of their own maximum. **B** Data are expressed as a percentage of the reference contraction to 50mM KCl. Each point represents mean ± s.e.mean. All CCRCs in the presence of 0.1mM L-NAME.

Effect of selective 5-HT receptor antagonists

L-NAME was shown to have a marked effect on the sensitivity to 5-HT (figure 3.14), the majority of antagonist studies were therefore performed in the presence of 0.1mM L-NAME to prevent any inhibitory effects of endothelium derived nitric oxide on 5-HT-receptor mediated responses.

The effect of ketanserin, GR55562, SB224289 and BRL15572 on 5-HT and 5-CT-induced vasoconstriction in PRAs from adult rabbits are shown in figures 3.16, 3.17, and 3.18 respectively. pEC_{10}, pEC_{25} and pEC_{50} values are summarised in table 3.6

5-HT vs ketanserin

Figure 3.16 shows the effect of the 5-HT_{2A} antagonist ketanserin on 5-HT-induced contractions in adult rabbit PRAs. In adult PRAs ketanserin (30nM) significantly inhibited 5-HT-evoked vasoconstriction only at 5-HT concentrations ≥ 0.1μM (see table 3.6). Responses to lower concentrations of 5-HT were resistant to ketanserin (Figure 3.16B). 0.1μM ketanserin did not significantly inhibit the 5-HT responses any further and the 'ketanserin-resistant' component observed at low concentrations of 5-HT was still present. In comparison, 1μM ketanserin did significantly inhibited the 5-HT CCRC at the level of the pEC_{10} value (see table 3.6). All three concentrations of ketanserin did significantly decrease the pEC_{50} value. The maximum contractile responses in the presence of ketanserin were 64 ±5%, 81 ±9% and 70 ± 8% (% response to reference 50mM KCl contractile response) for 30nM, 0.1μM and 1μM, ketanserin respectively; ketanserin did not significantly affect the maximum contractile response to 5-HT compared to the control response in the presence of 0.1mM L-NAME (Emax. = 75 ± 8%). The

estimated pK_B value for 1μM ketanserin was 6.98 ± 0.2. (% response to reference 50mM KCl contractile response).

5-HT vs. GR55562

The effect of the 5-$HT_{1B/1D}$ antagonist GR55562 on 5-HT-evoked vasoconstrictions in adult rabbit PRAs is shown in figure 3.17. pEC_{10}, pEC_{25} and pEC_{50} values are summarised in table 3.6. GR55562 (1μM) significantly inhibited the 5-HT-induced contractions at the level of the pEC_{10}, pEC_{25}, and pEC_{50} values, including the 'ketanserin-resistant' component (see figure 3.17). Higher concentrations of GR55562 (10μM), further inhibited responses to 5-HT but also significantly reduced the maximum response to 5-HT from 75 ± 8% to 41 ± 7% (% of response to 50mM KCl, $P < 0.005$).

5-CT vs. BRL15572 and SB224289

Figure 3.18 shows the effect of the selective 5-HT_{1B} antagonist SB224289 and the selective 5-HT_{1D} antagonist BRL15572 on 5-CT-induced contractions in adult rabbit PRAs. Table 3,6 summarises the pEC_{10}, pEC_{25} and pEC_{50} values for BRL15572. SB224289 (0.2μM) caused a marked inhibition of the CCRC to 5-CT, which did not achieve a maximum contractile response in the 5-CT concentration range studied. pEC values could not, therefore, be calculated. BRL15572 (0.5μM) caused a significant, but non parallel, rightward shift of the CCRC to 5-CT, significantly inhibiting the 5-CT-induced contractions in these vessels at 5-CT concentrations ≥ 1.0μM, and resulted in a significant decrease in the sensitivity to 5-CT at the levels of the pEC_{25} and pEC_{50} values. The maximum contractile response to 5-CT in the presence of BRL15572 (47 ± 8% 50mM KCl)

was not significantly different from the control response to 5-CT (60 ± 6% 50mM KCl).

	pEC_{10}	pEC_{25}	pEC_{50}	n/n
Ketanserin				
5-HT control	7.75 ± 0.2	7.21 ± 0.1	6.75 ± 0.1	8/8
+30 nM ketanserin	7.39 ± 0.3	6.70 ± 0.2*	5.95 ± 0.1**	7/7
+0.1μM ketanserin	7.63 ± 0.2	6.68 ± 0.3	6.04 ± 0.3*	7/7
+1.0 μM ketanserin	6.9 ± 0.2*	5.76 ±0.2***	5.73 ± 0.2***	6/6
GR55562				
5-HT control	7.75 ± 0.2	7.21 ± 0.1	6.75 ± 0.1	8/8
+ 1.0 μM GR55562	7.10 ± 0.2*	6.70 ± 0.1*	6.05 ± 0.2**	6/6
+10 μM GR55562	7.25 ± 0.2	6.20± 0.2***	5.54 ± 0.2***	6/6
5-CT control	7.3 ± 0.2	6.76 ± 0.2	5.85 ± 0.1	7/7
+0.2 μM SB224289	nc	nc	nc	7/7
+0.5 μM BRL15572	7.2 ± 0.2	6.08 ± 0.2*	4.82 ± 0.1***	7/7

Table 3.6 Effect of selective antagonists on the sensitivity to 5-HT in PRAs from the adult rabbit. Statistical comparisons were made by Students' unpaired *t*-test: antagonist vs. control *$P<0.05$, **$P<0.01$, ***$P<0.001$. Values are expressed as arithmetic mean ± s.e.mean., 5-HT, 5-hydroxytryptamine; n/n, number of ring preparations/ number of animals. All CCRCs constructed in the presence of 0.1mM L-NAME.

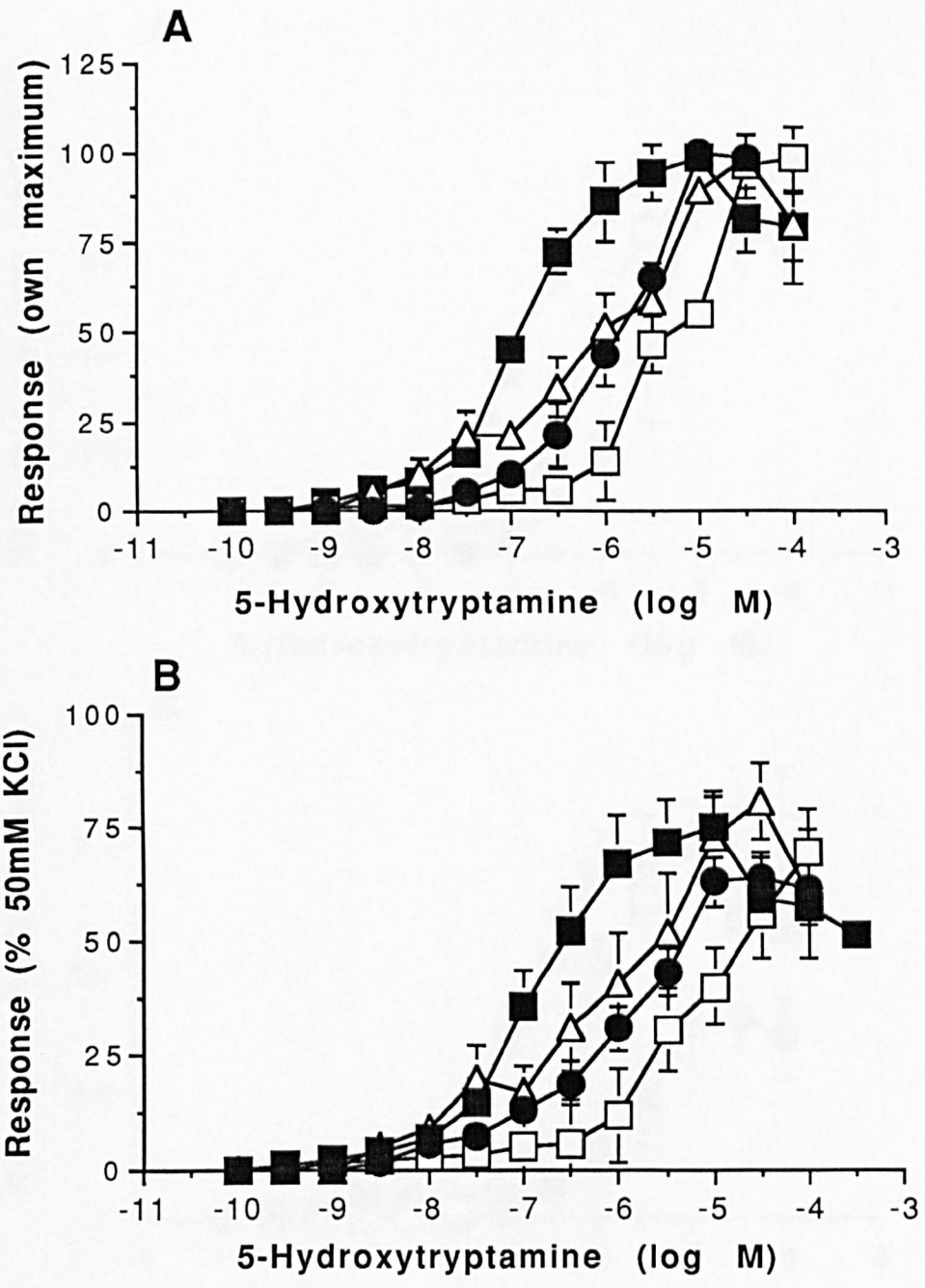

Figure 3.16 Effect of ketanserin on 5-HT-induced contraction in adult rabbit PRAs. CCRCs to 5-HT (■, n = 8/8), and in the presence of 30nM (△,n= 7/7), 0.1μM (●,n= 7/7) and 1.0μM (□, n= 6/6) ketanserin. A. Data are expressed as a percentage of their own maximum. **B** Data expressed as a percentage of the reference contraction to 50mM. Each point represents mean ± s.e.mean. All CCRCs in the presence of 0.1mM L-NAME.

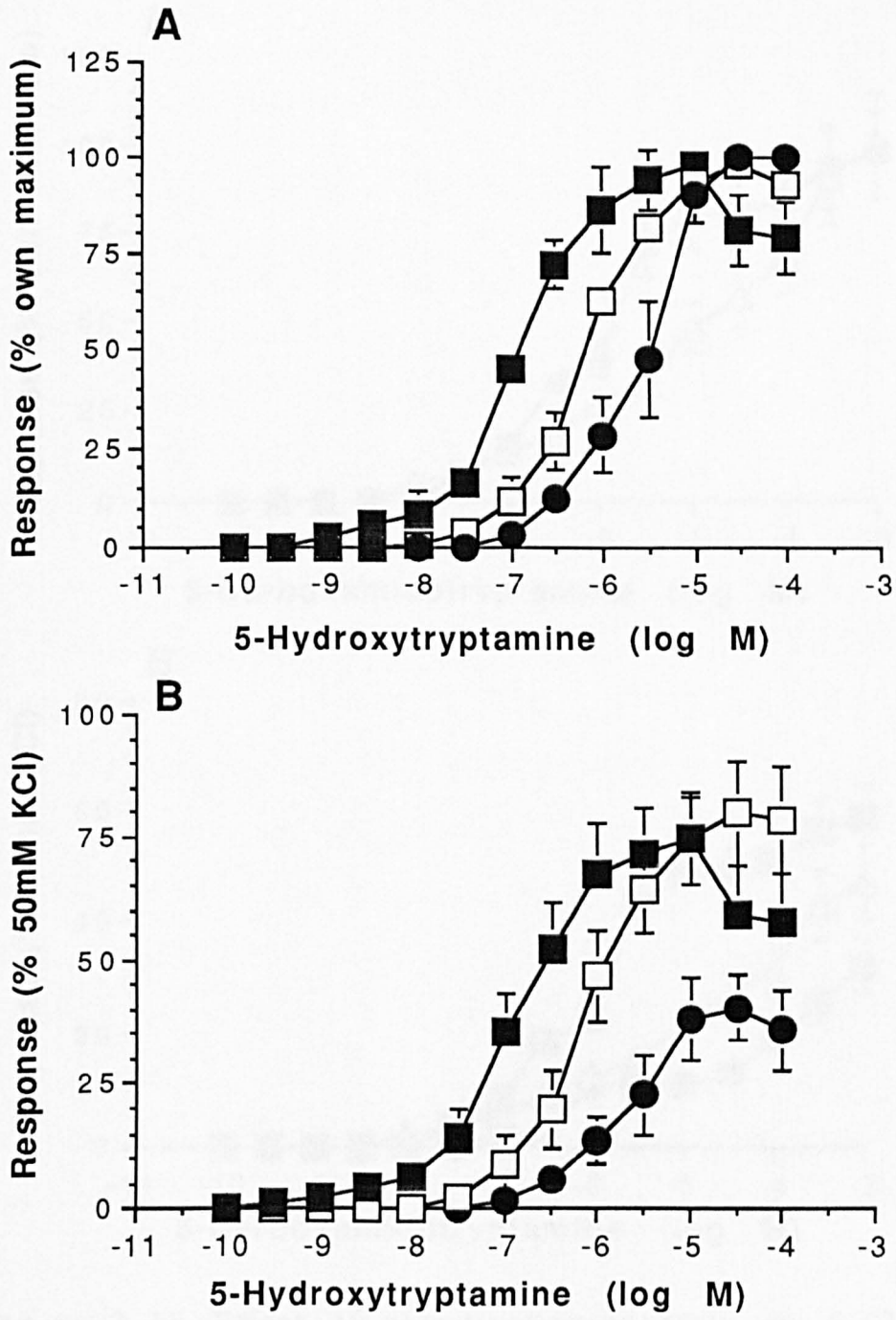

Figure 3.17 Effect of GR55562 on 5-HT-induced contraction in PRAs from adult rabbits. CCRCs to 5-HT (■, n = 8/8), and in the presence of 0.1μM (□, n= 6/6), and 1μM (●, n= 6/6) GR55562. **A**. Data are expressed as a percentage of their own maximum. **B** Data are expressed as a percentage reference contraction to 50mM KCl. Each point represents mean ± s.e.mean. All CCRCs constructed in the presence of 0.1mM L-NAME.

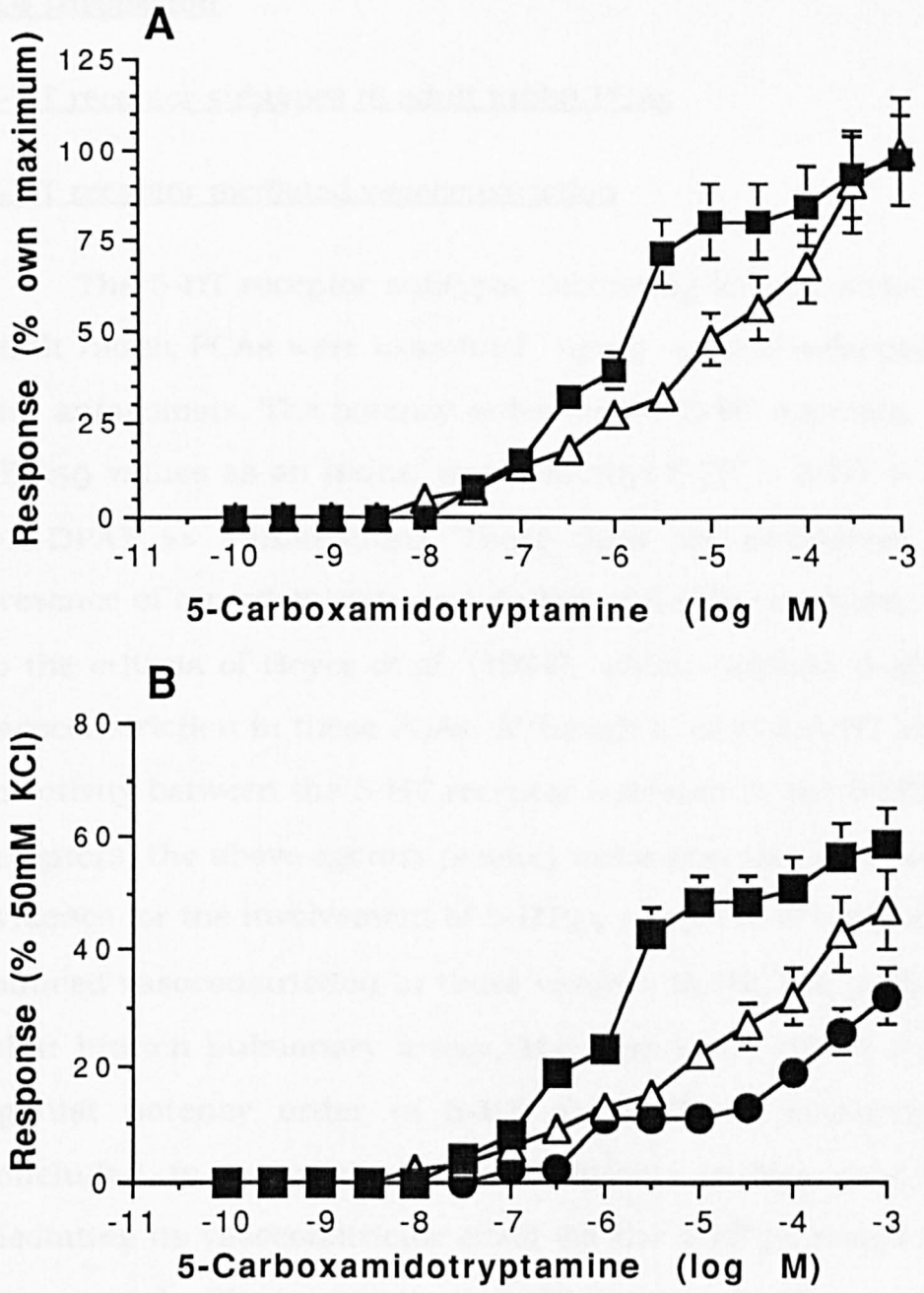

Figure 3.18 Effect of selective antagonists on 5-CT-induced contraction in adult rabbit PRAs. CCRCs to 5-CT (■, n = 7/7), and in the presence of 0.2μM SB224289 (●,n= 7/7), and 0.5μM BRL15572 (△,n= 7/7). **A**. Data are expressed as a percentage of their own maximum. **B** Data are expressed as a percentage reference contraction to 50mM KCl. Each point represents mean ± s.e.mean. All CCRCs in the presence of 0.1mM L-NAME.

3.4 Discussion

5-HT receptor subtypes in adult rabbit PCAs

5-HT receptor mediated vasoconstriction

The 5-HT receptor subtypes mediating vasoconstriction in the adult rabbit PCAs were examined using several selective agonists and antagonists. The potency order for the 5-HT agonists, using the *p*EC50 values as an index, was α-methyl 5-HT = 5-HT > 5-CT > 8-OH-DPAT >> sumatriptan. These data are consistent with the presence of a predominant population of 5-HT_2 receptors, according to the criteria of Hoyer *et al.* (1994), which mediate 5-HT-induced vasoconstriction in these PCAs. Although α-methyl-5-HT shows little selectivity between the 5-HT receptor subtypes in the 5-HT_2 class of receptors, the above agonist potency order provides some supporting evidence for the involvement of 5-HT_{2A} receptors in mediating 5-HT-induced vasoconstriction in these vessels. In the rat, isolated extra-lobar branch pulmonary artery, MacLean *et al.* (1996) reported an agonist potency order of 5-HT >> 5-CT >> sumatriptan and concluded, in conjunction with antagonist studies, that 5-HT was mediating its vasoconstrictor effect via the 5-HT_{2A} receptor class in these vessels. The sensitivity to 5-HT reported in this previous study was ~5.3, which is markedly less than the sensitivity to 5-HT in the studies with the adult rabbit large isolated pulmonary artery, highlighting a significant species difference in the sensitivity of capacitance pulmonary arteries to the vasoconstrictor action of 5-HT. Ketanserin in these vessels behaved as a competitive antagonist. The sub-nM affinities observed for ketanserin against 5-HT in the rabbit PCAs, where the estimated pK_B values ranged from 8.6-8.95, strongly suggest that the 5-HT receptor mediating 5-HT-

induced vasoconstriction in these vessels is the 5-HT_{2A} receptor subtype (Hoyer *et al.*, 1994). This range of apparent pK_B values is consistent with the affinity of ketanserin for the 5-HT_{2A} receptor in other rabbit vascular preparations including the rabbit aorta (pK_B ~8.6; Leff & Martin, 1986), and rabbit carotid artery (pK_B ~8.7; Black *et al.*, 1981). It has been suggested that ketanserin at μM concentrations has some affinity for the 5-HT_{1D} receptor (Kaumann *et al.*, 1993; 1994). The possibility that μM concentrations of ketanserin may inhibit 5-HT_{1D}receptor-mediated contractions of 5-HT in these adult rabbit PCAs is unlikely as the selective 5-$HT_{1B/1D}$ receptor antagonist GR55562 did not inhibit 5-HT-induced contractions in this study, suggesting an absence of 5-HT_{1D} receptors in this preparation.

MacLean *et al.* (1996), reported previously that the affinity for ketanserin against 5-HT in control rat extra-lobar pulmonary arteries was ~7.8. The authors concluded that in those vessels, the 5-HT_{2A} receptor was predominantly present but could not exclude the possibility of the presence of another 5-HT receptor subtype. In the left branch calf pulmonary artery, Frenken and Kaumann (1984) demonstrated that the high affinity of ketanserin (pK_B ~9.4) against 5-HT indicated the presence of 5-HT_{2A} receptors mediating 5-HT-induced vasoconstriction in this preparation. In human large isolated pulmonary arteries, ketanserin had no significant effect on the sensitivity of the preparation to 5-HT at the pEC_{50} level but did reduce the maximum contractile response to 5-HT (MacLean *et al.*, 1996b), suggesting the involvement of a 5-HT receptor subtype other than 5-HT_{2A} in mediating the vasoconstrictor actions of 5-HT in human pulmonary arteries and highlighting another species difference. GR55562 has previously been shown to be a selective

antagonist for the 5-HT_{1B} and 5-HT_{1D} receptor subtypes (Connor *et al.*, 1995), giving an affinity value of ~7.9 against sumatriptan-induced contractions of the monkey and dog isolated basilar arteries. In the study on isolated human PCAs, MacLean *et al.*(1996b) provided conclusive evidence that the 5-HT_1-like receptor mediating vasoconstriction in these vessels was either the 5-HT_{1B} or 5-HT_{1D} receptor as GR55562 produced a truly competitive antagonism against 5-HT with an affinity (pA_2) value against the 5-HT_{1D} and 5-HT_{1B} agonist sumatriptan of ~8.9. In the adult rabbit PCAs however, GR55562 had no effect on the 5-HT-induced vasoconstriction, showing that a 5-HT_{1B} or 5-HT_{1D} receptor does not mediate the contractile response.

Methiothepin has a pA_2 value of ~9 against 5-HT_{2A} receptors and about a 10-fold less affinity for 5-HT_1 receptors (Hoyer *et al.*, 1994). Here, methiothepin (1-10nM), significantly shifted the CCRC to 5-HT to the right, suggesting further the presence of a population of 5-HT_{2A} receptors, due to the non-competitive nature of methiothepin, a value for the affinity could not be calculated. 5-HT generally mediates its vasoconstrictor effect by activating 5-HT_{2A} or 5-HT_1 receptors. There is some evidence to suggest that 5-HT may mediate its vasoconstrictor actions indirectly in certain rabbit arteries (Black *et al.*, 1981; Grandaw and Purdy, 1996; Purdy *et al.*, 1987). Previous studies have shown that in rat pulmonary conduit arteries, 5-HT may activate α_1-adrenoceptors (Ogawa *et al.*, 1995), mediating an indirect vasoconstrictor action of 5-HT. This was recently confirmed by Shaw *et al* (2000) who reported that the α_1-receptor antagonist prazosin, at 10nM, produced a potent inhibition of 5-HT-induced contractions in the rat isolated extralobar pulmonary artery. In preliminary studies in the adult rabbit PCAs

investigated in this thesis, prazosin (1μM) did not have any significant effect on responses to 5-HT in these vessels (n = 5/5). Hence α_1-adrenoceptors are unlikely to contribute to the observed responses to 5-HT in this study even though a previous study has shown that this preparation contains a significant population of α_1 and α_2 -adrenoceptors which mediate the vasoconstrictor actions of noradrenaline (MacLean *et al.*, 1993). Taken together, the results of the current studies using isolated rabbit PCAs strongly suggest that in the adult rabbit PCA, vasoconstriction is mediated via the 5-HT_{2A} receptor subtype. This is consistent with the observations that 5-HT_{2A} receptors mediate the vasoconstrictor actions of 5-HT in a wide range of rabbit conduit arteries such as the carotid (Black *et al.*, 1981), aorta (Leff & Martin, 1986) and femoral (MacLennan & Martin, 1992) arteries. This study demonstrated a considerable species difference compared to human conduit pulmonary arteries where 5-HT mediates its potent vasoconstrictor action predominantly through 5-HT_{1B} or 5-HT_{1D} receptors. Ullmer *et al.* in 1995 identified the mRNA for 5-HT_{1B} receptors (as well as for 5-HT_{2A}, 5-HT_7 and 5-HT_{2B} receptors) using RT-PCR suggesting the importance of the 5-HT_{1B} receptor in the human PCAs. In comparison, the studies in chapter 4 of this thesis also provide evidence that the 5-HT_{1B} receptor mediates 5-HT-induced vasoconstriction in human PRAs, importantly, at physiological and pathophysiological concentrations of 5-HT.

Several studies on other rabbit isolated vessels including the rabbit saphenous vein (Valentin *et al.*, 1996) and rabbit basilar artery (Trezise, 1992) have demonstrated a profound inhibitory effect of the endothelium and endothelial-derived nitric oxide on contractions to 5-HT and 5-HT_{1B}/5-HT_{1D} agonists. More importantly for the

pulmonary circulation, in bovine isolated large pulmonary arteries, MacLean *et al.*, (1994) demonstrated that either removal of the endothelium or inhibition of NOS with L-NAME significantly increased the sensitivity to sumatriptan. The presence of L-NAME also significantly increased the maximum contractile response to sumatriptan. Here, this possibility was addressed by examining the effect of mechanical endothelial cell removal and eNOS inhibition using L-NAME on the vasoconstrictor responses to 5-HT, 5-CT and sumatriptan. Neither endothelial cell removal nor eNOS inhibition significantly affected the sensitivity to 5-HT, 5-CT or sumatriptan indicating that in contrast to bovine PCAs, NO or a functional endothelium did not mask any 5-HT_1 (specifically 5-HT_{1B} or 5-HT_{1D}) receptor mediated vasoconstriction in this preparation.

5-HT receptor mediated vasodilation

Here, 5-HT receptor-mediated vasodilation in pre-contracted adult rabbit PCAs using selective 5-HT agonists and antagonists was also studied. In 1975, Eyre *et al.*, first demonstrated that sub-μM concentrations of 5-HT evoked a relaxation in isolated sheep pulmonary vein pre-contracted with histamine, similarly this vasodilator effect of 5-HT was later confirmed in the goat pulmonary vein (Chand, 1981). More recently, 5-HT was shown to elicit an endothelium-dependent vasodilation in pig pulmonary arteries (Glusa & Richter *et al.*, 1993). 5-HT receptors are therefore, known to mediate vasodilation in blood vessels either directly via 5-HT receptors located on the vascular smooth muscle (Cocks & Arnold, 1992; Leung *et al.*, 1996) or indirectly via endothelial released NO (Bodelson *et al.*, 1993; Glusa & Roos, 1996; Glusa & Richter, 1993). In the studies where the 5-HT-induced vasoconstriction in rabbit

PCAs was investigated, the 5-HT_{2A} receptor was shown to mediate a potent vasoconstrictor effect of 5-HT. For the vasorelaxant studies described here, all experiments were conducted in the presence of 0.1μM ketanserin in order to inhibit any vasoconstrictor responses to 5-HT agonists. 5-CT and 5-HT both produced a significant concentration-dependent relaxation in the pre-contracted adult rabbit PCAs, suggesting an involvement of a 5-HT_1-like receptor. However the lack of vasodilator effect of the 5-HT_{1B} and 5-HT_{1D} receptor agonist sumatriptan suggests that these receptors are not involved in mediating 5-HT-receptor-induced vasodilation in this preparation. This was confirmed using the selective antagonist GR55562 which did not significantly affect the 5-CT-induced vasodilation in this preparation.

Glusa and Richter (1993) demonstrated endothelium-dependent relaxation in porcine pulmonary arteries and concluded that this vasodilation was mediated via what was termed the '5-HT_{1C}-like' receptor. This 5-HT receptor subtype in the porcine pulmonary artery displays a markedly similar pharmacological profile to the 5-HT_{2B} receptor. This was supported by the observations of Ullmer *et al.*, (1995) who demonstrated the presence of mRNA for this receptor in porcine pulmonary arteries. BW 723C86 has recently been described as the most selective 5-HT_{2B} receptor agonist to date (Kennet *et al.*, 1996). This compound did not elicit any significant vasorelaxation in the rabbit PCAs in these studies, suggesting a lack of this receptor in mediating vasodilation in this preparation, in direct contrast to the porcine pulmonary artery. The observation that endothelial cell removal did not affect 5-HT agonist-induced vasorelaxation in the rabbit PCAs in these studies also provides evidence against the involvement of any 5-HT receptor located on the

endothelium.

Recently, the novel 5-HT7 receptor has been described and has been found on the vascular smooth muscle of several preparations including the canine coronary (Terron, 1996) and cerebral arteries (Terron, 1998) and *Cynomolgus* monkey jugular vein (Leung *et al*, 1996). In the rabbit, this receptor is thought to mediate the direct relaxation of the femoral vein (Martin & Wilson, 1995). This receptor is thought to mediate smooth muscle relaxation directly being located on the vascular smooth muscle. Several binding studies have previously shown that 5-HT and 5-CT display nM affinities for rat and human cloned 5-HT7 receptors (Bard *et al.*, 1993; Ruat *et al.*, 1993). Their *p*IC50 values in the adult rabbit PCAs in the studies described here (~6.5 for 5-HT and ~7.6 for 5-CT) are similar to those mediating vasodilation in the canine coronary artery (~6.7 and ~7.6 for 5-HT and 5-CT respectively; Terron, 1996). This may highlight important differences between functional and cloned 5-HT7 receptors.

Several antipsychotic drugs have been shown to behave as antagonists at this receptor. Spiperone displays a high binding affinity for the cloned 5-HT7 receptor (Roth *et al.*, 1994) and 5-HT7 receptors located on vascular smooth muscle which stimulate adenylate cyclase and mediate vascular relaxation directly (Hoyer *et al.*, 1994; Leung *et al.*, 1996). There was good agreement between spiperones antagonist affinity against 5-CT (pK_B ~6.8) and the antagonist affinity of spiperone in both the primate jugular vein (pK_B ~7.3; Leung *et al.*, 1996) and the canine coronary artery (pK_B ~6.9; Terron, 1996), strongly indicating that 5-HT receptor-induced vasodilation in pre-contracted adult rabbit PCAs is mediated via the 5-HT7 receptor. Spiperone also has a high affinity for 5-HT_{2A} receptors (Leff & Martin, 1986) but the presence of ketanserin in

these studies rules out 5-HT_{2A} receptor-mediated effects. Further evidence for the presence of the 5-HT7 receptor in this study was provided by the antagonism by pimozide and risperidone of the 5-CT-induced relaxation. Risperidone markedly attenuated the response to 5-CT in these vessels and has been shown to exhibit a high selectivity for the cloned 5-HT7 receptor (Roth *et al.*, 1994). In addition, results from chapter 5 of this thesis demonstrate that PRAs from 7 day old rabbits exhibit direct relaxation of the pulmonary artery, possibly through the 5-HT7 receptor. In common with the 5-HT7 receptor, 5-HT_6 receptors have also been shown to activate adenylate cyclase (Ruat *et al.*, 1993), this second messenger action is consistent with direct smooth muscle relaxation. The presence of this receptor cannot be definitely ruled out because selective antagonists for the 5-HT_6 receptor are not currently available.

5-HT receptor-mediated responses in adult rabbit PRAs

Endothelial cell integrity

In contrast to the results of the studies in the adult rabbit PCAs, ACh was unable to evoke a relaxation response in the pre-contracted adult rabbit PRAs. In some vessels, ACh induced a small vasoconstrictor response. This is in agreement with Sada *et al.*, (1987) who reported ACh-induced contraction in rabbit small pulmonary vessels. One possibility for this is either a lack of endothelial muscarinic receptors which mediate the release of NO, or a predominance of contractile muscarinic receptors located on the pulmonary arterial smooth muscle which override any vasodilator effects of ACh in this preparation. Consistent with this, earlier studies, using *in situ* hybridisation and autoradiographic mapping, demonstrated a predominance of muscarinic M_4-receptors in

pulmonary vessels of rabbit lungs (Mak, *et al.*, 1993). Other agents known to cause endothelium-dependent relaxation such as substance P and A23187 were also without effect in the rabbit PRAs. The PRAs were not insensitive to NO however as the endothelium-independent, NO-donating, vasodilator SNP evoked a concentration-dependent relaxation in these vessels.

Vasoconstrictor response to 5-HT

The identity of the 5-HT receptors mediating 5-HT-induced vasoconstriction in adult rabbit pulmonary resistance arteries was investigated. In the absence of eNOS inhibition, the rank order of agonist potency was α-methyl-5-HT = 5-HT > 5-CT = sumatriptan which is indicative of the presence of 5-HT_2 receptors. The antagonist studies, however indicate that 5-HT may mediate its vasoconstrictor activity in adult rabbit PRAs via either a different receptor, or via a heterogeneous population of 5-HT receptors. Ketanserin normally has a nM affinity for the 5-HT_{2A} receptor, acting as a competitive antagonist. In this study, however, the inhibition in the presence of ketanserin (0.1μM; ~7-fold decrease in sensitivity) was not as marked as would be expected if 5-HT was interacting solely with the 5-HT_{2A} receptor. This is in contrast with the studies in the adult rabbit PCAs where ketanserin antagonised 5-HT-evoked contractions with nM affinity, and 5-HT mediated its effects exclusively via the 5-HT_{2A} receptor. This suggests that there is a transition in 5-HT receptor subtypes mediating pulmonary arterial vasoconstriction in the rabbit from a single population in the large conductance vessels to a mixed population in the smaller pulmonary resistance arteries. This is discussed further below.

One other possibility is that 5-HT may partly mediate

contraction in these vessels indirectly via activation of α-adrenergic receptors and this has been reported in rat pulmonary arteries (Ogawa *et al.*, 1995). Docherty and MacLean (1998) recently reported that in adult rabbit PRAs, of equivalent size to those in this present study, the endogenous α-adrenoceptor agonist noradrenaline does not cause vasoconstriction. This suggests a lack of functional α-adrenoceptors in these vessels. The involvement of these receptors in mediating the vasoconstrictor actions of 5-HT directly in the adult rabbit PRAs is, therefore unlikely.

Influence of endothelium derived nitric oxide on 5-HT-induced contractions

In this study, the results demonstrated that the NOS inhibitor L-NAME, increases the sensitivity to 5-HT and 5-CT in adult rabbit small muscular, pulmonary resistance arteries.

In previous studies, several investigators have reported an inhibitory effect of the endothelium and NO on 5-HT receptor mediated responses in several vascular preparations from the rabbit. In the rabbit isolated saphenous vein, for example, responses to 5-HT, 5-CT and sumatriptan were potentiated in the presence of the NOS inhibitor L-NAME (Valentin *et al.*, 1996) and in the absence of a functional endothelium, suggesting the endothelial release of NO counteracted the constrictor responses of 5-HT receptor agonists. A similar observation was evident in the rabbit basilar artery where the removal of the endothelium markedly increased the sensitivity and maximum contractile response to 5-HT (Trezise, *et al.*, 1992). L-NAME has been reported to elevate pulmonary artery pressure and pulmonary vascular resistance in rabbit lungs *in vitro* (Wiklund *et al.*, 1990) suggesting a role for basal release of NO in maintaining low

resting pulmonary vascular tone in the rabbit. This could explain why L-NAME potentiated the contractile response to the 5-HT agonists in this present study. In human pulmonary arteries, L-NAME has been shown to potentiate the vasoconstrictor responses to sumatriptan (MacLean *et al.*, 1993) and in bovine pulmonary arteries 5-HT_{1B} or 5-HT_{1D} receptor -mediated contractions with sumatriptan are also potentiated in the presence of L-NAME (MacLean *et al.*, 1994). One interpretation of the current studies is that 5-HT may cause release of NO in the adult rabbit PRAs via an endothelium-located receptor, thus masking the true vasoconstrictor effects in this preparation and in chapter 5 of this thesis this is demonstrated in 4 day and 7 day old neonatal rabbit PRAs. This interpretation, however, is unlikely as I was unable to demonstrate any vasodilator effects of 5-HT or 5-CT in pre-contracted adult rabbit PRAs.

The role of basal NO in the pulmonary circulation seems to be dependent on species. For example, in the dog, L-NAME does not have any effect on pulmonary vascular resistance (Nishiwaki *et al.*, 1992) whereas in the rabbit NOS inhibition was shown to increase the baseline pulmonary arterial pressure (Wiklund *et al.*, 1990). Importantly in man, infusing the NOS inhibitor L-NMMA results in an increased pulmonary vascular resistance (Stamler *et al.*, 1994) as well as a decrease in pulmonary blood flow (Celermajer *et al.*, 1994). This suggests that basal NO plays an important role in maintaining a low pulmonary vascular tone in man. NO mediates its effects predominantly via the generation of the cyclic nucleotide second messenger cyclic 3'5'-guanosine monophosphate (cGMP; Ignarro, 1989). It is possible that cGMP may play a crucial role in the responsiveness of pulmonary arteries to the activation of receptors coupled to G_i-proteins, for example NOS inhibition, resulting in

decreased generation of cGMP, causes an unmasking of α_2-adrenoceptor (G_i-linked receptor)-mediated vasoconstriction of rabbit isolated pulmonary arteries (MacLean *et al.*, 1993a,b). As described in chapter 1 of this thesis, 5-HT_1 receptors also mediate their effects via a G_i-coupled second messenger system. In accordance with this, responses to sumatriptan, working through G_i-coupled receptors, have been shown to be potentiated in both bovine and human pulmonary arteries when levels of cGMP are decreased through NOS inhibition (MacLean, *et al.*, 1994; MacLean *et al.*, 1993c). The results of the current studies also suggest that decreased cGMP levels are required to 'uncover' responses to 5-HT_1 receptor mediated vasoconstriction and produce a pharmacological 'synergism' increasing 5-HT-induced vasoconstriction (MacLean *et al.*, 1999). The importance of this pathophysiologically in pulmonary hypertension is highlighted by the fact that in the CHPHT rat, decreased levels of cGMP (along with elevated tone) have been reported (MacLean *et al.*, 1996) along with an increased 5-HT_{1B} receptor-mediated vasoconstriction in isolated pulmonary arteries. In chapter 6 of this thesis this phenomenon was investigated more fully in the normal and CHPHT rat.

In the presence of NOS inhibition, the agonist potency order in the adult rabbit PRAs was 5-HT = α-methyl-5-HT $\geq$ 5-CT $\geq$ sumatriptan which is not typical for a single population of 5-HT receptor subtype (Hoyer *et al.*, 1994). These findings are also in contrast to the 5-HT agonist potency order in the adult rabbit PCAs described earlier in this chapter, which, along with the antagonist studies, provided evidence for the presence of a single population of 5-HT receptors mediating 5-HT-induced vasoconstriction, namely the 5-HT_{2A} receptor. Even in the presence of L-NAME, the results with

ketanserin and GR55562 in the adult rabbit PCAs strongly suggested a single population of 5-HT_{2A} receptors exclusively mediated the vasoconstrictor action of 5-HT. The established criteria for 5-HT_1-receptor mediated responses, in terms of agonist potency order, is 5-CT > 5-HT ≥ sumatriptan ≥ α-methyl-5-HT (Hoyer *et al.*, 1994). Clearly, in the rabbit PRAs, this was not the case. It is possible that the adult rabbit PRAs express a heterogeneous population of 5-HT receptors contributing to 5-HT-evoked vasoconstriction. Evidence for this co-existence of different vasoconstrictor 5-HT receptor subtypes exists in other rabbit vascular tissues including the cerebral arteries (Deckert *et al.*, 1994; Ellwood & Curtis, 1997). It is shown here that in the adult rabbit PRAs, ketanserin inhibited responses to concentrations of 5-HT greater than 30nM. Responses elicited by lower concentrations of 5-HT were resistant to ketanserin (10nM & 0.1μM). This does suggest the involvement of two 5-HT receptor populations evoking vasoconstriction in these arteries; non-5-HT_{2A} receptors activated by low concentrations of 5-HT and 5-HT_{2A} receptors activated by higher concentrations. Furthermore, GR55562 antagonized responses to all concentrations of 5-HT, including those which were ketanserin resistant, suggesting that a significant population of 5-HT_{1B} or 5-HT_{1D} receptors contributes towards 5-HT-induced vasoconstriction. Hence, in the adult rabbit PRA, both the 5-HT_{2A} receptor and either the 5-HT_{1B} receptor or the 5-HT_{1D} receptor mediate vasoconstriction. Recently, in a study of cloned rabbit 5-HT_{1B} and 5-HT_{1D} receptors Bard *et al* (1996), reported that ketanserin has an affinity for 5-$HT_{1B/1D}$ receptors at submicromolar concentrations. Ketanserin showed approximately 23-fold selectivity for 5-HT_{1D} receptors over 5-HT_{1B} receptors. These findings indicate that, at the higher concentration of ketanserin used in the present

study (1μM), 5-HT_{1D} receptors would have been greatly antagonised. The observation that 1μM ketanserin antagonized the response to 5-HT in the PRAs suggests that a population of 5-HT_{1D} receptors may also be present in these vessels. The involvement of a population of 5-HT_{1D} receptors in mediating 5-HT-induced vasoconstriction in adult rabbit PRAs is discussed further below. The study by Bard *et al* (1996) also reported that human and rabbit recombinant 5-HT_{1B} and 5-HT_{1D} receptors display a significant interspecies similarity in their ligand binding profiles, suggesting that responses in rabbit preparations mediated by these two receptors may provide information that is relevant to the pharmacology of the 5-HT_{1B} or 5-HT_{1D} receptor in man. In human large pulmonary arteries strong evidence exists that it is the 5-HT_{1B} or the 5-HT_{1D} receptor which mediates vasoconstriction. Templeton *et al.*, (1993; 1994) have shown previously that the 5-HT_1 receptor agonist 5-CT is more potent than 5-HT and that sumatriptan is equipotent to 5-HT in these vessels, indicative of a 5-HT_{1B} receptor mediated response. More recently, the 5-HT_{1D} and 5-HT_{1B} receptor antagonist GR55562 was shown to inhibit both 5-HT and sumatriptan-induced contractions in a true competitive manner; whereas, the selective 5-HT_{2A} receptor antagonist ketanserin and the non-selective antagonist methiothepin did not affect the sensitivity of these agonists (MacLean *et al.*, 1996b). this was subsequently confirmed by Cortijo *et al.*, (1997).

In chapter 4 of this thesis, the importance of the 5-HT_{1B} receptor in mediating 5-HT-induced contractions in human small pulmonary arteries was demonstrated and recent RT-PCR analysis detected a strong signal of the mRNA transcript for the 5-HT_{1B}, but not the 5-HT_{1D} receptor, in these vessels (Morecroft *et al.*, 1999). In the adult rat PRA, both the 5-HT_{2A} receptor and the 5-HT_{1B} or the 5-

HT_{1D} receptor mediate vasoconstriction but it is the 5-HT_{2A} receptor which dominates (MacLean *et al.*, 1996). Hence, there is species variation in the 5-HT receptor/s mediating vasoconstriction in small muscular pulmonary arteries. In chapter 5 of this thesis, the results demonstrated that 5-HT mediates its vasoconstrictor action in PRAs of perinatal rabbits, predominantly via the 5-HT_{2A} receptor type, thus providing evidence for a maturational change in receptors mediating 5-HT-induced pulmonary arterial vasoconstriction within the same species.

Until recently, the pharmacological profile of the 5-HT_{1D} and 5-HT_{1B} receptor was almost identical, both acting as G_i-protein coupled receptors with the majority of agonists and antagonists being non-selective for both receptor subtypes. SB224289 has recently been reported as a selective antagonist with high affinity for the 5-HT_{1B} receptor (Roberts *et al.*, 1997; Verheggen *et al.*, 1998) and BRL 15572 as a selective antagonist at the 5-HT_{1D} receptor (Price *et al.*, 1997; DeVries *et al.*, 1999). In the results of the studies of this chapter, both compounds antagonised the 5-CT-induced contractions in the adult rabbit PRAs, SB224289 being more potent than BRL15572. It was difficult to obtain an accurate estimate of the affinity of either of these compounds from the experiments. Both displayed a non parallel rightward shift in the CCRC to 5-CT suggesting the presence of both the 5-HT_{1B} and 5-HT_{1D} receptors in this preparation. One interpretation of the data with SB224289 could be that this compound was inhibiting 5-HT_{2A} receptor-mediated effects at the higher concentrations of 5-CT. This is unlikely as this antagonist exhibits a low affinity for recombinant 5-HT_{2A} receptors (Roberts *et al.*, 1997).

The results in this chapter with BRL15572 are in contrast to

those described in chapter 4 in human PRAs where BRL15572 was without any significant effect. Some investigators have suggested that the predominant 5-HT_1 receptor mediating vascular contraction is the 5-HT_{1B} receptor subtype given that molecular studies display a predominance of expression of this subtype over that of the 5-HT_{1D} subtype in vascular tissue (Hamel *et al.*, 1993; Verheggen *et al.*, 1998; Nilsson *et al.*, 1999). Furthermore, DeVries *et al* (1998) have shown that SB224289 but not BRL15572 blocks 5-HT-induced decrease in canine external carotid blood flow *in vivo*. Coupled with evidence from *in vitro* pharmacology (Verheggen *et al.*, 1998); these findings provide strong evidence for the importance of the 5-HT_{1B} receptor over the 5-HT_{1D} receptor in mediating 5-HT_1 receptor activated vasoconstriction. In the studies in the adult rabbit PRAs however, involvement of the 5-HT_{1D} receptor can not be completely ruled out.

As mentioned earlier, the overall pulmonary arterial response to 5-HT will be dependent on the 5-HT receptor subtype, vascular location and underlying pulmonary vascular tone. As there was no evidence for 5-HT receptor mediated vasodilation in the adult rabbit PRAs, neither in the presence or absence of NOS inhibition, it is probable that the 5-HT-mediated contractile response would predominate over any vasodilator response *in vivo* in the rabbit pulmonary circulation. This has been confirmed by Deuchar *et al* (1998), who reported that i.v. administration of 5-HT resulted in a significant, concentration dependent increase in pulmonary artery pressure above baseline in the anaesthetised rabbit.

One well characterised model of left ventricular dysfunction (LVD) is the rabbit coronary-ligation model (Pye *et al.*, 1996; Denvir, *et al.*, 1996) which has recently been reported as a model for pulmonary hypertension secondary to LVD (Deuchar *et al.*, 1998)

where the authors reported elevated baseline pulmonary arterial pressure and a supersensitivity to 5-HT administration compared to control, sham-operated rabbits. Given that the studies in this chapter have demonstrated the presence of 5-HT_{1B}, also 5-HT_{2A} and possibly 5-HT_{1D} receptors in mediating vasoconstriction in the pulmonary resistance arteries of the adult rabbit, further examination, using the selective agonists and antagonists, of the role of 5-HT and its receptor subtypes in this model of PHT is warranted.

In conclusion, this study suggests that there is heterogeneity of 5-HT receptor subtypes mediating vasoconstriction in the pulmonary arteries of the adult rabbit along the pulmonary arterial tree. The larger conduit pulmonary arteries contain a single population of the 5-HT_{2A} receptor subtype which mediate a potent vasoconstriction in this preparation. 5-HT receptor-mediated vasoconstriction in these PCAs is not affected by endothelium derived NO or endothelium removal. The PCAs also contain a functional population of 5-HT receptors which mediate vasodilation in these vessels when pre-constricted and possibly mediate this action via the newly described 5-HT_7 receptor. In contrast, the results in the adult rabbit pulmonary resistance arteries are consistent with the mediation of 5-HT-induced vasoconstriction through two receptor populations, namely the 5-HT_{2A} receptor and 5-HT_{1B} receptor. The 5-HT_1 mediated responses are markedly influenced by the presence of endothelium-derived NO. This study highlights the importance of heterogeneity within the pulmonary circulation with regard to 5-HT receptors and underlines the importance of determining independently the 5-HT pharmacology of the pulmonary arteries that determine pulmonary vascular resistance especially in PHT, namely the small muscular pulmonary resistance arteries.

Chapter 4

5-Hydroxytryptamine (5-HT) receptors mediating contraction in human small muscular pulmonary arteries

4.1 Introduction

Several studies have suggested a role for 5-HT in the aetiology of pulmonary hypertension. As described in chapter 1 of this thesis, 5-HT is released from pulmonary neuroendocrine cells and from neuroepithelial bodies distributed throughout the airways. Secretion of large amounts of 5-HT from these cells has been shown to occur in response to airway hypoxia and therefore may contribute to secondary pulmonary hypertension (Johnson & Georgieff, 1989; Gould *et al.*, 1983). Elevated plasma levels of 5-HT have been reported in primary PHT (Hervé *et al.*, 1990; 1995) and isolated pulmonary arteries from PHT patients undergoing lung transplantation exhibit augmented vasoconstrictor responses to 5-HT (Brink *et al*, 1988). 5-HT is also linked to hypoxia-induced PHT in new-borns (Johnson & Georgieff, 1989) where a 34-fold increase in 5-HT-immunoreactive cells has been demonstrated and recently, increased 5-HT turnover has been observed in children with PHT secondary to congenital heart disease (Breuer *et al.*, 1996) as explained in chapter 1 of this thesis.

5-HT may promote pulmonary vascular smooth muscle cell proliferation as well as pulmonary arterial vasoconstriction and local micro thrombosis (Fanburg & Lee, 1997).

Ketanserin, the classic 5-HT_{2A} receptor antagonist, has had limited success in the treatment of pulmonary hypertension, for example in pulmonary hypertension secondary to platelet storage pool disease (Herve' *et al.*, 1990). Ketanserins' use, however, is based on the assumption that 5-HT_{2A} receptors mediate vasoconstriction in the human pulmonary circulation. A considerable amount of evidence exists to suggest that other 5-HT receptors may play an

important role in mediating 5-HT-induced pulmonary arterial vasoconstriction.

In 1992, McIntyre and colleagues studied the effect and duration of the 5-HT_{1B} and 5-HT_{1D} agonist sumatriptan on central haemodynamics and the coronary circulation in patients undergoing diagnostic coronary angiography. Systemic and pulmonary pressures were also measured. Sumatriptan produced a more pronounced vasopressor effect on the pulmonary pressure when compared with overall systemic pressure, suggesting the preferential involvement of the 5-$HT_{1B/1D}$ receptor in vasoconstriction of human pulmonary arteries. This was subsequently supported by MacLean *et al* (1996b) who provided evidence that 5-HT is a potent human pulmonary artery vasoconstrictor and that 5-HT-induced vasoconstriction in isolated human large pulmonary arteries is mediated, in part, through a 5-HT_1 receptor thought to be either the 5-HT_{1B} or the 5-HT_{1D} receptor (MacLean *et al.*, 1996b). In these human large PAs, the 5-HT_{1B} & $_{1D}$-selective antagonist GR55562 was the only antagonist tested that produced a true competitive antagonism of responses to both 5-HT and sumatriptan. These results were later supported by Cortijo *et al.*, (1997). Using RT-PCR, Ullmer *et al.*, (1995) identified the mRNA for 5-HT_{1B}, 5-HT_{2A}, 5-HT_7 and 5-HT_{2B} receptors in cells isolated from human large pulmonary arteries.

In the pulmonary hypertensive state, it is the small muscular pulmonary resistance arteries, however, which are the major contributors to increased pulmonary vascular resistance (Singhal *et al.*, 1973). Frid *et al.* (1997) demonstrated that the vascular smooth muscle cells of the human small muscular pulmonary resistance arteries are distinctly different phenotypically from those of the larger pulmonary arteries. In addition, McCulloch *et al.* (1996) and MacLean *et al.* (1994b) previously demonstrated a distinct heterogeneity

within the pulmonary circulation with regard to endothelin receptors, highlighting the observation that the pharmacology of small pulmonary arteries must be determined independently. Thus the aim of this chapter was to investigate the 5-HT receptor subtypes in human small muscular pulmonary arteries using several 5-HT receptor antagonists. The functional responses to 5-HT-receptor-evoked vasoconstriction in human small muscular pulmonary arteries (smPAs) were therefore examined using 5-HT, 5-CT (5-HT_1 non selective agonist) and sumatriptan. The 5-HT receptor antagonists used to characterise the 5-HT receptors mediating 5-HT-induced vasoconstriction were the 5-HT_{2A}-selective antagonist ketanserin (Leysen, *et al*,. 1982) the potent and selective 5-HT_{1B} and 5-HT_{1D} antagonist GR55562 (Connor *et al.*, 1995), the novel 5-HT_{1B}-selective antagonist SB224289 (Verheggen *et al.*, 1998) and the 5-HT_{1D} receptor selective antagonist BRL15572 (Price *et al.*, 1997). Using RT-PCR to compliment these pharmacological studies, Morecroft *et al.*, (1999) determined if the mRNA for the 5-HT_{2A}, 5-HT_{1B} and 5-HT_{1D} receptors was present in these vessels obtained from the same lung tissue samples. The results of that study are also presented here for direct comparison.

4.2 Methods

Human small muscular pulmonary arteries

Human peripheral lung tissue was obtained postoperatively from patients undergoing surgery for bronchial carcinoma (described in section 2.1.3) at the Western Infirmary, Glasgow and the Royal Infirmary, Glasgow. Human small muscular, intralobar pulmonary arteries (~ 250-300μm internal diameter) were promptly dissected out

from macroscopically normal samples of the lung tissue (see section 2.1.3). The smPAs were mounted as ring preparations (2mm long) on an isometric wire myograph, bathed at 37°C with a constant supply of 16%O_2/5%CO_2 (balance N_2). Using the procedures for normalisation explained in chapter 2, tension was applied to the vessels to give a transmural pressure equivalent of approximately 12-16 mmHg, which is similar to *in vivo* pressures of pulmonary arteries. All vessels were studied within a 12 hour postoperative time period.

Experimental Protocols

Following a 45-60 minute equilibration period under these conditions, the response of the smPAs to 50mM KCl was determined twice. When the contractile response to KCl had plateaued, the vessels were washed out a minimum of 6 times with fresh Krebs solution and further equilibration for 45-60 minutes. The endothelial cell integrity of the vessels was assessed by contraction with a sub-maximal concentration of phenylephrine (0.1μM) and subsequent addition of acetylcholine (ACh; 1 μM) once the contraction had plateaued. Only vessels displaying >70% relaxation were deemed as having an intact endothelium and used in the following studies. Upon washout (6 times with fresh Krebs) a further minimum 45 minutes equilibration was allowed either in the absence or presence of a selected concentration of a 5-HT antagonist. Cumulative concentration response curves (CCRCs) were then determined in the human smPAs for 5-HT, 5-carboxamidotryptamine, or sumatriptan. All CCRCs were performed in the ligand concentration range 1nM-300μM.

CCRCs to 5-HT and sumatriptan were determined in the presence of the 5-HT_{2A} receptor antagonist ketanserin (0.1μM), or the 5-$HT_{1B/1D}$ receptor antagonist GR55562 (1μM). CCRCs to

sumatriptan were determined in the presence of the 5-HT_{1B} receptor-selective antagonist SB224289 (0.2μM) or the 5-HT_{1D} receptor-selective antagonist BRL15572 (0.5μM). Concentrations of the various antagonists were selected from previous studies in other vascular preparations (see glossary III, p XXVII).

Note

Due to limitations of intermittent availability of tissue and in equipment and time it was not always possible to perform a control CCRC to a particular 5-HT agonist whilst studying the different antagonists. The data for the control curves have therefore been pooled over several protocols. No significant differences, however, were found in the control responses to the agonists when studied over the whole period of investigation. It was unfortunately not possible to study a range of antagonist concentrations in each tissue again due to the above mentioned constraints.

Analysis of data.

pEC_{50} values were calculated according to methods stated in chapter 2 and estimated pK_B values calculated assuming the antagonists behaves in a competitive manner and a maximum response to the agonist is achieved in the concentration range studied. Apparent pK_B values were calculated for a single stated concentration of antagonist according to the methods described in chapter 2. Result are expressed graphically as a percentage of their own maximum contraction, or as percentage of the reference contraction to the second application of 50mM KCl. Statistical analysis for the means of groups of data were made by Student's unpaired *t* test or where appropriate, one-way analysis of variance

(ANOVA) followed by an ad hoc post test; a $P<0.05$ was considered to be statistically significant. Throughout the results, data are expressed as mean ± SEM. n/n = number of ring preparations/ number of lung samples.

Results

The effective pressure to which the human smPAs were tensioned, along with the resulting measurement of internal diameter are shown in table 4.1.

	(n)	Internal diameter (μm)	Effective pressure (mmHg)
Human smPAs	(56)	243.9 ± 9.1	17.2 ± 0.5

Table 4.1 Internal diameter and effective pressure of human smPAs studied in this chapter. Values are mean ± s.e.mean. n = number of vessels.

50 mM KCl induced a contraction of 202 ± 21 mg wt tension (1.98 ± 0.21 mN) in a representative 36 vessels from at least n = 6 patients.

Endothelium integrity

1μM ACh evoked a 74 ± 7% reversal of 0.1μM PE-induced contraction; which induced a contractile response of 28 ± 4% of the reference contraction to 50mM KCl.

5-HT agonists

Figure 4.1 shows that the agonists 5-HT, 5-CT and sumatriptan produced concentration-dependent contractions in

human small muscular pulmonary arteries with pEC_{50} values of 7.0 ± 0.2, 7.1 ± 0.3 and 6.7 ± 0.1 respectively. There was no significant difference in the potency of these agonists in the smPAs. The corresponding maximum contractile responses for these agonists were 112 ± 19%, 62 ± 11% and 106 ± 10% (of the maximum response to 50mM KCl) and were not significantly different. The potency of the 5-HT_1 agonists strongly indicated the presence of vasoconstrictor 5-HT_1 receptors in these vessels.

Effect of 5-HT antagonists on 5-HT-receptor-induced contraction of human small muscular pulmonary arteries

All *p*EC values for 5-HT and sumatriptan in the presence of specific antagonists are summarised in Table 4.2.

Ketanserin (vs. 5-HT)

Figure 4.2 shows that ketanserin caused significant inhibition of 5-HT-evoked vasoconstriction at 5-HT concentrations > 0.1μM but was without effect at lower concentrations of 5-HT. This significant inhibition at 5-HT concentrations > 0.1μM can be clearly seen in Figure 4.2B ($P<0.05$ at 0.3, 1.0 and 10μM and $P<0.001$ at 3μM) where the maximum contraction evoked by 5-HT was reduced from 107 ± 11% (reference contraction to 50mM KCl (control)) to 49 ± 7% (in the presence of 0.1μM ketanserin); hence an estimate of the pK_B value could not be made for ketanserin against 5-HT-induced contraction in human smPAs. Ketanserin did not significantly affect the sensitivity to 5-HT (figure 4.2A; table 4.2) where the pEC_{50} value for

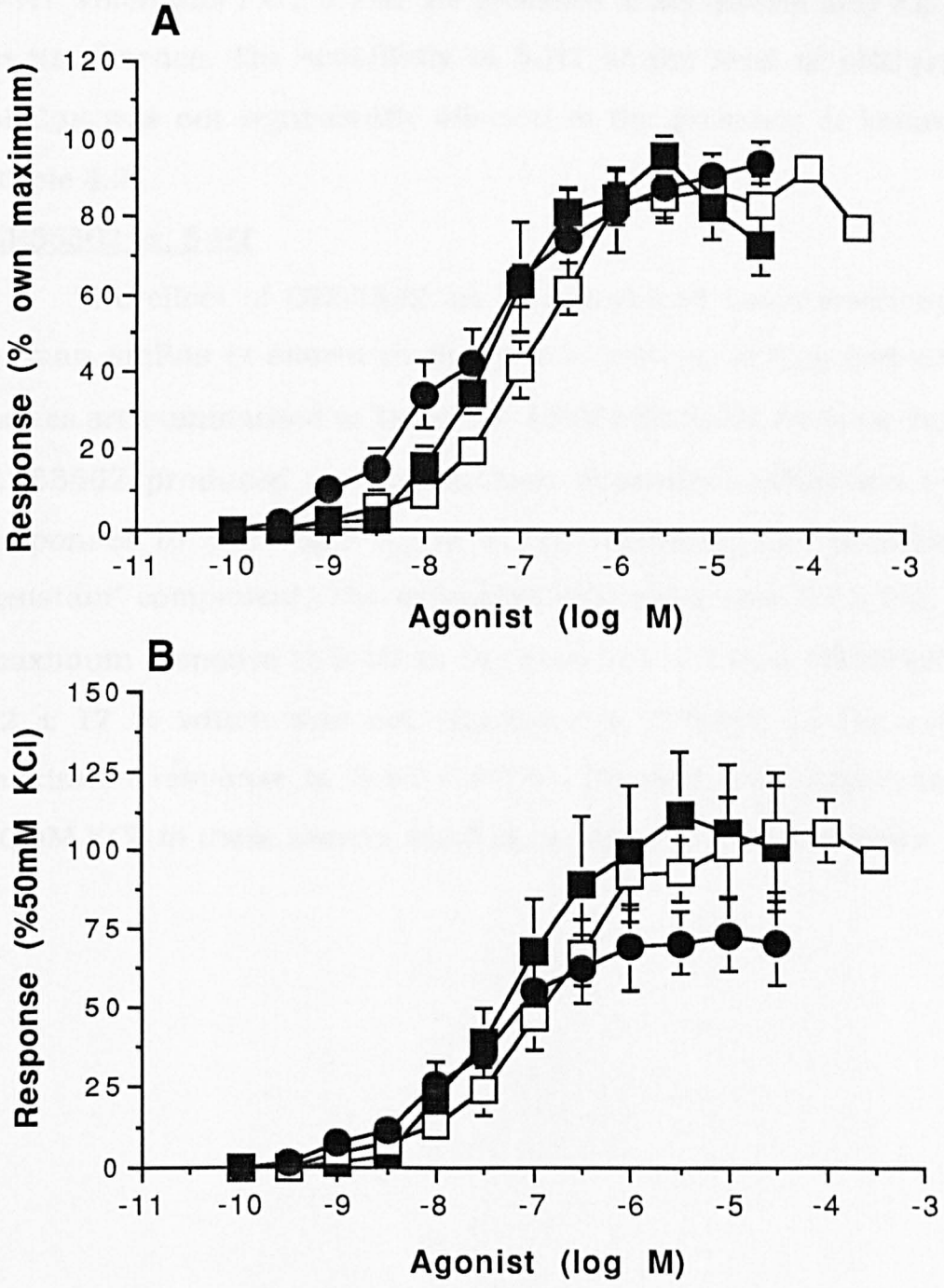

Figure 4.1 Vasoconstrictor responses to 5-HT receptor agonists in human smPAs. Responses to 5-HT (■, n = 9), 5-CT (●, n = 5), and sumatriptan (□, n = 11) are shown. A Data are expressed as a percentage own maximum contraction B Data are expressed as a percentage of the reference contraction to 50mM KCl in each vessel and shown as mean ± s.e.mean. n = number of patients.

5-HT which was 7.0 ± 0.1 in the presence of ketanserin and 7.0 ± 0.2 in its absence. The sensitivity of 5-HT at the level of pEC_{10} and pEC_{25} was not significantly affected in the presence of ketanserin (Table 4.2).

GR55562 vs. 5-HT

The effect of GR55562 on 5-HT-induced vasoconstriction in human smPAs is shown in figure 4.3. pEC_{10}, pEC_{25} and pEC_{50} values are summarised in Table 4.2. GR55562 (1μM). In these vessels, GR55562 produced a concentration dependent inhibition of the responses to 5-HT (see figure 4.3C), including the 'ketanserin-resistant' component. The estimated pK_B value was 7.7 ± 0.2. The maximum response to 5-HT in the presence of 1.0μM GR55562 was 72 ± 17 % which was not significantly different to the control maximum response to 5-HT (107 ± 11% reference contraction to 50mM KCl) in these human small muscular pulmonary arteries.

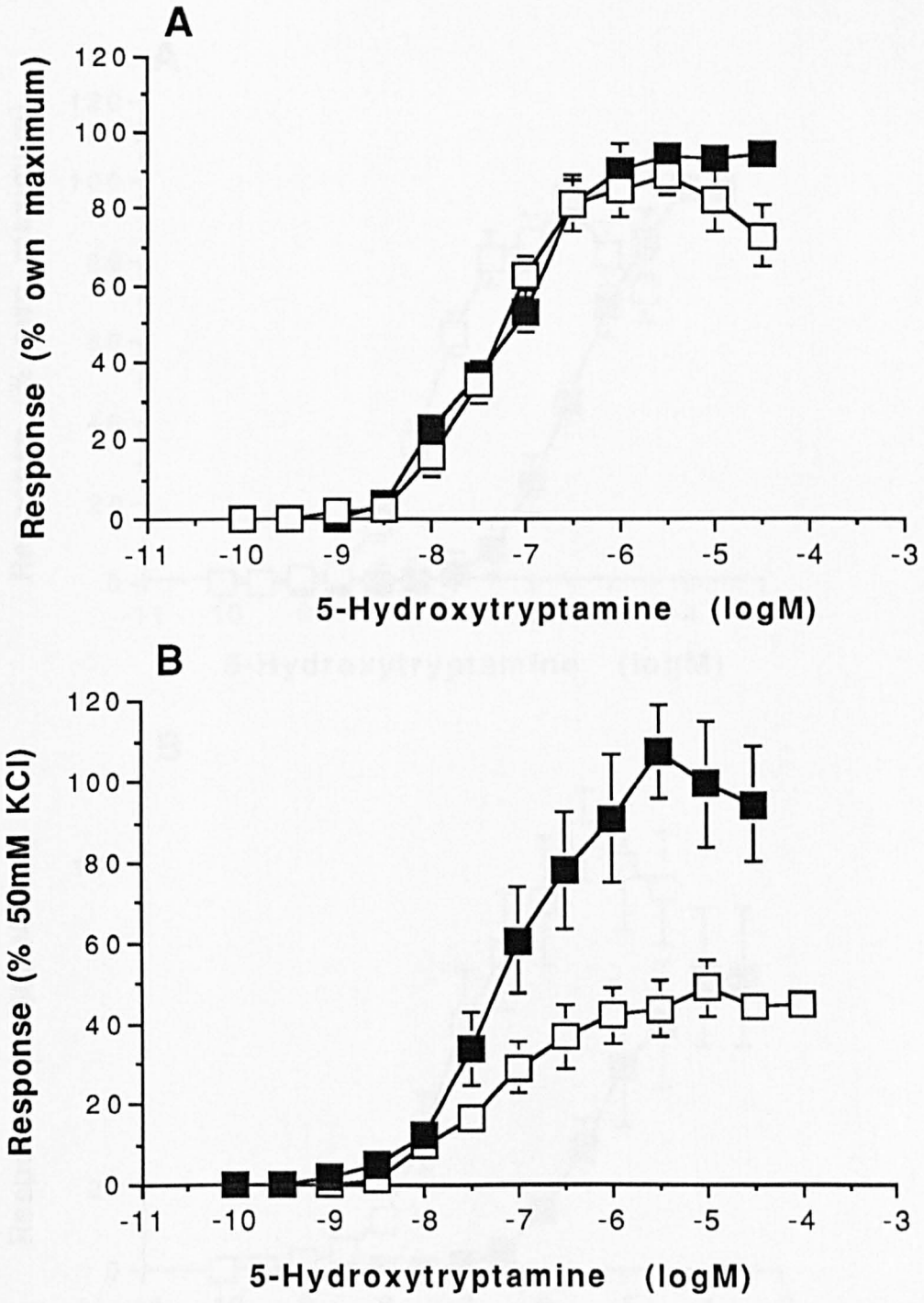

Figure 4.2 Effect of ketanserin on 5-HT-induced responses in human smPAs. CCRCs to 5-HT (■, n = 7/7) and in the presence of 1.0μM (□, n = 6/6) ketanserin. Data are expressed as a percentage own maximum contraction. B Data are expressed as a percentage reference contraction to 50mM KCl. Each point represents mean ± s.e.mean.

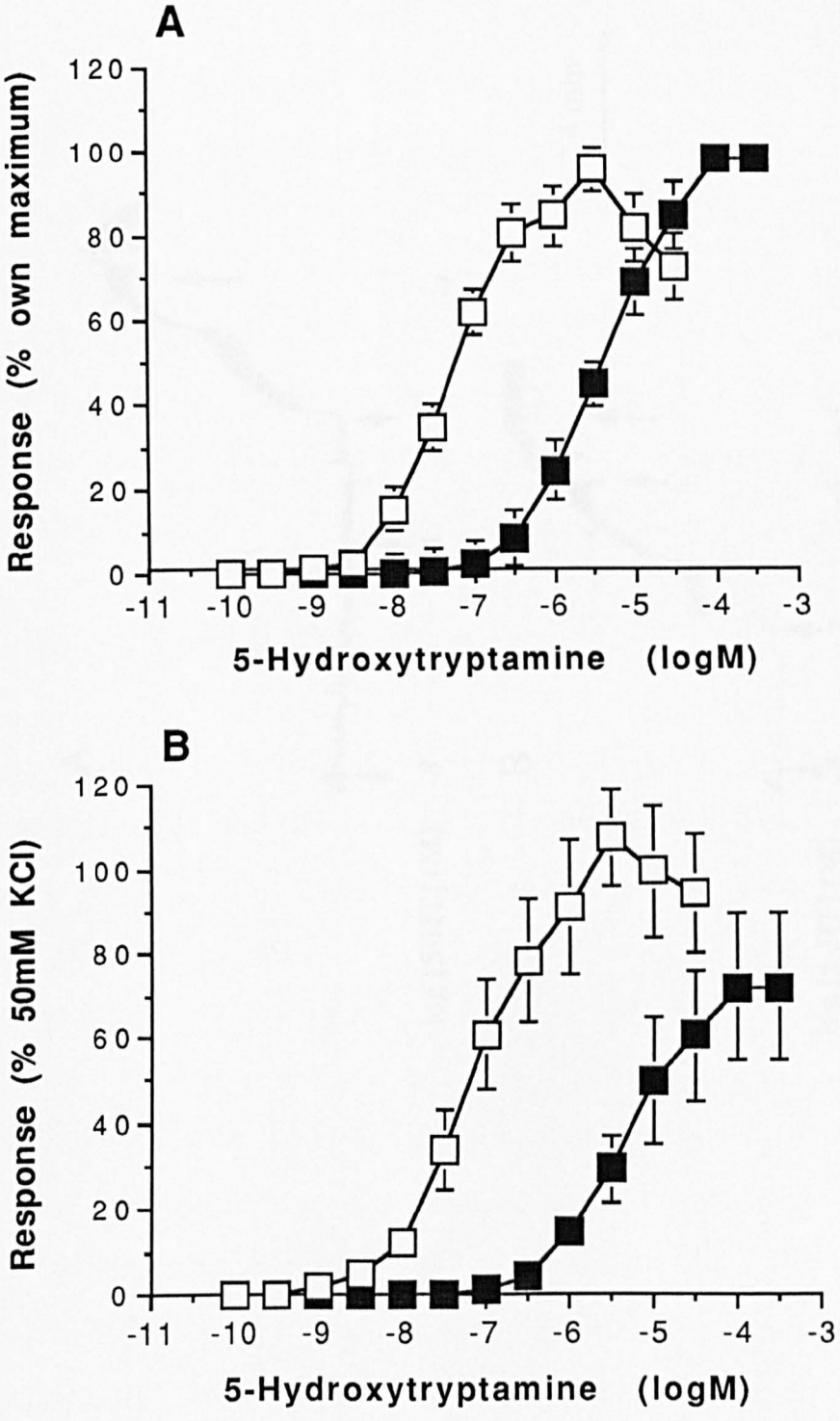

Figure 4.3 Effect of GR55562 on 5-HT-induced responses in human smPAs. CCRCs to 5-HT (□, n = 7/7) and in the presence of 1.0μM (■, n = 6/6) GR55562. Data are expressed as a percentage own maximum contraction. B Data are expressed as a percentage reference contraction to 50mM KCl. Each point represents mean ± s.e.mean.

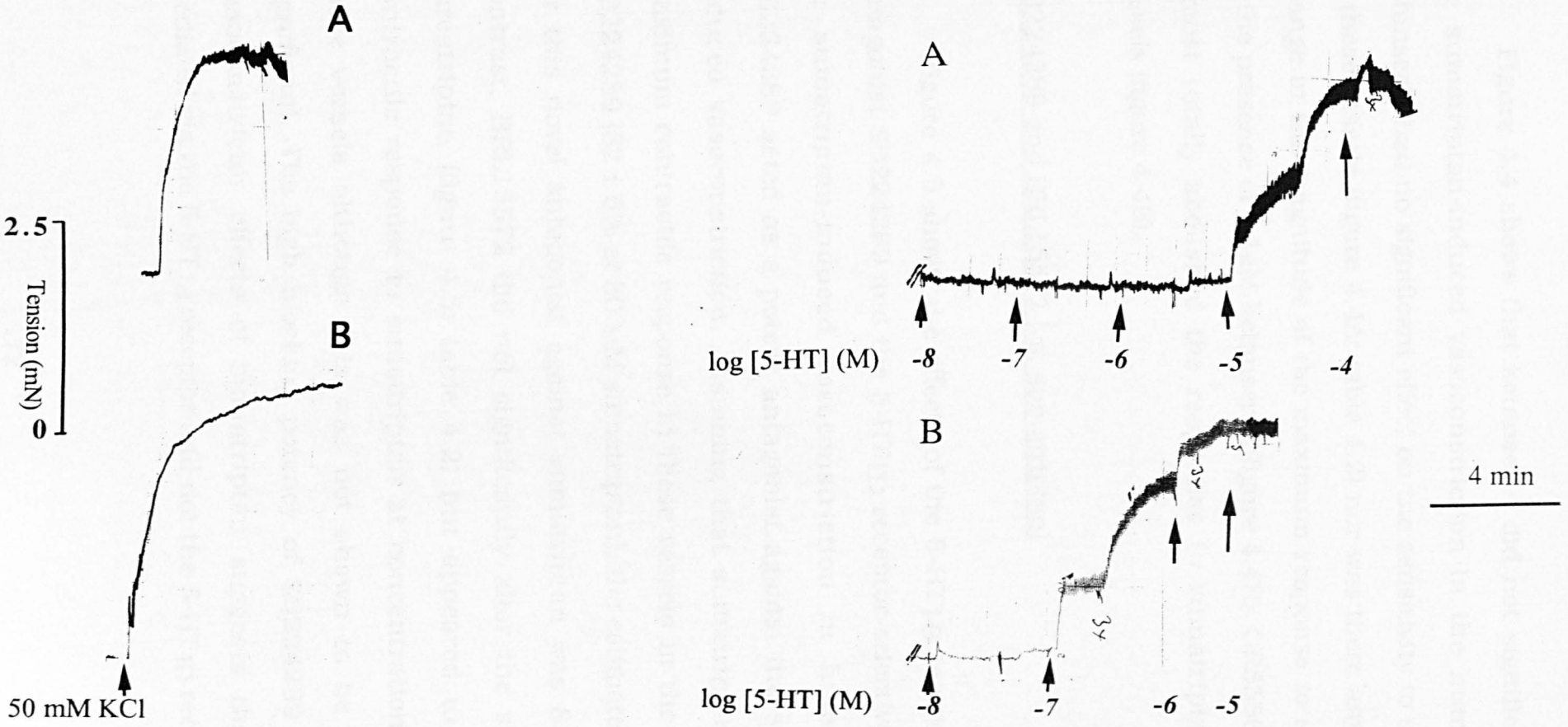

Figure 4.3C Representative trace showing the effect of GR55562 (1μM) on 5-HT-evoked contractions in human isolated smPRAs. **A.** In the presence and **B**. in the absence of GR55562. Responses are compared with response to 50mM KCl.

Ketanserin and GR55562 (vs. sumatriptan)

Figure 4.4 shows that ketanserin did not significantly affect the sumatriptan-induced vasoconstriction in the human smPAs. Ketanserin had no significant effect on the sensitivity to sumatriptan in these vessels (figure 4.4A; table 4.2) nor was there any significant change in the magnitude of the maximum response to sumatriptan in the presence of 0.1μM ketanserin (figure 4.4B). GR55562, however, almost totally abolished the responses to sumatriptan in these vessels (figure 4.4B).

SB224289 and BRL15572 (vs. sumatriptan)

Figure 4.5 shows the effects of the 5-HT_{1B} receptor selective antagonist SB224289 and the 5-HT_{1D} receptor selective antagonist on sumatriptan-induced vasoconstriction in human smPAs. SB224289 acted as a potent antagonist against the sumatriptan-induced vasoconstriction. Assuming that sumatriptan reached a maximum contractile response in these vessels in the presence of SB224289 (82 ± 6% at 300μM sumatriptan), the estimated pK_B value for this novel antagonist against sumatriptan was 8.4 ± 0.1. In contrast, BRL15572 did not significantly alter the sensitivity to sumatriptan (figure 4.5; table 4.2) but appeared to reduce the contractile response to sumatriptan at concentrations > 1μM in these vessels although this was not shown to be statistically significant. The high blocking potency of SB224289 against the vasoconstrictor effects of sumatriptan suggests that they are mediated via the 5-HT_{1B} receptor and not the 5-HT_{1D} receptor.

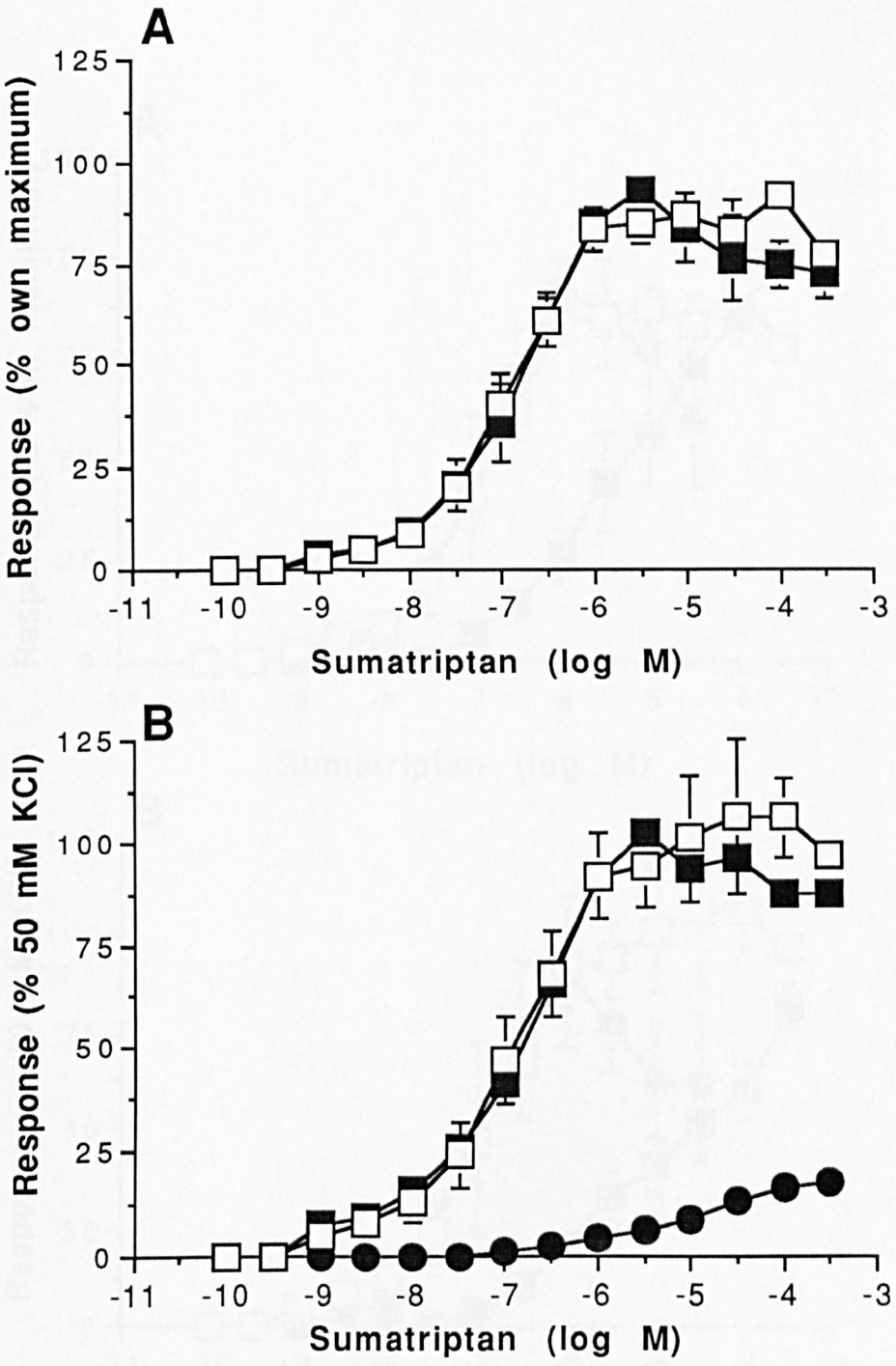

Figure 4.4 Effect of selective antagonists on sumatriptan-induced responses in human smPAs. CCRCs to sumatriptan (□, n = 11/11) and in the presence of 0.1μM (■, n = 6/6) ketanserin and 1.0μM (●, n = 5/5) GR55562. Data are expressed as a percentage own maximum contraction. B Data are expressed as a percentage reference contraction to 50mM KCl. Each point represents mean ± s.e.mean.

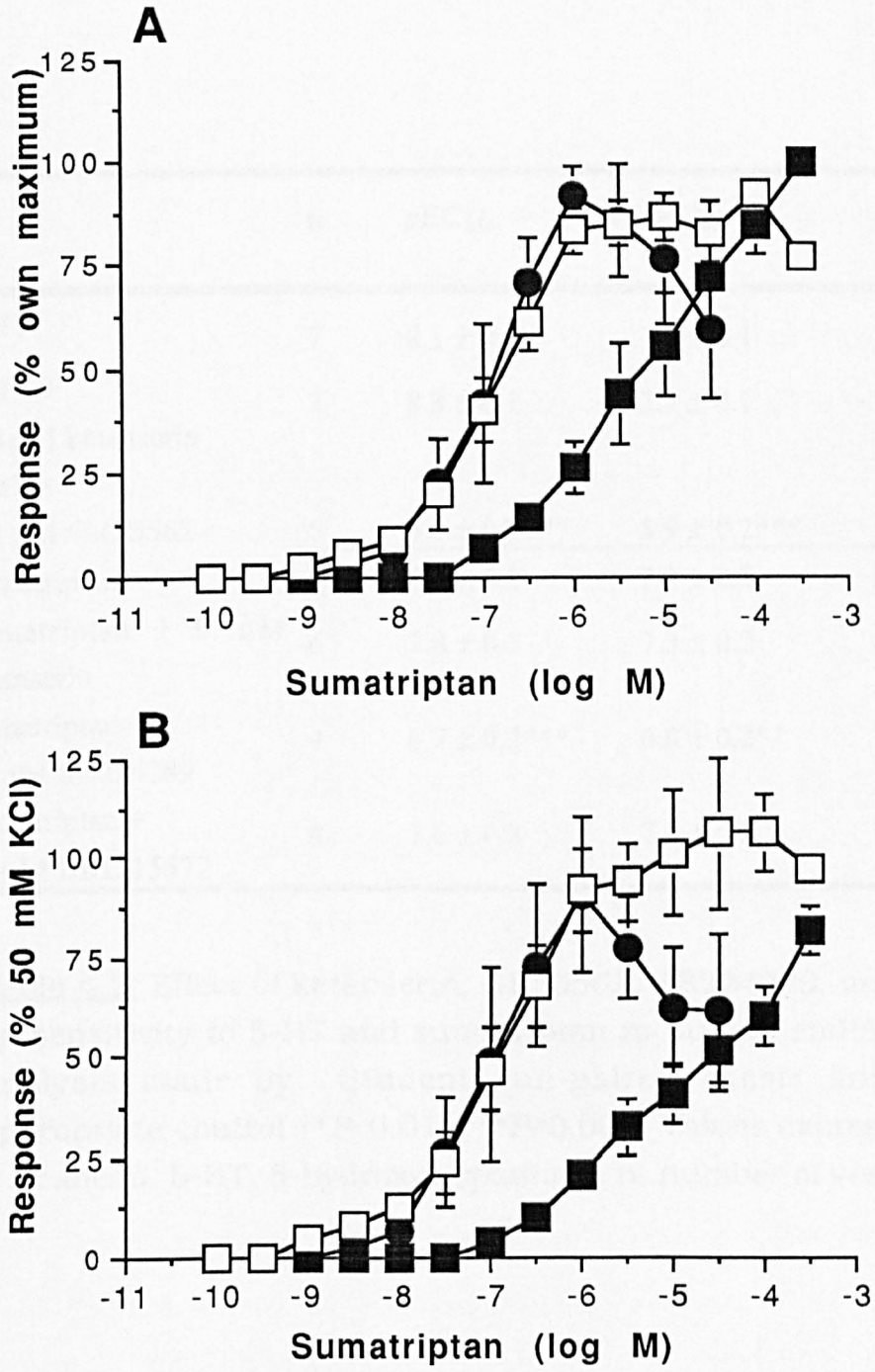

Figure 4.5 Effect of selective antagonists on sumatriptan-induced responses in human smPAs. CCRCs to sumatriptan (□, n = 11/11) and in the presence of 0.2μM (■, n = 4/4) SB224289 and 0.5μM BRL15572 (●, n = 4/4). **A** Data are expressed as a percentage own maximum contraction. **B** Data are expressed as a percentage reference contraction to 50mM KCl. Each point represents mean ± s.e.mean.

	n	pEC_{10}	pEC_{25}	pEC_{50}
5-HT	7	8.1 ± 0.1	7.6 ± 0.1	7.0 ± 0.2
5-HT + 0.1μM ketanserin	7	8.3 ± 0.1	7.7 ± 0.1	7.0 ± 0.1
5-HT + 1.0 μM GR55562	6	6.3 ± 0.2***	5.9 ± 0.2***	5.4 ± 0.2***
Sumatriptan	11	7.8 ± 0.2	7.3 ± 0.2	6.7 ± 0.1
Sumatriptan + 0.1μM ketanserin	6	7.8 ± 0.3	7.3 ± 0.2	6.8 ± 0.1
Sumatriptan + 0.2μM SB224289	4	6.7 ± 0.2***	6.0 ± 0.2**	5.1 ± 0.3***
Sumatriptan + 0.5μM BRL 15572	4	7.6 ± 0.3	7.2 ± 0.3	6.9 ± 0.3

Table 4.2 Effect of ketanserin, GR55562, SB224289, and BRL15572 on sensitivity to 5-HT and sumatriptan in human smPAs. Statistical analysis made by Students un-paired *t*-test; antagonist vs. appropriate control **P<0.01, ***P<0.001. Values expressed as mean ± s.e.mean. 5-HT, 5-hydroxytryptamine. n, number of vessels/lungs.

Discussion

The present results presented in this chapter are consistent with the 5-HT_{1B}- and 5-HT_{2A}-receptors mediating vasoconstriction in isolated human smPAs via two different receptor populations. 5-HT, 5-CT and sumatriptan are equipotent, confirming, and satisfying one of the criteria for, the presence of 5-HT_1-like receptors (Bradley *et al.*, 1986). This has also been shown by previous studies in human conduit pulmonary arteries (MacLean *et al.*, 1996b). Responses to 5-HT at concentrations < 0.1μM and to sumatriptan (1nM-300μM) were inhibited by GR55562 but resistant to ketanserin. responses to 5-HT at concentrations > 0.1μM were inhibited by ketanserin. Responses to sumatriptan were inhibited by the 5-HT_{1B} receptor-selective antagonist SB224289 but not by the 5-HT_{1D} receptor selective antagonist BRL15572. Substantial amounts of mRNA for 5-HT_{1B} and 5-HT_{2A} receptors has been detected in human smPAs isolated from lung tissue of the same patients (Morecroft *et al*; 1999). Figure 4.6, from this complimentary study, shows that signals of mRNA transcripts for all of the vasoconstrictor 5-HT receptor subtypes investigated in pharmacology experiments; 5-HT_{1D}, 5-HT_{1B} and 5-HT_{2A}, were found in intact human smPAs. 5-HT_{1B} and 5-HT_{2A} mRNA transcripts were consistently detected in all arteries studied. Figure 4.6 shows typical RT-PCR signals of the expected size for each receptor, which was similar in 6/6 patients. Compared to 5-HT_{1B} and 5-HT_{2A} receptors, only weak signals corresponding to the mRNA transcript for the 5-HT_{1D} receptor was detected.

In this chapter whole human small muscular pulmonary arteries comprised of a heterogeneous population of adventitial, smooth muscle and endothelial cells were studied. As 5-HT_1 receptors have previously been identified on vascular endothelium

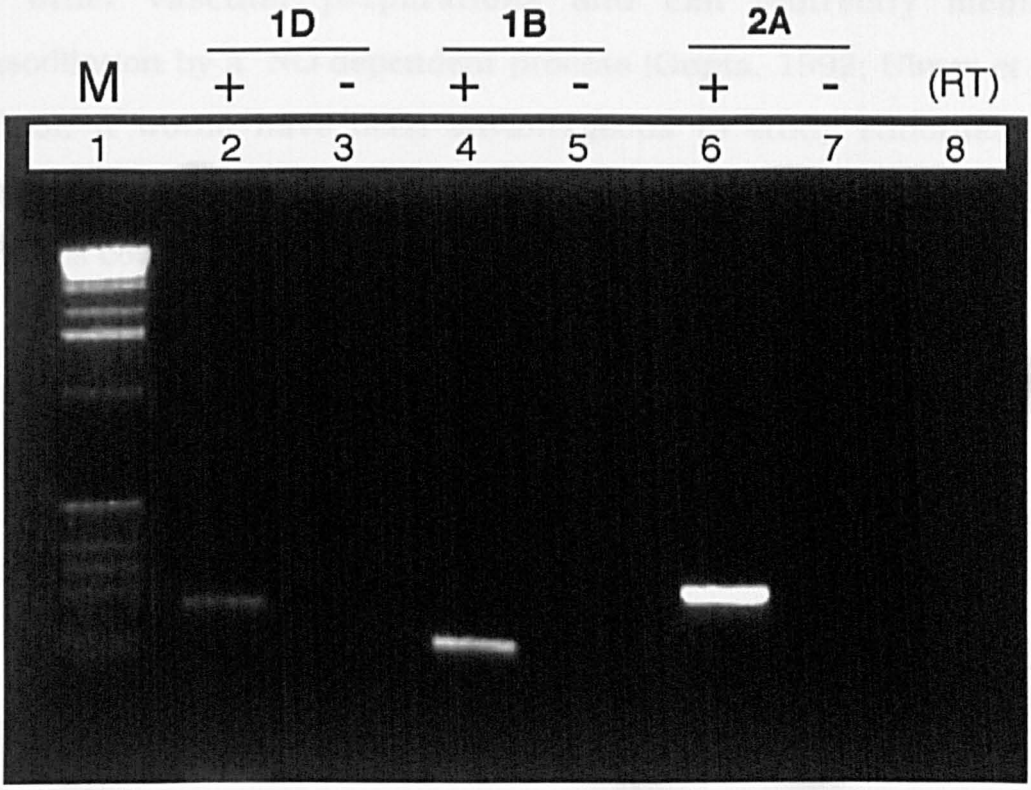

Figure 4.6 RT-PCR detection of selected 5-HT receptors expressed in human small muscular pulmonary arteries. Lanes 2, 4 and 6; 5-HT$_{1D}$ (239 bp), 5-HT$_{1B}$ (213 bp) and 5-HT$_{2A}$ (309 bp) receptor signals respectively. Lanes 3, 5 and 7, RT-negative controls: +, MMLV(+); -, MMLV (-). RT, reverse transcribed; MMLV, murine moloney leukaemia virus.

of other vascular preparations and can indirectly mediate vasodilation by a NO dependent process (Gupta, 1992; Ulmer *et al.*, 1995), it would have been advantageous to study endothelium-denuded vessels. The vascular endothelium of these small, delicate arteries could not be removed as experience dictates that any attempt to remove the endothelium by mechanical disruption results in significant damage to, and reduction of, the smooth muscle layer. This is due to the relative sparsity and fragility of the vascular smooth muscle cells in the pulmonary arterial circulation (Brenner 1935; Frid *et al.*, 1997). The lung tissue received for these studies was routinely taken from the lung periphery and therefore the arterial bed could not be perfused with detergents or other agents to successfully remove the endothelium. The RT-PCR experiments described in Morecroft *et al.*, (1999) were, however, able to confirm that 5-HT_{1B}, 5-HT_{2A} and 5-HT_{1D} receptor mRNA was transcribed in the whole human small muscular pulmonary arteries. Only trace levels of the 5-HT_{1D} receptor mRNA transcript appeared to be present compared to those of the 5-HT_{1B} and 5-HT_{2A} receptors. This is, therefore consistent with the pharmacological characterisation studies in this chapter and together provide strong evidence that both the 5-HT_{1B} receptor and 5-HT_{2A} receptor mediate vasoconstriction in these vessels. The receptor antagonist GR55562 is selective for both the 5-HT_{1B} and 5-HT_{1D} receptors. The PK_B value of 7.7 for GR55562 against 5-HT is consistent with previous observations in large human pulmonary arteries (MacLean *et al.*, 1996b) where GR55562 (at 1.0μM) was found to have a PK_B value of ~7.0, consistent with studies in other vascular preparations (Connor *et al.*, 1995). Cloned and transfected 5-HT_{1D} receptors have been shown to have a higher affinity for ketanserin with a K_D value about 200nM compared to the

5-HT_{1B} receptor with a K_D value of about 10 μM (Kaumann *et al.*, 1994). In a study of rabbit cloned 5-$HT_{1B/1D}$ receptors, Bard *et al.* (1996) reported that ketanserin displayed ~23-fold selectivity for 5-HT_{1D} receptors over 5-HT_{1B} receptors. In the studies described in this chapter, ketanserin did not, however inhibit the 5-HT-induced contraction at 5-HT concentrations < 0.1μM or the sumatriptan-evoked contraction, suggesting that 5-HT_{1D} receptors do not significantly contribute to vasoconstriction in preparations of human small muscular pulmonary artery. This is confirmed by the observation that BRL15572, the 5-HT_{1D} receptor selective antagonist, at 0.5μM, did not inhibit contractions to the 5-HT_{1B} and 5-HT_{1D} receptor agonist sumatriptan at concentrations < 1μM. At sumatriptan concentrations > 1μM, BRL15572 (0.5μM) appeared to cause a slight inhibition, the reason for this is uncertain. At high concentrations (> 1μM) sumatriptan may mediate vasoconstriction through the 5-HT_{1D} receptor and the presence of 5-HT_{1D} mRNA in these vessels, albeit in trace amounts, suggests this may be possible. However, as discussed below, it is the 5-HT_{1B} receptor that is of physiological importance in mediating 5-HT-induced vasoconstriction in these vessels. This concentration of BRL15572 is selective for the 5-HT_{1D} receptor with a 60 fold lower affinity for the 5-HT_{1B} receptor (Price *et al.*, 1997).

In view of the fact that the contractile effects of high concentrations of 5-HT in some blood vessels may be mediated by a direct action on α-adrenoceptors (Black, *et al.*, 1981; Fenuik, 1984) including pulmonary arteries (Ogawa, *et al.*, 1995), the possibility that 5-HT may mediate, at least partly, its contractile effects via α-adrenoceptors cannot be strictly ruled out in the human PRAs. However this is unlikely as sumatriptan and 5-CT, which are not

known to activate α-adrenoceptor, contracted the human PRAs in this present study with a similar potency and magnitude as 5-HT.

It has also been reported that high concentrations of 5-HT can have indirect sympathomimetic actions by releasing noradrenaline (McGrath, 1977; Saxena & Villalon, 1990). The possibility that 5-HT and its agonists elicit contractions at high concentrations by releasing NA from sympathetic nerve endings cannot be ruled out. It is probable, however, that 5-HT is not involved in the modulation of sympathetic neurotransmission in the human pulmonary vasculature as no pre-junctional 5-HT receptors have been identified in the human pulmonary artery (Freeman, Rorie & Tyce, 1981).

The estimated pK_B of 8.4 for SB224289 against sumatriptan in human small muscular pulmonary arteries is similar to the affinity (pK_i = 8.1) reported by Roberts *et al.*, (1997) for sumatriptan in binding to recombinant 5-HT_{1B} receptors. This provides further evidence that 5-HT_{1B} receptors play a significant role in mediating 5-HT-induced vasoconstriction in human small muscular pulmonary arteries. Although sumatriptan at very high concentrations, can mediate its vasoconstrictor effect through 5-HT_{2A} receptors (Peroutka & McCarthy, 1989), SB224289 at 0.2μM is unlikely to interact significantly with 5-HT_{2A} receptors in this study because SB-224289 has a low affinity (pK_i ~5.8) for recombinant human 5-HT_{2A} receptors (Roberts *et al.*, 1997).

The normal plasma concentration range for 5-HT in man has been reported as 1-2nM (Anderson *et al.*, 1987). Hervé *et al.*, (1990; 1995) recently reported that plasma concentrations of 5-HT can be significantly elevated to ~ 30nM in primary PHT. The results of this study show that 5-HT induced vasoconstriction in human small muscular pulmonary arteries only at concentrations greater than 3nM and that responses to 5-HT up to ~0.1μM are ketanserin

resistant. It is reasonable, therefore, that the 5-HT_{1B} receptor is likely to be responsible for mediating pulmonary arterial vasoconstriction in man at pathophysiological concentrations of 5-HT. This probably explains why ketanserin has been shown to be of very limited use in the treatment of both primary and secondary pulmonary hypertension patients (Dominighetti *et al.*, 1997; Herve´ *et al.*, 1990) where side effects such as marked systemic hypotension are common. The results presented in this chapter therefore highlight the possibility of a more 'pulmonary-selective' 5-HT-receptor subtype. The 5-HT_{1B} receptor may therefore provide an important therapeutic target in the treatment of PHT. From the results here, the 5-HT_{2A} receptor would only mediate pulmonary arterial vasoconstriction if local concentrations of 5-HT in the pulmonary vascular bed were to exceed 0.1μM in pulmonary disease. This possibility, however, cannot be completely ruled out if there is concomitant local platelet and neuroendocrine body release of 5-HT in the lung (Johnson & Georgieff, 1989).

MacLean *et al* (1996) demonstrated augmented responses to 5-HT at all levels of the pulmonary arterial vasculature in the chronic hypoxic rat model of pulmonary hypertension and, using various 5-HT antagonists, concluded that this increased response was a result of enhanced 5-HT_{2A} receptor stimulation in combination with an increased influence of r5-HT_{1B}-like receptor stimulation. There is an abundant expression of 5-HT_{1B} receptor mRNA in rat pulmonary arteries and that this expression is increased in the same chronic hypoxic rat model of pulmonary hypertension (Heeley *et al.*, 1998). Artificially induced tone 'uncovers' vasoconstrictor responses to sumatriptan in bovine pulmonary arteries (MacLean *et al.*, 1994; Sweeney *et al.*, 1995). Decreasing cyclic GMP levels by NOS inhibition also increases vasoconstrictor effect of sumatriptan in both bovine

and human large intralobar pulmonary arteries. (MacLean *et al.*, 1993; 1994). These effects became pathophysiologically relevant recently when MacLean *et al* (1996) provided evidence that vascular tone is increased and cyclic GMP levels are decreased in pulmonary arteries from the CH rat. Collectively, these observations, taken with the results presented in this chapter, suggest that 5-HT_{1B} receptor-mediated pulmonary vasoconstriction is likely to be increased and play an important significant role in the increased pulmonary vascular tone observed in pulmonary hypertension.

The studies in this chapter do not provide any indication of the second messenger system linking the pulmonary vascular smooth muscle contraction to 5-HT_{1B} receptor stimulation. Although it is well known that the ketanserin-sensitive 5-HT_{2A} receptors are linked to a increase in the activity of phosphoinositide C, generating IP_3 and diacylglycerol (Martin, 1994), the contractile pathways, via 5-HT_1 receptor stimulation are less well defined. Although in a number of vascular preparations the 5-$HT_{1B/1D}$ receptor is reported to be negatively coupled to the formation of cyclic AMP, contractions mediated via these receptors may well occur in a cyclic AMP-independent manner (Movahedi & Purdy, 1997; Parsons, *et al.*, 1996; Randall, *et al.*, 1996). Recently, Hill *et al.* (2000) demonstrated that contractions linked to 5-$HT_{1B/1D}$ receptor stimulation in the rabbit isolated renal artery can be explained by an influx of external Ca^{2+} through voltage-activated Ca^{2+} channels as well as Ca^{2+} sensitization of the contractile myofilaments (Hill *et al.*, 2000). In many vascular preparations, the contractile effect mediated through 5-$HT_{1B/1D}$ receptor stimulation only becomes apparent when the vessels are prestimulated with another contractile agent. Clearly, the results of the studies in this chapter show that 5-HT_{1B} receptor stimulation evokes contractions in the absence of a prestimulating

agonist. It would, therefore, be useful to investigate the contractile pathways involved in mediating 5-$HT_{1B/1D}$ receptor evoked contractions in human PRAs as this receptor subtype appears to mediate vasoconstriction in a complex way, which may involve the interaction of several intracellular signalling pathways.

Myofilament Ca^{2+} sensitization is an important component of the smooth muscle contraction elicited by a number of G-protein linked receptors, occurring through different kinase pathways (Somlyo & Somlyo, 1998). Recently, Evans *et al.* (1999) reported that ET-1 induced an increase in myofilament calcium sensitivity in rat pulmonary arteries via the activation of ET_A receptors and by a mechanism(s) independent of tyrosine kinase. This pathway has been proposed to mediate ET-1 induced Ca^{2+} sensitization in rat mesenteric arteries (Ohanian, *et al.*, 1997). From a clinical viewpoint, these observations may be of some importance, as the identification of a distinct signal transduction mechanism associated with the development of pulmonary hypertension could lead to the development of new more effective therapies for this disorder. Indeed, the possibility that physiologically relevant agonists such as ET-1 and 5-HT (as well as NA) may act in the pulmonary vasculature primarily via a mechanism that does not involve mobilisation of Ca^{2+} may explain why only a small percentage of patients with PHT respond to Ca^{2+} channel blocker therapy (Palevsky and Fishman, 1991). It would therefore, be useful to investigate possible 5-$HT_{1B/1D}$ receptor-induced Ca^{2+} sensitization of the contractile filaments to in the human PRAs.

In conclusion, 5-HT-induced contractions of human small muscular pulmonary arteries are mediated through two receptor populations. The main important finding of this work is that one of these receptor populations is the 5-HT_{1B} subtype which mediates 5-

HT-induced vasoconstriction in human smPAs at concentrations which are pathophysiologically relevant. The 5-HT_{1B} receptor therefore provides a potentially important new therapeutic target for pulmonary hypertension. Further support for this hypothesis will require both molecular biological and further pharmacological evidence about the nature and location of 5-HT receptor subtypes in the human pulmonary vasculature from patients with pulmonary hypertension.

Chapter 5

5-HT receptors in the perinatal rabbit pulmonary arteries.

5.1 Introduction

As previously explained in chapter 1, at birth, the pulmonary circulation undergoes important structural and functional changes (Haworth & Hislop, 1981; Hall & Haworth, 1987; Dunn *et al* 1989).The initiation of lung ventilation and concomitant increase in pulmonary arterial PO_2 results in a rapid fall in pulmonary arterial pressure (PAP) and pulmonary vascular resistance (PVR) through vasodilation of the small pulmonary arteries. This fall in PVR continues postnatally over the first few hours after birth and is essential to allow a ten-fold increase in pulmonary blood flow to occur in order to adapt to life outside the uterus and permit normal gas exchange, via the lungs, to begin (Heymann & Soifer 1989). In a significant number of neonates, PVR does not decrease at birth resulting in persistent pulmonary hypertension of the newborn (PPHN) (Gersony *et al* 1973; John *et al* 1988). This condition results in a considerably high mortality rate.

5-hydroxytryptamine (5-HT) is a potent pulmonary vasoconstrictor *in vitro* in several species including man (Raffestin *et al*, 1985; MacLean *et al*; 1996b) and several studies have implicated a role for 5-HT in the aetiology of pulmonary hypertension (PHT). Elevated plasma levels of 5-HT have been reported in primary PHT (Herve *et al*; 1995) and isolated pulmonary arteries from PHT patients undergoing transplantation exhibit exaggerated vasoconstrictor responses to 5-HT (Brink *et al*, 1988). With particular relevance to the perinatal pulmonary circulation, 5-HT has also been linked to PHT associated with hypoxia in the newborn infant. Johnson & Georgieff (1989) suggested that secretion of excessive amounts of serotonin, as well as other vasoactive agents, from pulmonary neuroendocrine cells following airway hypoxia may be associated with

the development of PHT in the newborn. Recently, 5-HT turnover has been shown to be increased in children with PHT secondary to congenital heart disease (Breuer *et al*, 1996). Hence, the 5-HT receptors mediating pulmonary vasoconstriction in the perinatal period are of interest.

5-HT interacts with a multiplicity of receptors causing either vasoconstriction or vasodilation depending on receptor subtype, species, pulmonary vascular location and underlying vascular tone (Hoyer *et al.*, 1994). The two variants of the 5-HT_{1D} receptor previously described as the 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptor (Weinshank *et al.*, 1992) have recently been renamed the 5-HT_{1D} and 5-HT_{1B} receptor respectively (Hartig *et al.*, 1996). Where it cannot be distinguished between the 5-HT_{1D} and 5-HT_{1B} receptor, the receptor involved will subsequently be referred to as the 5-$HT_{1D/1B}$ receptor.

5-HT causes vasoconstriction in isolated bovine intrapulmonary arteries via a 5-$HT_{1D/1B}$ receptor (MacLean *et al*, 1994) but induces endothelium-dependent relaxation in isolated porcine pulmonary arteries via the '5-HT_{1C}' receptor (Glusa & Richter, 1993). The 5-HT_{1C} receptor has subsequently been renamed the 5-HT_{2C} receptor (Hoyer *et al.*, 1994).

In human large pulmonary arteries, 5-HT-mediated vasoconstriction is via stimulation of 5-$HT_{1D/1B}$ receptors (MacLean *et al*, 1996b); and the studies in chapter 4 examined and discussed the importance of the 5-HT_{1B} receptor in mediating 5-HT-receptor-induced vasoconstriction in the pulmonary small muscular resistance arteries in man. In other vascular beds, 5-HT can induce endothelium-dependent vasodilation via the 5-HT_{2C} receptor (Bodelson *et al.*, 1993), the 5-HT_{2B} receptor (Glusa & Roos, 1996; Ellis *et al.*, 1995) and the 5-$HT_{1D/1B}$ receptor (Gupta, 1992). There is

also evidence that 5-HT can mediate vasodilation directly through 5-HT4 and 5-HT7 receptors located on vascular smooth muscle (Cocks & Arnold, 1992; Leung *et al.*, 1996; Terron, 1996).

The receptors mediating vasoconstriction and vasodilation in perinatal pulmonary arteries have not previously been studied. The neonatal lung is still undergoing development and maturation at birth (Hislop & Reid, 1972; Meyrick & Reid, 1982), and very little is known at present about the actions of 5-HT on perinatal pulmonary resistance vasculature.

In order to understand more fully the possible role of 5-hydroxytryptamine (5-HT) in both the ordinary adaptation of the perinatal pulmonary vasculature at birth and in the pathophysiological state of PPHN, it is important to elucidate the 5-HT receptor(s) present in the pulmonary vasculature at different developmental ages. The results from chapter 3 of this thesis suggest that in the adult rabbit pulmonary arteries, 5-HT-induced vasoconstriction is mediated solely via the 5-HT_{2A} receptor in the large capacitance PAs, but by a heterogeneous population of both the 5-HT_{2A} and 5-HT_{1B} or 5-HT_{1D} receptor in the pulmonary resistance arteries (PRAs). In adult human PRAs, 5-HT-evoked vasoconstriction also appears to be mediated via stimulation of both 5-HT_{2A} and 5-HT_{1B} receptors, with the 5-HT_{1B} receptor being of more importance at physiological concentrations of 5-HT (Chapter 5 of this thesis). Although, as previously mentioned, it is the PRAs which are the important determinants of pulmonary vascular resistance and pulmonary hypertension *in vivo* in the adult (Staub, 1985), it has been suggested that in the perinatal period, the pulmonary capacitance arteries (PCAs) may also play a significant role in the maintenance of PVR (Belik, 1994, 1995). Hence, The studies in this

chapter examined the functional responses to 5-HT, the influence of endothelium-derived NO and the 5-HT receptor subtype(s) involved in mediating 5-HT-induced vasoconstriction and vasodilation, in both PCAs and PRAs from near term (2 days pre-term) foetal and neonatal rabbits.

5.2 Materials and methods

Rabbit pulmonary arteries

New Zealand White rabbits were studied at the following ages: foetal (2 days pre-term), 0-24 hrs, 4 days, and 7 days after birth. They were killed by sodium pentobarbitone (200mgkg^{-1}) and the lungs removed. The main pulmonary and 1st branch, extralobar, pulmonary conduit arteries (PCAs) were dissected out. The PCAs from the 2 day pre-term foetal (1-2mm i.d.), 0-12 hour (1-2mm i.d.), 4 day (1.5-2.5mm i.d.), and 7 (2-3.5 mm i.d.)) day old NZW rabbits were set up in 5 ml organ baths. Under a dissecting microscope, intralobar pulmonary resistance arteries ([PRAs], ~250-300μm i.d.) were carefully dissected out and cleaned of surrounding parenchyma. Two mm long segments of the arteries were threaded onto 40μm stainless steel wires and mounted as ring preparations in isometric wire myographs.

All vessels were bathed in Krebs-buffer solution (pH 7.4) (composition (mM): NaCl 118.4, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 0.6, $CaCl_2$ 2.5, glucose 11.0, and EDTA 23.0) at 37°C. Foetal vessels were bubbled with 3%O_2/5%CO_2 balance N_2 in order to mimic *in utero* partial oxygen pressures (pO_2~18-23mmHg, pCO_2~35-40mmHg), and the neonatal vessels bubbled with 16%O_2/5%CO_2 balance N_2 to give values similar to those found *in vivo* (pO_2~120mmHg, pCO_2~35-40mmHg).

PCAs were set up under tensions at which maximum responses to 50mM KCl were obtained (0.8 -1.25 g).

Tension was then applied to the PRAs to give a transmural pressure equivalent of approximately 12-16mmHg, similar to *in vivo* pressures of pulmonary arterioles after birth. Foetal PRAs were also tensioned to this pressure equivalent as this was the minimum tension at which reproducible, consistent contractile responses could be observed.

Experimental protocol

Following a 45 minute equilibration period, the response to 50mM KCl was determined twice; once the contractile response had reached a plateau, the vessels were washed out 8 times with fresh Krebs solution.

Pulmonary conduit arteries (PCAs)

In any experiments involving the PCAs, no attempt was made to remove the endothelium as previous experience dictates that, with mechanical disruption, there is significant damage to the smooth muscle layer of these vessels.

The vessels were preconstricted with 1μM NA and once the contraction had levelled off, 1μM ACh was added on top of the NA-induced tone to assess endothelial integrity.

(In separate experiments in the extralobar branch pulmonary arteries (bPAs) (see Morecroft & MacLean, 1998), the pulmonary arterial rings were pre-contracted with a concentration of phenylephrine which in a preliminary experiment produced 70% maximum contraction (0.1-0.3μM). Once a stable, plateau had been reached, cumulative concentration-dependent-response curves

(CCRCs) to ACh (1nM-10mM) were constructed in half log increments, each increment added when response to previous addition had plateaued (about 2 minutes)).

Following washout and sufficient time for return to baseline tension the vessels were subject to one of the following protocols:

In the PCAs, cumulative concentration-response curves (CCRCs) to 5-HT (1nM-0.1mM) were constructed, following washout with fresh Krebs and re-equilibration, the CCRCs to 5-HT were repeated in the presence of 100μM L-NAME, a non-selective NO synthase inhibitor. Appropriate 'time control' CCRCs to 5-HT were also constructed in each age group which showed no change in sensitivity or magnitude of contractile response to 5-HT (not shown).

In order to evaluate the 5-HT receptor(s) involved in the pulmonary vasoconstrictor activity in the extralobar branch PAs, CCRCs were also constructed to the 5-HT agonists 5-carboxamidotryptamine (5-CT, 1nM-0.1mM; 5-HT_1 receptor agonist), α-methyl-5-HT (5-HT_2-selective, 1nM-0.1mM) and the 5-$HT_{1B/1D}$ receptor agonist, sumatriptan.

For antagonist studies, CCRCs were constructed to 5-HT (1nM-0.1mM) and following washout and re-equilibration, these were repeated in the presence of an appropriate antagonist which was allowed to equilibrate for 45 minutes before the commencement of the second CCRC to 5-HT. The antagonists used were ketanserin (selective for 5-HT_{2A} receptors) and GR55562 (3-[3- (dimethylamino) propyl]-4-hydroxy-N- [4-(4pyridinyl)phenyl]benzamide) (selective 5-$HT_{1B/1D}$ antagonist). Appropriate time controls for the second CCRC to 5-HT were always performed.

A second protocol examined the 5-HT receptor(s) mediating vasodilation in the bPAs and these experiments were conducted in

the presence of 0.1μM ketanserin to exclude any 5-HT_{2A} -receptor mediated effects. Vessels were contracted with phenylephrine (PE ~0.1μM) as it reliably induces a maintained contraction in these arterial segments. Vessels were pre-contracted to give a contraction approximately 50-70% of the maximal contractile response to this PE as preliminary studies indicated that contraction above this level actually decreased the sensitivity of vasodilators including 5-CT. Vasodilator responses to 5-CT, 5-HT, sumatriptan and BW 723C86 (selective 5-HT_{2B} agonist) were studied.

Pulmonary resistance arteries(PRAs)

Cumulative concentration-response curves (CCRCs) to 5-HT (1nM-0.1mM) were constructed either in the presence or absence of 100μM L-NAME, a non-selective NO synthase inhibitor.

In order to evaluate the 5-HT receptor(s) involved in the pulmonary vasoconstrictor activity in the PRAs, CCRCs were also constructed to the 5-HT agonists 5-carboxamidotryptamine (5-CT, 1nM-0.1mM; 5-HT_1 receptor agonist), α-methyl-5-HT (5-HT_2-selective, 1nM-0.1mM) and the 5-$HT_{1B/1D}$ receptor agonist, sumatriptan. Previous experience dictates that attempts at mechanical removal of the vascular endothelium in these arteries damages the thin and fragile underlying smooth muscle. These experiments were therefore, carried out in the presence of 100μM L-NAME to prevent any inhibitory role of NO release on constrictor responses. CCRCs to 5-HT are not reproducible in each tissue as, unlike the conduit vessels, desensitization often occurs with a second CCRC, therefore, only one CCRC to 5-HT was constructed either in the absence or presence of antagonist in each vessel. The antagonists used were ketanserin (selective for 5-HT_{2A} receptors) and

GR55562 (selective 5-$HT_{1B/1D}$ antagonist). All antagonists studies were also carried out in the presence of L-NAME (100μM).

The second protocol examined the 5-HT receptor(s) mediating vasodilation in perinatal rabbit PRAs and these experiments were conducted in the presence of 0.1μM ketanserin to exclude any 5-HT_{2A}-receptor mediated effects. Vessels were contracted with endothelin-1 (ET-1) (0.1-10nM) as it reliably induces a maintained contraction in these vessels (Docherty & MacLean, 1998). Vessels were pre-contracted to give a contraction approximately half the maximal contractile response to this ET-1 as preliminary studies indicated that contraction above this level actually decreased the sensitivity of vasodilators including 5-CT. We studied vasodilation in responses to 5-CT as preliminary results showed that 5-HT gave a variable vasodilator response with a tendency to cause contraction at higher concentrations in preconstricted PRAs. A CCRC to 5-CT was constructed in each vessel either in the presence or absence of one of the following; GR55562 (1μM), 100μM L-NAME, 100μM L-NAME + spiperone (1μM, 5-HT_7 receptor antagonist). Antagonists were allowed a 45 minute equilibrium period prior to constructing the CCRCs to 5-CT.

Data analysis

Contractile responses are expressed as a percentage of the second reference contraction to 50mM KCl determined at the start of the experiment in each preparation. The results are shown as the mean ± s.e.mean. Dilator responses are expressed as the % reduction of ET-1-induced tone. *p*EC_{50}s and *p*IC_{50}s were calculated by computer interpolation from individual CCRCs. Statistical comparison of the means of groups of data were made by Student's t-

test or one way analysis of variance where appropriate. $P < 0.05$ was considered statistically significant. n = number of vessels/number of animals. In antagonist studies, estimated pK_B values for antagonists were calculated whenever possible according to the methods described in chapter 2.

5.3 Results

5.3.1 Pulmonary conduit arteries

Assessment of endothelial integrity

Figure 5.1 shows the response to 1μM ACh in 1 μM NA-pre-contracted foetal and perinatal rabbit **A.** main and **B.** first branch extralobar pulmonary arteries (PAs). Levels of preconstriction to 1μM NA were not significantly different between age groups being (% response to 50mM KCl): 97 ± 8% (foetal), 101 ±11% (0-24 hour), 106 ± 5.6% (4 day) and 92 ± 7% (7 day) for the main PA and 89 ± 11% (foetal), 103 ± 14% (0-24 hour) 92 ±8% (4 day) and 111 ± 9% for the branch PAs. No significant relaxation was observed in the 0-24 hr rabbit main PA. The greatest relaxation was observed in the 4 day main PAs. In the branch PAs, the greatest relaxation was observed in the 7 day old rabbits, where vessels relaxed slightly below the baseline tension. A substantial relaxation was also observed in the foetal and 4 day rabbit branch vessels but ACh-induced relaxation was virtually non-existent in the 0-24 hr rabbit vessels. The response at the foetal, 4 day and 7 days age points was significantly greater than that of the 0-24 hour rabbit PAs.

Figure 5.1C illustrates the effect of ACh on pre-contracted branch PAs. Pulmonary arteries from the 0-24hr neonate did not relax to ACh. ACh caused vasodilation in all other vessels with the

following order of potency: adult > 4 day = 2 day pre-term as pIC_{50} values for the ACh-induced vasodilation were 7.40 ± 0.1, 7.38 ± 0.20 and 7.73 ± 0.04 for the foetal, 4 day old and adult rabbit bPAs respectively. Maximum vasodilation was in the following order (% preconstriction): 4 day (105.4 ± 3.0%) = 2 day pre-term (86.4 ± 4.2%) > adult (67.4 ± 6.6%, $P < 0.01$ cf. 4 day).

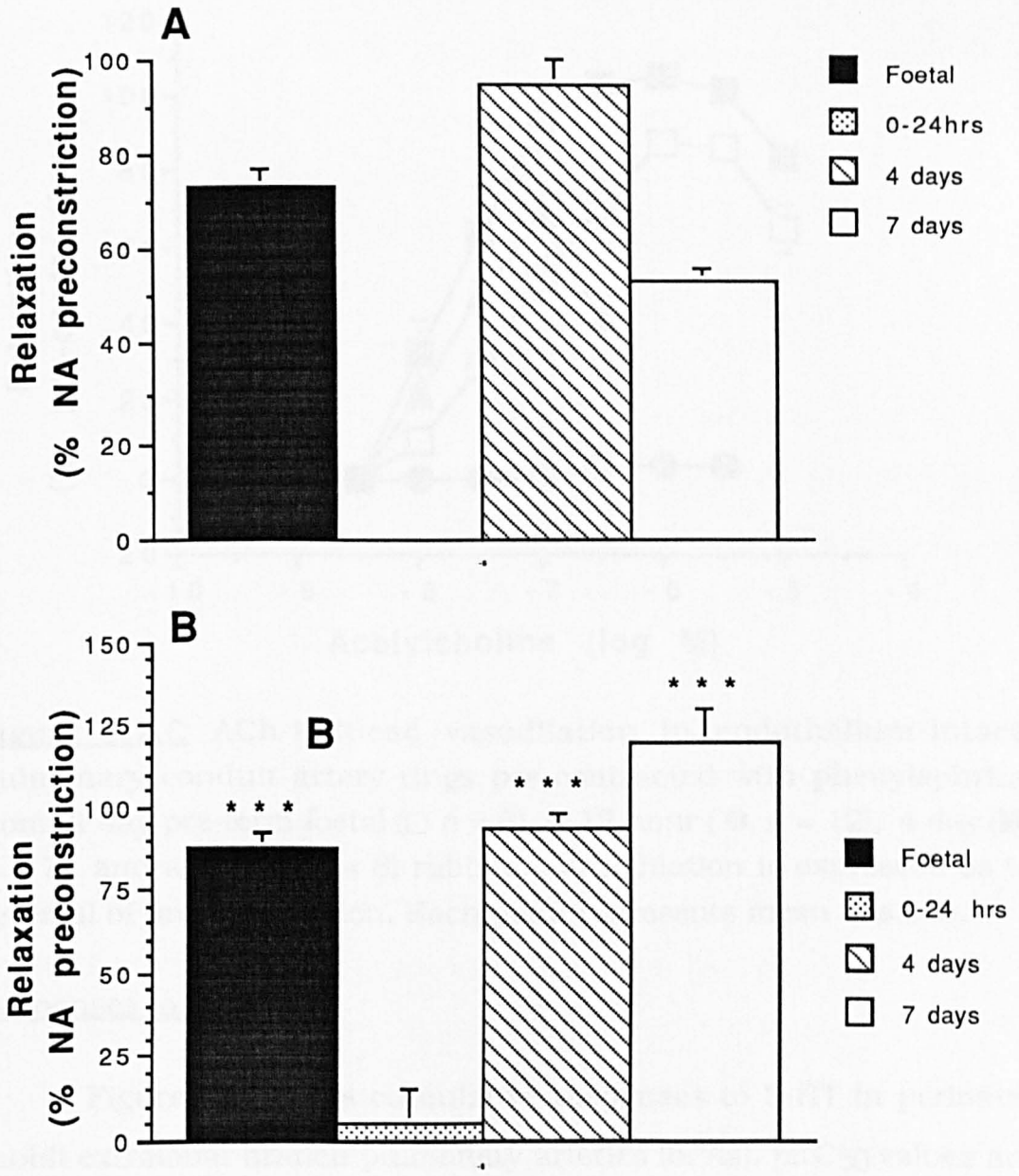

Figure 5.1 ACh-induced vasorelaxation of perinatal rabbit PCAs. Response to 1μM ACh in **A** main and **B** extralobar branch pulmonary artery expressed as percentage of NA (1μM)-induced tone. ***P<0.001 foetal, 4 day and 7 day cf. 0-24 hr response. n > 10/10 for each age. Statistical comparisons were made using ANOVA.

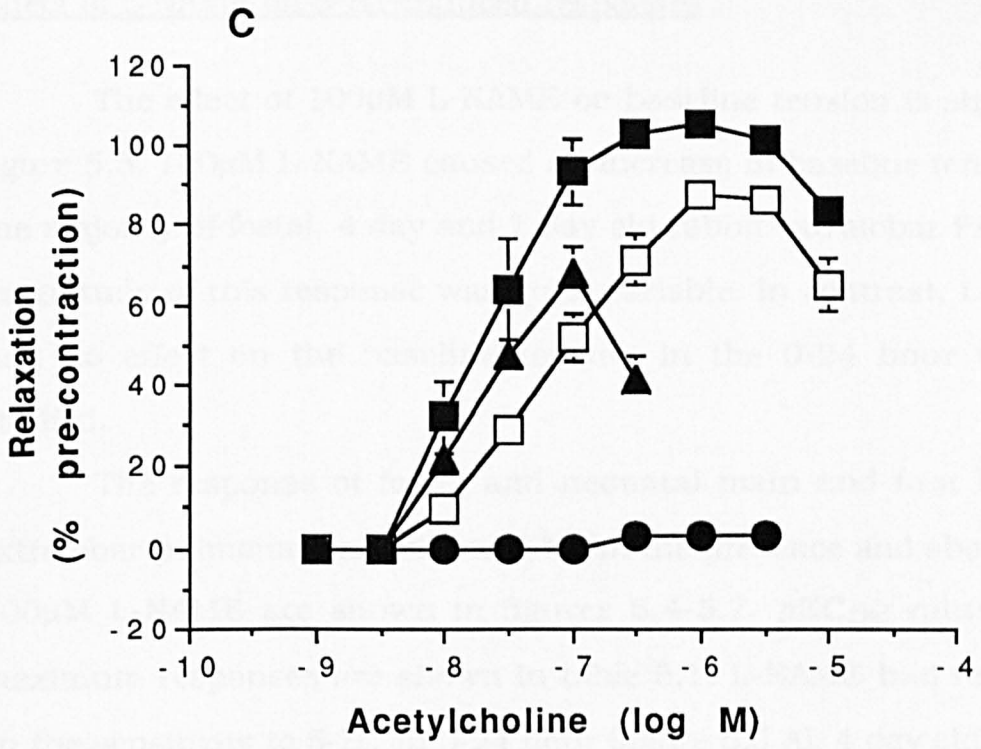

Figure 5.1.C ACh-induced vasodilation in endothelium-intact, pulmonary conduit artery rings pre-contracted with phenylephrine from 2 day pre-term foetal (□ n = 6), 0-12 hour (●, n = 12), 4 day (■, n = 7), and adult (▲, n = 8) rabbits. Vasodilation is expressed as % reversal of pre-constriction. Each point represents mean ± s.e.m.

Responses to 5-HT

Figure 5.2 shows cumulative responses to 5-HT in perinatal rabbit extralobar branch pulmonary arteries (bPAs). *p*EC50 values are summarised in table 5.1. 5-HT induced a concentration dependent contraction in the vessels at all age points studied. The sensitivity to 5-HT was similar in the bPAs from the foetal, 4 day old and 7 day old rabbits, which, in turn were all significantly more sensitive than the vessels from the 0-24 hour rabbit. The maximum contractile responses to 5-HT were similar at all age points studied being 115 ± 10%, 127 ± 17%, 129 ± 13% and 131 ± 13% (% 50mM KCl response) for the foetal, 0-24 hour, 4 day and 7 day rabbit bPAs respectively. There was no significant difference between these responses.

Effect of L-NAME on 5-HT-induced responses

The effect of 100μM L-NAME on baseline tension is shown in figure 5.3. 100μM L-NAME caused an increase in baseline tension in the majority of foetal, 4 day and 7 day old rabbit extralobar PAs. The magnitude of this response was quite variable. In contrast, L-NAME had no effect on the baseline tension in the 0-24 hour vessels studied.

The response of foetal and neonatal main and first branch extralobar pulmonary arteries to 5-HT in the presence and absence of 100μM L-NAME are shown in figures 5.4-5.7. pEC_{50} values and maximum responses are shown in table 5.1. L-NAME had no effect on the sensitivity to 5-HT in 0-24 hour (figure 5.5 A), 4 day old (figure 5.6 A) or 7 day old (figure 5.7 A) rabbit main PAs (table 5.1). In comparison, a significant increase in sensitivity was evident in vessels from foetal rabbit main PAs (figure 5.4 A, table 5.1). The magnitude of the maximum contractile response to 5-HT was increased by ~180% in the foetal main PAs (figure 5.4 A, table 5.1) but there was no significant effect of L-NAME on the maximal 5-HT-induced vasoconstriction in the other age groups. In the extralobar pulmonary arteries, a marked significant increase in sensitivity to 5-HT was evident in the 0-24 hour rabbit vessels in the presence of L-NAME (figure 5.5 B, table 5.1), but not in the extralobar PAs from the foetal, 4 day old and 7 day old rabbits (figures 5.4 B, 5.6 B & 5.7 B, table 5.1). The magnitude of the maximal 5-HT-induced contraction was significantly increased in the presence of L-NAME in the foetal (figure 5.4 B), 0-24 hour (figure 5.5 B), 4 day old (figure 5.6 B) and the 7 day old (figure 5.7 B) rabbit extralobar PAs. The greatest increase was observed in 4 day rabbit vessels, where the maximum contractile response to 5-HT was augmented by ~250% (figure 5.6 B,

table 5.1). In all experiments with L-NAME, 'time control' responses to a second CCRC to 5-HT in the same vessel did not significantly differ in sensitivity or maximal contractile response from the first 5-HT CCRC at all age points studied.

Preparation	*pEC50*		*n/n*	*Emax.* (+ L-NAME)
	Control	+ L-NAME		
Foetal				
-main	6.6 ± 0.1	7.0 ± 0.1*	6/6	280 ± 60***
-extra-lobe	6.9 ± 0.1††	7.1 ± 0.1	6/6	267 ± 32***
0-24 hours				
-main	6.8 ± 0.1	6.8 ± 0.1	12/12	109 ± 6
-extra-lobe	6.5 ± 0.05	7.2 ± 0.02***	12/12	142 ± 18**
4 day				
-main	6.8 ± 0.1	6.9 ± 0.1	8/8	104 ± 7
-extra-lobe	6.7 ± 0.07	6.8 ± 0.1	8/8	349 ± 41***
7 day				
-main	7.2 ± 0.2	7.2 ± 0.2	6/6	115 ± 6
-extra-lobe	7.0 ± 0.1††	7.2 ± 0.1	6/6	135 ± 9**

Table 5.1 *p*EC50 values for 5-HT in the presence and absence of 100μM L-NAME and maximum responses in the presence of L-NAME in perinatal rabbit pulmonary arteries. $E_{max.}$ = maximum response expressed as % maximum control 5-HT response in the same preparation. n/n = number of preparations/number of animals. Statistical comparisons were made by ANOVA : 0-24 hour control vs. other age points ††P<0.01; †††P<0.001; and Students paired t-test 5-HT EC50 or maximum response vs. + L-NAME.

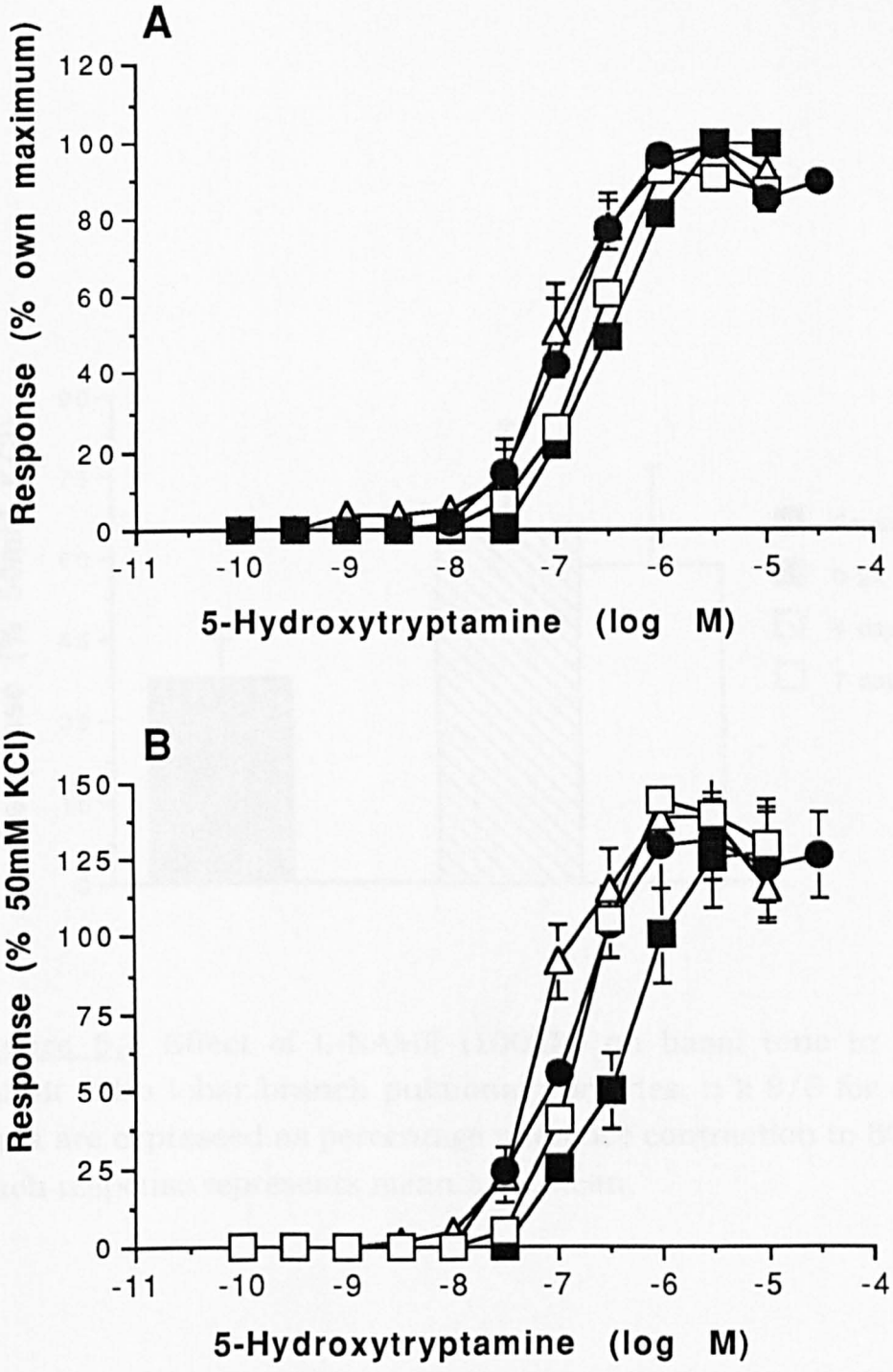

Figure 5.2 5-HT-induced contractions in perinatal rabbit extralobar branch pulmonary arteries. 5-HT CCRCs; foetal (△, n = 18/9), 0-24 hour (■, n = 18/9), 4 day (□, n = 18/9) and 7 day (●, n = 18/9) rabbits. **A** Data are expressed as percentage own maximum contraction. **B** Data are expressed as percentage reference contraction to 50 mM KCl. Each point represents mean ± s.e.mean.

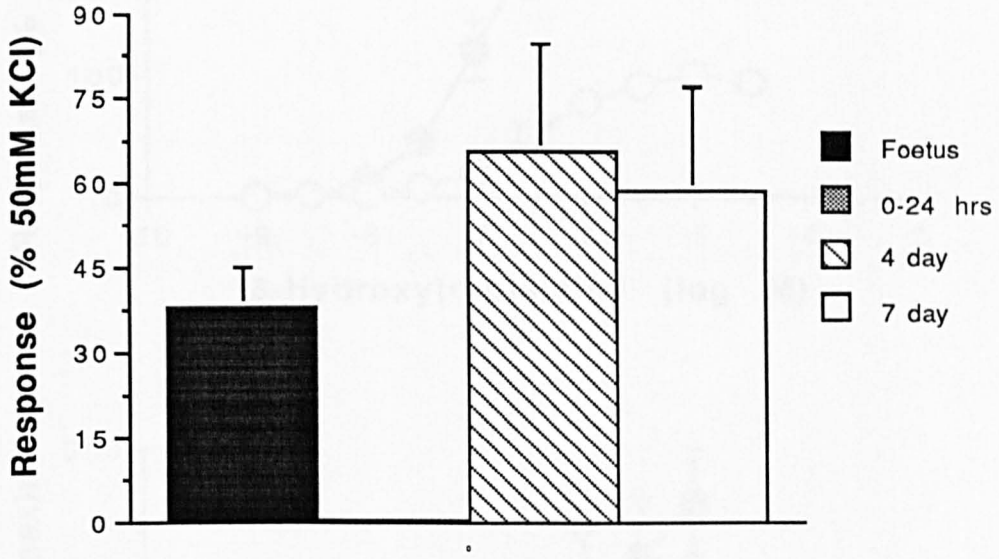

Figure 5.3 Effect of L-NAME (100μM) on basal tone in perinatal rabbit extra lobar branch pulmonary arteries. n ≥ 8/8 for each age. Data are expressed as percentage reference contraction to 50mM KCl. Each response represents mean ± s.e.mean.

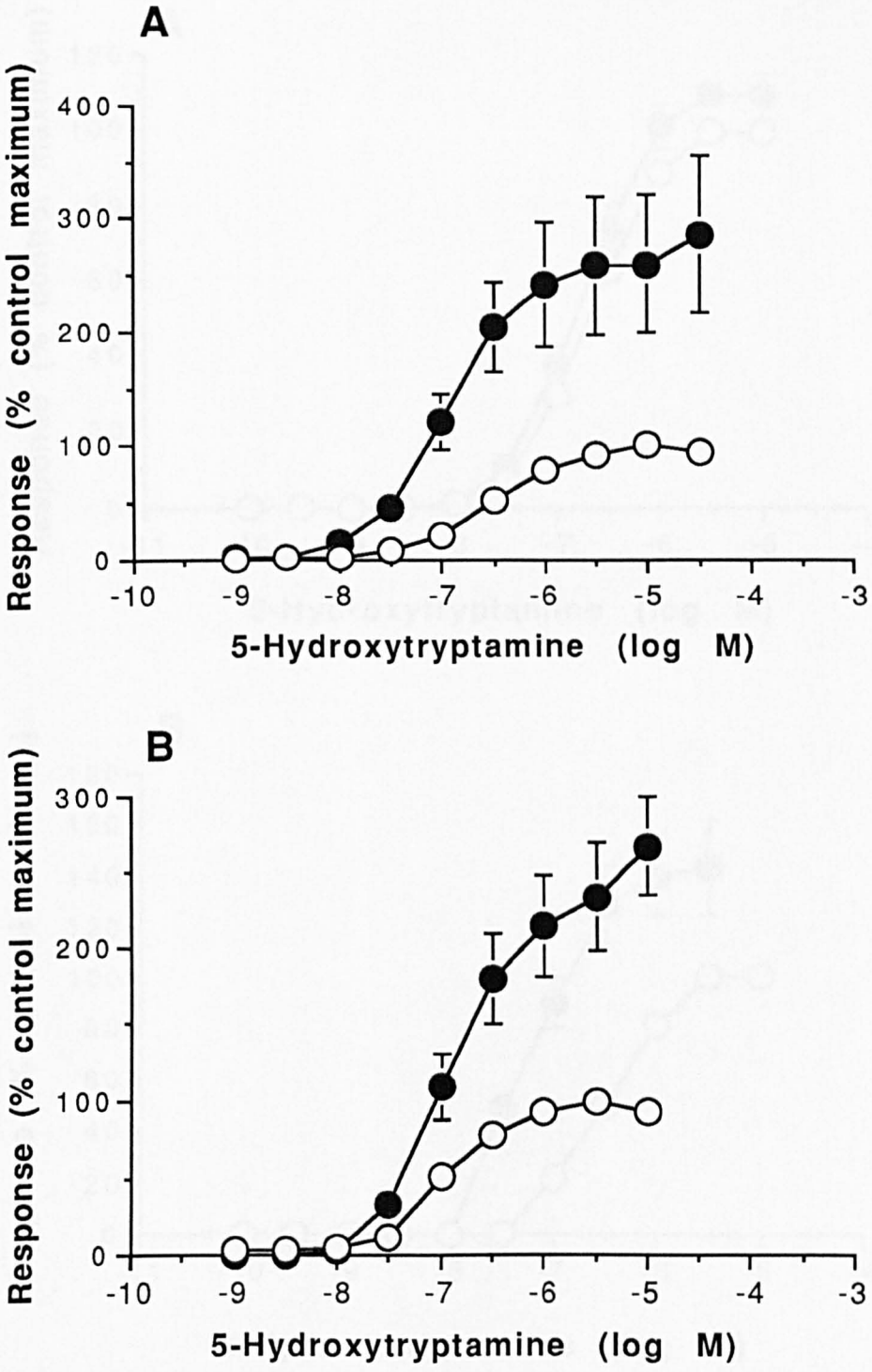

Figure 5.4 Effect of L-NAME (100μM) on 5-HT responses in foetal rabbit pulmonary arteries. CCRCs; control (O,n = 6/6) and in the presence of L-NAME (●,n = 6/6) in **A** main and **B** extralobar branch pulmonary arteries. Data are expressed as percentage control 5-HT maximum contractile response. Each point represents mean ± s.e.mean. n = number of vessels/number of animals.

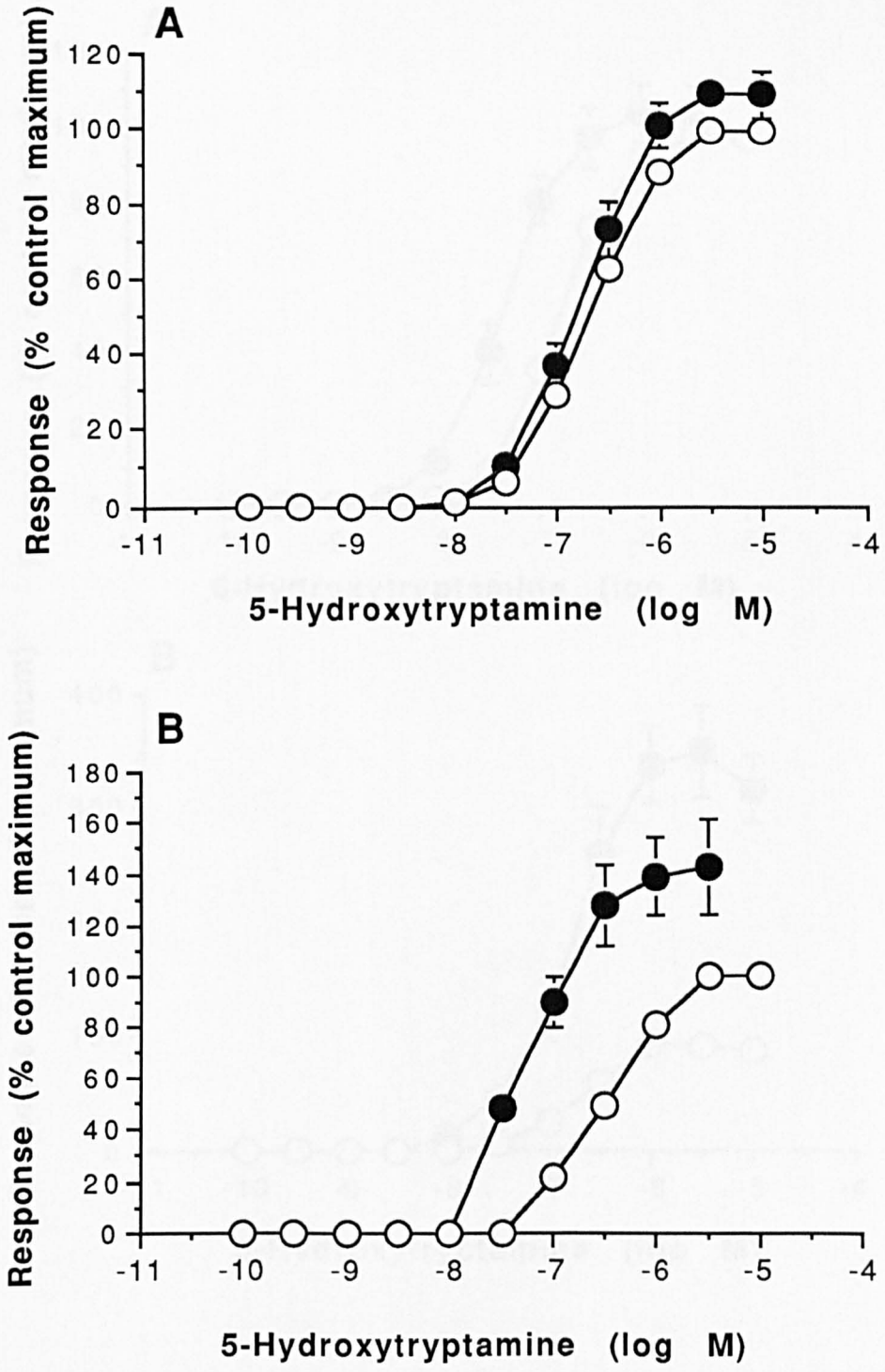

Figure 5.5 Effect of L-NAME (100μM) on responses to 5-HT in pulmonary arteries from 0-24 hr rabbits. CCRCs; control (O, n = 12/12) and in the presence of L-NAME (●, n = 12/12) in **A** Main and **B** extralobar branch pulmonary arteries. Data are expressed as percentage control 5-HT maximum contractile response. Each point represents mean ± s.e.mean. n = number of vessels/number of animals.

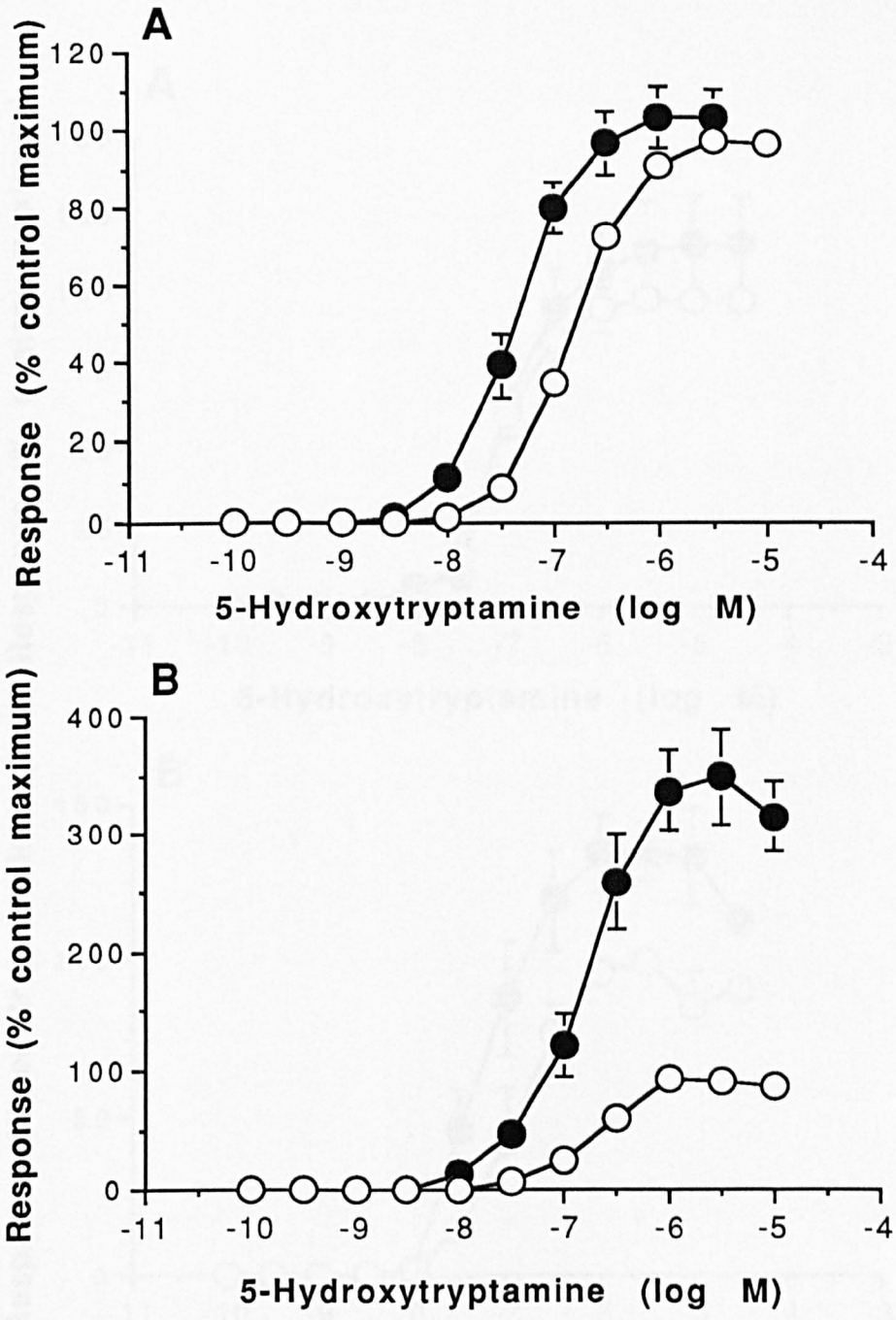

Figure 5.6 Effect of L-NAME (100μM) on responses to 5-HT in pulmonary arteries from 4 day rabbits. CCRCs; control (O, n = 8/8) and in the presence of L-NAME (●, n = 8/8) in **A** Main and **B** extralobar branch pulmonary arteries. Data are expressed as percentage control 5-HT maximum contractile response. Each point represents mean ± s.e.mean. n = number of vessels/number of animals.

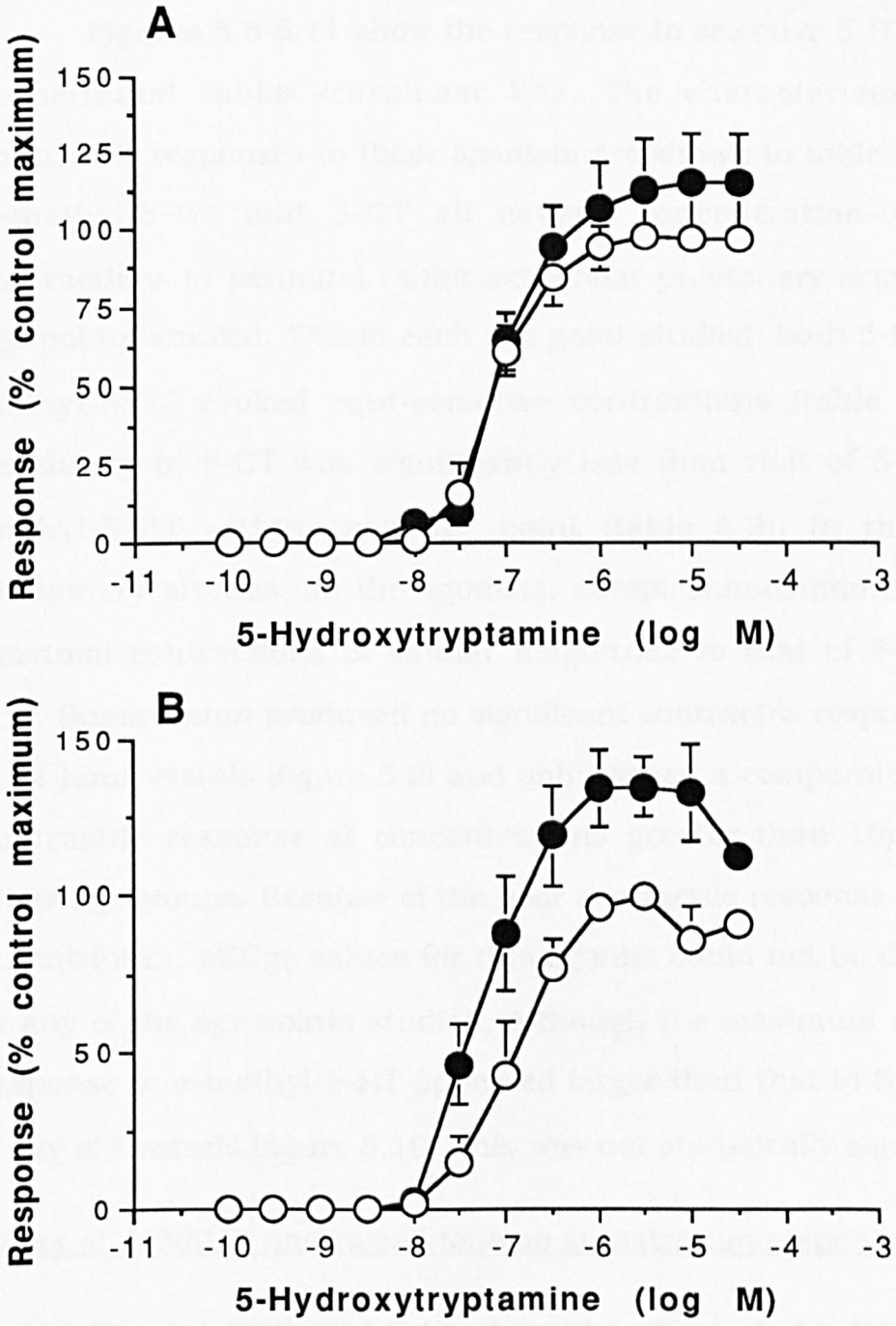

Figure 5.7 Effect of L-NAME (100μM) on responses to 5-HT in pulmonary arteries from 7 day rabbits. CCRCs; control (O, n = 6/6) and in the presence of L-NAME (●, n = 6/6) in **A** Main and **B** extralobar branch pulmonary arteries. Data are expressed as percentage control 5-HT maximum contractile response. Each point represents mean ± s.e.mean.

Responses to 5-HT agonists

Figures 5.8-5.11 show the response to selective 5-HT agonists in perinatal rabbit extralobar PAs. The characteristics of the contractile responses to these agonists are shown in table 5.2. 5-HT, α-methyl-5-HT and 5-CT all caused concentration-dependent contractions in perinatal rabbit extralobar pulmonary arteries at all age points studied. Within each age point studied, both 5-HT and α-methyl-5-HT evoked equi-sensitive contractions (table 5.2). The sensitivity to 5-CT was significantly less than that of 5-HT or α-methyl-5-HT within each age point (table 5.2). In the branch pulmonary arteries, all the agonists, except sumatriptan, produced maximal contractions of similar magnitude to that of 5-HT (table 5.2). Sumatriptan produced no significant contractile response in the 0-24 hour vessels (figure 5.9) and only evoked a comparatively small contractile response at concentrations greater than 10μM in the other age groups. Because of the poor contractile response elicited by sumatriptan, pEC_{50} values for this agonist could not be determined at any of the age points studied. Although the maximum contractile response to α-methyl-5-HT appeared larger than that to 5-HT in the 4 day old vessels (figure 5.10), this was not statistically significant.

Effect of L-NAME and raised tone on sumatriptan responses

Figures 5.12 and 5.13 show the effect of the NO synthase inhibitor L-NAME and the effect of U46619-induced tone on sumatriptan induced vasoconstriction in perinatal rabbit extralobar branch pulmonary arteries. In the foetal vessels (figure 5.12 A), neither 100μM L-NAME or U46619-induced tone had any significant effect on the poor contractile response to sumatriptan. In the 0-24 hour vessels (figure 5.12 B), both 100μM L-NAME and U46619-

induced tone 'uncovered' contractile responses to sumatriptan at concentrations greater than 10μM, this response was very variable. In the 4 day old vessels, both L-NAME and U146619-induced tone significantly increased the contractile response to sumatriptan at 1μM-10μM and decreased the threshold for sumatriptan-induced contraction from 10μM to 1μM (figure 5.13 A), the overall contractile response, however was still poor compared to that of 5-HT.

	*p*EC50	Emax (%)	n
Foetal			
5-HT	6.92 ± 0.1	138 ± 8	6/6
α-methyl-5-HT	6.76 ± 0.19	115 ± 18	6/6
5-CT	6.15 ± 0.2**	103 ± 13	6/6
0-24 hour			
5-HT	6.51 ± 0.05	116 ±19	7/6
α-methyl-5-HT	6.56 ± 0.11	105 ± 11	6/6
5-CT	5.67 ± 0.21**	114 ± 18	6/6
4 day			
5-HT	6.71 ± 0.14	128 ± 19	7/7
α-methyl-5-HT	6.91 ± 0.18	163 ± 27	6/6
5-CT	5.61 ± 0.2***	132 ± 13	6/6
7 day			
5-HT	7.19 ± 0.14	148 ± 21	6/6
α-methyl-5-HT	7.03 ± 0.11	152 ± 13	6/6
5-CT	6.13 ± 0.15***	119 ± 18	6/6

Table 5.2 *p*EC50 values and maximum responses for 5-HT agonists in perinatal rabbit extralobar PAs. Emax. = maximum response expressed as % contraction to 50mM KCl. Statistical comparisons were made by Students unpaired t-test: pEC50 for 5-HT vs agonist pEC50 at each respective age point $^{**}P< 0.01$, $^{***}P<0.001$. 5-HT, 5-hydroxytryptamine; 5-CT, 5-carboxamidotryptamine. Data are expressed as mean ± s.e.mean.

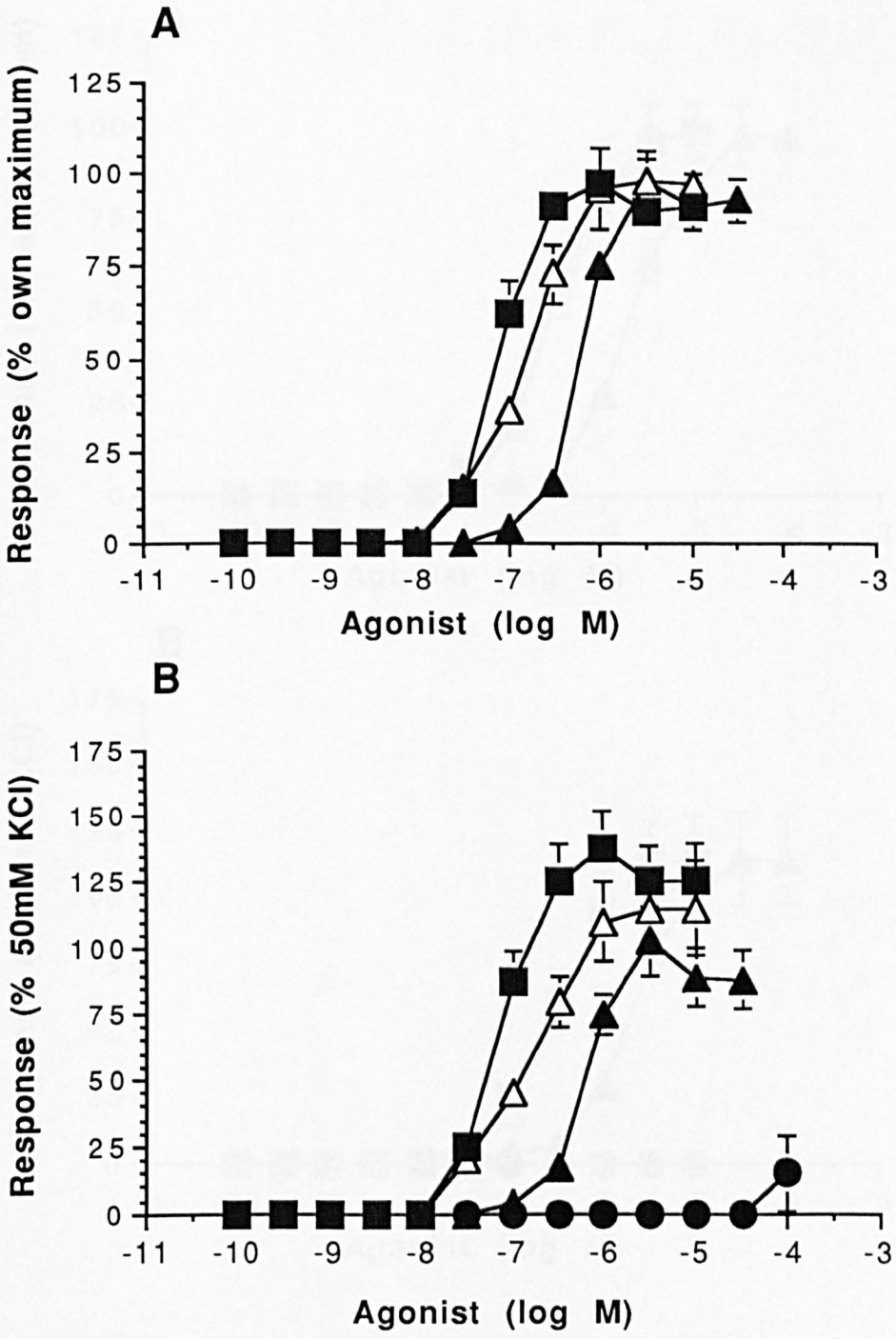

Figure 5.8 Response to selective agonists in foetal rabbit extralobar branch pulmonary arteries. CCRCs; 5-HT (■,n = 6/6), α-methyl-5-HT (△,n = 6/6), 5-CT (▲,n = 6/6) and sumatriptan (●,n = 6/6). **A** Data are expressed as percentage own maximum contraction. **B** Data are expressed as percentage reference contraction to 50 mM KCl. Each point represents mean ± s.e.mean.

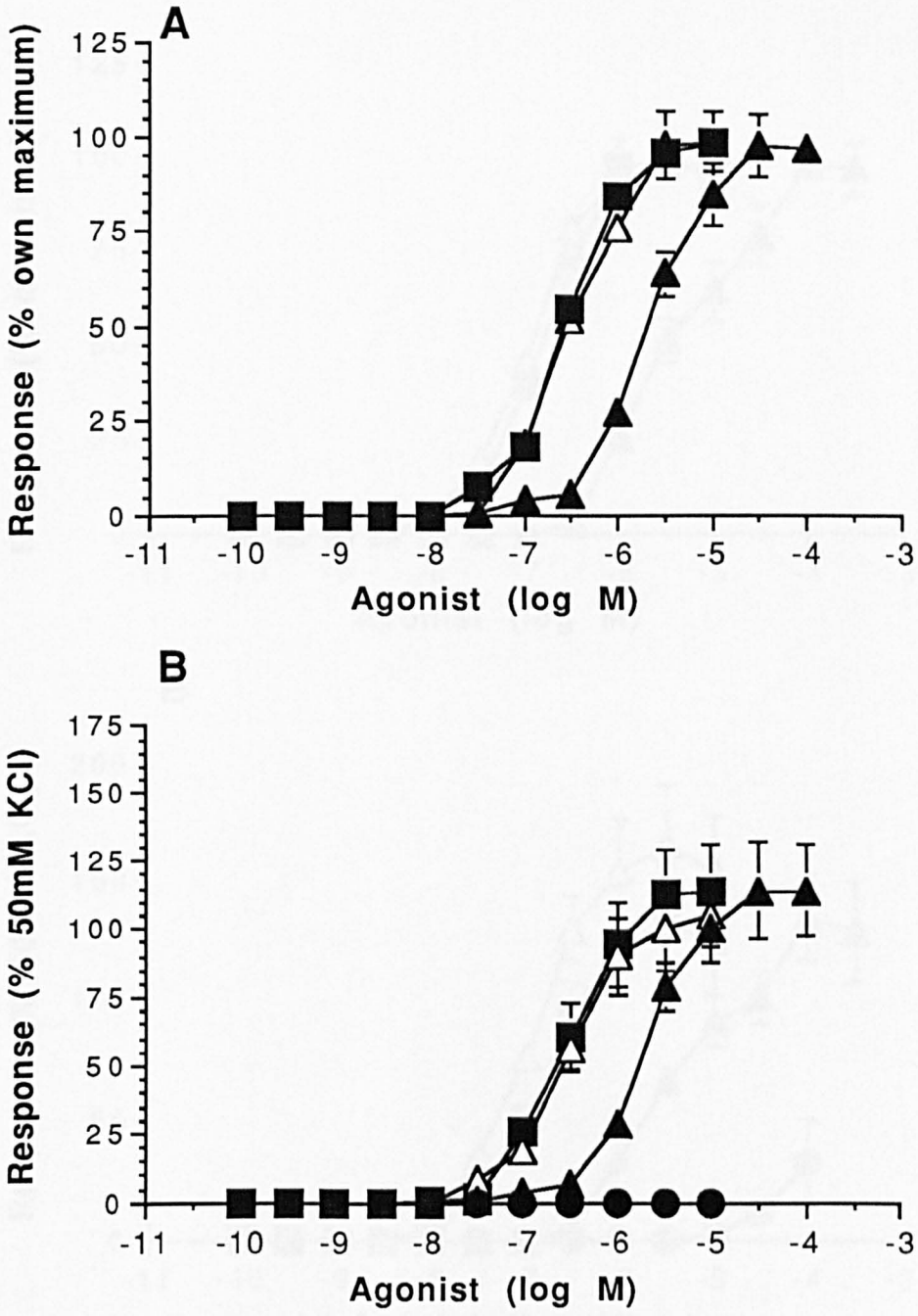

Figure 5.9 Response to selective agonists in 0-24 hr rabbit extralobar branch pulmonary arteries. CCRCs; 5-HT (■,n = 7/6), α-methyl-5-HT (△,n = 6/6), 5-CT (▲,n = 6/6) and sumatriptan (●,n = 6/6). **A** Data are expressed as percentage own maximum contraction. **B** Data are expressed as percentage reference contraction to 50 mM KCl. Each point represents mean ± s.e.mean.

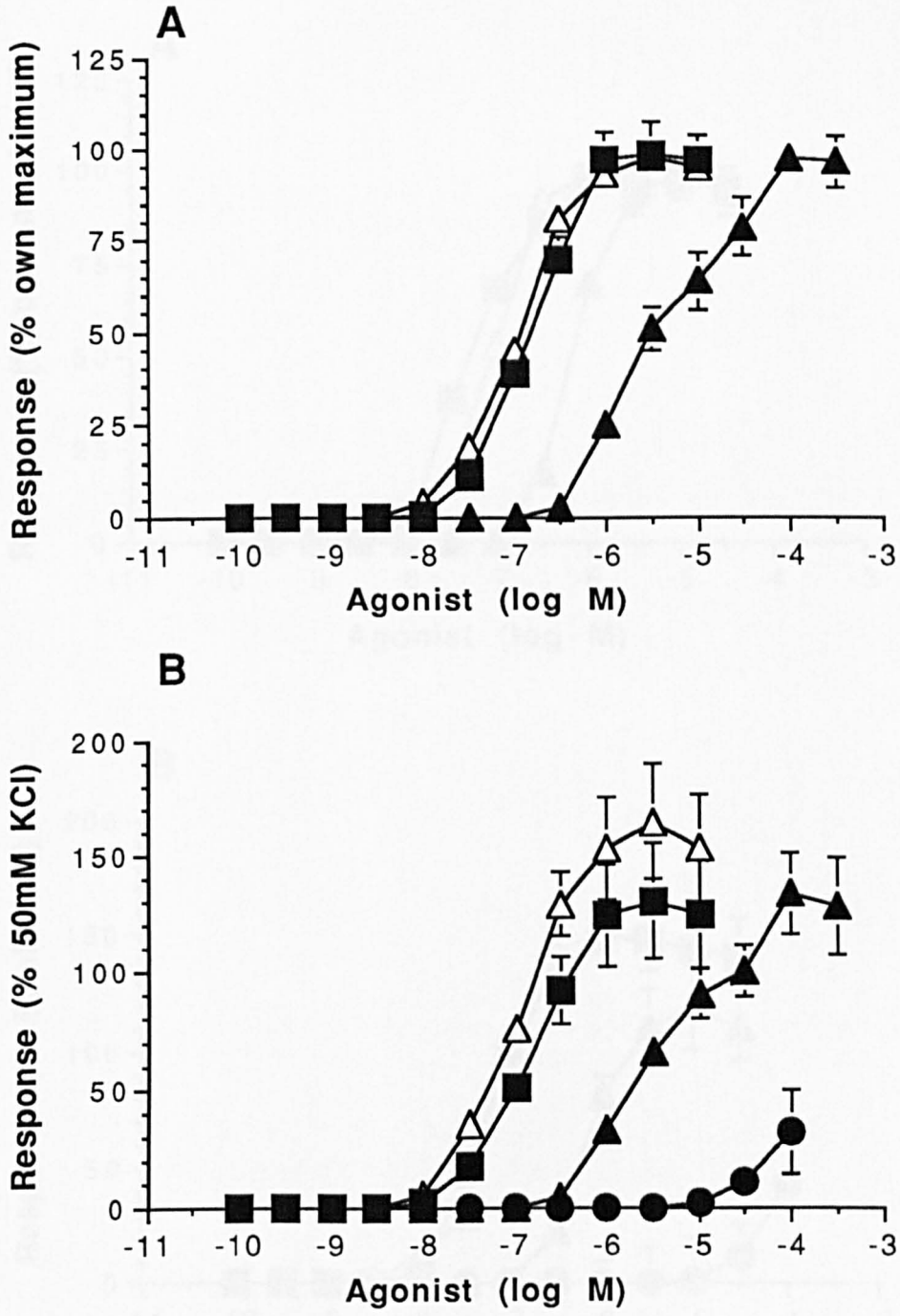

Figure 5.10 Response to selective agonists in 4 day rabbit extralobar branch pulmonary arteries. CCRCs; 5-HT (■,n = 7/7), α-methyl-5-HT (△,n = 6/6), 5-CT (▲,n = 6/6) and sumatriptan (●,n = 6/6). **A** Data are expressed as percentage own maximum contraction. **B** Data are expressed as percentage reference contraction to 50 mM KCl. Each point represents mean ± s.e.mean.

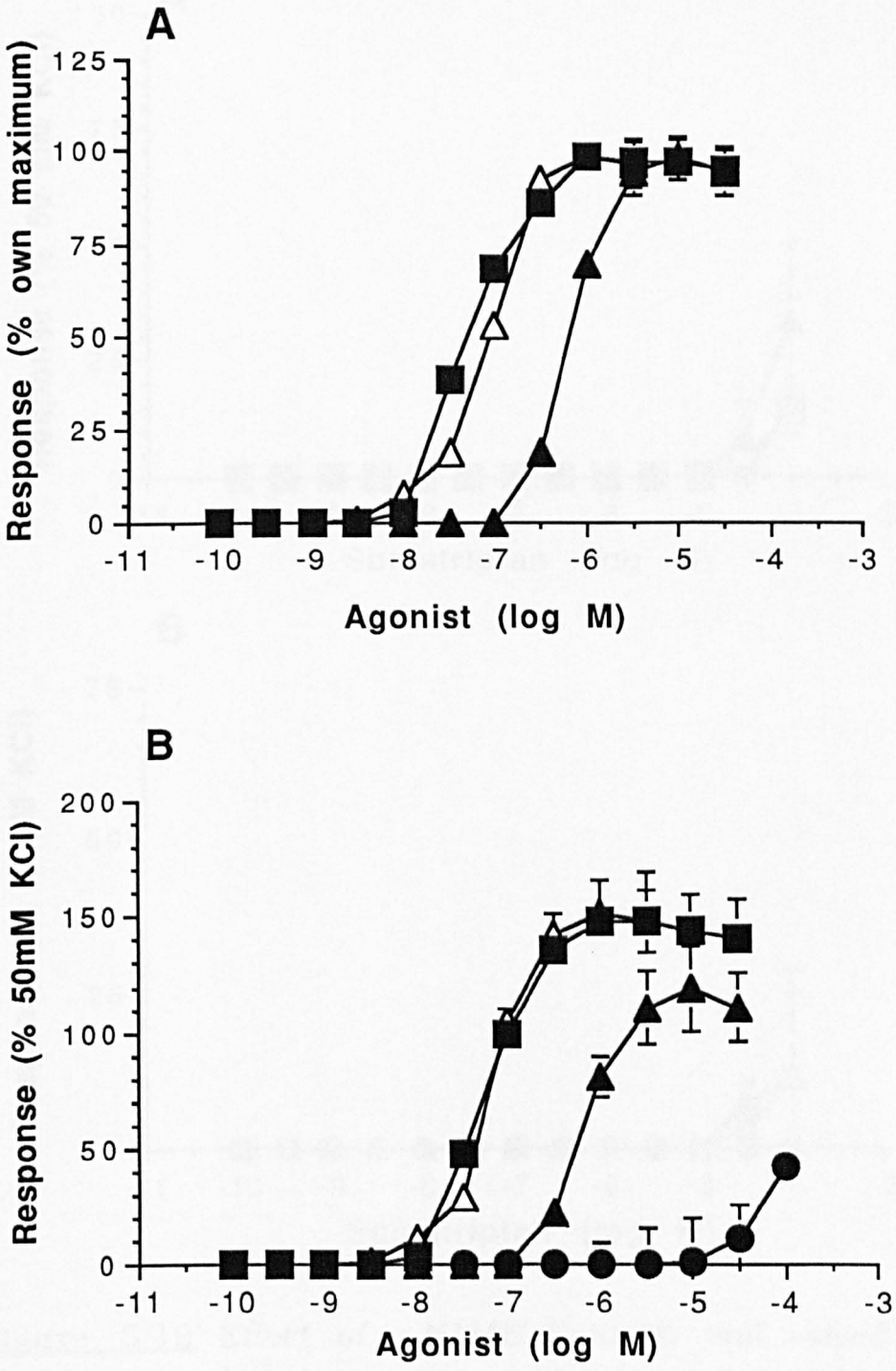

Figure 5.11 Response to selective agonists in 7 day rabbit extralobar branch pulmonary arteries. CCRCs; 5-HT (■,n = 6/6), α-methyl-5-HT (△, n = 6/6), 5-CT (▲, n = 6/6) and sumatriptan (●, n = 6/6). **A** Data are expressed as percentage own maximum contraction. **B** Data are expressed as percentage reference contraction to 50 mM KCl. Each point represents mean ± s.e.mean.

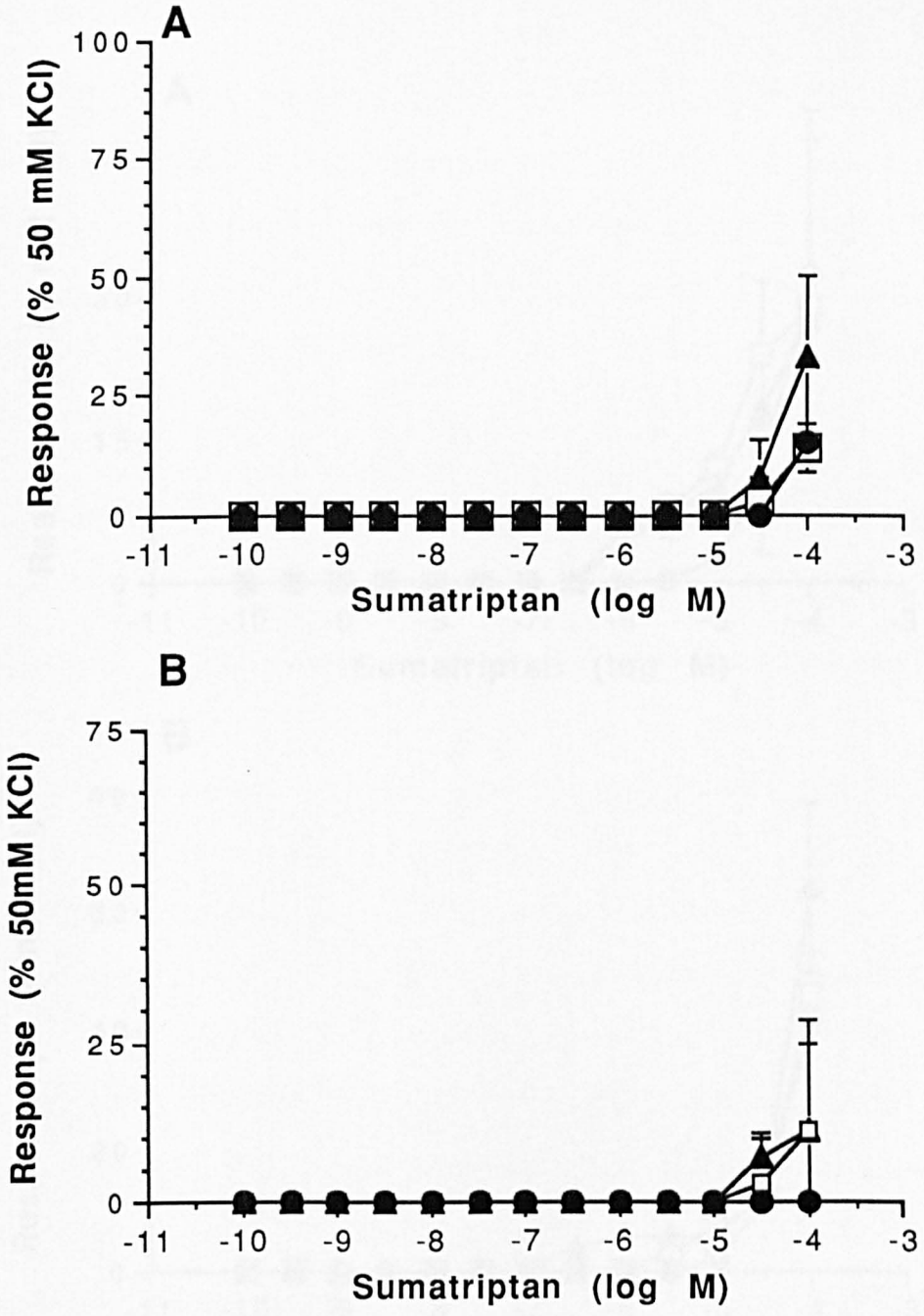

Figure 5.12 Effect of L-NAME (100μM) and raised tone on sumariptan-induced vasoconstriction in **A** foetal and **B** 0-24 hr rabbit extralobar pulmonary arteries. CCRCs to sumatriptan; control (●, n = 6/6), plus 100μM L-NAME (▲, n = 6/6) and plus U46619 (1-3nM)-induced tone (□, n = 6/6). Data are expressed as percentage reference contraction to 50 mM KCl. Each point represents mean ± s.e.mean. n = number vessels/number animals.

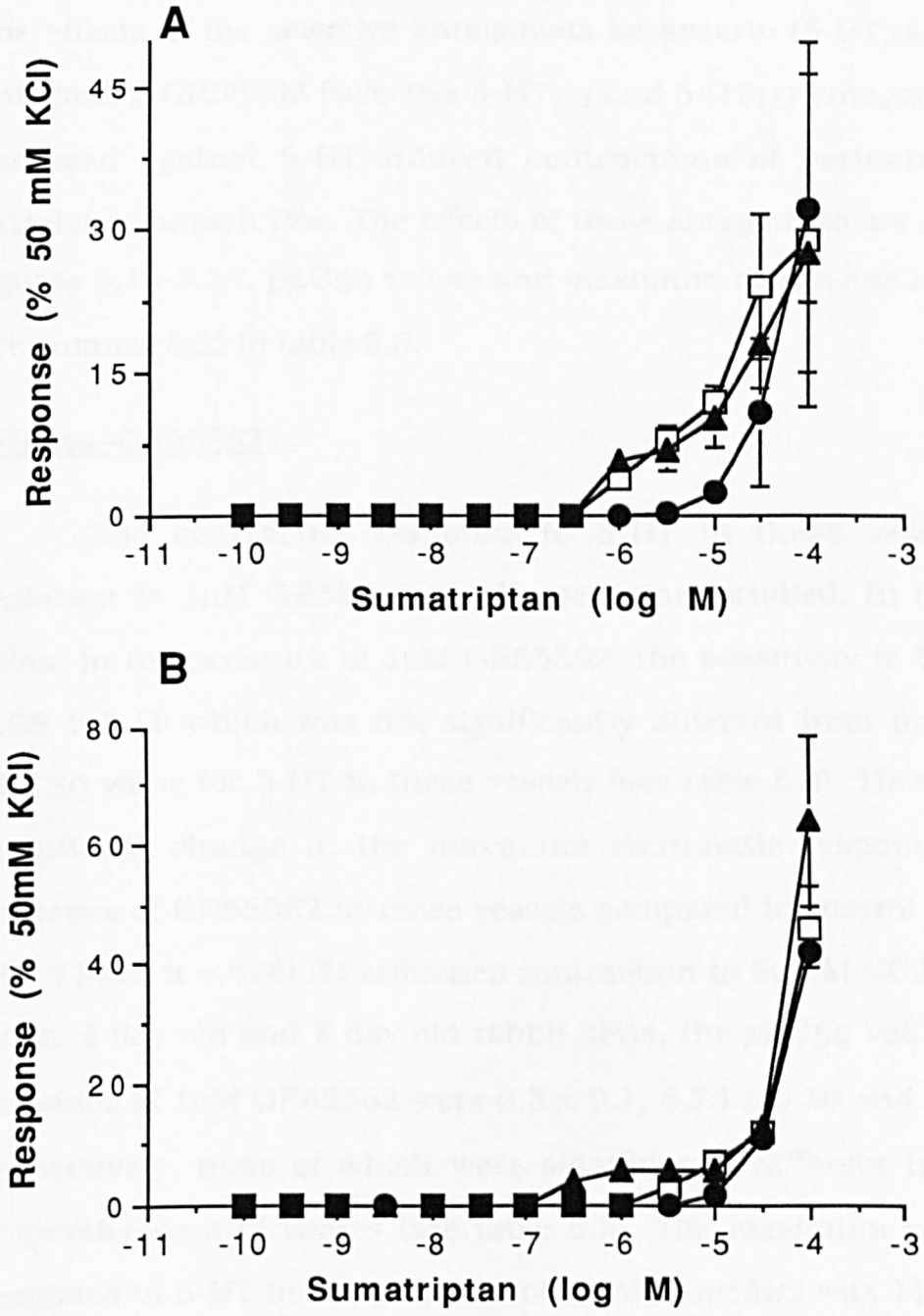

Figure 5.13 Effect of L-NAME (100μM) and raised tone on sumariptan-induced vasoconstriction in **A** 4 day and **B** 7 day rabbit extralobar pulmonary arteries. CCRCs to sumatriptan; control (●, n = 6/6), plus 100μM L-NAME (▲, n = 6/6) and plus U46619 (1-3nM)-induced tone (□, n = 6/6). Data are expressed as percentage reference contraction to 50 mM KCl. Each point represents mean ± s.e.mean. n = number vessels/number animals.

Antagonist studies

The effects of the selective antagonists ketanserin (5-HT_{2A} receptor antagonist) GR55562 (selective 5-HT_{1B} and 5-HT_{1D} antagonist) were assessed against 5-HT-induced contractions of perinatal rabbit extralobar branch PAs. The effects of these antagonists are shown in figures 5.14-5.17. pEC_{50} values and maximum contractile responses are summarised in table 5.3.

5-HT vs -GR55562

The contractile response to 5-HT in these vessels was resistant to 1μM GR55562 at all age points studied. In the foetal bPAs, in the presence of 1μM GR55562, the sensitivity to 5-HT was 6.98 ± 0.15 which was not significantly different from the control pEC_{50} value for 5-HT in these vessels (see table 5.3). There was no significant change in the maximum contractile response in the presence of GR55562 in these vessels compared to control (Emax. = 169 ±14%; n = 6/6) (% reference contraction to 50mM KCl). In 0-24 hour, 4 day old and 7 day old rabbit bPAs, the pEC_{50} values in the presence of 1μM GR55562 were 6.3 ± 0.1, 6.73 ± 0.16 and 7.1 ± 0.2 respectively, none of which were significantly different from their respective control values (see table 5.3). The maximum contractile response to 5-HT in the presence of 1μM GR55562 was 115 ± 15%, 138 ± 17% and 185 ± 21% (% reference contraction to 50mM KCl) in the 0-24 hour, 4 day and 7 day old rabbit vessels respectively. None of these values was significantly different to their respective control values (see table 5.3).

5-HT vs. ketanserin

At all age points studied, 30nM ketanserin, a selective 5-HT_{2A}

receptor antagonist, behaved as a potent antagonist causing a concentration-dependent parallel rightward shift of the CCRC to 5-HT with no significant reduction of the maximal contractile response (figures 5.14-5.17, table 5.3). In the foetal bPAs, the apparent pK_B for 30nM and 0.1μM ketanserin was 8.4 ± 0.2 and 9.3 ± 0.2 respectively. In the 0-24 hour rabbit bPAs, the apparent pK_B for ketanserin at 30nM was 8.7 ± 0.1. In the presence of 0.1μM ketanserin, in the 0-24 hour vessels, the CCRC was shifted rightward but did not achieve a maximal contractile response within the concentration range studied (figure 5.15 B). In the 4 day old rabbit vessels, although the CCRC to 5-HT in the presence of 30nM ketanserin appeared to evoke a lower maximal contraction than in control CCRCs to 5-HT, this was not statistically significant (figure 5.16 B, table 2). The apparent pK_B value in these vessels was 8.4 ± 0.2. In the presence of 0.1μM ketanserin, a maximal response to 5-HT was not achieved in these vessels. Concentration-dependent, rightward parallel shifts of the 5-HT CCRC were also observed for ketanserin in the 7 day old rabbit bPAs. The apparent pK_B values were 8.8 ± 0.2 and 9.2 ± 0.2 for 30nM and 0.1μM ketanserin respectively.

	pEC_{50}	$E_{max.}$ (%)	n
Foetal			
5-HT control	6.96 ± 0.15	153 ± 4	18/9
+ 30nM ketanserin	6.1 ± 0.1**	152 ± 10	6/6
+ 0.1μM ketanserin	5.12 ± 0.10***	149 ± 14	6/6
0-24 hours			
5-HT control	6.51 ± 0.05	114 ± 17	18/9
+ 30nM ketanserin	5.25 ± 0.1***	128 ± 12	6/6
+ 0.1μM ketanserin	nc	nc	6/6
4 day old			
5-HT control	6.76 ± 0.16	157 ± 16	21/12
+ 30nM ketanserin	5.85 ± 0.21**	115 ± 21	7/7
+ 0.1μM ketanserin	nc		8/8
7 day old			
5-HT control	7.02 ± 0.1	161 ± 18	20/11
+ 30nM ketanserin	5.7 ± 0.2***	174 ± 21	6/6
+ 0.1μM ketanserin	4.78 ± 0.15***	198 ± 26	8/8

Table 5.3 Effect of ketanserin on 5-hydroxytryptamine (5-HT)-induced contraction of perinatal rabbit extralobar branch pulmonary arteries. Data are shown as mean ± s.e.mean. Emax. = maximum contractile response expressed as a percentage of the reference contraction to 50mM KCl. Statistical comparisons were made by Students' paired t-test **P<0.01, ***P<0.001; compared with respective control 5-HT $pEC50$ data. n = number of vessels/number of animals. nc = not calculated.

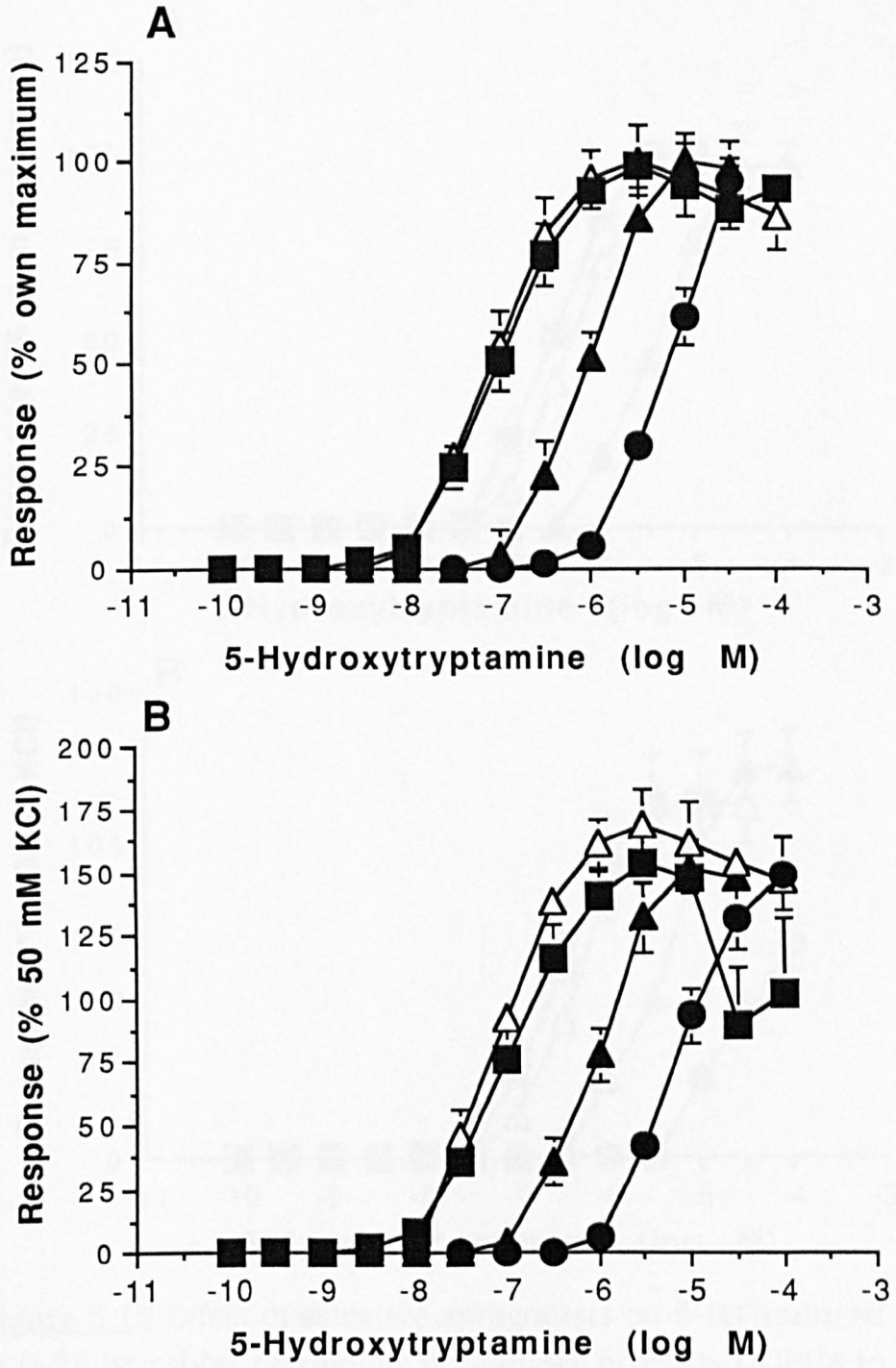

Figure 5.14 Effect of selective antagonists on 5-HT-induced responses in foetal rabbit extralobar pulmonary arteries. CCRCs to 5-HT (■,n = 18/9) and in the presence of 30nM ketanserin (▲,n = 6/6), 0.1μM ketanserin (●,n = 6/6) and 1μM GR55562 (△,n= 6/6).**A** Data are expressed as percentage own maximum contraction. **B** Data are expressed as percentage reference contraction to 50 mM KCl. Each point represents mean ± s.e.mean. n = number vessels/number animals.

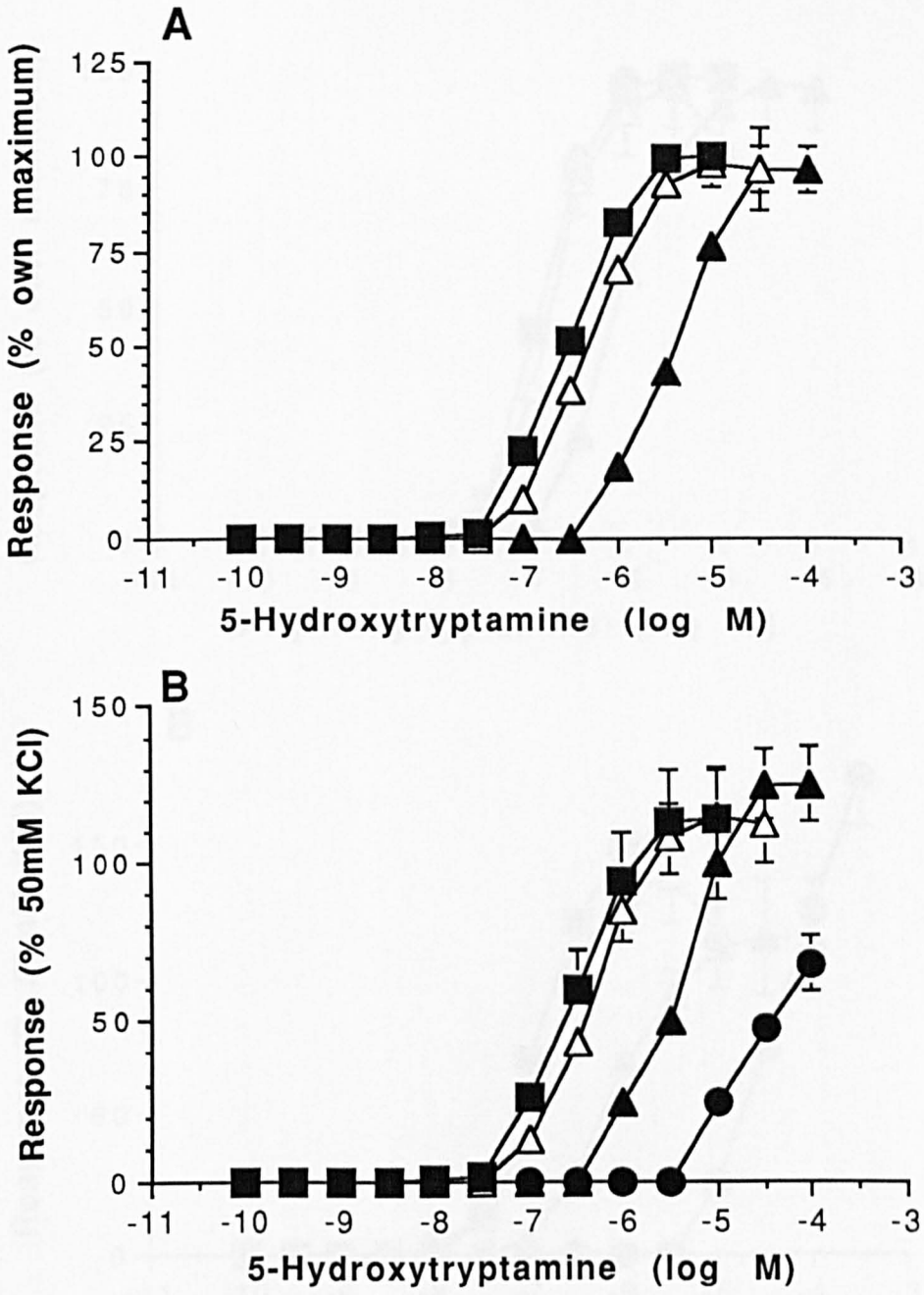

Figure 5.15 Effect of selective antagonists on 5-HT-induced responses in 0-24 hr rabbit extralobar pulmonary arteries. CCRCs to 5-HT (■,n = 18/9) and in the presence of 30nM ketanserin (▲,n = 6/6), 0.1μM ketanserin (●,n = 6/6) and 1μM GR55562 (△,n= 6/6).**A** Data are expressed as percentage own maximum contraction. **B** Data are expressed as percentage reference contraction to 50 mM KCl. Each point represents mean ± s.e.mean. n = number vessels/number animals.

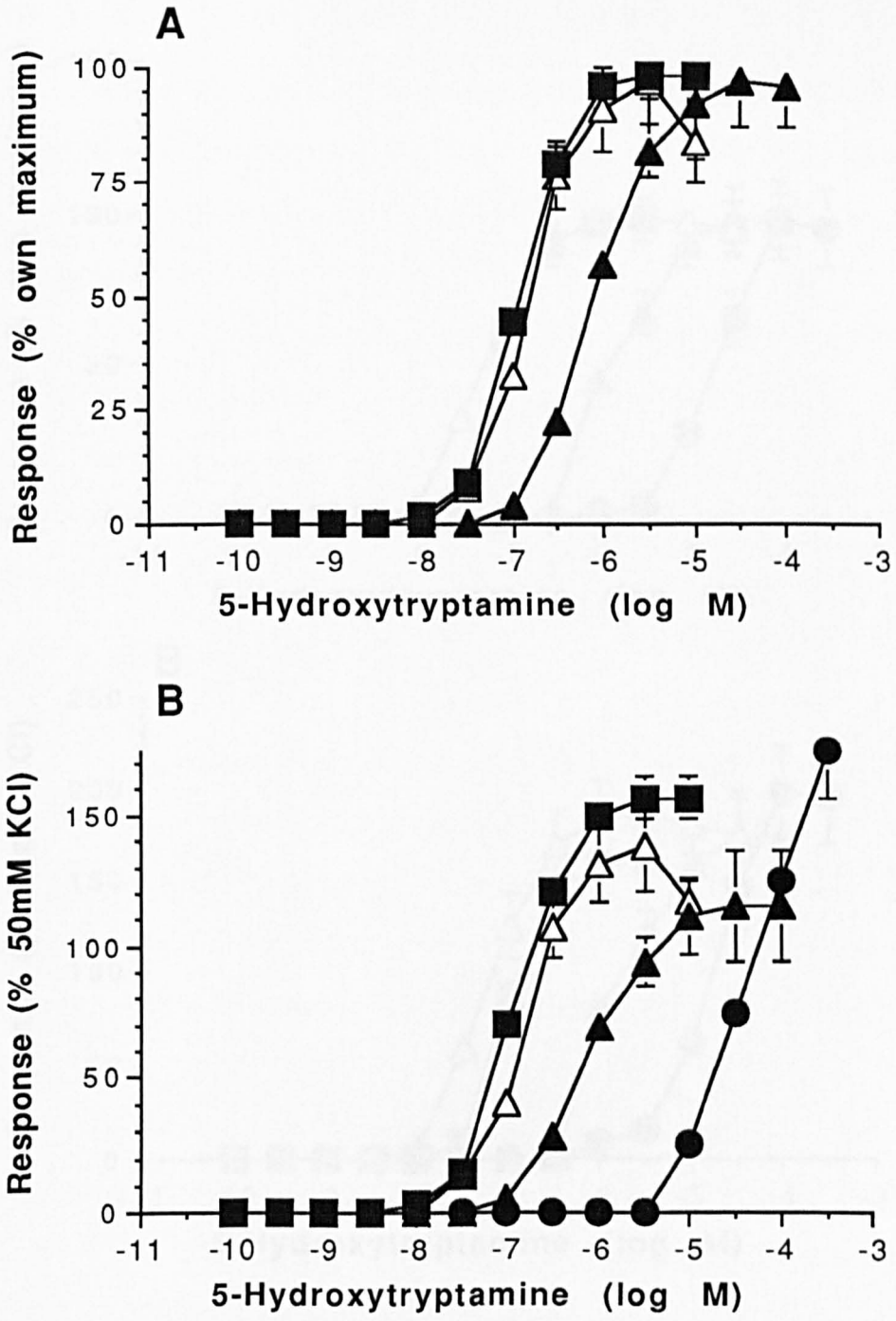

Figure 5.16 Effect of selective antagonists on 5-HT-induced responses in 4 day rabbit extralobar pulmonary arteries. CCRCs to 5-HT (■,n = 21/12) and in the presence of 30nM ketanserin (▲,n = 7/7), 0.1μM ketanserin (●,n = 8/8) and 1μM GR55562 (△,n= 6/6).**A** Data are expressed as percentage own maximum contraction. **B** Data are expressed as percentage reference contraction to 50 mM KCl. Each point represents mean ± s.e.mean. n = number vessels/number animals.

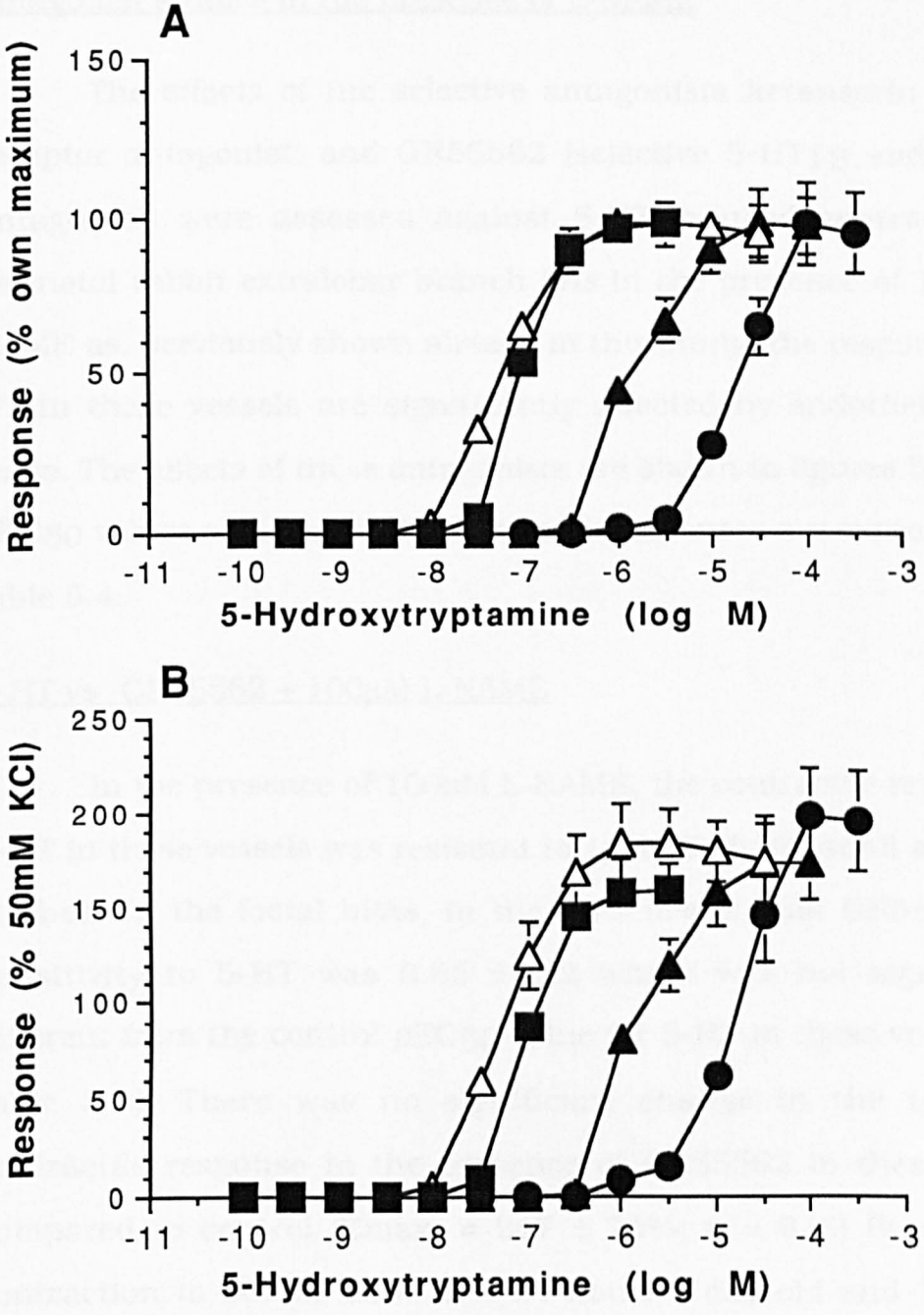

Figure 5.17 Effect of selective antagonists on 5-HT-induced responses in 7 day rabbit extralobar pulmonary arteries. CCRCs to 5-HT (■,n = 20/11) and in the presence of 30nM ketanserin (▲,n = 6/6), 0.1μM ketanserin (●,n = 8/8) and 1μM GR55562 (△,n= 6/6).**A** Data are expressed as percentage own maximum contraction. **B** Data are expressed as percentage reference contraction to 50 mM KCl. Each point represents mean ± s.e.mean. n = number vessels/number animals.

Antagonist studies in the presence of L-NAME

The effects of the selective antagonists ketanserin (5-HT_{2A} receptor antagonist) and GR55562 (selective 5-HT_{1B} and 5-HT_{1D} antagonist) were assessed against 5-HT-induced contractions of perinatal rabbit extralobar branch PAs in the presence of 100μM L-NAME as, previously shown already in this study, the responses to 5-HT in these vessels are significantly affected by endothelial nitric oxide. The effects of these antagonists are shown in figures 5.18-5.21. pEC_{50} values and maximum contractile responses are summarised in table 5.4.

5-HT vs -GR55562 + 100μM L-NAME

In the presence of 100μM L-NAME, the contractile response to 5-HT in these vessels was resistant to 1μM GR55562 at all age points studied. In the foetal bPAs, in the presence of 1μM GR55562, the sensitivity to 5-HT was 6.85 ± 0.2 which was not significantly different from the control pEC_{50} value for 5-HT in these vessels (see table 5.4). There was no significant change in the maximum contractile response in the presence of GR55562 in these vessels compared to control (Emax. = 217 ± 25%; n = 6/6) (% reference contraction to 50mM KCl). In 0-24 hour, 4 day old and 7 day old rabbit bPAs, the pEC_{50} values in the presence of 1μM GR55562 were 7.1 ± 0.1, 6.89 ± 0.15 and 7.01 ± 0.15 respectively, none of which were significantly different from their respective control values (see table 5.4). The maximum contractile response to 5-HT in the presence of 1μM GR55562 was 129 ± 11%, 174 ± 18% and 1226 ± 31% (% reference contraction to 50mM KCl) in the 0-24 hour, 4 day and 7 day old rabbit vessels respectively. None of these values was significantly different to their respective control values (see table 5.4).

5-HT vs ketanserin + 100μM L-NAME

Ketanserin, in the presence of L-NAME, caused concentration-dependent rightward shifts of the CCRC to 5-HT in perinatal rabbit bPAs at all age points studied (figures 5.18-5.21). In the foetal vessels (figure 5.18), the pK_B value for ketanserin was 8.57 ± 0.2 and 9.00 ± 0.21 for 30nM and 0.1μM ketanserin respectively. There was no change in the maximum contractile response to 5-HT in the presence of ketanserin in these vessels (figure 5.18 B, table 5.4). In the 0-24 hour rabbit vessels (figure 5.19), the pK_B value for ketanserin was 8.8 ± 0.19 and 8.9 ± 0.2 for 30nM and 0.1μM ketanserin respectively. The maximum contractile response to 5-HT in the 4 day old rabbit bPAs appeared to be greater in the presence of 30nM ketanserin than in control response to 5-HT (figure 5.20 B), this was not statistically significant; ketanserin at this concentration had an apparent pK_B of 8.7 ± 0.18. At 0.1μM ketanserin, the apparent pK_B was 8.57 ± 0.2 in these vessels. In the 7 day old rabbit bPAs, ketanserin (30nM) significantly inhibited 5-HT responses (figure 5.21), giving an apparent pK_B value of 8.96 ± 0.19, this value and pEC_{50} value could not be calculated at 0.1μM ketanserin since the responses to 5-HT in the presence of this concentration of antagonist did not reach a maximum (figure 5.21 B).

5-HT receptor-mediated vasodilation

At all the age points studied, 5-HT, 5-CT, sumatriptan and BW 723C86, did not cause any significant relaxation in bPAs precontracted with phenylephrine (0.1-0.3μM). The level of precontraction was similar at each age point being 78 ± 12%, 91 ±13%, 81 ± 6% and 102 ± 14% (% contraction to 50mM KCl) in the foetal, 0-24 hour, 4 day old and 7 day old rabbit bPAs respectively.

	pEC_{50}	$E_{max.}$ (%)	n
Foetal			
5-HT + 100μM L-NAME	7.21 ± 0.15	207 ± 13	6/6
+ 30nM ketanserin	6.1 ± 0.2***	225 ± 24	6/6
+ 0.1μM ketanserin	5.20 ± 0.15***	200 ± 17	6/6
0-24 hours			
5-HT + 100μM L-NAME	7.10 ± 0.08	106 ± 13	6/6
+ 30nM ketanserin	5.78 ± 0.1***	119 ± 14	6/6
+ 0.1μM ketanserin	5.21 ± 0.16***	128 ± 14	6/6
4 day old			
5-HT + 100μM L-NAME	6.92 ± 0.13	223 ± 25	6/6
+ 30nM ketanserin	5.70 ± 0.15***	297 ± 32	6/6
+ 0.1μM ketanserin	5.35 ± 0.15***	189 ± 21	8/8
7 day old			
5-HT + 100μM L-NAME	7.23 ± 0.2	275 ± 27	6/6
+ 30nM ketanserin	5.75 ± 0.2***	220 ± 28	6/6
+ 0.1μM ketanserin	nc	nc	6/6

Table 5.4 Effect of ketanserin on 5-hydroxytryptamine (5-HT)-induced contraction of perinatal rabbit extralobar branch pulmonary arteries in the presence of 100μM L-NAME. Data are shown as mean ± s.e.mean. Emax. = maximum contractile response expressed as a percentage of the reference contraction to 50mM KCl. Statistical comparisons were made by ANOVA **P<0.01, ***P<0.001; compared with respective control 5-HT pEC_{50} data. n = number of vessels/number of animals. nc = not calculated.

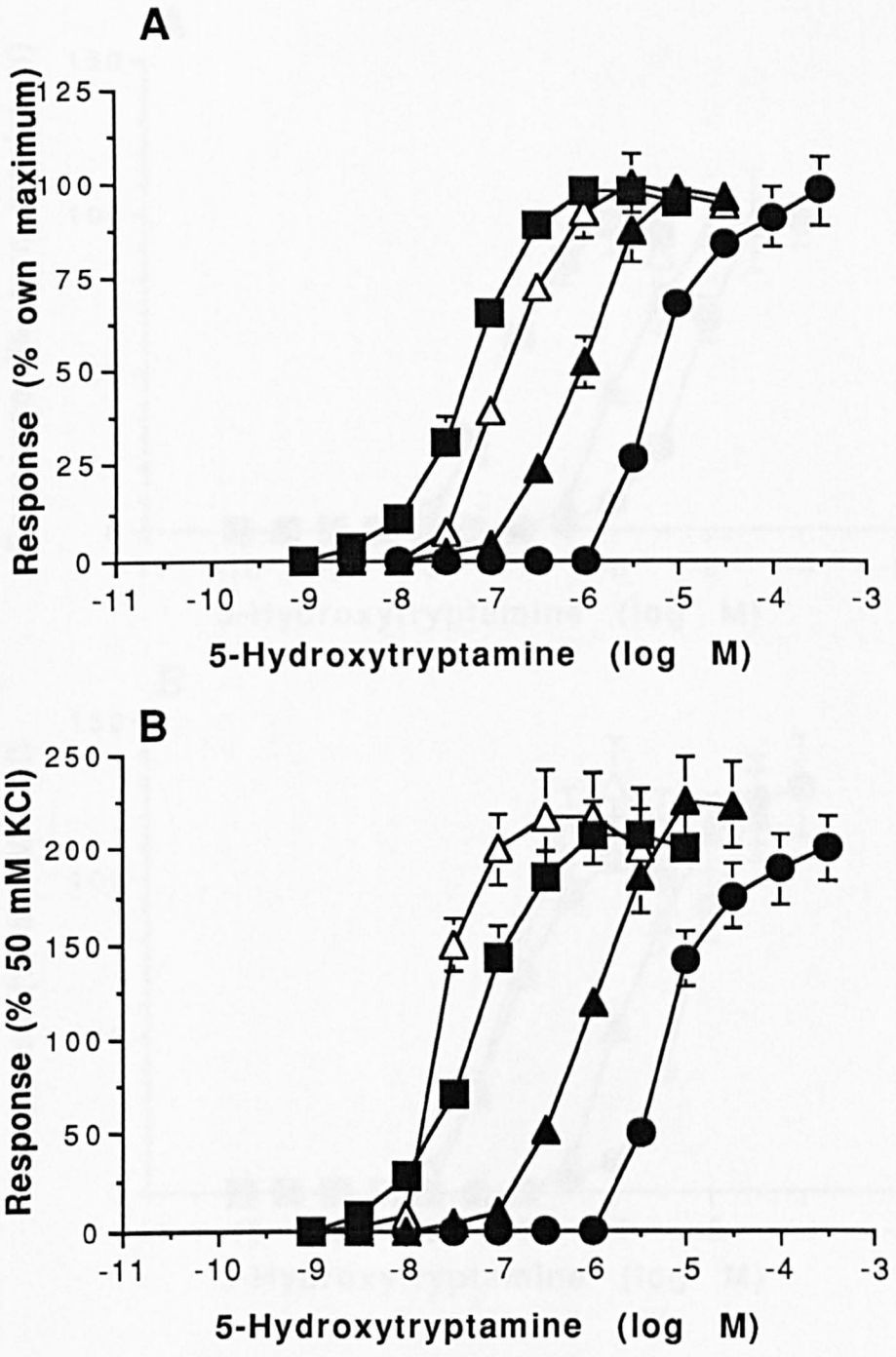

Figure 5.18 Effect of selective antagonists on 5-HT-induced responses in foetal rabbit extralobar pulmonary arteries in the presence of 100μM L-NAME. CCRCs to 5-HT (■,n = 6/6) and in the presence of 30nM ketanserin (▲,n = 6/6), 0.1μM ketanserin (●,n = 6/6) and 1μM GR55562 (△,n= 5/5).**A** Data are expressed as percentage own maximum contraction. **B** Data are expressed as percentage reference contraction to 50 mM KCl. Each point represents mean ± s.e.mean. n = number vessels/number animals.

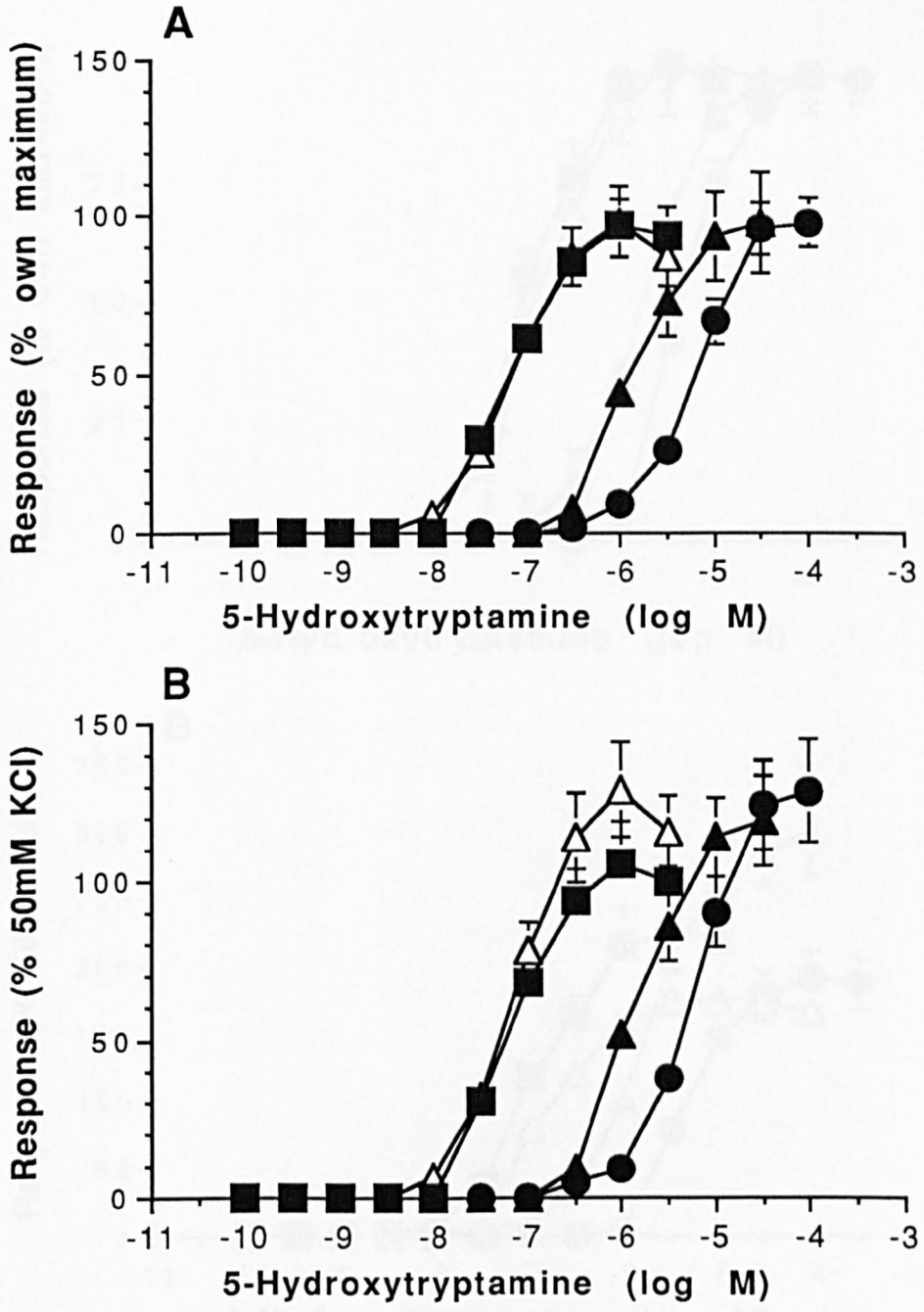

Figure 5.19 Effect of selective antagonists on 5-HT-induced responses in 0-24 hr rabbit extralobar pulmonary arteries in the presence of 100μM L-NAME. CCRCs to 5-HT (■,n = 6/6) and in the presence of 30nM ketanserin (▲,n = 6/6), 0.1μM ketanserin (●,n = 6/6) and 1μM GR55562 (△,n= 6/6).**A** Data are expressed as percentage own maximum contraction. **B** Data are expressed as percentage reference contraction to 50 mM KCl. Each point represents mean ± s.e.mean. n = number vessels/number animals.

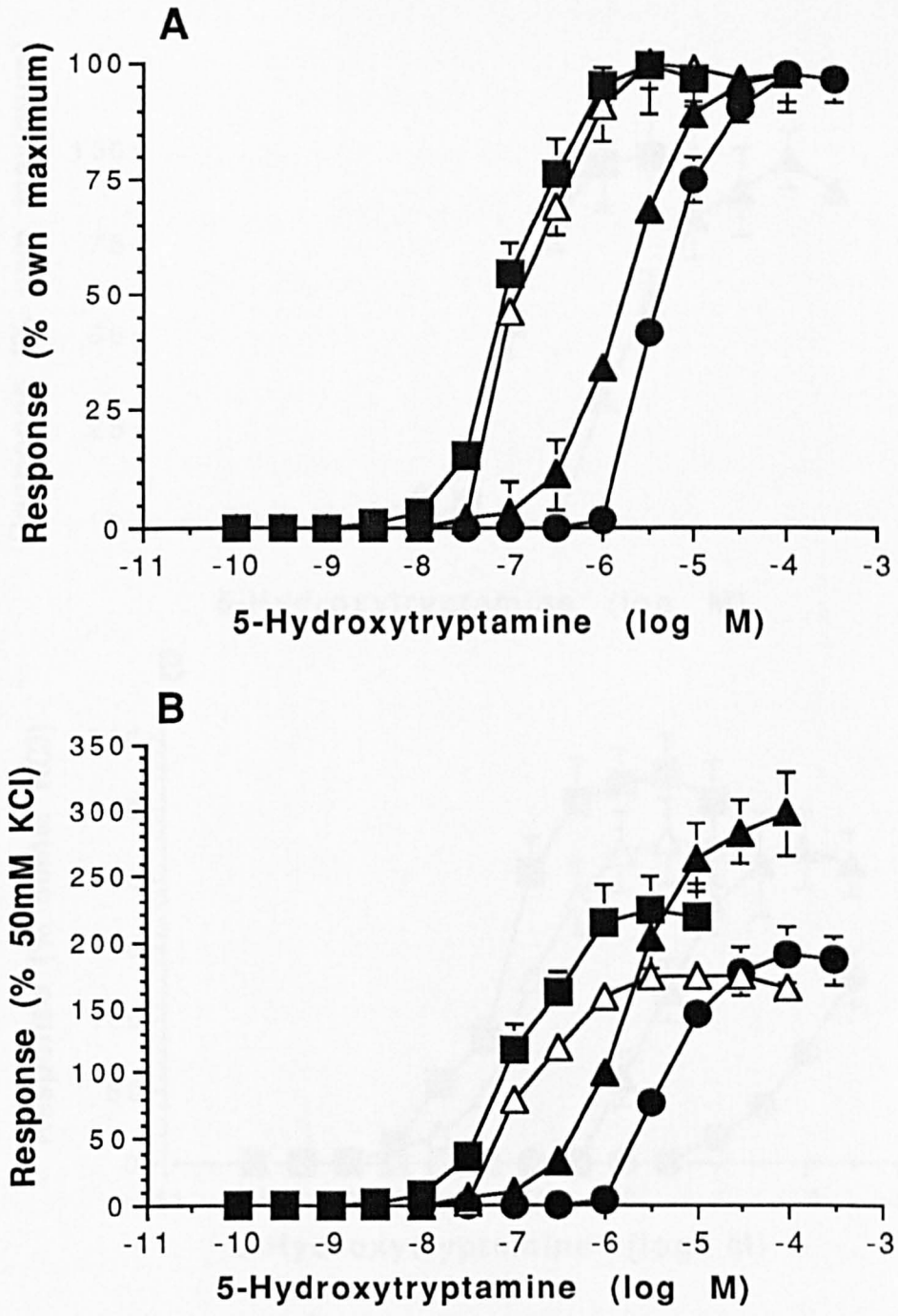

Figure 5.20 Effect of selective antagonists on 5-HT-induced responses in 4 day rabbit extralobar pulmonary arteries in the presence of 100μM L-NAME. CCRCs to 5-HT (■,n = 8/8) and in the presence of 30nM ketanserin (▲,n = 6/6), 0.1μM ketanserin (●,n = 6/6) and 1μM GR55562 (△,n= 6/6). **A** Data are expressed as percentage own maximum contraction. **B** Data are expressed as percentage reference contraction to 50 mM KCl. Each point represents mean ± s.e.mean. n = number vessels/number animals.

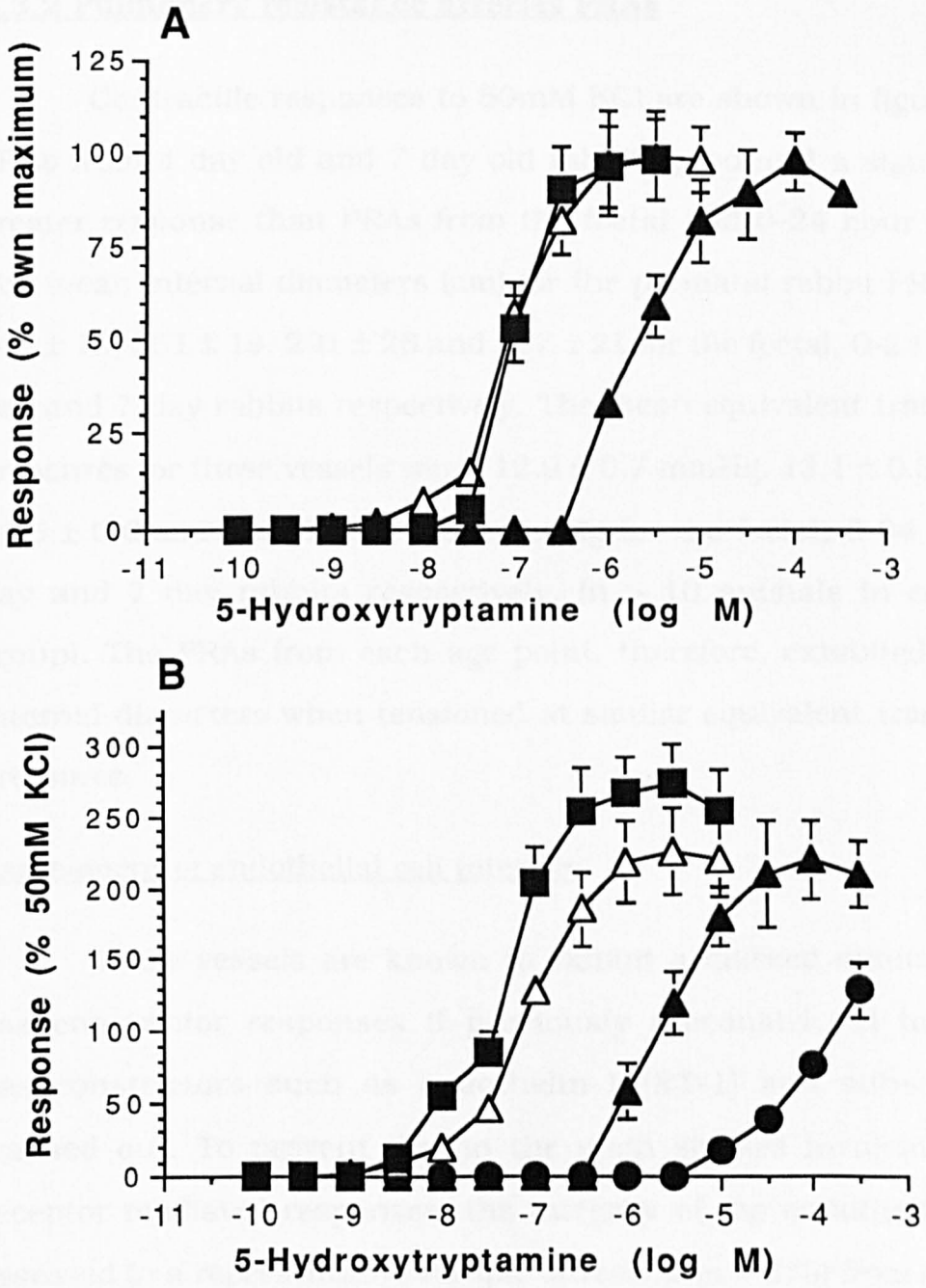

Figure 5.21 Effect of selective antagonists on 5-HT-induced responses in 7 day rabbit extralobar pulmonary arteries in the presence of 100μM L-NAME. CCRCs to 5-HT (■,n = 6/6) and in the presence of 30nM ketanserin (▲,n = 6/6), 0.1μM ketanserin (●,n = 6/6) and 1μM GR55562 (△,n= 6/6). **A** Data are expressed as percentage own maximum contraction. **B** Data are expressed as percentage reference contraction to 50 mM KCl. Each point represents mean ± s.e.mean. n = number vessels/number animals.

5.3.2 Pulmonary resistance arteries PRAs

Contractile responses to 50mM KCl are shown in figure 5.22. PRAs from 4 day old and 7 day old rabbits produced a significantly greater response than PRAs from the foetal and 0-24 hour rabbits. The mean internal diameters (μm) for the perinatal rabbit PRAs were 243 ± 16, 261 ± 19, 291 ± 26 and 287 ± 21 for the foetal, 0-24 hour, 4 day and 7 day rabbits respectively. The mean equivalent transmural pressures for these vessels were 12.9 ± 0.7 mmHg, 13.1 ± 0.3mmHg, 12.5 ± 0.6mmHg and 13.3 ± 0.9 mmHg for the foetal, 0-24 hour, 4 day and 7 day rabbits respectively. (n > 10 animals in each age group). The PRAs from each age point, therefore, exhibited similar internal diameters when tensioned at similar equivalent transmural pressures.

Assessment of endothelial cell integrity

These vessels are known to exhibit a marked diminution in vasoconstrictor responses if previously preconstricted to known vasoconstrictors such as endothelin-1 (ET-1) and subsequently washed out. To prevent this in the main studies involving 5-HT-receptor mediated responses, the integrity of the endothelium was assessed in a representative sample of vessels (n = 5/5) from each age point. 1μM ACh elicited a marked vasodilator response in ET-1 (10nM) precontracted vessels (> 80% reversal of ET-1-induced contraction) in all the representative vessels from each age point indicating the presence of a functional endothelium (see table 5.4i). Addition of 1μM SNP to similarly precontracted vessels resulted in relaxation of the vessels to baseline tension at all age points.

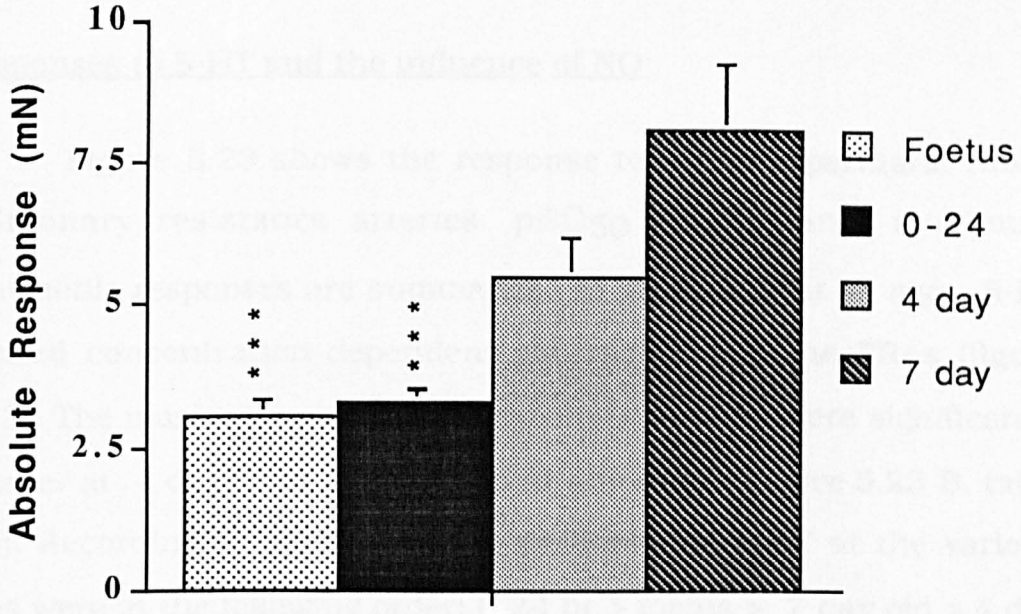

Figure 5.22 Contractile responses to 50mM KCl in PRAs from perinatal rabbits. n = > 10 lungs for each age. Data are expressed as absolute force contraction in mN. Each column represents the mean ± s.e.mean. Statistical comparisons were made using ANOVA followed by Tukey's post test; ***$P < 0.001$ cf. 7 day response.

AGE	ET-1 induced tone (% 50mM KCl)	Relaxatory response to 1μM ACh (% ET-1 induced tone)
Foetal	67.6 ± 3.6	97 ± 6
0-24 hour	62.5 ± 4.5	92 ± 8
4 days	61.3 ± 5.5	86 ± 6
7 days	71.6 ± 7.8	95 ± 5

Table 5.4i Relaxatory responses of perinatal rabbit PRAs to 1μM ACh following preconstriction with 10nM ET-1.

Responses to 5-HT and the influence of NO

Figure 5.23 shows the response to 5-HT in perinatal rabbit pulmonary resistance arteries. pEC_{50} values and maximum contractile responses are summarized in Table 5.5. At all ages, 5-HT elicited concentration-dependent contractions in the PRAs (figure 5.23). The maximum contractile responses to 5-HT were significantly smaller at 4 days and 7 days than at other ages (figure 5,23 B, table 5.5). According to pEC_{50} values, potencies of 5-HT at the various ages were in the following order: 0-24 hr > foetus ≥ 7 day old > 4 day old.

The response of perinatal PRAs to 5-HT in the presence and absence of 100μM L-NAME are shown in figures 5.24-5.27. pEC_{50} values and maximum contractile responses are summarised in table 5.5. In the foetal PRAs, NOS inhibition with L-NAME did not significantly alter the contractile response to 5-HT either at the pEC_{50} level or the maximum contractile response (figure 5.24, table 5.5). There was an increase in maximum contractile response to 5-HT in the presence of L-NAME in the 0-24 hour PRAs (figure 5.25 B, table 5.5) but there was no significant change in 5-HT potency (figure 5.25 A, table 5.5). In both the 4 day old and 7 day PRAs, inhibition of NOS resulted in profound increases in the sensitivity and in the maximum contractile responses to 5-HT (figures 5.26 and 5.27, table 5.5.

The order of 5-HT potency was altered in the presence of L-NAME: 7 day >> 0-24 hour = fetus > 4 day. In the 0-24hr and 4 day vessels, in the presence of L-NAME, the maximum responses to 5-HT were significantly greater than at other age points (Table 5.5).

5-HT receptor agonist studies

The selective 5-HT_{2A} receptor agonist α-methyl-5-HT (in the presence of L-NAME) elicited concentration-dependent contractions in rabbit PRAs at all ages (Table 5.5). There was no significant difference in sensitivity or maximum response to α-methyl-5-HT when compared to 5-HT at any other age. However, at 0-24hrs and 4 days (as with 5-HT in the presence of L-NAME) the maximum responses to α-methyl-5-HT was significantly greater than at other age points (Table 5.5).

5-CT only caused contractions at very high (>10mM) concentrations, in the foetal to 7 day period, and the size of these contractions were extremely variable and never greater than ~15% of the response to 50mM KCl. Sumatriptan did not contract vessels from the foetal-7 day period.

	5-HT Control		5-HT+ 0.1mM L-NAME		α-methyl-5-HT (+0.1mM L-NAME)	
Age	pEC_{50}	E_{max}	pEC_{50}	E_{max}	pEC_{50}	E_{max}
Foetus (n = 6/6)	6.47 ± 0.11	42 ± 8	6.79 ± 0.17	66 ± 9§	6.80 ± 0.19	65 ± 7§
0-24 h (n = 7/7)	6.93 ± 0.08†	63 ± 15	6.98 ± 0.15	102 ± 8*	6.79 ± 0.16	100 ± 9
4 days (n = 6/6)	5.99 ± 0.03†	15 ± 1	6.38 ± 0.12*	95 ± 20**	6.48 ± 0.19	95 ± 9
7 days (n = 6/6)	6.32 ± 0.04	7 ± 1§§	7.73 ± 0.07***	64 ± 2***§§§	7.57 ± 0.05	66 ± 4§§

Figure 5.5 pEC_{50} and E_{max} values for 5-hydroxytryptamine (5-HT) and α-methyl-5-HT in perinatal rabbit pulmonary resistance arteries in the presence and absence of 100μM L-NAME. E_{max}: maximum contractile response (% of response to 50mM KCl); L-NAME: N^{ω}-nitro-L-arginine methylester. Statistical comparison using ANOVA: compared with 5-HT controls $^{*}P<0.05$, $^{**}P<0.01$; $^{***}P<0.001$; compared with foetal response, $^{\dagger}P<0.05$; compared with 0-24 h data. $^{\S}P<0.05$, $^{\S\S}P<0.01$, $^{\S\S\S}P<0.001$ (ANOVA). n = number of vessels/number of rabbits.

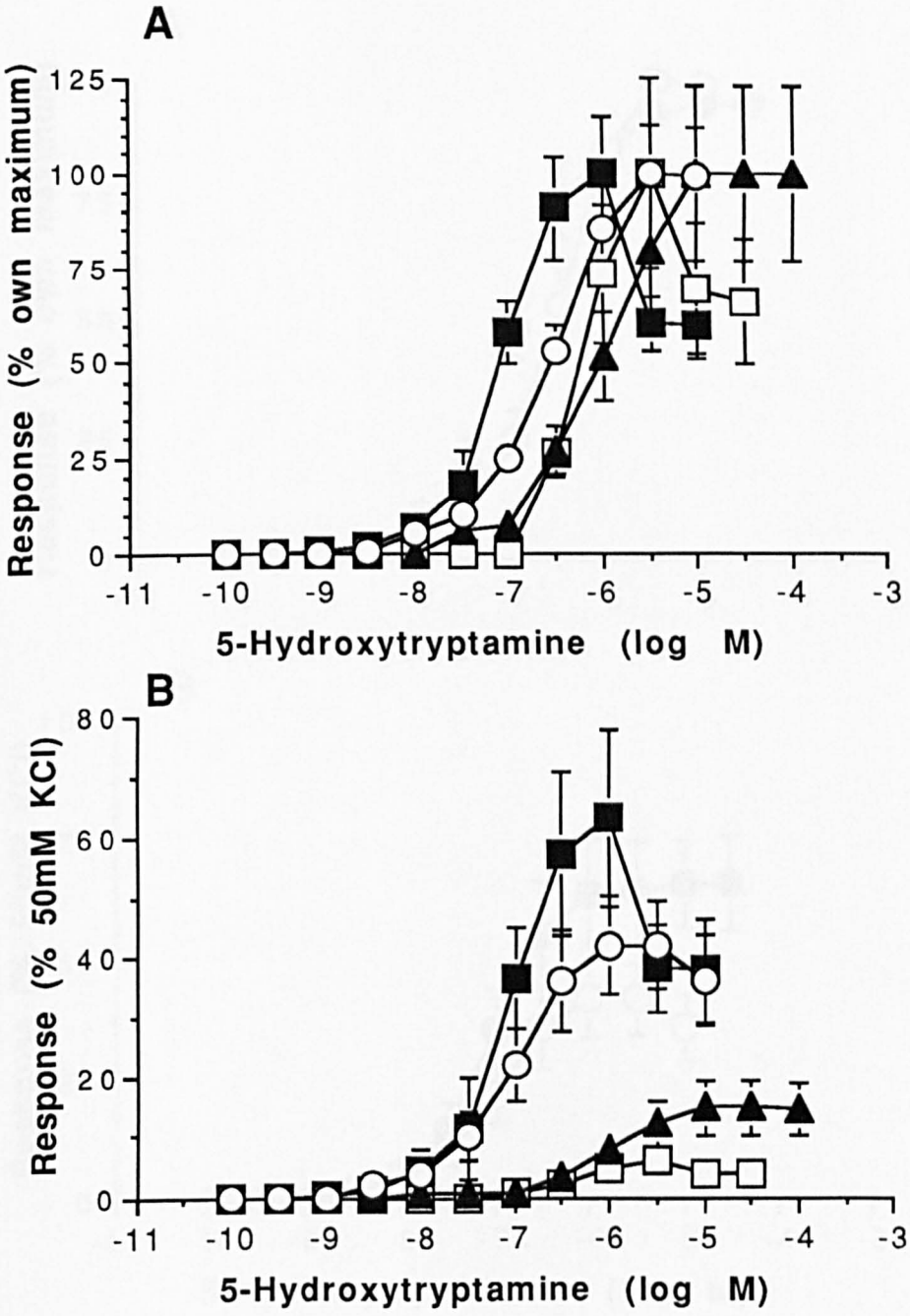

Figure 5.23 Contractile responses to 5-HT in perinatal rabbit PRAs.CCRCs to 5-HT in foetal (O,n = 6/6), 0-24 hour (■,n = 6/6), 4 day (▲,n = 6/6) and 7 day old (□,n = 6/6) rabbit PRAs. **A** Data are expressed as percentage own maximum contraction. **B** Data are expressed as percentage reference contraction to 50 mM KCl. Each point represents mean ± s.e.mean. n = number vessels/number animals.

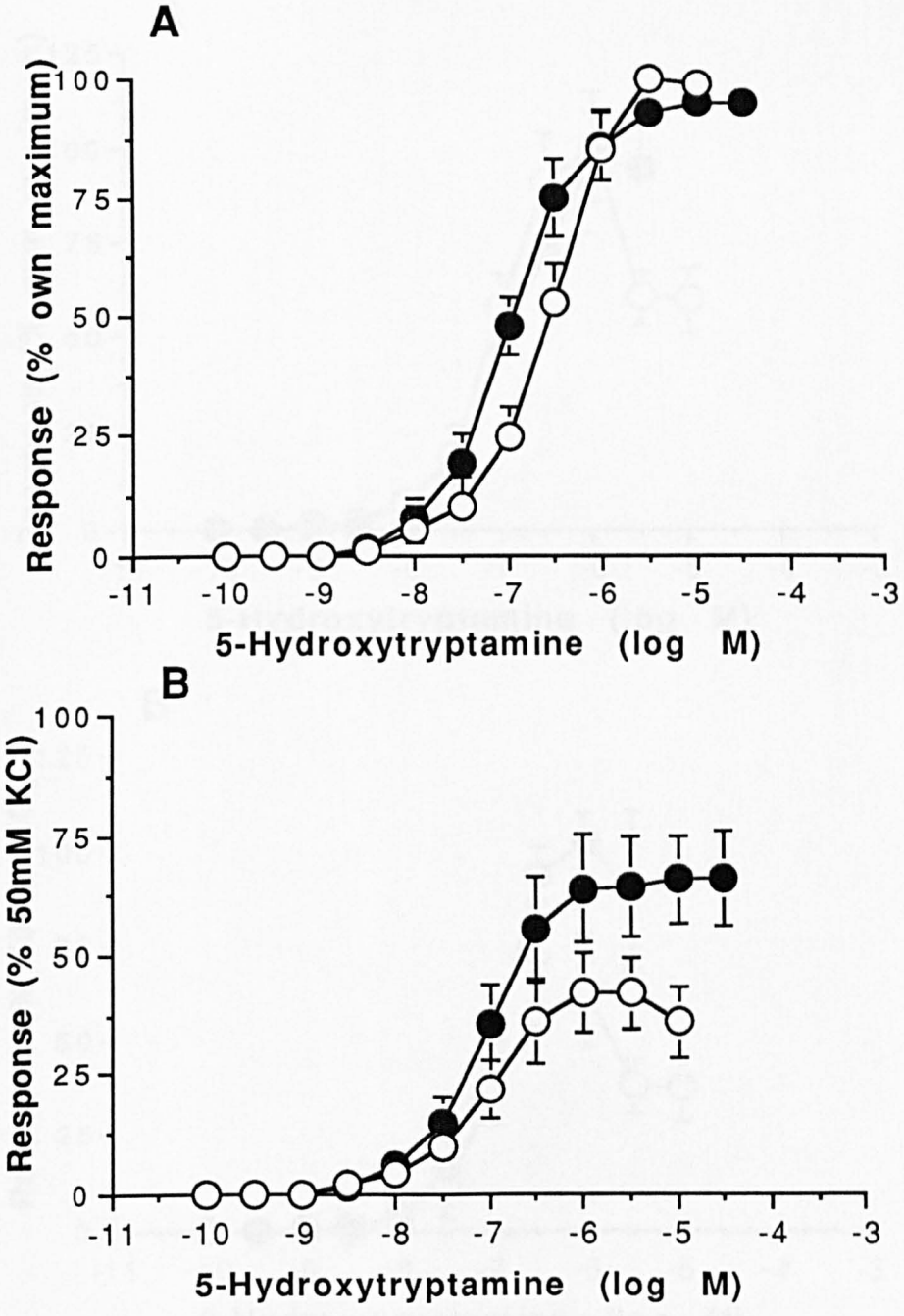

Figure 5.24 Effect of L-NAME (100μM) on responses to 5-HT in foetal rabbit PRAs. 5-HT CCRCs; control (O, n= 6/6) and in the presence of L-NAME (●, n= 6/6). **A** Data are expressed as percentage own maximum contraction. **B** Data are expressed as percentage reference contraction to 50 mM KCl. Each point represents mean ± s.e.mean. n = number vessels/number animals.

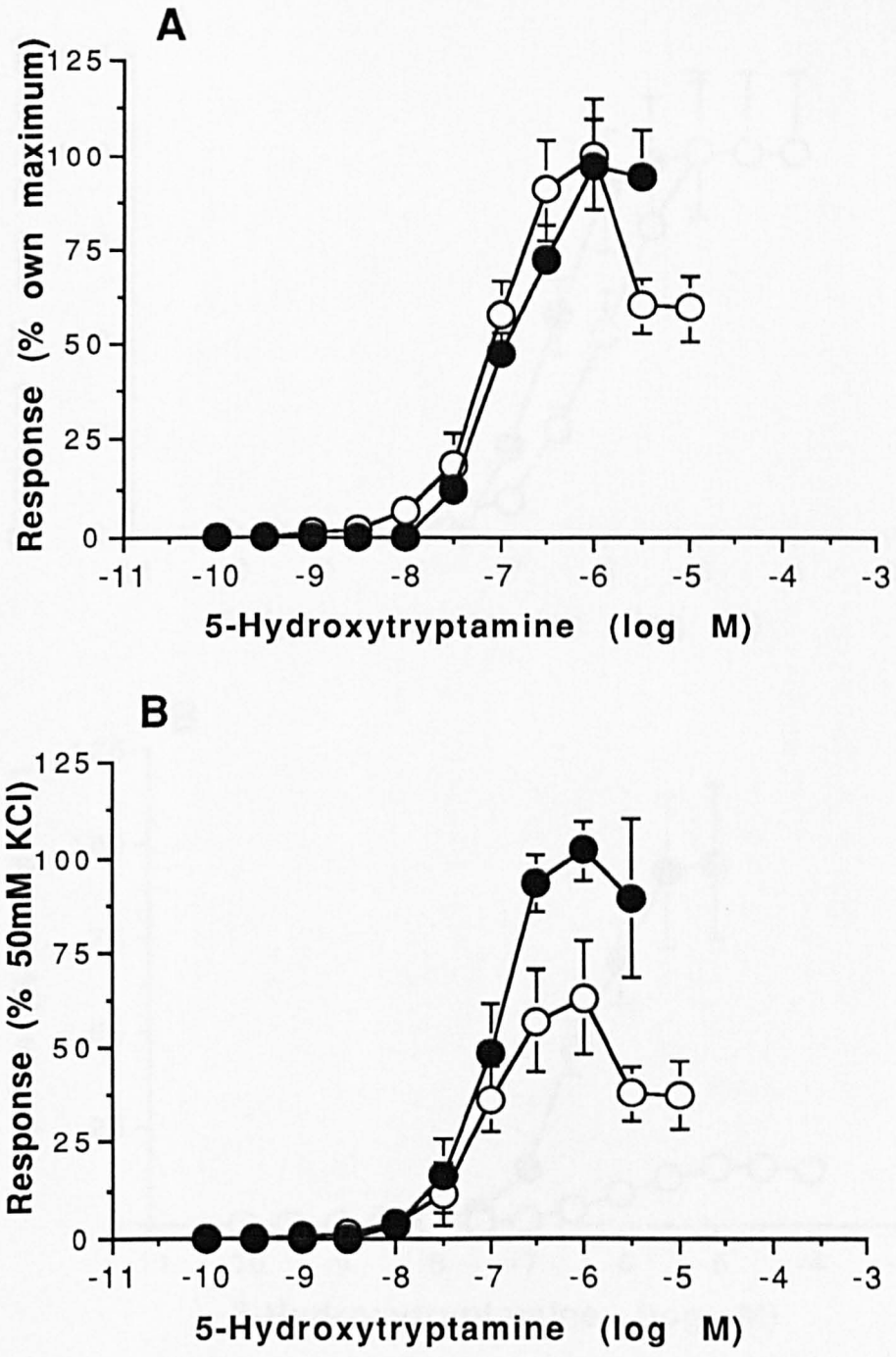

Figure 5.25 Effect of L-NAME (100μM) on responses to 5-HT in0-24 hrs rabbit PRAs. 5-HT CCRCs; control (O, n= 6/6) and in the presence of L-NAME (●, n= 7/7). **A** Data are expressed as percentage own maximum contraction. **B** Data are expressed as percentage reference contraction to 50 mM KCl. Each point represents mean ± s.e.mean. n = number vessels/number animals.

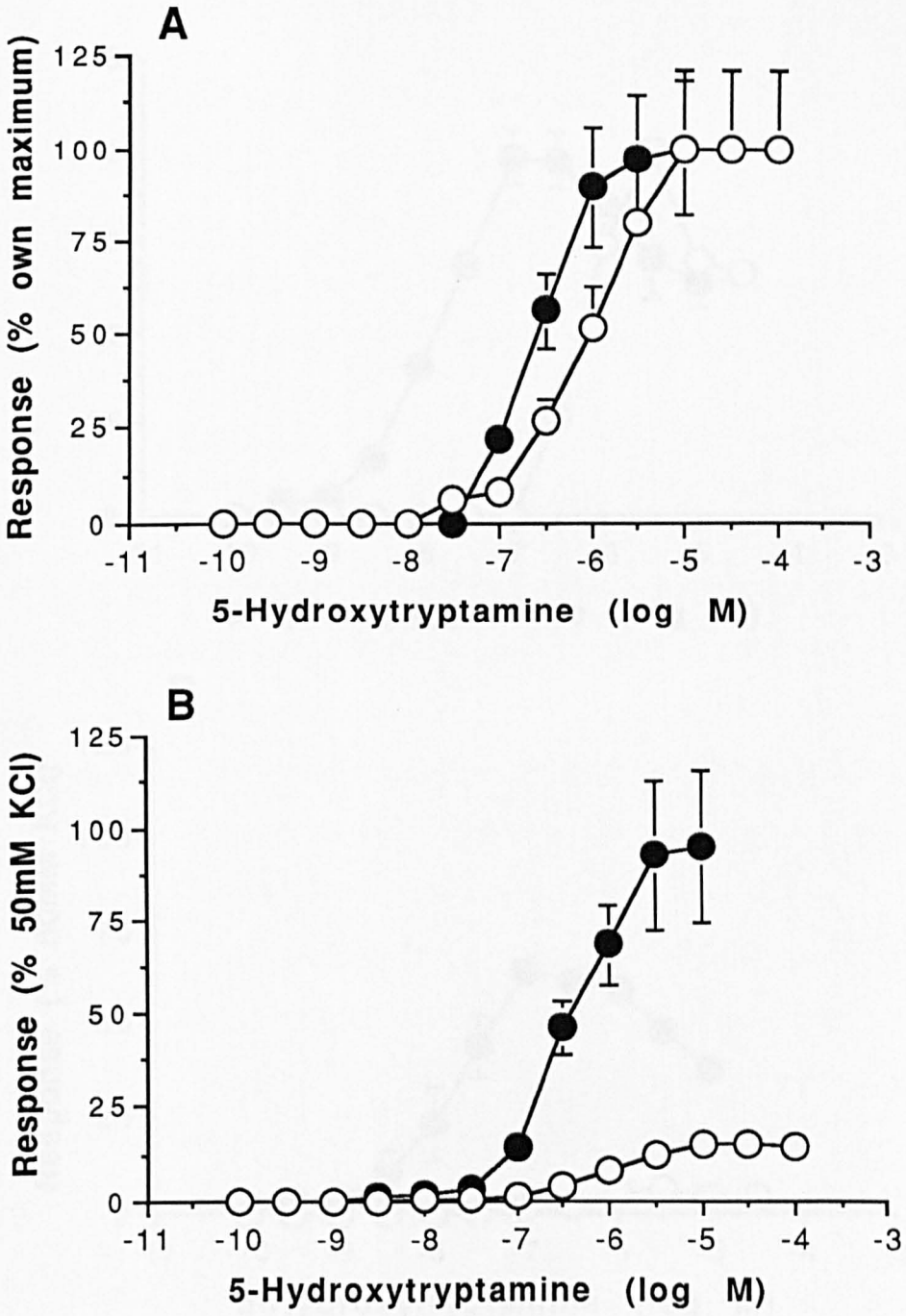

Figure 5.26 Effect of L-NAME (100μM) on responses to 5-HT in 4 day old rabbit PRAs. 5-HT CCRCs; control (O, n= 6/6) and in the presence of L-NAME (●, n= 6/6). **A** Data are expressed as percentage own maximum contraction. **B** Data are expressed as percentage reference contraction to 50 mM KCl. Each point represents mean ± s.e.mean. n = number vessels/number animals.

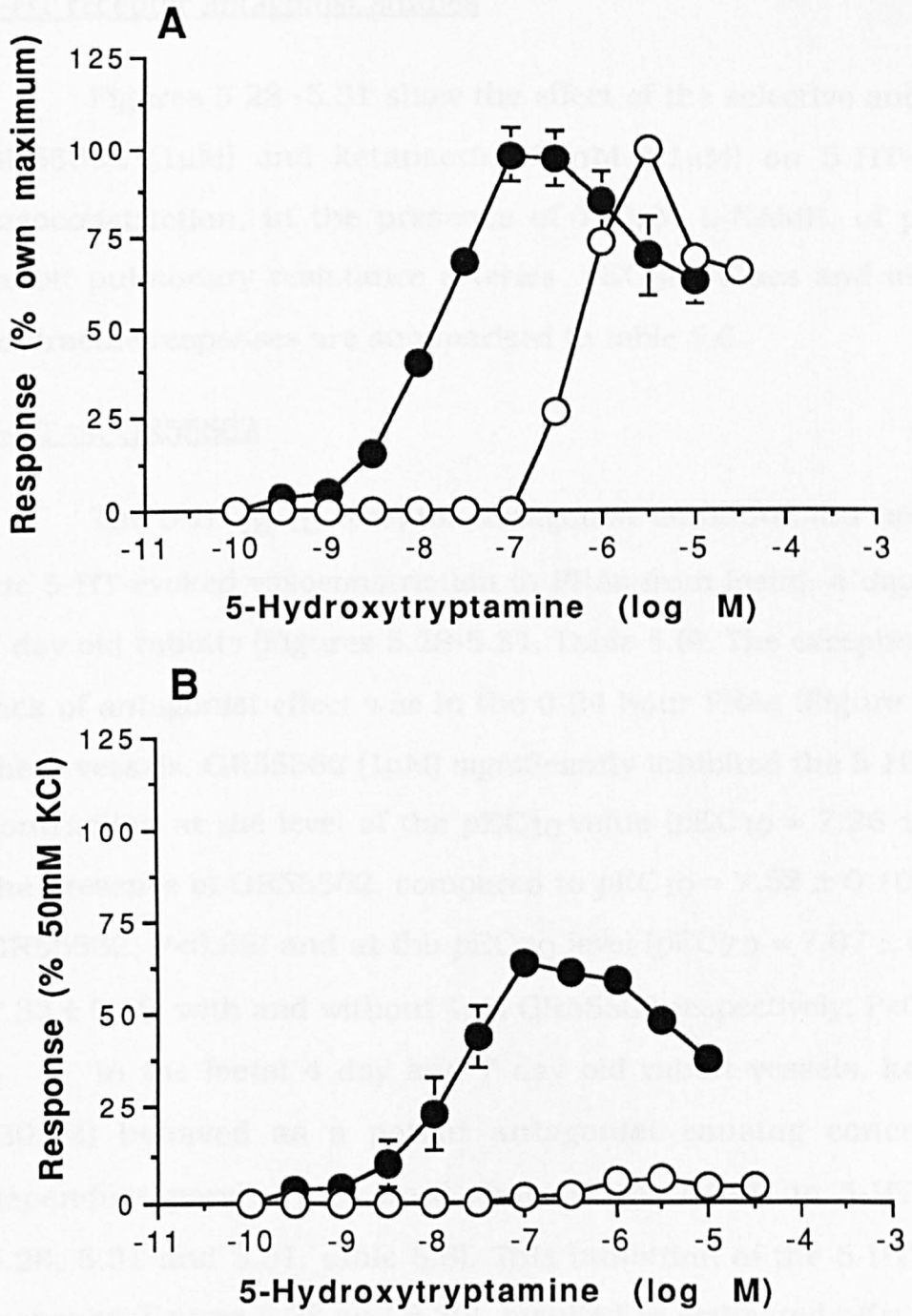

Figure 5.27 Effect of L-NAME (100μM) on responses to 5-HT in 7 day old rabbit PRAs. 5-HT CCRCs; control (O, n= 6/6) and in the presence of L-NAME (●, n= 6/6). **A** Data are expressed as percentage own maximum contraction. **B** Data are expressed as percentage reference contraction to 50 mM KCl. Each point represents mean ± s.e.mean. n = number vessels/number animals.

5-HT receptor antagonist studies

Figures 5.28 -5.31 show the effect of the selective antagonists GR55562 (1μM) and ketanserin (30nM-0.1μM) on 5-HT-induced vasoconstriction, in the presence of 100μM L-NAME, of perinatal rabbit pulmonary resistance arteries. pEC_{50} values and maximum contractile responses are summarised in table 5.6.

5-HT vs. GR55562

The $5\text{-HT}_{1B/1D}$ receptor antagonist GR55562 did not inhibit the 5-HT-evoked vasoconstriction in PRAs from foetal, 4 day old and 7 day old rabbits (Figures 5.28-5.31, Table 5.6). The exception to this lack of antagonist effect was in the 0-24 hour PRAs (Figure 5.29). In these vessels, GR55562 (1μM) significantly inhibited the 5-HT-evoked contraction at the level of the pEC_{10} value (pEC_{10} = 7.26 ± 0.03 in the presence of GR55562, compared to pEC_{10} = 7.52 ± 0.10 without GR55562; $P<0.05$) and at the pEC_{20} level (pEC_{20} = 7.07 ± 0.05 and 7.33 ± 0.10, with and without 1μM GR55562 respectively; $P<0.05$).

In the foetal 4 day and 7 day old rabbit vessels, ketanserin (30nM) behaved as a potent antagonist causing concentration dependent parallel rightward shifts of the CCRC to 5-HT (figures 5.28, 5.31 and 5.31, table 5.6). This inhibition of the 5-HT-induced response (figures 5.28 and 5.30), resulted in estimated pK_B values of 9.06 ± 0.2 and 8.5 ± 0.2 for foetal and 4 day old vessels respectively, where maximum responses to 5-HT were achieved in the presence of this antagonist. In contrast, in the 0-24 hour rabbit PRAs (Figure 5.29), ketanserin caused a non-parallel rightward shift of the CCRC for 5-HT and only inhibited the contractile response to 5-HT at concentrations ≥0.1μM although this ketanserin resistant

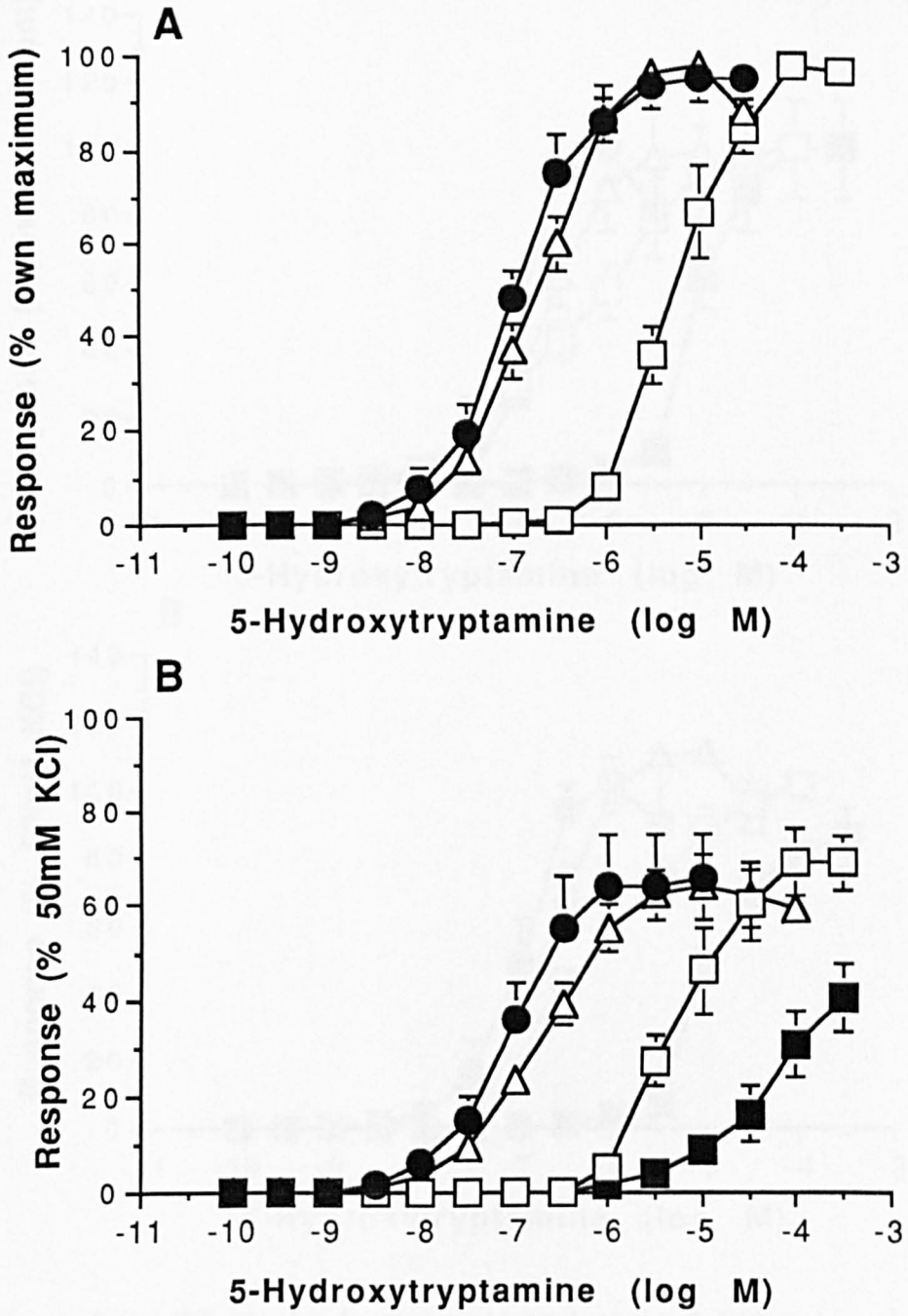

Figure 5.28 Effect of selective antagonists on 5-HT-induced responses in foetal rabbit PRAs. CCRCs to 5-HT (●,n = 6/6) and in the presence of 30nM ketanserin (□,n = 6/6), 0.1μM ketanserin (■,n = 6/6) and 1μM GR55562 (△,n= 6/6). **A** Data are expressed as percentage own maximum contraction. **B** Data are expressed as percentage reference contraction to 50 mM KCl. All CCRCs in the presence of 100μM L-NAME. Each point represents mean ± s.e.mean. n = number vessels/number animals.

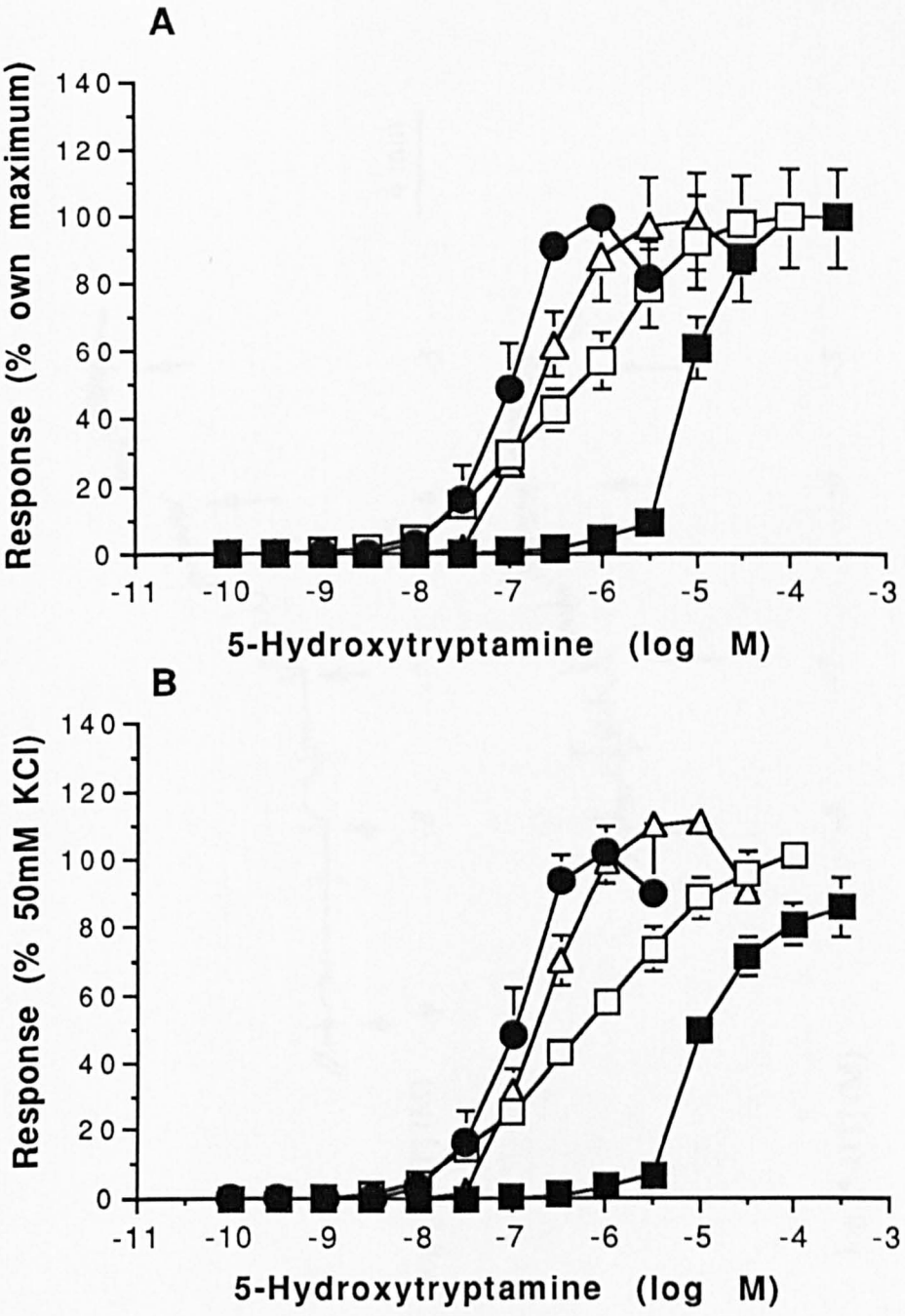

Figure 5.29 Effect of selective antagonists on 5-HT-induced responses in 0-24 hr rabbit PRAs. CCRCs to 5-HT (●,n = 7/7) and in the presence of 30nM ketanserin (□,n = 6/6), 0.1μM ketanserin (■, n = 6/6) and 1μM GR55562 (Δ ,n= 6/6). **A** Data are expressed as percentage own maximum contraction. **B** Data are expressed as percentage reference contraction to 50 mM KCl. All CCRCs in the presence of 100μM L-NAME. Each point represents mean ± s.e.mean. n = number vessels/number animals.

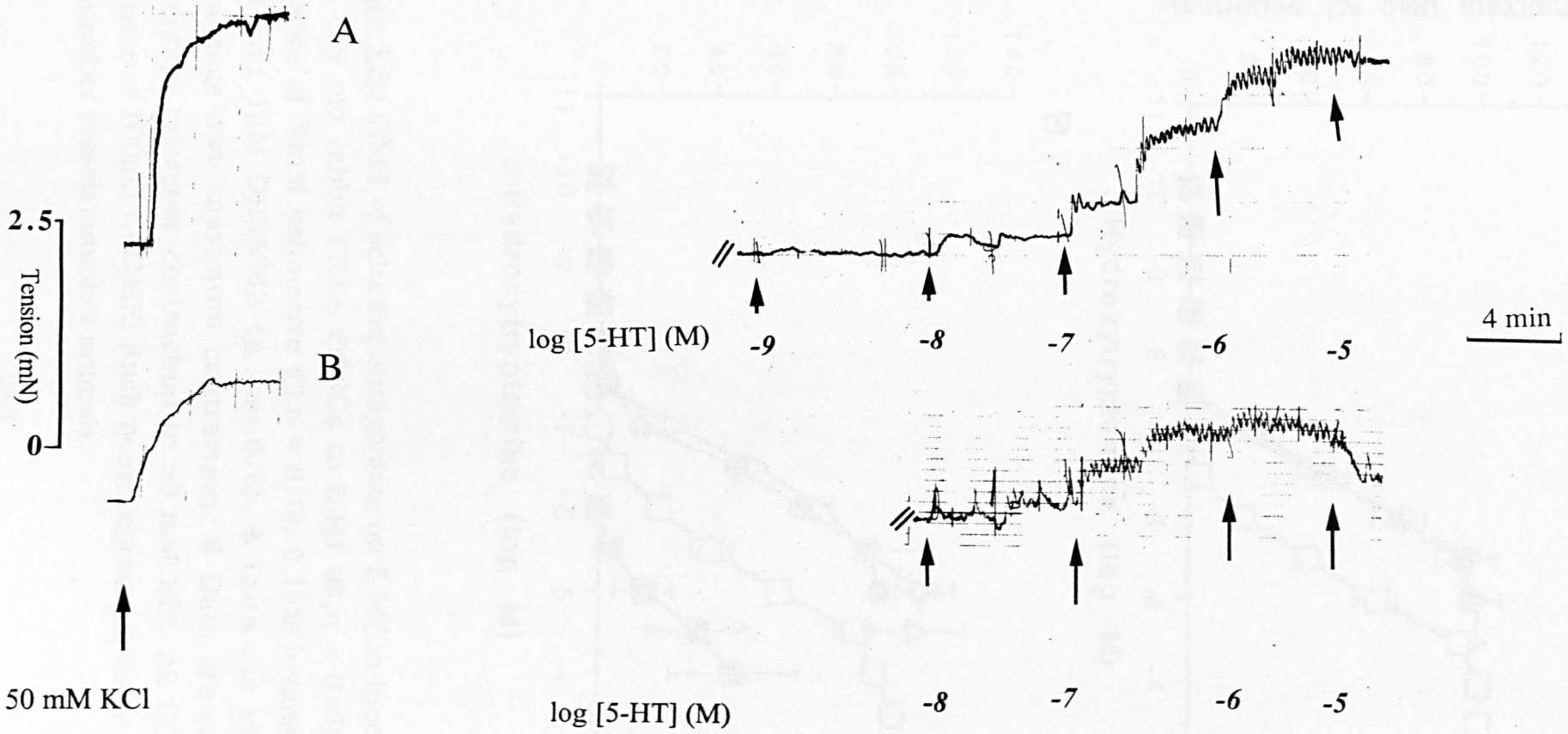

Figure 5.29C Representative trace showing the effect of ketanserin (30nM) on 5-HT-evoked contractions in 0-24 hour old rabbit isolated PRAs. **A.** In the presence and **B.** in the absence of ketanserin. Responses are compared with response to 50mM KCl. Vessels were incubated with 100μM L-NAME prior to and throughout the cumulative addition of 5-HT. Responses are compared with response to 50mM KCl.

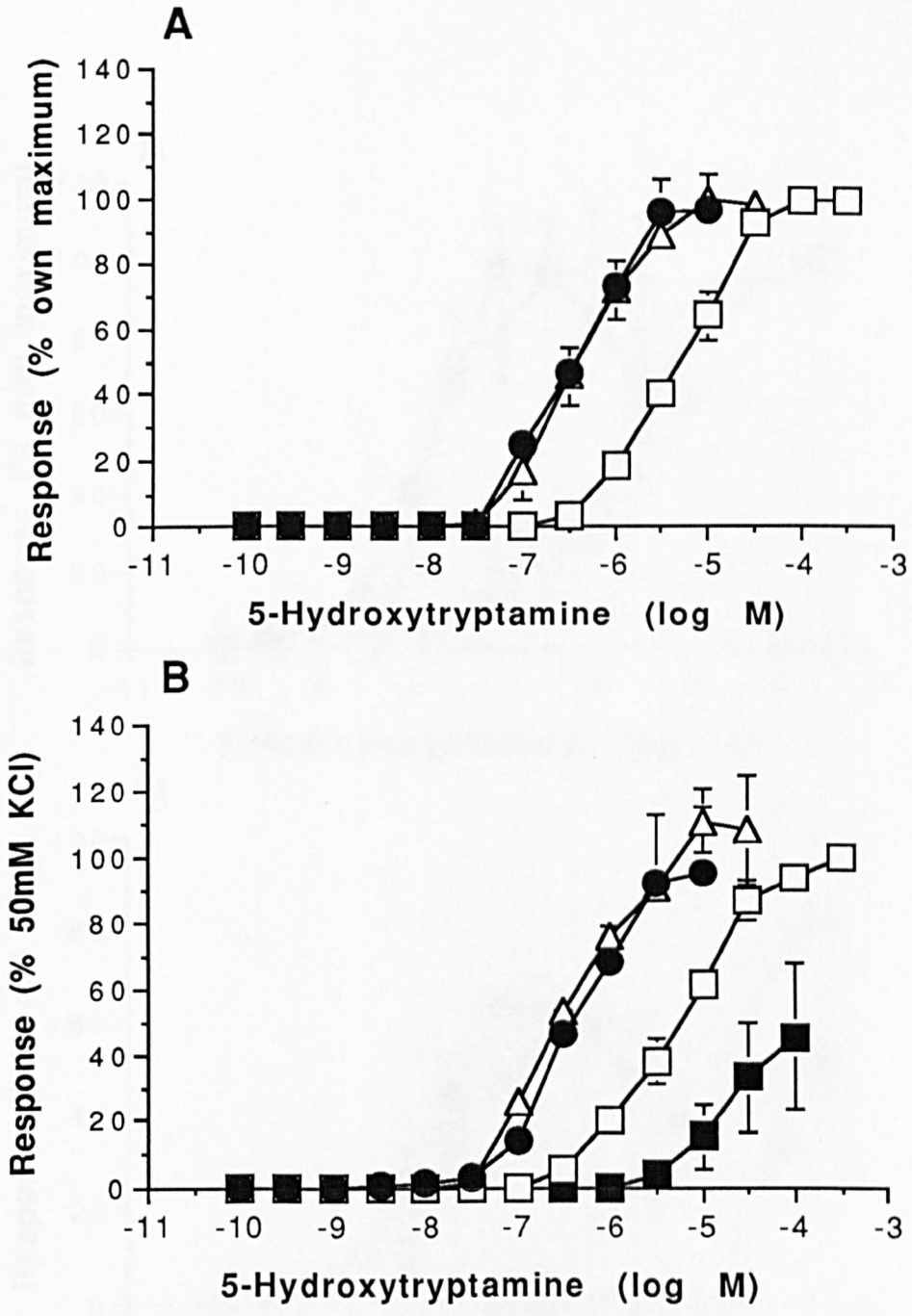

Figure 5.30 Effect of selective antagonists on 5-HT-induced responses in 4 day old rabbit PRAs. CCRCs to 5-HT (●,n = 6/6) and in the presence of 30nM ketanserin (□,n = 6/6), 0.1μM ketanserin (■, n = 6/6) and 1μM GR55562 (△, n= 6/6). **A** Data are expressed as percentage own maximum contraction. **B** Data are expressed as percentage reference contraction to 50 mM KCl. All CCRCs in the presence of 100μM L-NAME. Each point represents mean ± s.e.mean. n = number vessels/number animals.

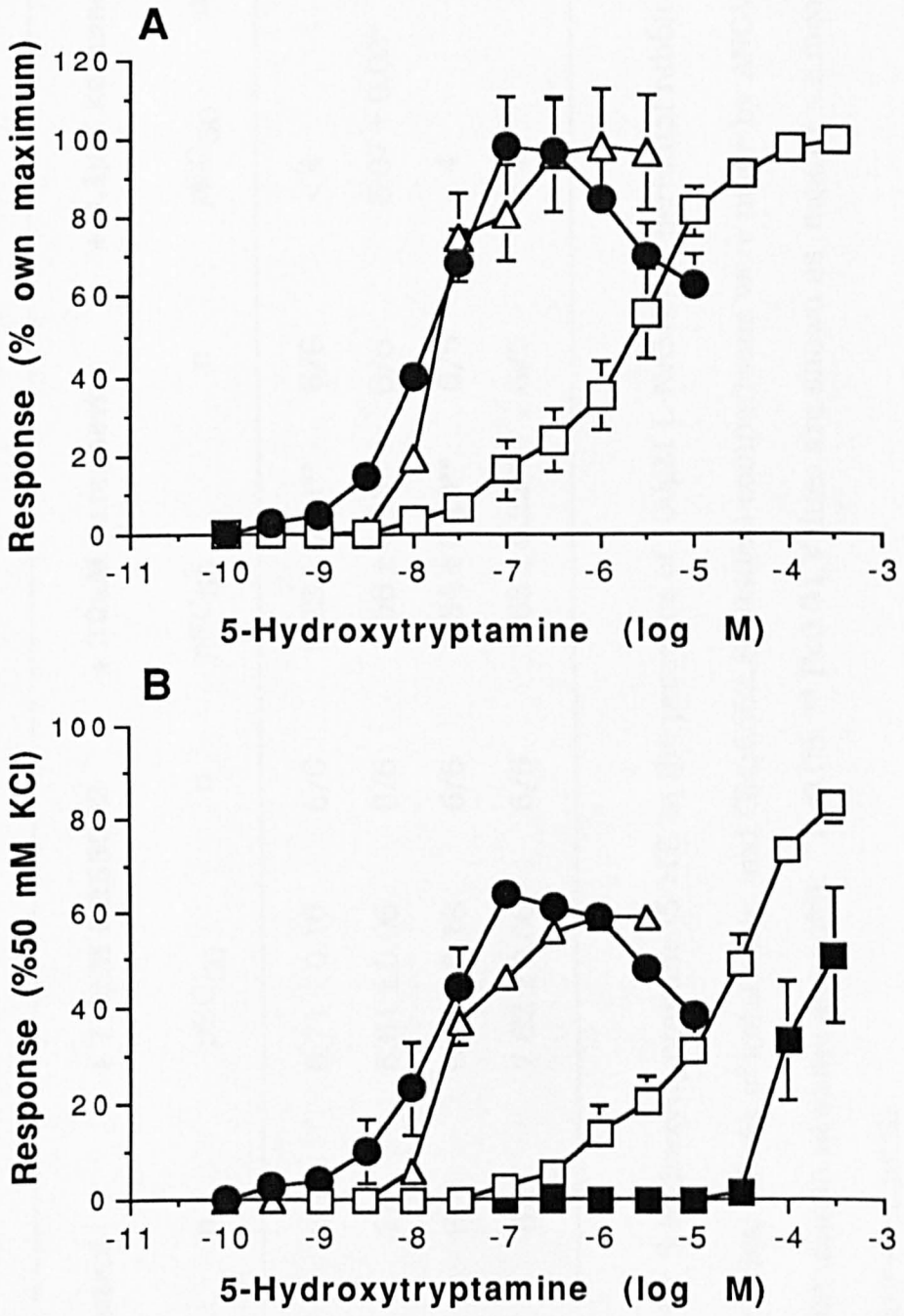

Figure 5.31 Effect of selective antagonists on 5-HT-induced responses in 7 day old rabbit PRAs. CCRCs to 5-HT (●, n = 6/6) and in the presence of 30nM ketanserin (□,n = 6/6), 0.1μM ketanserin (■, n = 6/6) and 1μM GR55562 (△,n= 6/6). **A** Data are expressed as percentage own maximum contraction. **B** Data are expressed as percentage reference contraction to 50 mM KCl. All CCRCs in the presence of 100μM L-NAME. Each point represents mean ± s.e.mean. n = number vessels/number animals.

	5-HT Control		+ 1.0μM GR55562		+ 30nM ketanserin		+ 0.1μM ketanserin	
Age	pEC_{50}	n	pEC_{50}	n	pEC_{50}	n	pEC_{50}	n
Fetus	6.79 ± 0.17	6/6	6.71 ± 0.10	6/6	5.22 ± 0.14**	6/6	< 4	6/6
0-24 hour	6.98 ± 0.15	7/7	6.64 ± 0.06	6/6	6.56 ± 0.13	6/6	5.07 ± 0.02*	6/6
4 day	6.38 ± 0.12	6/6	6.40 ± 0.18	6/6	5.34 ± 0.10**	6/6	< 4	6/6
7 day	7.73 ± 0.07	6/6	7.62 ± 0.06	6/6	5.63 ± 0.22**	6/6	< 4	6/6

Table 5.6 pEC_{50} values for 5-hydroxytryptamine (5-HT, in the presence of 100μM L-NAME) in perinatal rabbit pulmonary resistance arteries: effects of ketanserin and GR55562.Statistical comparisons were made by ANOVA : 5-HT pEC50 vs 5-HT + ketanserin at same age point; * P<0.05, ** P<0.01.Values are shown as mean ± s.e.mean.n = number of vessels/number of animals.

component formed only ~ 20-30% of the total contractile response to 5-HT. Although this concentration of ketanserin did not significantly affect the pEC_{50} in these vessels (Table 5.6), the pEC_{75} and pEC_{80} values (5.64 ± 0.2 & 5.45 ± 0.21 respectively) were significantly less ($P< 0.01$) than their respective control values of 6.76 ± 0.12 & 6.72 ± 0.12). In these 0-24 hr vessels, 100nM ketanserin did inhibit responses to 5-HT in a competitive fashion with a parallel rightward shift in the CCRC giving an estimated pK_B of 8.91 ± 0.2. At 7 days (Figure 5.31), 30nM ketanserin inhibited responses to 5-HT with an estimated pK_B = 9.6 ± 0.2. The CCRC to 5-HT in the presence of 0.1μM ketanserin, failed to reach a maximum within the concentration range examined in vessels from foetal (figure 5.28 B), 4 day old (figure 5.30 B) and 7 day old (figure 5.31 B) animals, pK_B and pEC_{50} values could therefore not be calculated at this antagonist concentration.

5-CT-mediated vasodilation

The contractile response to ET-1 (0.1-10nM) in the perinatal rabbit PRAs was similar in each age group being 47 ± 12%, 51 ± 15%, 44 ± 9% and 53 ±13% (% contraction to 50mM KCl) in the foetal, 0-24 hour, 4 day old and 7 day old rabbit PRAs respectively.

Figure 5.32 shows the vasodilator effect of 5-CT in perinatal rabbit PRAs precontracted with ET-1. 5-CT evoked concentration-dependent vasodilation in the vessels of both the 4 day and 7 day old animals (figure 5.32). The pIC_{50} values were 7.1 ± 0.2 and 7.5 ± 0.2 for the 4 day old rabbit (n = 7/7) and 7 day old rabbit (n = 6/6) respectively. The respective maximal relaxations were 91 ± 5% and 91 ± 2 % (% relaxation of ET-1-induced tone). 5-CT failed to elicit relaxation of any significance in the foetal and 0-24 hour rabbit

vessels (figure 5.32). In the 4 day old rabbit PRAs, L-NAME virtually abolished vasodilation induced by 5-CT, significantly reducing the maximal vasodilator response to 21 ± 6 ($P<0.01$; cf. control), suggesting an indirect, NO-dependent dilation at this age (figure 5.33). In these 4 day vessels, GR55562 (1μM) inhibited 5-CT-induced vasodilation (see figure 5.33C) significantly increasing the pIC_{50} value to 5.7 ± 0.2 ($P < 0.001$ cf. control 5-CT response) without affecting the maximal vasodilator response (98 ± 5%). This gave an apparent pK_B value of 7.36 ± 0.3 (figure 6.33) in these vessels.

Figure 5.34 shows the effect of NO synthase inhibition and GR55562 on 5-CT-induced vasodilation in ET-1 precontracted PRAs from the 7 day rabbits. 100μM L-NAME significantly reduced the maximum dilation to 5-CT (43 ± 5%; $P<0.01$) compared to control responses to 5-CT but there was no significant change in sensitivity in terms of the pIC_{50} value (7.3 ± 0.3, figure 6.34 A). This suggests that L-NAME only partly blocked the 5-CT-induced dilation at this age (figure 6.34). In these 7 day vessels, GR55562 (1μM) only inhibited the dilation evoked by high concentrations of 5-CT (figure 6.34 B). In the presence of 100μM L-NAME and 0.1μM ketanserin, the 5-HT_7 receptor antagonist spiperone (1μM) was a potent inhibitor of the vasodilator responses to 5-CT causing a concentration-dependent, parallel rightward shift in the CCRC to 5-CT in the 7 day vessels (figure 7.35). The pIC_{50} value was significantly reduced to 5.6 ± 0.3 ($P<0.001$; cf. 5-CT pIC_{50} in the presence of 100μM L-NAME) and the estimated pK_B for spiperone was 7.64 ± 0.3.

Time control data are also shown (figures 5.33-5.35) and illustrate the fall off in vascular tone during the time course of each experiment. This was significantly different from the control 5-CT response data at every time point of 5-CT administration where 5-CT

induced a relaxation.

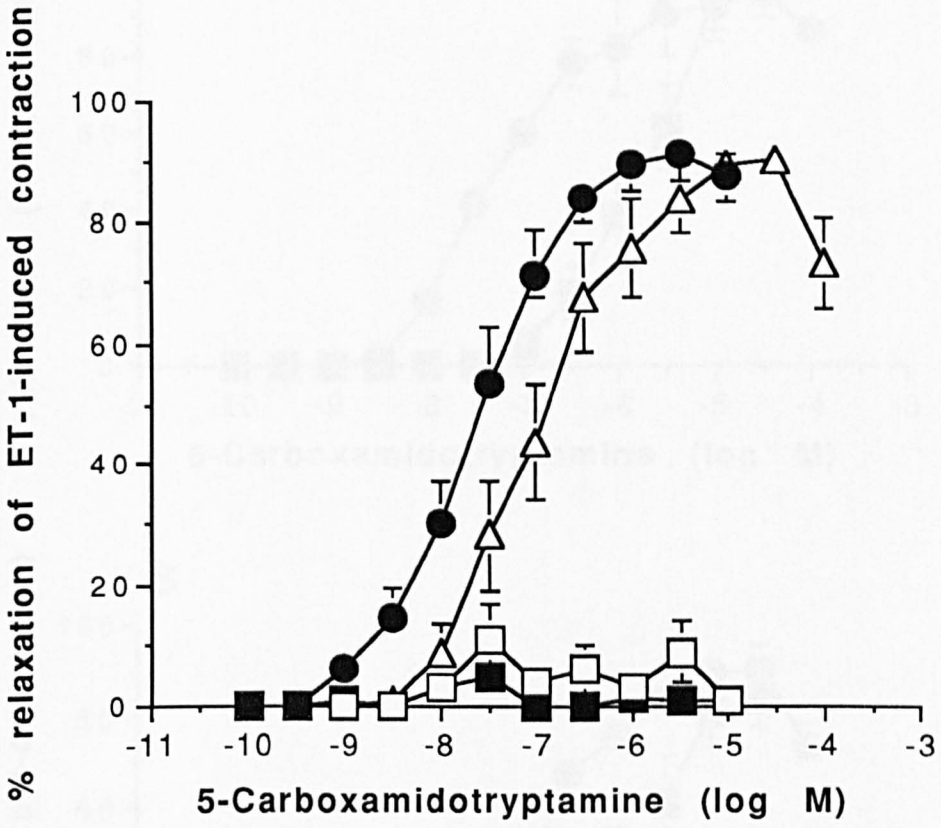

Figure 5.32 Relaxation by 5-carboxamidotryptamine, of ET-1 pre-contracted foetal (□,n = 6/6), 0-24 hr (■, n = 6/6), 4 day (△, n = 7/7) and 7 day (●, n = 6/6) rabbit PRAs in the presence of 0.1μM ketanserin. Data are expressed as % relaxation of the ET-1-induced pre-contraction. Each point represents mean ± s.e.mean. n = number vessels/number of animals.

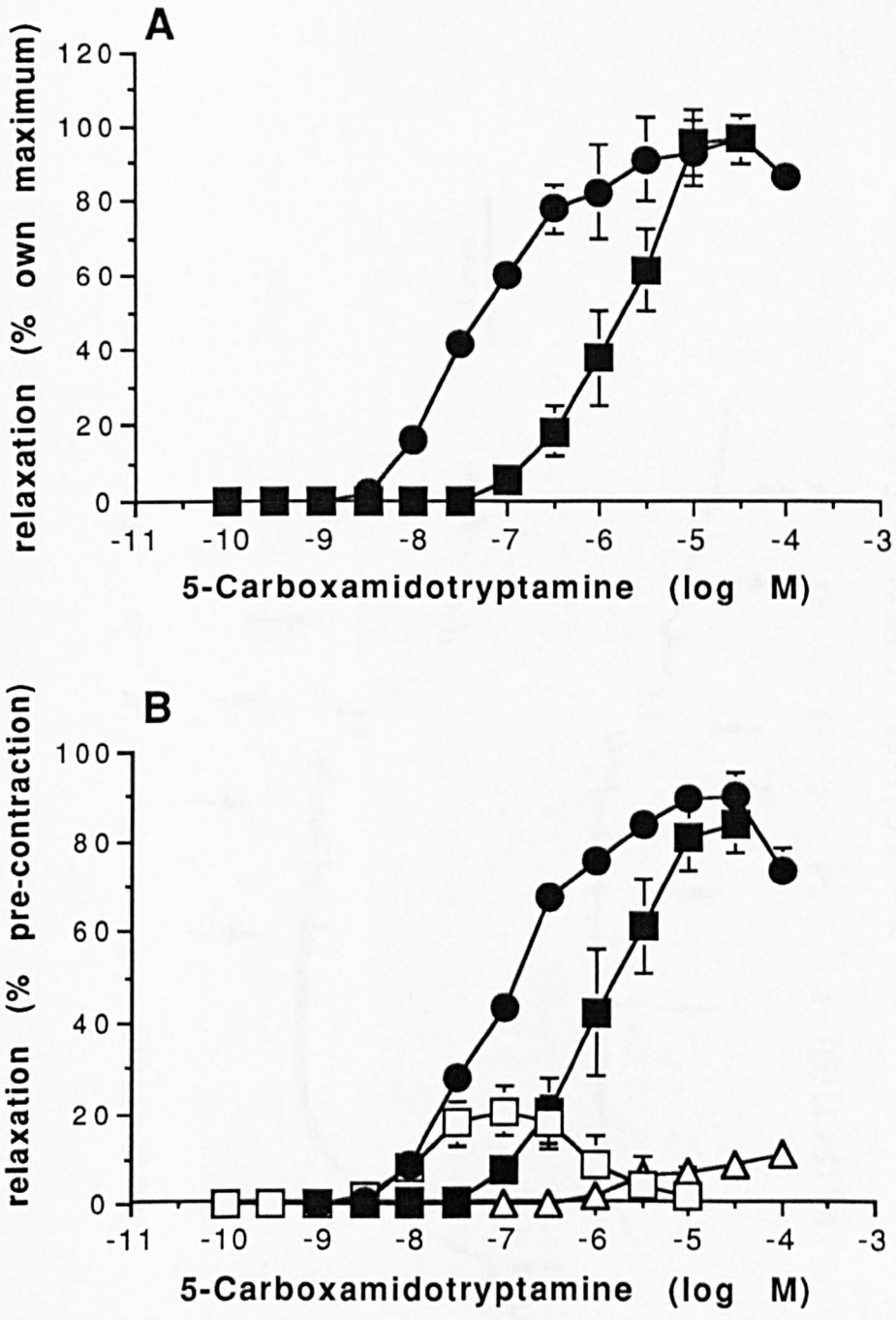

Figure 5.33 Effect of L-NAME (100μM) and GR55562 (1μM) on 5-CT-induced relaxation in ET-1 pre-contracted 4 day old rabbit PRAs in the presence of 0.1μM ketanserin. CCRCs to 5-CT; control (●, n = 6/6) and in the presence of L-NAME (□,n = 6/5) and GR55562 (■, n = 6/5). Time control pre-contractile data are also shown (△, n = 6/6). **A** Data are expressed as percentage own maximum relaxation. **B** Data are expressed as percentage relaxation of the ET-1 precontraction. Each point represents mean ± s.e.mean. n = number vessels/number animals.

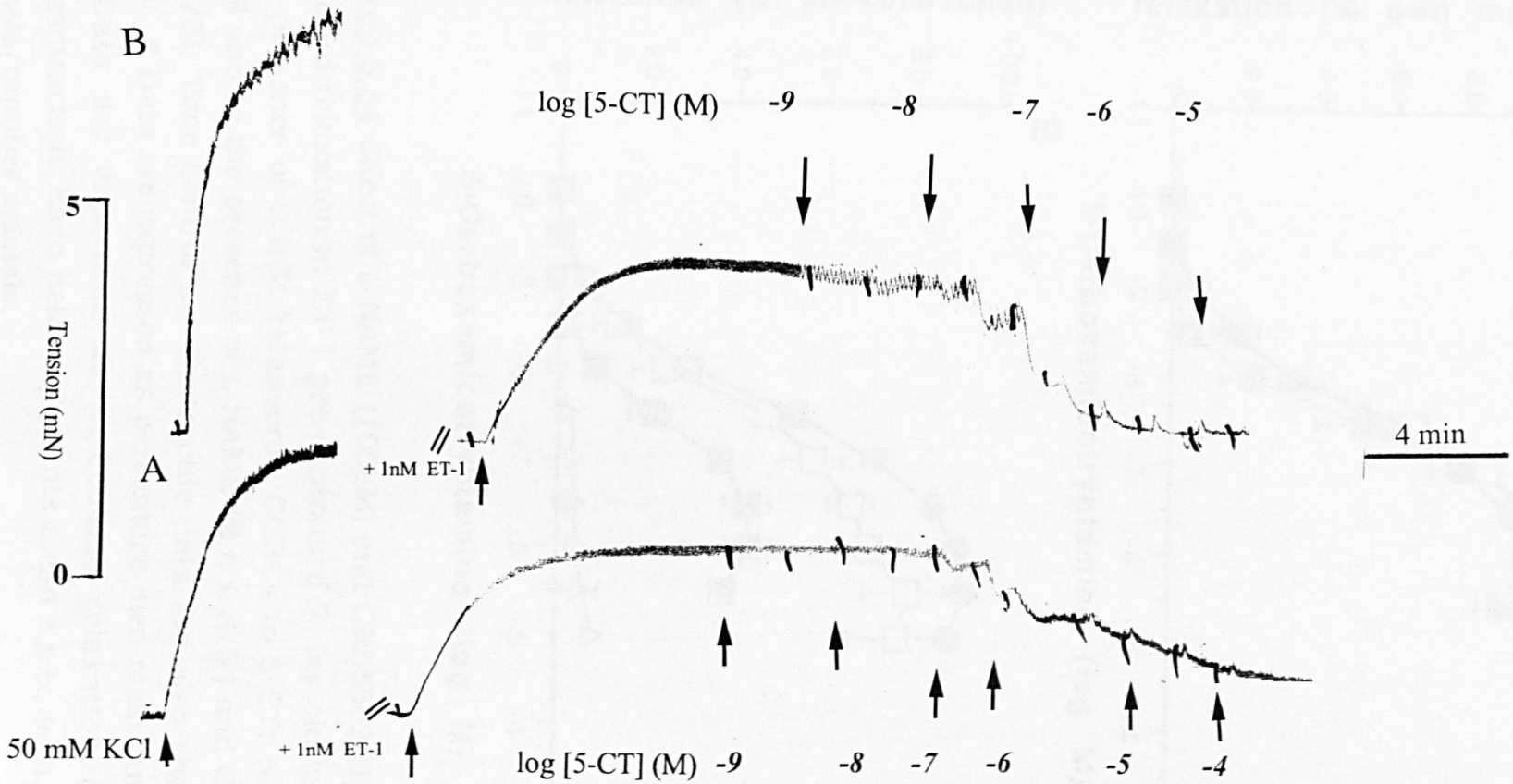

Figure 5.33C Representative trace showing the effect of GR55562 (1μM) on 5-CT evoked relaxations in 4 day old rabbit isolated PRAs. **A.** In the presence and **B**. in the absence of GR55562. Vessels were pre-contracted with ET-1 (1nM) prior to the cumulative addition of 5-CT.

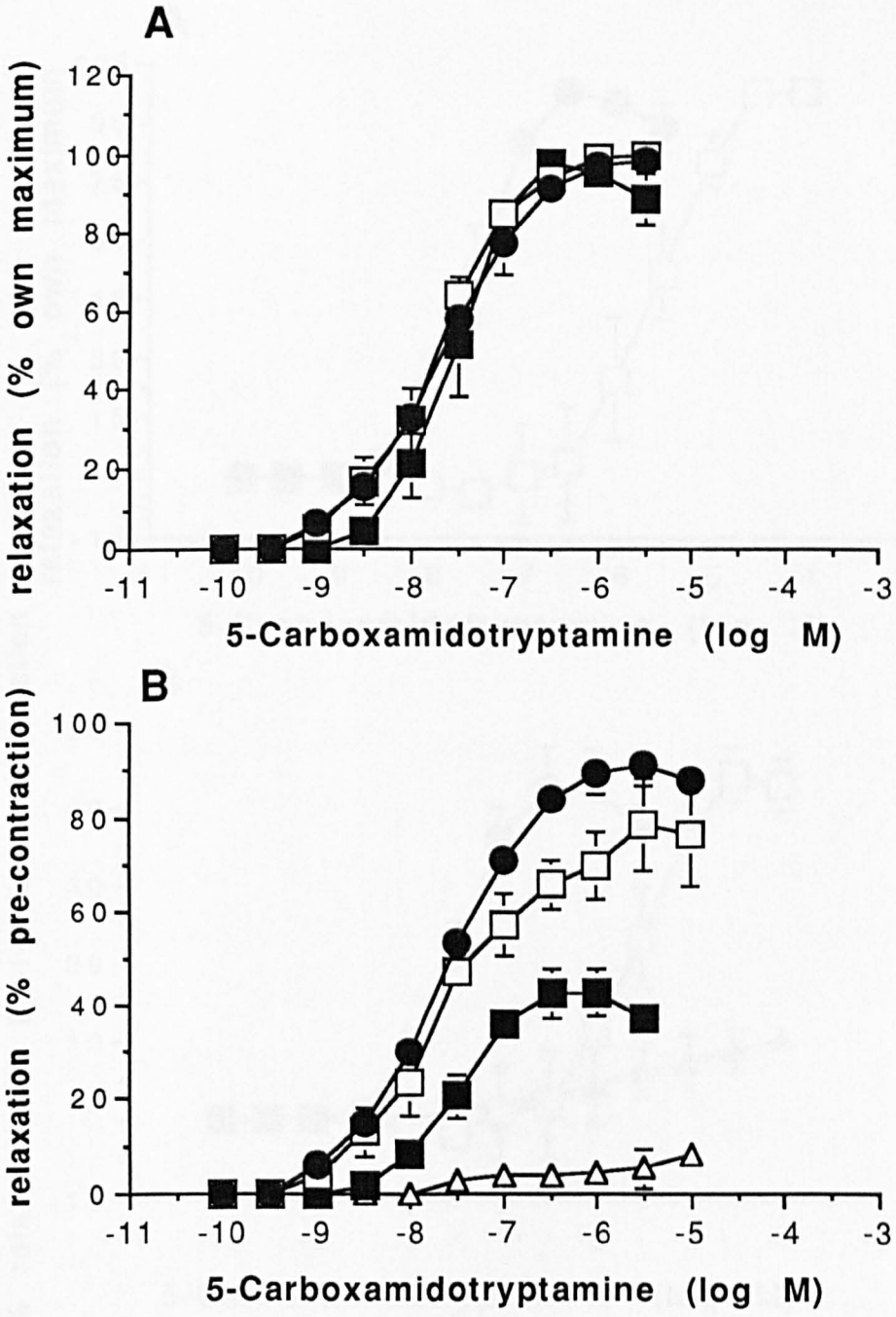

Figure 5.34 Effect of L-NAME (100μM) and GR55562 (1μM) on 5-CT-induced relaxation in ET-1 pre-contracted 7 day old rabbit PRAs in the presence of 0.1μM ketanserin. CCRCs to 5-CT; control (●, n = 6/6) and in the presence of L-NAME (■,n = 6/5) and GR55562 (□,n = 6/5). Time control pre-contractile data are also shown (△, n = 6/6). **A** Data are expressed as percentage own maximum relaxation. **B** Data are expressed as percentage relaxation of the ET-1 precontraction. Each point represents mean ± s.e.mean. n = number vessels/number animals.

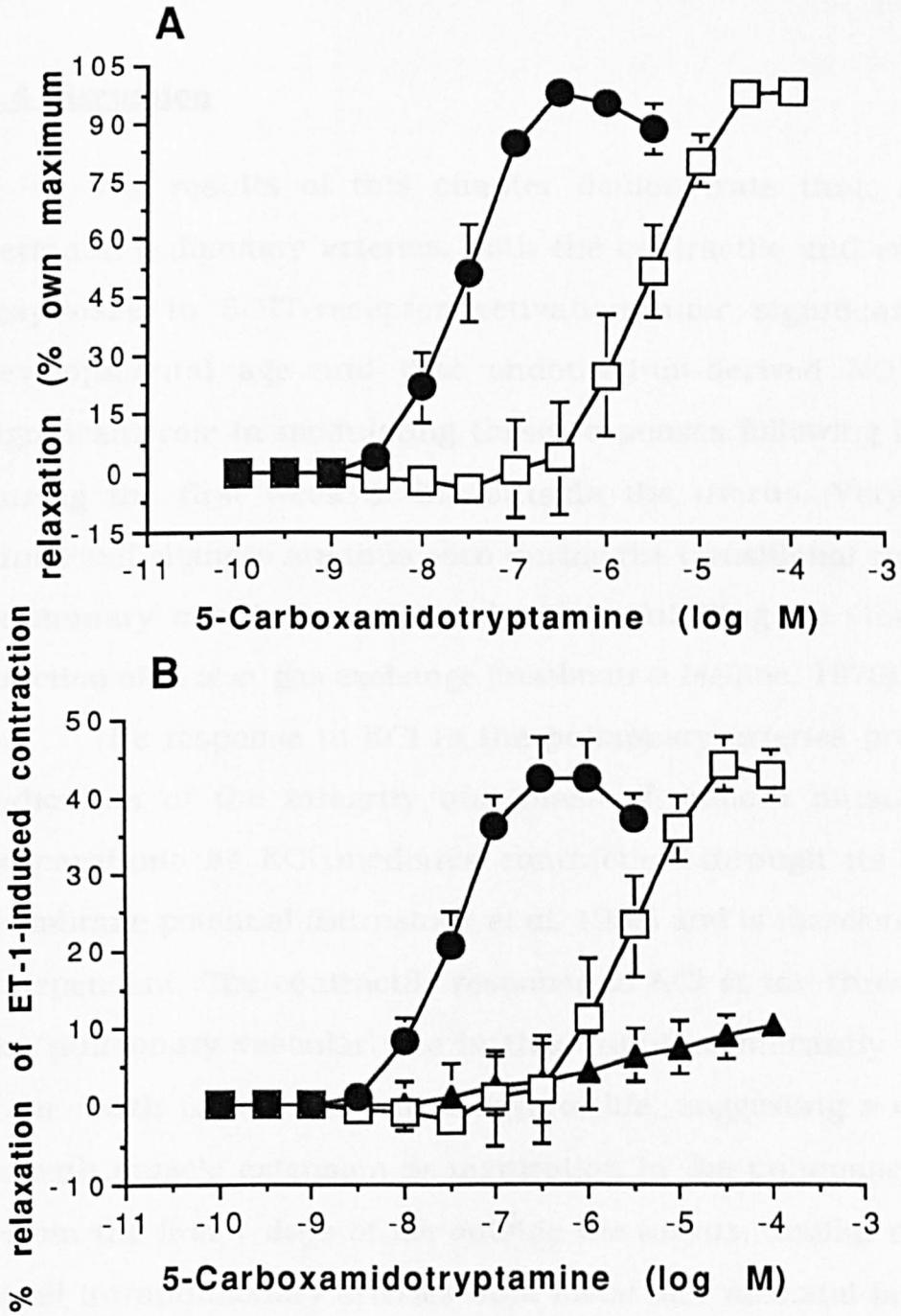

Figure 5.35 Effect of spiperone (1μM) on 5-CT-induced relaxation in ET-1 precontracted 7 day rabbit PRAs, in the presence of 100μM L-NAME and 0.1μM ketanserin. CCRCs to 5-CT; control (●,n = 6/5) and in the presence of spiperone (□,n = 6/5). Time control pre-contractile data are also shown (▲, n = 6/6).**A** Data are expressed as percentage own maximum relaxation. **B** Data are expressed as percentage relaxation of the ET-1 precontraction. Each point represents mean ± s.e.mean. n = number vessels/number animals.

5.4 Discussion

The results of this chapter demonstrate that, in rabbit perinatal pulmonary arteries, both the contractile and vasodilator responses to 5-HT-receptor activation alter significantly with developmental age and that endothelium-derived NO plays a significant role in modulating these responses following birth and during the first week of life outside the uterus. Very marked functional changes are thus seen during the transitional stage of the pulmonary circulation as it adapts to fulfilling its vital normal function of *in vivo* gas exchange (Inselman & Mellins, 1976).

The response to KCl in the pulmonary arteries provides an indication of the integrity and mass of smooth muscle in the preparations as KCl mediates contraction through its effect on membrane potential (Burnstock *et al*, 1963) and is therefore receptor independent. The contractile response to KCl at the three levels of the pulmonary vascular tree in this study, significantly increased from birth to the first seven days of life, suggesting a continued smooth muscle extension or maturation in the pulmonary arteries within the first 7 days of life outside the uterus. Similar results for small intrapulmonary arteries from foetal and neonatal lambs have been previously reported (Dunn *et al*, 1989), although in that study, vessels with a larger internal diameter (530-1000μm) demonstrated no significant change in KCl-induced contraction. This may reflect a species difference in the maturation of the pulmonary arteries.

Pulmonary conduit arteries

Endothelium-dependent vasodilation

In assessing the integrity of the endothelium in the PCAs, a marked alteration in the response to ACh in NA pre-constricted vessels was noted indicating developmental changes in the ACh-induced vasodilation of rabbit conduit pulmonary arteries during the perinatal period into the first week of extra-uterine life. ACh-induced vasodilation was substantial in the 2 day preterm foetal vessels, absent in the 0-24 hour vessels and restored by 4 days. The loss of ACh-induced vasodilation in pulmonary arteries immediately after birth suggests NO activity is transiently compromised immediately after birth and is in agreement with previous reports in newborn isolated pulmonary arteries from other species (Lui *et al.*, 1992; Steinhorn *et al.*, 1993). This could reflect changes in expression of eNOS, ACh-induced prostaglandin release, changes in muscarinic receptor number or decreased sensitivity of the tissue to NO at birth (Lui *et al.*, 1992; Steinhorn *et al.*, 1993). The 'transient loss' in these vessels was extensively investigated further in a separate study not presented in this thesis (see Morecroft & MacLean, 1998) where the findings are discussed more extensively. Those findings suggested that ACh-induced NO activity may be compromised by endogenous superoxide anion production and deficiencies in endogenous superoxide dismutase (SOD) activity at birth as pre-incubation with SOD uncovered ACh-induced vasodilation in the 0-24 hour vessels (Morecroft & MacLean, 1998).

Besides the release of NO, ACh can induce pulmonary vasodilation via the production and release of prostacyclin and EDHF from endothelial cells (Chand & Altura, 1981, Nagao & Vanhoutte, 1993). The NOS- and prostacyclin-independent component of the relaxation response to ACh is accompanied by an endothelium-dependent hyperpolarization of vascular smooth

muscle, criteria which define EDHF (Chen, *et al.*, 1988; Zygmunt, *et al.*, 1997). In rat pulmonary arteries, for example, EDHF is thought to mediate about 20-25% of the relaxation induced by ACh (Chen, *et al.*, 1988). EDHF has been reported to contribute to the variability in ACh-induced relaxation of rabbit isolated arteries from different vascular beds (Triggle, *et al.*, 1999). It is unlikely, however, that EDHF (or prostacyclin) plays any major role in mediating the vasodilator response to ACh in the perinatal rabbit PCAs. L-NAME abolishes ACh-induced vasodilation in these vessels suggesting that NO is the main endothelium-derived factor responsible for ACh-induced relaxation (Morecroft & MacLean, 1998).

Vasoconstrictor response to 5-HT and the influence of NO

5-HT was a potent vasoconstrictor of perinatal rabbit pulmonary conduit arteries at all age points studied with a potency order of 7 day = foetus = 4 days > 0-24 hours. Dunn and co-workers (1989) also observed a similar fall in sensitivity to 5-HT-evoked contractions in 1 day old lambs compared to older neonates and suggested that this may be an important, selective mechanism in the newborn in adjusting to the stress of birth, allowing the maintenance of the marked drop in PVR vital at birth, and in spite of the presence of high circulating levels of vasoconstrictor agents.

Several studies have suggested that spontaneously released NO plays an active role in aiding the maintenance of the pulmonary vasculature at a low state of tone in adult pulmonary arteries (Cremona *et al*, 1994; Steeds *et al*, 1997) including man (Cremona *et al*; 1994); and in the adaptation of the perinatal pulmonary circulation to extra-uterine life (Davidson & Eldermerdash, 1991). An

increase in pulmonary vascular NOS enzyme activity is thought to occur after birth. For example Hislop *et al* (1993) have shown an abundance of endothelial NOS at birth in the intra-pulmonary arteries of neonatal piglets, using immunocytochemistry, which reaches a peak by 2-3 days old and then decreases to adult levels by the second week of extra-uterine life. Similarly, in the newborn rat, the highest levels of constitutive endothelial NOS (eNOS) mRNA were detected by 24 hours after birth (Kawai *et al*, 1995). In human neonates, an increase in serum NO metabolites was observed between birth and 5 days of age (Endo *et al.*, 1996). The possibility that NO was having an inhibitory effect on the 5-HT-evoked contractile responses in the perinatal rabbit pulmonary arteries was investigated using the eNOS enzyme inhibitor L-NAME (Rees, *et al*, 1990) as this was demonstrated in adult rabbit PRAs (see chapter 3). Studies using isolated vessels from other vascular beds in the rabbit have also demonstrated a marked inhibitory effect of NO on the contractile responses to 5-HT (Valentin *et al.*, 1996).

L-NAME alone produced an increase in baseline tension in the foetal, 4 day and 7 day old rabbit vessels highlighting a significant contribution of endogenous NO to basal tone in these vessels. In the foetal vessels, the response to L-NAME appeared to be smaller than in the 4 and 7 day old bPAs. The lack of contraction to L-NAME in the 0-24 hour vessels suggests that basal NO was compromised at this age; subsequent studies were carried out and showed this may be due to an excess of superoxide anion production at birth (Morecroft & MacLean, 1998). In contrast, from the results of chapter 3, in adult rabbit extralobar arteries, L-NAME did not cause a significant, sustained contraction highlighting a maturational change in these vessels to a non-existent basal NO release in the adult bPAs.

Inhibition of NO synthesis with L-NAME significantly potentiated the magnitude of the maximal response to 5-HT in the extralobar branch pulmonary arteries at all age points studied suggesting a marked depressant effect of NO on contractions to 5-HT in these vessels.

The most pronounced effects in terms of maximal response were shown in the bPAs from the 4 day old rabbit. In contrast, the presence of L-NAME had no effect on maximal contractile response to 5-HT in the main pulmonary artery from birth onwards. In the foetal main pulmonary artery however, NO synthase inhibition did result in a marked augmentation of the contractile response to 5-HT as well as an increased sensitivity to 5-HT. These findings indicate functional differences between vessels of different sizes in the transitional pulmonary circulation. In the absence of endogenous NO, in the presence of L-NAME, the potency of 5-HT in the bPAs at the various ages was 0-24 hour = foetus = 7 day >4 day old rabbits.

Interestingly, in the 0-24 hour rabbit bPAs, NOS inhibition resulted in a marked increase in sensitivity to 5-HT which was not observed at any other developmental age up to the first week of extra-uterine life. These observations suggest that the sensitivity to 5-HT is normally attenuated by NO in 0-24 hour vessels. It is unclear, however, why this marked increase is observed in these vessels in the presence of L-NAME despite the observations that L-NAME produced no observed increase in the baseline tension of the 0-24 hour rabbit bPAs, indicating a lack of basal NO release. One possible explanation could be that in these vessels, a population of 5-HT receptors may exist on the endothelium which mediate release of NO as this has been shown recently in the porcine pulmonary artery (Glusa, *et al*, 1993; Glusa & Pertz, 2000) where activation of endothelial 5-HT_{2B} receptors is thought to stimulate NO release, leading to vascular

relaxation. This has also been demonstrated in the rat jugular vein (Ellis, *et al*, 1995). A greater contractile response to 5-HT would therefore be expected in these 0-24 hour vessels in the presence of L-NAME if these receptors were present. This explanation, however seems unlikely as no evidence was found for 5-HT agonist-mediated relaxation of pre-contracted bPAs from the 0-24 hour rabbit. Indeed, no evidence for 5-HT receptor mediated vasodilation could be found at any of the developmental ages studied. The results in chapter 3 of this thesis, show that the adult rabbit extralobar pulmonary arteries contain a population of 5-HT receptors (probably the 5-HT_7 type) which directly mediates vasorelaxation in these precontracted vessels. The results in this chapter, therefore, highlight a maturational change in vasodilator 5-HT receptors which appear after the first week of life and by adulthood in the rabbit extralobar branch pulmonary arteries.

Another potential explanation exists. Plasma ET-1 levels in the newborn piglet have been shown to be greater at birth than at 3 days old or later (Levy, *et al*, 1995) and a similar occurrence has been shown in the human infant (Malamitsi *et al*, 1993; Endo, *et al*, 1996). Threshold concentrations of ET-1 are known to potentiate the contractile response to 5-HT in rat mesenteric arteries (Hempelmann, *et al*, 1999) and contractions to other vasoconstrictors such as noradrenaline (Tabuchi, *et al.*, 1989). The results in chapter 6 of this thesis also indicate that this 'synergism' occurs in rat PRAs where low concentrations of ET-1 produced a significant potentiation of 5-HT receptor mediated contraction and this is fully discussed in that chapter. A synergistic potentiation of the sensitivity to 5-HT in the 0-24 hour rabbit vessels, in the presence of L-NAME, may therefore occur at a time when plasma ET-1 levels are known to be

elevated. No evidence, however, of potentiated 5-HT_1 receptor-mediated contractions could be found either in the presence of raised tone or L-NAME in these vessels, making this explanation unlikely.

It could be concluded that at this age point there are changes in the coupling of the second messenger system with the contractile apparatus in the presence of NOS inhibition, a possibility that would require further investigation.

Several studies have reported that, at high concentrations (>1μM), 5-HT can mediate an indirect vasoconstrictor effect via activation of α-adrenoceptors in some rabbit large blood vessels including the femoral artery (Grandaw & Purdy, 1996), ear artery (Black *et al.*, 1981) and aorta (Purdy *et al.*, 1987). In the rabbit isolated femoral artery, for example, 5-HT elicits a biphasic concentration response curve. The first phase being sensitive to antagonism by ketanserin and the second phase blocked by the selective α_1-adrenoceptor antagonist benextramine (Grandaw & Purdy, 1996). The involvement of an α_1-adrenoceptor in the contractile response to 5-HT in the rat pulmonary conduit artery has also been reported (Ogawa *et al.*, 1995). Noradrenaline has previously been shown to be a very poor vasoconstrictor of perinatal rabbit PRAs and actually fails to contract PRAs from adult rabbits (Docherty & MacLean, 1998). In addition, the preliminary experiments in adult rabbit conduit pulmonary arteries in chapter 3 of this thesis failed to demonstrate any effect of the α_1-adrenoceptor antagonist prazosin on contractile responses to 5-HT. Hence a contribution of α_1-adrenoceptors to 5-HT-induced responses in these perinatal vessels is unlikely although cannot be strictly ruled out without the use of selective α_1-adrenoceptor antagonists.

It has also been reported that high concentrations of 5-HT

can have indirect sympathomimetic actions by releasing noradrenaline from perivascular sympathetic nerve endings (McGrath, 1977; Saxena & Villalon, 1990). Further studies would need to be performed to clarify if this occurs in perinatal rabbit pulmonary arteries.

One of the most important determinants of vascular reactivity to a particular agonist is the receptor subtype to which the agonist binds. It is therefore possible that any changes in the sensitivity and reactivity to 5-HT related to perinatal development in the pulmonary arteries may be associated with changes in 5-HT receptor type. This possibility was investigated in the perinatal rabbit bPAs (and later the pulmonary resistance arteries) using selective 5-HT receptor agonists and antagonists.

5-HT receptor agonist and antagonist studies

As L-NAME did not significantly affect the contractile response to 5-HT in the main pulmonary artery of the perinatal rabbits (the foetal vessels being the exception) compared to its effect on the bPAs, the agonist and antagonist studies in the pulmonary arteries focused on the bPAs.

In the bPAs from all age points studied, 5-HT was more potent in inducing contraction than either 5-CT or sumatriptan and was equipotent to the 5-HT_2 receptor agonist α-methyl-5-HT. These data, with an agonist potency order of 5-HT= α-methyl-5-HT > 5-CT >> sumatriptan are consistent with the mediation of 5-HT-induced contraction via a 5-HT_2 receptor type according to the criteria established by Hoyer *et al.*(1994). 5-CT displayed a low potency (pEC_{50}s ~5.6-6.2) in bPAs at all age points studied, suggesting that these contractile responses to 5-CT were mediated via 5-HT_2 receptor

stimulation as 5-CT has previously been shown to typically display pEC_{50} values of ~7.9-8.6 at most 5-HT_1 receptors (Hoyer, *et al*, 1994). The virtual absence of any contractile response to sumatriptan, known to be a selective agonist at 5-HT_{1B} and 5-HT_{1D} receptors (Peroutka & McCarthy, *et al*, 1989), also strongly suggests that these receptor subtypes are absent in perinatal rabbit extralobar branch pulmonary arteries.

Several studies on isolated pulmonary arteries have shown that contractile responses to sumatriptan can be augmented or 'uncovered' by the eNOS inhibitor L-NAME and by raised tone. For example, in isolated bovine pulmonary arteries, MacLean *et al*, (1994) demonstrated that in the presence of L-NAME, which effectively decreases cGMP levels, or in the presence of raised tone, the sensitivity to sumatriptan were significantly increased ~3 fold and ~15 fold respectively. A marked increase in the maximum contraction to sumatriptan was also observed. The same authors have also observed a significant increase in the sensitivity of this agonist in isolated human pulmonary arteries in the presence of L-NAME (MacLean, *et al*, 1993). This may be pathophysiologically important in pulmonary hypertension where both a decrease in cGMP levels and increase in vascular tone, alongside potentiated responses to 5-HT_{1B} receptor-induced contraction, have been observed in pulmonary arteries from the chronic hypoxic rat model of pulmonary hypertension (MacLean, et al, 1996). This phenomenon may also be of pathophysiological importance in PPHN when considering the observation that in the prenatal model of PPH in the lamb, impaired production of cGMP is evident (Steinhorn *et al*, 1995) as well as decreased endothelial NO synthase gene expression. The possibility of augmented responses to sumatriptan under conditions of raised

pulmonary vascular tone and decreased cGMP levels through NOS inhibition was investigated in the bPAs from perinatal rabbits.

In the foetal, 0-24 hour and 7 day vessels, there was no significant effect of either raised tone or eNOS inhibition with L-NAME, suggesting an absence of 5-$HT_{1B/1D}$ receptors in these vessels. In contrast, in the 4 day old vessels, a small but significant reduction in the threshold concentration for contraction to sumatriptan was evident in the presence of L-NAME and in the presence of raised pulmonary arterial tone suggesting an 'uncovering' of a small population of 5-HT_1 receptors, this however requires further pharmacological investigation with appropriate antagonists.

Studies with the selective antagonists provide further evidence that in the perinatal extralobar branch pulmonary arteries, the predominant 5-HT receptor type mediating 5-HT-induced vasoconstriction is the 5-HT_{2A} receptor. The near nM affinities observed for ketanserin against 5-HT in these vessels (pK_B ~8.6-9) is consistent with 5-HT mediating its vasoconstrictor effects via the 5-HT_{2A} receptor (Van Neuten, *et al*, 1981; Leff & Martin, 1986). Ketanserin has also been shown to display 5-HT_{1D} receptor antagonist activity, but only in the micromolar range (Kaumann *et al*, 1993). The marked inhibition of 5-HT-induced contractions by concentrations of ketanserin below this micromolar range, implies that ketanserin is mediating its antagonist effect at the 5-HT_{2A} receptor rather than the 5-HT_{1D} receptor in perinatal rabbit PCAs.

In chapter 3 of this thesis, in the adult rabbit extralobar branch pulmonary artery, the affinity for ketanserin was ~9, similar to that observed in these perinatal vessels and suggesting that there is no significant maturational change in the 5-HT receptor subtype mediating 5-HT-induced vasoconstriction in rabbit conduit

pulmonary arteries. The lack of any antagonist effect of GR55562, the selective antagonist at 5-HT_{1B} and 5-HT_{1D} receptors, again provides further strong evidence against the presence of either of these receptors in these vessels. In the presence of L-NAME, there was no alteration in the affect of the selective antagonists ketanserin or GR55562 on the 5-HT-induced contractions in the perinatal rabbit vessels, underlining the proposition that perinatal rabbit pulmonary conduit arteries contain a single population of 5-HT receptors, namely the 5-HT_{2A} receptor, which mediate the contractile effects of 5-HT and that there is no significant shift in receptor subtype mediating these effects with developmental age.

Together, these findings suggest that the changes in the branch pulmonary arteries reactivity to 5-HT during the first week of extra-uterine life in the rabbit probably do not involve changes in 5-HT receptor subtype, but rather alterations in the coupling of the receptor to the contractile apparatus.

Pulmonary resistance arteries

Vasoconstrictor response to 5-HT and the influence of NO

The results of this chapter demonstrate a marked alteration in 5-HT-induced vasoconstriction in perinatal rabbit PRAs between near term foetal life, birth and during the first 7 days of extra-uterine life, with the 0-24 hour vessels displaying the greatest sensitivity to 5-HT.

Several studies have suggested that NO is released under basal conditions to maintain the pulmonary vasculature under a low state of tone in both adults (Wiklund *et al.*, 1990; Steeds *et al.*, 1997) and in neonatal guinea pigs (Davidson *et al.*, 1991). The possibility that NO was having an inhibitory effect on the 5-HT-induced contractile response was investigated as this has been demonstrated

in the perinatal rabbit conduit pulmonary arteries (described earlier in this chapter) and in other rabbit vascular preparations such as the isolated basilar artery and saphenous vein (Trezise, *et al*, 1992; Valentin *et al*, 1996). Inhibition of NO synthesis with L-NAME significantly potentiated the maximum contractile response to 5-HT at all ages, except the foetus. In the foetal PRAs the presence of L-NAME had no effect on sensitivity or magnitude of the maximal response of 5-HT. At this age, the sensitivity of the PRAs to 5-HT is ~2.5 fold greater than in adult rabbit PRAs (see chapter 3 of this thesis). One of the bioactive secretory products of pulmonary neuroendocrine cells (PNECs) in the lung is 5-HT. In the developing human lung, these PNECs have been shown to be immunoreactive for 5-HT as early as 8 weeks gestation (Cutz, *et al*, 1985), to increase in number towards term and they are greatest in number in the foetus and newborn (Johnson & Georgieff, 1989). These observations, coupled with the results in this chapter, which show 5-HT to be a potent vasoconstrictor of foetal PRAs, suggest the possibility that this bioamine could contribute directly to the elevated pulmonary vascular tone of the foetus. Vessels of similar order of magnitude examined in this study make a significant contribution to *in vivo* PVR (Staub, 1985).

PRAs from the 0-24 hour rabbits also demonstrated no alteration in sensitivity to 5-HT, whilst a marked augmentation of the contractile response was observed. In the absence of endogenous NO, in the presence of L-NAME, the potency of 5-HT at the various ages was: 7 day>>0-24 hr = foetus > 4 day. This suggests variable developmental changes in 5-HT receptor sensitivity or changes in the coupling of the second messenger system with the contractile apparatus.

One possibility could be developmental changes in Ca^{2+} sensitivity of the contractile myofilaments of the PRAs as recent studies clearly establish Ca^{2+} sensitivity as a variable determinant of vascular reactivity in many vascular beds (Nishimura, *et al.*, 1989; Somlyo and Somlyo, 1994). It is now well established that receptor-mediated activation of vascular smooth muscle elicits a proportionally smaller change in cytosolic Ca^{2+} for a given production of force than does depolarization with K^+ (Rembold & Murphy, 1988), suggesting that modulation of the Ca^{2+} sensitivity of the contractile apparatus is a key mechanism governing overall reactivity (Somlyo & Somlyo, 1994). Moreover, agonist-induced Ca^{2+} sensitization is responsible for a significant portion of the increase in contractile tone resulting from activation of 5-HT receptors (Parsons, *et al.*, 1996).

Ca^{2+} sensitivity and/or its alteration by G protein-dependent mechanisms may determine variations in pulmonary vascular reactivity associated with differences developmental age. Developmental changes in baseline Ca^{2+} sensitivity have recently been demonstrated in rabbit common carotid, basilar and femoral arteries (Akopov, et al., 1998). In the same study, 5-HT demonstrated an increase in Ca^{2+} sensitivity via a G-protein coupled mechanism which was more effective in neonatal than adult arteries. It would therefore be useful to investigate contractile myofilament sensitivity during perinatal development.

Frid *et al* (1994) indentified phenotypic alterations of distinct smooth muscle cell populations with development. It would also be useful to investigate contractile protein regulation during perinatal development in these vessels. Docherty & MacLean, (1998) recently reported a similar phenomenon for ET-1 induced contraction in the

same preparations.

The effect of NOS inhibition was most pronounced in the vessels removed from the 4 and 7 day old rabbits where the contractile response to 5-HT, in the absence of L-NAME, was extremely small. This suggests that significant responses to 5-HT in these vessels are normally masked by endogenous NO production in these vessels at this age and that sensitivity to 5-HT is normally attenuated by NO in the 4 day and especially in the 7 day rabbit vessels. These results suggest that there is an increase in NO production associated with birth in these vessels. One interpretation of this data is that 5-HT can cause release of NO in 4 and 7 day old vessels but is less able, or unable, to do this in the vessels from the foetus and 0-24 hr old rabbits. This is confirmed by the studies discussed later in this chapter on 5-HT receptor-induced, NO-dependent vasodilation, which was only present in the vessels from the 4 and 7 day old rabbits. In the 7 day old vessels, in the presence of L-NAME, a fall in tension at the higher concentrations of 5-HT was observed. One explanation is that, in these vessels, 5-HT may activate a 5-HT receptor subtype which causes direct pulmonary arterial smooth muscle relaxation. This receptor has been shown to directly mediate smooth muscle relaxation in a variety of isolated vascular tissues such as the neonatal pig vena cava (Trevethick, *et al*, 1986) and the canine coronary artery (Terron, 1996) and has been classified as the 5-HT_7 receptor which is positively coupled to adenylate cyclase. The results described later in this chapter provide evidence that this may be so, via a 5-HT_7 receptor population. Other possibilities, however cannot be ruled out including the actions of mediators such as PGI_2 or EDHF, or that the fall in tension may be due to desensitisation of the 5-HT-induced response.

The response of the fetal PRAs to NO synthase inhibition differs from that of the rabbit foetal pulmonary conduit arteries where L-NAME markedly potentiated the size of the 5-HT vasoconstrictor response, this is discussed earlier in this chapter. This comparison suggests heterogeneity of NO activity throughout the pulmonary arterial bed in the foetal rabbit, with 5-HT-sensitive, endogenous NO activity being present in the large conduit arteries but not in the small muscular pulmonary arteries. Such heterogeneity has been observed in the adult rat where eNOS can be detected in large conduit arteries but not small muscular vessels (Le Cras *et al.*, 1996).

In comparison to neonatal PRAs, the lack of effect of eNOS inhibition on 5-HT-induced responses of foetal vessels suggests there are diminished NO levels in the pulmonary resistance vasculature in near term foetal compared with extra-uterine life in the first week. These results are similar to previous studies in pigs and sheep, showing a diminution of basal and stimulated NO release from isolated pulmonary arteries of the near term foetus compared to neonatal animals (Abman, *et al*, 1991; Zellers & Vanhoutte, 1991; Lui, *et al*, 1992; Steinhorn, *et al*, 1993). In the previous study on foetal rabbit conduit pulmonary arteries, these were exposed to relatively low oxygen tensions, hence this excludes the possibility that these low oxygen tensions had inhibited NO production in the present study. The foetal PRAs were therefore maintained at the oxygen tension expected *in utero*. As Docherty and MacLean (1998) have shown that these foetal PRAs demonstrate a marked relaxation response to ACh, this suggests that there are differential effects on the control of agonist-induced NO release (by ACh) and basal release of NO in these pre-term vessels. This has also been suggested by

Cremona *et al* (1994) who investigated the inhibition of NO release in isolated human, ovine, porcine and canine lungs.

5-HT receptor agonist studies

Due to the inhibitory role of NO on constrictor responses to 5-HT at some age points, the response to the selective 5-HT agonists (and antagonists) were investigated in the presence of L-NAME.

5-HT and α-methyl-5-HT were equipotent in their ability to induce vasoconstrictor responses in the PRAs from the perinatal rabbits at all age points studied. As previously described, an agonist potency order of 5-HT = α-me-5-HT > 5-CT satisfies one of the criteria for classifying 5-HT_2 receptors (Hoyer *et al.*, 1994). In this study, the rank order of agonist potency in the PRAs from foetal rabbits to 7 days of age was 5-HT = α-me-5-HT >> 5-CT. Sumatriptan did not induce contraction in these vessels. In the absence of any contractile response to sumatriptan, and only weak and variable contractions to 5-CT, suggests that the 5-HT_{1B} and 5-HT_{1D} receptors are unlikely to be present in the perinatal rabbit PRAs. This suggests that the predominant receptor mediating 5-HT-induced vasoconstriction in these vessels, at the perinatal ages studied, is of the 5-HT_2 class. The maximum responses to both 5-HT and α-me-5-HT were significantly higher in the 0-24 hr and 4 day rabbit vessels. This may be due to a comparatively greater number of 5-HT receptors present in the newly born compared to the foetal and older neonatal lung. Indeed the results from chapter 3 in the adult rabbit PRAs also show a decreased maximal contraction to these two agonists compared to the newborn PRAs, suggesting there are developmental differences in 5-HT_2 receptor density. Similarly, evidence for developmental changes in ET-1 receptor density also

exists in the same preparation (Docherty & MacLean, 1998).

Ketanserin, at submicromolar concentrations, shows selectivity for 5-HT_{2A} receptors (Hoyer *et al.*, 1994). 30nM Ketanserin inhibited responses to 5-HT in a competitive manner in the fetal, 4 day and seven day old rabbit vessels confirming that 5-HT_{2A} receptors mediate the contractile response to 5-HT in these vessels. The results in chapter 3 of this thesis showed that in the rabbit PRAs, 30nM ketanserin only antagonised 5-HT-induced vasoconstriction at the higher concentration range of 5-HT. This further indicates an alteration in the distribution of 5-HT receptor subtypes with developmental age.

The estimated pK_B values for ketanserin in the present study were 8.6-9.6 and are similar to the affinity values obtained for ketanserin in other systems where a 5-HT_{2A} subtype is described (Hoyer *et al.*, 1994). From the results in chapter 4 of this thesis, in human PRAs, both the 5-HT_{2A} receptor and 5-HT_{1B} receptor mediate vasoconstriction, but it is the 5-HT_{1B} receptor which appears to be physiologically important. Whether this simply reflects a species difference or is also due to a maturational change in the 5-HT receptors mediating vasoconstriction from perinatal human pulmonary arteries to those in adulthood remains unclear.

In the vessels from the 0-24 hr old rabbits, 30nM ketanserin caused a non-parallel rightward shift of the 5-HT CCRC revealing small, ketanserin-resistant contractions to low concentrations of 5-HT. These were inhibited by the 5-$HT_{1D/1B}$ receptor antagonist GR55562 and by higher concentrations of ketanserin. This suggests that, immediately after birth, there is transitory expression of a small population of contractile 5-$HT_{1D/1B}$ receptors. One possible explanation for this could be the pharmacological synergism

described earlier in this chapter, where threshold concentrations of ET-1 or decreased NO may augment 5-HT-induced vasoconstriction in these vessels and uncover, if only to a small degree, a 5-HT_1 receptor-mediated response. This explanation takes on more importance given that several reports indicate the highest levels of plasma ET-1 are found in the neonate at birth in humans (Malamitsi Puchner, *et al*, 1993; Endo *et al*, 1996) and the pig (Levy *et al.*, 1995) and that decreased endothelium-derived NO has been observed in these neonatal rabbit vessels at birth (Docherty & MacLean, 1998). In the newborn rabbit, therefore, both an increase in the sensitivity of 5-HT and an uncovering of $5\text{-HT}_{1B/1D}$ receptor mediated responses were observed at a time when high tissue and plasma levels of ET-1 may occur. Noguchi *et al*, (1994) have reported a transient appearance of the ET_B receptor on the endothelium in 2-3 day old piglet peripheral pulmonary arteries.

GR55562 has recently been shown to be selective for the 5-HT_{1B} and 5-HT_{1D} receptors (Connor *et al.*, 1995). In recent studies this compound was used to identify a functional population of $5\text{-HT}_{1B/1D}$ receptors in human pulmonary conduit arteries (MacLean *et al.*,1996b) and, from the results of the studies in chapter 4, in human pulmonary resistance arteries. However, in the rabbit, GR55562 did not inhibit the 5-HT-mediated vasoconstriction at the foetal, 4 day and 7 day age points and only inhibited responses to 5-HT in the 0-24 hour vessels at the pEC_{10} and pEC_{20} levels. Any major involvement of $5\text{-HT}_{1B/1D}$ receptor subtype in mediating 5-HT-induced vasoconstriction in the rabbit perinatal small pulmonary artery can therefore be ruled out. Thus the combined agonist and antagonist data are consistent with the interpretation that the dominant receptor in mediating vasoconstriction in these vessels is

the 5-HT_{2A} receptor. In contrast, the results from chapter 3, provide evidence that the adult rabbit PRAs, contain a heterogeneous population of 5-HT receptors contributing to 5-HT-evoked vasoconstriction. The agonist and antagonist studies in the adult provide strong evidence that this vasoconstriction is mediated by the 5-HT_{2A} receptor and 5-HT_{1B} receptor. Taken together, the results from both the adult and perinatal studies highlight a maturational change in 5-HT receptor type mediating 5-HT-induced vasoconstriction in rabbit PRAs.

Interestingly, pulmonary resistance arteries in the rabbit have been shown to rely more on extracellular calcium for activation compared to larger arteries which rely more on intracellular calcium release through inositol trisphosphate (IP_3) (Franco-Obergón & López-Barneo, 1996). This is in accordance with reports using other vascular preparations (Cauvin, *et al*, 1984, 1985). Thus, from the studies in chapter 3, the 5-HT receptor type mediating vasoconstriction in the adult rabbit pulmonary arteries is closely related to the relative dependence on a particular arteries source of activator calcium. Hence, in the large, conduit, PAs 5-HT mediates its vasoconstrictor action predominantly through the 5-HT_{2A} receptor which is known to be coupled to the formation of IP_3 and ultimate release of intracellular calcium (Roth, *et al.*, 1986). In contrast, the PRAs contain a 5-$HT_{1B/1D}$ receptor. In the perinatal PRAs, however, 5-HT still mediates its vasoconstrictor action solely (the 0-24 hour vessels not withstanding) via a 5-HT_{2A} receptor. Theoretically, several possibilities could explain this. It is possible that the perinatal PRAs contain greater intracellular stores of calcium compared to adult PRAs. Alternatively, as diacylglycerol is concomitantly released with IP_3 during 5-HT_{2A} receptor stimulation (Berridge, 1983; Martin,

1994), it may cause increased calcium sensitivity in perinatal vessels compared to adults, as shown in ovine cerebral vessels (Akopov, *et al*, 1997). Das *et al* (1995), recently provided evidence for increased protein kinase C activity, which is dependent on diacylglycerol, in foetal compared to adult pulmonary arteries. It is therefore possible, that in the perinatal rabbit PRAs, diacylglycerol release, through 5-HT_{2A} receptor stimulation, plays a greater role in increasing extracellular calcium entry than in the adult vessels. Experiments investigating signal transduction mechanisms of 5-HT receptor stimulation during the perinatal period in the first week of life and into adulthood would help clarify this.

5-HT receptor-induced vasodilation

5-HT has previously been shown to elicit endothelium-dependent relaxation in porcine pulmonary arteries (Glusa & Richter, 1993) as well as other, systemic preparations (Bodelson *et al.*, 1993). Using 5-CT, it was established that there is 5-HT-receptor-induced vasodilation present in the vessels from the 4 and 7 day old rabbits. Other studies have previously shown that pre-constriction of bovine pulmonary arteries can 'uncover' 5-HT_1-receptor-mediated vasoconstriction (MacLean *et al.*, 1994; Sweeney *et al.*, 1995). It is of interest, therefore, that no contractile responses to 5-CT were observed in the present study, indicating that under the conditions used, there was no uncovering of 5-HT_1-receptor-mediated vasoconstriction these perinatal rabbit pre-constricted vessels.

Vasodilations to 5-CT were not observed in the fetal, 0-24 hour (or from the results in chapter 3, adult) rabbit PRAs again demonstrating rapid developmental changes in the rabbit pulmonary circulation. It is possible that there is a maturational alteration in

the ability of the pulmonary vascular smooth muscle to relax to NO as shown in porcine perinatal pulmonary arteries (Wilson *et al.*, 1993). This explanation is, however, unlikely in this rabbit preparation as ACh-induced relaxation has been demonstrated in the foetal and 0-24 hour rabbit PRAs (Docherty & MacLean, 1998). 5-HT receptors are known to mediate vasodilation in blood vessels either directly via 5-HT receptors located on the vascular smooth muscle (Cocks & Arnold, 1992; Leung *et al.*, 1996) or indirectly via endothelium released NO (Bodelson *et al.*, 1993; Glusa & Roos, 1996; Glusa & Richter, 1993). The results in this study show that, in 4 and 7 day rabbit small pulmonary arteries, 5-CT-evoked vasodilation is inhibited by the NO synthase inhibitor L-NAME. This suggests that 5-HT-induced vasodilation in these vessels involves release of the endothelium-derived relaxing factor NO. The endothelial cell location of the receptor could not be confirmed by experiments in which the endothelium is removed as previous experience dictates that any attempt to remove the endothelium greatly damages the thin and fragile underlying smooth muscle in this preparation. Endothelial cell, vasodilator 5-HT receptors have previously been identified in arteries and veins as being similar to either the 5-HT_{2B} subtype or the $5\text{-HT}_{1D/1B}$ subtype (Glusa *et al.*, 1996; Gupta, 1992). In the present study, in the 4 day old rabbit vessels, GR55562 inhibited 5-CT-induced vasodilation suggesting that the $5\text{-HT}_{1B/1D}$ subtype mediated NO-dependent vasodilation in these vessels. In contrast, in the 7 day old rabbit vessels, GR55562 only caused a small inhibition of 5-CT induced vasodilation at high concentrations of 5-CT. In these vessels L-NAME significantly reduced the maximum 5-CT-elicited relaxation and so NO does contribute to 5-CT-induced vasodilation in these vessels. Spiperone, however, potently inhibited 5-CT-induced

vasodilation in these vessels. Recently, the novel 5-HT_7 receptor has been described and has been found on the vascular smooth muscle of several preparations including the dog coronary artery (Terron, 1996) and *Cynomolgus* monkey jugular vein (Leung *et al*, 1996). This receptor is thought to mediate smooth muscle relaxation directly. 5-CT is known to be a potent agonist at this receptor (Raut, et al, 1993; Schoeffter *et al*, 1996). Several antipsychotic drugs, including spiperone have been shown to behave as antagonists at this receptor. Spiperone displays a high binding affinity for the cloned 5-HT_7 receptor (Roth *et al.*, 1994) and 5-HT_7 receptors located on vascular smooth muscle stimulate adenylate cyclase and mediate vascular relaxation directly (Hoyer *et al.*, 1994; Leung *et al.*, 1996). The pK_B of spiperone against 5-CT mediated vasodilation was 7.6 which is comparable with its pK_B of 7.1-7.8 at 5-HT_7 receptors in the monkey jugular vein, rabbit femoral vein and dog coronary artery (Leung *et al.*, 1996; Martin, 1994; Terron, 1996). Spiperone has a high affinity for 5-HT_{2A} receptors (Leff & Martin, 1986) but the presence of ketanserin in these studies rules out 5-HT_{2A} receptor effects and 5-HT_{2A} receptors are highly unlikely to mediate any 5-HT-induced vasodilator effects. Hence it is likely that, in the vessels from the 7 day old rabbits, 5-HT mediates NO-independent vasodilation through the 5-HT_7 receptor located on the vascular smooth muscle as well as NO-dependent vasodilation through the 5-$HT_{1D/1B}$ receptor. Involvement of other 5-HT receptors cannot be completely ruled out, for example the 5-ht_6 receptor is known to stimulate adenylate cyclase (Hoyer, *et al*, 1994), a signal transduction mechanism identical to that of the 5-HT_7 receptor and one which favours direct smooth muscle relaxation. 5-CT, however has low affinity for 5-ht_6 receptors (Sebben, *et al*, 1994), and given the potency of 5-CT in the 4

and 7 day old vessels, it is unlikely that the 5-ht_6 receptor is mediating 5-CT-induced relaxation in these vessels.

Endothelium involvement in the L-NAME resistant component of 5-CT-induced relaxation in the 7 days vessels could not be ruled out however. One possible explanation could be that 5-CT mediates relaxation, in part, in these 7 day resistance vessels via EDHF. In addition to NO (and prostacyclin), EDHF appears to be an important mediator of vasodilation in various vascular beds by hyperpolarizing the vascular smooth muscle through K^+ channel activation (Garland *et al.*, 1995) including pulmonary arteries (Chen, *et al.*, 1988). EDHF is now thought to assume a greater importance in vascular resistance beds. Shimokawa *et al.* (1996) reported that the contribution of EDHF to endothelium-dependent relaxation in rat mesenteric arteries is greater in the smaller diameter vessels. The contribution of the EDHF response in human arteries is also significantly greater in small blood vessels compared to large arteries (Urakami-Harasawa, *et al.*, 1997). Involvement of EDHF in 5-CT-induced relaxation in the 7 day rabbit vessels is unclear, however, as no documented evidence for 5-HT agonist-evoked EDHF production is currently available. One other possibility is that 5-CT may induce PGI_2 release in these vessels, causing relaxation. The ability of 5-HT to stimulate vascular synthesis and release of prostacyclin was first reported by Coughlin *et al.* (1981) in bovine aorta. Moreover, Demolle *et al.* (1989) demonstrated that 5-HT-induced release of was mimicked with greater potency by 5-CT in aorta.

The presence of 5-HT receptor-induced vasodilation between 4 and 7 days is consistent with previous studies in the pig where endothelium-dependent vasodilation is most marked 3-10 days after birth, declining with increasing age (Liu *et al.*, 1992). After birth,

there is a reduction in muscularisation of the pulmonary circulation between 24 hrs and two weeks (Haworth & Hislop., 1981). The presence of pulmonary 5-HT vasodilator mechanisms at this age may, therefore, facilitate such changes. The current advancement in molecular techniques, particularly in the development of specific monoclonal antibodies for the different 5-HT receptor subtypes (eg Wu, *et al*, 1998) will aid the future study of these receptors in terms of their expression and distribution in newborn pulmonary resistance arteries.

The role of 5-HT in PPHN is uncertain but given the fact that this bioamine is a potent pulmonary vascular smooth muscle cell mitogen (Lee, *et al.*, 1991; Pitt, *et al.*, 1994), it may contribute to increased pulmonary vascular remodelling associated with smooth muscle migration and proliferation. It is also likely that the balance of 5-HT receptor subtypes in the perinatal pulmonary vasculature would determine the end functional response to 5-HT, thus affecting pulmonary vascular resistance. A reduction of 5-HT receptors mediating 5-HT-induced relaxation, such as the endothelium-located 5-$HT_{1B/1D}$ receptor which mediates indirect vasodilation via NO release or the 5-HT_7 receptor, which mediates direct relaxation of the vascular smooth muscle would presumably augment responses to endogenous 5-HT. The results of this chapter provides strong evidence for the existence of both these receptors in the 4 and 7 day old rabbit PRAs. If these receptors exist in human neonatal pulmonary arteries, one could speculate that in the instance of the endothelial 5-$HT_{1B/1D}$ receptor for example, a reduction of this receptor type may impair vasodilation by decreased release of endothelium-derived NO. In PPHN, a reduction, or absence of these receptors in the pulmonary vasculature may contribute to the

maintenance of abnormally high pulmonary arterial pressure after birth, characteristic of this disease. Concerning functional vasoconstrictor 5-HT receptors, a similar effect would probably be observed if there was an elevation in the number of these receptors in PPHN. In the chronic hypoxic rat model of PHT, for example, elevated vasoconstrictor responses to 5-HT are observed, thought to be partly due to increased expression of contractile 5-HT_{2A} and 5-HT_{1B} receptors (MacLean, *et al*, 1996).

In conclusion, these studies have demonstrated marked developmental changes in the 5-HT receptors involved in mediating vasoconstriction and vasodilation in rabbit small pulmonary arteries. The predominant receptor mediating vasoconstriction is the 5-HT_{2A} receptor in fetal to 7 day old rabbits. In contrast, in adult vessels both 5-HT_{2A} and $5\text{-HT}_{1D/1B}$ receptors contribute towards 5-HT-induced vasoconstriction. 5-HT receptor-mediated vasodilation is absent in the fetus, 0-24 hour and adult rabbits. In the 4 day old rabbit vessels 5-HT-mediated vasodilation is mediated indirectly via NO release following $5\text{-HT}_{1D/1B}$ receptor activation. In 7 day rabbit vessels, however, vasodilation is also mediated directly by a 5-HT_7 receptor.

Chapter 6

Effect of increased tone and cGMP reduction on 5-HT1-receptor-induced vasoconstriction in rat small muscular pulmonary arteries; evidence for pharmacological synergism

6.1 Introduction

Pharmacological synergism occurs when a greater than additive effect ensues as a result of two agonists acting at different receptors on the same cell. This phenomenon has been observed in several systemic blood vessels including the rabbit femoral artery and rabbit ear artery (Movahedi & Purdy, 1997; MacLennan & Martin, 1992) as well as in the guinea-pig iliac (Sahin-Erdemli *et al*, 1991) and human coronary (Cocks *et al.*,1993) arteries. This involves G_i-coupled 5-HT_1-receptor induced vasoconstriction being 'uncovered' or greatly potentiated by increasing vascular tone through a G_q-coupled receptor pathway or by elevations in extracellular K^+.

The members of the 5-HT_1 receptor family are coupled to the inhibition of adenylate cyclase through pertussis toxin-sensitive Gi/o proteins (Dickenson and Hill; 1995; Sumner and Humphries, 1990). However reductions in the cytoplasmic concentration of cyclic AMP may not *per se* be responsible for ensuing smooth muscle contraction (Randall *et al.*, 1996). There is substantial evidence that 5-HT_{1B} receptors play an important role in mediating 5-HT-induced vasoconstriction in human large pulmonary arteries (MacLean *et al* 1999., 1996a; Cortijo *et al.*, 1997). More recently, this has been demonstrated in human small muscular pulmonary arteries (see chapter 4 of this thesis and Morecroft *et al.*, 1999). In rat pulmonary arteries, 5-HT-induced vasoconstriction is mediated entirely by the $G_{q/11}$-coupled 5-HT_{2A} receptor (MacLean, *et al.*, 1996). However, pulmonary arterial responses to 5-hydroxytryptamine (5-HT) are augmented in the chronic hypoxic rat model of pulmonary

hypertension where the increased vasoconstriction to 5-HT is mediated by both the G_q-coupled 5-HT_{2A} and the G_i-linked 5-HT_{1B} receptors (MacLean *et al.*, 1996). It has also been demonstrated in the chronic hypoxic pulmonary hypertensive rat, that at all levels of the pulmonary vascular bed, increased endogenous vascular tone is evident (MacLean *et al.*, 1996). This may be mediated by an elevation of vasoconstrictors such as ET-1 (MacLean *et al.*, 1999), depolarisation via inactivation of K^+ channels (Osipenko et al., 1998) or decreased levels of cyclic nucleotides (MacLean *et al.*, 1996). Given these findings and that 5-HT has recently been implicated in the aetiology of pulmonary hypertension (Herve *et al.*, 1995), any evidence for pharmacological synergism and the effect of increased vascular tone on 5-HT receptor induced vasoconstriction in the pulmonary arteries that are the major determinants of pulmonary vascular resistance is of interest.

Here the effect of increasing vascular tone via different second messenger signalling pathways on 5-carboxamidotryptamine (5-CT, 5-HT_1 agonist)-induced vasoconstriction was examined on small muscular pulmonary arteries (smPAs) from normoxic (control) rats. The effect of decreasing cGMP levels on these responses was also examined. Any key observations were further investigated in age-matched chronic hypoxic pulmonary hypertensive rats.

6.2 Methods

Chronic hypoxic rats

The chronic hypoxic rat model of pulmonary hypertension is fully described in chapter 2, section 2.1.3 of this thesis. Briefly, male Wistar rats aged 28 days were placed in a specially designed perspex

hypobaric chamber. This was depressurised, over 2 days, to 550 mbar (oxygen concentration equivalent to 10%). The temperature of the chamber was maintained at 21-22^{0}C, and the chamber was ventilated with air at approximately 45 lmin^{-1}. Rats were maintained in these hypobaric/hypoxic conditions for 2 weeks in order to develop pulmonary hypertension (PHT). Age-matched normoxic animals were held in room air. The right ventricle (RV) was carefully dissected free from the septum and left ventricle (LV), and both were blotted lightly and weighed. PHT was assessed by measuring the ratio of RV weight to total ventricular (TV) weight (see section 2.1.4.2). RV/TV ratio is an established and reliable index for PHT in this model (Herget *et al.*,1978; Wanstall *et al.*, 1991; Sheedy *et al.*, 1996).

Intrapulmonary arteries

Rats from both experimental groups were killed by an overdose of sodium pentobarbitone (60 mgkg^{-1}) and the lungs promptly removed. Small, intralobar pulmonary arteries (smPAs) with an internal diameter of between 150-250μm were dissected out according to the same methods stated in section 2.3.4.1 for the adult rabbit vessels, These were mounted on a wire myograph, bathed in Krebs solution (pH 7.4) at 37°C and bubbled with 16% O_2/6%CO_2 balance N_2. The gas mixtures were chosen as they are similar to those which the control rat vessels would be exposed to *in vivo*. The smPAs from the CH rats were also bubbled with the same gas mixture for direct comparison, thus minimising variables between the two groups. Some difficulty is encountered when trying to control the pO_2 of the myograph baths at values equivalent to the CH rats breathing 10% O_2. In preliminary studies, bubbling CH vessels with a 10% O_2/6%CO_2 balance N_2 gas mixture still produced pO_2 values of ~100mmHg, probably due to the influence of atmospheric O_2. Using

the normalisation process explained in section 2.3.6, smPAs from control rats were tensioned to an equivalent transmural pressure of ~15mmHg in order to mimic *in vivo* pulmonary arterial transmural pressure in the rat. Vessels from the chronic hypoxic rats were tensioned to a transmural pressure equivalent of ~34mmHg to mimic pulmonary artery and arteriolar pressure of chronic hypoxic animals *in vivo*. No attempt was made to remove the endothelium in any vessel.

Experimental protocol

The smPAs were allowed to equilibrate for 1 hour prior to the addition of any drugs. The response of the vessels to 50mM KCl was determined twice; once the contractile response had plateaued, the vessels were washed out a minimum of 6 times with fresh Krebs solution. Vessels were then preconstricted with a threshold concentration of either endothelin-1 (ET-1, G_q-linked receptor activation) (0.1-1.0nM), 10mM KCl (opening of calcium channels and subsequent raising of intracellular calcium concentration), Neuropeptide Y (NPY, G_i-linked receptor activation), A23187 (calcium ionophore) or 4-aminopyridine (4-AP, K_V channel blocker). Once a stable plateau had been reached, cumulative concentration response curves (CCRCs) to 5-CT (1nM-0.1mM) were obtained either in the presence or absence of N^W-nitro-L-arginine methyl ester (L-NAME) (100μM) to decrease levels of cGMP. In some vessels, the CCRCs to 5-CT, both in the presence or absence of 100μM L-NAME, were constructed without prior addition of threshold concentrations of contractile agent.

Data analysis

Contractile responses are expressed as a percentage of the reference contraction to the second application of 50mM KCl. pEC_{10}, pEC_{25}, and pEC_{50} values for the hypoxic groups were calculated, according to the methods described in section 2.5.2, from individual CCRCs. Statistical analysis of the means of groups of data were made by Student's unpaired t-test when two groups of data were compared, one-way analysis of variance (ANOVA) followed by Tukeys post test when three groups or more were compared. $P < 0.05$ was considered to be statistically significant. Throughout, data are expressed as mean ± s.e.mean and n/n = number of vessels/number of animals.

6.3 Results

Assessment of PHT

The RV/TV ratio of the control rats was 0.251 ± 0.009 and of the chronic hypoxic rats was 0.387 ± 0.008 ($P<0.001$; unpaired Student's t test, n = 6-12); this is indicative of severe pulmonary hypertension and in agreement with previous findings of others (MacLean *et al.*, 1996).

Wire myography

The effective pressure to which the vessels from normoxic rats were tensioned was 14.6 ± 0.3 mmHg and the resulting estimate of internal diameter for these vessels was 169.7 ± 9.8μm. These two measurements for the CH vessels were 34.5 ± 0.5 mmHg and 172 ± 11.1μm respectively. For both groups n > 20/10. There was no significant difference between the internal diameters from the two

groups.

Response to KCl

50mM KCl-induced contractions were of the same magnitude in both the normoxic (control) and chronic hypoxia-treated groups. These were 2.53 ± 0.28 mN and 2.75 ± 0.37 mN respectively.

Responses to 5-CT and 5-HT

Figure 6.1 demonstrates CCRCs to 5-HT and 5-CT in normoxic and chronic hypoxic rat smPAs. In normoxic rat smPAs, 5-CT evoked a small, concentration-dependent vasoconstriction only at 5-CT concentrations > 1μM, and did not produce a maximum response in the concentration range studied, thus a pEC_{50} value could not be accurately determined. The response at the maximum concentration of 5-CT used ([5-CT]max.) was 33.3 ± 5.3 % 50mM KCl reference contraction. Exposure to chronic hypoxia markedly increased (pEC_{50} = 5.6 ± 0.2) the response to 5-CT in the smPAs. 5-HT evoked a potent, concentration dependent contraction in both the normoxic and CH rat vessels. Figure 6.1 shows that exposure to chronic hypoxia markedly increased ($P<0.01$; Student's unpaired t-test) the maximum contractile response to 5-HT ($E_{max.}$ = 35 ± 6% and 118 ± 26 % for normoxic and chronic hypoxic vessels respectively). There was no significant difference between the 5-HT pEC_{50} values for the normoxic (pEC_{50} = 5.8 ± 0.2) and CH rat smPAs (pEC_{50} = 6.4 ± 0.2). The maximum contractile responses to 5-CT and 5-HT in the CH rat vessels were similar (figure 6.1).

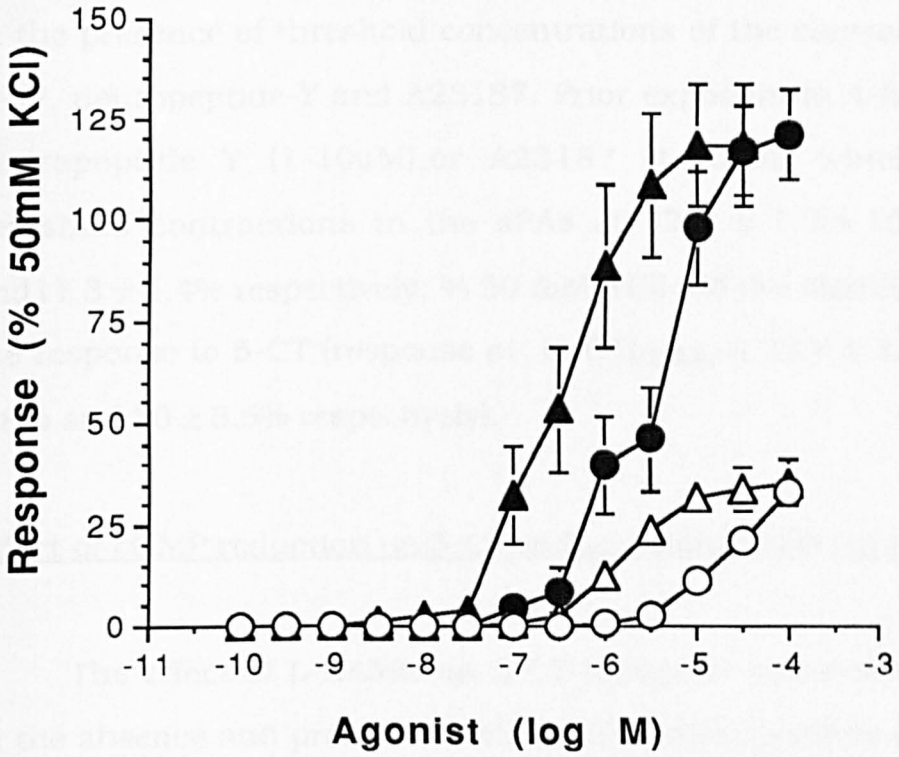

Figure 6.1 Effect of 2 weeks chronic hypoxia on contractile responses to 5-HT and 5-CT in rat smPRAs. CCRCs to 5-CT in control (O, n = 12/12) and in chronic hypoxic (●, n = 8/8) rats. CCRCs to 5-HT in control (△, n = 6/6) and in chronic hypoxic (▲, n = 6/6) rats. Data are expressed as a percentage of reference contraction to 50mM KCl. Each point represents mean ± s.e.m.

Normoxic rat sPAs

Figures 6.2 and 6.3 and Table 6.1 demonstrate the effects of elevated tone on the CCRCs to 5-CT in smPAs from normoxic rats. ET-1 (1-3nM) and KCl (10mM) induced threshold contractions (12.5 ± 1.2 %; 12.6 ± 1.4% respectively, % 50mM KCl reference contraction) of the smPAs. Prior exposure to these threshold concentrations of either ET-1 or KCl significantly augmented the 5-CT-induced

contraction (Figure 6.2 and Table 6.1) by decreasing the threshold concentration for 5-CT 10-100 fold. KCl pre-contraction significantly potentiated the response to 5-CT at all concentrations (response at $[5\text{-CT}]_{max.}$ = 49 ± 3%; P <0.05). Figure 6.3 shows the CCRCs to 5-CT in the presence of threshold concentrations of the contractile agents 4-AP, neuropeptide-Y and A23187. Prior exposure to 4-AP (1-3mM), neuropeptide Y (1-10μM),or A23187 (1-10μM) which induced threshold contractions in the sPAs of 12.7 ± 1.2%,10.7 ± 0.5% and11.3 ± 1.4% respectively; % 50 mM KCl) did not significantly alter the response to 5-CT (response at $[5\text{-CT}]_{max.}$ = 32.7 ± 3.3%, 30.8 ± 2.8% and 30 ± 5.5% respectively).

Effect of cGMP reduction on 5-CT-induced contraction of rat smPAs

The effect of L-NAME on 5-CT-induced vasoconstriction both in the absence and presence of threshold concentrations of ET-1 and KCl are shown in figure 6.4 and table 6.1. Inhibition of nitric oxide synthase with 100μM L-NAME, in order to reduce cGMP levels, did not significantly alter the response to 5-CT (figure 6.4A and table 6.1). In the presence of 100μM L-NAME, the 5-CT-induced contraction was further significantly augmented by threshold concentrations of ET-1 or KCl (Figure 6.4B and table 6.1).

Group	-L-NAME E_t	n	+L-NAME E_t	n
5-CT	0.4 ± 0.3	12/12	3.2 ± 1.3	7/7
+ET-1 tone	6.8 ± 1.1*	8/8	14.4 ± 2.0**††	6/6
+ KCl tone	7.4 ± 3.4**	9/9	33.0 ± 6.0*†	8/8
+ 4-AP tone	0.2 ± 0.2	6/6		
+ NPY tone	0.7 ± 0.5	6/6		
+ A23187 tone	0.5 ± 0.3	6/6		

Table 6.1 Effect of elevated tone and L-NAME on 5-CT-induced contractions in normoxic rat smPAs. Statistical comparisons were made by ANOVA followed by Tukey's post test: 1) control vs. presence of threshold concentration of contractile agent *P<0.05, **P<0.01. 2) response in absence of L-NAME vs. respective response in presence of 100μM L-NAME †P<0.05, ††P<0.01. Data shown as % response to 50mM KCl reference contraction. Values are mean ± s.e.m. E_t, Response at control 5-CT threshold concentration (1μM). n, number of vessels/number of animals.

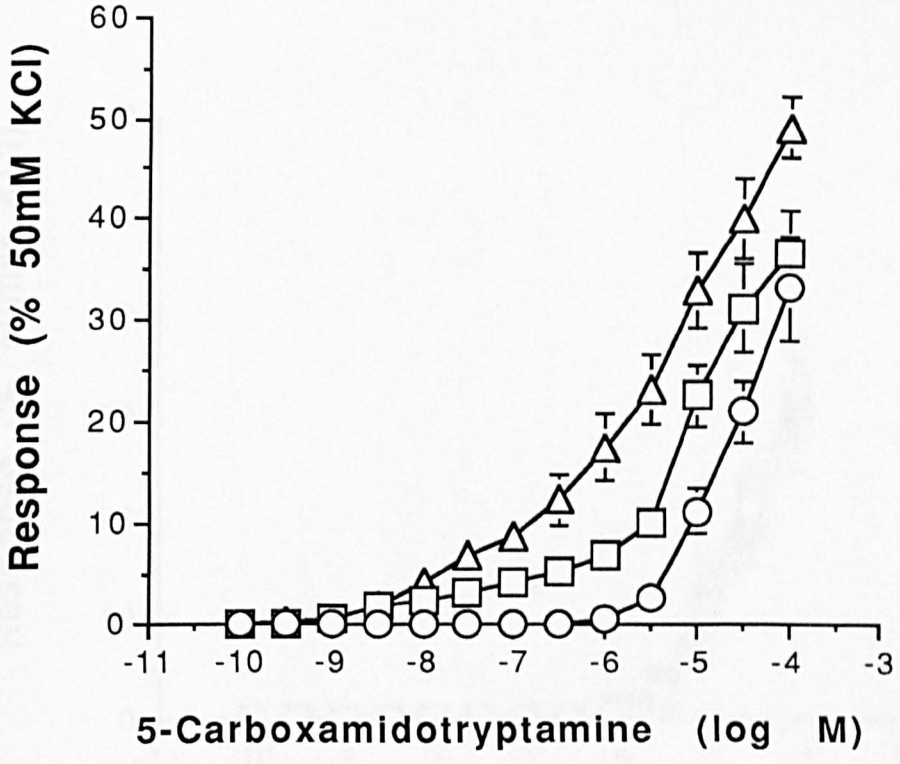

Figure 6.2 Effect of threshold concentrations of ET-1 and KCl on 5-CT-induced vasoconstriction in normoxic rat smPRAs. CCRCs to 5-CT (O, n = 12/12) and in the presence of ET-1(□, n = 8/8) and KCl (△, n = 9/9). Data are expressed as a percentage of reference contraction to 50mM KCl. Each point represents mean ± s.e.m.

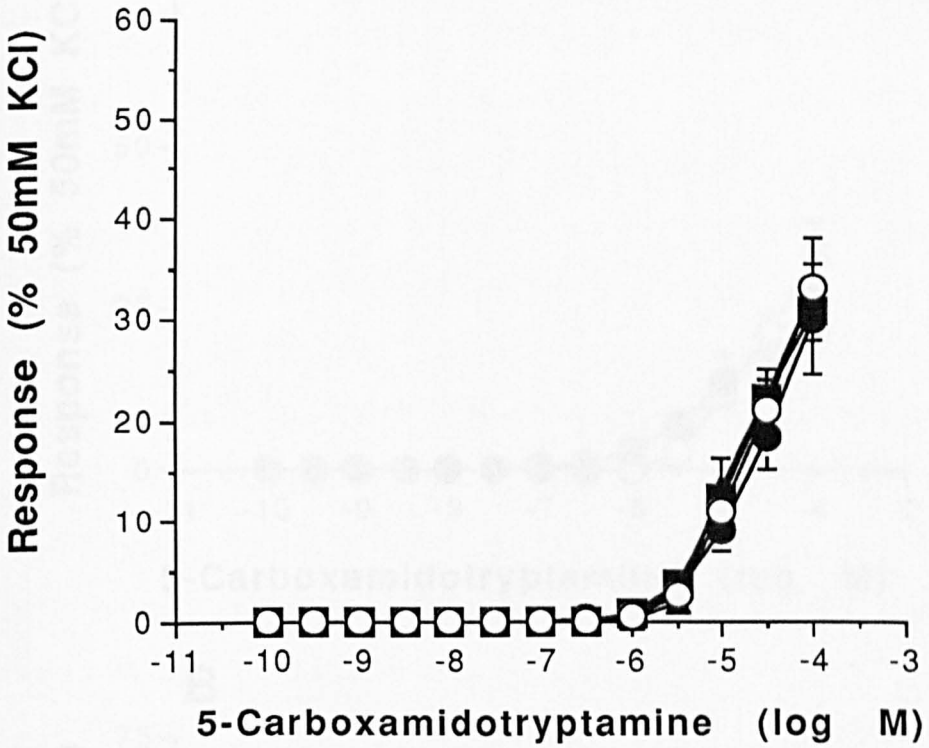

Figure 6.3 Effect of threshold concentrations of neuropeptide Y (NPY), 4-aminopyridine (4-AP) and A23187 on 5-CT-induced vasoconstriction in normoxic rat smPRAs. CCRCs to 5-CT (O, n = 12/12) and in the presence of NPY (■, n = 6/6), 4-AP (▲, n = 6/6) and A23187 (●, n = 6/6). Data are expressed as a percentage of reference contraction to 50mM KCl. Each point represents mean ± s.e.m.

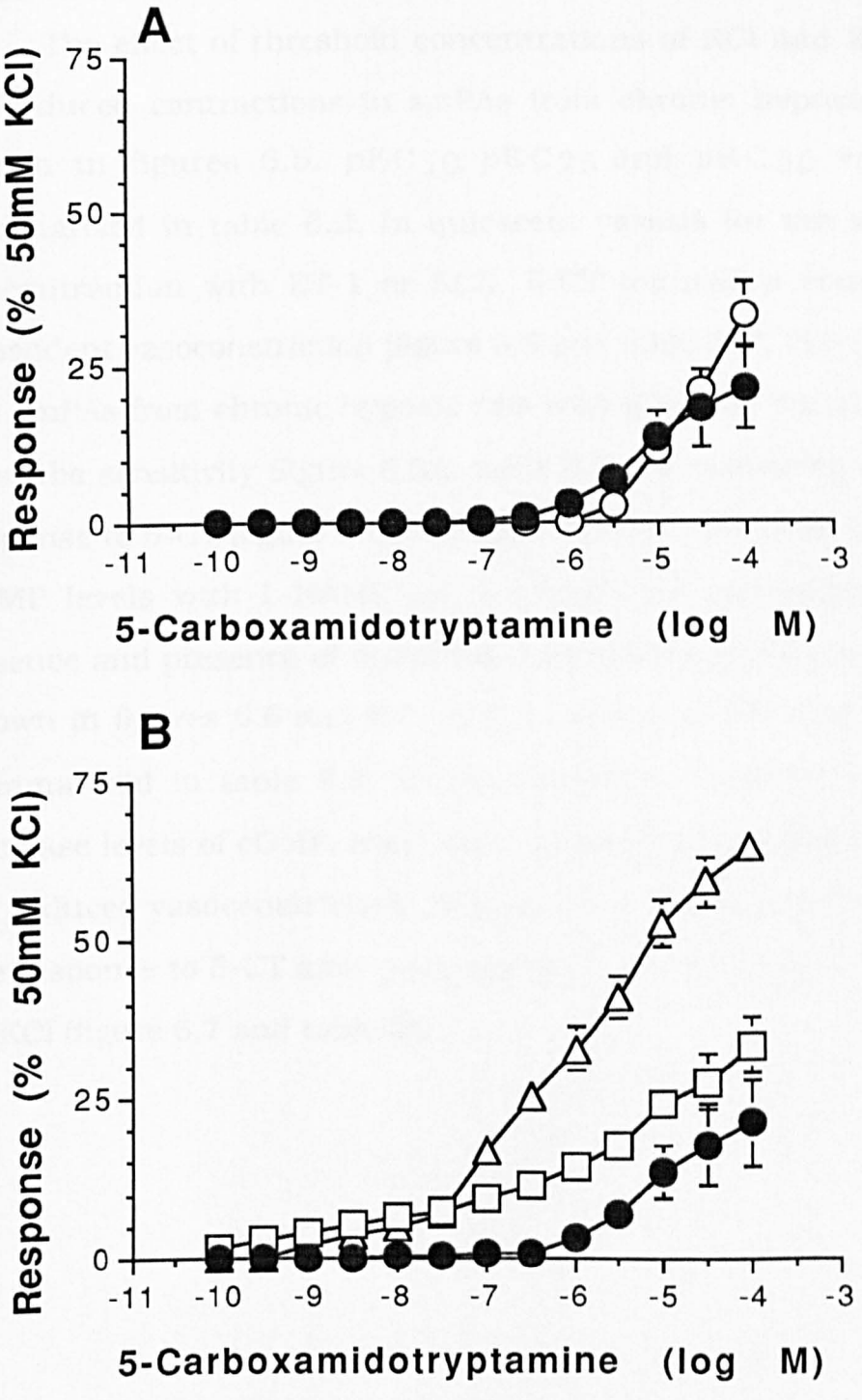

Figure 6.4 Effect of 100μM L-NAME (to reduce cGMP levels) on 5-CT-induced vasoconstriction in normoxic rat smPRAs. **A** CCRCs to 5-CT (○, n = 12/12) and in the presence of 100μM L-NAME (●, n = 7/7). **B** CCRCs to 5-CT (●, n = 7/7) and in the presence of threshold concentrations of ET-1 (□, n = 6/6) and KCl (△, n = 8/8); all in the presence of 100μM L-NAME. All data are expressed as a percentage of reference contraction to 50mM KCl. Each point represents mean ± s.e.m.

Chronic hypoxic rat smPAs

The effect of threshold concentrations of KCl and ET-1 on 5-CT-induced contractions in smPAs from chronic hypoxic rats are shown in figures 6.5. pEC_{10}, pEC_{25} and pEC_{50} values are summarised in table 6.2. In quiescent vessels (in the absence of precontraction with ET-1 or KCl), 5-CT induced a concentration dependent vasoconstriction (figure 6.5 and table 6.2). Pre-contracting the smPAs from chronic hypoxic rats with ET-1 did not significantly alter the sensitivity (figure 6.5A; table 6.2) or maximum contractile response to 5-CT (figure 6.5B) in these vessels. The effect of reducing cGMP levels with L-NAME on 5-CT-induced contractions in the absence and presence of threshold contractions of ET-1 and KCl are shown in figures 6.6 and 6.7. pEC_{10}, pEC_{25} and pEC_{50} values are summarised in table 6.2. In the presence of 100μM L-NAME to decrease levels of cGMP, there was no significant alteration in the 5-CT induced vasoconstriction, neither did L-NAME significantly alter the response to 5-CT after prior exposure to threshold levels of ET-1 or KCl (figure 6.7 and table 2).

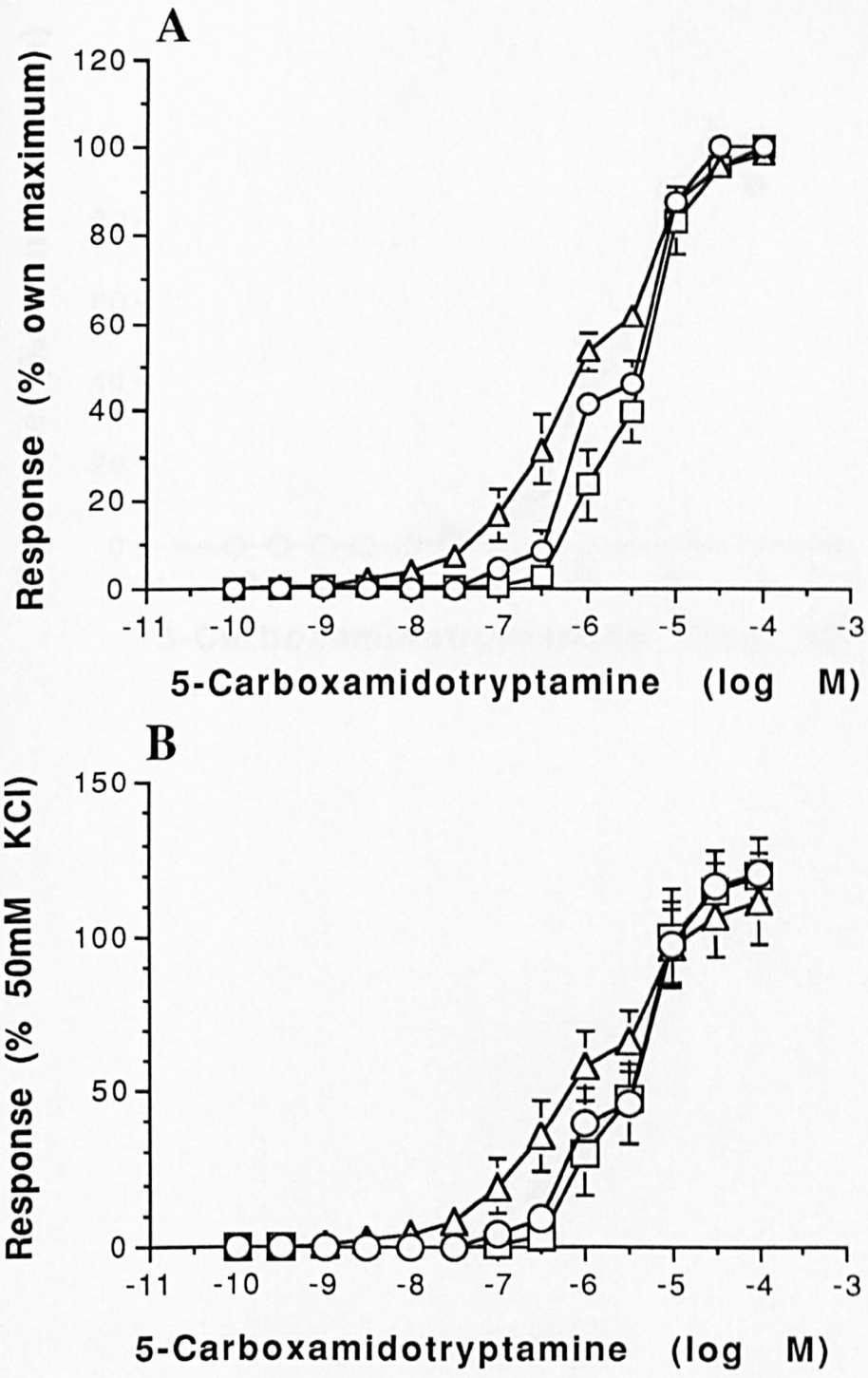

Figure 6.5 Effect of threshold concentrations of ET-1 and KCl on 5-CT-induced vasoconstriction in chronic hypoxic rat smPRAs. CCRCs to 5-CT (O, n = 6/6) and in the presence of ET-1 (□, n = 6/6) and KCl (△, n = 6/6). **A** Data are expressed as a percentage own maximum contraction. **B** Data are expressed as a percentage of reference contraction to 50mM KCl. Each point represents mean ± s.e.m.

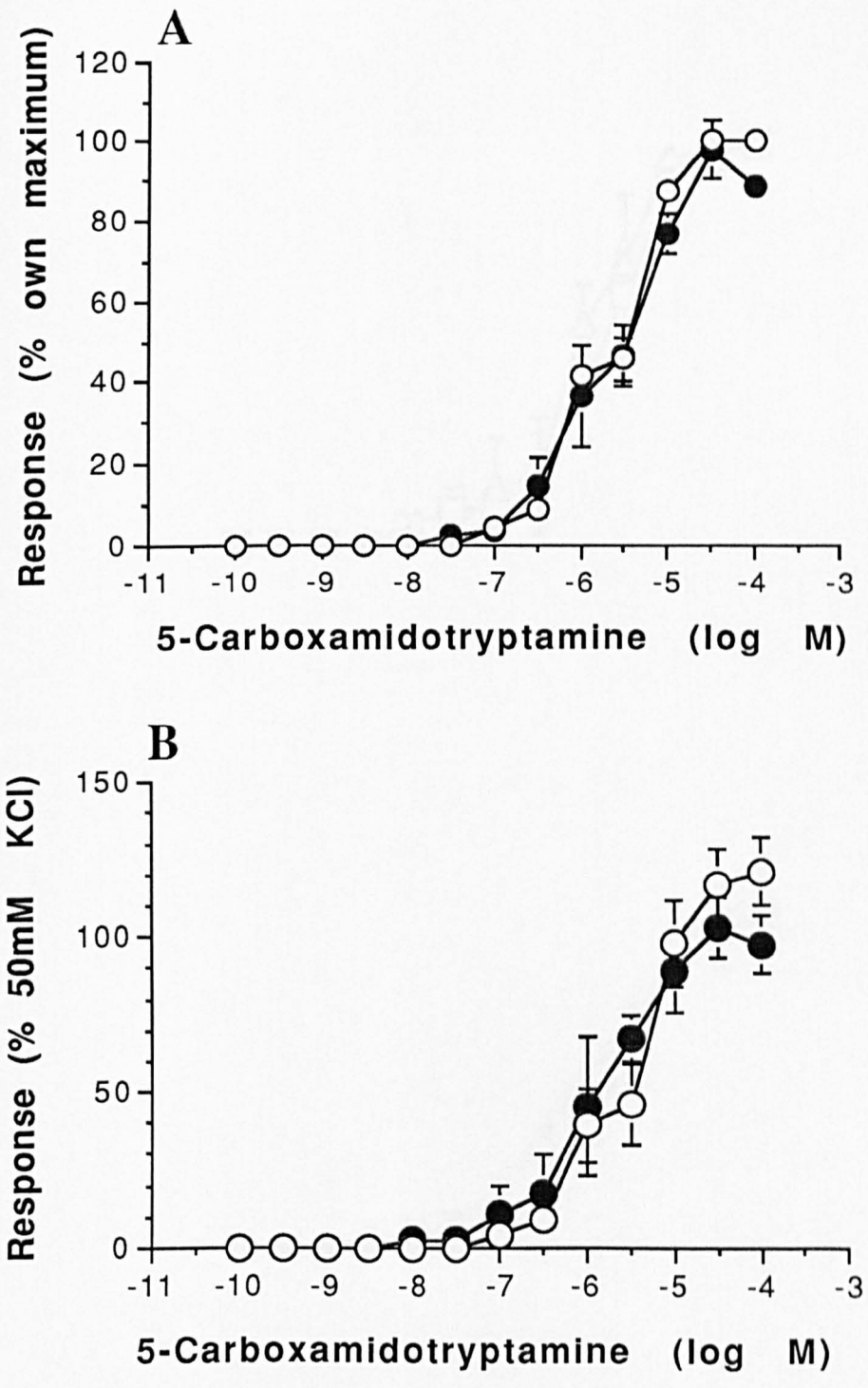

Figure 6.6 Effect of 100μM L-NAME (to reduce cGMP levels) on 5-CT-induced vasoconstriction in chronic hypoxic rat smPRAs. CCRCs to 5-CT (O, n = 6/6) and in the presence of 100μM L-NAME (●, n = 6/6). **A** Data are expressed as a percentage own maximum contraction. **B** Data are expressed as a percentage of reference contraction to 50mM KCl. Each point represents mean ± s.e.m.

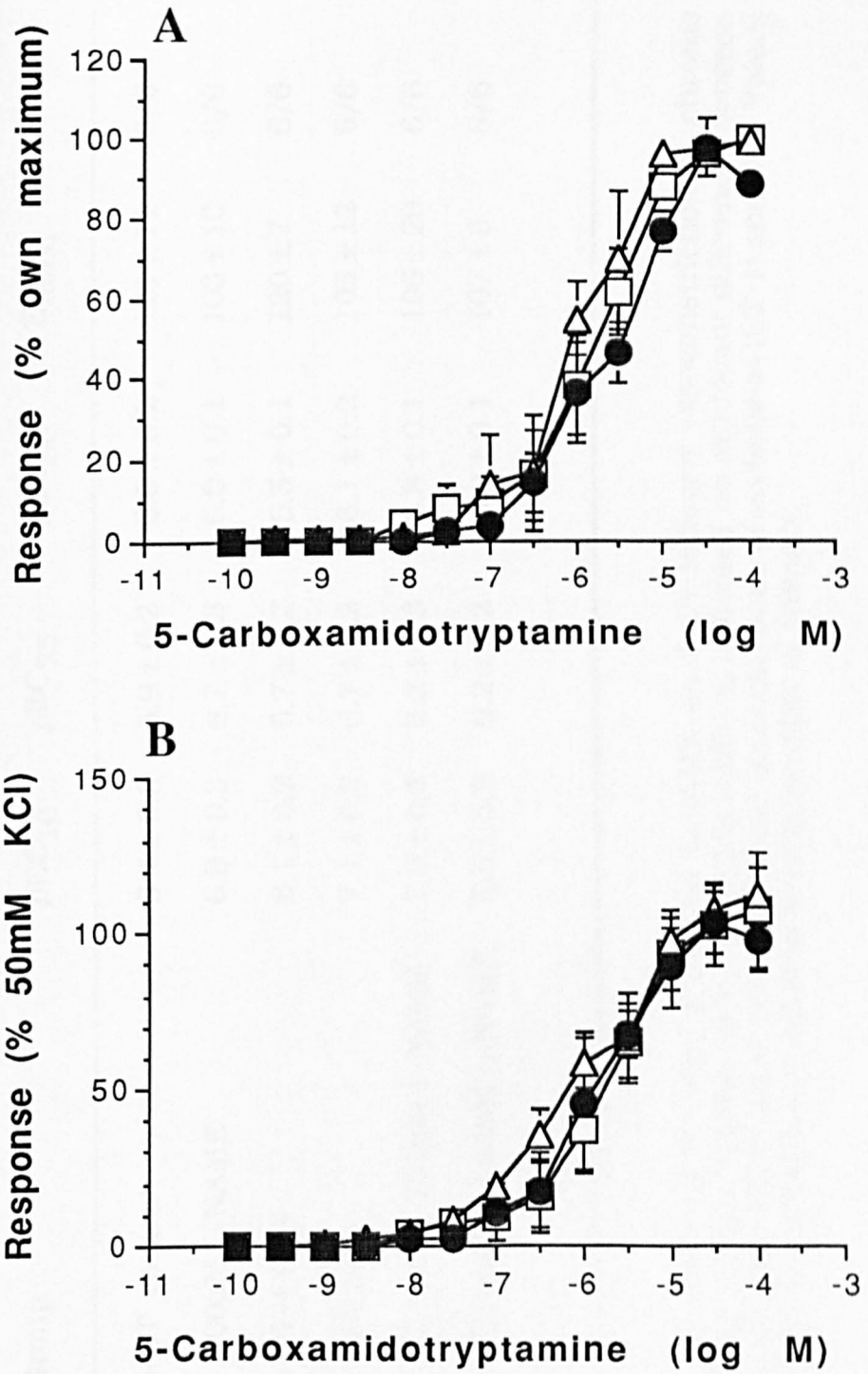

Figure 6.7 Effect of threshold concentrations of ET-1 and KCl on 5-CT-induced vasoconstriction in chronic hypoxic rat smPRAs in the presence of 100μM L-NAME. CCRCs to 5-CT (●, n = 6/6) and in the presence of ET-1 (□, n = 6/6) and KCl (△, n = 6/6). **A** Data are expressed as a percentage own maximum contraction. **B** Data are expressed as a percentage of reference contraction to 50mM KCl. Each point represents mean ± s.e.m.

Group	pEC_{10}	pEC_{25}	pEC_{50}	$E_{max.}$	n
5-CT control	6.4 ± 0.3	5.9 ± 0.2	5.6 ± 0.2	121 ± 11	6/6
+ 100μM L-NAME	6.9 ± 0.3	6.7 ± 0.3	6.0 ± 0.1	103 ± 10	6/6
+ ET-1 tone	6.1 ± 0.2	5.7 ± 0.2	5.5 ± 0.1	120 ± 7	6/6
+ KCl tone	7.1 ± 0.3	6.7 ± 0.2	6.1 ± 0.2	105 ± 12	6/6
+ ET tone + 100μM L-NAME	7.0 ± 0.4	6.3 ± 0.3	5.8 ± 0.1	106 ± 20	6/6
+ KCl tone + 100μM L-NAME	6.5 ± 0.3	6.2 ± 0.2	6.0 ± 0.1	107 ± 9	6/6

Table 6.2 Effect of elevated tone and L-NAME on 5-CT-induced vasoconstriction in chronic hypoxic rat smPAs. Statistical comparisons (ANOVA) indicated no significant differences between relevant groups. Tone raised using threshold concentrations of endothelin (ET-1) and KCl. Values are mean ± s.e.mean.n = number of vessels/number of animals.

6.4 Discussion

The very marked augmentation of vasoconstrictor responses to 5-CT and to 5-HT in the pulmonary hypertensive chronic hypoxic rat intralobar small pulmonary arteries is in agreement with previous observations (MacLean *et al.*, 1996b) where an 'uncovering' of G_i-linked 5-HT_{1B} receptor-mediated vasoconstriction was observed (along with an enhanced 5-HT_{2A} receptor mediated contraction). Although the intracellular signalling pathways mediating pulmonary arterial responses to ETs are still to be fully elucidated, several studies *in vitro* have shown ET-1 to mediate its effects through $G_{q/11}$-protein coupled receptors and subsequent activation of phospholipase C (Resink *et al*, 1988; Ohlstein *et al.*, 1995). The augmented response to 5-CT in normoxic rat smPAs observed in this study, in the presence of ET-1 is consistent with several reports showing that pre-contraction with agonists that act via $G_{q/11}$-coupled receptors causes an augmentation or uncovering of G_i-coupled receptor responses such as 5-HT_1 receptor mediated vasoconstriction (Selbie & Hill, 1998). In the rat tail artery, for example, responses to the α_2-adrenoceptor agonist UK14304, which mediates its effects via a G_i-coupled receptor, are uncovered by threshold G_q-receptor-induced vasoconstriction (MacLean, *et al.*,1990). MacLennan and Martin in 1992 reported that threshold contractions to the thromboxane A_2-mimetic, U46619 (acting via a G_q-coupled receptor), increased the sensitivity of the rabbit femoral artery to 5-HT and uncovered a 5-HT_1 receptor-mediated contractile response in those vessels. This synergistic interaction has mainly been demonstrated in large systemic arteries as well as in isolated bovine large pulmonary arteries (MacLean, *et al.*, 1994;1999). The

results presented in this chapter are the first to demonstrate this phenomenon in the small muscular pulmonary arteries, which are the major contributors to increased pulmonary vascular resistance in the pulmonary hypertensive state (Singhal *et al.*, 1973). This suggests that crosstalk between two G-protein coupled receptors may result in the amplification of signals and lead to an uncovering of otherwise silent contractile receptors in these pulmonary resistance vessels as well as in larger systemic vessels (Martin *et al.*, 1996). Systemic arteries *in vivo* are under a certain amount of tonic sympathetic tone and therefore, the synergistic interactions between receptors in these vessels may be normally operative *in vivo*. In contrast, pulmonary arteries in the normal lung are fully dilated and therefore synergistic effects may only operate when the pulmonary arteries are under the influence of increased vascular tone as in pulmonary hypertension. Importantly, in pulmonary hypertension, there is evidence for an increased production of endothelin-1 (MacLean *et al.*, 1999) as well as thromboxane (Christman, 1998) and angiotensin-II (Cargill and Lipworth, 1995). These all are agonists acting via Gq-coupled receptors and collectively, supports the possibility that increased G_q-coupled-receptor activation may synergise with G_i-coupled responses in pulmonary hypertension.

The potentiation of 5-CT-induced vasoconstriction in the normoxic sPAs by threshold concentrations of KCl suggests that direct elevation of intracellular Ca^{2+} augments responses to G_i-coupled agonists. Several studies have demonstrated a crucial role for Ca^{2+} influx through voltage-operated Ca^{2+} channels in the mediation of 5-HT_1 receptor-mediated contraction (Hill *et al.*, 2000; Movahedi & Purdy, 1997; Sumner *et al.*, 1992). Although 5-$HT_{1B/1D}$ receptor activation may not activate voltage-operated Ca^{2+} channels in quiescent vessels, in depolarised arteries when the channel open

probability has been increased, such activation of these receptors may occur, leading to contraction. In fact, the 5-HT_1-receptor mediated vasoconstriction was augmented at all concentrations of 5-CT and the potentiation was greater than that of ET-1. In addition to membrane depolarisation, KCl has been shown to stimulate phospholipase C (PLC) (Phillipe & Chien, 1995) and may, therefore, facilitate entry to 5-HT_1 receptor stimulation by activation of the PLC signalling pathway. Jin *et al.*, 1993 have shown that KCl can also increase the formation of inositol (1,4,5) triphosphate (IP_3) in rat pulmonary arteries, this may explain the strong synergistic interaction observed here. Decreased levels of cGMP have previously been shown to potentiate α_2-adrenoceptor ($G_{i/o}$-coupled receptor) induced vasoconstriction in rabbit large pulmonary arteries (MacLean *et al.*, 1993) and, in bovine pulmonary arteries, responses to the 5-$HT_{1B/1D}$ -G_i-linked receptor agonist sumatriptan are potentiated by decreased cGMP (MacLean *et al.*, 1994). The results of this present study, show that a decrease in cGMP levels via the inhibition of NOS with L-NAME further potentiates the augmented responses to 5-CT but only in the presence of ET-1- and KCl-induced tone. This suggests that levels of the cyclic nucleotide cGMP may also play an important synergistic role in the response of pulmonary resistance arteries to G_i-coupled receptor-induced vasoconstriction. In pulmonary arteries removed from chronic hypoxic, pulmonary hypertensive rats, decreased levels of cGMP, as well as decreased cAMP, along with increased vascular tone have been observed (MacLean *et al.*, 1996). The observed decrease in the cyclic nucleotides is thought to be caused by increased phosphodiesterase activity (MacLean, *et al.*, 1997). The observation here that G_q and G_i-coupled interactions can be further modulated by cyclic nucleotide levels may therefore be of pathophysiological importance in

pulmonary hypertension.

Pulmonary arterial cell membrane depolarisation is known to occur in the CH rat model of pulmonary hypertension (Osipenko *et al.*, 1998) and this is possibly due to a down regulation of K channels in these cells. Based on these results, the observed augmented response to 5-HT receptor-induced vasoconstriction in pulmonary hypertension would be expected to be mimicked by blocking Kv channels with 4-aminopyridine. Surprisingly, the findings in this did not support this hypothesis, suggesting that down regulation of Kv channels may not be involved in the observed augmentation of 5-HT_1 receptor-mediated vasoconstriction in small muscular pulmonary arteries from the chronic hypoxic rat model of pulmonary hypertension. Similarly, Doggrell *et al.*, 1999 observed that 4-AP did not augment responses to 5-HT in the rat intralobar small pulmonary artery, but did in the main pulmonary artery, suggesting membrane depolarisation plays a more important role in 5-HT-induced vasoconstriction in large, conduit pulmonary arteries.

Neuropeptide Y (NPY) mediates its vasoconstrictor effects, like 5-HT_1 receptor mediated stimulation, via activation of a G_i-coupled receptor (Lundberg *et al.*, 1988). Not surprisingly, prior exposure to threshold concentrations of NPY did not significantly alter the response to 5-CT in the rat sPAs. The majority of blood vessels in which 5-HT_1 mediated smooth muscle contraction has been described require prestimulation, either with another agonist or with a slight increase in the extracellular $[K^+]$, before 5-HT_1 receptor stimulation can elicit a contraction (Movahedi et al., 1997; Randall, *et al.*, 1996). The suggestion has been that prestimulation somehow increase $[Ca^{2+}]_i$ to a 'threshold' value such that contraction to 5-HT_1 receptor activation is facilitated. A23187 mediates its vasoconstrictor effects as a calcium ionophore (Yamashita *et al.*, 1994), however it

was also unable to significantly alter the response to 5-CT. This suggests that simply elevating $[Ca^{2+}]_i$ does not produce synergistic amplification of vasoconstriction, at least in rat PRAs. Although we have demonstrated here that increasing vascular tone and decreasing cGMP levels significantly potentiate the response to 5-CT in rat sPAs, none of these were able to augment the response to the same degree as that observed in sPAs from the chronic hypoxic rat. Other amplification factors such increased expression of $5\text{-}HT_{1B}$ receptors, are likely to be present which can influence the magnitude of the response. Increased mRNA for the $5\text{-}HT_{1B}$ receptor has been recently demonstrated in pulmonary arteries from this rat model of PHT (Heeley, *et al.*, 1998).

The fact that increasing vascular tone and/or decreasing levels of cGMP in the sPAs from chronic hypoxic pulmonary hypertensive rats did not significantly change the sensitivity or maximum contraction to 5-CT provides further evidence that the amplification factors necessary for pharmacological synergism already exist in these vessels and collectively provide an 'optimum' environment for $5\text{-}HT_1$-G_i-protein coupled receptor mediated vasoconstriction. This synergism may play an important role in pulmonary vasoconstriction associated with pulmonary hypertension.

In conclusion, these results show that in rat smPAs, increased vascular tone via either G_q-coupled receptor activation or increased KCl can potentiate G_i-coupled $5\text{-}HT_1$ receptor-induced vasoconstriction. Pulmonary arteries *in vivo* are normally fully dilated. This synergism may, therefore, become pathophysiologically important in pulmonary hypertension which is characterised by elevated pulmonary vascular tone. The observation that decreasing cGMP (via NOS inhibition) can further augment the Gq-Gi interactions in these smPAs strengthens the suggestion that

pharmacological synergism is pathophysiologically important in pulmonary hypertension. A greater understanding of the key signalling events occurring in pulmonary hypertension should provide new therapeutic strategies in treating this disease.

Chapter 7

General Discussion

The results from all the studies have been extensively discussed in the relevant chapters of this thesis. This final chapter will focus on drawing on the findings from the preceding chapters, in order to speculate on the significance of the results and suggest future studies.

The vascular reactivity of rabbit pulmonary arteries (both conduit and resistance vessels) were examined particularly under the important transitional state from foetal to neonatal life and in adulthood. These studies focused on 5-HT receptor mediated responses and the influence of developmental age and endothelial NO on these responses.

Primarily, the results from the studies in chapter 3 and 5 demonstrate that in rabbit pulmonary arteries, especially resistance arteries, both the contractile and vasodilator responses to 5-HT alter significantly with developmental age and that endothelial NO plays a significant role in modulating these responses.

The predominant 5-HT receptor mediating vasoconstriction in the foetal to 7 day old rabbit pulmonary arteries (both conduit and resistance vessels) was the 5-HT_{2A} receptor characterised by the nanomolar affinity of ketanserin against 5-HT. An exception to this was the transitory expression of a small population of contractile 5-$HT_{1B/1D}$ receptors occurring immediately after birth in the neonatal resistance vessels. The reason for this may reflect a pharmacological synergism as the 'uncovered' 5-$HT_{1B/1D}$ receptor mediated response seems to exist at a time when plasma levels of the potent pulmonary vasoconstrictor ET-1 have been shown to be elevated in both human and animal neonates (Endo *et al.*, 1994; Noguchi *et al.*, 1997). As discussed in chapter 6, a pharmacological synergistic action was demonstrated between ET-1 and 5-HT receptor mediated responses in the rat smPRAs. It would be useful in further studies at this age

point to focus on mimicking intracellular changes thought to occur in the neonatal pulmonary vasculature in PPHN with the hypothesis that this may amplify the 5-$HT_{1B/1D}$ receptor mediated vasoconstriction. Earlier studies have reported the transient appearance of the ET_B receptor in neonatal piglet peripheral pulmonary arteries.

Other studies in chapter 5 demonstrated rapid developmental changes in 5-HT receptor mediated relaxation in rabbit PRAs. There was a lack of 5-CT-induced vasodilation in the foetal and 0-24 hour rabbits, however 5-CT-induced vasodilation was present by 4 days old being mediated by an endothelium-dependent 5-$HT_{1B/1D}$ receptor. By 7 days old however this vasodilation was also mediated by the 5-HT_7 receptor. The presence of pulmonary vasodilator mechanisms at these neonatal age points is consistent with previous studies in the piglet where endothelium-dependent vasodilation is most marked 3-10 days after birth (Liu *et al.*, 1992). The presence of pulmonary vasodilator mechanisms at this time may therefore facilitate such changes. 5-HT receptor localisation studies using receptor subtype specific monoclonal antibodies would aid in clarifying the identity and location of these receptors on the pulmonary vasculature. The findings of the studies in chapter 5 are of great importance in gaining an understanding of the effects of 5-HT in the normal adaptation of the pulmonary circulation to extra uterine life and is essential before examining any alterations in pathologic conditions. In speculating the possible influence of 5-HT in PPHN, the potent effects of 5-HT observed in the perinatal pulmonary vasculature suggests that the balance of 5-HT receptor subtypes would determine the overall functional response. To speculate, a lack or significant reduction of endothelial 5-HT vasodilator receptors such as the 5-$HT_{1B/1D}$ receptor and/or similar decrease in arterial smooth muscle

vasodilator 5-HT$_7$ receptors would presumably enhance the vasoconstrictor actions of 5-HT. Similarly, any increase in functional contractility mediated by contractile 5-HT$_{1B}$, 5-HT$_{1D}$ or 5-HT$_{2A}$ receptors, would contribute to the maintenance of abnormally high pulmonary arterial pressure after birth which occurs in the pathological state.

Endothelium-derived NO was observed to play a significant role in modulating the 5-HT receptor mediated responses in the perinatal and adult PRAs (chapters 3 & 5). The comparatively greater role of NO on functional responses in the neonatal vessels is consistent with its putative role in the rapid and progressive reduction of PVR following birth as the system takes on its normal role characterised by low pressure and low resistance.

In contrast to the perinatal PRAs, in adult vessels both 5-HT$_{2A}$ and 54-HT$_{1B/1D}$ receptors contribute towards the observed 5-HT-induced vasoconstrictor response as discussed in chapter 3. A useful study in the future would be to examine the effect of PHT on 5-HT receptor mediated responses in rabbit pulmonary arteries. A previous, *in vivo*, study using the well established rabbit coronary ligation model of PHT secondary to left ventricular dysfunction, demonstrated a marked increase in pulmonary pressor response to i.v. administered 5-HT in 8 week ligated rabbits when compared to control, sham operated animals (Deuchar, *et al.*, 1997).

A further aim of this project was to investigate the possibility that pharmacological synergism could be observed in the smPRAs in the rat given that this phenomenon may contribute to the increased 5-HT$_{1B}$ receptor mediated vasoconstriction observed in the CH rat model of PHT (MacLean *et al.*, 1996; 1999). The results from chapter 6 demonstrated that agonist-induced tone, potentiated vasoconstrictor responses to 5-HT$_1$ receptor stimulation in rat smPRAs and that

altering cyclic nucleotide levels, by decreasing cGMP content led to a further augmentation of the 5-HT_1 receptor mediated response in these vessels. The results from this study strengthen the suggestion that pharmacological synergism is pathophysiologically important in PHT where increased endogenous tone and decreased cGMP levels are known to prevail (MacLean *et al.*, 1996; Sweeney *et al.*, 1995). Immediate future functional studies would be the use of selective 5-HT receptor antagonists, including SB224289, to conclusively identify the 5-HT_1 receptor subtype responsible for the observed augmentation of the 5-CT-induced response in these vessels. Investigation of the key intracellular signalling events occurring during pharmacological synergy in these vessels would be of great importance in helping to provide new therapeutic strategies in treating PHT. An initial investigation could involve the use of routine signalling assays for cyclic nucleotide and phospholipase C signalling in pulmonary arterial homogenates from snap frozen vessels to determine the signalling elements implicated in $G_{i/Go}$ synergy. This technique has been successfully used in examining intracellular messenger changes in these vessels (MacLean *et al.*, 1996; Mullaney *et al.*, 2000).

It is important to compare the responses obtained in animal vessels with those in similar preparations obtained from human tissue. As discussed in chapter 4, investigation of human smPAs demonstrated a similar sensitivity to 5-HT and 5-CT as observed in adult rabbit pulmonary arteries of similar internal diameter. Due to the infrequency of human tissue available, only key studies with 5-HT receptor antagonists could be carried out, again focusing on those compounds examined in the animal vessels. Primarily, the results of this study suggest that 5-HT-induced vasoconstriction is mediated through both 5-HT_{2A} receptors and 5-HT_{1B} receptors and

significantly highlight the importance of the 5-HT_{1B} subtype in mediating this vasoconstriction. These conclusions were indicated by the following observations: 1) equipotency of 5-HT, 5-CT and sumatriptan, confirming the presence of 5-HT_1-like receptors. 2) responses to lower 5-HT concentrations (<0.1μM) and to sumatriptan (1nM-300μM) being resistant to ketanserin inhibition but being significantly inhibited by GR55562, the 5-HT_{1B} and 5-HT_{1D} receptor antagonist. 3) Responses to sumatriptan were inhibited by the selective 5-HT_{1B} receptor antagonist SB224289 but not BRL15572 (5-HT_{1D} receptor antagonist). The results of these functional studies were confirmed by RT-PCR, showing the presence of 5-HT_{2A} and 5-HT_{1B} receptor mRNA transcripts in human pulmonary arteries of similar size (Morecroft *et al.*,1999). Given that the normal plasma concentrations of 5-HT are between 1-2 nM and can rise to 30nM in PHT (Herve, *et al.*, 1990; 1995; Anderson *et al.*, 1987), the results of these functional studies provide evidence that the 5-HT_{1B} receptor plays a significant role in mediating 5-HT-induced vasoconstriction in human small PAs. Essential future studies in the human smPAs would have to include an investigation of the possible existence of 5-HT-receptor mediated vasorelaxations. Although these appear to be absent in human larger, capacitance PAs (Cortijo *et al.*, 1997), the pharmacology of the small pulmonary arteries must be determined separately as explained in chapter 4. Secondly the results from chapter 4 suggests that this receptor may provide an important new therapeutic target in PHT.

The profile of 5-HT receptor mediated responses in human PHT is unknown. Although isolated pulmonary arteries from PHT patients undergoing lung transplantation demonstrate augmented vasoconstrictor responses to 5-HT (Brink *et al.*, 1988), it is not yet clear how significant a role the 5-HT_{1B} receptor may play in human

pulmonary hypertensive states. Of immense interest for future studies, would be to examine 5-HT receptor mediated responses in pulmonary resistance arteries obtained from patients with PHT. These studies would assess any changes in the pulmonary arterial vascular reactivity to 5-HT, and would also characterise the 5-HT receptor populations involved in these responses as well as highlight any possible changes in the 5-HT receptor subtypes present which may occur.

Finally, these studies have demonstrated marked developmental changes in the 5-HT receptors involved in mediating vasoconstriction and vasodilation in rabbit pulmonary arteries. These studies have also highlighted the importance, in particular of the 5-HT_{1B} receptor in the control of pulmonary vascular tone and suggest this receptor may be a pulmonary-selective target for PHT both in the adult and newborn.

Research Summary and Conclusions

Responses to 5-HT, and the receptor subtypes involved were studied in foetal, 0 - 24 h, 4 day, 7 day and adult rabbit pulmonary conduit arteries (PCAs). 5-HT was a potent vasoconstrictor of perinatal and adult rabbit PCAs with a potency order of adult = 7 days = foetus = 4 days > 0-24 hours and investigation of 5-HT-receptor mediated responses showed an agonist potency profile of 5-HT = α-methyl-5-HT > 5-carboxamidotryptamine (5-CT) >> sumatriptan at all ages. This indicates a predominant role of contractile 5-HT_{2A} receptors in these vessels.

In PCAs at all age points, ketanserin (5-HT_{2A} receptor antagonist) displayed near nanomolar affinities against 5-HT in these vessels. GR55562 (5-$HT_{1B/1D}$ receptor antagonist) lacked any inhibitory effect. These findings are consistent with 5-HT contractions mediated via 5-HT_{2A} receptors and suggest no significant maturational alteration in the 5-HT receptor subtype mediating 5-HT-induced vasoconstriction in rabbit PCAs.

The influence of nitric oxide (NO) on 5-HT-receptor mediated contractile responses was examined in PCAs from adult and perinatal rabbit. The NO synthase inhibitor L-NAME potentiated 5-HT responses in vessels from all perinatal rabbits but not in adult vessels. The most pronounced effect was observed in the 4 and 7 day rabbit vessels suggesting 5-HT-induced vasoconstriction is normally attenuated by NO in perinatal rabbit vessels, accompanied by maturational changes in the magnitude of this attenuation.

5-HT-receptor mediated relaxation was examined in pre-contracted PCAs from perinatal and adult rabbits. 5-CT and 5-HT both induced relaxation of adult vessels (pEC_{50} s ~7.6 and ~6.5 respectively). Spiperone, pimozide and risperidone inhibited the 5-CT-induced responses in the adult vessels. These findings indicate that

5-HT receptor induced vasodilation is mediated via 5-HT_7 receptors in adult rabbit PCAs. No evidence for 5-HT-receptor induced vasodilation was observed in the perinatal vessels.

5-HT agonist responses were studied in the perinatal and adult rabbit isolated pulmonary resistance arteries (PRAs), alone and in the presence of L-NAME. The effect of the selective 5-HT receptor antagonists ketanserin and GR55562 on vasoconstrictor responses to 5-HT were studied in the presence of L-NAME. Vasodilator responses to 5-CT were studied in pre-contracted PRAs. 5-HT and α-methyl-5-HT were equipotent in the PRAs at each age. In the perinatal PRAs, sumatriptan and 5-CT produced negligible contractions, but in adult PRAs, 5-CT and sumatriptan were potent agonists with *p*EC_{50}s of 6.05 ± 0.3 and 5.70 ± 0.20 respectively. These findings indicate maturational changes in the 5-HT receptor mediating contractions in these vessels.

L-NAME markedly increased the maximum response to 5-HT in the 0 -24 h, 4 day and 7 day vessels and increased 5-HT potency in the 4-7 day-old and adult rabbit vessels. These findings indicate that endothelial NO markedly attenuates 5-HT-induced responses and provide evidence for diminished NO levels in the pulmonary vasculature in foetal compared to neonatal life where increased NO associated with early neonatal life aids the continued fall in PVR observed following birth.

In perinatal vessels, responses to 5-HT, with L-NAME present, were antagonised by ketanserin (30 nM and 0.1 μM) but not GR55562 (1 μM). A small ketanserin-resistant, GR55562-sensitive component was observed at 0-24 h. In adult vessels, both ketanserin and GR55562 inhibited 5-HT-induced responses. The predominant receptor mediating vasoconstriction is the 5-HT_{2A} receptor in foetal to 7-day old rabbits. In the adult rabbit PRA, both the 5-HT_{2A}

receptor and the 5-$HT_{1B/1D}$ receptor mediate vasoconstriction.

Vasodilator responses to 5-CT were observed in pre-contracted PRAs from 4- and 7-day-old rabbits but not in the foetus, 0-24 h old or adult rabbit vessels. At 4 days the vasodilator response was inhibited both by L-NAME and GR55562. At 7 days the response was only partly blocked by L-NAME and resistant to GR55562. The L-NAME resistant component was antagonised by the 5-HT_7 receptor antagonist spiperone (1 μM). The 5-HT_{1D} or 5-HT_{1B} receptor mediates NO-dependent vasodilation in vessels from rabbits at 4 days of age whilst 5-HT_7 receptors mediate NO-independent vasodilation by 7 days.

Collectively, the results demonstrate marked developmental changes in the 5-HT receptors involved in mediating vasoconstriction and vasodilation in rabbit pulmonary arteries and that endothelium-derived NO plays a significant role in modulating these responses.

The 5-HT receptors mediating vasoconstriction in isolated human small muscular pulmonary arteries (smPAs) was determined. The agonists 5-HT, 5-CT, and sumatriptan all caused contraction and were equipotent (pEC_{50}s were 7.0 ± 0.2, 7.1 ± 0.3 & 6.7 ± 0.1, respectively), suggesting the presence of a 5-HT_1 receptor.

Ketanserin (0.1μM) inhibited 5-HT contractions only at non-physiological/pathological concentrations of 5-HT (>0.1μM) whilst GR55562 (1μM) inhibited 5-HT contractions at all concentrations of 5-HT (estimated pK_B = 7.7 ± 0.2). SB224289 (5-HT_{1B}-selective antagonist, 0.2μM) inhibited sumatriptan-induced contractions (estimated pK_B = 8.4 ± 0.1). Whilst these were unaffected by the 5-HT_{1D}-selective antagonist BRL15572 (0.5μM) suggesting that the 5-HT_{1B} receptor mediates vasoconstriction in these vessels.

These findings suggest that a heterogeneous population of 5-HT_{2A} and 5-HT_{1B} receptors co-exist in human small muscular

pulmonary arteries but the 5-HT_{1B} receptor normally mediates the 5-HT induced vasoconstriction at physiological and pathophysiological concentrations of 5-HT. These results have important implications for the treatment of pulmonary hypertension in which the 5-HT_{1B} receptor may provide a novel and potentially important therapeutic target.

The effect of raised tone and reduced cGMP levels on 5-HT_1 receptor mediated vasoconstriction was studied in isolated PRAs from control and chronic hypoxic pulmonary hypertensive (CH) rats.

The CH rats displayed right ventricular hypertrophy, consistent with the development of severe PHT. Responses to 5-CT and 5-HT were augmented in the isolated PRAs compared to control animals.

Threshold concentrations of endothelin-1 (ET-1) and KCl significantly augmented 5-CT-induced contractions in the PRAs from normal but not from CH animals. L-NAME further augmented the contractile response to 5-CT but only in the presence of ET-1 or KCl-increased tone.

These results suggest that inhibition of NOS combined with KCL- or ET-1-induced vascular tone potentiates responses to 5-CT in rat PRAs in a synergistic fashion and mimics the effects of chronic hypoxic exposure. This synergism may therefore become pathophysiologically important in PHT which is characterised by elevated pulmonary vascular tone, increased Gq-coupled receptor activation and membrane depolarisation.

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