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The Roles of Serotonin, Human Urotensin II and Calcium Sensitivity in the Regulation of Pulmonary Arterial Tone

By

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Abstract

Background

Pulmonary arterial hypertension (PAH) is a rare, progressive fatal disease characterised by persistent vasoconstriction and vascular remodelling of the small pulmonary arteries. 1) The endogenous peptide human urotensin II (hU-II) has recently been found to be a potent vasoconstrictor of the rat main pulmonary artery and the response was reported to be augmented in the chronically hypoxic rat model of PAH. 2) Increased basal Ca^{2+} sensitivity and enhanced contractile responses to 5-HT, have been reported to be augmented in the chronically hypoxic rat model of PAH. Additionally expression of the 5-HT transporter, 5-HTT, has been reported to be enhanced following exposure to chronic hypoxia and its over-expression linked to PAH. These reports are controversial.

Aims

1) To profile the vasoconstrictor and vasodilator responses to hU-II along the C57BL/6×CBA mouse and Wistar rat pulmonary arterial trees (extra/intra-lobar) and determine if the contractile activity of hU-II is augmented in rodent models of PAH. 2) To determine if basal Ca^{2+} sensitivity in the Wistar rat small pulmonary artery is augmented following exposure to chronic hypoxia. 3) To determine the influence of 5-HT and 5-HTT over-expression on Ca^{2+} sensitivity in Wistar rat, and C57BL/6×CBA mouse, small pulmonary arteries respectively.

Results

hU-II caused a potent but low efficacy contractile response in Wistar rat extra-lobar (Main pulmonary artery: pEC_{50}; 8.64 ± 0.17, Emax: 19 ± 4% of the response to 50mM KCl n=9, 1st branch: pEC_{50}; 8.57 ± 0.16, Emax: 9 ± 2%, n=13) and 1st

generation intra-lobar (pEC_{50} 8.54 ± 0.19, Emax: 13 ± 3%, n=14) vessels but had essentially no action on the smaller pulmonary arteries. hU-II failed to dilate any pre-constricted rat vessel tested and the contractile responses to hU-II were not augmented in rat models of PAH. Furthermore, hU-II had no effect on any mouse pulmonary vessel tested. 2) Basal Ca^{2+} sensitivity in β-escin permeabilised, Wistar rat small pulmonary arteries was found to be slightly attenuated (pEC_{50}: Control; 6.33 ± 0.02 vs CH; 6.24 ± 0.02, n=10-11) following exposure to 2weeks chronic hypoxia, consistent with a decrease in Rho Kinase activity/Rho A expression. Neither 5-HT, nor 5-HTT over-expression augmented Ca^{2+} sensitivity.

Conclusion

hU-II can increase pulmonary vascular tone in the normal Wistar rat but the data was not consistent with a role in the pathogenesis of PAH in rodents. Altered Ca^{2+} sensitivity was not found to be responsible for the persistent elevated basal tone of the chronically hypoxic rat model of PAH. Furthermore 5-HT and 5-HTT over-expression did not exert their effects on vasomotor tone through altered Ca^{2+} sensitivity.

Abbreviations

Ach	Acetylcholine
AM	Age matched
αMe 5-HT	α Methyl 5-hydroxytryptamine
ANP	Atrial natiuretic peptide
ANOVA	One way analysis of variance
AP-1	Activating protein-1
APAH	PAH occurring in association with a known risk factor
AVD	Apoptotic volume decrease
A23187	Calcium ionophore
BMP-R2	Bone morphogenetic protein receptor 2
B/T	Basal tone
Ca^{2+}	Calcium
$[Ca^{2+}]$	Calcium concentration
$[Ca^{2+}]_{cyt}$	Cytosolic Ca^{2+} concentration
CaM	Calmodulin
CaMKII	Ca^{2+} /calmodulin-dependant protein kinase II
CaS	Calcium sensitivity
CCE	Capacitive Ca^{2+} entry
CCRC	Cumulative concentration response curve
CH	Chronic hypoxia
Cit	Citalopram hydrobromide
CO	Cardiac output
CP 94253	Selective 5-HT_{1B} receptor agonist
EDHF	Endothelial derived hyper-polarising factor
EDRF	Endothelial derived relaxing factor
*E*m	Membrane potential
Emax	Maximum efficacy
e_{max}	Extra-cellular $[Ca^{2+}]$ at which an unpermeabilised vessel produces maximum force whilst depolarised
ET-1	Endothelin 1
exp	Exposure
Fe_{max}	Force at e_{max}
Fi_{max}	Force at i_{max}
F/O	Fall off (Decline in tone between P/R and T/R)
FPAH	Familial (genetically inherited) PAH
F_{90}	90% of the maximum force
GPCR	G-protein coupled receptor
IP_3	Inositol trisphosphate
IPAH	Idiopathic (unexplained) PAH
HCO_3^-	Bi-carbonate

HPV	Hypoxic pulmonary vasoconstriction
hU-II	Human urotensin II
i_{max}	Intra-cellular Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) at which a permeabilised vessel produces maximum force
MAO	Monoamine oxidase
MAP	Mean arterial pressure
MI	Mock intra-cellular (solution)
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
MRNA	Messenger RNA
NANC	Nonadrenergic, noncholinergic
NO	Nitric oxide
NOS	Nitric oxide synthase
NOS III	Endothelial nitric oxide synthase
NPH	Non-pulmonary hypertensive (patient)
PAP	Pulmonary arterial pressure
PAEC	Pulmonary arterial endothelial cell
PASMC	Pulmonary arterial smooth muscle cell
PDGF	Platelet derived growth factor
PE	Phenylephrine
PGI_2	Prostacyclin
PH	Pulmonary hypertension
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PLC	Phospholipase C
PPH	Primary (idiopathic/essential) pulmonary hypertension
P/R	Phasic response
Pre-pro hU-II mRNA	Precursor hU-II mRNA
P/T	Pre-tone (A stable sub-maximal pre-constriction determined by the pCa of the MI solution (pre-agonist addition tone)
P/V	Permeabilised vessel
PVR	Pulmonary vascular resistance
ROC	Receptor operated channels
ROK	Rho kinase
RV	Right ventricular
RVP	Right ventricular pressure
RV/TV	Right ventricular/total ventricular & septum ratio
SERCA	SR Ca^{2+}-Mg^{2+} ATPase
SMC	Smooth muscle cell
SOC	Store operated channels
SPA	Small pulmonary artery

SPH	Secondary pulmonary hypertension
SR	Sarcoplasmic reticulum
T/C	Time control
TG	Transgenic
TGF-β	Transforming growth factor β
Time to F/O_{90}	Time taken to return to within 10% of new basal tone (i.e. Time taken for 90% fall off)
T/R	Tonic response
TRPC	Canonical transient receptor potential channels
TV	Total ventricular & septum weight
TXA_2	Thromboxane A2
U-II	Urotensin II
UCE	U-II converting enzyme
UT-II	Human urotensin II (hU-II) receptor (GPR14)
U/V	Unpermeabilised vessels
VDCC	Voltage dependant Ca^{2+} channels
VEGF	Vascular endothelial cell growth factor
VIP	Vasoactive intestinal polypeptide
WT	Wild type
Y27632	Rho kinase inhibitor
2APB	2-Aminoethoxydiphenylborate
5-CT	5-Carboxytryptamine
5-HT	5-Hydroxytryptamine (Serotonin)
5-HTT	5-HT transporter

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Chapter 1

General Introduction

The Pulmonary Circulation in Health and Disease

1.1 The pulmonary circulation

All but the simplest multi-cellular animals require a mechanism to distribute oxygen, nutrients and hormones to the respiring tissues and to collect from them the products of metabolism. In vertebrates, this task is the responsibility of the cardiovascular system. The cardiovascular system comprises a muscular pump, the heart, and two continuous systems of tubular vessels, the systemic and pulmonary circulations (Bloom and Fawcett, 1975). Whilst the primary job of the systemic circulation is one of delivery and removal of the blood to and from the respiring tissues, the primary job of the pulmonary circulation is recycling, thus ensuring the correct composition of the blood being delivered (Fig 1.1).

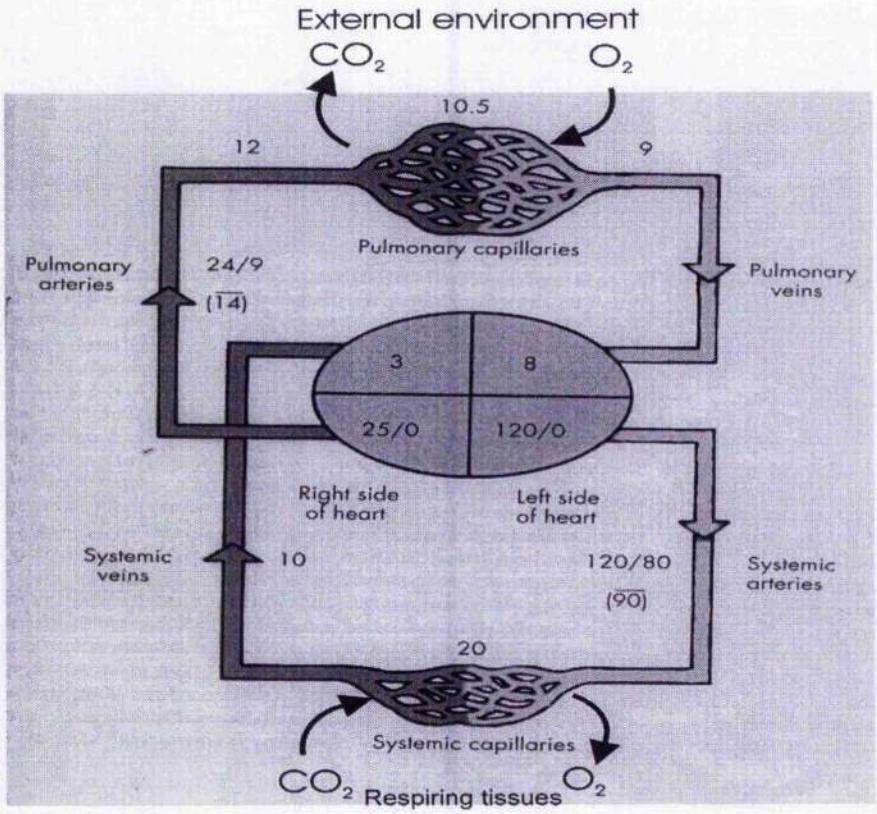


Figure 1.1 Graphic depiction of the relationship between the pulmonary and systemic circulations (Adapted from Berne and Levy, 1998)

Single numbers represent mean vascular and cardiac blood pressures (mmHg). Dual numbers represent systolic/diastolic pressures (mmHg). Oxygen (O_2), carbon dioxide (CO_2).

1.1.1 Structural overview of the pulmonary circulation

The pulmonary circulation encompasses those vessels that conduct blood from the right side of the heart through the lungs, which are the site of gaseous exchange and then return it to the left side of the heart in readiness for delivery to the systemic tissues. It begins with the main pulmonary artery, which bifurcates before entering the left and right lungs. Upon entry into the lungs the pulmonary arteries align themselves adjacent to the airways forming bronchio-vascular bundles, which begin to branch repeatedly creating some 15 orders of pulmonary arteries before ultimately forming vast sheet like, interdigitating networks of capillary beds which are the site of gaseous exchange. The capillaries then unite to form the venules and then the veins, which eventually leave the lungs via four pulmonary veins, which empty into the left atrium (Berne and Levy, 1998;Mandegar *et al.*, 2004).

1.1.1.1 Vessel structure

The structural components of the arteries, capillaries and veins, which make up the pulmonary circulation, are no different to those of the systemic circulation. However in keeping with a low-pressure system the pulmonary arteries have much thinner walls. Adjacent to the vessel lumen, the pulmonary arteries are lined by a single layer of endothelial cells which, are backed by a thin basement membrane. The endothelium is known as the tunica intima and structurally is the same as in systemic arteries. The next layer, is the smooth muscle layer or tunica media, which is bounded by the internal and external elastic laminae. In normal pulmonary arteries the media is substantially thinner than in corresponding systemic arteries, in keeping with a low pressure system. The outer coat, the tunica externa (adventitia), is composed principally of fibroblasts and elastic and collagen fibres and is also less thickly developed than in the systemic arteries (Tortora, 1995) (Fig 1.2).

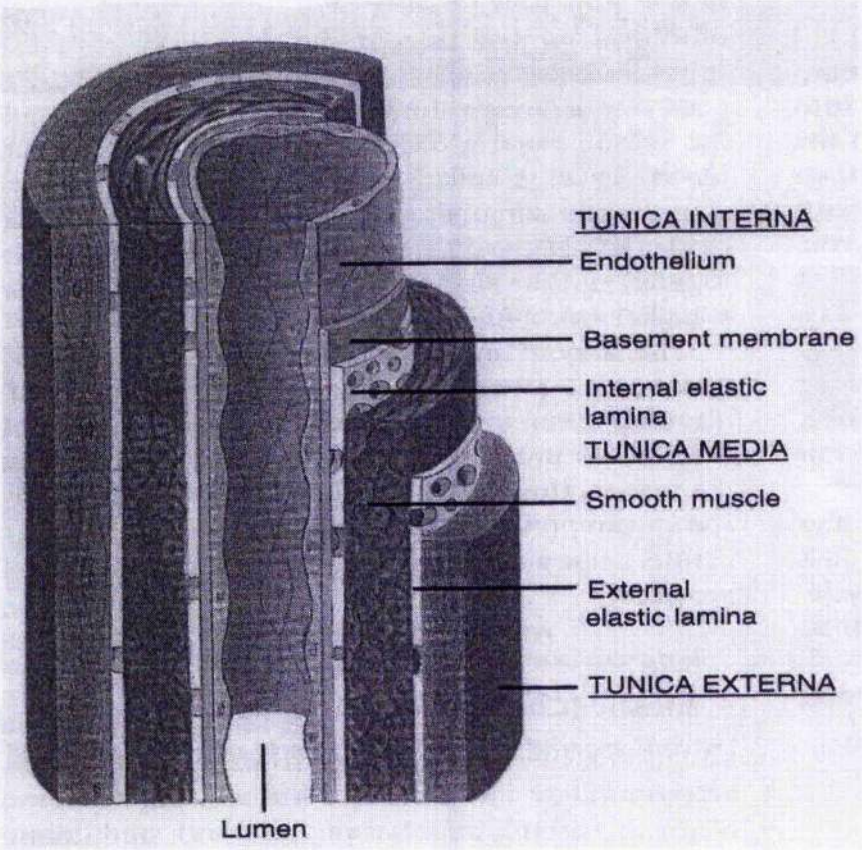


Figure 1.2 The structural components a pulmonary artery

(Reproduced from Tortora, 1995)

The large conducting arteries of the pulmonary system are elastic in nature with well-developed internal and external elastic laminae, which define the borders of the medial smooth muscle layer. Structurally they are similar to systemic conducting arteries but have a less distinct medial smooth muscle layer. Distal to the respiratory bronchioles however, the smooth muscle layer becomes markedly reduced and unlike the noticeably muscular pre-capillary arteries of the systemic circulation, the pulmonary pre-capillary arteries are only partially muscularised or may lack smooth muscle altogether (Meyrick & Reid, 1983).

1.1.2 Physiology of the pulmonary circulation

The primary role of the pulmonary circulation is one of gaseous exchange and because of this, the lungs constitute the only organ in the body that receives the entire cardiac output at all times, following birth. As gaseous exchange is the result of diffusion, the tissues that make up the blood gas interface are required to be very thin and in close opposition. In accordance, the pulmonary circulation is therefore normally a high-flow, low-pressure system. Pulmonary arterial pressure (PAP) is the product of cardiac output (CO) and pulmonary vascular resistance (PVR) (Eq 1). As cardiac output can vary up to five-fold depending on the metabolic requirements of the body, the key to maintaining high flow without a concomitant increase in pressure is maintaining a low PVR (West, 1995).

Eq 1 $$PAP = CO \times PVR$$

Equation 1) The determinants of pulmonary arterial pressure

PAP = pulmonary arterial pressure, CO = cardiac output, PVR = total pulmonary vascular resistance.

As shown in equation 2, resistance is inversely proportional to radius to the power of 4. The internal radius of a vessel is therefore the prime determinant of resistance.

Eq 2 $$PVR = \frac{8L\eta}{\pi r^4}$$

Equation 2) The determinants of pulmonary vascular resistance

PVR = pulmonary vascular resistance, L = vessel length, η = viscosity of the blood, r = internal radius of the vessel. NB) This equation assumes blood to be a Newtonian fluid. Whilst this is a simplification of the true fluid mechanics of blood, the equation remains an adequate descriptor of the relative importance of the components which determine resistance to flow.

The conducting arteries of the pulmonary circulation are highly compliant and have a large internal diameter conferring them with low resistance. However, as the vessels repeatedly branch in order to supply the various regions of the lung with blood, the diameter of the arteries decreases exponentially with each succeeding order of branching. Given this, one may expect resistance to increase dramatically. However the reduction in internal diameter is more than offset by the overall increase in cross-sectional area as the number of arteries (resistors) in parallel increases exponentially. This is further aided by the low basal tone of the vessels, which maximises the internal diameter. As a consequence, in the normal pulmonary circulation PVR is ~$1/10^{th}$ that of the systemic circulation (Mandegar *et al.*, 2004).

1.1.3 Regulation of pulmonary blood flow

1.1.3.1 Passive regulation of pulmonary blood flow

Regulation of the pulmonary vasculature involves both passive and active control (Daly and Hebb, 1966). The main passive factors are those, which change resistance and/or flow independently of changes in vascular tone and include cardiac output, gravity, distension and recruitment. As cardiac output is primarily determined by systemic demand the following discussion will focus on the effects of gravity, distension and recruitment, however, it should be recognised that passive regulation is the integration of all of the aforementioned factors.

Because the lungs are located both above and below the point at which blood leaves the heart, the effects of gravity create a differential hydrostatic pressure which in turn causes an uneven distribution of blood flow. The lung can thus be divided into 3 zones.

Zone 1: At the very apex of the lung, alveolar pressure may exceed PAP causing the capillaries to collapse thus occluding the vessel lumen and increasing resistance; no flow

occurs. This generally only occurs during haemorrhage, as PAP is usually just sufficient to maintain some flow to the top of the lung.

Zone 2: Alveolar pressure has not changed, but PAP now exceeds it, as the blood has less vertical distance to overcome. However alveolar pressure may still exceed venous pressure (e.g. upon exhalation) so flow is determined by the difference between arterial and alveolar pressures. The vessels act as starling resistors and in times of increased flow, previously closed vessels are recruited. The greater the number of resistors in parallel, the smaller the resistance to flow. Hence recruitment of these vessels allows the pulmonary circulation to increase flow with little or no increase in PVR. Similarly, distension of already patent vessels increases their internal radii thus further lowering resistance to flow. This zone comprises the middle and upper regions of the lungs.

Zone 3: At or near the basilar area of the lungs, PAP exceeds pulmonary venous pressure, which constantly exceeds alveolar pressure. Flow is now continuously driven by the arterial venous pressure gradient (West, 1995).

Distension and recruitment are so effective at decreasing PVR that even a five-fold increase in CO causes only a trivial change in PAP so long as a low basal tone is maintained.

1.1.3.2 Active regulation of pulmonary blood flow

Active factors are those which change resistance and/or flow by altering vascular tone through contraction or relaxation of the vascular smooth muscle which makes up the medial layer of the vessel wall. Active regulation of flow can to some extent be achieved by changing compliance of the large conduit vessels. However, by far the more important mechanism is the active regulation of the small pre-capillary arteries. Active regulation is therefore the sum of the influences of 1) the autonomic nervous system, 2) humoral agents, 3) respiratory gas concentrations and 4) the endothelium (which may

influence tone directly or indirectly by modifying the input of the other active factors) (Barnes & Liu, 1995).

Autonomic control

The influence of the autonomic nervous system on pulmonary vascular physiology is not well understood. Although the pulmonary vascular bed is innervated by both sympathetic (adrenergic) and parasympathetic (cholinergic) nerves (Fishman and Hecht, 1969), the distribution of innervation between species and between vessels of different orders of branching is extremely varied (Barnes and Liu, 1995).

Adrenergic control; Generally speaking the extra pulmonary arteries and veins of mammals show a dense network of adrenergic fibres which can elicit both α adrenoceptor (primarily α_1) mediated vasoconstriction and β adrenoceptor (primarily β_2) mediated vasodilatation in response to circulating catecholamines and neurally released norepinephrine (Ingram *et al.*, 1968;Ingram, Jr. *et al.*, 1970;Kadowitz *et al.*, 1973;Kadowitz & Hyman, 1973;Boe & Simonsson, 1980;Hyman *et al.*, 1981;Hyman *et al.*, 1986;Docherty, 1988;Hyman *et al.*, 1990;Hussain & Marshall, 1997). However intrapulmonary sympathetic innervation is highly variable, being almost absent in the rat and mouse but fairly intensive in man (Cech, 1969;Kai, 1969;Bradley *et al.*, 1970;Neild *et al.*, 1986). Overall, sympathetic nerve stimulation seems to cause an increase in PVR and a decrease in compliance resulting in an increased PAP. It is thought that sympathetic output may create a small degree of basal tone under normal physiological conditions, (Pace, 1971;Piene, 1976;Nishiwaki *et al.*, 1993), although this is far lower than in the systemic circulation because vasodilators usually have little or no effect on PAP in patients with normal lung function (Barnes & Liu, 1995).

Cholinergic control; Cholinergic innervation of the pulmonary vasculature is also highly species and regionally specific but in general seems to be less dense than adrenergic

innervation. In the rat and mouse, cholinergic innervation is limited to the extra-pulmonary vessels and in humans it is thought to be entirely absent (Cech, 1973;Bradley *et al.*, 1970;Partanen *et al.*, 1982). Cholinergic nerves do not seem to be important in the maintenance of low basal tone, because even where present their blockade does not result in increased PAP (Murray *et al.*, 1986). However the pulmonary vasculature of many mammals including the mouse, rat and humans is responsive to acetylcholine (Ach). The response to Ach in mammals can depend on the level of pre-existing tone; vasoconstriction occurring if no tone was previously present and vasodilatation if pre-existing tone was present (Boc *et al.*, 1980;Fritts, Jr. *et al.*, 1958;McLean, 1986;Cherry & Gillis, 1987;Hyman & Kadowitz, 1988;Hyman & Kadowitz, 1989). Responses to exogenous or vagally released Ach are mediated through muscarinic receptors located both on the smooth muscle and on endothelial cells, although the subtypes are still unclear. Those mediating dilatation seem to be located on the endothelial cells whilst those mediating vasoconstriction are located on the smooth muscle cells (SMCs) (Greenberg *et al.*, 1987;Norel *et al.*, 1996;McMahon *et al.*, 1992). This knowledge is utilised by investigators to confirm the presence of a functional endothelium in isolated vessel preparations (i.e. pre-constricted vessels with a functional endothelium relax to Ach).

Nonadrenergic, noncholinergic (NANC) control; In addition to classical adrenergic and cholinergic innervation there also exist neural control mechanisms that are not inhibited by adrenergic or cholinergic blockade. These are known as nonadrenergic, noncholinergic (NANC) nerves (Barnes *et al.*, 1991;Barnes & Liu, 1995). Both vasoconstrictor (excitatory-eNANC) and vasodilator (inhibitory-iNANC) responses have been attributed to NANC nerves in mammalian pulmonary arteries although their physiological significance remains speculative (Inoue & Kannan, 1988;Maggi *et al.*, 1990;Liu *et al.*, 1992a). In the rat pulmonary artery eNANC vasoconstriction has been

reported to be mediated by ATP whilst in the guinea pig pulmonary artery both nitric oxide (NO) and ATP have been reported to mediate iNANC vasodilatation (Inoue & Kannan, 1988;Liu *et al.*, 1992a;Liu *et al.*, 1992b).

Humoral control

The pulmonary vasculature is continuously exposed to a vast array of humoral and autacoid factors and it has been proposed that the maintenance of low basal tone is the result of the balance of vasoconstrictors and vasodilators being tipped in favour of the dilators (Robin, 1982). In pulmonary arterial hypertension it is thought that this balance is upset in favour of the vasoconstrictors, resulting in sustained vasoconstriction and vascular remodelling.

Substances known to induce only vasoconstriction include angiotensin II (Boe & Simonsson, 1981), and a number of protanoids such as thromboxane A_2 (TXA_2) (Buzzard *et al.*, 1993), Whilst substances known to cause only vasodilatation include the prostanoid, prostacyclin (PGI_2) (Hyman & Kadowitz, 1979), atrial natiuretic peptide (ANP) (Lindberg & Andersson, 1988) and vasoactive intestinal polypeptide (VIP) (Nandiwada *et al.*, 1985). However many substances can cause both vasodilatation and vasoconstriction the net response being dependent on the pre-existing level of tone. These substances include; endothelin 1 (ET-1) (McKay *et al.*, 1991) serotonin (5-HT) (Neely *et al.*, 1993), ATP (Liu *et al.*, 1989) bradykinnin (Pang *et al.*, 1982;Lippton *et al.*, 1984) and histamine (Okpaka, 1972;Abacioglu *et al.*, 1987). In some cases substances may even act synergistically to cause substantial elevation in tone (e.g. TXA_2 and 5-HT (MacLean, 1999). However the relative importance of each of these factors seems to vary between species and anatomical location within the pulmonary vasculature and therefore the relative contributions of these agents to normal pulmonary vascular tone are still being elucidated.

Control by the respiratory gases

Given that the primary function of the pulmonary circulation is gaseous exchange, it is not surprising that the respiratory gases actively influence pulmonary vascular tone. Both hypoxia and hypercapnia induce vasoconstriction of the pulmonary circulation (Fishman, 1961). However, whilst increased mixed venous CO_2 is the predominant stimulus for the hypercapnic response, it is alveolar hypoxia that is the main stimulus for the hypoxic response, with mixed venous partial pressure of O_2 (P_{O2}) being only a contributory factor (Duke, 1954;Marshall & Marshall, 1983).

Hypoxic pulmonary vasoconstriction; Hypoxic pulmonary vasoconstriction (HPV) is perhaps the most powerful regulatory mechanism of the pulmonary circulation. Unique to pulmonary vessels, HPV is a physiological response that causes vasoconstriction of poorly ventilated regions of the lung thus redirecting blood flow to the better ventilated regions resulting in an optimisation of ventilation/perfusion matching (Leach & Treacher, 1995). HPV primarily occurs in the small pre-capillary arterioles of the hypoxic region particularly when alveolar P_{O2} drops below 70mmHg (Von Euler & Liljestrand, 1947;Kato & Staub, 1966;Nagasaka *et al.*, 1984). The HPV response incorporates the acute hypoxic vasoconstrictor response and a chronic vascular remodelling phase associated with prolonged alveolar hypoxia (Leach & Treacher, 1995). However, despite intensive investigation, the identity of the oxygen sensor remains elusive. It has been shown that the acute response is not dependent on the central nervous system, as the response is still present in the isolated lung and even in excised segments of pulmonary artery (Hauge, 1968a;Dawson *et al.*, 1979;Madden *et al.*, 1985). Two main hypotheses for the acute intrinsic mechanisms have been proposed. 1) The mediator hypothesis suggests that endogenous vasoactive factors are released or suppressed in response to hypoxia. However, despite extensive investigations no such factors have been identified as being essential to HPV (Hauge, 1968a;Hauge & Melmon, 1968;Hauge,

1968b;Fishman, 1976;Roos *et al.*, 1976;Tucker *et al.*, 1976;Weir *et al.*, 1976a;Weir *et al.*, 1976b). Furthermore it has been reported that the acute response is endothelium independent making it more likely to be an intrinsic property of pulmonary arterial smooth muscle cells (PASMCs) (Yuan *et al.*, 1990) 2) The second hypothesis is that hypoxia has a direct effect on the pulmonary vascular smooth muscle cells. Whilst cultured PAMSCs have been shown to contract to hypoxia (Murray *et al.*, 1990), the molecular mechanism, which remains to be identified would have to account for the diametrically opposed responses of pulmonary and systemic vessels (which dilate to hypoxia). Furthermore, whilst the initiation of the acute HPV response may be endothelium independent some investigators have reported a need for an intact endothelium for the response to be maintained for more than 20minutes (see- *The endothelium and HPV,* for explanation and refs). Hence it is not inconceivable that both hypotheses have merit, that is an endothelium independent acute response, which triggers a sub-acute endothelium dependant/facilitated response (Aaronson *et al.*, 2002).

If the hypoxic challenge is sustained (i.e. chronic hypoxia) HPV results in vascular remodelling (See 1.2 Pulmonary arterial hypertension) which leads to a sustained elevation of PAP and the development of secondary pulmonary hypertension. However, the relationship between chronic hypoxia (CH) and HPV are less than clear cut as some investigators report attenuated HPV and agonist induced vasoconstrictor responses following chronic hypoxia (McMurtry *et al.*, 1980;Hill & Ou, 1984) whilst others report increased HPV and agonist induced vasoconstrictor responses (Emery *et al.*, 1981;Park *et al.*, 1977). Chronic hypoxia also alters the balance between endogenous vasoconstrictors and vasodilators and may also increase vascular reactivity (Shimoda *et al.*, 2000). Although the signalling pathways that mediate the response to chronic hypoxia may be somewhat more varied than the those that mediate the acute HPV response,

fundamentally they are both mediated by an elevation of cytosolic free Ca^{2+} (See 1.1.4 Smooth muscle contraction).

Endothelial control

Furchgott and Zawadski (1980) brought to light the importance of the endothelium in the regulation of vascular tone, when they demonstrated that Ach induced relaxation was an endothelium dependant phenomenon. The nonprostanoid substance responsible, subsequently termed endothelium derived relaxing factor (EDRF) (Cherry *et al.*, 1982), was found to mediate smooth muscle relaxation in a wide range of arterial and venous preparations in response to a diverse range of physiological and pharmacological stimuli (Furchgott & Zawadzki, 1980;Furchgott *et al.*, 1984;Furchgott & Vanhoutte, 1989). EDRF is now accepted to be nitric oxide (NO) (Moncada *et al.*, 1991;Nathan, 1992) although a second EDRF known as endothelium derived hyperpolarising factor (EDHF) has also been proposed (Komori & Vanhoutte, 1990). In addition to NO, the prostanoid, prostacyclin (PGI_2) is also synthesised and released by the endothelium and plays an important role in the endothelium mediated vasodilator response to increased shear stress (Frangos *et al.*, 1985).

The endothelium and pulmonary tone; A large number of humoral factors and autacoids have been shown to induce pulmonary vasodilatation through endothelium mediated NO release. Some of these substances have already been introduced in the nervous and humoral control sections and include; norepinephrine (Liu *et al.*, 1991), Ach (McMahon *et al.*, 1992;McMahon *et al.*, 1991), ATP (Liu *et al.*, 1992a), bradykinin (Ignarro *et al.*, 1987), histamine (Szarek *et al.*, 1992), ET-1 (Namiki *et al.*, 1992) and 5-HT (Glusa & Richter, 1993). In addition, the endothelial cells also generate, metabolise and degrade many of these vasoactive substances, some of which can also mediate vasoconstriction. Hence, the endothelium may play a pivotal role in the maintenance of low basal tone

through its modulation and mediation of the many nervous, humoral and mechanical influences.

The relative importance of NO and PGI_2 with regards to maintaining pulmonary basal tone shows variation from species to species but in the rat, rabbit, cat, guinea pig, and sheep, NO appears to be more important than PGI_2, as inhibition of NO under normal or hypertensive conditions results in an increase in PAP and PVR, which does not occur with the block of PGI_2 (Oka *et al.*, 1993;Davidson & Eldemerdash, 1991;Persson *et al.*, 1990;Fineman *et al.*, 1991). However this situation is completely reversed in the dog with PGI_2 inhibition causing increased PVR but NO inhibition having no effect (Barnard *et al.*, 1993). In humans the degree to which NO regulates pulmonary vascular tone under basal conditions is still being debated (Celermajer *et al.*, 1994;Stamler *et al.*, 1994;Hampl, 2000), but NO production has been shown to be augmented in times of elevated PAP or PVR and is thought to provide a tonic antagonism until the point at which endothelial damage or pathological changes limit its effectiveness (Seghaye *et al.*, 1997;Takaya *et al.*, 1998;Hampl, 2000).

The endothelium and HPV; The role of the endothelium in hypoxic pulmonary vasoconstriction (HPV) is controversial. HPV has been demonstrated in both pulmonary arteries where the endothelium has been removed and isolated PASMCs, suggesting it is an endothelium independent mechanism (Harder *et al.*, 1985;Yuan *et al.*, 1990;Marshall & Marshall, 1992;Wang *et al.*, 1995;Gelband & Gelband, 1997). However a number of investigators have also shown either partial (Rodman *et al.*, 1989;Zhang & Morice, 1994;Dipp *et al.*, 2001) or complete dependence on an intact endothelium for sustained HPV of more than 20minutes duration (Holden & McCall, 1984;Demiryurek *et al.*, 1991;Kovitz *et al.*, 1993;Leach *et al.*, 1994;Leach *et al.*, 2001). It has been suggested that the discrepancy for these findings may be that acute HPV consists of 2 phases; an

initial transient, endothelium independent vasoconstriction (phase 1) superimposed on a more slowly developing but sustained (sub acute) endothelium dependent/facilitated vasoconstriction (phase 2) (Kovitz *et al.*, 1993;Leach *et al.*, 1994;Zhang & Morice, 1994;Dipp *et al.*, 2001).

Active regulation favours low pulmonary tone in the normal lung

Whilst much still remains to be understood about the relative roles and interplay of the active mechanisms which influence pulmonary vascular tone, it is widely recognised that in the normal pulmonary circulation the balance of these influences is such that the arterial basal tone and hence PVR is very low. In disease states such as pulmonary hypertension however it is thought that the balance of the active regulatory mechanisms is disturbed leading to an elevation of basal tone (vasoconstriction) and vascular remodelling. The combination of these two factors causes an increase in PVR, which in turn requires an elevation in PAP to maintain the required blood flow through the lung. A key regulator in both vasoconstriction and the cellular proliferation of vascular remodelling, is cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_{cyt}$) (Mandegar *et al.*, 2004).

1.1.4 Smooth muscle contraction and pulmonary arterial smooth muscle cells

1.1.4.1 Cytoplasmic Ca^{2+} ($[Ca^{2+}]_{cyt}$) regulation

The low basal tone of PASMCs is primarily a function of low ($[Ca^{2+}]_{cyt}$). The appropriate $[Ca^{2+}]_{cyt}$ is maintained by the relative activities of those events which cause an increase in $[Ca^{2+}]_{cyt}$ (i.e. trans-sarcolemmal influx of Ca^{2+} and/or the mobilisation of Ca^{2+} from the sarcoplasmic reticulum (SR)) and those which reduce $[Ca^{2+}]_{cyt}$ (i.e. extrusion of Ca^{2+} through the plasma membrane Ca^{2+}-Mg^{2+}ATPase pump, and sequestration of Ca^{2+} into the SR by its Ca^{2+}-Mg^{2+} ATPase pumps (SERCA)) (Berridge, 1993;Blaustein, 1993;Blaustein & Lederer, 1999).

1.1.4.2 Elevation of $[Ca^{2+}]_{cyt}$; the signal for contraction

Ca^{2+} influx through the membrane can involve up to 3 types of channel depending on the stimulus. 1) Voltage dependant channels (VDCC) which are regulated by membrane potential (*E*m), 2) receptor operated channels (ROC) which are activated by the interaction of agonists and membrane receptors, and 3) store operated channels (SOC) which are activated by depletion of Ca^{2+} from the intra-cellular stores (SR) (Nelson *et al.*, 1990;Parekh & Penner, 1997).

The SR is the predominant intra-cellular Ca^{2+} store, and can be functionally divided into two pools based on the Ca^{2+} release caused by inositol trisphosphate (IP_3) or caffeine (or sensitivity to ryanodine). Both mechanisms have been identified in vascular smooth muscle cells (SMCs) (Berridge, 1993;Gurney & Allam, 1995). Caffeine activated (ryanodine sensitive) stores are involved in Ca^{2+}-induced Ca^{2+} release, whilst IP_3 SR store release is triggered by the interaction of agonists and membrane receptors although this response can be modulated by a change in *E*m (Ganitkevich & Isenberg, 1993).

1.1.4.3 Smooth muscle contraction; the mechanism

Smooth muscle contraction is directly triggered by a rise in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) which occurs in response to stimuli such as membrane depolarisation or the binding of an agonist to cell surface receptors (Somlyo & Himpens, 1989). Calmodulin (CaM), an intra-cellular Ca^{2+}-binding protein, binds free Ca^{2+} as $[Ca^{2+}]_{cyt}$ rises. The Ca^{2+}/CaM complex then activates myosin light chain kinase (MLCK), which, in turn, phosphorylates the myosin light chain (MLC). The phosphorylated MLC stimulates the activity of myosin ATPase, hydrolysing ATP to release energy for the subsequent cycling of the myosin cross-bridges with the actin filament. The formation of these cross-bridges underlies smooth muscle cell contraction, prompting vasoconstriction (Somlyo & Somlyo, 1994) (Fig 1.3). Conversely, relaxation is achieved by those

mechanisms that remove Ca^{2+} from the cytosol; extrusion of Ca^{2+} through the plasma membrane Ca^{2+}-Mg^{2+}ATPase pump, and sequestration of Ca^{2+} into the SR by its Ca^{2+}-Mg^{2+} ATPase pumps (SERCA) (Berridge, 1993; Blaustein, 1993).

1.1.4.4 Smooth muscle contraction and Ca^{2+} sensitisation

Although elevation of $[Ca^{2+}]_{cyt}$ triggers contraction and hence vasoconstriction, the actual force produced is dependent on 2 factors. 1) The $[Ca^{2+}]_{cyt}$ and 2) the Ca^{2+} sensitivity (CaS) of the contractile apparatus. These two factors determine the level of phosphorylation of the MLC, which is the key event regulating the resulting level of contraction (Kamm & Stull, 1985). Under some circumstances thin filament regulation, which is independent of MLC phosphorylation may also modify the final response, but less is known about the physiological relevance of this mechanism (Arner & Pfitzer, 1999). Phosphorylation of the MLC is mediated by myosin light chain kinase (MLCK) whilst dephosphorylation is mediated by myosin light chain phosphatase (MLCP) and both are targets for intra-cellular signalling cascades. It is the influence of these signalling cascades on the relative activities of MLCK and MLCP that modulate force independent of $[Ca^{2+}]_{cyt}$ (Pfitzer, 2001;Somlyo & Somlyo, 2003).

Signalling cascades that increase Ca^{2+} sensitivity (CaS)

Of the several agonists which have now been found to increase CaS in a variety of smooth muscle preparations, most seem to do so by G-protein mediated inhibition of MLCP. It was originally thought that inhibition of MLCP occurred via phosphorylation of its regulatory myosin-binding subunit (Kimura *et al.*, 1996;Feng *et al.*, 1999). However recent reports are conflicting, with both an increase and no change in phosphorylation of the regulatory myosin-binding subunit at the Thr695 residue being reported following agonist stimulation which resulted in MLCP inhibition (Shin *et al.*, 2002;Niiro *et al.*, 2003).

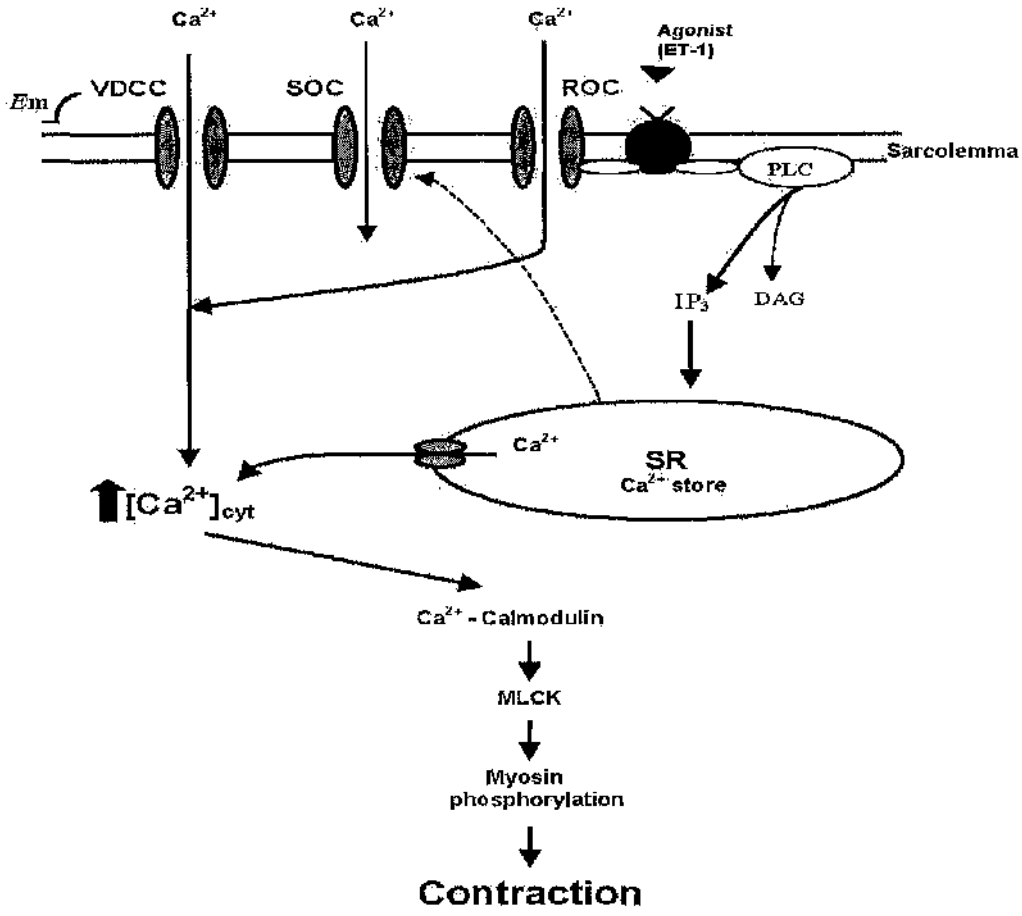


Figure 1.3 Smooth muscle contraction

The mechanisms underlying vasoconstriction. The relative contribution of each route of Ca^{2+} entry varies depending on the stimulus involved. Voltage dependant channels (VDCC) are regulated by membrane potential (*Em*), whilst receptor operated channels (ROC) and phospholipase C (PLC) are activated by the interaction of agonists and membrane receptors. Inositol trisphosphate (IP_3) and diacyiglycerol (DAG) are produced from phosphatidyl inositol bisphosphate during PLC activation. IP_3 is the intra-cellular signal for sarcoplasmic reticulum (SR) Ca^{2+} store release. Store operated channels (SOC) are activated by depletion of Ca^{2+} from the SR. Elevated cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) increases the activity of myosin light chain kinase (MLCP) via the Ca^{2+}/calmodulin complex, resulting in the phosphorylation of the myosin light chain and contraction.

A second, indirect mechanism has also been identified, with inactivation of MLCP occuring following phosphorylation of protein kinase C-potentiated inhibitory protein

(CPI-17). CPI-17 is a smooth muscle-specific protein inhibitor of MLCP (Eto *et al.*, 2001). However, the exact details of how CPI-17 phosphorylation inhibits MCLP remain to be elucidated. Presently, there are two well described G-protein mediated, MLCP inhibitory pathways and recent evidence suggests they may both be influenced by complex signalling cascades and may invole convergence.

The Rho kinase pathway; The monomeric Rho GTPase has been found to activate Rho kinase (ROK)(Matsui *et al.*, 1996) which causes phosphorylation of the regulatory sub unit of MLCP resulting in its inactivation (Kimura *et al.*, 1996;Feng *et al.*, 1999). ROK activation may also indirectly inactivate MLCP via CPI-17 phoshorylation (Eto *et al.*, 2001). It has since been shown using the specific ROK inhibitor Y27632 that the tonic component of the contraction caused by agonists, which utilise the ROK pathway could be inhibited, but that the initial phasic response remained (Uehata et al., 1997;Fu et al., 1998;Yoshii et al., 1999;Sward et al., 2000). Thus, some agonists may increase SMC tone by both triggering Ca^{2-} influx and by increasing CaS through ROK. However, it has also been reported that the concentration (10μM) of Y27632 used by most investigators for inibition of ROK, may also partially inhibit atypical protein kinase C (Eto *et al.*, 2001) and therefore its action may not be entirely specific for ROK.

The ROK pathway has also recently been implicated in both phase 2 of HPV (Wang *et al.*, 2001;Wang *et al.*, 2003;Ward *et al.*, 2004) and the development of pulmonary hypertension occuring secondary to chronic hypoxia, although its role in the latter is less clear (Sauzeau *et al.*, 2003a;Nagaoka *et al.*, 2004b).

The protein kinase C pathway; The second major pathway that has been proposed is the phosphorylation of CPI-17, a smooth muscle-specific protein inhibitor of MLCP by protein kinase C (PKC). It has been shown that the PKCs involved are atypical with conventional PKCs playing only a transient, small role in CaS (Gailly *et al.*, 1997;Iizuka

et al., 1999;Eto *et al.*, 2001). It has been suggested that either a convergence of, or cross-talk between the PKC pathway and the ROK pathway exists, because CPI-17 phosphorylation can be inhibited by both PKC inhibitors and the specific ROK inhibitor Y 27632 (Kitazawa et al., 2000). However the specificity of Y27632 has been questioned at the concentration (10μM) used by Kitazawa (Eto *et al.*, 2001). Interestingly, several $G\alpha_q$-coupled receptors (such as ET-1 and purinergic receptors) are known to activate both ROK and PKC (Somlyo & Somlyo, 2003).

Signalling cascades that decrease Ca^{2+} sensitivity (CaS)

Both cAMP and cGMP have been shown to decrease CaS in permeabilised and intact preparations (Kerrick & Hoar, 1981;Morgan & Morgan, 1984;Karaki *et al.*, 1988;Van Riper *et al.*, 1995;Seguchi *et al.*, 1996;Gailly *et al.*, 1997).

***cAMP desensitisation*;** Protein kinase A (PKA) has been shown to phosphorylate 2 sites on MLCK, although only one of these phosphorylated sites (site A) was associated with decreased CaS (Conti & Adelstein, 1981). However, agents that elevate cAMP *in vivo* do not seem to have significant effect at this site so it is possible that cAMP causes decreased CaS through some, as yet undefined mechanism, although antagonism of ROK has been proposed (Miller *et al.*, 1983;Stull *et al.*, 1990;Somlyo & Somlyo, 2003). As several effects of cAMP are mediated by cross activation of protein kinase G (PKG) it is also possible that PKG may be involved in the cAMP associated Ca^{2+} desensitisation.

cGMP desensitisation; PKG which is activated by cGMP, has no effect on MLCK activity but has been shown to activate MLCP. The mechanism of cGMP/PKG action is still speculative but there is evidence that it can both increase activity of MLCP and independently antagonise ROK (Surks *et al.*, 1999;Sauzeau *et al.*, 2000a). ROK antagonism has been reported to occur following increased endothelial nitric oxide synthase (NOS III) expression and resulted in reduced contraction to 5-HT and PE in the

mouse aorta (Budzyn *et al.*, 2004). It has also been reported that the NO stimulated cGMP/PKG desensitisation pathway, rather than reduced $[Ca^{2-}]_{cyt}$, is responsible for vasodilatation in resistance arteries and that this pathway is antagonised by ROK (Bolz *et al.*, 2003). Hence the findings suggest that both the NO/cGMP and ROK pathways are key players in the regulation of vascular tone and that the CaS of the contractile apparatus may primarily be the net result of these two opposing pathways.

Ca^{2+} /calmodulin-dependant protein kinase II; It has been reported that some agents which induce a substantial increase of $[Ca^{2+}]_{cyt}$ also cause a significant phosphorylation of site A on MLCK thus causing a decrease in CaS (Stull *et al.*, 1990). Inhibition of Ca^{2+} /calmodulin-dependant protein kinase II (CaMK II) has been reported to abolish this phosphorylation of site A leading to the suggestion that CaMK II might mediate a negative feedback mechanism which protects from high levels of MLC phosphorylation (Tansey *et al.*, 1994;Stull *et al.*, 1990) (Fig 1.4).

1.1.4.5 HPV involves both elevated $[Ca^{2+}]_{cyt}$ and, Ca^{2+} sensitisation

The acute HPV response to hypoxia is characterised by a bi-phasic contraction consisting of a rapid initial phasic response (lasting ~ up to 20minutes) followed by a lesser but sustained sub acute tonic response, which is maintained until the hypoxic challenge is ceased (Bennie *et al.*, 1991;Jin *et al.*, 1992;Leach *et al.*, 1994). It is now known that the phasic response coincides with an elevation in $[Ca^{2+}]_{cyt}$ but that the tonic response shows a dissociation between force and $[Ca^{2+}]_{cyt}$ (Robertson *et al.*, 1995). This dissociation is believed to be due to a change in the CaS of the contractile apparatus. Several signalling pathways have been proposed to mediate the CaS response of phase 2 of HPV, including PKC and ROK (Robertson *et al.*, 1995;Robertson *et al.*, 2001;Wang *et al.*, 2001;Wang *et al.*, 2003;Fagan *et al.*, 2004;Ward *et al.*, 2004), however it has been shown the response can occur in the presence of PKC block (Robertson *et al.*, 2001). It now thought that the

ROK pathway is primarily responsible for mediating the sub-acute response (phase 2) of HPV (Wang *et al.*, 2001;Wang *et al.*, 2003) and that ROK is activated by some endothelial derived factor (Aaronson *et al.*, 2002;Dipp *et al.*, 2001). If the hypoxic challenge is sustained (i.e. chronic hypoxia), HPV persists, and in conjunction with a series of other influences results in the development of secondary pulmonary hypertension (SPH). PH secondary to chronic hypoxia is a widely used model for the study of pulmonary arterial hypertension (PAH) (see chapter 5).

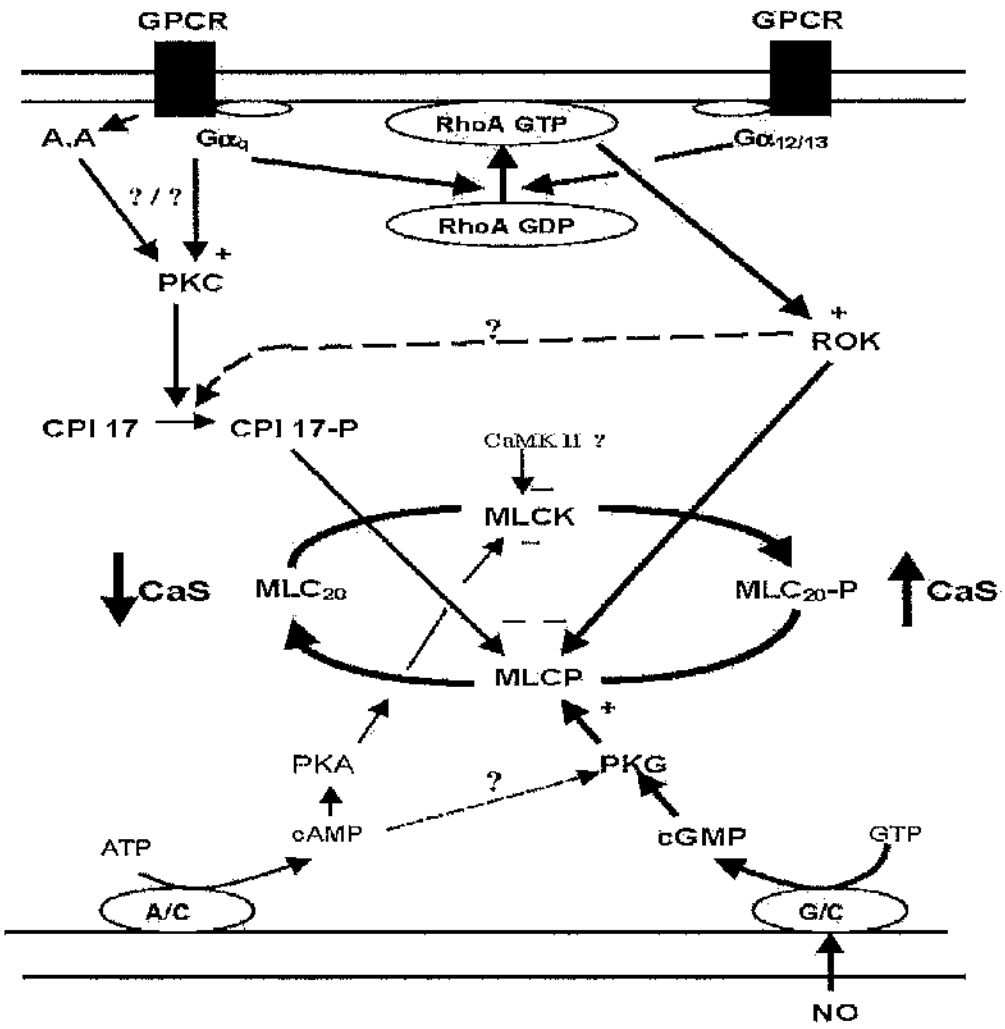


Figure 1.4 The proposed pathways of smooth muscle Ca^{2+} sensitisation/desensitisation

The Rho kinase (ROK) pathway is activated by the small RhoA GTPase whose activity is mediated by both Gαq and $G\alpha_{12/13}$ G-protein coupled receptors (GPCR). ROK increases Ca^{2+} sensitivity (CaS) by inhibition of myosin light chain phosphatase (MLCP), resulting in increased phosphorylation of the myosin light chain (MLC_{20}). ROK may also promote CPI-17 phosphorylation, which can also result in the inhibition of MLCP. CPI-17 has been shown to be activated by the protein kinase C (PKC) pathway, although the PKCs involved are thought to be atypical and may be the product of arachidonic acid (A.A) metabolism. Nitric oxide (NO) causes a decrease in CaS via gaunyiyi cyclase (G/C) production of cyclic GMP (cGMP). cGMP augments protein kinase G activity which activates MLCP thus causing dephosphorylation of MLC_{20}. Cyclic AMP (cAMP) and Ca^{2+}/calmodulin dependent kinase (CaMK II) have both been implicated in decreased CaS through their inhibition of MLCK. + Stimulation, - Inhibition, ? Possible pathway convergence, ?/? Relative contribution to pathway stimulation requires further clarification.

1.2 Pulmonary hypertension

Pulmonary hypertension (PH) is defined as a sustained elevation in pulmonary arterial pressure (PAP) above 25mmHg at rest and 30mmHg during exercise (Rich *et al.*, 1987). However, this is a general term, which can be applied to over 30 conditions, many of which have different aetiologies. Until the 1998 WHO sponsored World symposium on primary pulmonary hypertension, one of the fundamental problems in tackling what is in effect a range of complex and multi-factorial diseases, was the classification of its various forms.

1.2.1 Classification of pulmonary arterial hypertension (PAH)

Prior to the 1998 WHO classification, PH was described either as Primary (idiopathic/essential) pulmonary hypertension (PPH) or Secondary pulmonary hypertension. PPH was diagnosed when the disease occurred spontaneously without any known insult, whilst secondary PH was used as a blanket term to describe PH occurring secondary to or in association with, some other known condition. Unfortunately these generalisations grouped together patients who may have had little similarity in the development of their PH whilst also separating others who may have been found to have a shared aetiology. The revisions of 1998 and then 2003 have since produced a classification system, which reflects advances in the understanding of pulmonary hypertensive diseases and recognises those varieties of the condition, which demonstrate similarities in their aetiologies. The diseases are thus defined into 5 main categories based upon their common clinical features;

1) Pulmonary arterial hypertension (PAH)

2) Pulmonary hypertension with left heart disease

3) Pulmonary hypertension associated with lung diseases and/or hypoxaemia

4) Pulmonary hypertension due to chronic thrombotic and/or embolic disease

5) Miscellaneous

In essence however, PH is physiologically divided into two broad categories: 1) Pulmonary arterial hypertension (PAH) encompasses those intrinsic diseases of the pulmonary vascular smooth muscle and endothelial cells which, due to abnormalities at cellular and molecular levels result in an obliteration of the vessels lumen (Mandegar *et al.*, 2004), 2) Secondary pulmonary hypertension (SPH) now covers all those conditions in which pulmonary hypertension occurs due to a physiological abnormality that arose independently and in the absence of any intrinsic pulmonary vascular disease (Mandegar *et al.*, 2004).

In our laboratory we are primarily concerned with PAH. This group of clinically and pathologically associated conditions includes those not related to any known previous insult, idiopathic PAH (IPAH), cases with a known genetic /familial history (FPAH) and those in which PAH occurs in association with, or secondary to, some additional known risk factor (APAH). Severe PAH has been associated with collagen vascular disease, congenital systemic to pulmonary shunts, portal hypertension, HIV, drug (anorexigen & amphetamines) and toxin exposure and a number of other conditions (Rich *et al.*, 1987).

The new classification system should help identify common aspects of the disease, which may provide clues to its aetiology. For example, up to 40% of patients with the CREST variant of systemic sclerosis (grouped in the collagen vascular disease bracket) will die of PAH (Lee *et al.*, 1992). Interestingly ~ 10% of IPAH and FPAH patients also report symptoms of Raynauds phenomenon (collagen vascular disease). This highlights the benefits of the new classification system and simplifies identifying possible avenues of investigation, as it is becoming apparent that IPAH and FPAH lie at the far end of a spectrum of diseases which cause PAH (Peacock, 1999).

1.2.2 The epidemiology of pulmonary arterial hypertension (PAH)

IPAH and FPAH are rare (combined incidence of 1-2/million), progressive and usually fatal conditions. Although they can occur at any age, the mean age at diagnosis is 36 years old and the gender ratio shows a greater prevalence for females (~2:1) (Rich *et al.*, 1987). Death is typically the result of right heart failure, which despite significant hypertrophy can no longer prevail over the inexorable rise in pulmonary vascular resistance (PVR). Due to the relatively non-specific early symptoms such as dyspnea, fatigue, angina and sometimes exercise syncope, mean time form onset of symptoms to diagnosis is about 2 years (Rich *et al.*, 1987) and the median survival time after diagnosis is reported as 2.5 years in the American National institutes of health registry (D'Alonzo *et al.*, 1991). Clinically, PAH is characterised by a marked derangement of pulmonary haemodynamics such as a three-fold or greater rise in pulmonary arterial pressure (PAP) and increased right atrial pressure but is not usually associated with changes in pressures on the left side of the heart. Hence, a definitive diagnosis could only formerly made by right heart catheterisation, an invasive procedure, not without risk. However, the recent advent of trans-thoracic echocardiography has provided an important step forward in non-invasive patient screening making earlier diagnosis likely.

1.2.2.1 The genetic link

Approximately 6% of all formerly classed PPH cases are classified as familial (FPAH), but the true figure may be higher as the disease is an autosomal dominant trait with incomplete penetrance and shows a pattern of genetic anticipation, a worsening of the disease in subsequent generations manifested by greater severity or earlier onset (Loyd *et al.*, 1995). Fortunately, children of an affected parent only have a 5-10% lifetime risk of developing the disease.

It is suspected that at least 2 genes are involved in FPAH. The first gene to be ascribed to FPAH was designated PPH 1 and has a locus at 2q31-32. More recently the bone morphogenetic protein receptor 2 (BMP-R2), a member of the transforming growth factor (TGF-β) superfamily has been found to show mutations in most cases of FPAH (Machado *et al.*, 2001).

IPAH displays a virtually identical clinical progression and histopathology to the familial form and whilst a family history cannot be demonstrated it is now considered by many that the development of severe PAH requires a genetic predisposition (Voelkel & Tuder, 1995;Voelkel, 1997). However, the BMP-R2 mutation has been found to be present in only 15- 25% of IPAH patients and as only ~ 10-20% of people with a BMP-R2 mutation develop PPH it is likely that other genetic influences and/or specific environmental triggers are necessary to initiate the pathological sequence that leads to the onset of the disease (Newman *et al.*, 2001). Another mutation associated with IPAH is the polymorphism of the 5-HT transporter (5-HTT). The L-allelic variant has been found to be ~two-fold more common in IPAH patients than in controls (Eddahibi *et al.*, 2001b) and is reported to enhance 5-HTT transcription in animal models (Eddahibi *et al.*, 1999) and cause vascular proliferation in human PASMCs (Eddahibi *et al.*, 2001b). However the nature of its role in PH is controversial and it is more thoroughly discussed later in this chapter.

1.2.3 The pathology of pulmonary arterial hypertension; vascular remodelling

Autopsies have identified that PAH patients all show a histological remodelling of the small pulmonary arteries (SPA's). The most obvious change is the distal "migration" or progression of smooth muscle into small peripheral normally non-muscular arteries (Rich et al, 2003). This migration may also be observed in SPH such as that occuring secondary

to chronic hypoxia (Hislop & Reid, 1976;Smith & Heath, 1977;Meyrick & Reid, 1978) (Fig 1.5).

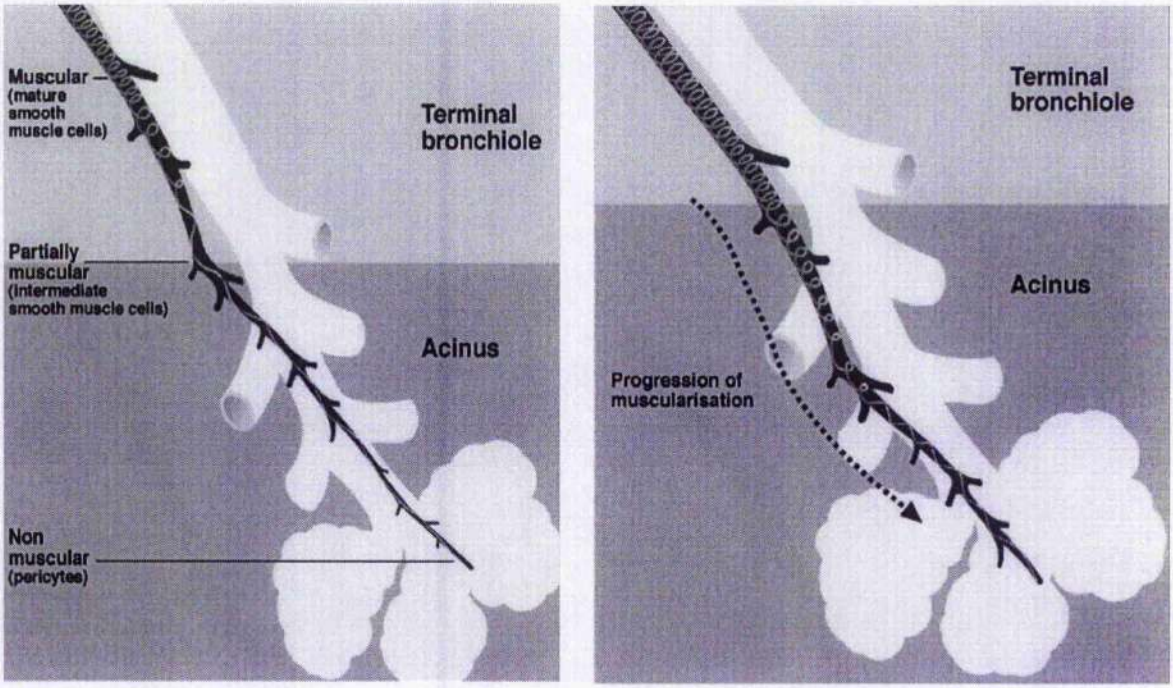


Figure 1.5 The distal progression of smooth muscle into the small pulmonary arteries

The diagram on the left shows the normal distribution of smooth muscle along the pulmonary arterial tree. The diagram on the right shows the progression of smooth muscle into the normally non-muscular arteries and arterioles.

Within these remodelled arteries some or all of the following pathological changes are observed; 1) Intimal thickening and formation of plexiform lesions. 2) Muscularisation and thickening of the media; 3) Thickening of the adventitia (Rich et al, 2003). Some of these changes can to a degree, be replicated in the chronically hypoxic animal model of PH, making this a useful tool for the study of PH (Smith & Heath, 1977;Meyrick & Reid, 1980a;Meyrick & Reid, 1980b). However the phenotypical changes that occur in SPH may not be typical of those that occur in PAH (Lee *et al.*, 1998).

1.2.3.1 Intimal thickening and formation of the plexiform lesion

In the normal pulmonary circulation the intima consists of a single layer of endothelial cells and is separated from the media by a thin elastic basal lamina (Tortora, 1995)(Fig 1.2). The endothelium shows a marked heterogeneity although the relationship between the phenotype and functions of these multiple cell types is not yet fully understood. In PAH endothelial proliferation causes a thickening of the intima, resulting in occlusion of the vessel lumen. The thickening is further exaggerated by the formation of the neointima, which consists of a layer of fibroblasts and extra-cellular matrix proteins (Rich et al, 2003) . In the chronically hypoxic (10days) rat model of PH, the intima has been reported to be thickened upto three-fold by endothelial cell hyperplasia and hypertrophy of the cytoplasmic organelles (Meyrick & Reid, 1980a). However not all studies have found evidence of intimal proliferation following exposure to chronic hypoxia (Smith & Heath, 1977)

In some cases of PAH and SPH, endothelial proliferation may also result in the formation of the plexiform lesion. The lesion is an unusual and pathological glomerular-like mass of disorganised capillary formations, which occurs within an anuerysmal dilatation of an arterial branch, distal to an obstructed larger artery. The vessels that comprise the lesion are primarily composed of endothelial channels supported by a stroma containing matrix proteins and myofibroblasts. However, plexiform lesions in IPAH are monoclonal whilst those from SPH patients are polyclonal (Lee *et al.*, 1998) leading to speculation of a fundamental abnormality of the endothelium in IPAH. Recently mutations in the TGF-βR2 gene as well as dysregulation by vasculotropic viruses have been implicated (Yeager *et al.*, 2001;Cool *et al.*, 2003).

1.2.3.2 Muscularisation and thickening of the media

The media supplies the mechanical strength and contractile power of the vascular wall. It is composed of heterogeneous subpopulations of SMCs with marked differences in the phenotype, growth, and matrix-producing capabilities (Dempsey *et al.*, 1997;Frid *et al.*, 1997). It is thought that these differences are intrinsic to the cell type and can determine the pattern of abnormal cell proliferation and matrix protein synthesis observed in the pathogenesis of vascular disease. For example, sub-populations from the outer media have an augmented proliferative response to hypoxia relative to those of the middle and inner media (Dempsey *et al.*, 1997;Frid *et al.*, 1997). Although not completely understood, it is thought that in PAH, the media of the normally non-muscular distal arteries undergoes hyperplasia of favoured cell types causing them to become muscularised. This not only thickens the medial wall, but as the different phenotypes respond differentially to vaso-active factors it can also change the vessels contractile profile (Rich et al, 2003). In rat vessels, remodelled as a result of exposure to 10days chronic hypoxia, the media is typically thickened by more than two-fold as a result of SMC hypertrophy and an increase in extra-cellular connective tissue and oedema like fluid (Meyrick & Reid, 1980b). The thickening of medial layer also causes the internal and external elastic laminae, which are themselves thickened two-fold by hypoxic exposure, to be further separated and this can be used as a means of identifying remodelled vessels (Meyrick & Reid, 1980b) (Fig 1.6).

1.2.3.3 Adventitial thickening

The adventitia is a sheath of connective tissue with no distinct outer border, which is composed mainly of fibroblasts and elastic and collagen fibres. Fibroblasts being less differentiated than endothelial or SMCs, demonstrate a tremendous capacity for rapid migration, proliferation and trans-differentiation into other cell types (Sartore *et al.*, 2001). In animal models of PH secondary to hypoxia, adventitial fibroblasts appear to be

the first cells to proliferate and increase synthesis and in conjunction with increased collagen synthesis, result in a two-fold thickening of the adventitia (Meyrick & Reid, 1980b).

Adventitial fibroblasts can also migrate to the media (and ultimately the intima) and contribute to remodelling in these regions of the vascular wall. Additionally, as the vessel wall thickens, the vasa vasorum, which is responsible for the blood supply to the vessels themselves, undergoes neovascularisation (Rich et al, 2003).

The comined effects of remodelling lead to an occlusion of the vessel lumen and increased vascular tone. This manifests as an increase in resistance to flow, and a decrease in compliance, forcing the right side of the heart to increase its workload in an attempt to maintain flow. The result is pulmonary arterial hypertension.

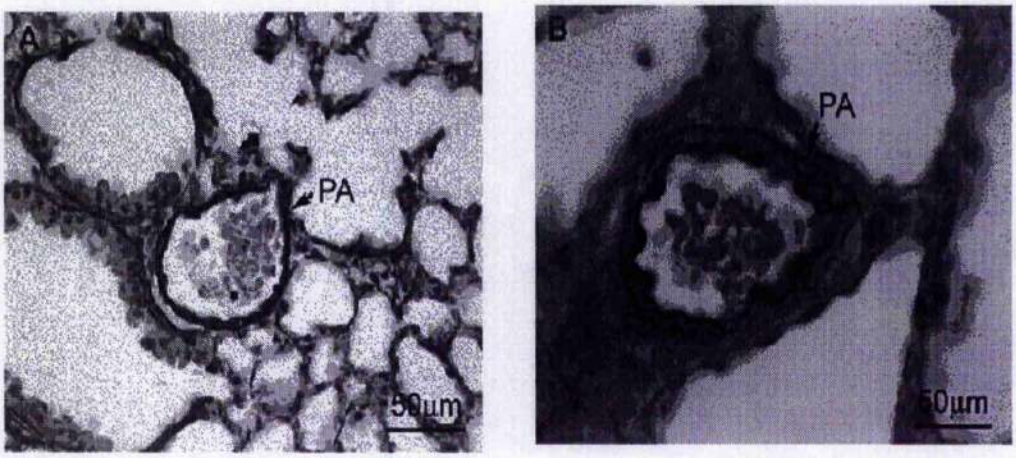


Figure 1.6 Medial thickening of the small pulmonary artery in the chronically hypoxic rodent model of pulmonary arterial hypertension.

A) A normal rat small pulmonary artery (SPA). The internal and external elastic laminae have been stained black (Van-Geeson staining) and appear as a single band due to the very thin layer of smooth muscle separating them. B) A remodelled rat SPA following 2weeks exposure to chronic hypoxia. The medial (tunica media) thickening is evident from the now clearly separated internal and external elastic laminae, which are also thickened (Keegan *et al*, 2001).

1.2.4 The pathophysiology of pulmonary arterial hypertension; The mechanisms behind PAH

The pathological changes just described have been associated with the presence of one or more of four predominating mechanisms; 1) vasoconstriction; 2) vascular wall remodelling; 3) inflammation and 4) thrombosis.

1.2.4.1 Vasoconstriction

Vasoconstriction has been favoured as a candidate for the initiating mechanism since (Wood, 1958) demonstrated vasodilatation in response to an infusion of acetylcholine. Vasoconstriction causes decreased vascular compliance and increased PVR, which if sustained lead to PAH. The most convincing evidence supporting the role of vasoconstriction as an important player in the development of PAH is that ~ ¼ of all IPAH patients show a 15-20% decrease in PVR and PAP in response to application of acute vasodilators such as nitric oxide (NO) (Pepke-Zaba *et al.*, 1991) or prostacyclin (PGI_2) (Rubin *et al.*, 1982). These observations actually form the basis of one aspect of the current clinical treatment of PAH. For example, those patients who respond to the acute vasodilator challenge are typically prescribed Ca^{2+} channel blockers such as nifedipine or diltiazem. In this group of patients there is a 95% survival rate at 5years following diagnosis (Rubin, 1997). However no evidence as yet can unequivocally confirm vasoconstriction as the initiating stimulus and the fact that ~ ¾ of patients do not respond to the acute vasodilator challenge suggests an alternative mechanism may be responsible for initiating the early lesion. Current consensus is that vasoconstriction is more likely to be a contributing factor rather than the initial cause and much effort has recently been directed towards greater understanding of vascular remodelling.

1.2.4.2 Remodelling

In the healthy pulmonary circulation, small pulmonary arteries are usually thin walled, highly compliant vessels. The optimum wall thickness and overall tissue mass of these vessels is maintained by a delicate balance between proliferation and apoptosis of its cellular components such as fibroblasts, SMCs and endothelial cells. Upsetting this balance in favour of proliferation leads to SMC hypertrophy and/or luminal occlusion which results in decreased vascular compliance and increased PVR, which in turn lead to the onset and progression of PAH (Rubin, 1997). However, considering vasoconstriction and cellular proliferation as completely separate entities may be counterproductive. For example, it has been shown that both elastic stretch of PASMCs, (which has been shown to occur during cross-sectional vasoconstriction) and elevation of PAP, promoted pulmonary arterial cellular growth (Hishikawa *et al.*, 1994). Most experts now agree that PASMC proliferation and to some extent, vasoconstriction lead to the vascular remodelling processes that underlie severe PAH (Mandegar *et al.*, 2004).

Elevation in $[Ca^{2+}]_{cyt}$ mediates vasoconstriction and proliferation of pulmonary arterial smooth muscle cells

It seems increasingly likely that vasoconstriction and cellular proliferation may both involve common signalling processes that may result in parallel cytosolic events. Findings have shown that elevations in $[Ca^{2+}]_{cyt}$ not only initiates SMC contraction but also result in a rapid increase in nuclear $[Ca^{2+}]$ which is an essential component of proliferation in SMCs (Allbritton *et al.*, 1994). In neuronal cells this $[Ca^{2+}]_{cyt}$ elevation has been shown to stimulate the quiescent cells into a state of mitosis, resulting in proliferation (Hardingham *et al.*, 1997). It now seems that at least four steps in the cell cycle are sensitive to the activation of the Ca^{2+}/calmodulin complex; 1) G0 to G1 (resting state to the beginning of DNA synthesis) 2) transition from G1 to S phase (DNA synthesis) 3) transition of G2 to M phase (mitosis) and 4) Mitosis itself (Steps 1-4 quoted

direct form Mandegar *et al.*, (2004)). This is entirely consistent with the report that resting $[Ca^{2+}]_{cyt}$ is significantly elevated in proliferating human PASMCs when compared to growth arrested cells (Golovina *et al.*, 2001). In addition the signal transduction protein, MAP kinase II, which is involved in the initiation of DNA synthesis has also been shown to be activated by a rise in $[Ca^{2+}]_{cyt}$. It would thus appear that elevation of $[Ca^{2+}]_{cyt}$ is a fundamental part of the proliferation, signalling cascade.

Elevation in $[Ca^{2+}]_{cyt}$ mediates proliferation of pulmonary arterial endothelial cells

Elevated $[Ca^{2+}]_{cyt}$ has also been shown to increase activating protein-1 (AP-1) activity and growth factor synthesis in pulmonary arterial endothelial cells (PAECs). AP-1's are transcription factors that regulate expression of genes such as those regulating ET-1, vascular endothelial cell growth factor (VEGF) and platelet derived growth factor (PDGF) production. These findings are therefore consistent with the report that over-expression of AP-1 accelerates tumour cell growth, as ET-1, VEGF and PDGF are known to be involved in aspects of cell proliferation, migration and apoptosis (Mathas *et al.*, 2002).

Apoptosis and vascular remodelling

The changes caused by increased proliferation are often mirrored and further exaggerated by a decrease in apoptosis, which has been shown to be markedly inhibited in PASMCs from IPAH patients (Zhang *et al.*, 2003). However, this aspect of remodelling appears to be independent of changes in $[Ca^{2+}]_{cyt}$. Instead reduced apoptosis has been suggested to be linked to mutations in the BMP-R2 gene which exerts its influence through its indirect effects on K^+ channels in the sarcolemmal membrane. (Mandegar *et al.*, 2004). Apoptosis is associated with apoptotic volume decrease (AVD) and an increase in caspases and nucleases (Remillard & Yuan, 2004), (Bortner & Cidlowski, 1999) Maintenance of a high $[K^+]_{cyt}$ maintains cell volume and suppresses caspases and

nucleases, hence inhibition or decreased activity of K^+ channels causes an accumulation of $[K^+]_{cyt}$ and a reduction in apoptosis. It has been hypothesised that the mutations in the BMP-R2 gene prevent the usual down regulation of Bcl-2 by the BMP protein (Mandegar *et al.*, 2004). Bcl-2 is an antiapoptotic membrane protein which, reduces apoptosis by a number of mechanisms one of which is inhibition of K^+ channels (Ekhterae *et al.*, 2001).

1.2.4.3 Inflammation

Mediators of inflammation such as TGF-β, interleukin 6, 5-LO and FLAP have been shown to cause vasoconstriction and may induce proliferation in animal models of PH (Voelkel *et al.*, 1996;Bhargava *et al.*, 1999). Elevated levels of these mediators have also been reported in patients formerly classed as PPH. However, inflammation of the vascular wall is neither a consistent nor prominent feature of the histologic findings at autopsy and when present is thought to be a complication of the disease rather than an initiating mechanism (Fishman, 1998).

1.2.4.4 Thrombosis

Currently, whilst in situ thrombosis is recognised to contribute to the progression of PAH it is not generally thought to be responsible for initiating the early lesion and is thought to occur secondary to an interplay between the abnormal endothelium and blood borne constituents (Fishman, 1998). However, the fact that a number of studies of PAH patients treated with the anti-coagulant Warfarin have demonstrated a significant increase in the 3 and 5 year survival rates suggests that thrombosis does play an important role in the progression of the disease (Frank *et al.*, 1997;Ogata *et al.*, 1993).

1.2.5 The cellular and molecular mechanisms associated with vasoconstriction and vascular remodelling in pulmonary arterial hypertension

The review so far has shown that the development of PAH is characterised by 3 structural and/or functional abnormalities, which result in remodelling of the small pulmonary arteries, these are; vasoconstriction, proliferation and apoptosis. Thrombosis and inflammation, which increase the risk of additional complications, may also accompany these abnormalities (Fishman, 1998). Whilst it is considered that a genetic predisposition is probably necessary for these changes to occur in such a way as to result in the development of severe PAH, there is substantial evidence to suggest some additional trigger is usually required (Mandegar *et al.*, 2004). Whilst this trigger, known as the initiating lesion remains a mystery, a number of pathological cellular and molecular changes have been repeatedly observed in PAH which are believed to be linked to the processes of remodelling.

1.2.5.1 Endothelial cell dysfunction

The normal pulmonary vasculature is highly distensible with little or no basal tone and the endothelial cells play an integral role in maintaining this state by producing a wide array of vasoactive mediators and growth factors, which mediate PASMC growth and contractility (Mandegar *et al.*, 2004). In the normally functioning endothelium the balance of vasoactive mediators and growth factors is maintained so that PAP can be kept relatively constant through distension and recruitment, even during a five-fold increase in cardiac output as may occur with intense exercise. However when this balance is disturbed, vasomotor tone, PAEC and PASMC proliferation, and thrombosis have all been shown to be increased. Given the marked heterogeneity in the endothelium of the vascular bed and the lack of understanding in the relationship between phenotype and function, it is difficult for pathologists to determine the extent of the role which

endothelial dysfunction has played in a given case of PAH (Rich et al, 2003). However, immunohistochemical studies have shown endothelial production of vasoconstrictors and co-mitogens such as ET-1 and 5-HT to be increased and vasodilators and antiproliferatives such as nitric oxide (NO) and prostacyclin (PGI_2) to be decreased in PAH patients (Christman *et al.*, 1992;Giaid *et al.*, 1993;Giaid & Saleh, 1995;Giaid, 1998). This is consistent with reports that both ET-1 and 5-HT plasma levels are elevated in PAH (Herve *et al.*, 1990;Herve *et al.*, 1995;Stewart *et al.*, 1991).

Increased Endothelin 1 (ET-1)

In IPAH patients, the high arterial levels of ET-1 relative to venous levels, initially suggested to researchers that increased pulmonary production of ET-1 contributed to the development of the disease (Stewart *et al.*, 1991). ET-1 is now considered to play a crucial role in vascular remodelling and the development of PAH (Kirchengast & Munter, 1999). In short term human clinical trials, bosentan, a competitive antagonist of ETA and ETB receptors, has been shown to cause reversal of IPAH and regression of its associated histological changes (Rubin & Roux, 2002;Rubin *et al.*, 2002) although the long term outcome remains to be seen. Bosentan has now been adopted as the front line approach in the treatment of grade 3 PAH patients (moderate symptoms) and is marketed as Tracleer. Interestingly the company that produces Tracleer claim it acts as an anti-mitogen rather than a vasodilator. However the disease is not solely attributable to ET-1 abnormalities and some patients fail to respond. Furthermore, Tracleer is associated with a relatively high risk of liver toxicity and therefore it is not suitable for all patients (Rich & McLaughlin, 2003).

Decreased Nitric oxide (NO)

NO, which has been widely shown to inhibit SMC proliferation and vasoconstriction also inhibits platelet aggregation (Singh & Evans, 1997). PAH patients with severe

morphological changes to their pulmonary arteries have been found to have a reduced expression of endothelial nitric oxide synthase (NOS III) (Giaid & Saleh, 1995). This is corroborated by experiments using NOS III mutant mice which found an elevated PAP and an impaired vasodilator response to Ach (Steudel *et al.*, 1997). NO is also known to regulate apoptosis via its effects on K_V channels (Krick *et al.*, 2002)) (see 1.2.5.2 Down regulation of K_V channels), hence decreased synthesis of NO would be expected to result in decreased apoptosis and increased remodelling. NO is used clinically as one means of identifying those patients who are likely to respond to Ca^{2+} channel blockers.

Change in the prostanoid balance; The thromboxane: prostacyclin ratio

Originally linked to endothelial dysfunction, is the reported increase in thromboxane A_2 (TXA_2) production. In IPAH and SPH patients TXA_2 metabolites where found to increase where the metabolites of prostacyclin (PGI_2) were decreased, (Christman *et al.*, 1992). TXA_2 is a vasoconstrictor and SMC mitogen and is thought to play a role in the vascular remodelling associated with IPAH. However, the site of increased synthesis in IPAH has not been determined although activated alveolar macrophages and platelets have been suggested (Robbins *et al.*, 2001).

PGI_2 is a powerful vasodilator and inhibits platelet adhesion and cell growth. In IPAH and PAH patients expression of the PGI_2 synthase has been shown to be significantly reduced (Tuder *et al.*, 1999). PGI_2 promotes the production of vascular endothelial cell growth factor (VEGF) which is an angiogenic factor (Machado *et al.*, 2001). Growth factors such as VEGF and TGF-β are crucial to lung development and play a protective role by modulating adaptation to abnormal conditions such as chronic hypoxia. Defects in the function of these factors have implicated them in the development of PAH. Experimental research appears to corroborate this. In the rat, studies where VEGF receptor blockade was performed, resulted in vascular remodelling (characterised by

endothelial proliferation) and mild PH in normoxic rats and severe irreversible PH in chronically hypoxic rats (Taraseviciene-Stewart *et al.*, 2001).

PGI_2 as a therapeutic tool: PGI_2 is an important therapeutic tool, which was originally tried because it was observed to produce acute heamodynamic effects in a number of PAH patients (Rubin *et al.*, 1982). However PGI_2 was also subsequently found to considerably improve the survival time of patients who do not show an acute vasodilator response and in these patients it is thought that it may inhibit or perhaps even reverse remodelling (Peacock, 1999;Rubin, 1997). In fact, some patients have shown sustained heamodynamic and clinical effects even after 10years of continuous infusion, although most do develop a tolerance to its effects. Unfortunately PGI_2 requires continuous intravenous infusion due to its short half-life. The inconvenience, high risk of infection and also the cost, ~£50,000/year/patient, mean this is far from an ideal treatment (Peacock, 1999).

PGI_2 and gene therapy: An exciting recent development in PGI_2 treatment came in 2002 when gene therapy was successfully used to increase PGI_2 synthase expression in the liver of rats suffering from PH induced by monacrotaline. The rats treated with gene therapy showed decreased right atrial pressure, decreased lung ET-1 levels and a significant increase in survival rate when compared to monacrotaline induced PH rats which had not received gene therapy (Suhara et al., 2002).

The loss of the endothelium as a physical barrier

The endothelium also acts as a physical barrier between the fibroblasts and PASMCs of the vascular wall and numerous circulating blood-borne factors. Damage to the endothelium allows contact of circulating factors, which can stimulate PASMC and fibroblast proliferation resulting in medial and adventitial hypertrophy, both of which are features of IPAH. Additionally, endothelial damage can also result in the development

of thrombosis due to the contact between platelets and the sub-endothelial components of the vascular wall (Ruf & Morgenstern, 1995). Thrombosis causes further occlusion of the vessel and exacerbate PAH.

1.2.5.2 Downregulation of voltage gated potassium (K_V) channels

K_V influences vasoconstriction and proliferation

The transmembrane K^+ current (I_k) through voltage-gated K^+ (K_V) channels plays an important role in regulating the resting membrane potential (*E*m) in PASMCs (Yuan, 1995). As such, the down regulation of K_V would cause a reduction in I_K and result in depolarisation of the cell. This in turn could cause increased vasoconstriction and proliferation via its direct action on VDCCs and indirectly by its effect on the SR, via modulation of IP_3 production. As already explained, elevated $[Ca^{2+}]_{cyt}$ is a critical signal in both vasoconstriction and proliferation. Evidence for the involvement of K_V downregulation comes from IPAH patients who show a decreased amplitude in $I_{K(V)}$ and mRNA/protein expression of K_V channel subunits (K_V1.2 and K_V1.5) relative to non-pulmonary hypertensive (NPH) and SPH patients (Yuan *et al.*, 1998a;Yuan *et al.*, 1998b). It has also been reported that the genes for the inward rectifier potassium channels are upregulated in PAH (Geraci *et al.*, 2001) These findings are consistent with reports that resting $[Ca^{2+}]_{cyt}$ is substantially higher in PASMCs obtained from IPAH patients than PASMCs obtained from NPH or SPH patients (Yuan *et al.*, 1998a)). At present it is not known whether K_V channel abnormalities are the result of genetic mutations or are acquired, but APAH following anorexigen use could be associated with a reduction in I_K as aminorex fumarate, fenfluramine and dexfenfluramine are known K^+ channel blockers (Weir *et al.*, 1996).

K_V influences apoptosis

Downregulation or inhibition of K_V channels may also be associated with the reduction in apoptosis in IPAH. Cell shrinkage, known as apoptotic volume decrease (AVD), is a hallmark of apoptosis. However, a reduction in I_K has been found to preserve cell volume and thus slow AVD (Remillard & Yuan, 2004). Furthermore, a high cytoplasmic $[K^+]$ also suppresses cytoplasmic caspases and nucleases which are thought to be the final mediators of apoptosis (Bortner & Cidlowski, 1999;Krick *et al.*, 2001). Reduced apoptosis via downregulation of K_V channels has been shown to occur as a result of up-regulation of the Bcl-2 antiapoptotic membrane protein (Ekhterae *et al.*, 2001). Although Bcl-2 is down-regulated by the BMP protein in normal PASMCs, it is hypothesised that in IPAH mutations in the BMP-R2 gene lead to a dysfunction in BMP mediated signalling as BMP-mediated apoptosis is significantly inhibited in IPAH but not in SPH (Zhang *et al.*, 2003).

K_V as a therapeutic target

As downregulation of K^+ channels appears to be involved in vasoconstriction, proliferation and apoptosis, augmentation of K^+ channel function is a logical therapeutic target. Oral dichloroacetate has been found to increase K_V 2.1 channel expression, decrease remodelling and lower PVR in rats with SPH following exposure to chronic hypoxia (Michelakis *et al.*, 2002). As this drug has already been tested and found to be safe in heart failure studies it may be a useful therapeutic tool for the treatment of PAH.

1.2.5.3 Up-regulation of canonical transient receptor potential (TRPC) channels

In addition to store release and Ca^{2+} influx through VDCCs, $[Ca^{2+}]_{cyt}$ can also be elevated by influx through receptor operated (ROC) and store operated channels (SOC) (Fig 1.3). As already mentioned, elevation of $[Ca^{2+}]_{cyt}$ is an important signal for enhanced proliferation, which in PAECs can result in intimal thickening and plexiform lesions and in PASMCs results in medial hypertrophy. Depletion of store Ca^{2+}, triggers capacitive

Ca^{2+} entry (CCE) through SOCs in order to maintain Ca^{2+} influx and refill intra-cellular stores. Canonical transient receptor potential (TRPC) genes have been shown to encode for SOCs and are believed to be responsible for CCE (Birnbaumer *et al.*, 1996;Boulay *et al.*, 1999;Zhu *et al.*, 1996). Evidence for this in human PAECs comes from experiments in which PEACs were exposed to chronic hypoxia. Increases in SOC current and CCE induced by hypoxia were found to be paralleled by TRPC mRNA and protein expression and the use of TPRC interfering RNA was found to attenuate CCE (Fantozzi *et al.*, 2003). In PASMCs increased CCE and proliferation is also associated with TRPC channel expression (Golovina *et al.*, 2001) and inhibition of TRPC expression decreases the amplitude of CCE and inhibits proliferation. The elevated $[Ca^{2+}]_{cyt}$ observed in IPAH would appear to be at least partially linked to up-regulated TPRC channel expression as CCE has been shown to be significantly elevated in PASMCs from these patients (Mandegar *et al.*, 2004). The evidence suggests that up-regulation of TRPC (SOC) channels may contribute to the pathogenesis of PAH and could therefore be a suitable target for therapeutic intervention.

1.2.5.4 The 5-HT hypothesis

The possible role of serotonin (5-HT) in the development of PAH was first hinted at in the late 1960's when an epidemic of PAH followed the over the counter sale of the anorexigen (diet drug), aminorex fumurate (Kay *et al.*, 1971). Aminorex increases 5-HT availability by inducing platelet release of 5-HT, inhibiting its re-uptake and by inhibiting its breakdown by monamine oxidase (MAO) (MacLean *et al.*, 2000b). In European countries where the drug had been sold, cases of PAH increased ten-fold and the epidemic continued until shortly after the drug was withdrawn in 1972 (Fishman, 1999). In the 1990's a new epidemic of "dietary" PAH occurred following the combined prescription of fenfluramine/dexfenfluramine and phentermine.

Fenfluramine/dexfenfluramine selectively act on the 5-HT transporter (5-HTT) to induce 5-HT release whilst simultaneously inhibiting 5-HT uptake (McTavish & Heel, 1992;Garattini *et al.*, 1989). The inhibition of the 5-HTT has been shown to occur in serotinergic neurones of the brain, circulating platelets, PAECs and PASMCs (Lesch *et al.*, 1994), but interestingly the use of anorexigens have not been associated with remodelling in any other organ system but the lungs. Phentermine on the other hand, can inhibit MOA and has been shown to increase the vasoconstrictor effects of 5-HT in the rat lung (Seiler *et al.*, 1976). In combination these drugs had a similar effect as the banned aminorex fumurate. It has since been determined that use of anorexigen drugs causes a thirty-fold increase in the risk of developing IPAH (Fishman, 1999). These observations and findings lead to the 5-HT hypothesis of PAH.

5-HT acts as a vasoconstrictor and mitogen

In comparison to control subjects, some PAH patients have been shown to have reduced platelet 5-HT storage, increased platelet 5-HT release during aggregation and an increased 5-HT plasma concentration. These include those formerly classed as PPH as well as those with PAH associated with anorexigen use and portal hypertension (Herve *et al.*, 1995;Herve *et al.*, 1990;Laffi *et al.*, 1992;Liu & Lee, 1999). Hence elevation of plasma 5-HT and possible localised 5-HT elevations due to abnormal functioning of the 5-HTT could cause the pulmonary circulation to increase its tone. This would not only increase PVR but could also increase the reactivity of the vessels to other agonists through synergistic interactions between Gi and Gq proteins (MacLean, 1999)

Unlike a number of other mammals such as the rat, dog and cow where 5-HT induced vasoconstriction is mediated primarily through the 5-HT_{2A} receptor, in the human pulmonary circulation, vasoconstriction seems to be mediated primarily through the 5-HT_{1B} receptor (Macintyre *et al.*, 1992;Morecroft *et al.*, 1999;MacLean *et al.*, 1996a).

However rat models of chronic hypoxia induced SPH, show increased 5-HT_{1B} mRNA and an increased contribution of the 5-HT_{1B} receptor to the contractile response (MacLean *et al.*, 1996b). Furthermore, treatment with the 5-$HT_{1B/D}$ antagonist GR 127935 substantially reduces the development of right ventricular hypertrophy in these animals (Keegan *et al.*, 2001). These findings suggest the 5-HT_{1B} receptor may play a role in the development PAH. Therapeutically this would be an attractive target as unlike the 5-HT_{2A} receptor, which also mediates vasoconstriction in systemic vessels, the 5-HT_{1B} receptor is confined to the pulmonary circulation. This would avoid the problems of systemic hypo-tension associated with drugs like the 5-HT_{2A} receptor antagonist ketanserin, which has proven to be relatively ineffective in the treatment of PAH.

However, perhaps the more important role of 5-HT in the development of PAH is related to its mitogenic activity. The mitogenic effects of 5-HT have been demonstrated in rat, bovine and human PASMCs (Lee *et al.*, 1991;Pitt *et al.*, 1994;Eddahibi *et al.*, 2001b) although 5-HT induced proliferation does not seem to occur in PAECs (Lee *et al.*, 1989;Lee *et al.*, 1994b;Lee *et al.*, 1997).

Mitogenesis and the 5-HT transporter (5-HTT)

There is an increasing body of evidence, which suggests that the mitogenic effects of 5-HT primarily occur through the internalisation of 5-HT by the 5-HT transporter (5-HTT) although mitogenesis may also be mediated through cell surface receptors. (Lee *et al.*, 1989;Lee *et al.*, 1991;Lee *et al.*, 1994a;Lee *et al.*, 1994b;Pitt *et al.*, 1994;Lee *et al.*, 1997;Eddahibi *et al.*, 1999;Eddahibi *et al.*, 2001b). For example, the proliferative effect of 5-HT on bovine and rat PASMCs has been shown to be dose dependently inhibited by the 5-HTT selective inhibitors paroxetine and fluoxetine (Lee *et al.*, 1991;Eddahibi *et al.*, 1999). Furthermore, paroxetine and fluoxetine inhibit [^{3}H]5-HT uptake and 5-HT

induced cell proliferation at concentrations which suggest the phenomena are linked (Eddahibi *et al.*, 1999).

The relationship between 5-HTT, chronic hypoxia and vascular medial remodelling

Whilst increased proliferation may be associated with the 5-HTT, there is however, a significant contradiction in the literature as to how up-regulation of the 5-HTT is involved in PH. One research group has consistently reported the 5-HTT to be essential to the medial proliferation associated with chronic hypoxia induced SPH. Chronic hypoxia, an intervention which can be used experimentally to cause vascular medial remodelling of PASMCs was found to cause a transient two and a half-fold increase in 5-HTT mRNA and a sustained three-fold increase in 5-HT uptake *in vitro* (Eddahibi *et al.*, 1999). *In vivo,* 5-HTT mRNA expression was also been found to be enhanced in the lungs of rats exposed to chronic hypoxia (Eddahibi *et al.*, 1999) and reduced in transgenic mice which, under-express 5-HTT (Eddahibi *et al.*, 2000). At first these findings may seem to contradict the anorexigens having a role in the development of PAH, as the anorexigens are generally associated with inhibition of 5-HTT uptake and it might be expected that their use would therefore attenuate 5-HTT associated mitogenesis. However, it has been shown that discontinuation of dexfenfluramine treatment can also trigger an increase in 5-HTT expression. The increased expression resulted in significantly enhanced remodelling and right ventricular hypertrophy in rats, which were subsequently exposed to chronic hypoxia relative to those that had not been treated with dexfenfluramine (Eddahibi *et al.*, 2001a). Furthermore the anorexigens may themselves act as a substrate for the 5-HTT and it is possible they could be accumulated inside the cell, having effects similar to or greater than the 5-HT whose uptake they are preventing (Rothman *et al.*, 1999). It has also been proposed that the anorexigens can cause inhibition of K^+ channels leading to elevated $[Ca^{2+}]_{cyt}$ the effects of which have already been discussed.

Although the exact mechanism by which the anorexigens trigger increased remodelling is not known, the findings that 5-HTT over-expression or increased activity play a critical role in the development of PH secondary to chronic hypoxia, naturally suggests the possibility of a role in PAH. Recent studies have demonstrated an increased expression of 5-HTT in lung tissues and pulmonary arteries isolated from patients with IPAH. PASMCs from these patients demonstrated a marked enhancement of proliferative growth to 5-HT, which did not occur in response to other growth factors. Furthermore the enhancement was abolished by the presence of 5-HTT inhibitors (Eddahibi *et al.*, 2001a). Thus it is possible that anorexigens may contribute to the development of "dietary" PAH by 1) elevating $[5\text{-HT}]_{plasma}$ 2) acting as a substrate for 5-HTT (which then mimics the effect of internalised 5-HT) 3) affecting 5-HTT expression.

However, a number of other investigators strongly disagree that increased 5-HTT expression is involved in chronic hypoxia induced SPH. A number of studies have in fact reported a marked decrease in the expression, protein and activity of the 5-HTT following chronic hypoxia (Jeffery *et al.*, 2000;Hoshikawa *et al.*, 2003;MacLean *et al.*, 2004). In one *in-vivo* study, genetically induced over-expression of the 5-HTT was found to elevate PAP but did not cause remodelling or right ventricular hypertrophy, implying that over-expression of the 5-HTT caused vasoconstriction prior to the onset of morphological change. The study also found that following chronic hypoxia, expression and activity of the 5-HTT were significantly attenuated despite the symptoms of PH being exacerbated (MacLean *et al.*, 2004). Hence whilst the 5-HTT may well be involved in the development of PAH its role is presently far from clear.

5-HTT gene polymorphism and IPAH

It is thought that a 5-HTT gene polymorphism, could be responsible for the susceptibility of some IPAH patients. For example, a patient diagnosed with PAH associated with

anorexigen use may have had an underlying 5-HTT polymorphism which on its own was not sufficient to trigger the onset of PAH but was subsequently exacerbated by the effect of anorexigen use.

A polymorphism in the promoter region of 5-HTT has been shown to alter transcriptional activity and has been associated with increased proliferation in human PASMC (Eddahibi *et al.*, 2001b). The L-allelic variant of the 5HTT gene promoter was present in homozygous form in 65% of IPAH patients but in only 27% of controls and has a two to three-fold greater 5-HTT transcription relative to the S-allelic variant. As normal signalling from the BMP-R2 is associated with suppression of PAMSC proliferation and promotion of apoptosis, it has been hypothesised that it may antagonise the proliferative effects of 5-HT (Morrell *et al.*, 2001;Zhang *et al.*, 2003). If a BMP-R2 mutation is present, the antagonism to the proliferative effects of 5-HT (or substrates which can mimic its action) may be lost resulting in proliferation and increased expression of the 5-HTT. If this were to occur in patients who also posses the homologous L-allelic variant of the 5HTT gene promoter then the proliferative response and expression of the 5-HTT would be particularly enhanced (Mandegar *et al.*, 2004). However it must be pointed out that at present there is no direct evidence for this but no doubt this avenue will be fully explored in the very near future.

1.2.5.5 Human urotensin II (hU-II), a role in pulmonary arterial hypertension?

A recent development in the field of research into PAH, was the report by MacLean *et al.*, (2000a) that contractile responses to the endogenous peptide human urotensin II (hU-II), were enhanced in the rat main pulmonary artery by conditions which mimic aspects of PAH, such as nitric oxide synthase (NOS) inhibition and exposure to chronic hypoxia. The same group also reported that NOS inhibition unmasked a highly variable response to hU-II in 30% of human small pulmonary arteries tested. hU-II is the most potent

peptide identified to date (Douglas & Ohlstein, 2000) and has been likened to ET-1, as initial studies suggested a number of similarities between these G-protein stimulating agonists (Maguire & Davenport, 2002). Hence it has been suggested that hU-II may be involved in the aetiology of PAH (MacLean *et al.*, 2000a). However, although hU-II has been linked to some aspects of systemic cardiovascular disease (Lapp *et al.*, 2004;Thanassoulis *et al.*, 2004), there is currently no evidence from studies involving PAH patients. Furthermore, other studies using modelled conditions of PAH in rats and isolated human lungs and vessels, have failed to substantiate this hypothesis (Zhang *et al.*, 2002;Bennett *et al.*, 2004). Consequently, the role of hU-II in the regulation of pulmonary arterial tone and the pathogenesis of PAH requires further investigation and constitutes one of the aims of this thesis. A thorough review of hU-II is presented in the introduction to chapter 3.

1.2.5.6 Additional mechanisms

A number of studies using tissue obtained from PAH patients have demonstrated persistent matrix protein synthesis. This observation has led to a number of animal studies, which have shown promising results, but require further investigation.

Endogenous vascular elastase and vascular remodelling

In rats with chronic hypoxia or monocrotaline induced PH, a characteristic of remodelling is the thickening of the elastic laminae, which define the boundaries of the medial smooth muscle layer (Finlay *et al.*, 1986;Todorovich-Hunter *et al.*, 1992). The formation of the laminae are characterised by an early increase in serine elastase activity and the proteinase-dependent deposition of the extra-cellular matrix smooth muscle cell survival factor tenascin-C (Jones *et al.*, 1997) . Tenascin-C amplifies the response of SMCs to growth factors, which are liberated through matrix proteolysis. *In vitro* studies using hypertrophied rat pulmonary arteries have shown that elastase inhibitors suppress

tenascin-C and induce SMC apoptosis resulting in the complete regression of the hypertrophied vessel wall (Cowan *et al.*, 1999). It has since been shown *in vivo* using the monacrotaline rat model, that inhibition of serine elastase resulted in 86% survival and a regression of structural changes associated with PH, compared with a 100% mortality in the group that did not receive the elastase inhibitor (Cowan *et al.*, 2000). This is corroborated by experiments in transgenic mice which show that over-expression of the serine elastase inhibitor elafin protected the mice from developing PH secondary to chronic hypoxia (Zaidi *et al.*, 2002). These findings suggest that elastase inhibitors may be useful in halting or reversing aspects of the vascular remodelling associated with PAH.

Statins

When activated, Rho GTPases, which are fundamental components of G protein coupled receptor transduction mechanisms, couple membrane growth factor receptors to the intra-cellular MAP/ERK kinase signalling pathways that influence proliferation. In addition Rho GTPase activation also inhibits endothelial NO production. Statins which are HMG-CoA reductase inhibitors better known for there cholesterol reducing effects, prevent the isoprenylation of Rho GTPases, thus reducing proliferation and augmenting vasodilatation through increased endothelial NO synthesis (Laufs *et al.*, 2000;Eto *et al.*, 2002). Simvastatin has subsequently been shown to reverse PH and increase survival in both monacrotaline and chronically hypoxic rat models of PAH (Nishimura *et al.*, 2003;Girgis *et al.*, 2003). Hence these findings suggest that statins may provide yet another possible avenue for treating PAH.

1.3 Aims

Recently the peptide, human urotensin II (hU-II), has been reported to be capable of causing both vasoconstriction (in the presence of L-NAME) and vasodilatation in human small pulmonary arteries (MacLean *et al.*, 2000a;Stirrat *et al.*, 2001). The study by MacLean *et al.*, (2000) also found that contractile responses to hU-II were enhanced in the main pulmonary artery of rats suffering from PH secondary to chronic hypoxia. However, other studies have found no response to hU-II in human vessels with endothelial dysfunction (Bennett *et al.*, 2004) and that hU-II does not increase PAP in the chronically hypoxic rat *in-vivo* (Deuchar *et al.*, 2005)) (submitted for publication). Hence, the first aim of this thesis was to determine the role of hU-II in the regulation of pulmonary arterial tone and whether hU-II could be involved in the aetiology of PAH.

Another recent development in the pulmonary vascular field has been the report that ET-1, which is widely implicated in the development of PAH, can modulate Ca^{2+} sensitivity (CaS) in the rat small pulmonary artery (Evans *et al.*, 1999). Furthermore, the Rho kinase (ROK) pathway, which is known to mediate increases in CaS, has also been implicated in the elevated basal tone of PH occurring secondary to chronic hypoxia, although the limited number of reports are conflicting (Sauzeau *et al.*, 2003a;Nagaoka *et al.*, 2004b). The second aim of this thesis was therefore to determine the role of CaS in the regulation of pulmonary vascular tone and to determine whether modulations in CaS could be involved in the aetiology of PAH. The two areas chosen for study where PH secondary to chronic hypoxia and the 5-HT system. However only one previous permeabilisation study has been performed in the rat small pulmonary artery (Evans *et al.*, 1999) and the technique used was not found to produce a sustainable preparation using the tools available to the author of this thesis. Hence it was first necessary to develop a suitable protocol.

Chapter 2

General Methods

The common methodology underlying the studies

2.1 Tissue preparation

2.1.1 The chronically hypoxic model (rat)

Male Wistar rats aged 28 days were divided into 2groups, age matched control (AM) and those to be exposed to 14-15days chronic hypoxia. The chronically hypoxic (CH) rats were placed in a hypobaric chamber (Royal Hallamshire Hospital Sheffield, U.K.) which was depressurised to 550mbar over two days and then maintained at this pressure for a further 12days. The chamber was ventilated with room air at ~ 45 l min^{-1}. Environmental conditions (e.g. temperature and light exposure) and dietary composition were identical for both groups. The chronically hypoxic rat is an accepted model for the study of pulmonary hypertension (PH) (Rabinovitch *et al.*, 1988;Suggett & Barer, 1988;Eddahibi *et al.*, 1997;McCulloch *et al.*, 1998;MacLean *et al.*, 2004).

2.1.2 The 5-HHT over-expression model (mouse)

Female C57BL/6×CBA wild type (WT) mice were used to generate transgenic (TG) mice over-expressing the human 5-HT transporter (5-HTT). The transgene was a 500-kb yeast artificial chromosome (YAC35D8) containing the human 5-HTT gene and its presence was confirmed after weaning (~1month) by tail tipping and RT-PCR. Transgenic mice yielded a 240bp product (2% agarose, ethidium bromide gel) which was absent in wild type mice. The forward and reverse primers were 5'-TTC TTT CCCT TAC TAA GTT GAG AAC G- and 3'5'-TGG CAT GCA ATT GTA GTC TC-3' respectively. Environmental conditions and dietary composition were identical for both wild type and transgenic groups. This model has been previously used to study 5-HTT over-expression (MacLean *et al.*, 2004).

2.2 Sacrifice and tissue procurement

2.2.1 Animal tissue

Rats were killed at 42-45 days old (180-250g), and mice at 5months (20-25g) by cervical dislocation followed by exsanguinatinon. The heart and lungs were removed *en bloc* and immediately placed in ice-cold Krebs buffer solution. The arteries (see vessel identification) were then dissected free from the surrounding lung parenchyma and sectioned into 2mm lengths ready for mounting.

2.2.2 Human tissue

2mm long segments of intra-lobar pulmonary arteries (see 2.2.3 vessel identification) were dissected free (see appendix) from macroscopically normal pieces of lung tissue obtained following bronchial carcinoma removal. The tissue was obtained from three female and two male patients aged between 54 and 78 years. The study was approved by the Research and Ethics Committee for North Glasgow Hospitals University Trust and all patients had given written informed consent (see appendix).

2.2.3 Vessel identification

Both extra-lobar and intra-lobar, rat and mouse pulmonary arteries were used in the hU-II study, whilst permeabilisation studies were limited to the rat and mouse intra-lobar, small pulmonary artery (SPA). The vessels were defined as follows:

Rat: Extra-lobar; main pulmonary artery (2-3mm i.d.) (Referred to as A-class vessels) and the first branch, pulmonary artery (1-2mm i.d.) (B-class). Intra-lobar; the 1st generation intra-lobar, pulmonary artery (0.75 –1.25mm i.d.) (C-class) and the 2nd generation intra-lobar, pulmonary artery (150-350μm i.d.) (D-class, also known as small pulmonary arteries (SPA's)) (Fig 2.1).

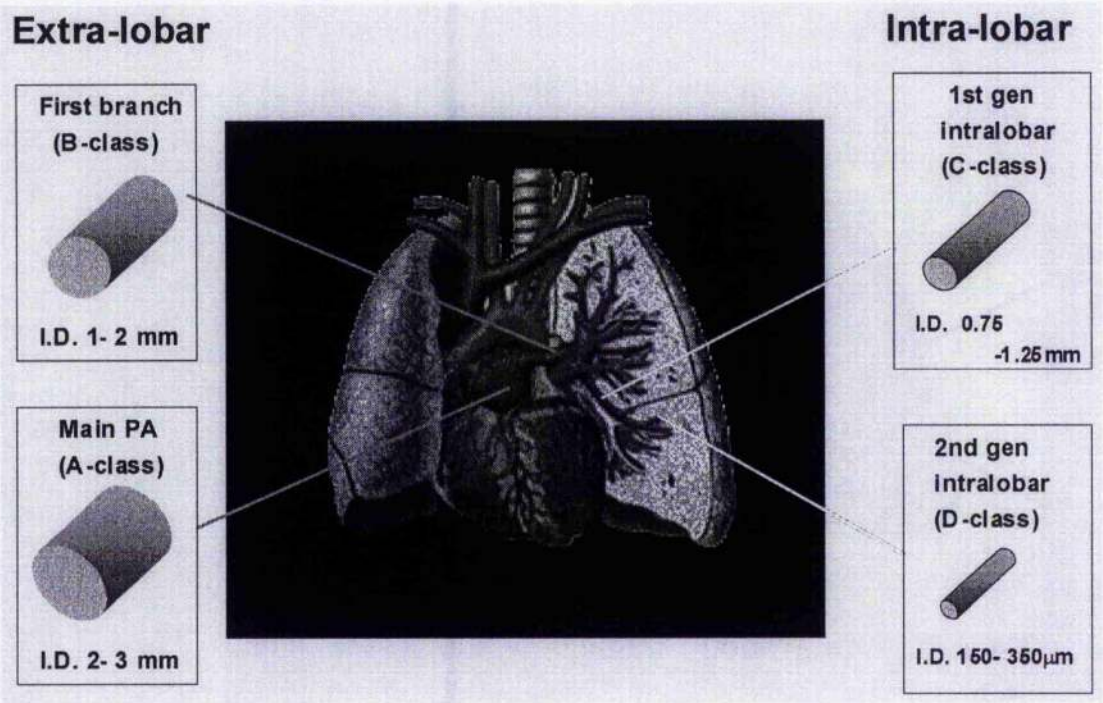


Figure 2.1 Vessel guide

The *in situ* location of the various classes of pulmonary arteries (sizes quoted are typical for rat vessels).

Mouse: Extra-lobar; main pulmonary artery (0.75-1.25mm i.d.) (Referred to as A-class vessels) and the first branch, pulmonary artery (0.5-1.0mm i.d.) (B-class). Intra-lobar; the 1st generation intra-lobar, pulmonary artery (250 –550μm i.d.) (C-class also known as SPA's) (Anatomical locations as in Fig 2.1).

Human: Intra-lobar; 750-1500μm (size equivalent to C-class rat vessels) and 150-300μm (size equivalent to D-class rat vessels).

2.2.4 Assessment of pulmonary hypertension

The development of pulmonary hypertension (PH) was assessed by measuring the ratio of the right ventricular (RV) to total ventricular weight (TV). The heart was dissected free from the lung lobes, and the atria and all great vessels removed. The right ventricle was then dissected free of the septum and left ventricle, blotted lightly and weighed. The left

ventricle was incised to remove any remaining blood clots and the left ventricle/septum then blotted lightly and weighed along with the right ventricle. The RV/TV ratio is a reliable and accepted index for the development of PH in rats (Herget *et al.*, 1978;Sheedy *et al.*, 1996;Keegan *et al.*, 2001).

2.3 Vessel set up

The isolated 2mm long vessel sections were mounted on a Mulvaney-Halpern isometric wire myograph (model 610M, JP Trading, Aarhus, Denmark) (Fig 2.2) and bathed in a Krebs-Hanseleit buffer solution of the following composition (mM): NaCl 118, $NaHCO_3$ 25, D-glucose 11, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2 (pH 7.4) at 24^0C (permeabilisation studies) or 37^0C (hU-II studies). The preparation was constantly gassed (16% O_2, 5% CO_2, balance N_2) to mimic normotensive, *in vivo* pulmonary arterial gas tensions.

Following 45minutes equilibration the vessels were tensioned in a stepwise manner until a stable tension, equivalent to a transmural pressure of 12-16mmHg was obtained. This procedure was intended to mimic the physiological *in vivo* pulmonary arterial transmural pressure and is known as normalisation. Normalisation is based on the law of Laplace (see Eq 2.1 and 2.2) for a thin walled tube. The procedure is adapted from the technique described by Mulvany and Halpern (1977), which is a reliable and accepted normalisation procedure in systemic vessels.

Eq 2.1 $$\mathbf{T = r_i P}$$

Equation 2.1 The law of Laplace for a thin walled tube

T = wall tension, r_i = internal radius, P = transmural pressure.

Eq 2.2 $$P = \frac{T}{r_i}$$

Equation 2.2 Normalisation of pressure in a thin walled vessel

Where wall tension is calculated as (Force recorded /2)×vessel length and r_i is calculated from the micrometer readings (Mulvany & Halpern, 1977).

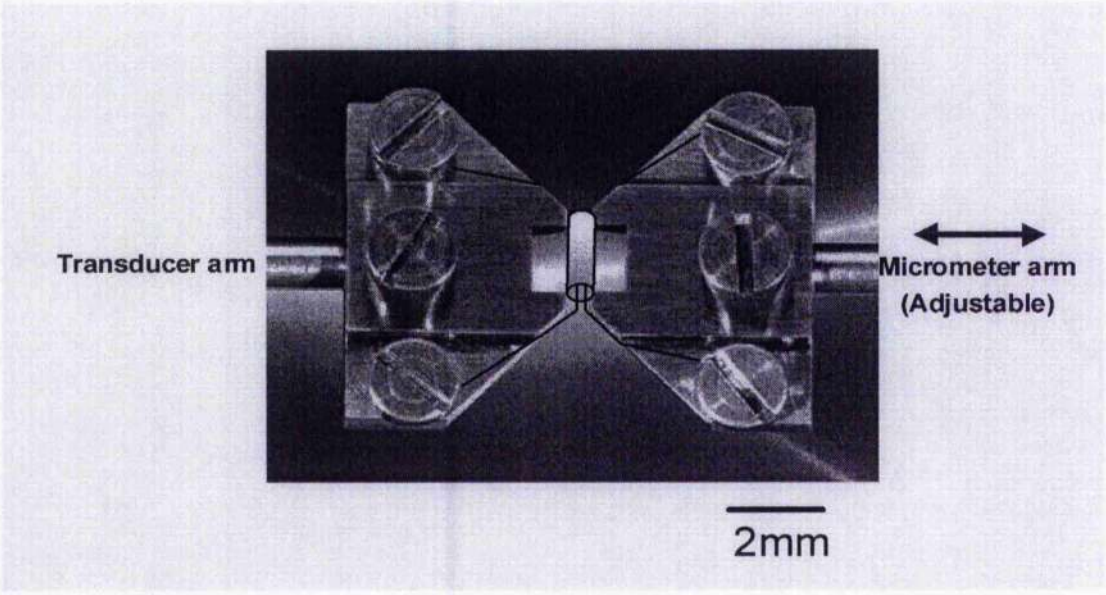


Figure 2.2 Isolated small pulmonary artery (rat D-class 150-350μm i.d.) mounted between the adjustable jaws of an isometric wire myograph unit

2.4 Determining the tension standard

Following the normalisation of the replicated transmural pressure, each vessels response to either a nominally iso-osmotic 120mM KCl solution exchange (permeabilisation studies) or a hyper-osmotic 50mM KCl addition (hU-II and 5-HT general pharmacology studies in unpermeabilised vessels) was determined. Any vessel which did not produce a maximum force of > 1mN was discarded. The composition of the nominally iso-osmotic KCl solution was as follows (mM): KCl 120, $NaHCO_3$ 25, D-glucose 11, $CaCl_2$ 2.5,

KH_2PO_4 1.2, $MgSO_4$ 1.2 (pH 7.4). Following the ascertainment of a stable maximal response to the chosen depolarising solution, vessels were thoroughly washed (>6 times) in Krebs buffer and allowed to return to baseline in readiness for use in their specific protocol.

2.5 Assessment of endothelial function (hU-II studies only)

Following determination of the vessels tension standard response, the integrity of the vessels vascular endothelium was established by the ability of acetylcholine (Ach) (1 μM) to cause a relaxation of >20% in vessels pre-constricted with either phenylephrine (PE) or 5-HT (AM; 1μM, CH; 0.1μM). All vessels were then thoroughly washed again and allowed to return to baseline prior to commencing the individual protocols.

2.6 Specific protocols

Specific protocols are presented in the methods section of each results chapter.

2.7 Data analysis

Data were captured on Myodaq 2.01 multi and analysed using Myodata 2.02 (JP Trading, Aarhus, Denmark). All force data were recorded in mN and time data in seconds. The manner in which data are expressed (i.e. normalised, when this occurred) is described in the methods section of each results chapter. Where efficacy (E_{max}) is reported, it is expressed as a percentage of the tension standard. Logarithmic concentration-response plots were fitted by non-linear regression using the Hill equation (Eq 2.3) and potency (pEC_{50} values) obtained from the lines of best fit using Graphpad Prism 3.00. All data

are presented as the mean ± s.e.m. and n represents the number of animals/patients sampled unless otherwise stated. Statistical analyses were performed using Graphpad Prism 3.00 and original traces transferred for presentation using Origin pro 6.1. Data were compared using the, students t-test or one way analysis of variance (ANOVA) followed by a multiple comparisons test (Tukey's), but where appropriate, normalised data were compared using a non-parametric, Mann Whitney test, a Wilcoxon matched pairs test, or a Kruskal Wallis test. $P<0.05$ was considered to be statistically significant.

Eq 2.3

$$E = \frac{E_{max}}{[1+(D/K_D)]^n}$$

Equation 2.3 The Hill equation

E = response, E_{max} = maximal response, D = drug concentration, K_D = dissociation constant, n = Hill coefficient

2.8 Solutions and Drugs

2.8.1 Mock intra-cellular solutions (permeabilisation studies)

The free Ca^{2+} concentration of the mock intra-cellular (MI) solutions was regulated by the ratio of K_2EGTA/Ca^{2+}EGTA present in the solution. Stock MI solutions of the following composition (mM) were produced fresh each day; KCH_3COO 100, HEPES 25, $MgCl_2$ 5.5, CrP 10, ATP 5, DTT 0.1, K_2/Ca EGTA 10. All MI solutions were adjusted to pH 7.1 using KOH, in order to mimic actual intra-cellular pH. Temperature was maintained at 24^0C in order to maximise the stability of endogenous high-energy compounds such as ATP and creatine phosphate. To preserve pH in the absence of bi-

carbonate (HCO_3^-) all gassing was ceased immediately upon exposure to the MI solutions. ØATP MI solutions were of the same composition as the normal MI solutions but contained no ATP and therefore had their Mg^{2+} content reduced to 1mM.

2.8.2 Preparation of drugs used in permeabilisation studies

β-escin was dissolved in a pCa 5.7 MI solution, whilst all 5-HT agonist stock solutions were dissolved in a pCa 6.64 MI solution. All agonist solutions were adjusted to pH 7.1 at 24^{0}C where required (except for general pharmacology experiments).

2.8.3 Drug procurement

ATP, A 23187, β-escin, Caffeine, L-NAME, 5-HT, 5-CT, and αme 5-HT were obtained from SIGMA (Poole, Dorset, U.K.). Citalopram, Fluoxetine, CP 94253, Thapsigargin and Y-27632 were obtained from Tocris (Avonmouth, Bristol U.K.). ET-1 was obtained from Scientific Marketing Associates (Barnet, Hertfordshire, U.K.). LY 393588 was obtained from Eli Lillcy (Windlesham, Surrey, U.K.). Human urotensin II was a gift from E.H. Ohlstein, GlaxoSmithKline.

Chapter 3

Results Part I

The Role of Human Urotensin II (hU-II) in the Regulation of Pulmonary Arterial Tone

3.1 Introduction

Urotensin II (U-II) was first discovered in extracts from the urophysis of teleost fish just over 35 years ago (Bern & Lederis, 1969). Teleosts are the dominant super-order of bony fish and their urophysis is functionally analogous to the mammalian posterior pituitary. U-II was subsequently isolated and sequenced from the Goby, *Gillichthys mirabilis*, and found to be a "somatostatin like" dodecapeptide (12 amino acid peptide) with a 6 amino acid cyclic region near the C-terminal (Pearson *et al.*, 1980). The extract was found to be vasoactive generally causing smooth muscle contraction including vasoconstriction in the Goby (Bern *et al.*, 1985). In mammalian preparations, U-II has been found to cause both endothelium dependant vasodilatation (Gibson *et al.*, 1986;Gibson, 1987) and endothelium independent contraction (Itoh *et al.*, 1987;Ames *et al.*, 1999;Douglas *et al.*, 2000). It has been reported to be the most potent vasoconstrictor identified to date, being ten-fold more potent than ET-1 in the rat aorta and more than one hundred-fold more potent in the primate femoral artery, however its efficacy is often much lower than that of ET-1 (Ames *et al.*, 1999;Douglas *et al.*, 2000). The vasoactive properties of U-II have been found to be highly variable, demonstrating both species and anatomical variation and have generated a number of conflicting reports (Gibson *et al.*, 1986;Itoh *et al.*, 1987;Itoh *et al.*, 1988;Ames *et al.*, 1999;Douglas *et al.*, 2000). To date there have been fewer than 10 studies that have reported on the effects of U-II pulmonary circulation. Of these, 6 have involved the use of the human isoform

3.1.1 Homologues of urotensin II (U-II)

The strong vasoactive properties of U-II led to the search for homologues in other species and in 1992 a similar but unique form of U-II was isolated and sequenced from frog brain (Conlon *et al.*, 1992). Subsequently, mouse, rat, (Coulouarn *et al.*, 1999) porcine (Mori *et al.*, 1999) and human (Coulouarn *et al.*, 1998) isoforms of U-II have all been cloned.

All forms of U-II demonstrate absolute conservation of the C-terminal cyclic hexapeptide sequence, with the homologues predominantly differing from one another in the first four to seven amino acids of the N-terminal (Douglas & Ohlstein, 2000). The conserved cyclic sequence is thought to be responsible for ligand binding and hence for U-II's biological activity as the different isoforms exhibit similar potencies both *in vitro* (Ames *et al.*, 1999;Liu *et al.*, 1999;Mori *et al.*, 1999;Russell *et al.*, 2001) and *in vivo* (Gardiner *et al.*, 2001). Furthermore, substitution of residues outside of the ring has little effect on the biological activity of the various isoforms, whereas substitution of residues within the conserved ring or disruption of the disulphide-bridge markedly attenuates their biological activity (Brkovic *et al.*, 2003;Labarrere *et al.*, 2003).

3.1.2 Human urotensin II (hU-II)

Human urotensin II (hU-II) is the shortest of the U-II homologues and is an eleven amino acid peptide (Coulouarn *et al.*, 1998). As with all of the homologues of U-II so far identified it retains the conserved cyclic hexapeptide region (-Cys-Phe-Trp-Lys-Tyr-Cys-) of the C-terminal (Fig. 3.1). Reports from several species and various tissues suggest the potency and general effects of hU-II are very similar to that of the other homologues and most animal studies now use hU-II rather than the animals own endogenous homologue (Ames *et al.*, 1999;Liu *et al.*, 1999;Mori *et al.*, 1999;Gardiner *et al.*, 2001;Russell *et al.*, 2001).

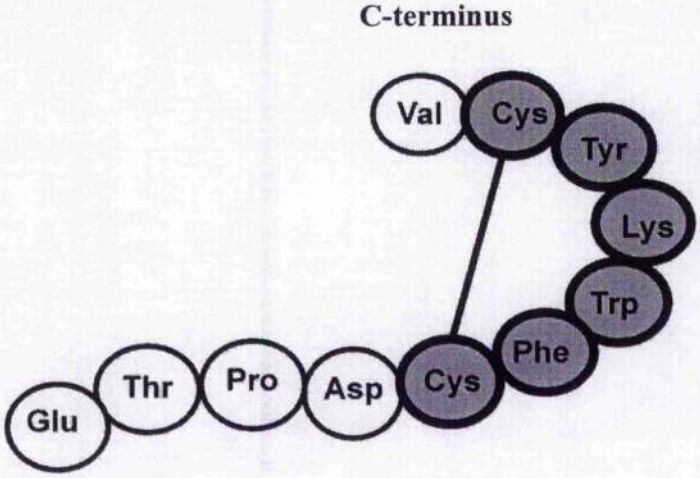


Figure 3.1 The structure of human urotensin II (hU-II) showing the conserved cyclic hexapeptide sequence near the C-terminus

3.1.3 Human urotensin II (hU-II) synthesis

Cloning studies in human tissues have shown high concentrations of precursor hU-II mRNA (Pre-pro hU-II mRNA) in the medulla oblongata and motoneurones of the spinal cord (Coulouarn *et al.*, 1998;Ames *et al.*, 1999;Nothacker *et al.*, 1999;Coulouarn *et al.*, 2001). Additionally, lower concentrations of prepro hU-II mRNA were also found in peripheral tissues such as the kidneys (Coulouarn *et al.*, 1998;Nothacker *et al.*, 1999) adrenals and spleen (Coulouarn *et al.*, 1998), although initially none was reported in cardiovascular tissues (Ames *et al.*, 1999;Nothacker *et al.*, 1999). However more recently, quantitative RT PCR has shown hU-II mRNA to be abundantly expressed in the adrenals and kidney, as well as in the pituitary, placenta, and colonic mucosa (Totsune *et al.*, 2001). Furthermore, lower levels of expression were also reported in the heart, aorta, thoracic arteries and saphenous vein (Matsushita *et al.*, 2001;Totsune *et al.*, 2001) and in coronary artery and umbilical vein endothelial cells (Totsune *et al.*, 2003). It is interesting to note that three of the sites implicated in hU-II synthesis, the medulla, the kidneys and the adrenals, play important roles in the regulation of blood pressure.

In humans, two pre-pro hU-II peptides have been identified and are 124 and 139 residue alternate spliced variants (Coulouarn *et al.*, 1998;Ames *et al.*, 1999). Both of these variants encode identical mature hU-II, which is thought to be liberated following the processing of a putative polybasic KKR site (Ames *et al.*, 1999;Chartrel *et al.*, 2004). Recent evidence suggests that pre-pro hU-II undergoes post-translational cleavage within the cells that produce it by a putative, U-II converting enzyme (UCE). One of the proteolytic enzymes proposed to be UCE is Furin, a type I endoprotease which is also involved in the cleavage of Pre ET-1 (Russell *et al.*, 2004).

3.1.4 Human urotensin II (hU-II) localisation

Immunohistochemical studies using human and monkey tissues, have shown U-II like immunoreactivity not only in the spinal cord motoneurones but also in vascular and cardiac tissue, including human cardiomyocytes and coronary atheroma (Ames *et al.*, 1999). This has been corroborated by Maguire *et al.*, (2000) who found the highest levels of immunoreactivity in skeletal muscle, and low levels in the left ventricle, aorta and coronary arteries. The presence of hU-II in the vasculature has been reported both in endothelial cells (Maguire & Davenport, 2002;Maguire *et al.*, 2004) and in SMCs (Ames *et al.*, 1999). Circulating plasma levels of hU-II are relatively low (5 –70pM) (Totsune *et al.*, 2001;Dschietzig *et al.*, 2002;Totsune *et al.*, 2003;Totsune *et al.*, 2004), well below reported pEC_{50} values, which are typically in the low nM range. Hence it is thought that, like ET-1, hU-II may principally act as a paracrine/autocrine agent rather than as a circulating hormone (Totsune *et al.*, 2001). Furthermore, like ET-1, elevated plasma levels of hU-II have been reported in conditions such as diabetes mellitus and cardiovascular disease (Totsune *et al.*, 2003;Lapp *et al.*, 2004;Thanassoulis *et al.*, 2004;Totsune *et al.*, 2004).

3.1.5 Human urotensin II receptor(s) (UT-II)

The first receptor for U-II was recently discovered by Ames *et al.*, (1999), using what has been termed as reverse pharmacology. Whilst attempting to identify novel G-protein coupled receptors (GPCR) a human seven transmembrane orphan GPCR with 75% homology to the rat GPR14 (SENR), was isolated and labelled human GPR14 (now known as UT-II). Within two months of these findings, three other independent groups (Liu *et al.*, 1999;Mori *et al.*, 1999;Nothacker *et al.*, 1999) had reported the action of various U-II homologues (fish, frog, porcine and human) on the rat GPR14 confirming U-II as the ligand for GPR14. UT-II is thought to be selective for U-II homologues as out of the 700 or so ligands tested, not even somatostatin, which shares the closest structural similarity to U-II, competed for binding, although it should be noted that ET-1 was not tried (Ames *et al.*, 1999).

UT-II mRNA has been found to be widely expressed both within the central nervous system and peripheral tissues of mammals (Ames *et al.*, 1999;Liu *et al.*, 1999;Nothacker *et al.*, 1999;Gartlon *et al.*, 2001;Maguire & Davenport, 2002). In human peripheral tissues, UT-II mRNA has been reported in the heart (atria and ventricles), aorta, thoracic arteries as well as in the lung, kidneys, pancreas, adrenals and tumour cell lines (Douglas & Ohlstein, 2000;Matsushita *et al.*, 2001;Totsune *et al.*, 2001;Maguire & Davenport, 2002;Takahashi *et al.*, 2003). On a cellular level, UT-II mRNA was found to be present both in arterial smooth muscle and endothelial cells consistent with hU-II's action as an endothelium independent vasoconstrictor and an endothelium dependant vasodilator (Ames *et al.*, 1999;Maguire, 2003). These findings are consistent with UT-II receptor protein expression which was also detected in human vascular smooth muscle and endothelial cells and cardiac myocytes (Maguire *et al.*, 2000;Douglas *et al.*, 2002)

3.1.6 hU-II signal transduction

The UT-II receptor is believed to be a G-protein coupled receptor ($G_{q/11}$ family) as its activation is linked to PLC stimulated phosphoinositide turnover and IP_3 production (Sactrum *et al.*, 2000). Binding of hU-II to UT-II has been shown to cause both Ca^{2+} mobilisation (Ames *et al.*, 1999;Liu *et al.*, 1999;Nothacker *et al.*, 1999;Sauzeau *et al.*, 2001) and Ca^{2+} sensitisation in rat systemic arterial SMCs through activation of the small GTPase RhoA and its down-stream effector, Rho kinase (ROK) resulting in vasoconstriction and proliferation (Sauzeau *et al.*, 2001). Additionally activation of the UT-II receptor has also been associated with activation of PLA_2 and arachidonic acid metabolism (Mori *et al.*, 1999). Vasodilatation is presumed to occur in as a result of Ca^{2+} mobilisation in endothelial cells triggering the release of endothelial derived relaxing factors, however the specific nature of the agents released seems to vary between vascular beds. NO, PGI_2 and endothelial derived hyperpolarising factor (EDHF) have all been implicated (Bottrill *et al.*, 2000;Gardiner *et al.*, 2004).

3.1.7 The pharmacological profile of hU-II as a vasoactive agent

Most of the *in vitro* responses to hU-II observed to date have been limited to the arterial side of the vasculature and are consistent with the initial findings of UT-II/GPR 14 expression by RT PCR (Ames *et al.*, 1999) (Table 1). This has led to hU-II being labelled as an arterial selective spasmogen (Newby & Jalan, 2000). Although a human (Maguire *et al.*, 2000), and a primate (Hay *et al.*, 2000) study have reported venous responses to hU-II, the constriction was of very low efficacy (<20% of the tension standard). Furthermore, in the case of the human saphenous vein there have also been two contradictory reports that found no response to hU-II in this vessel (Hillier *et al.*, 2001;Paysant *et al.*, 2001).

hU-II also demonstrates species selectivity. For example, in the Cynomolgus monkey (old world primate)(Douglas *et al.*, 2000) and the cat (Behm *et al.*, 2004), every arterial vessel tested to date has demonstrated a potent and very efficacious vasoconstrictor response, whereas in the marmoset (new world primate), mouse (Douglas *et al.*, 2000) and human vessels (Maguire *et al.*, 2000;Hillier *et al.*, 2001;Paysant *et al.*, 2001), contractile responses have either been entirely absent or where present, of very low efficacy (Table 1). This species selectivity is not simply the result of vasoconstriction being offset by an opposing vasodilator action as in many of these vessels the responses were unaffected by removal of the endothelium (Douglas *et al.*, 2000).

Consistent with the earliest *in vivo* report of the vasodilator action of U-II in the anaesthetised rat (Gibson *et al.*, 1986), *in vitro* hU-II has been reported to cause endothelial dependant vasodilatation in rat and human isolated arteries (Bottrill *et al.*, 2000;Stirrat *et al.*, 2001). However, in keeping with the complicated pattern of mammalian hU-II pharmacology, the mechanism by which this occurs seems to vary between vascular beds, with NO reported to be the mediator in rat coronary arteries, EDHF thought to be responsible in rat small mesenteric arteries (Bottrill *et al.*, 2000), and prostanoids being reported to be involved in hindquarter vasodilatation in the rat *in vivo* (Gardiner *et al.*, 2004).

In vivo studies of hU-II have been performed in the rat, Cynomolgous monkey and humans. The findings of these studies highlight the species-specific response to hU-II and seem to be broadly consistent with what has been established from *in vitro* studies so far. In the rat, hU-II infusion caused an initial reduction (at high concentrations ~3nM) in mean arterial pressure (MAP), which was characterised by mesenteric and hindquarter vasodilatation and non-reflex tachycardia. However this was subsequently followed by a

Table 1

Vessels	Mouse	Rat	Cat	Dog	Pig	Marmoset	Cynomolgus monkey	Human
Systemic arteries								
Thoracic aorta	0/	+++/0	+++/	0/	++?/	+/	+++/	
Common carotid		++/	+++/	0/	++?/	+?/	+++/	
Abdominal aorta	0/	+/		0/	+?/	+?/	+++/	
Femoral		0/---?	+++/	0/	+?/	0/	+++/	
LCX coronary				+++/	0/		+++/	+?/
LAD coronary		++?/--		+++/			+++/	0/
Basilar		+/-		0/			+++/	
Internal mammary				0/	0/		+++/	+?/
Renal		0/	+++/	0/	0/		+++/	
Mesenteric		0/---	+++/	0/	++?/	+?/	+++/	
Radial								+?/
Subcutaneous								0/
Abdominal arterioles			+++/					/--
Pulmonary arteries								
Extra-lobar		++/		0/	++?/		+++/	
Intra-lobar		0/						0/--
Systemic Veins								
Saphenous				0/	0/		0/	+?/
Jugular		0/		0/			0/	
Portal							0/	
Subcutaneous								0/
Umbilical		0/		0/			0/	+/
Pulmonary veins								
Adjacent to atria								+/

Table 1. Summary of the *in vitro* responses to the peptide human urotensin II (hU-II) performed in isolated vessels with an intact endothelium, taken from various species and vascular beds

+ vasoconstriction, - vasodilatation, 0 no response, blank response not tested.

+ < 25% of the tension standard, low efficacy, ++ 25%-75% moderate efficacy, +++ >75% high efficacy, same scale for dilatation.

? variable response, where vessels have regularly failed to respond within a study and/or reports suggesting no response from other authors.

slow onset increase in MAP after ~30 –120mins (Gardiner *et al.*, 2001;Gardiner *et al.*, 2004).

In contrast, hU-II infusion in the Cynomolgous monkey caused a massive increase in peripheral resistance and cardiac dysfunction resulting in circulatory collapse, which is entirely consistent with the constrictor profile form *in vitro* studies (Ames *et al.*, 1999). *In vivo* responses in humans are conflicting with localised vasoconstriction (Bohm & Pernow, 2002) and vasodilatation (Lim *et al.*, 2004) reported with administration of hU-II to specific vascular beds. However, general i.v. infusion of hU-II has been reported to have no effect on haemodynamics or arterial compliance even at high concentrations (Affolter *et al.*, 2002;Wilkinson *et al.*, 2002) and seems to be consistent with the generally low efficacy or unresponsive reports from *in vitro* studies.

3.1.8 hU-II, pulmonary vascular regulation and pulmonary arterial hypertension

hU-II has recently been reported to cause vasodilatation (Stirrat *et al.*, 2001) but not vasoconstriction of normal human small pulmonary arteries (MacLean *et al.*, 2000a;Bennett *et al.*, 2004). Additionally, vasoconstriction of the main pulmonary artery has been reported in the Cynomolgous monkey, pig (Douglas *et al.*, 2000) and rat (MacLean *et al.*, 2000a). These findings suggest a role for hU-II in the regulation of pulmonary arterial tone and raise the question of whether hU-II could be involved in the pathological changes of PAH. Models, which mimic the pathological changes of PAH such as medial remodelling due to chronic hypoxia, replicated endothelial dysfunction due to inhibition of NOS by L-NAME and elevated tone using ET-1, have all been reported to enhance responses to endogenous vasoconstrictors in the pulmonary circulation (MacLean, 1999). It is therefore interesting to note that in human small pulmonary arteries pre-treated with the NOS inhibitor L-NAME, a highly variable

contractile response has been reported in 30% of vessels tested, suggesting a possible role for hU-II in the pathogenesis of PAH, although the vessels did not respond in the presence of ET-1 (MacLean *et al.*, 2000a). However, the dysfunctional endothelial hypothesis of hU-II's action has recently been questioned, with evidence that neither hU-II nor gU-II caused increased constriction of human pulmonary vessels obtained from 20 patients who might be expected to exhibit some degree of endothelial dysfunction, including those with hypertension, coronary artery disease, peripheral vascular disease, or diabetes (Bennett *et al.*, 2004). Although the vessels obtained were not from PAH patients, no contractile effects to hU-II were observed despite some vessels having a dysfunctional endothelium as indicated by the absence of vasodilatation to Ach in pre-constricted vessels. The same group also reported that hU-II failed to induce either vasoconstriction or vasodilatation in the isolated perfused lung (Bennett *et al.*, 2004).

In addition its action on arterial preparations, hU-II has also been reported to be a potent vasoconstrictor of primate airways and pulmonary veins. However only those veins which were directly adjacent to the atria responded and hU-II had no effect on the more distal veins (Hay *et al.*, 2000).

Plasma levels of hU-II have not been measured in PAH patients to date, but in chronic heart failure patients (left heart failure), reports are conflicting with both an elevation (Douglas *et al.*, 2002;Russell *et al.*, 2003) and no change of hU-II plasma levels being reported (Dschietzig *et al.*, 2002). Most PAH patients eventually die of right heart failure.

In the rat, it has been reported that the efficacy but not the potency of the main pulmonary artery's contractile response to hU-II was increased ~ 60% by incubation with the NOS inhibitor L-NAME. However it was also reported that removal of the endothelium by mechanical denudation or raising the vessel tone with ET-1 prior to hU-II exposure did

not increase the efficacy of the hU-II response, although it did increase its potency some two and a half-fold (MacLean *et al.*, 2000a).

The chronically hypoxic rat model of PAH, which exhibits medial hypertrophy, increased PAP and right-sided heart failure, has also been reported to show an enhanced contractile response to hU-II with an ~80% increase in efficacy but no change in potency (MacLean *et al.*, 2000a). However, although hU-II content has been reported to be increased two-fold in the right ventricle of chronically hypoxic rats consistent with the development of right ventricular hypertrophy, it was also reported that there was no increase in either plasma hU-II levels or lung tissue concentration of hU-II following exposure to chronic hypoxia (Zhang *et al.*, 2002). These findings would suggest that any pathogenic changes in the hU-II response of the chronically hypoxic rat, may occur at a receptor or intra-cellular signalling level, for example through increased receptor expression or increased efficiency of G-protein coupling.

3.1.9 Aims

The aims of this chapter were three-fold. 1) To pharmacologically profile the vasoconstrictor and vasodilator responses to hU-II in the mouse, rat and human, pulmonary arterial systems. 2) To determine if responses to hU-II are augmented in "single aspect" models of PAH. 3) To determine if responses to hU-II are further enhanced in combined models of PAH (e.g. combinations of chronic hypoxia, NOS inhibition and ET-1 raised tone).

3.2 Methods

Mouse, rat and human pulmonary arteries were dissected out and set up as described in the general methods section. PH was confirmed in animals exposed to 2weeks chronic hypoxia by assessment of the RV/TV ratio (see chapter 2). Vessels were normalised using tensions, which created the equivalent of a transmural pressure of either 12-16mmHg (to mimic normotensive *in vivo* pressures) or 30–35mmHg (to mimic hypertensive *in vivo* pressures). Following normalisation, the response of each vessel to 50mM KCl was established and used as the tension standard. Endothelial function was then established as described in chapter 2. The following studies where then performed:

3.2.1 Responses to hU-II in control, isolated rodent and human pulmonary arteries

3.2.1.1 Vasoconstriction

To determine the vasoconstrictor profile of hU-II, cumulative concentration response curves (CCRCs) (1pM - 1μM) were constructed using the following vessels; Mouse; extra-lobar A and B-class, intra-lobar C-class. Rat; extra-lobar A and B-class, intra-lobar C and D-class. Human; intra-lobar 750-1500μm (size equivalent to C-class rat vessels) and 150-300μm (size equivalent to D-class rat vessels).

3.2.1.2 Vasodilatation

To determine the dilator profile of hU-II, tone was raised using 0.1μM PE (mouse) or 1-10nM ET-1 (rat) in paired vessels from the same animal, taken from the following sources. Mouse; extra-lobar A and B-class, intra-lobar C-class. Rat; extra-lobar A and B-class, intra-lobar C and D-class. Following establishment of a stable raised tone, CCRCs to hU-II (1pM - 1μM) were constructed using one of each of the paired vessels, whilst the remaining vessel of the pair acted as a time control to determine the natural

rate of deterioration of the raised tone. The hU-II dilator response in the human small pulmonary artery has previously been reported (Stirrat *et al.*, 2001).

3.2.2 Contractile responses to hU-II in single aspect models of pulmonary arterial hypertension

3.2.2.1 Wistar rat pulmonary arteries

To determine if the contractile effects of hU-II could be enhanced in PAH, CCRCs to hU-II (1pM - 1μM) were constructed under 3 separate conditions (each modelling a single aspect of PAH) in the following rat pulmonary arteries; extra-lobar A and B-class, intra-lobar C and D-class. The conditions were. 1) Inhibition of nitric oxide synthase (NOS) with 100μM L-NAME (20mins incubation), to mimic endothelial dysfunction. 2) ET-1 (1-3nM) induced tone to mimic elevated basal tone. 3) Vascular remodelling by using vessels from animals that had been exposed to 2weeks chronic hypoxia.

3.2.2.2 Human pulmonary arteries

To confirm reports that contractile responses are uncovered by NOS inhibition in human intra-lobar pulmonary arteries and determine their suitability for receptor characterisation, hU-II, CCRCs (1pM - 1μM) were constructed using the following vessels which had been incubated (20mins) with 100μM L-NAME; human intra-lobar; 750-1500μm (size equivalent to C-class in rat vessels) and 150-300μm (size equivalent to D-class rat vessels).

3.2.3 Contractile responses to hU-II in Wistar rat pulmonary arteries in the presence of increased resting tension

Observations from the models of PAH suggested that increased tension might unmask an augmented contractile response to hU-II. To investigate this hypothesis further hU-II,

CCRCs (1pM - 1μM) were constructed using the following rat pulmonary arteries; extra-lobar A and B-class, intra-lobar C and D-class, both in the presence and absence of L-NAME at tensions which mimicked a transmural pressure equivalent to 30-35mmHg.

3.2.4 Contractile responses to hU-II in combination models of pulmonary arterial hypertension in the Wistar rat

As some individual A-class rat pulmonary arteries had shown a modified response to hU-II under the single aspect models of PAH and all had demonstrated a marked increase in efficacy under conditions of elevated tension, it was hypothesised that these responses may be further enhanced in a combination model of PAH, which simultaneously mimicked several aspects of PAH. Similarly the uncovering of a small contractile response in the chronically hypoxic rat, D-class (SPA) vessels suggested a possible role in the increased vasoconstriction of PAH, which predominantly occurs at this site. To determine if the contractile effects of hU-II could be enhanced in combination models of PAH, CCRCs to hU-II (1pM - 1μM) were constructed in the combined presence of NOS inhibition and ET-1 raised tone using A and D-class vessels from chronically hypoxic rats.

3.3 Results

3.3.1 Responses to hU-II in control, isolated rodent and human pulmonary arteries

The aim of the first series of experiments was to systematically determine the vasoactive profile of hU-II in the mouse and rat pulmonary arterial circulations. This approach was intended to identify which animal(s) and vessel class(es) would be suitable for further study under modelled conditions of PAH.

3.3.1.1 Mouse

hU-II failed to induce either vasoconstriction or vasodilatation in any of the vessels studied (Vasoconstriction; A, B, C-class (all groups) n=8, vasodilatation; all groups n=4). However, hU-II did cause a potent (pEC_{50} 7.53 ± 0.20) and sustained contraction in the aorta that was 21± 6% (n=8) of the 50mM KCl tension standard response, confirming the hU-II peptide was active.

The mean internal diameter and replicated transmural pressure of each vessel class was as follows: A-class; 1037 ± 41μm / 15.1 ± 0.4mmHg, B-class; 759 ± 44μm / 14.7 ± 0.4mmHg, C-class; 383 ± 18μm / 13.5 ± 0.3mmHg (all groups n=12). Endothelial function of A-class vessels used in the vasodilatation study was confirmed by Ach induced relaxation (30 ± 5%, n=4) of 0.1μM PE induced tone. However, endothelial function could not be routinely confirmed in the B and C-class vessels. The internal diameter, replicated transmural pressure and PE raised tone used during the hU-II vasodilatation CCRC, was not different between the time control and response vessels in any group (P>0.05).

3.3.1.2 Rat

hU-II had a potent, but low efficacy, constrictor action on A-class (Emax: 19 ± 4% (of the response to 50mM KCl); pEC_{50} 8.64 ± 0.17, n=9 rats), B-class (Emax: 9 ± 2%; pEC_{50} 8.57 ± 0.16, n=13) and C-class vessels (Emax: 13 ± 3%; pEC_{50} 8.54 ± 0.19, n=14), but had essentially no action on the D-class vessels (n=14) (Fig 3.2 & 3.3).

hU-II failed to elicit a dilator response in any of the ET-1 pre-constricted rat vessels (n≥6, (all groups (A,B,C,D-class)). Endothelial function of all A, B, C-class and 3/6 D-class response vessels in the vasodilatation study was confirmed by Ach induced relaxation of PE raised tone prior to the hU-II CCRC (A-class relaxation to Ach; 58 ± 12% of the phenylephrine induced tone, B-class; 53 ± 13%, C-class; 63 ± 9%, D-class; 16 ± 5%, n=6-7 all groups). Furthermore the ET-1 raised tone was found to be attenuated by Ach in a dose dependant manner in randomly selected vessels following the hU-II CCRC. There was no difference in internal diameter, replicated transmural pressure or the magnitude of the ET-1 raised tone between the time control and response vessels in any group (P>0.05).

The mean internal diameter and replicated transmural pressure of each vessel class was as follows: A-class; 2433 ± 25μm / 15.9 ± 0.2mmHg, n=15, B-class; 1426 ± 41μm / 13.8 ± 0.2mmHg, n=19, C-class; 959 ± 31μm / 13.5 ± 0.2mmHg, n=22, D-class; 217 ± 12μm / 14.7 ± 0.3mmHg, n=21.

3.3.1.3 Human

hU-II failed to cause a constrictor response in any of the vessels tested (750-1500μm C-class size equivalent n=3, 150-300μm D-class size equivalent n=4). The mean internal diameter and replicated transmural pressure of the vessels was as follows: C-class size

equivalent; 874 ± 76μm / 14.0 ± 0.7mmHg, n=3, D-class size equivalent; 270 ± 9μm / 14.8 ± 0.4mmHg, n=4.

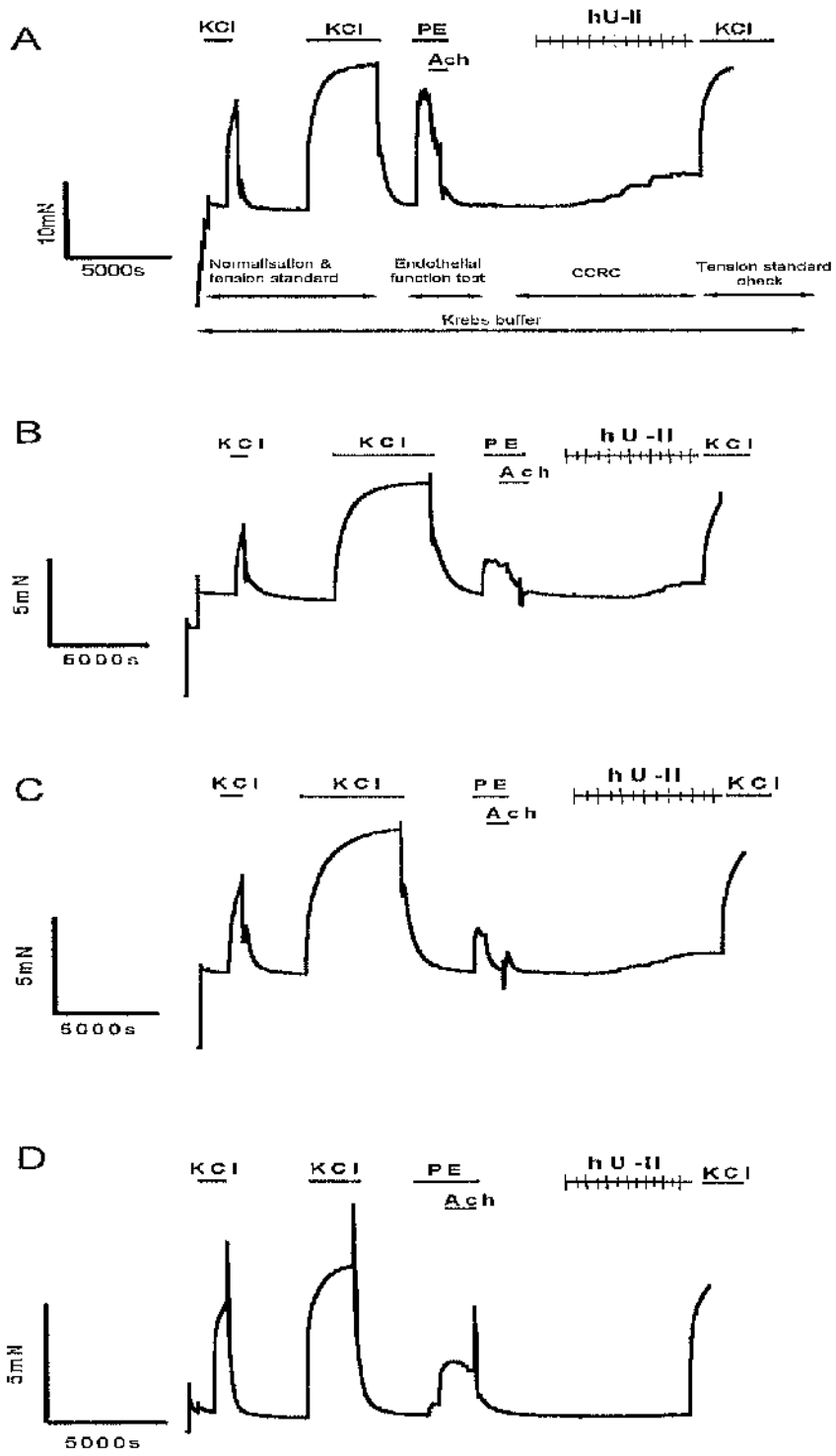


Figure 3.2 The contractile profile of hU-II in the pulmonary circulation of the Wistar rat

Experimental traces showing the protocol used to construct hU-II CCRCs in A) Extra-lobar A-class vessels B) Extra-lobar B-class vessels C) Intra-lobar C-class vessels and D) Intra-lobar D-class vessels. hU-II was added in 13 incremental steps over the range 10^{-12} – 10^{-8}M.

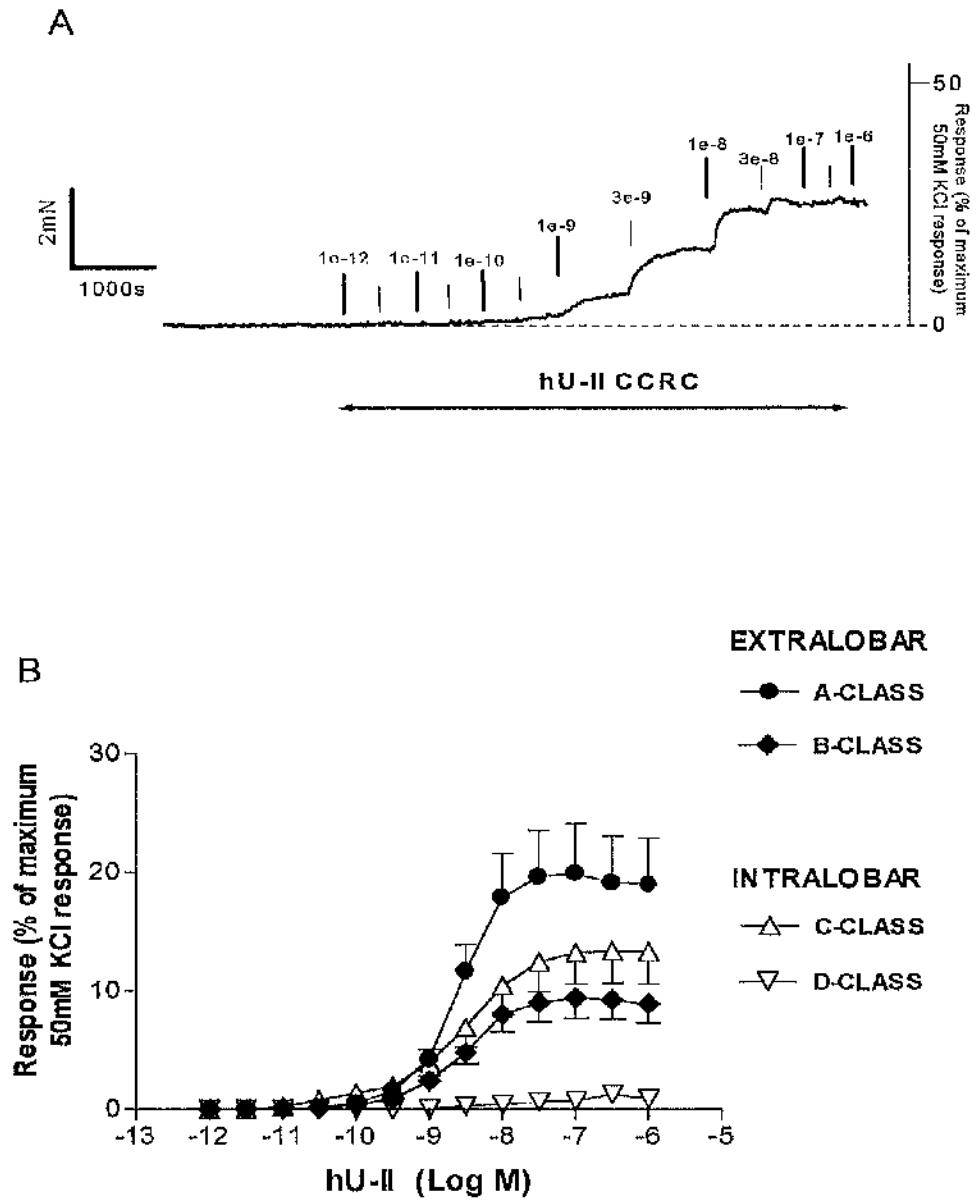


Figure 3.3 hU-II CCRCs in Wistar rat pulmonary arteries

A) Experimental trace showing a typical hU-II CCRC in an A-class vessel. B) Graph showing the potent, but low efficacy constrictor profile of hU-II in extra-lobar and intra-lobar vessels. Extra-lobar; A-class n=9, B-class n=13, Intra-lobar; C-class n=14, D-class n=14.

3.3.2 Contractile responses to hU-II in single aspect models of pulmonary arterial hypertension

3.3.2.1 Wistar rat pulmonary arteries

Inhibition of nitric oxide synthase (NOS) using L-NAME

Incubation with the nitric oxide synthase inhibitor L-NAME (100μM) caused an increase in tone in A, B and C-class vessels, but only elvated tone in 3/11 D-class vessels (L-NAME tone: A-class; 3 ± 1% of the 50mM KCl response, B-class; 5 ± 1%, C-class; 15 ± 3%, D-class; <1 ± 1%, n ≥ 10 all groups). The presence of L-NAME induced tone failed to significantly enhance either the efficacy or potency of the contractile response to hU-II in any vessel class (n ≥ 10 all groups, P>0.05, all unpaired t-test), although a small but rapid spiking phenomenon was uncovered in 3/10 A-class vessels. This phenomenon, was not observed in any other vessel group (Fig 3.4).

Elevated vascular tone induced by endothelin 1 (ET-1)

Elevating vascular tone by 10-20% (of the 50mM KCl tension standard) with ET-1 (1-3nM) failed to significantly increase either the efficacy or potency of the contractile response to hU-II in any vessel class tested (n ≥ 6 vessel groups A, B, D, P>0.05, all unpaired t-test). However the presence of ET-1 induced tone did uncover a rapid spiking phenomenon in 4/6 A-class and 1/8 B-class vessels challenged with hU-II. This occurred over the hU-II concentration range of 3nM - 0.1μM. In 2 A-class vessels the ET-1 associated spiking phenomenon was substantial (~80-90% of the 50Mm KCl tension standard), however it should also be noted a similar unusually large response also occurred in 1 control vessel. The frequency of the phenomenon did not appear to be modulated by hU-II (Fig 3.5).

Vascular remodelling associated with chronic hypoxia

2weeks chronic hypoxia failed to enhance either the efficacy or potency of the contractile response to hU-II in A, B and C-class vessels (A and B-class; n≥7, $P>0.05$, unpaired t-test). In fact, hypoxia caused a significant attenuation in the C-class vessel response to hU-II (AM C-class Emax; 11 ± 3% of the 50mM KCl response, n=7 vs CH; 3 ± 2%, n=8, $P<0.05$, unpaired t-test). 2weeks chronic hypoxia did however, cause a very small but significant increase in the D-class contractile response to hU-II (AM; <1 ± 0.4% vs CH; 6 ± 2%, n=8, $P<0.05$ unpaired t-test) (Fig 3.6).

Vessel's from chronically hypoxic rats, which did not stabilise to a true basal tone by the 3rd KCl exposure where discarded from the study. Unstable, A-class vessels from chronically hypoxic animals demonstrated a rapid spiking behaviour, which spontaneously and progressively increased basal tone until the vessels were often near maximal contraction. When these vessels were incubated with 2 APB, which competitively blocks IP_3 receptors, the spiking phenomenon immediately ceased and the vessels returned to a stable basal tone. Studies performed in A-class vessels in the presence of 2 APB, found that unstable vessels from hypoxic rats did not show an enhanced response to hU-II relative to control vessels under identical conditions (AM; 7 ± 2% of the 50mM KCl tension standard response, n=5 vs CH; 5 ± 2%, n=6, $P>0.05$ unpaired t-test). The RV/TV ratio was significantly larger in the chronically hypoxic groups and was consistent with the development of PH secondary to chronic hypoxia (AM; 0.217 ± 0.002 n=12 vs CH; 0.318 ± 0.008, n=12, $P<0.0001$, unpaired t-test).

The internal diameters and replicated transmural pressures of the age matched control vessels and those vessels incubated with L-NAME or ET-1, were not different and were comparable with those of the vessels reported in results section 3.3.1.2 (n ≥ 6 all groups, $P>0.05$, unpaired t-test). Although the internal diameters of the age matched control and

chronically hypoxic vessels were not different (n ≥ 7 all groups, P>0.05, unpaired t-test), the initial replicated transmural pressure was at least two-fold greater in all chronically hypoxic vessel goups relative to the control vessel groups (A-class; 33.3 ± 0.9 mmHg vs 15.8 ± 0.4mmHg, B-class; 33.9 ± 0.6mmHg vs 13.4 ± 0.3mmHg, C-class; 32.4 ± 0.8mmHg vs 13.2 ± 0.2mmHg, D-class; 32.9 ± 0.8mmHg vs 15.1 ± 0.5mmHg, n ≥ 7 all groups, P<0.0001, unpaired t-tests).

3.3.2.2 Human pulmonary arteries

Inhibition of nitric oxide synthase (NOS) with L-NAME

Inhibition of NOS using L-NAME failed to unmask a contractile response to hU-II in any of the human pulmonary arteries tested (750-1500μm C-class size equivalent n=3, 150-300μm D-class size equivalent n=4). The internal diameters and replicated transmural pressures of those vessels incubated with L-NAME, were comparable with those of the control vessels (n =3-4, P>0.05, unpaired t-test).

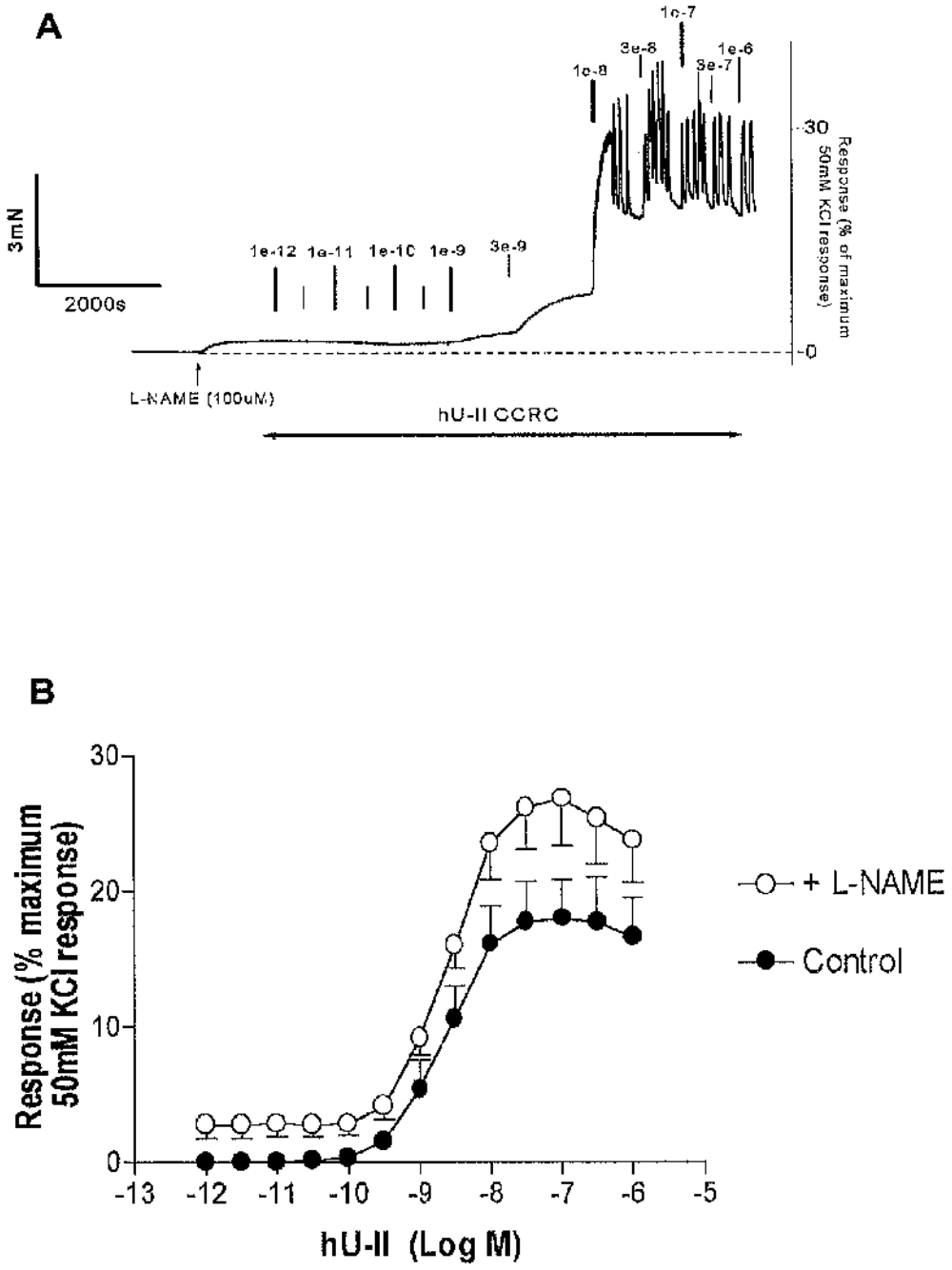
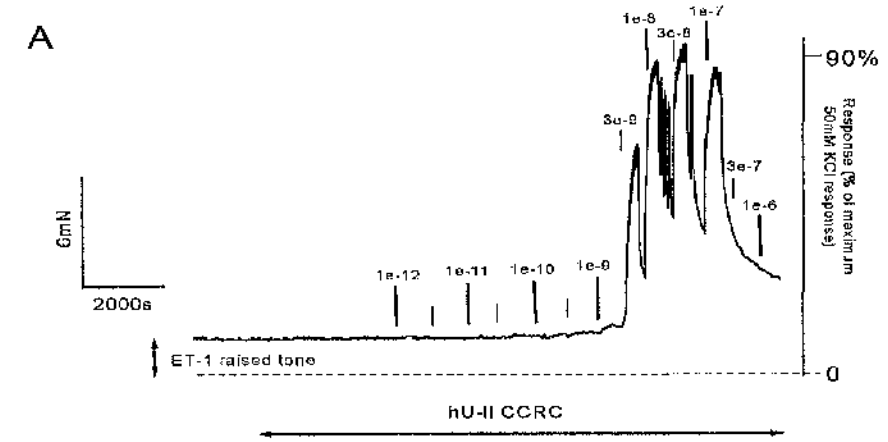


Figure 3.4 The effect of nitric oxide synthase (NOS) inhibition on the hU-II response in Wistar rat, A-class (extra-lobar) pulmonary arteries

A) Experimental trace showing the small spiking phenomenon observed in 3/10 vessels during the performance of the hU-II CCRC. B) Graph comparing hU-II CCRCs performed in A-class extra-lobar vessels in the presence and absence of the NOS inhibitor L-NAME (n=10/10, $P>0.05$). The tone associated with L-NAME incubation, which is shown in the figure was subtracted prior to statistical comparison.



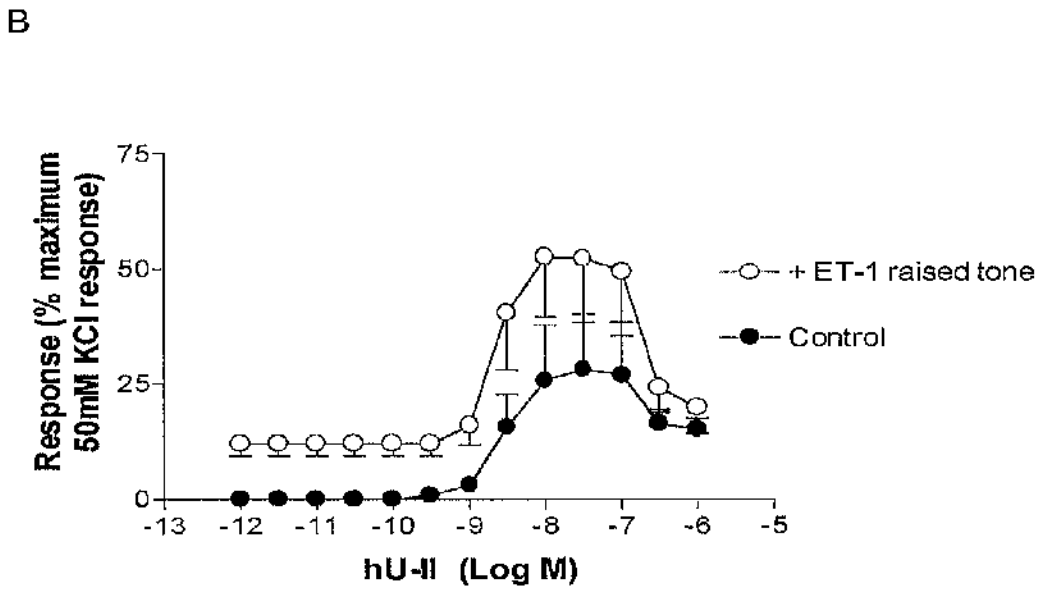


Figure 3.5 The effect of ET-1 raised tone on the hU-II response in Wistar rat, A-class (extra-lobar) pulmonary arteries

A) Experimental trace showing the unusually large spiking phenomenon observed in 2/6 vessels during the performance of the hU-II CCRC in the presence of ET-1 raised tone. A similar response was also observed in 1 control vessel. B) Graph comparing hU-II CCRCs performed in A-class extra-lobar vessels in the presence and absence of ET-1 raised tone (n=6/8, $P>0.05$). The tone associated with ET-1 which, is shown in both the trace and the graph was subtracted prior to statistical comparison.

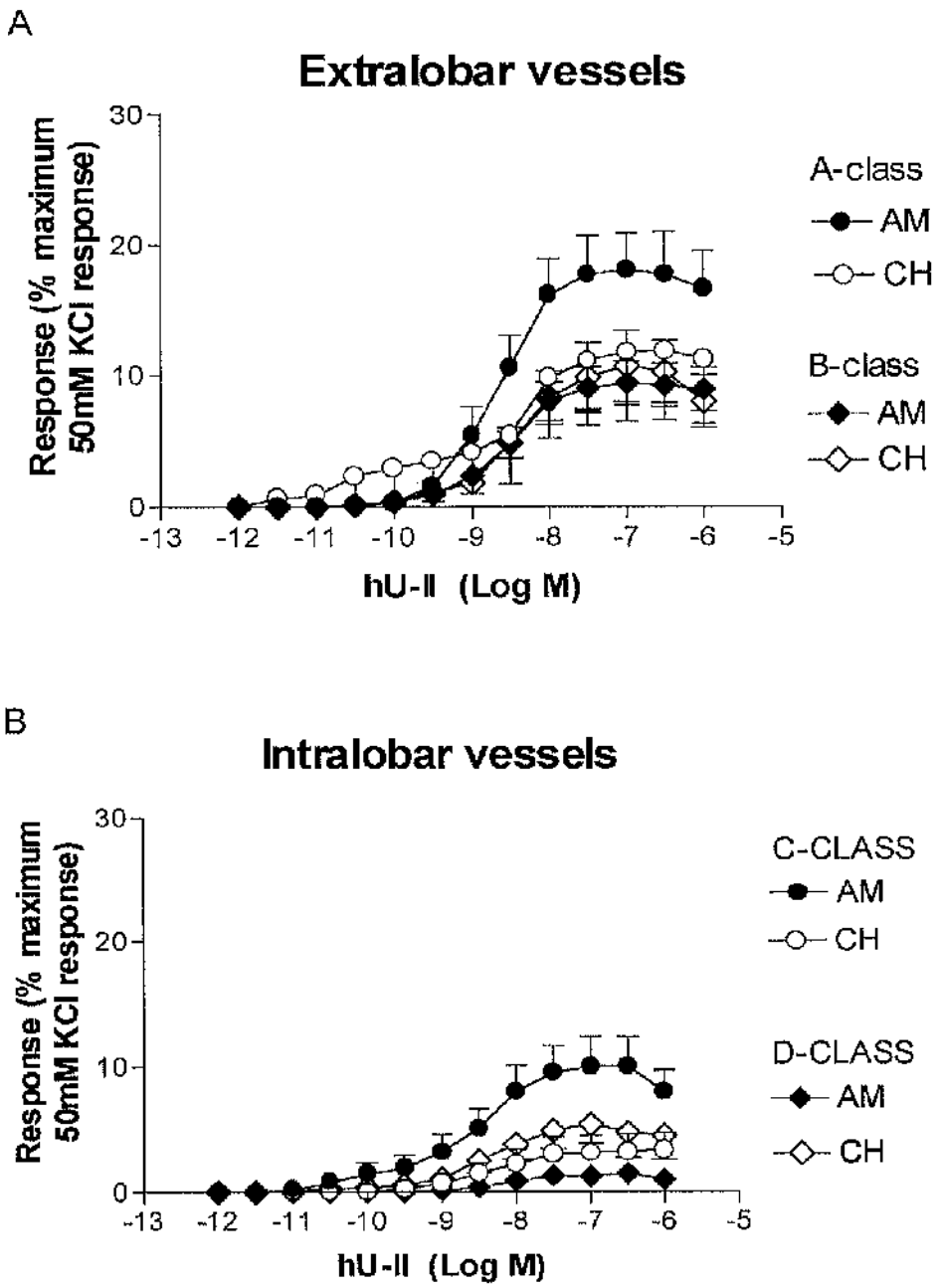


Figure 3.6 The effect of 2weeks chronic hypoxia on the hU-II response in Wistar rat, extra-lobar and intra-lobar pulmonary arteries.

A) Extra-lobar vessels; A-class (n=8/8, $P>0.05$), B-class (n=8/7, $P>0.05$). B) Intra-lobar vessels C-class (n=7/8, $P<0.05$), D-class (n=8/8, $P<0.05$). AM = age matched control vessels - Black symbols, CH = vessels from rats exposed to chronic hypoxia – White symbols.

3.3.3 Contractile responses to hU-II in Wistar rat pulmonary arteries in the presence of increased resting tension

Increasing resting tension by additional stretch of the vessel wall, mimicked a two-fold increase in transmural pressure (30-35mmHg) but failed to significantly enhance either the efficacy or potency of the contractile response to hU-II in any vessel class (n ≥6 all groups (A,B,C,D class), P>0.05 unpaired t-test). However the increased tension did uncover a small but rapid spiking phenomenon at hU-II concentrations above 3-10nM in 4/7 A-class vessels. This phenomenon was also observed in 1/12 B-class group vessels (not shown).

In contrast, the combination of increased resting tension and NOS inhibition by L-NAME caused a substantial three-fold increase in the efficacy, but not the potency of the A-class hU-II contractile response. The response was characterised by the onset of a large rapid spiking phenomenon in 7/7vessels at hU-II concentrations of ≥3nM (AM control hU-II response Emax; 18 ± 3% of the 50mM KCl response, n=7 vs AM 35mmHg + L-NAME hU-II response Emax; 65 ± 5%, n=7, P<0.0001, unpaired t-test) (Fig 3.7). Incubation with 2 APB completely inhibited both the L-NAME induced elevation in basal tone and the hU-II CCRC associated spiking phenomenon in these vessels. In the presence of 2 APB, efficacy was no longer significantly different from that of control vessels treated with 2 APB (AM 16mmHg control response Emax; 7 ± 2% of the 50mM KCl response, n=5 vs AM 35mmHg + L-NAME response Emax; 9 ± 1%, n=8, P>0.05, unpaired t-test).

The combination of increased resting tension and NOS inhibition did not significantly increase the efficacy or potency of the hU-II contractile response in B, C or D-class vessels (n≥7, all groups, P>0.05) although the spiking phenomenon was observed more frequently in B and C-class vessels.

The internal diameters and replicated transmural pressures were significantly larger in the A, B and C-class elevated tension vessels relative the the controls (A-class; 3703 ± 60μm/ 32.7 ± 0.5 mmHg vs 2597 ± 28μm/ 14.7 ± 0.2mmHg, B-class; 2142 ± 115μm/ 33.5 ± 0.5mmHg vs 1383 ± 15μm/ 13.8 ± 0.2mmHg, C-class; 1183 ± 57μm/ 33.2 ± 0.8mmHg vs 948 ± 39μm 13.3 ± 0.3mmHg, $n \geq 12$, all groups, $P<0.005$, unpaired t-tests). In D-class vessels however, whilst the replicated elevated transmural pressure was more than two-fold greater in the elevated tension vessels relative to control vessels, the internal diameter was not significantly different between the two conditions (D-class; 32.6 ± 0.5mmHg vs 15.2 ± 0.3mmHg, $n = 12$, $P<0.0001$, unpaired t-test).

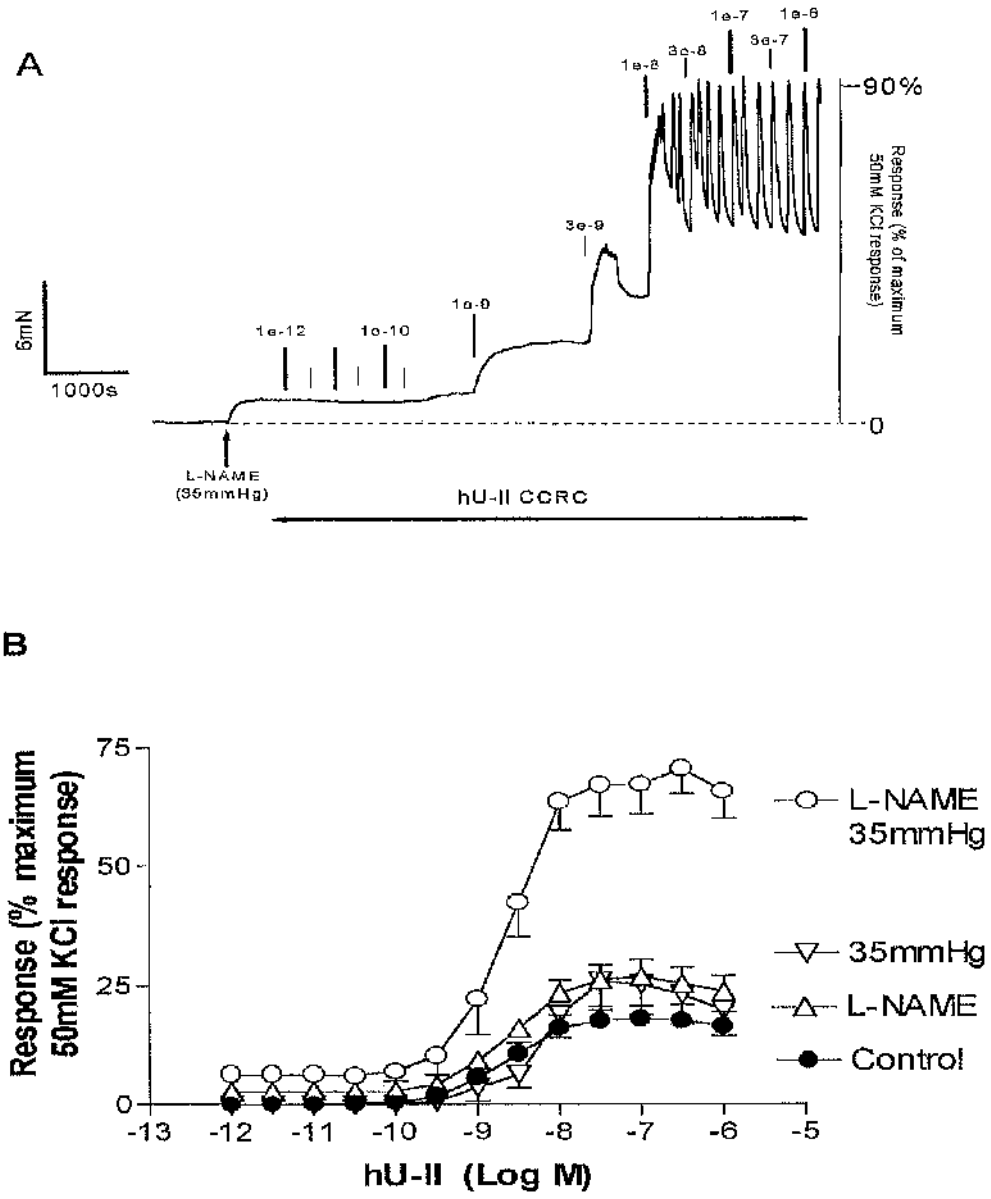


Figure 3.7 The effect of increased resting tension on the hU-II response in the presence and absence of nitric oxide synthase inhibition in Wistar rat, A-class (extra-lobar) pulmonary arteries.

A) Experimental trace showing the large spiking phenomenon observed in all 7 A-class vessels during the performance of the hU-II CCRC in the presence of both increased resting tension (replicating a transmural pressure of 30-35mmHg) and NOS inhibition by L-NAME. B) Graph comparing hU-II CCRCs performed in A-class extra-lobar vessels in the absence (n=7) and presence of increased tension (n=7 $P>0.05$), NOS inhibition using L-NAME (n=7, $P>0.05$) and a combination of both (n=7, $P<0.0001$).

3.3.4 Contractile responses to hU-II in combination models of PAH in Wistar rat pulmonary arteries

The previous series of experiments suggested that in models of dysfunction, individual extra-lobar (A-class vessels) could show a modified contractile response to hU-II. Furthermore, the combination of, increased resting tension (due to stretch of the vessel wall) and NOS inhibition unmasked a strong contractile response in all A-class vessels tested. However the A-class response seemed to be attenuated, though not significantly in vessels remodelled by 2weeks exposure to chronic hypoxia. Conversely whilst the control D-class vessels showed no contractile activity under any of the models or conditions of increased resting tension, the D-class vessels which came from rats exposed to chronic hypoxia did develop a small contractile response to hU-II. To help determine the relevance of these observations to PAH where these conditions occur together, the responses to hU-II of A and D-class vessels, obtained from chronically hypoxic rats, where studied in a combined model of PAH, in which all of the abnormalities were mimicked simultaneously. As the B and C class vessels showed no significant increase in contractile activity under any of the previous PAH models or the elevated transmural studies, these vessels were not studied further.

3.3.4.1 Extra-lobar main pulmonary artery (A-class vessels)

The combined effects of replicated endothelial dysfunction (NOS inhibition) and ET-1 raised tone could not be studied in A-class vessels obtained from chronically hypoxic rats. Vessels set up under this combination of conditions where inherently unstable often spontaneously contracting to near maximal tone before the commencement of the hU-II CCRC (Fig 3.9).

3.3.4.2 Intra-lobar small pulmonary arteries (D-class vessels)

The combined effects of replicated endothelial dysfunction (NOS inhibition), ET-1 raised tone and increased resting tension (replicating a transmural pressure of 30-35mmHg) did not elicit any contractile response to hU-II in the age matched control rat D-class vessels. As in the previous group of chronically hypoxic rats, exposure to 2weeks hypoxia was associated with the development of a small but significant contractile response in these vessels (AM hU-II response Emax; 1 ± 0.5% of the 50mM KCl response vs CH; 10 ± 2%, n=6, $P<0.05$ unpaired t-test). However, neither the efficacy nor the potency of this response was further significantly enhanced by either the individual or combined effects of replicated endothelial dysfunction (NOS inhibition) and ET-1 raised tone (CH; 10 ± 2% of the 50mM KCl response, n=6 vs CH + L-NAME; 8 ± 2%, n=6 vs CH + ET-1; 12 ± 2%, n=6 vs CH + ET-1 + L-NAME; 18 ± 5%, n=7, $P>0.05$, unpaired ANOVA) (Fig 3.8).

The RV/TV ratio was significantly larger in the chronically hypoxic group consistent with the development of PH (AM; 0.207 ± 0.003 n=16 vs CH; 0.285 ± 0.003, n=12, $P<0.0001$, unpaired t-test). All vessels obtained from chronically hypoxic rats were normalised to an initial resting tension which replicated a transmural pressure of 30–35mmHg, which was two-fold greater than the replicated trasmural pressure of the vessels from the age matched control rats. However the internal diameter was not significantly different between any of the chronically hypoxic or age matched control vessels (n ≥6, $P>0.05$, unpaired ANOVA).

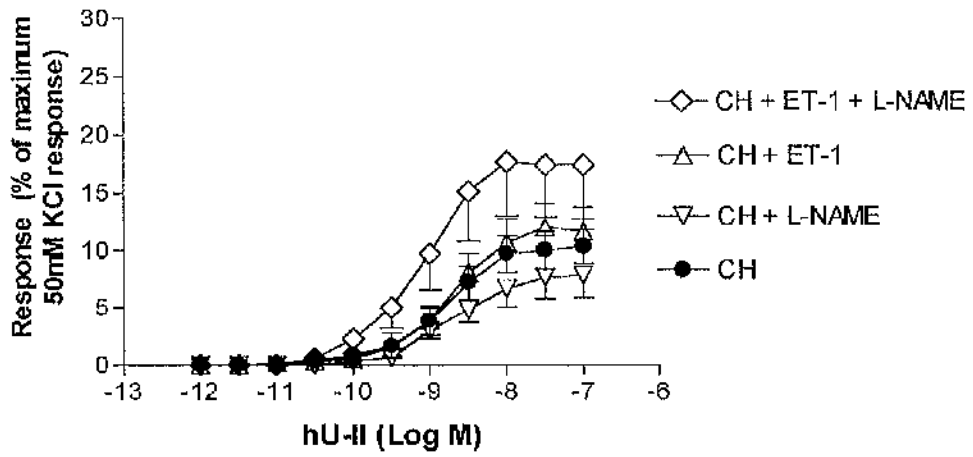


Figure 3.8 The effect of combined models of PAH on the contractile response to hU-II in Wistar rat, small pulmonary arteries (SPA's) (D-class).

CH = Vessels from chronically hypoxic rats (n=6), CH + L-NAME (n=6), CH+ ET-1 raised tone (r/t) (n=6), CH + ET-1r/t + L-NAME = combined model of PAH (n=7) (P>0.05). For ease of comparison the ET-1 and L-NAME tone is not shown.

3.4 Discussion

3.4.1 hU-II does not regulate pulmonary arterial tone in the mouse

hU-II was found to have no vasoactive effects in any mouse pulmonary vessel tested, despite the biological activity of the peptide being confirmed in the mouse aorta where it caused a reliable and potent (IC_{50} ~30nM) but low efficacy response (~20% of the tension standard). Interestingly the response in the aorta is in contrast to previous reports that hU-II had no effect in this vessel (Douglas *et al.*, 2000). Although endothelial function could only be consistently confirmed in the mouse extra-lobar (A-class) main pulmonary artery there was no reason to suspect that failure of the smaller vessels to relax to acetylcholine or subsequently to hU-II was due to endothelial damage incurred by the mounting procedure. Vessels of a similar size were routinely mounted and demonstrated to have an intact endothelium in the rat. The findings suggest that hU-II does not influence pulmonary vascular tone in the mouse and therefore this species was unsuitable for further study.

3.4.2 hU-II is a potent but low efficacy vasoconstrictor of rat pulmonary arteries but does not cause vasodilatation

In the rat, hU-II was found to be a potent but low efficacy vasoconstrictor of the extra-lobar (A and B-class) and 1st generation intra-lobar (C-class) vessels but not the 2nd generation intra-lobar (D-class) vessels. A general pattern of decreasing efficacy along the arterial tree was observed (Fig 3.2), which suggested a similarity with the contractile profile reported in the systemic circulation of the rat (Bottrill *et al.*, 2000;Douglas *et al.*, 2000;Maguire *et al.*, 2000) (Table 1). The potency of the response in the main pulmonary artery (A-class) was found to be virtually identical (~3nM) to the previous report by MacLean *et al.*, (2000), although the efficacy of the response was ~50% less.

The previous study had reported no response in the more distal vessels, however with the very low efficacy reported in the present study this discrepancy could be due to biological variability. Inconsistency of low and moderate efficacy responses have been reported in both pig and human vessels (Douglas *et al.*, 2000;Maguire *et al.*, 2000;Hillier *et al.*, 2001;Paysant *et al.*, 2001). The very similar pEC_{50} values of the responsive A, B and C class vessels in the present study suggested mediation by a single receptor type, presumably the UT-II, although as there are presently no selective UT-II antagonists available, this could not be confirmed. No hU-II induced vasodilator action could be demonstrated in any of the vessel classes (A, B, C or D-class) despite these vessels showing a consistent relaxation to Ach both prior to and after challenge with hU-II. Hence the vessels were functionally capable of relaxing the ET-1 raised tone. Presently no suitable animal models of hU-II pulmonary vasodilatation have been identified.

3.4.3 hU-II does not cause vasoconstriction of human intra-lobar pulmonary arteries

Neither of the human intra-lobar vessel groups tested (750-1500μm size equivalent to C-class rat vessels, 150 –300μm D-class equivalent), responded to hU-II. These findings were consistent with both previous studies in human vessels which also found no response in intra-lobar small pulmonary arteries (150-500μm) (MacLean *et al.*, 2000a;Bennett *et al.*, 2004). However, inhibition of NOS using L-NAME did not uncover a contractile response as has been previously reported (MacLean *et al.*, 2000a). Bennett *et al.*, (2004) also failed to demonstrate a contractile response to hU-II in human vessels which lacked a dilator response to acetylcholine. However, as only 3/10 vessels in the study by MacLean *et al.*, (2000) responded in the presence of L-NAME it is conceivable that failure to uncover this response could have been a function of sample size. One other possible explanation for the discrepancy exists. The samples from both

the study by MacLean *et al.*, (2000) and the present study came from patients with lung carcinoma. Both hU-II localisation and UT-II receptor expression have been found in human renal and adrenocortical tumours and their associated vasculature (Takahashi *et al.*, 2001;Shenouda *et al.*, 2002;Takahashi *et al.*, 2003) so it is not inconceivable that UT-II receptor expression could have been affected in some of these samples even though they were believed to be from tissues that were macroscopically normal. Unfortunately human tissue became unavailable shortly after beginning this study making a larger sample impossible to achieve. The dilator response to hU-II in human small pulmonary arteries has already been reported (Stirrat *et al.*, 2001).

3.4.4 hU-II contractile responses are not enhanced in rat models of pulmonary arterial hypertension

The relatively consistent findings in the rat suggested possible a role for hU-II in the regulation of pulmonary arterial tone and raised the question of whether hU-II could be involved in the aetiology of PH in this species. Models, which mimic individual aspects of the pathological changes of PAH such as medial remodelling resulting from exposure to chronic hypoxia, replicated endothelial dysfunction due to inhibition of NOS and elevated tone using ET-1, have all been reported to enhance responses to endogenous vasoconstrictors in the pulmonary circulation of the rat (MacLean, 1999). In a previous study, it was reported that NOS inhibition by L-NAME and 2 weeks chronic hypoxia both increased the efficacy, but not the potency of the rat main (A-class) pulmonary artery contractile response to hU-II and that endothelial removal and ET-1 raised tone both increased the potency but not the efficacy of the response (MacLean *et al.*, 2000a). In the present study however, no significant augmentation of the hU-II contractile response took place in the extra-lobar (A and B-class) or 1st generation intra-lobar (C-class) vessels in any of the models of PAH which mimicked individual aspects of the

disease. In fact, some B and C-class responses appeared attenuated although these were generally not significant changes. The only significant increase was observed in the formerly unresponsive small pulmonary arteries (D-class) following 2weeks of chronic hypoxia. These vessels showed a very small but significant contractile response to hU-II (~5-8% of the tension standard). This was consistent with the "migration" of smooth muscle distally into the small pulmonary arteries, which is a hallmark of vascular remodelling associated with exposure to chronic hypoxia (Meyrick & Reid, 1978). Conversely, in the more proximal vessels there was a general pattern of decreased responsiveness to hU-II following exposure of the rats to 2weeks hypoxia, although this was only significant in the 1st generation intra-lobar (C-class) vessels. This had the overall effect of creating a very weak (≤10% of the tension standard) but uniform contractile response along the arterial tree. These findings are consistent with a recent *in vivo* study in the rat (Deuchar *et al.*, 2005) (submitted for publication), which found that hU-II caused a very small increase in right ventricular pressure (RVP) (which is indicative of PAP) in the control rat and that following treatment with L-NAME or 2 weeks chronic hypoxia, the hU-II response was no longer observed.

3.4.5 hU-II responses in rat main pulmonary arteries are augmented by a combination of increased tension and NOS inhibition

The data from the present study appeared to contradict the earlier *in vitro* study by MacLean *et al.*, (2000a). However it was observed that some individual extra-lobar (A-class) vessels in the present study had shown a modified response under the modelled conditions, but that these responses were generally not large or consistent enough to be of significance. This suggested that in the previous study, an additional stimulus/factor might have unmasked the enhanced contractile response. The most obvious explanation would be that of variability of UT-II receptor expression. Inconsistent responses have

been widely reported in hU-II studies in several species even when the experiments have been performed in the same lab using identical technique (Douglas *et al.*, 2000).

A second variable was also investigated. The initial study had been performed using the traditional organ bath set up in which all vessels were tensioned to a fixed, pre-determined level of force (1.5g) as is accepted pharmacological practice in these vessels. However at a uniform predetermined level of force, the replicated transmural pressure each vessel wall experiences will depend on its internal diameter. Hence given the range of internal diameters in normal, main pulmonary arteries, the final replicated transmural pressure of a vessel set up in the organ bath can vary by a factor of nearly 2, with some vessels being set to nearly 30mmHg. In contrast, the myograph set up allows the operative to set the replicated transmural pressure of each vessel individually thus closely mimicking actual *in vivo* pressure. The actual variation in the replicated pressure of the vessels set up on the myograph is usually less than 20% (14-16mmHg in extra-lobar vessels, 12-14mmHg in intra-lobar vessels).

When the main pulmonary artery, NOS inhibition study was repeated on the myograph under conditions of increased tension (~two-fold increase in replicated transmural pressure), the contractile response to hU-II in the presence of L-NAME was enhanced some three-fold to levels similar to that reported previously in the earlier organ bath studies (MacLean *et al.*, 2000a). The enhanced response was characterised by a large but now consistent spiking phenomenon, which could be completely abolished by 2 APB. Interestingly following incubation with 2 APB, responses to hU-II in the presence of L-NAME and increased tension were indistinguishable from control responses obtained following incubation with 2 APB. Control responses were themselves reduced by ~ 50% by the presence of 2 APB ($P<0.05$). This implied that ~ 50% of the control response to hU-II in the main pulmonary artery was associated with IP_3 mediated Ca^{2+} store release

and that the augmented responses could not occur in the absence of IP_3 mediated Ca^{2+} store release. Increased tension also appeared to modify the contractile response to sub-maximal concentrations of ET-1 (used to raise tone in these vessels), making a stable raised tone unachievable. These observations suggested that stretch of the vascular wall increased the likelihood of Ca^{2+} store release in response to both (hU-II and ET-1) of these G-protein linked agonists. The mechanism underlying the stretch induced response was not determined (see next section).

3.4.6 Increased tension may account for previous reports of enhanced hU-II contractile responses following chronic hypoxia in the rat

The findings of the increased tension study may also have relevance to the discrepancy between the present study and the previously reported, augmented hU-II response of the main pulmonary artery following exposure of the rats to 2weeks of chronic hypoxia (MacLean *et al.*, 2000a) as a somewhat similar phenomena was observed in these vessels.

Vessels from chronically hypoxic rats were initially normalised to a tension equivalent to 30-35mmHg to replicate *in vivo* hypertensive pressures. However unless these vessels fully dilated to their own true basal tone (~12-20mmHg) following repeated exposures to KCl, spontaneous, rhythmic, contractions caused the vessel tone to become progressively elevated (Fig 3.9). This often resulted in a near maximal contraction, despite the absence of any challenge. This spontaneous contractile behaviour was unpredictable and made it impossible to differentiate spontaneous activity from agonist induced tone. The spontaneous activity of these unstable vessels was immediately and completely abolished by 2 APB suggesting it to be at least partly mediated via IP_3 triggered Ca^{2+} store release. Interestingly phospholamban the protein, which regulates SERCA 2 mediated Ca^{2+} uptake into the SR has been found to demonstrate, increased phosphorylation in rats exposed to chronic hypoxia (J. Millen, Glasgow, unpublished observations).

Phosphorylation of phospholamban increases SERCA 2 activity and would therefore be expected to increase Ca^{2+} uptake by the SR, perhaps resulting in overloading and instability. Spontaneous rhythmic contractions have been previously reported in the chronically hypoxic rat main pulmonary artery (Wanstall *et al.*, 1997;Bonnet *et al.*, 2001b). Occurring with a 90% incidence following 2weeks chronic hypoxia, these spontaneous rhythmic contractions are superimposed on any existing tone and are associated with a reduction in resting *E*m, due to down regulation of Kv channels. The contractions involve both L-type Ca^{2+} channels and SR Ca^{2+} store release (Bonnet *et al.*, 2001b) and may be the result of an amplification loop. Interestingly by the third week of exposure to chronic hypoxia their prevalence has reduced to 20% and by 4weeks are absent, suggesting they may be part of an adaptive process (Bonnet *et al.*, 2001b)

As the results of the chronically hypoxic rat study were in direct opposition to the previous report, it was considered that the few persistently unstable vessels, which had been discarded from the study, could have included vessels, which may have been more reactive to hU-II. However, unstable hypoxic and stable age matched control vessels treated with 2 APB showed no difference in either the efficacy or potency of their response to hU-II. This suggested that ROC mediated Ca^{2+} sarcolemmal flux was not increased and therefore there was no suggestion of increased UT-II expression/signalling, although this assumption needs to be confirmed using molecular techniques. Given these findings and the difficulty of interpreting any hU-II response data from these vessels it was considered justified to eliminate these vessels from the study.

No attempt was made to reset the tension in stabilised vessels obtained from hypoxic rats as to do so invariably caused the instability to return making further study impossible. It was thus assumed that the initial tension (equivalent to a transmural pressure of 30-35mmHg) recreated the previously existing *in vivo* transmural pressure and that

resistance under these conditions was the result of both passive elastic recoil and some element of vasoconstriction. Hence relaxation following KCl responses, should not have changed the component of the replicated transmural pressure which was caused by passive recoil of the vascular wall and that any attempt to readjust the tension back to the equivalent of 30-35mmHg would have increased this component well beyond its normal *in vivo* value. This had already been demonstrated to effect SR discharge in response to hU-II (study 3.4.5). The approach was corroborated by the aforementioned *in vivo* chronically hypoxic rat study, which showed no response to hU-II infusion despite basal RVP being two and a half-fold higher in these animals (Deuchar *et al.*, 2005).

3.4.7 hU-II contractile responses are not enhanced in the rat small pulmonary artery in the combined model of pulmonary arterial hypertension

A-class (main) pulmonary arteries were too unstable to study, under the conditions created by the combined model and vessels typically maximally contracted before the hU-II challenge could be performed. D-class vessels (SPA's) were more stable, but failed to show any significant augmentation of their contractile response to hU-II under the combined model conditions. Furthermore, hU-II contractile responses in these vessels were not enhanced even when the tension was readjusted to an equivalent of 30-35mmHg following any fall off.

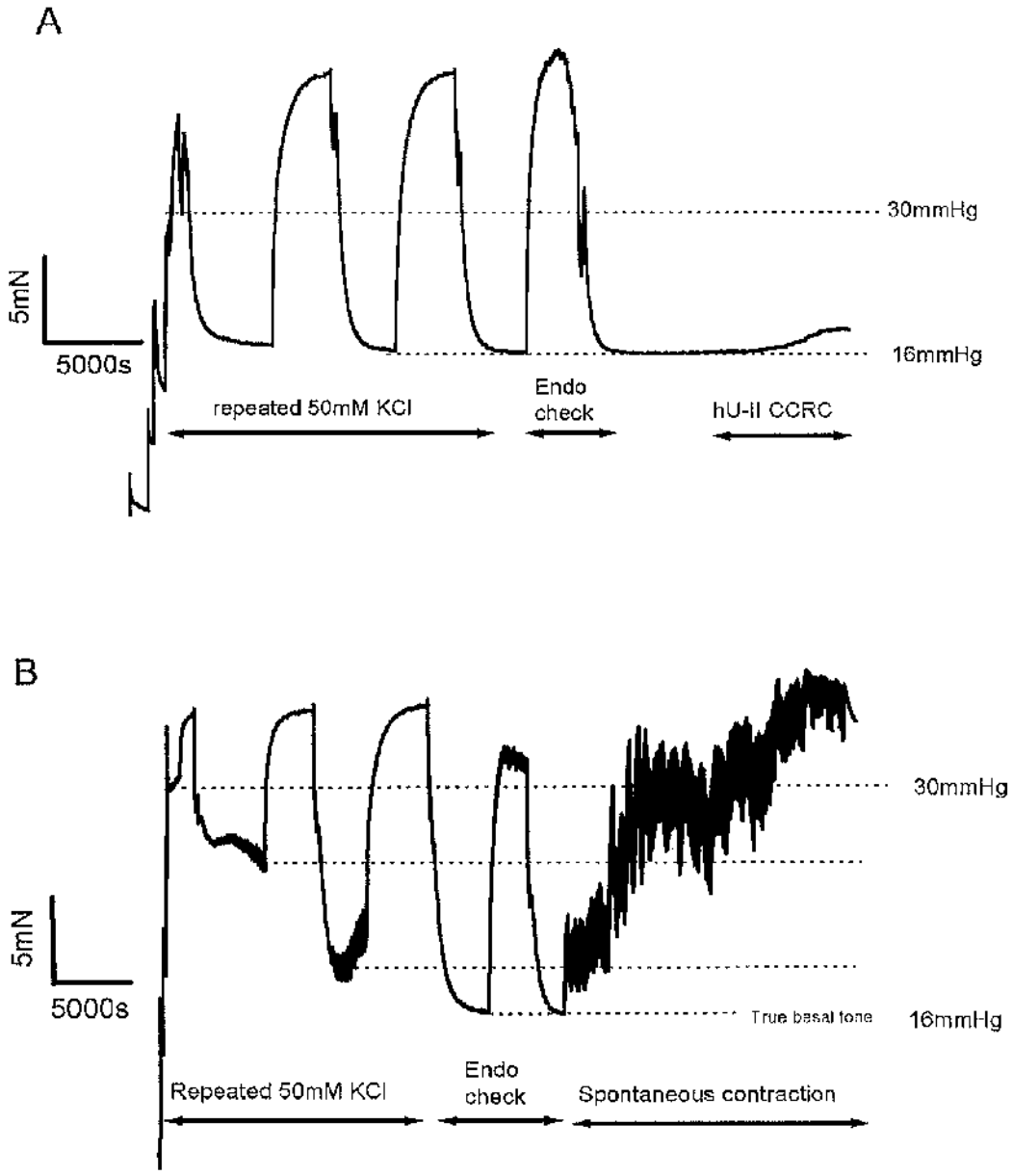


Figure 3.9 Spontaneous contractile activity in a main pulmonary artery (A-class) from a Wistar rat exposed to 2weeks chronic hypoxia

Experimental traces showing A) a stable and B) an unstable A-class vessel form chronically hypoxic (CH) Wistar rats. Both vessels were initially normalised to an equivalent transmural pressure of 30mmHg. This instability also occurred in previously stable A-class vessels from CH rats, when attempting to recreate the conditions necessary for the combined model of PAH.

3.5 Conclusion

The data suggested that hU-II was not involved in the regulation of pulmonary arterial tone in the mouse and this animal was therefore unsuitable for further study. Similarly, hU-II had no vasoconstrictor effect in any of the human intra-lobar vessels tested regardless of the presence or absence of a functional endothelium. In control rat extra-lobar (A and B-class) and 1st generation intra-lobar (C-class) vessels, hU-II caused a potent but low efficacy response, which at high concentration has been shown to result in a small pressor response *in vivo* (Deuchar *et al.*, 2005). However, hU-II did not cause vasodilatation in any rat pulmonary artery.

The models of PAH suggested that the hU-II contractile response in extra-lobar branch and intra-lobar pulmonary arteries (B, C and D-class vessels) was not a contributory factor in the elevated PVR that is central to the development of PAH. In PAH it is primarily the pathological changes of the small pulmonary arteries (D-class vessels) that are associated with elevated PVR and hence elevated pressure. The main pulmonary artery (A-class) contractile response to hU-II was not significantly augmented in any of the single aspect models of PAH, but was enhanced by the specific conditions of increased tension (caused by stretch) combined with NOS inhibition. Contraction of large conduit vessels is associated with decreased vascular compliance and elevation in systolic pressure (Mandegar *et al.*, 2004). However the combined model of PAH, suggested that the main pulmonary artery was so unstable under these conditions that it would be strongly constricted even in the absence of any other challenge and therefore the influence of hU-II on this vessel may not be of consequence. The physiological relevance of this finding would however, need to be confirmed by an *in vivo* study in the chronically hypoxic rat, using a selective hU-II antagonist (when one becomes available) which would determine if the elevated basal tone associated with chronic hypoxia was being contributed to by endogenous hU-II production.

The findings of the present study suggested that in the normal rat pulmonary circulation, hU-II has a low efficacy constrictor effect, which may slightly increase pulmonary arterial tone. However the evidence was generally not consistent with hU-II contributing to the development of PAH in the mouse, rat or human.

Chapter 4

Results Part II

The Role of Calcium Sensitivity in the Regulation of Pulmonary Arterial Tone

i) Protocol development

4.1 Introduction

4.1.1 G-protein mediated smooth muscle contraction; a role for the modulation of Ca^{2+} sensitivity (CaS) in the pulmonary circulation?

Smooth muscle contraction is the basis for the active regulation of blood pressure and flow within organ beds. The key event coupling the extra-cellular signal to contraction in smooth muscle is the elevation in cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) and the relationship between $[Ca^{2+}]_{cyt}$ and force production is known as the Ca^{2+} sensitivity (CaS) of the contractile apparatus (Somlyo & Himpens, 1989). It has become evident however that this relationship can be modified by some endogenous circulating agonists (Kitazawa *et al.*, 1989). A review of this phenomena reported that in swine carotid artery a general pattern was apparent: Agonists causing G-protein activation and concomitant release of intra-cellular Ca^{2+} stores mediated via IP_3, were associated with an increase in CaS, whilst those agonists that only promoted Ca^{2+} influx via the sarcolemma were associated with a decrease in CaS (Rembold, 1992). More recently the G-protein linked receptor agonist, endothelin 1 (ET-1), which is known to be an important modulator of pulmonary vascular tone and has been implicated in the development of pulmonary arterial hypertension (PAH) in humans (Giaid *et al.*, 1993;Stewart *et al.*, 1991), has been shown to cause an increase in CaS in the rat small pulmonary artery (SPA) (Evans *et al.*, 1999). This raises the question of whether other G-protein activating agonists, which play a role in the regulation of pulmonary vascular tone, such as 5-HT, influence the CaS of the contractile apparatus? Furthermore, does the modulation of CaS play a role in the aetiology of PAH and is this change observed in models of PAH such as the chronically hypoxic rat model where the effects of these G-protein activating agonists have been reported by some, to be enhanced (McCulloch *et al.*, 1998).

4.1.2 Techniques for studying Ca^{2+} sensitivity (CaS) in isolated vessels

The techniques that have been applied to the study of Ca^{2+} sensitisation of the contractile apparatus can be broadly classified into two categories; 1) Those that selectively permeabilise the smooth muscle cell membrane and permit $[Ca^{2+}]_{cyt}$ to be clamped by the choice of the extra-cellular bathing solution and 2) those that use fluorescent imaging techniques to measure $[Ca^{2+}]_{cyt}$ and force simultaneously in unpermeabilised vessels. Imaging techniques have the advantage of causing minimal anatomical and physiological disruption to the SMCs, however, whilst they can accurately measure $[Ca^{2+}]_{cyt}$ they cannot control it (Sato *et al.*, 1988). The technique is ideal for temporal Ca^{2+} measurement but cannot buffer local intra-cellular Ca^{2+} gradients and is therefore not so suited to spatial Ca^{2+} measurement (Somlyo & Himpens, 1989). In the present series of experiments where whole segments of vessels were to be studied, any fluorescent measurement would be a reflection of the mean $[Ca^{2+}]_{cyt}$ at a given time and may therefore not necessarily be an accurate reflection of the actual $[Ca^{2+}]$ responsible for a given force. In contrast, permeabilisation techniques allow the investigator to precisely control the $[Ca^{2+}]_{cyt}$ and also prevent localised Ca^{2+} gradients (Himpens *et al.*, 1990). The disadvantages of permeabilisation techniques, are the possibility of disruption to the 2nd messenger systems and the gradual loss of intra-cellular regulatory factors/proteins following permeabilisation (Iizuka *et al.*, 1994;Fujita *et al.*, 1995;Konishi & Watanabe, 1995;Akopov *et al.*, 1998), however these problems can be minimised if the protocol is optimised for the tissues in which it is to be used.

As the effects of chronic hypoxia and the 5-HT system on the CaS of the contractile apparatus of the rat small pulmonary artery had never been previously studied it was decided to use a chemical permeabilisation technique to be certain that any response was indeed due to a change in CaS and not just the action of some localised Ca^{2+} gradient. Of the various chemical permeabilisation techniques available, two have been most widely

used in intact smooth muscle preparations; α-toxin and β-escin. Although both options were considered, financial restraints precluded using the somewhat more precise bacterial pore forming *Staphylococcus aureus* α-toxin.

4.1.3 β-escin

β-escin, a triterpeniod saponin derived from the seed of the horse-chestnut tree -*Aesculus hippocastanum*, has been successfully used to permeabilise a wide range of smooth muscle preparations in order to study CaS (Itoh *et al.*, 1992;Iizuka *et al.*, 1994;Savineau & Marthan, 1994;Yoshida *et al.*, 1994;Lee *et al.*, 1996;Iizuka *et al.*, 1997;Akopov *et al.*, 1998;Kitazawa *et al.*, 2004;Soloviev *et al.*, 2004). It was originally believed to aggregate only the cholesterol component of the sarcolemma, resulting in the formation of aqueous pores, whilst retaining receptor coupling (Kobayashi *et al.*, 1989). However, more recent reports have also suggested a more complex but as yet undefined action. For example, it has been shown to inhibit loading of the SR (which has a low cholesterol content) in skeletal muscle (Launikonis & Stephenson, 1999). Additionally, its effects on the sarcolemma occur in a concentration dependant manner (Iizuka *et al.*, 1994;Konishi & Watanabe, 1995) rather than at some critical concentration. It is therefore best suited to studies in which preservation of sarcolemmal receptor coupled mechanisms is paramount but preservation of SR function is not (Launikonis & Stephenson, 1999).

4.1.4 Optimising the β-escin permeabilisation protocol for use in the rat small pulmonary artery

To date, only one publication has reported on the phenomenon of Ca^{2+} sensitisation in rat small pulmonary arteries using a chemical permeabilisation technique (Evans *et al.*, 1999). Evans *et al.*, (1999) reported that both β-escin and α-toxin produced a functioning permeabilised preparation but that the β-escin preparation was subject to significant "run

down" within 30minutes. However the protocol described was originally employed for use in strips of rabbit portal vein and guinea pig ileum (Iizuka *et al.*, 1994) and made no mention of being optimised for use in the rat small pulmonary artery. Although β-escin permeabilised preparations have been shown to "leak" proteins of a molecular weight up to 150kD, contractile function and receptor-coupled signal transduction can be adequately preserved if the β-escin is correctly applied (Iizuka *et al.*, 1994). This may suggest that some aspect of the β-escin protocol used by Evans et al., was responsible for the "run down". Furthermore, even when using α-toxin, which reduces the loss of these high molecular weight proteins, the published maximum tone achieved by Evans *et al.*, (1999) following permeabilisation was only 0.28mN at a maximal $[Ca^{2+}]_{cyt}$ of pCa 4.5. This raised an additional concern; Although the KCl tension standard responses for this study were not reported, a typical response for a 2mm length of Wistar or Sprague Dawley rat small pulmonary artery (150-350μm i.d.) to a KCl depolarisation obtained by the author of this thesis would be around 5-6mN. This would suggest that the α-toxin permeabilised vessels had retained only ~ 5% of their unpermeabilised force production potential.

Preliminary experiments based upon β-escin permeabilisation methods applied to systemic arterial smooth muscle preparations also yielded very poor responses. For example, when a constant exposure time for β-escin was applied to the Wistar rat small pulmonary artery, the results suggested the presence of unpermeabilised SMCs in a number of vessels and over permeabilisation characterised by a loss of signal transduction in others. This method had been successfully applied to ovine cerebral arteries (Akopov *et al.*, 1997), but was clearly less than optimal in the rat small pulmonary artery, possibly due to the significant variation in the amount of smooth muscle present in these vessels as suggested by the huge variation contractile force generated in response to KCl depolarisation.

Of even greater concern was that some component of the mock intra-cellular (MI) solutions was acting as an agonist and seemed to be causing a substantial and prolonged activation of the 2nd messenger systems in the unpermeabilised Wistar rat small pulmonary artery. Naturally this raised concerns as to whether CaS was being modulated by the MI solution. Evans et al., (1999) had not reported this response, but it was consistently observed in the present study. ATP was considered the most likely culprit as several P2 receptors have been reported in the rat small pulmonary artery (Chootip *et al.*, 2002). Furthermore it has also recently been reported that multiple P2Y receptors, some of which are activated by ATP are capable of activating Rho kinase (ROK) in vascular myocytes (Sauzeau *et al.*, 2000b). ROK is an important component of a Ca^{2+} sensitising pathway, which has recently received much attention. Due to these concerns it was decided that the protocol should be redeveloped to specifically adapt the method for use in the Wistar rat small pulmonary artery.

4.1.5 Overview of the protocol development

The development of the protocol covered 5 main areas 1) Selection of an optimum tension standard for use in assessing contractile force. This would be important in assessing how detrimental the permeabilisation procedure was to the preparation, given that the action of β-escin is now known to be concentration dependant (Iizuka *et al.*, 1994). 2) Determination of the composition of the MI solution in which the vessels were to be bathed during and following permeabilisation. An appropriate solution would sustain the permeabilised vessels and maintain normal contractile function but not modulate CaS of the contractile apparatus. 3) Selection of an appropriate sensitising agonist standard to assess the effect of permeabilisation on the normal functioning of the receptor-coupled signal transduction mechanisms. 4) Determination of the most appropriate method of β-escin permeabilisation and the optimum concentration for its

use. 5) Selection of the best way to prevent the SR from compromising the clamped $[Ca^{2+}]_{cyt}$ in response to agonist or other stimulation.

Following protocol development in the Wistar control rat small pulmonary artery, the protocol was then tested on chronically hypoxic rat small pulmonary arteries and wild type and transgenic mouse small pulmonary arteries and adapted where necessary.

Due to the extensive number of experiments and broad range of inquiries involved in the protocol development it was decided that each of the studies would be presented as a discrete section. Each study therefore comprises a brief background to the problem, specific methods, results and a short explanation of how the findings were applied to the evolving protocol. This was intended to help present the protocol development as a series of logical and progressive steps.

4.2 Study 1) Selecting the optimum tension response standard

4.2.1 Background

The aim of this study was to determine the most appropriate standard for assessing changes in force during the development and subsequent application of the permeabilisation protocol. The initial question this study was intended to address was; is an iso-osmotic depolarising solution significantly superior (with regard to magnitude and rate of force development) to the more straight forward, hyper-osmotic addition of KCl. If not, what is the optimum concentration for a hyper-osmotic KCl addition?

Hyper-osmotic additions are simple to perform only requiring the addition of a predetermined amount of stock KCl to the bathing solution. However, whilst this results in depolarisation and hence contraction, it also increases the osmolarity of the extra-cellular medium and this has been associated with a decreased maximum force and a slowed rate of force production (Kent *et al.*, 1983;Karaki *et al.*, 1984). For example, even the addition of just 50mM KCl would increase the osmolarity of the bathing solution from ~300mOsmoles to ~400mOsmoles, a 33% increase. It was important that the chosen standard consistently yielded a true maximum response so that any deleterious effects of exposure to the MI solutions or the permeabilisation procedure could be accurately assessed.

The alternative was to use a nominally iso-osmotic solution exchange, which maintains osmotic balance, thus the magnitude and rate of force production should be an accurate reflection of the vessels normal physiological response to a depolarisation.

4.2.2 Methods

Small pulmonary arteries from Wistar rats were procured, set up and normalised to 12-14mmHg at 24^0C, as described in the general methods. The following questions were then addressed:

4.2.2.1 Are nominally iso-osmotic tension response standards superior to hyper-osmotic tension response standards?

Following normalisation, the maximum responses to 2 repeated KCl exposures (separated by washout and return to baseline) were ascertained. The following challenges were examined; 50, 100 or 120mM hyper-osmotic KCl additions or a nominally iso-osmotic 120mM KCl solution exchange. A hyper-osmotic addition consisted of adding a predetermined amount of KCl to the Krebs-buffer solution in which the vessels were bathed. An iso-osmotic solution exchange involved draining the Krebs-buffer solution and then replacing it with a K^+ based buffer solution which was nominally iso-osmotic (~300mOsm) to the cytoplasm of the SMCs. After washout of the second selected KCl challenge and return to baseline tension, the vessels were challenged with 2 repeated exposures (separated by washout and return to baseline) of a nominally iso-osmotic 120mM potassium acetate ($K^+CH_3COO^-$) solution (Fig.4.1).

The series of experiments was then repeated using fresh vessels, but this time performing the exposures to the iso-osmotic 120mM $K^+CH_3COO^-$ solution prior to the selected KCl challenges.

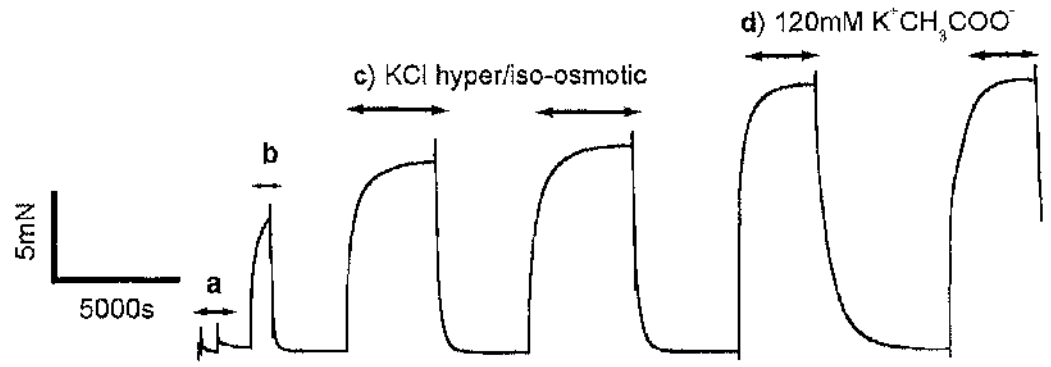


Figure 4.1 Experimental trace showing the protocol to determine if iso-osmotic depolarising solution exchanges are superior to hyper-osmotic depolarising additions

a) Normalisation; setting the tension to replicate the equivalent *in vivo* pressure b) "Wake up" depolarisation followed by re-adjustment of tension if required c) Repeated depolarisations using hyper-osmotic KCl additions or an iso-osmotic 120mM KCl solution exchange d) Repeated depolarisations using an iso-osmotic 120mM $K^+CH_3COO^-$ solution exchange.

4.2.2.2 Does acetate/chloride substitution affect contractile function?

The results of the previous experiments raised the question of whether the replacement of the chloride ion with the acetate (CH_3COO^-) anion was deleterious to the un-permeabilised vascular preparation. Hence, fresh vessels were challenged with either 4 repeated exposures (separated by washout) to iso-osmotic 120mM $K^+CH_3COO^-$ or 4 repeated exposures to iso-osmotic 120mM KCl.

Data analysis

As the MI solutions all use the impermeant acetate (CH_3COO^-) ion as the dominant anion, all depolarisation challenges were normalised against the iso-osmotic 120mM $K^+CH_3COO^-$ response of each vessel. The magnitude of force developed was expressed in mN as 90% of the maximum force (F_{90}) and the time taken to reach F_{90} in seconds.

4.2.3 Results

4.2.3.1 Are nominally iso-osmotic tension response standards superior to hyper-osmotic tension response standards?

During these measurements it became immediately obvious that challenges pre $K^+CH_3COO^-$ exposure showed different kinetics to those performed post $K^+CH_3COO^-$ exposure, therefore pre and post exposures were considered as different experiments so that the effect of acetate/chloride substitution could be determined.

Hyper-osmotic vs iso-osmotic KCl standards (pre $K^+CH_3COO^-$ exposure)

The responses to hyper-osmotic (H) KCl additions took 2-4 times longer to reach F_{90}, than those produced by a nominally iso-osmotic 120mM $K^+CH_3COO^-$ solution (50mM H; 1308 ± 144secs vs 729 ± 109secs, n= 4, $P<0.005$) (100mM H; 2608 ± 80secs vs 682 ± 111secs, n=4, $P<0.0005$) (120mM H; 3259 ± 337secs vs 782 ± 33secs, n=3, $P<0.05$) (all paired t-test). It was also observed that when the hyper-osmotic responses were standardised to their paired iso-osmotic 120mM $K^+CH_3COO^-$ responses, time to F_{90} was significantly increased with the increase in osmolarity from 50mM to 100mM KCl (50mM H; 182 ± 8% of the iso-osmotic 120mM $K^+CH_3COO^-$ response time vs 100mM H; 400 ± 53%, n=4, $P<0.05$) (unpaired ANOVA).

Additionally, the hyper-osmotic 100 and 120mM KCl additions both produced an attenuated force (F_{90}) relative to the nominally iso-osmotic 120mM $K^+CH_3COO^-$ responses to which they were paired (100mM H; 1.74 ± 0.41mN vs 2.37 ± 0.36mN, n=4, $P<0.05$) (120mM H; 2.77 ± 0.83mN vs 4.06 ± 1.06mN, n=3, $P<0.05$, all paired t-test).

In contrast, the responses to a nominally iso-osmotic 120mM KCl solution were virtually identical to those produced by the iso-osmotic 120mM $K^+CH_3COO^-$ solution (Time to F_{90}; 732 ± 32secs vs 741 ± 94secs, n=4, $P>0.05$, paired t-test) (F_{90}; 5.07 ±0.40mN vs 5.07 ± 0.43mN, n=4, $P>0.05$, paired t-test) (Fig 4.2 "PRE").

Hyper-osmotic vs iso-osmotic KCl standards (post $K^+CH_3COO^-$ exposure); effect of acetate/chioride substitution

Exposure to $K^+CH_3COO^-$ prior to KCl challenge caused a decrease in force (F_{90}) in all subsequent hypertonic and iso-osmotic challenges (% Pre F_{90}; 50mM H: pre; 82 ± 6%, n=4 vs post; 66 ± 2%, n=4, $P<0.05$) (100mM H; 72 ± 3% vs 62 ± 2%, n=4, $P<0.05$) (120mM H: pre; 67 ± 3%, n=3 vs post; 51 ± 2%, n=4, $P<0.005$) (120mM I: pre; 100 ± 1%, n=4 vs post; 93 ± 2%, n=4, $P<0.05$) (all unpaired t-test) (all vessels standardised to their paired iso-osmotic 120mM $K^+CH_3COO^-$ solution responses). Additionally there was also an increase the time taken to reach F_{90} in the hyper-osmotic 50mM and iso-osmotic 120mM KCl challenges (50mM H: pre; 182 ± 8%, n=4 vs post; 401 ± 42%, n=4, $P<0.005$) (120mM KCl I: pre; 104 ± 14%, n=4 vs post; 170 ± 10%, n=4, $P<0.05$, unpaired t-test) (Fig 4.2 "POST").

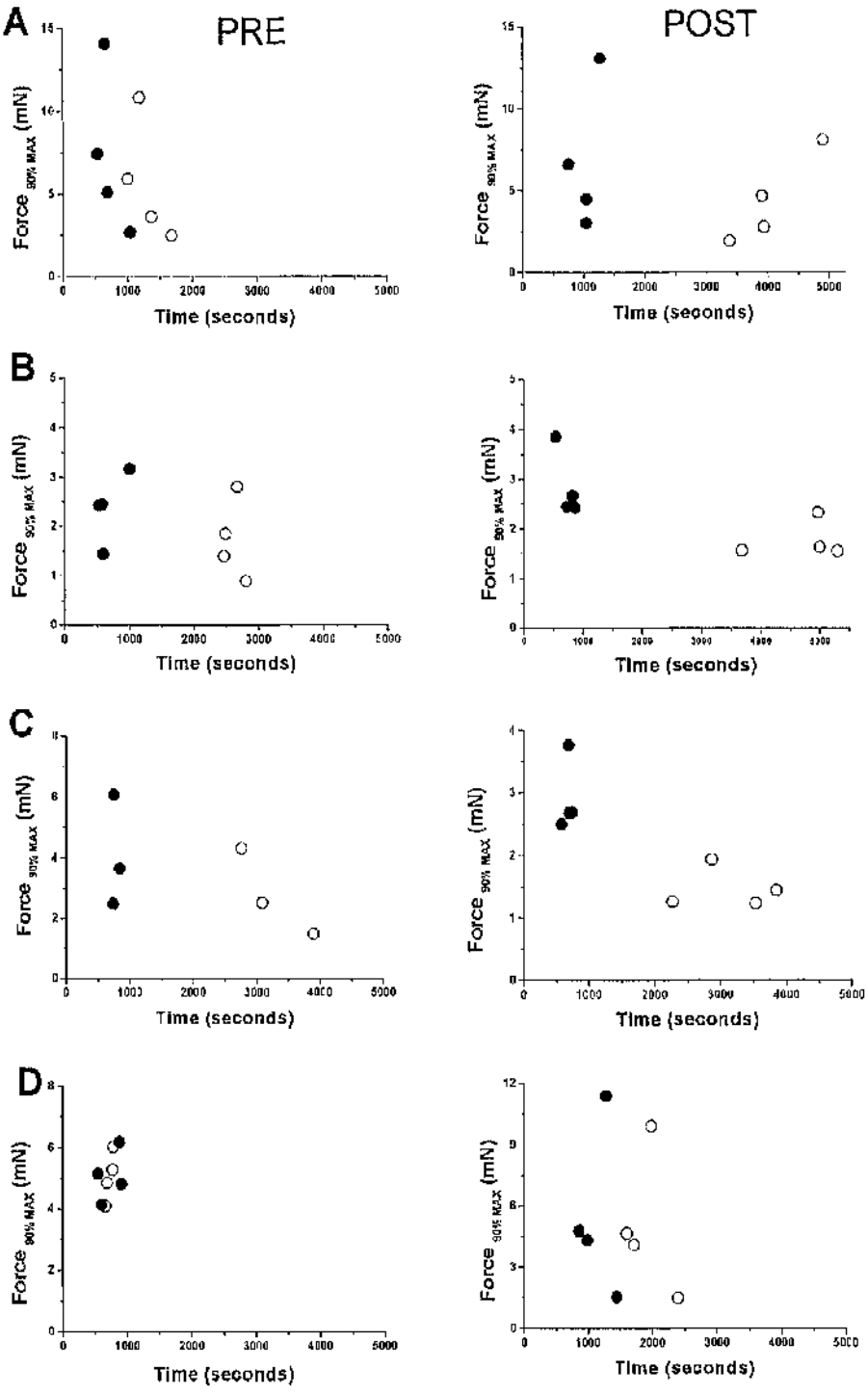


Figure 4.2 The effect of osmolarity and acetate/chloride substitution on tension standard contractile responses in the Wistar rat small pulmonary artery

Plots of 90% maximum force (F_{90}) against time to F_{90} for hyper-osmotic (A; 50mM, B; 100mM, C; 120mM) and iso-osmotic (D;120mM) KCl depolarisations (O - White circles) relative to each vessels paired response to iso-osmotic (A-D;120mM) $K^+CH_3COO^-$ depolarisations (● - Black circles). PRE; left hand column - KCl challenges pre-ceded $K^+CH_3COO^-$ challenge. POST; right hand column shows responses where KCl challenges followed $K^+CH_3COO^-$ challenge.

4.2.3.2 Does acetate/chloride substitution affect contractile function?

All vessels challenged with repeated iso-osmotic 120mM $K^+CH_3COO^-$ exposures demonstrated a progressive increase in their time to F_{90} with each subsequent exposure until the fourth challenge, where a decrease in F_{90} became the significant trend. (Time to F_{90}; exp 1; 820 ± 58secs vs exp 2; 1190 ± 60secs vs exp 3; 1524 ± 86secs, n=7, $P<0.01$) paired ANOVA) (F_{90}; exp 1; 6.54 ± 1.33mN vs exp 4; 5.28 ± 1.14mN, n=7, $P<0.05$, paired ANOVA) (Fig 4.3).

Similarly, there was also a successive increase in the fall off (F/O) period (Time to F/O_{90}), which was the time taken to return to 10% above basal tone following washout. This, was also accompanied by a progressive elevation in basal tone (B/T), which was significant following the third exposure (exp) to 120mM $K^+CH_3COO^-$ (Time to F/O_{90}; post exp 1; 1844 ± 138secs vs post exp 2; 2914 ± 364secs vs post exp 3; 3184 ± 546, n=7, $P<0.005$, paired ANOVA). (B/T; pre exp 1; 0.68 ± 0.15mN vs post exp 3; 1.37 ± 0.21mN, n=7, $P<0.01$, paired ANOVA) (Fig 4.3 B).

Repeated exposures to iso-osmotic 120mM KCl demonstrated no significant changes over the course of the four repeated challenges (n=4, $P>0.05$, all paired ANOVA) (Fig 4.3 A).

The vessels from all groups were of a comparable internal diameter and replicated transmural pressure (P>0.05, unpaired ANOVA). The mean internal diameter and replicated transmural pressure of the vessels used in study 1 was 253 ± 7μm and 13.6 ± 0.2mmHg respectively (n=42 vessels).

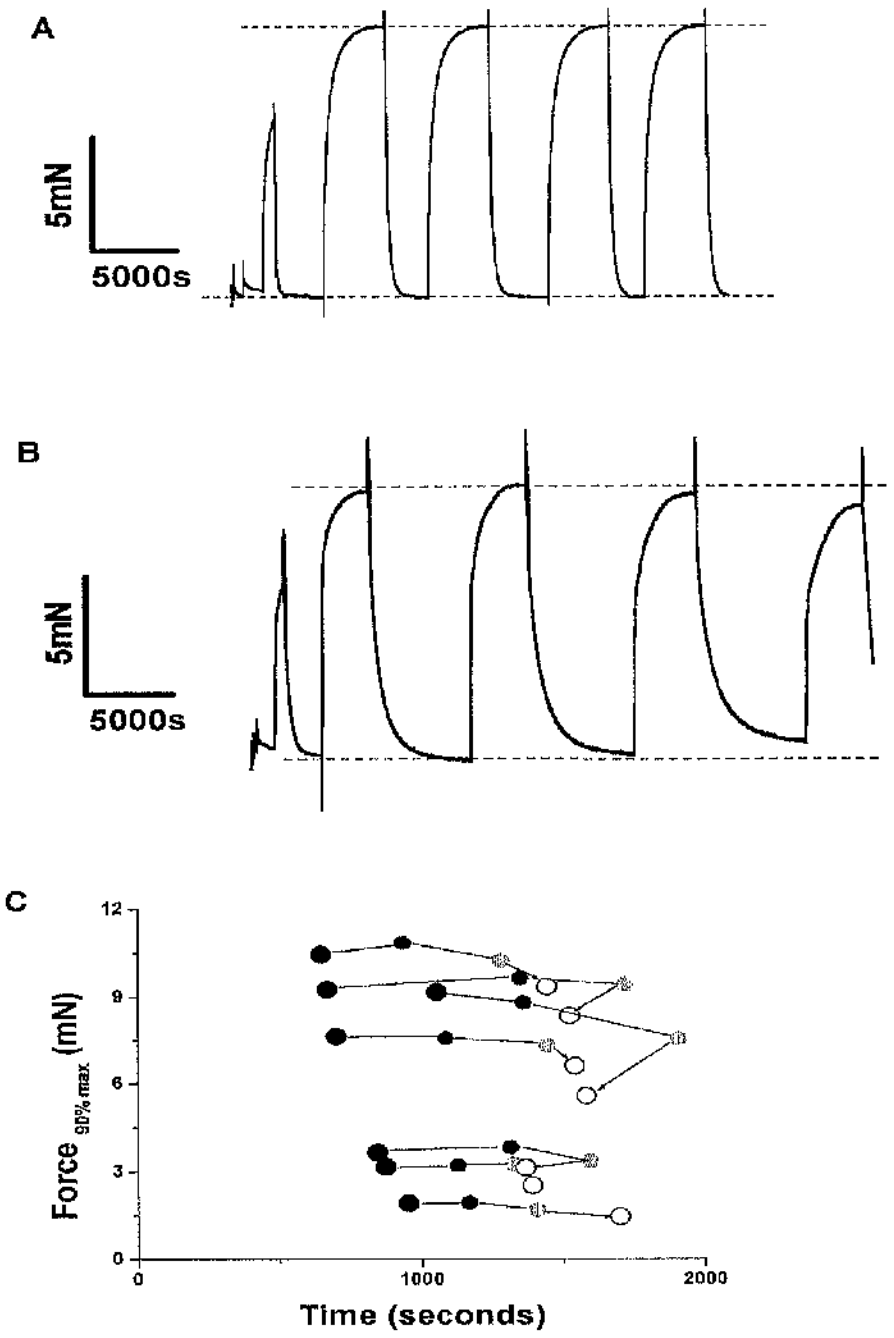


Figure 4.3 The effect of acetate/chloride substitution on tension standard contractile responses in the Wistar rat small pulmonary artery

Experimental traces showing responses to A) 4 repeated iso-osmotic 120mM KCl challenges and B) 4 repeated iso-osmotic 120mM $K^+CH_3COO^-$ challenges. C) Plots of 90% maximum force (F_{90}) against time to F_{90} for 4 repeated iso-osmotic 120mM $K^+CH_3COO^-$ challenges. (●) Black circles - 1st Challenge. (●) Dark grey circles - 2nd challenge. (●) Light grey circles - 3rd challenge. (O) White circles - 4th challenge.

4.2.4 Application to protocol

Hyper-osmotic additions and acetate/chloride substitution were both found to affect the magnitude and rate of force development. Hence, vessels in all subsequent studies were normalised to 12-14mmHg and then challenged with a nominally iso-osmotic, 120mM KCl solution exchange to determine their standard response whilst minimising exposure of the unpermeabilised vessel to acetate. However, as there was no evidence in the literature to suggest the effects of acetate were detrimental following permeabilisation, it was provisionally retained in the mock intra-cellular (MI) solution.

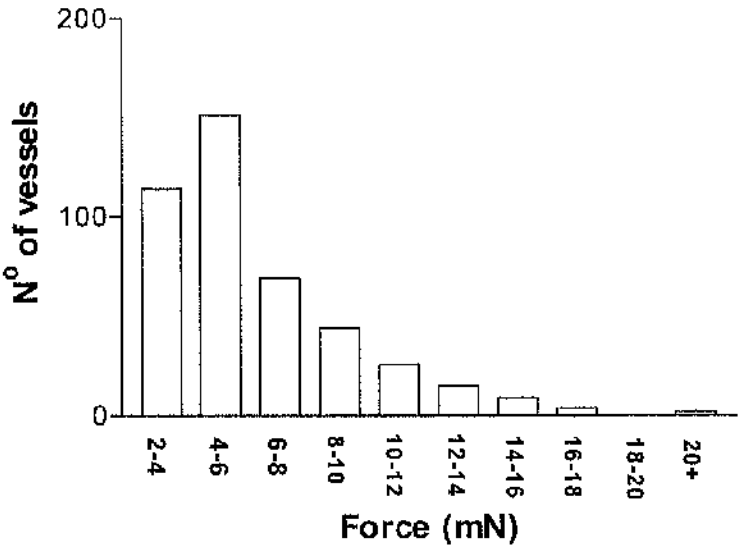


Figure 4.4 The range of nominally iso-osmotic 120mM KCl tension standard responses obtained during protocol development experiments

4.3 Study 2) Investigation into the agonist action of the pCa9.9 mock intra-cellular (MI) solution in un-permeabilised vessels

4.3.1 Background

Upon exposure of the Wistar rat small pulmonary arteries vessels to the "Ca^{2+} free", pCa 9.9 MI solution it was immediately apparent that one or more of its components was causing a large, possibly bi-phasic contractile response. The response rapidly peaked (phasic response) at ~ 50% of the 120mM KCl tension standard response, then deteriorated exponentially (fall off) over ~90minutes until it eventually stabilised (tonic response) at ~5-10% above the normalised basal tone (Fig 4.5).

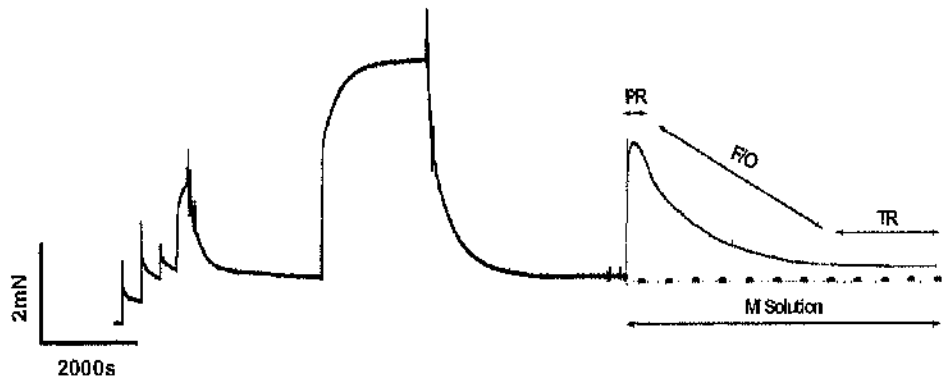


Figure 4.5 Experimental trace showing the "bi-phasic" response caused by a mock intra-cellular (MI) solution exchange

PR - Phasic response, TR - tonic response, F/O - fall off.

Although the presence of K^+ in the MI solution would cause immediate depolarisation of the vessels, it seemed highly unlikely that the response was solely due to sarcolemmal flux of unbuffered interstitial Ca^{2+}, as the tone remained elevated above baseline for several hours. A similar response was observed in the Sprague Dawley rat small

pulmonary artery, although this had not been reported by Evans *et al.,* (1999). It was considered that the tonic response may have been an artefact of the acetate/chloride substitution as had been observed in study 1. However, the response was ablated by a single addition of the Rho kinase (ROK) inhibitor Y 27632 (10μM), suggesting that the ROK pathway was activated in the presence of the MI solution, and raising the possibility that some component of the solution was modulating CaS (Fig 4.6).

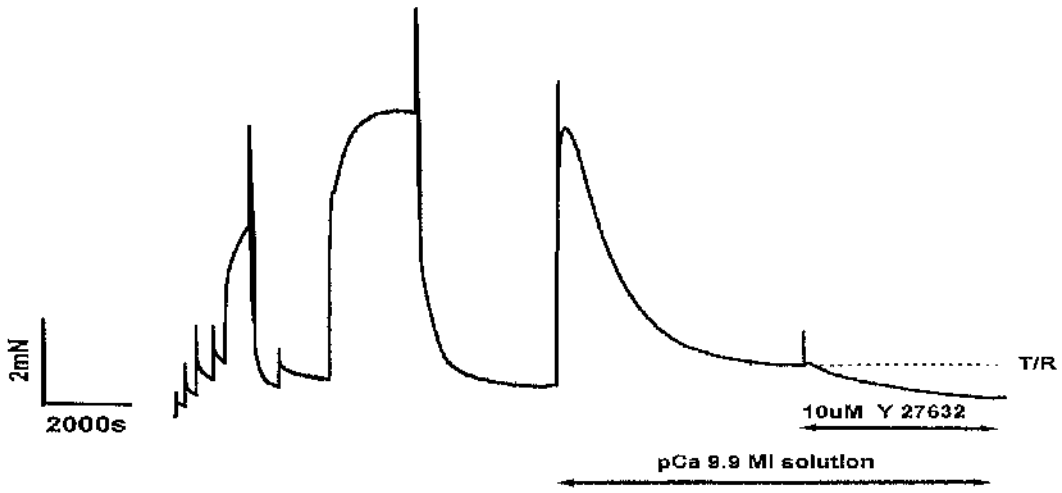


Figure 4.6 The effect of Rho kinase inhibition on the tonic response (T/R) caused by the solution exchange to a pCa 9.9 mock intra-cellular (MI) solution

It was therefore necessary to identify the component of the solution that was causing the response and determine what impact this may have on the preparation, especially with regard to the CaS of the contractile apparatus. Unfortunately, background reading suggested the most likely candidate was ATP (Chootip *et al.,* 2002). Purinergic (P2) receptors, some of which are strongly activated by ATP, have recently been linked to ROK activation and the ability to influence CaS in rat aortic myocytes (Sauzeau *et al.,* 2000). If ATP was modulating CaS this would pose a serious problem, as ATP would be necessary to supply the energy needs of the permeabilised SMCs and was therefore a

fundamental component of the MI solution. Preliminary permeabilisation studies using the technique of Akopov *et al.*, (1997) (50-200μM β-escin, for 20mins in a pCa 9.9 MI solution) found that reducing the concentration of ATP in the MI solution to a level (100μM ATP) that did not activate 2nd messenger systems (as implied by the absence of a tonic response upon exposure to the MI solution), was detrimental to both maximal force and rate of force development (~50% decrease) when these vessels were subsequently placed in a pCa 4.55 MI solution. The findings suggested that endogenous production by ATP synthases was not sufficient to maintain normal contractile function following permeabilisation and was consistent with a previous report which found that reducing exogenous ATP to 100μM in permeabilised guinea-pig taenia coli, reduced force and rate of contraction by half (Arner & Hellstrand, 1985). Unfortunately, pharmacological manipulation of the P2 receptors involved was not feasible due to the high concentration of ATP (5mM) used, the lack of non-competitive antagonists and the large number or P2 receptors reported to be present in rat small pulmonary artery (Chootip *et al.*, 2002). Hence the aims of this study were to determine: 1) What component of the MI solution is responsible for the observed response? 2) Does the tonic response involve mobilisation of Ca^{2+} stores? 3) Does the component modulate CaS?

4.3.2 Methods

Two main types of protocol were used in the following experiments 1) Cumulative Concentration Response Curves (CCRCs) and 2) Single dose additions to create a fixed bath concentration. Following set up and determination of their 120mM KCl tension standard response, all vessels were equilibrated in Krebs-buffer until their tensions had returned to baseline levels (~1hour). The following questions were then addressed.

4.3.2.1 Is ATP responsible for the contractile response to the mock intra-cellular bathing solution?

It was necessary to compare the contractile profile of ATP to that witnessed upon exposure to the pCa 9.9 MI solution. This would not only confirm whether ATP was the responsible agonist but also help to determine if the response was modified in any way by other components of the MI solution. To achieve this, vessels set up as previously described were placed in one of 3 solutions. 1) Krebs-buffer 2) Ca^{2+} free ($\emptyset Ca^{2+}$) Krebs-buffer 3) ØATP, pCa 9.9 MI solution. The vessels were then challenged with a single dose addition of 5mM ATP. A fourth vessel was simultaneously challenged with exposure to a 5mM ATP, pCa 9.9 MI solution to act as a control. All vessels were observed until the responses had fully stabilised.

4.3.2.2 How Does 5mM ATP effect Ca^{2+} store release?

It was considered useful to further elucidate the role of the SR and the relative contribution of IP_3 sensitive and ryanodine sensitive Ca^{2+} stores in the ATP associated response.

Vessels were set up as previously described and then placed in a ØATP, pCa 9.9 MI solution. Four conditions were studied; 1-3) The addition of a single dose of 5mM ATP to vessels incubated with 1) thapsigargin (2μM, 10mins incubation) to inhibit SR Ca^{2+} uptake, 2) 2 APB (100μM, 30mins) to block IP_3 mediated store release, or 3) nothing, to act as a control. 4) Rapid application of 10mM Caffeine to determine the potential contribution of the ryanodine sensitive stores.

4.3.2.3 Does 5mM ATP modulate Ca^{2+} sensitivity of the contractile apparatus?

Due to the effects of ATP and Y27632 on sarcolemmal Ca^{2+} permeability and contractile kinetics respectively, it was impossible to answer this question conclusively using a single experimental protocol. Instead evidence was gathered using 2 different approaches

centring on the use of the known ROK inhibitor Y27632. The ROK pathway was selected for study first in preference to other proposed sensitisation pathways as it had already been shown to be active in the presence of the MI solution (see introduction). Once the effect of 5mM ATP on the ROK pathway had been established the intention was then to study the action of 5mM ATP on the other proposed sensitisation pathways such as the PKC pathway.

Ca^{2+} CCRCs in unpermeabilised vessels in the presence and absence of 5mM ATP and ROK inhibition

Thapsigargin (2μM) was added to all vessels 10mins prior to a solution exchange from Krebs-buffer to a 5mM ATP, pCa 9.9 MI solution to deplete the SR. Following SR depletion, some vessels were bathed in a ∅ ATP MI solution and some in a 5mM ATP MI solution. Half of the vessels from each group were then incubated with 10μM of the selective ROK inhibitor Y27632 for 45mins. The following solution exchanges were performed to produce the cumulative exposure to Ca^{2+}; pCa; 9.9, 5.7, 4.55, 4.0, 3.5, 3.0, 2.5, 2.0, 1.5. During solution exchange, vessels were washed twice with the following solution to minimise the possibility of dilution by the previous solution.

Single addition of the ROK inhibitor Y-27632 in unpermeabilised vessels with a sub-maximally raised tone in the absence of ATP

Following equilibration in a ØATP pCa 9.9 MI solution the depolarised vessels were exposed to an increasing extra-cellular [Ca^{2+}] by increasing the ratio of Ca^{2+}EGTA/K_2EGTA in the MI solution until a stable tone which was ~25% of the 120mM KCl response was achieved. 10μM Y27632 was then added to one of each of the paired vessels, whilst the remaining vessel acted as a time control. The responses were observed for 1 hour or until the response had stabilised, whichever was the shorter.

4.3.2.4 Do the remaining components of the mock intra-cellular solution contribute to the observed contractile response?

Whilst the previous experiments addressed the action of ATP, it still remained important to determine if the other components of the MI solution where contributing to the contractile response which occurred upon the vessels exposure to the pCa 9.9 MI solution. The most likely cause of any remaining response upon exposure to the MI solution in the absence of ATP was residual interstitial Ca^{2+} originating from the previous Krebs-buffer solution. To determine if this was the case some vessels were first washed in $\emptyset Ca^{2+}$ Krebs-buffer solution prior to the ∅ ATP pCa 9.9 MI solution exchange. The procedure was then repeated with fresh vessels either using a Na^+ based pCa 9.9 MI solution (substitution of K^+) and also a K^+ based pCa 9.9 MI solution using Cl^- as the dominant anion (substitution of CH_3COO^-). Finally, fresh vessels bathed in a ØATP pCa 9.9 MI solution were challenged with either a single addition of 10mM Creatine phosphate or a 100μM DDT addition to determine if these components were involved in any part of the observed response.

4.3.3 Results

4.3.3.1 Is ATP responsible for the contractile response to the mock intra-cellular bathing solution?

Exchanging a $\emptyset Ca^{2+}$ Krebs solution for a 5mM ATP pCa 9.9 MI solution produced a bi-phasic contraction, consisting of a rapid phasic response (P/R; 50 ± 6% of the 120mM KCl response, n=7) and a sustained tonic response (T/R; and 9 ± 4%, n=7), separated by a slow exponential fall off (F/O). The responses were not different in either magnitude or profile to that produced by a single addition of 5mM ATP to vessels bathed in a ØATP, pCa 9.9 MI solution (n=7, $P>0.05$, unpaired t-tests). However, although adding a single dose addition of 5mM ATP to vessels bathed in a $\emptyset Ca^{2+}$ Krebs solution did not alter the

magnitude of the phasic and tonic responses (n=5-7, $P>0$ .05, unpaired test), the 90% fall off period (Time to F/O_{90}), as measured in seconds from the peak of the phasic response until the force reached a point at which it was 10% of the difference between P/R and T/R, was substantially reduced (922 ± 109secs, n=5 vs 4023 ± 305secs, n=8 $P<0.0001$, unpaired t-test). The presence of 2.5mM free Ca^{2+} in the Krebs buffer solution enhanced the magnitudes of the phasic and tonic responses to a single dose of 5mM ATP (P/R; 76 ± 7% vs 50 ± 6%, T/R; 41 ± 7% vs 9 ± 4%, n=7, $P<0.0001$, unpaired t-tests) but did not further increase the rate of fall-off beyond that which had been observed in the $ØCa^{2+}$ Krebs condition (n=7, $P>0.05$, unpaired test) (Fig 4.7).

4.3.3.2 How does 5mM ATP affect SR Ca^{2+} stores?

Incubation with 2μM thapsigargin, a specific inhibitor of the SERCA 2 Ca^{2+} ATPase, which regulates Ca^{2+} uptake into the SR, did not attenuate the phasic response of a 5mM ATP single dose addition. However the phasic response was almost completely abolished by incubation with 100μM 2 APB, a known inhibitor of IP_3 mediated Ca^{2+} store release. Rapid application of 10mM caffeine, which causes SR Ca^{2+} release via activation of Ryanodine sensitive channels, caused only a very small phasic response relative to the single 5mM ATP addition (P/R: control; 45 ± 5% of the 120mM KCl response, n=7 vs + Thaps; 39 ± 9%, n=5 vs +2 APB; 2 ± 1%, n=7 vs Caffeine; 3 ± 1%, n=7, $P<0.001$, unpaired ANOVA).

In contrast, the tonic response was completely ablated by thapsigargin but was enhanced by 2 APB. Rapid application of Caffeine failed to produce a tonic response (T/R: control; 7 ± 2% vs + Thaps; <1 ± 1%, n=5, +2 APB; 15 ± 2%, n=7 vs Caffeine; -1 ± 1% $P<0.001$, unpaired ANOVA) (Fig 4.8).

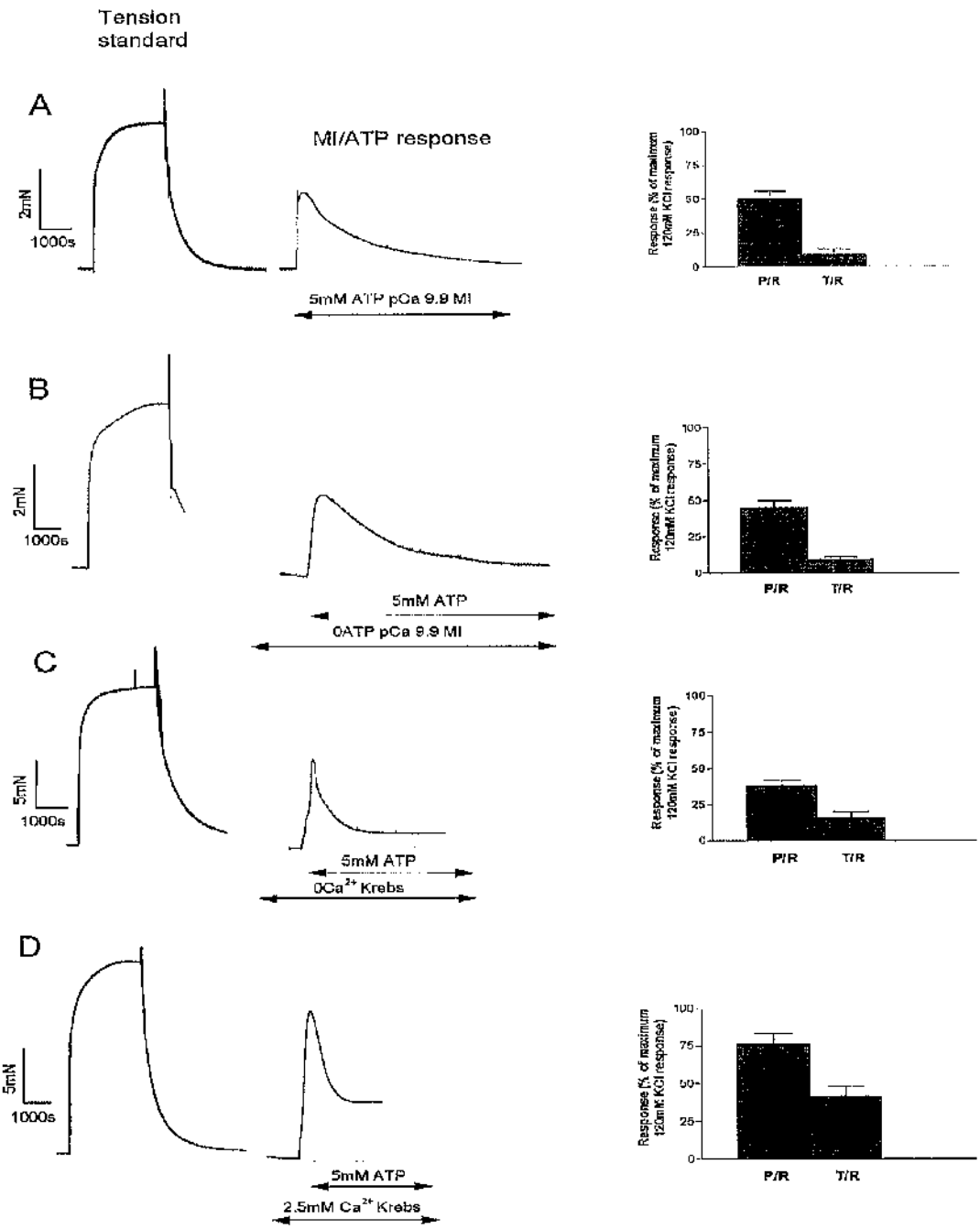


Figure 4.7 Identification of 5mM ATP as the component of the MI solution responsible for 2nd messenger activation

The phasic (P/R) and tonic (T/R) responses observed upon: A) Solution exchange from a $\emptyset Ca^{2+}$ Krebs buffer to a pCa 9.9 MI solution containing 5mM ATP (n=7). B-D) Single addition of 5mM ATP to vessels bathed in a B) ØATP pCa 9.9 MI solution (n=7). C) $\emptyset Ca^{2+}$ Krebs buffer solution (n=5). D) 2.5mM free Ca^{2+} Krebs buffer solution (n=7).

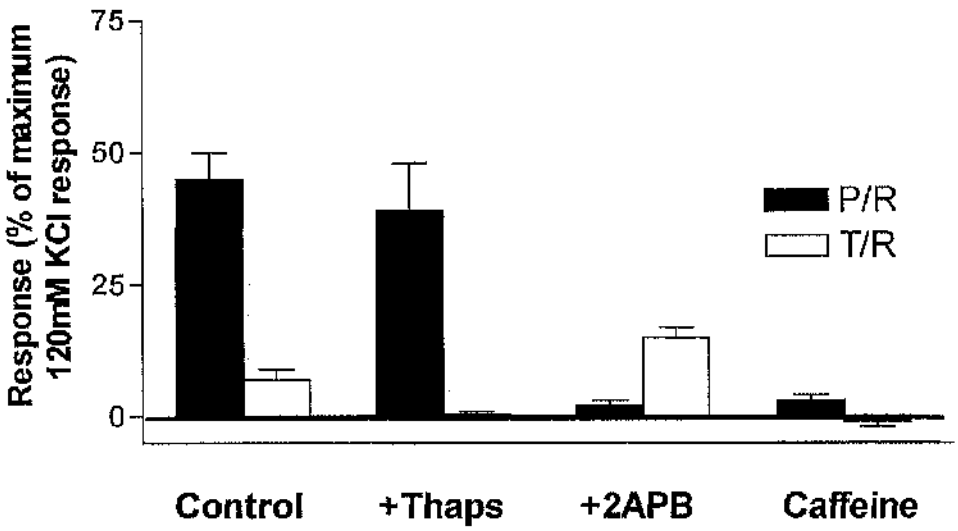


Figure 4.8 The role of the SR and the relative contribution of IP_3 and ryanodine sensitive Ca^{2+} stores to the phasic and tonic response caused by a single dose of 5mM ATP

The phasic (P/R) and Tonic (T/R) responses observed upon a single addition of 5mM ATP to vessels bathed in a ØATP pCa 9.9 MI solution in the absence (Control, n=7) and presence of either 2μM Thapsigargin (n=5) or 100μM 2 APB (n=7). Rapid application of 10mM caffeine (n=7) was used in place of 5mM ATP to assess the contribution of ryanodine sensitive stores.

4.3.3.3 Does 5mM ATP modulate Ca^{2+} sensitivity of the contractile apparatus?

Ca^{2+} CCRCs in the presence and absence of 5mM ATP and ROK inhibition

In vessels pre-treated with 2μM thapsigargin (to eliminate modulation of intra-cellular Ca^{2+} levels by the SR Ca^{2+} release) the presence of 5mM ATP in the MI solutions caused a markedly significant left shift in the Ca^{2+} CCRC relative the Ca^{2+} CCRC performed in the ØATP MI solution. This shift was partly, but not entirely reversed by incubation with 10μM of the ROK inhibitor Y27632 (pEC_{50} values: ØATP; 3.04 ± 0.07 vs 5mM ATP; 3.95 ± 0.07 vs, 5mM ATP + Y27632; 3.48 ± 0.08, n=8, P<0.01, unpaired ANOVA). In the 5mM ATP condition at pCa 4.55 the presence of Y27632 was associated with a 50% reduction in tone (33 ± 5% of own maximum vs 16 ± 4%, n=8, P<0.05, unpaired t-test) (Fig 4.9).

Incubation of vessels bathed in ∅ ATP MI solutions with 10μM Y27632 caused a substantial slowing of the rate of force production making the time required to perform the CCRC prohibitively long. The magnitude of the shift caused by the presence of 10μM Y27632 in the ∅ and the 5mM ATP MI solutions could therefore not be compared. However, the relative reduction in tone caused by Y27632 at pCa 4.55 in the 5mMATP condition was comparable to the reduction in tone caused by Y27632 in the ØATP condition in the single addition experiment (see next section).

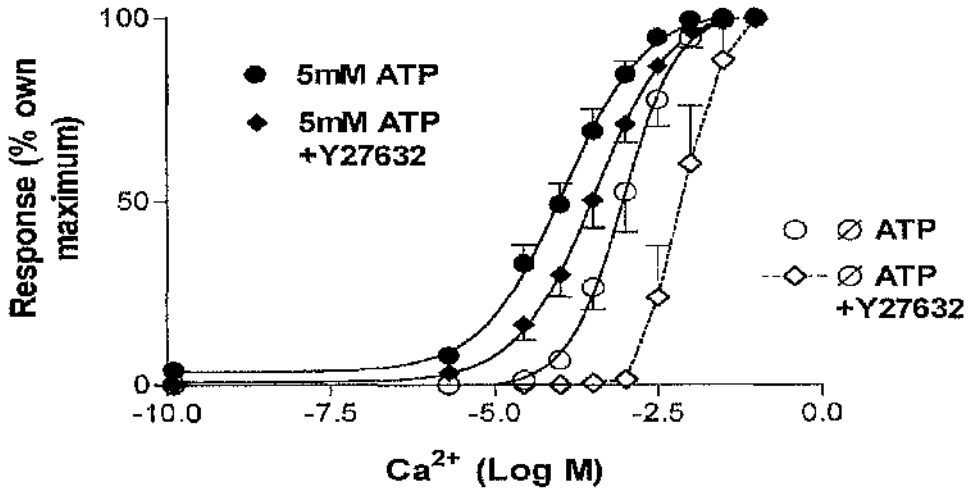


Figure 4.9 The effect of Rho kinase inhibition on Ca^{2+} CCRCs in the presence and absence of 5mM ATP performed in depolarised intact (unpermeabilised) vessels

Black symbols - vessels bathed in a 5mM ATP based MI solution, White symbols - vessels bathed in a ∅ ATP MI solution. Control vessels – circles, + Y27632 – diamonds. All groups n=8 (except ØATP MI +10μM Y 27632 (◇) Open diamonds, n=2).

Single addition of the ROK inhibitor Y-27632 in unpermeabilised vessels with a sub-maximally raised tone in the absence of ATP

A single addition of 10μM Y27632 to unpermeabilised vessels with a sub-maximally (pCa 4.55) raised tone caused a 47 ± 16% (P<0.001, unpaired t-test) reduction in tone relative to the time control vessels. Mean pre-tone (P/T) was not significantly different

between the response and control groups suggesting the comparison was valid (P/T; 30 ± 7% of the 120mM KCl response, n=5 vs 35 ± 9%, n=3, P>0.05, unpaired t-test) (Fig 4.10). Unfortunately the experiment could not be satisfactorily repeated in vessels bathed in a 5mM ATP MI solution. These vessels were subject to an unpredictable but sustained decline in tension, which prevented the attainment of a stable sub-maximal pre-tone. However, the reductions in tone at pCa 4.55 caused by Y27632 in the present ØATP experiment and the previous 5mM ATP Ca^{2+} CCRC were comparable.

4.3.3.4 Do the remaining components of the mock intra-cellular solution contribute to the observed contractile response?

In the absence of ATP, the solution switch from Krebs buffer to a ØATP pCa 9.9 MI solution still produced a phasic response that was 25 ± 4% of the 120mM KCl response (n=7). This phasic response was significantly attenuated to 6 ± 2% (n=6, $P<0.05$, unpaired t-test) by first switching to a $\varnothing Ca^{2+}$ Krebs solution (which had Na^+ as the dominant cation) for 10mins prior to switching to the Ø ATP MI solution. Substituting the K^+ cation with Na^+ in the MI solution (thus preventing depolarisation), completely abolished the remaining response (n=4). Substitution of the acetate anion with Cl^- however, had no effect (n=4, $P>0.05$, unpaired t-test). There was no tonic response associated with the Krebs to MI solution switch in the absence of ATP regardless of the dominant cation present or whether or not the vessels had first been washed in $\varnothing Ca^{2+}$ Krebs. Neither Phosphocreatine (n=4) nor DTT (n=4) was found to cause any response in unpermeabilised vessels.

The vessels from all groups were of a comparable internal diameter and replicated transmural pressure (P>0.05, unpaired ANOVA). The mean internal diameter and replicated transmural pressure of the vessels used in study 2 was 239 ± 6μm and 14.0 ± 0.2mmHg respectively (n=113 vessels).

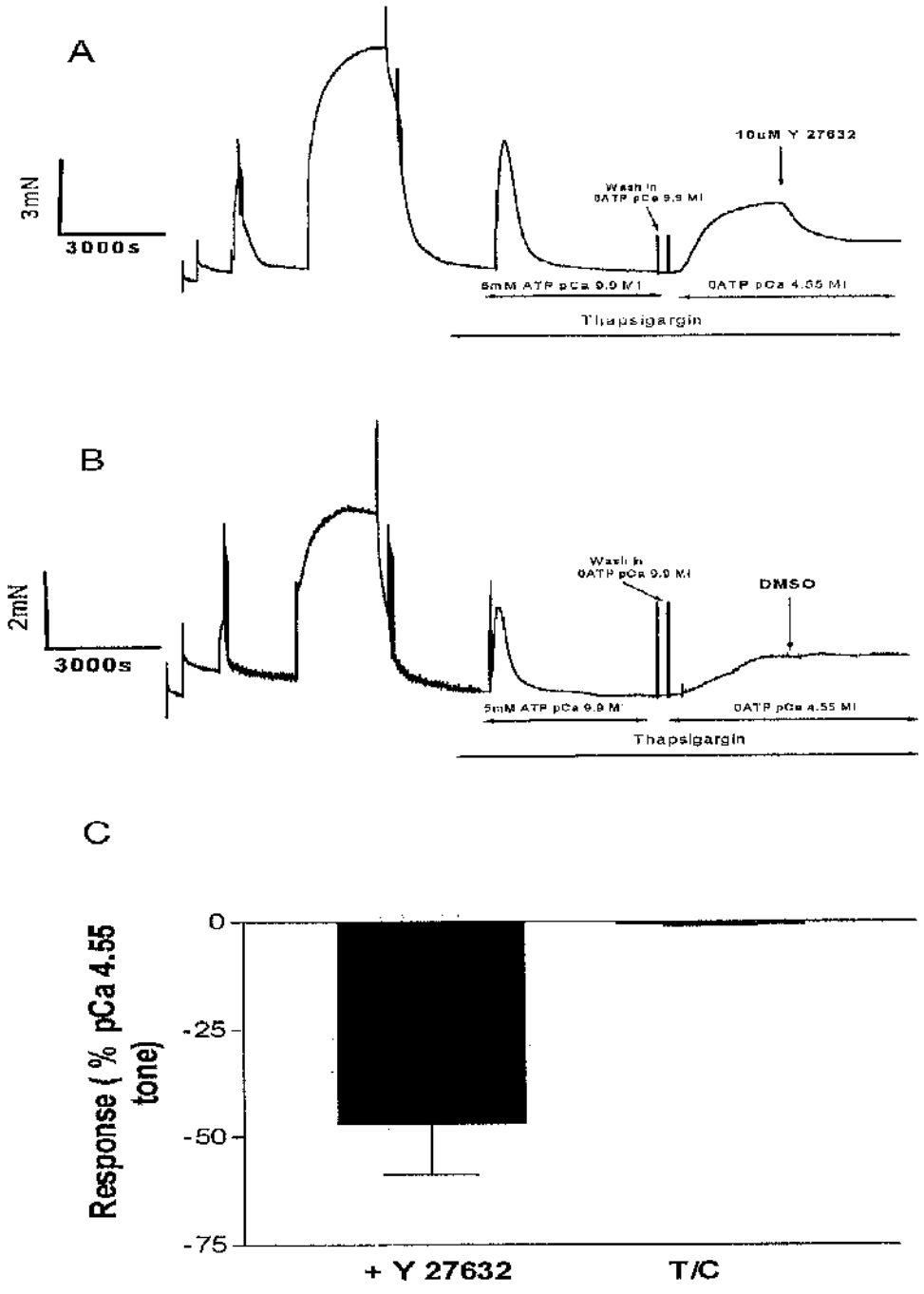


Figure 4.10 Confirmation of the constitutive activity of Rho kinase in the absence of exogenous ATP

Experimental traces showing: A) The effect of a single dose addition of the Rho kinase inhibitor Y 27632 (10μM) to an intact depolarised vessel with a sub-maximal raised tone B) The paired time control vessel. C) Bar graph showing the mean reduction in tone caused by a single dose addition of 10μM Y27632 (n=5) relative to the time control vessels (n=3) ($P<0.001$, unpaired t-test).

4.3.4 Application to protocol

Although the study could not conclusively prove whether the presence of 5mM ATP in the MI solution modulated the CaS of the contractile apparatus, it did clearly show that the ROK pathway was active both in its presence and its absence. Furthermore the magnitudes of the effect of ROK inhibition (~50% decrease in tone) at sub-maximal tone (pCa 4.55) both in the presence and absence of 5mM ATP were comparable and suggested any effects of ATP on the ROK pathway were minimal. 5mM ATP was therefore included in all subsequent MI solutions unless stated otherwise.

4.4 Study 3) Selection of a sensitising agonist as a standard

4.4.1 Background

Before a comparison of permeabilisation techniques and agent concentrations could occur it was necessary to establish a sensitising agent, which would act as a standard against which post permeabilised responses could be compared. The standard would be invaluable in assessing how well the receptor-coupled second messenger systems tolerated a chosen permeabilisation procedure and would allow the investigator to create a valid index for comparison. As ET-1 has previously been shown to increase CaS in the Sprague Dawley rat small pulmonary artery (Evans *et al.*, 1999) it was the obvious choice for further study. In the α-toxin permeabilised Sprague Dawley rat small pulmonary artery, 1μM ET-1 was reported to be capable of causing a contractile response, which was ~50% of the maximum permeabilised response to pCa 4.5. This response could not be attributed to any mechanism other than the sensitisation of the contractile apparatus.

The aim of this study was therefore to establish whether ET-1 was a suitable sensitising agonist in the Wistar rat small pulmonary artery and to determine the most appropriate concentration for its use.

4.4.2 Methods

4.4.2.1 What is the concentration range of the ET-1 induced vasoconstrictor response in the Wistar rat small pulmonary artery?

To establish the concentration range for the action of ET-1 and the concentration at which its maximal effect occurred, CCRCs to ET-1 (1pM-1μM) were performed in vessels bathed in Krebs-buffer.

4.4.2.2 What is the concentration range and Emax of the assumed ET-1 induced Ca^{2+} sensitisation response in the Wistar rat small pulmonary artery?

It was also necessary to establish whether ET-1 was likely to have a sensitising effect in the Wistar rat small pulmonary artery as had been reported in the Sprague Dawley (Evans *et al.*, 1999). To determine the approximate magnitude and concentration range of any such response, CCRCs to ET-1 (1pM-1μM) were performed in vessels with a negated SR (2μM thapsigargin) under 3 separate conditions: 1) pCa 9.9; the virtual absence of extra-cellular Ca^{2+} and Ca^{2+}_{cyt}. 2) pCa 6.64; the $[Ca^{2+}]$ predicted to cause a raised tone of ~ 25% in permeabilised vessels but no induced tone in unpermeabilised vessels. 3) pCa 4.55; the $[Ca^{2+}]$ predicted to cause a raised tone of ~ 25% in unpermeabilised vessels and maximal tone in permeabilised vessels .

Under these conditions the vessels were depolarised, extra-cellular Ca^{2+} levels were clamped and the SR's Ca^{2+} stores negated. It was considered that any subsequent response to ET-1 would be indicative of its sensitising capabilities. However, it was recognised that ET-1 could modulate the activity of sarcolemmal Ca^{2+} channels leading to a change in Ca^{2+} permeability, thus compromising the $[Ca^{2+}]_{cyt}$ and that this could influence the response, particularly in the pCa 4.55 condition.

4.4.2.3 Does 5mM ATP affect the ET-1 induced response in the Wistar rat small pulmonary artery bathed in a ØCa^{2+} mock intra-cellular solution?

It was suspected that 5mM ATP was causing chronic activation of the SR and that in conjunction with 10mM EGTA, this action may effectively prevent the SR from modulating $[Ca^{2+}]_{cyt}$ in permeabilised vessels, thus negating the need for additional intervention. To determine whether the presence of 5mM ATP in the MI solution could drain the SR as effectively as thapsigargin inhibition of Ca^{2+} uptake, ET-1 CCRCs (1pM-1μM) were repeated in Ø and 5mM ATP pCa 9.9 MI solutions in the absence of thapsigargin.

4.4.3 Results

4.4.3.1 Over what concentration range does the ET-1 induced vasoconstrictor response occur in the Wistar rat small pulmonary artery?

Cumulative doses of ET-1 to vessels bathed in Krebs-buffer solution produced a sigmiodal dose dependent response curve with a threshold at 1nM and an Emax of 128 ± 8% at 1μM. The pEC_{50} was 7.81 ± 0.07 (n=7) (Fig 4.11).

4.4.3.2 Does ET-1 induced sensitisation occur in the Wistar rat small pulmonary artery? What is the concentration range and magnitude of the effect?

Exposure of vessels (pre-treated with thapsigargin to negate the SR) to the 5mM ATP MI solutions caused a sustained elevation in tone, which appeared to be dependent on the level of free Ca^{2+} in the solutions. The pCa 4.55 MI solution initially produced a sub-maximal raised tone (pre-tone) that was 40 ± 3% (n=5) of the 120mM iso-osmotic KCl response. However, this was not stable and diminished to 27 ± 8% (n=5) before any response to ET-1 was observed. Relative to the pCa 4.55 MI solution, the pCa 9.9 and pCa 6.64 MI solutions produced attenuated ($P<0.05$, unpaired ANOVA) but entirely stable pre-tones, which were not significantly different from one another (2 ± 1%, vs 3 ± 2% n=5, $P>0.05$, unpaired t-test).

The concentrations at which the threshold and maximum response to ET-1 occurred were 10nM and 1μM respectively, regardless of the pCa of the depolarising MI solution and its associated level of pre-tone. The efficacy and potency of the ET-1 induced response was not different in the pCa 9.9 and pCa 6.64 conditions. However, the efficacy but not the potency of ET-1 was significantly enhanced in the pCa 4.55 condition (Emax: in pCa 9.9; 11 ± 2% of the120mM KCl response vs pCa 6.64; 11 ± 2% vs pCa 4.55; 21 ± 4%, n=5, $P<0.05$, unpaired ANOVA) (pEC_{50}: pCa 9.9; 7.20 ±. 0.16 vs pCa 6.64; 7.24 ± 0.18 vs pCa 4.55; 7.27 ± 0.27, n=5, $P>0.05$, unpaired ANOVA). Expressing the 1μM ET-1

response in pCa 4.55 as a percentage of the (pCa 4.55) pre-tone indicated a 74 ± 20% increase (n=5) (Fig 4.11).

4.4.3.3 Does 5mM ATP affect the ET-1 induced response in the Wistar rat small pulmonary artery bathed in a mock intra-cellular solution in the absence of extra-cellular Ca^{2+}?

The efficacy of the response to ET-1 in the 5mM ATP MI solution was unchanged by the absence of thapsigargin pre-treatment. However in the absence of ATP and thapsigargin, the efficacy of the response was significantly augmented (Emax; in 5mM ATP pCa 9.9 + Thaps; 11 ± 2% of the 120mM KCl response vs 5mM ATP pCa 9.9; 13 ± 3% vs 0ATP pCa 9.9; 32 ± 7%, n=5, $P<0.05$, unpaired ANOVA).

In contrast, the absence of thapsigargin increased potency of the ET-1 response, both in the presence and absence of ATP (pEC_{50}: 5mM ATP pCa 9.9 + Thaps; 7.20 ± 0.16 vs 5mM ATP pCa 9.9; 8.03 ± 0.14 vs 0ATP pCa 9.9; 8.07 ± 0.14, n=5, $P<0.005$, unpaired ANOVA) (Fig 4.11).

The vessels from all groups were of a comparable internal diameter and replicated transmural pressure ($P>0.05$, unpaired ANOVA). The mean internal diameter and replicated transmural pressure of the vessels used in study 3 was 231 ± 10μm and 13.7 ± 0.2mmHg respectively (n=32 vessels).

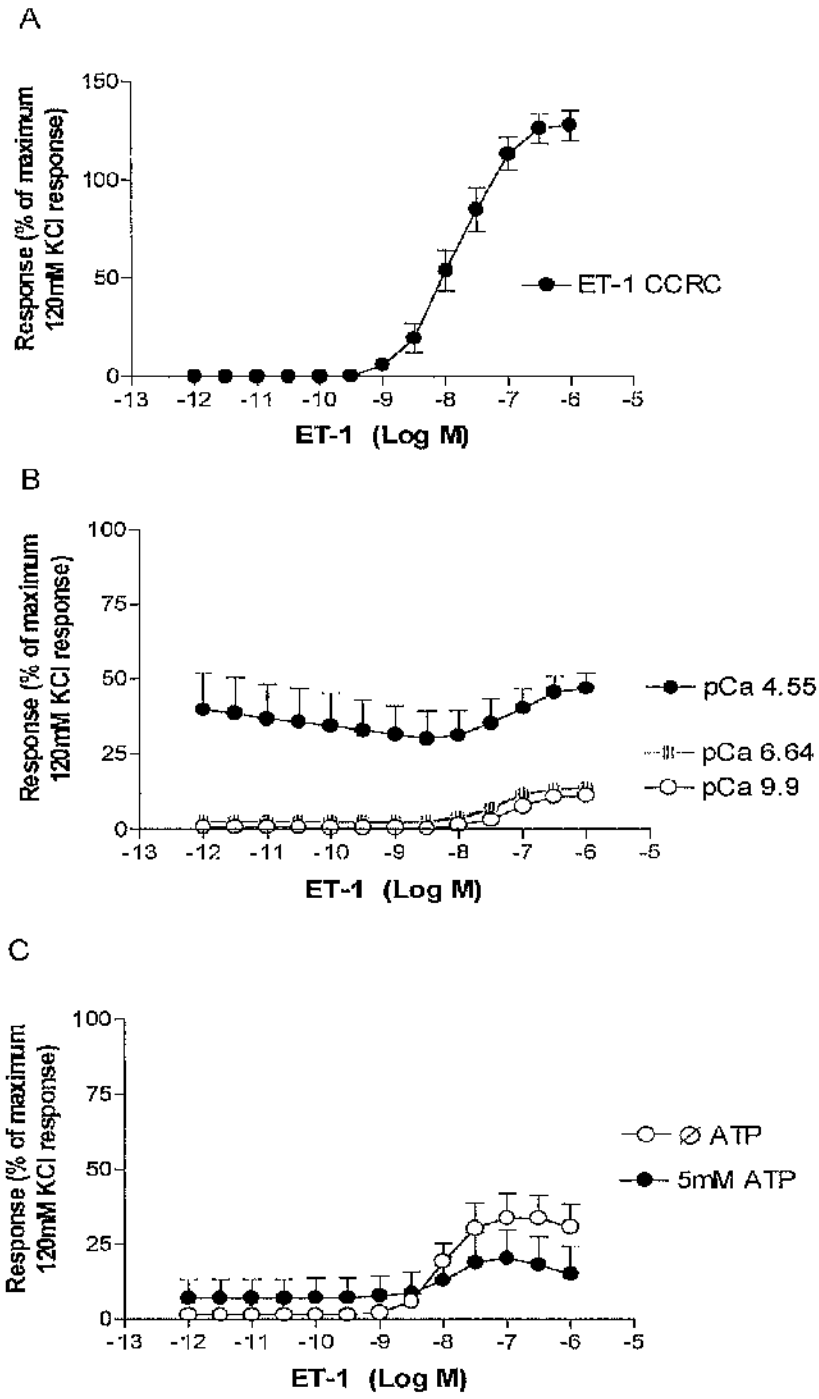


Figure 4.11 ET-1 is a suitable sensitising agent standard

A) CCRC to ET-1 in Krebs buffer (n=7). B) CCRCs to ET-1 in 5mM ATP based pCa 9.9 mock intra-cellular (MI) solutions in the presence of thapsigargin (ali n=5, $P<0.05$). C) CCRCs to ET-1 in Ø and 5mM pCa 9.9 ATP MI solutions in the absence of thapsigargin (all n=5, $P<0.05$). The pre-tone was subtracted prior to statistical comparison.

4.4.4 Application to protocol

The data suggest that in optimally permeabilised vessels, with a sub-maximal raised tone (~ 25% of max response), a single addition of an optimum concentration of ET-1 should increase tone by ~10-20% of the vessels own maximum response. The optimum concentration of ET-1 was found to occur between 0.1 and 1μM depending on the presence of thapsigargin/DMSO.

Additionally the response in the pCa 9.9 MI solution was recognised as a useful index of permeabilisation, which permitted the identification of intact SMC populations following permeabilisation. Furthermore, as the ET-1 responses in pCa 9.9 and pCa 6.64 MI solutions in unpermeabilised vessels were virtually identical, it was considered that following permeabilisation any response observed to ET-1 in pCa 9.9 would be indicative of the potential error (caused by the presence of unpermeabilised SMCs) in the ET-1 response at pCa 6.64.

The augmented response to ET-1 in the absence of thapsigargin in the ØATP pCa 9.9 MI solution also suggested that 5mM ATP caused a chronic activation of the SR, which effectively drained the SR to levels comparable with thapsigargin pre-treatment. It was recognised that this may negate the need for the additional treatment of the SR in permeabilised vessels and that this approach should be investigated further.

4.5 Study 4) Selecting the optimum β-escin concentration for permeabilisation

4.5.1 Background

To ensure uniformity in the permeabilised preparations, each vessel was exposed to β-escin in a pCa 5.7 MI solution. This solution caused no change in tone in the unpermeabilised SMCs but near maximal contraction of the permeabilised SMCs. Hence the development of tone reported on the progress of the permeabilisation procedure, and when tone finally stabilised this suggested the procedure was complete. Using this method, each vessel would therefore be exposed to a given concentration of β-escin for an optimum period of time (Fig 4.12).

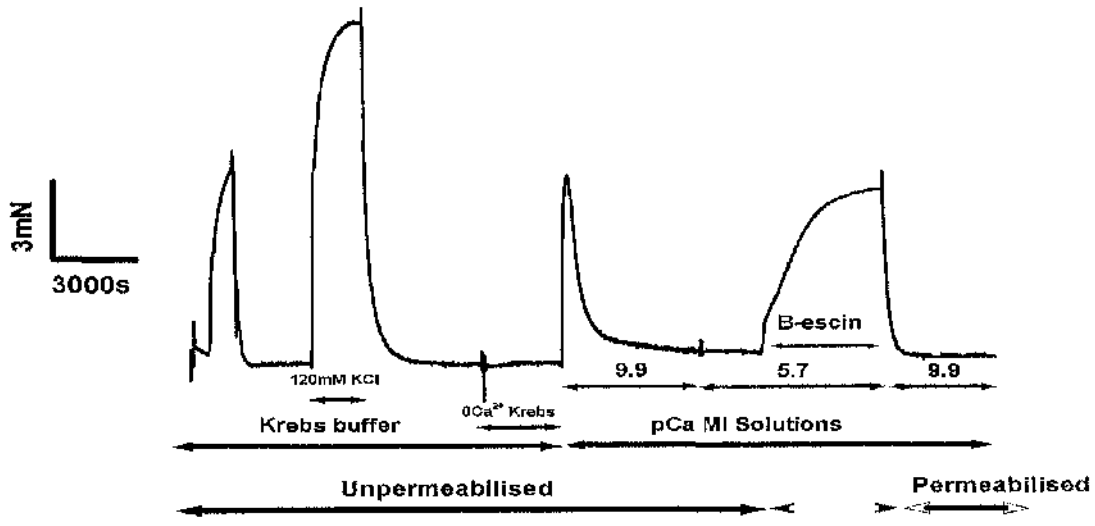


Figure 4.12 Experimental trace of the permeabilisation protocol

This technique uses Ca^{2+} entry into the permeabilised cells to report on the progress of the procedure, thus optimally permeabilising each vessel.

However, the dose dependant nature of β-escin also meant that whilst above a certain threshold, all concentrations would produce a fully permeabilised preparation, only a certain range of concentrations would also preserve the second messenger systems that were necessary to future studies. Hence, the aim of this study was to determine the optimum concentration of β-escin for use in the permeabilisation protocol.

This required the development of an index of permeabilisation and also a separate index, which reported on the preservation of signal transduction systems. The permeabilisation index would allow an assessment of the degree of permeabilisation achieved for each concentration of β-escin tested and hopefully provide a useful quality control test that could be applied at the end of future experiments. The previous study had already provided one index of permeabilisation, the response to ET-1 in a pCa 9.9 MI solution. However, whilst this was acceptable for the purpose of the protocol development it could not be used routinely, as ET-1 is resistant to washout making any vessels tested useless for further study. Furthermore it could not be confidently applied following the application of the sensitising agonists if these were resistant to washout. A second index of permeabilisation was therefore required, preferably one, which could be applied at the end of the protocol and one, which would not be invalidated by the action of the sensitising agonists under study. The second index was originally based on the hypothesis that permeabilised and unpermeabilised SMCs would respond to Ca^{2+} in the MI solutions over separate, distinct [Ca^{2+}] ranges. Hence, a partially permeabilised vessel exposed to a [Ca^{2+}] sufficient to cause maximum contraction of its permeabilised SMC population but no activation of its unpermeabilised SMC population, would show an additional increase in tone when it was subsequently exposed to a [Ca^{2+}] that would cause maximal activation of both permeabilised and unpermeabilised SMCs. The 2 concentrations were termed the intra-cellular maximum (i_{max}) and the extra-cellular

maximum (e_{max}) respectively, and when force at e_{max} (Fe_{max}) no longer exceeded force at i_{max} (Fi_{max}), the vessel was considered to be fully permeabilised. In practice it was found that the two ranges did marginally overlap, but that the contractile reserve of the unpermeabilised population of SMCs at i_{max} was more than sufficient to distinguish their presence at e_{max} (Fig 4.13).

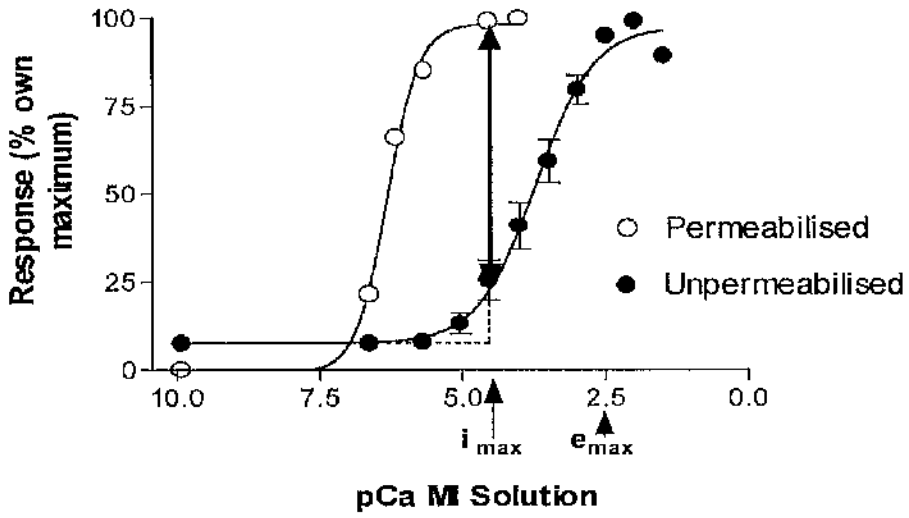


Figure 4.13 The rationale behind e_{max} index of permeabilisation

Ca^{2+} CCRCs in permeabilised and unpermeabilised vessels showing the predicted contractile reserve (vertical arrow) remaining in unpermeabilised SMCs at pCa 4.55 (i_{max}). Upon exposure to a pCa 2.5 (e_{max}) MI solution this contractile potential is realised, thus reporting the presence of any unpermeabilised SMCs.

Hence in vessels which were incompletely permeabilised the presence of unpermeabilised SMCs was clearly observed as an increase in tone following the switch from a pCa 4.55 (i_{max}) MI solution to a pCa 2.5 (e_{max}) MI solution. This increase in tone was absent in fully permeabilised vessels. This was known as the e_{max} index of permeabilisation.

The effect on the signal transduction mechanisms was determined by potency and efficacy of the ET-1 induced response in permeabilised vessels at a sub-maximally raised tone. This was known as the ET-1 index of signal transduction

4.5.2 Methods

Vessels were set up, normalised and their 120mM KCl tension standard response obtained as previously described. The following experiments were then performed.

4.5.2.1 Over what range of concentrations does ß-escin fully permeabilise the Wistar rat small pulmonary artery?

Following equilibration in a pCa 9.9 MI solution, vessels were bathed in a pCa 5.7 MI solution and β-escin added at one of the four following concentrations, 25, 50, 100 or 200μM. When each vessel reached its individual optimum exposure time it was washed in a pCa 9.9 MI solution and allowed to return to baseline. Each vessel was then exposed a pCa 4.55 (i_{max}) MI solution until the response stabilised and then the vessel was exposed to a pCa 2.5 (e_{max}) MI solution. The procedure was then repeated in fresh vessels, however this time the vessels were challenged with 1μM ET-1 in a pCa 9.9 MI solution (ET-1 index of permeabilisation) prior to performing the i_{max} and e_{max} challenges.

4.5.2.2 What concentration of ß-escin best preserves the receptor coupled signal transduction systems?

Vessels were permeabilised as previously described using one of four different concentrations of β-escin; 25, 50, 100 or 200μM. Following permeabilisation, vessels were washed in a pCa 9.9 MI solution and 10μM A 23187 (30mins incubation) was added to permeabilise the SR to Ca^{2+}, thus preventing agonist induced SR Ca^{2+} release which could influence tone. Once the vessels had returned to baseline, they were placed

in a pCa 6.64 MI solution in order to create a sub-maximal raised tone (~25% of $\mathbf{Fi}_{max}$). Upon stabilisation of the sub-maximal pre-tone, CCRCs to ET-1 (10nM-1μM) were performed. Following the performance of the CCRC the vessels were assessed using the e_{max} index of permeabilisation to determine the presence of any unpermeabilised SMCs.

4.5.3 Results

4.5.3.1 Over what range of concentrations does ß-escin fully permeabilise the Wistar rat small pulmonary artery?

$\mathbf{Fe}_{max}$ was not significantly different between any of the groups when expressed as a percentage of each vessels own pre-permeabilisation 120mM KCl response (all groups 49-50 ± 2%, n=6, P>0.05, unpaired ANOVA). However, when the data were expressed as a percentage of $\mathbf{Fi}_{max}$, $\mathbf{Fe}_{max}$ was significantly augmented in the 25μM group ($\mathbf{Fe}_{max}$: 50, 100, 200μM β-escin; 101 ± 1% of $\mathbf{Fi}_{max}$ vs 25μM β-escin; 107 ± 2%, all n=6, P<0.001, unpaired ANOVA) (Fig 4.14).

Vessels permeabilised with 50, 100 or 200μM β-escin showed essentially no response to 1μM ET-1 in the pCa 9.9 MI. However some vessels permeabilised in 25μM β-escin retained the ability to respond to 1μM ET-1, producing a significant elevation in tone at pCa 9.9 (ET-1 Emax: 50, 100, 200μM β-escin; <1 ± 1% of $\mathbf{Fi}_{max}$ vs 25μM β-escin; 5 ± 2%, n=5-6, P<0.05, unpaired ANOVA). Quantitatively the e_{max} index did not show a significant increase in $\mathbf{Fe}_{max}$ in any of the groups following exposure to ET-1 (P>0.05, n=5-6, unpaired ANOVA). However, qualitatively, vessels from the 25μM β-escin group which demonstrated a noticeable response to ET-1 in the pCa 9.9 MI solution also produced an e_{max} response that was visibly larger than the i_{max} response (Fc_{max}: 50, 100, 200μM β-escin; 101 ± 1% of $\mathbf{Fi}_{max}$ vs 25μM β-escin; 105 ± 3%, n = 5-6, P>0.05, unpaired ANOVA) (Fig 4.15).

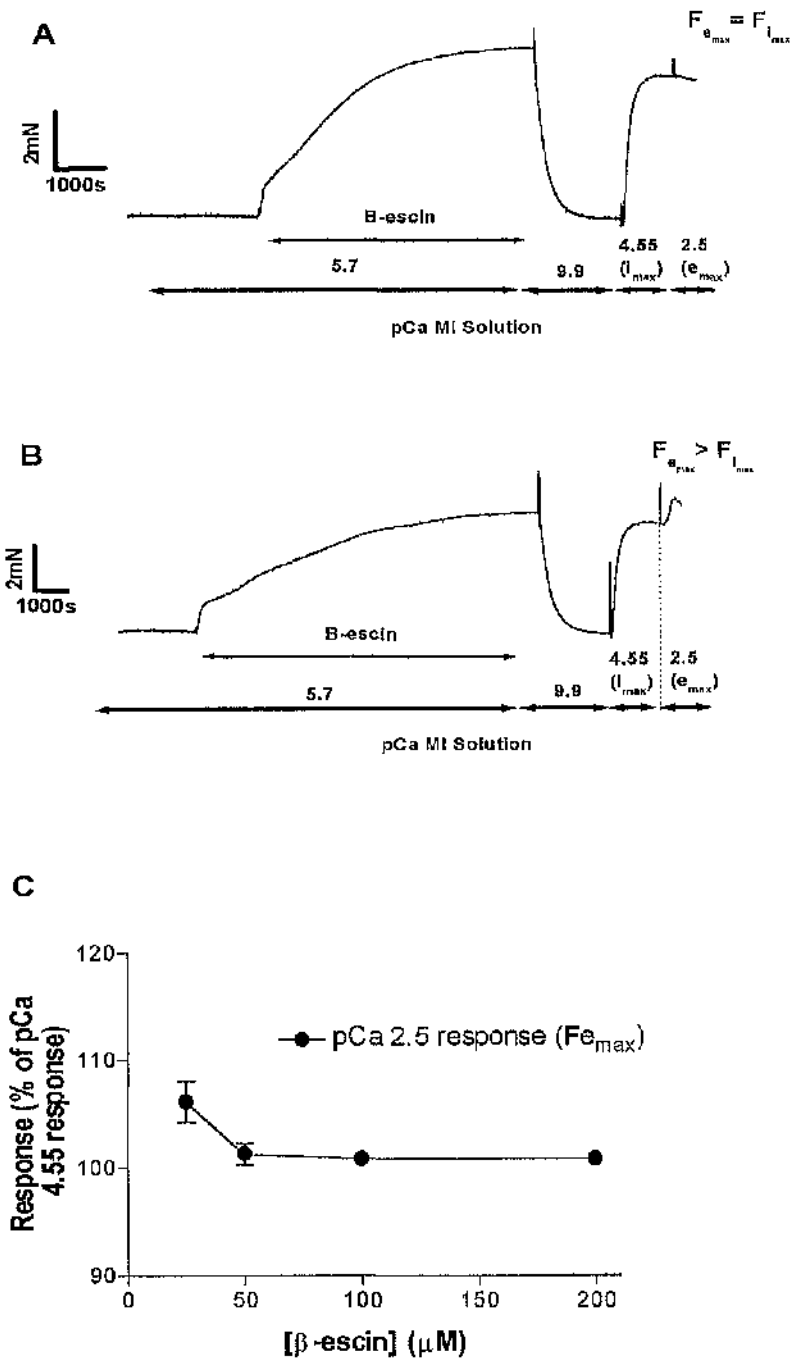


Figure 4.14 The e_{max} index of permeabilisation

Experimental traces showing the course of permeabilisation and the subsequent e_{max} index response of; A) a fully permeabilised vessel B) an incompletely permeabilised vessel. C) The pCa 2.5 (e_{max}) response (Fe_{max}) expressed as a percentage of the pCa 4.55 (i_{max}) response (Fi_{max}) for vessels permeabilised at 25, 50, 100 or 200 μM β-escin. (all groups n=6, P<0.01, unpaired ANOVA).

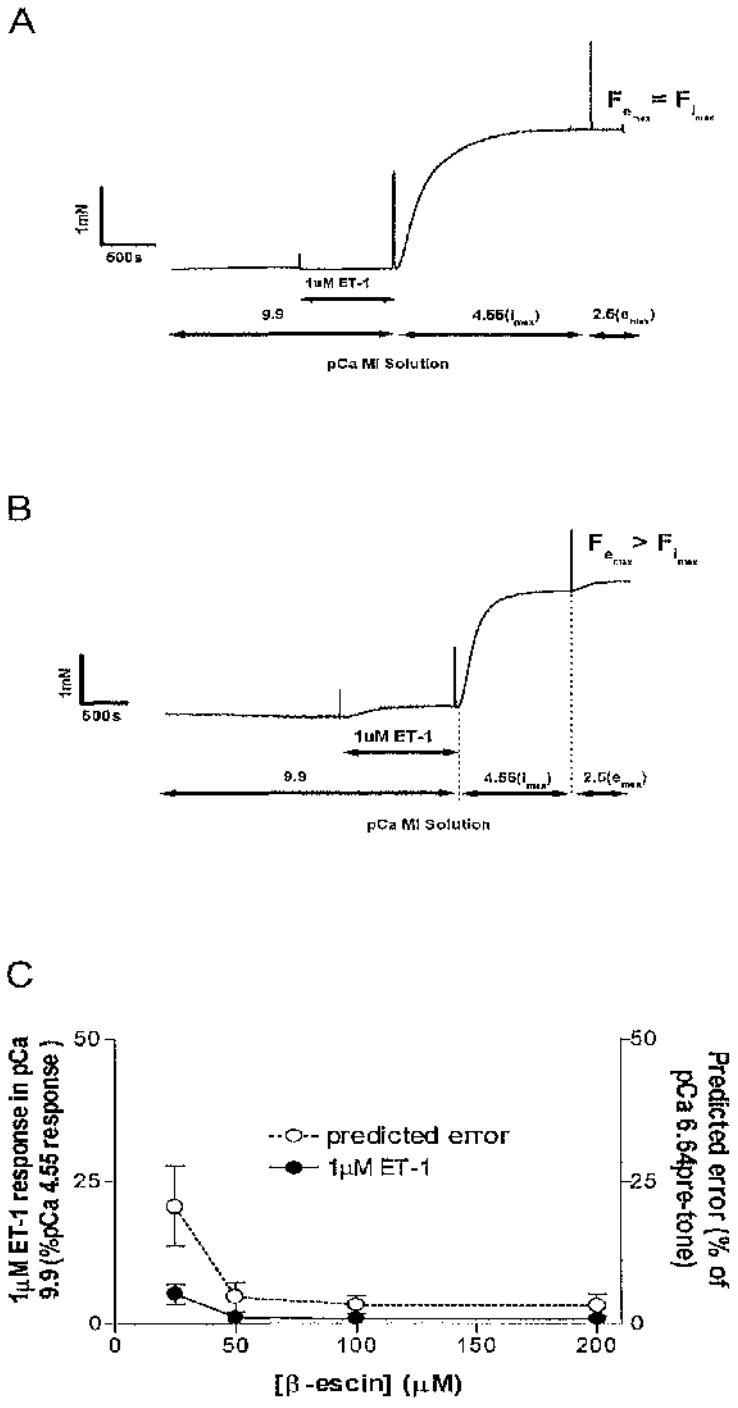


Figure 4.15 The ET-1 index of permeabilisation

Experimental traces showing the ET-1 response of; A) a fully permeabilised vessel B) an incompletely permeabilised vessel in a range of MI solutions. C) The ET-1 response expressed as a percentage of the pCa 4.55 response (Fi_{max}) for vessels permeabilised with 25, 50, 100 or 200 μM β-escin. (all groups n=5-6 ,P<0.01, unpaired ANOVA). Also shown is the predicted error of a maximal 1μM ET-1 response caused by the presence of unpermeabilised SMC's. The error is expressed as a % of the pCa 6.64 pre-tone.

4.5.3.2 What concentration of ß-escin best preserves the receptor coupled signal transduction systems?

ET-1 induced a maximum response at 1μM in all groups and significantly increased pre-tone (P/T) in vessels permeabilised with 25, 50 or 100μM β-escin (25μM β-escin: P/T; 0.45 ± 0.08mN vs 1μM ET-1 response; 0.68 ± 0.10mN, 50μM β-escin; 0.36 ± 0.08mN vs 0.53 ± 0.12mN, 100μM; 0.30 ± 0.04 vs 0.37 ± 0.04 mN, $P<0.01$, all n=5, paired t-test) but not in vessels permeabilised with 200μM β-escin ($P>0.05$, n=5, paired t-test). Neither the efficacy nor the potency of the ET-1 response was significantly different between the 25, 50 or 100μM β-escin groups. Efficacy data are expressed both as a % of the pCa 6.64 pre-tone (Emax: 25μM β-escin; 55 ± 12% of pCa 6.64 P/T vs 50μM; 45 ± 7% vs 100μM; 26 ± 9%, n=5, $P>0.05$, unpaired ANOVA) and as a % of Fi_{max} (Emax: 25μM β-escin; 13 ± 2% of Fi_{max} vs 50μM; 11 ± 2% vs 100μM; 6 ± 2%, n=5, $P>0.05$, unpaired ANOVA). Potency expressed as pEC_{50} values (pEC_{50}: 25μM β-escin; 7.10 ± 0.19 vs 50μM; 6.98 ± 0.11 vs 100μM; 6.72 ± 0.24, n=5, $P>0.05$, unpaired ANOVA) (Fig 4.16).

The vessels from all groups were of a comparable internal diameter and replicated transmural pressure (P>0.05, unpaired ANOVA). The mean internal diameter and replicated transmural pressure of the vessels used in study 4 was 271 ± 11μm and 13.4 ± 0.3mmHg respectively (n=66 vessels).

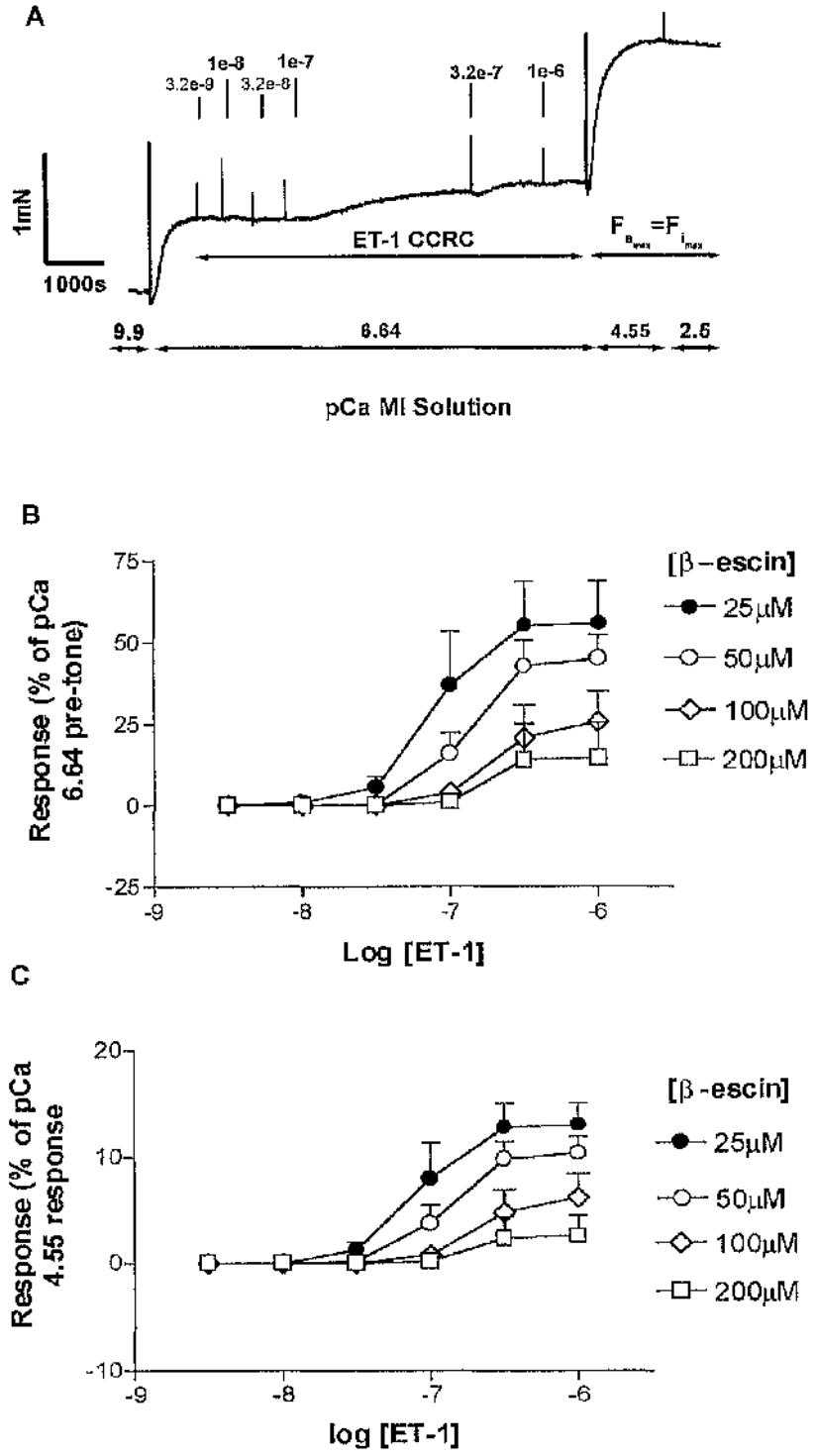


Figure 4.16 The ET-1 signal transduction index

A) Experimental trace showing an ET-1 CCRC in an optimally permeabilised vessel. ET-1 CCRCs for vessels permeabilised at 25, 50, 100 or 200μM β-escin expressed as a percentage of B) the pCa 6.64 pre-tone C) the pCa 4.55 response (Fi_{max}) (all groups n=5).

4.5.4 Application to protocol

25μM β-escin failed to consistently produce a fully permeabilised preparation, whilst 200μM β-escin failed to preserve receptor coupled signal transduction mechanisms. Both 50 and 100μM β-escin fully permeabilised the vessels whilst preserving the signal transduction mechanisms. Although the difference in efficacy was not significant between 50 and 100μM, vessels permeabilised in 50μM β-escin produced a response that was most compatible with that observed in unpermeabilised vessels. Therefore all subsequent permeabilisation studies were performed at this concentration. The e_{max} index of permeabilisation was found to be a useful indicator of the presence of unpermeabilised SMCs and was therefore applied at the end of all future permeabilisation experiments as a quality control measure.

4.6 Study 5) "Knocking out" the SR

4.6.1 Background

The final aspect of the protocol development that required investigation was to establish whether the SR could still influence $[Ca^{2+}]_{cyt}$ in response to an agonist in the permeabilised preparation. Previous studies (Akopov *et al.*, 1997;Akopov *et al.*, 1998;Kitazawa *et al.*, 1989;Evans *et al.*, 1999) had all used the DMSO based ionophore A 23187 (10μM) to irreversibly deplete the SR. In fully permeabilised preparations with the SR depleted and extra-cellular $[Ca^{2+}]$ (and hence $[Ca^{2+}]_{cyt}$) clamped by the EGTA based MI solution, any subsequent response to an agonist would have been expected to be caused by a change in CaS. However, DMSO may adversely affect the contractile apparatus in rat pulmonary arteries (unpublished observations). Furthermore, the obvious alternative to this, thapsigargin (which uses a lower [DMSO]), had been shown to cause a decrease in the potency of ET-1 in study 3. In light of the evidence suggesting that the presence of 5mM ATP in the MI solutions may deplete SR Ca^{2+}, it was considered that the SR may not require additional intervention in permeabilised vessels.

4.6.2 Methods

Vessels were set up and permeabilised using 50μM β-escin as previously described

4.6.2.1 Does the SR require additional intervention in permeabilised vessels?

Following permeabilisation, vessels were washed in a pCa 9.9 MI solution and 10μM A 23187, 2μM thapsigargin or nothing was added and the vessels allowed to return to baseline, whilst the drugs incubated. The vessels pre-treated with thapsigargin, A 23187 and one with no pre-treatment were then placed in a pCa 6.64 MI solution in order to create a sub-maximal tone (~25% of Fi_{max}), whilst a fourth vessel which had underwent

no additional pre-treatment of the SR was placed in a 50μM K_2EGTA based nominally Ca^{2+} free 5mM ATP, MI solution to act as a positive control. Upon stabilisation all vessels were challenged with 30μM IP_3 to trigger SR store release. Following any response to IP_3, vessels bathed in the 50μM K_2EGTA pCa 9.9 MI solution were washed and then exposed to a normal 10mM EGTA pCa 6.64 MI solution to determine what the vessels sub-maximal tone (P/T) would have been. Finally, all vessels were exposed to an i_{max} (pCa 4.55) MI solution to determine their $\mathbf{Fi}_{max}$.

4.6.3 Results

4.6.3.1 Does the SR require additional intervention in permeabilised vessels?

A 30μM single addition of IP_3 (the dominant intra-cellular signal for agonist induced SR Ca^{2+} release in Wistar rat small pulmonary arteries - see study 2) had essentially no effect on vessels incubated with 2μM thapsigargin, 10μM A-23187 or nothing in a pCa 6.64 MI solution (IP_3 response: all groups; <1 ± 1% of the pCa 6.64 P/T, n=5, P>0.05 unpaired ANOVA). This equated to a change in force of <0.25 ± 0.25% (all groups) of the $\mathbf{Fi}_{max}$.

Performing the challenge in the presence of a 50μM EGTA based, nominally Ca^{2+} free MI solution yielded a small, transient contraction that was 8 ± 4%, (n=4) of the subsequent response to a 10mM EGTA based pCa 6.64 MI solution. This equated to a change in force of <2 ± 1% of $\mathbf{Fi}_{max}$. Mean tone at pCa 6.64 was not significantly different between any of the groups (P>0.05, unpaired ANOVA) suggesting the comparison was valid (Fig 4.17).

The vessels from all groups were of a comparable internal diameter and replicated transmural pressure (P>0.05, unpaired ANOVA). The mean internal diameter and replicated transmural pressure of the vessels used in study 5 was 287 ± 7μm and 13.2 ± 0.2mmHg respectively (n=19 vessels).

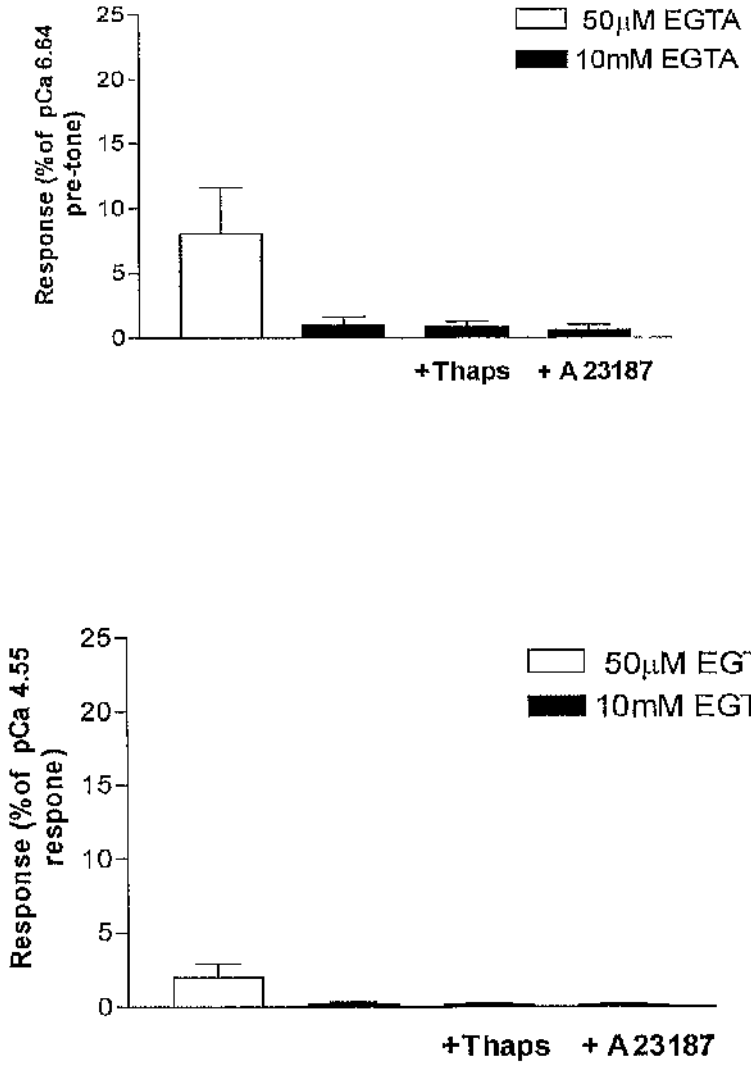


Figure 4.17 Establishing the SR buffering requirement

The response to a single dose addition of 30μM IP_3 in vessels bathed in either a 50μM (Transparent bars, n=4) or 10mM (Solid bars, n=5 all groups) EGTA based MI solution in the presence and absence of 2μM thapsigargin or 10μM A 23187. Results expressed as a percentage of A) the pCa 6.64 pre-tone and B) pCa 4.55 response (Fi_{max}).

4.6.4 Application to protocol

The results of study 5 confirmed that the SR of permeabilised vessels did not require additional intervention prior to their use in G-protein linked agonist studies performed in a pCa 6.64 MI solution.

4.7 Adaptation of the protocol for use with the chronically hypoxic rat and the control mouse.

The protocol was found to be compatible for use with the chronically hypoxic Wistar rat small pulmonary artery and required no further modification. The protocol was also compatible with the mouse C-class intra-pulmonary artery (SPA). In this vessel however the optimum concentration of β-escin was found to be 10μM.

4.8 Discussion

4.8.1 Study 1) Selecting the optimum tension response standard

Small pulmonary resistance arteries (SPAs) of a similar internal diameter and normalised to the equivalent of 12–14mmHg, regularly demonstrate a substantial variation in the absolute force they generate in response to a given stimulus (Fig 4.4). In order to compare the action of an agonist or other stimuli in these vessels, it is therefore necessary to express the response data as a percentage of some quantifiable and repeatable standard. Traditionally this has been achieved by depolarising the cell using potassium chloride (KCl) at a predetermined concentration to produce a maximum "un-sensitised" contraction which can be fully reversed by washout, with no detriment to the vessel.

4.8.1.1 Nominally iso-osmotic KCl depolarising solutions are superior to hyper-osmotic KCl additions

Hyper-osmotic KCl additions were associated with a 20–30% reduction in F_{90} and a two to four-fold increase in the time to F_{90} relative to nominally iso-osmotic $K^+CH_3COO^-$ depolarisations. However acetate/chloride substitution was also found to be detrimental to the normal contractile responses of the cell during repetitive exposure. This finding was consistent with previous reports that inhibition of chloride channels in rat PASMCs has been shown to slow the removal of Ca^{2+} from the cytosol following application of caffeine and in the absence of extra-cellular Ca^{2+}, significantly increase basal $[Ca^{2+}]_{cyt}$ (Cruickshank *et al.*, 2003).

Based on these findings a nominally iso-osmotic 120mM KCl solution was adopted as the protocols tension standard. The data showed that a nominally iso-osmotic KCl solution was a fast and reliable standard and had the least deleterious effect on force, rate of force production and basal tone despite repetitive and prolonged exposures.

However, it was noted that the response times of the pulmonary vessels to KCl were far slower those reported for systemic resistance vessels (Ghisdal *et al.*, 2003). A comparison of the time course of a nominally iso-osmotic KCl contraction with that of a maximal pCa 4.55 contraction in optimally permeabilised pulmonary vessels from study 4 found that the unpermeabilised response was ~ 50% slower. This suggested that even when using an iso-osmotic KCl solution, some process other than the responsiveness of the contractile proteins was limiting the rate of contraction. However this alone was not sufficient to explain the substantial difference in the response times between systemic and pulmonary vessels. A search of the literature failed to provide a suitable comparison of the time courses of pulmonary *in vitro* and *in vivo* depolarisation responses. Although the extra-lobar pulmonary vessels of the rat are known to be well innervated, sympathetic innervation is absent in the intra-lobar pulmonary arteries (Bradley *et al.*, 1970). However, *in vitro* and *in vivo* agonist responses to hU-II at 37^0C demonstrate a comparable time course (5-10mins) (Deuchar *et al.*, 2005) suggesting the tissue is not adversley affected by the *in vitro* technique. Furthermore the vessels from the current study which underwent repeated exposure to iso-osmotic KCl showed no sign of deterioration over the course of the experiment, again suggesting the vessels were not damaged by the procedure.

4.8.2 Study 2) Investigation into the agonist action of the pCa9.9 MI solution in un-permeabilised vessels

In selecting the bathing solution composition, 3 factors were considered of paramount importance: 1) The solution should mimic as closely as practicably possible the normal ionic internal environment so that the results were physiologically relevant. 2) It should be capable of sustaining the vessels without detriment for extended periods of time; i.e.

up to 2 hours following permeabilisation. 3) It should not appreciably affect Ca^{2+} sensitivity.

4.8.2.1 Composition of the MI solution

The MI solution was an adaptation of that used by Horiuti *et al.*, (1986) which has been widely used with α-toxin permeabilised smooth muscle preparations (Horiuti, 1986;Kitazawa *et al.*, 1989;Himpens *et al.*, 1990;Iizuka *et al.*, 1994;Evans *et al.*, 1999). The only major compositional difference was the substitution of acetate for methansulphonate. Acetate has been used as the dominant anion in MI solutions in studies which have successfully employed β-escin as the permeabilising agent (Akopov *et al.*, 1997;Akopov *et al.*, 1998). The MI solution was therefore believed to be a suitable replication of the cells internal environment, although this could only be confirmed once the permeabilisation stage was reached. However the observation that the MI solution consistently induced a bi-phasic contraction in the rat small pulmonary artery was of significant concern, especially as no mention had been made of this response previously. Hence the main question study 2 was required to address was, did the MI solution affect CaS?

4.8.2.2 The MI solution does not appear to modulate CaS

ATP mediates the contractile response associated with the MI solution through both sarcolemmal Ca^{2+} flux and Ca^{2+} store release

The single dose addition studies suggested that 5mM ATP was the only component of the MI solution responsible for the bi-phasic response. The rate of fall off between the phasic and tonic responses was modified by the MI solution, presumably due to acetate/chloride substitution (Cruickshank *et al.*, 2003), but the solution did not significantly alter the magnitude of the 5mM ATP responses. 5mM ATP was associated with both sarcolemmal Ca^{2+} flux and Ca^{2+} store mobilisation, consistent with the

activation of both P2Y and P2X receptor subgroups (Pediani *et al.*, 1999;Chootip *et al.*, 2002). In the permeabilised preparation, P2X activation, which causes sarcolemmal Ca^{2+} entry (Dubyak & el Moatassim, 1993) but not G-protein activation (Harden *et al.*, 1995), would not be of consequence. However P2Y activation, which has been linked to G-protein mediated Ca^{2+} store release (Pediani *et al.*, 1999) and ROK activation (Sauzeau *et al.*, 2000b), further suggested the possibility 5mM ATP modulating CaS in the permeabilised preparation.

The phasic response was almost entirely the result of IP_3 mediated Ca^{2+} store release, whilst the tonic response appeared independent of IP_3 production and was not associated with caffeine sensitive stores. However the tonic response, was dependent on SR Ca^{2+}, as the response was ablated by pre-treatment with thapsigargin. 2 APB has been reported to inhibit distinct IP_3 pathways with different sensitivity (Kukkonen *et al.*, 2001) thus suggesting that the ATP induced Ca^{2+} store release associated with the tonic response was mediated by a different pathway to the phasic response. These findings where consistent with $P2Y_2$ and $P2Y_4$ receptor activation. Both subtypes have been reported in rat isolated pulmonary vessels and can be activated by ATP (Bogdanov *et al.*, 1998;Chootip *et al.*, 2002). Additionally, they are G protein coupled receptors that can mediate Ca^{2+} store release through both Gq and Gi proteins (Pediani *et al.*, 1999;Zeng *et al.*, 2003) and of greatest concern, both have been shown to cause ROK activation in vascular myocytes (Sauzeau *et al.*, 2000b). The possibility of ATP modulating CaS through the ROK pathway seemed to be further corroborated by the observation that the tonic response was ablated by a single addition of the ROK inhibitor Y27632.

5mM ATP cannot be effectively blocked by pharmacological antagonism

Pharmacological antagonism was not a practical solution to the problem of multiple P2Y receptor activation by 5mM ATP, as only the $P2Y_2$ receptor has an effective antagonist,

Suramin. However Suramin is reported to be a competitive antagonist (Communi *et al.*, 1999) and as the MI solution contained 5mM ATP, it would not be effective at this concentration. Although PPADS and RB2 have been reported to be effective $P2Y_4$ antagonists by some investigators, both have also been reported to be ineffective in the rat isolated pulmonary artery (Rubino *et al.*, 1999;Chootip *et al.*, 2002).

5mM ATP does not appear to modulate CaS

As pharmacological antagonism was not feasible, it was necessary to determine if 5mMATP modulated CaS of the preparation. The relative potencies of Ca^{2+} CCRCs performed in unpermeabilised vessels suggested that 5mM ATP caused an eight-fold increase in sensitivity to extra-cellular Ca^{2+} and this was partially, but not entirely reversed by the ROK inhibitor Y27632, possibly implying changes in both CaS and sarcolemmal Ca^{2+} flux. However, Ca^{2+} CCRCs performed in the absence of ATP also suggested a decrease in CaS when performed in the presence of Y27632. Unfortunately this aspect of the study had to be abandoned as responses in the presence of Y27632 were slowed to such an extent that a single step of the CCRC could take up to 3hours. These findings suggested that ROK may have been constitutively active and therefore the shift caused by 5mM ATP may have been solely due to enhanced sarcolemmal Ca^{2+} flux caused by P2X activation (Chootip *et al.*, 2002). The suspected constitutive activity of ROK was corroborated by the single dose addition of Y27632 to unpermeabilised vessels with a sub-maximally raised tone in a ØATP MI solution. Unfortunately the presence of 5mM ATP in the MI solution prevented the attainment of a stable sub-maximal raised tone (presumably due to P2X desensitisation (Chootip *et al.*, 2002)), so a direct comparison of the effect of ATP could not be made in this experiment either. However when the magnitudes of the shifts caused by Y27632 at pCa 4.55, were compared for the 5mM ATP Ca^{2+} CCRC and the ØATP sub-maximal tone experiments, they were found to be comparable, with both experiments showing a decrease in tone of ~50% following

ROK inhibition. This suggested that ROK was constitutively active in the Wistar rat small pulmonary artery but was not substantially modulated by the presence of ATP.

4.8.3 Study 3) Selection of a sensitising agonist as a standard

4.8.3.1 ET-1 is a suitable standard

The efficacy of the ET-1 CCRCs in Krebs buffer demonstrated that ET-1 was capable of a causing a contraction greater than the tension standard in these vessels. Repeating the CCRCs in depolarised vessels with a clamped extra-cellular [Ca^{2+}] and a negated SR, confirmed that ET-1 was indeed capable of modulating tone in the absence of sarcolemmal Ca^{2+} flux and Ca^{2+} store release, consistent with its reported ability to modulate CaS (Evans *et al.*, 1999). The modulation in tone was found to be ~10-20% of the tension standard response, which was somewhat smaller than the effect of ET-1 on CaS in the Sprague Dawley rat, which was reported as ~50% of the maximum response (Evans *et al.*, 1999). The figure of 10-20% was arrived at based on the following assumptions; The response in the pCa 9.9 MI solution should not have been influenced by sarcolemmal flux or Ca^{2+} store release (because extra-cellular Ca^{2+} was absent and the SR had been negated by thapsigargin) hence this would only represent a modulation of CaS, but only in those fibres that had some pre-existing level of phosphorylation (Somlyo & Somlyo, 2003). The response in the pCa 4.55 MI solution would represent the modulation of CaS in all fibres but may have also been augmented by enhanced sarcolemmal Ca^{2+} flux. Hence, the true magnitude of the CaS response would lie somewhere between these two values.

The data suggested that ET-1 was a viable standard sensitising agonist in the Wistar rat small pulmonary artery. Based on the studies findings it was predicted that an optimally

permeabilised preparation should be capable of producing a response to 1μM ET-1 with an efficacy of 10-20% and a pEC_{50} in the range of 10-50nM.

4.8.3.2 A role for ET-1 as an Index of permeabilisation

As pre-treatment with thapsigargin had ablated the tonic response, the effect of ET-1 in the pCa 9.9 MI solution had not been expected. The response suggested that there was a residual $[Ca^{2+}]_{cyt}$ in these vessels. This finding was therefore used as a means of identifying the presence of populations SMCs that had been resistant to the permeabilisation procedure. The response, which occurred in the virtual absence of any pre-tone, also suggested it was important to express CaS modulation in relation to the maximum post permeabilisation response as well as a percentage of the change in pre-tone. This was because the magnitude of a CaS response may not be proportional to the pre-existing level of tone.

4.8.3.3 ET-1 responses suggest pharmacological blockade of the SR may be unnecessary in the permeabilised preparation

The study also found that the presence of 5mM ATP in the MI solution attenuated the contribution of store Ca^{2+} release (in response to ET-1) to a level that was indistinguishable from that caused by pre-treatment with thapsigargin. Furthermore, the potency of the response was increased ~seven-fold when Ca^{2+} store depletion was achieved solely by 5mM ATP in the MI solution as opposed to also using thapsigargin. This suggested that additional pharmacological treatment of the SR might be both unnecessary and even counterproductive. As a consequence, the possibility of relying solely on the combination of 5mM ATP and 10mM EGTA (in the MI solution) to counter agonist induced SR store release, was investigated in study 5.

4.8.4 Study 4) Selecting the optimum β-escin concentration for permeabilisation

4.8.4.1 The variable time method of permeabilisation

The most widely used method of chemical permeabilisation, exposes a vessel to the permeabilising agent for a pre-determined, fixed period of time in a pCa 9.9 MI solution (Iizuka *et al.*, 1994;Akopov *et al.*, 1997;Akopov *et al.*, 1998). However, whilst preliminary studies found that this technique could produce a functional, permeabilised preparation, vessels permeabilised at the same [β-escin] showed marked heterogeneity in their ability to respond to the sensitising standard ET-1. Furthermore, the permeabilisation indices sometimes showed these vessels to be compromised by a population of unpermeabilised SMCs. Hence the threshold concentration at which β-escin fully permeabilised the preparation was somewhat ill defined. Because of these observations, it was decided to adapt the method used by Kitazawa *et al.*, (1989) and permeabilise the vessels in a pCa 5.7 MI solution. Ca^{2+} entry, which therefore concomitantly occurred as a consequence of the procedure, caused a progressive elevation in tone and thus reported on the progression of the permeabilisation. Hence, using this technique each vessel was believed to be optimally permeabilised. The indices of permeabilisation reported the standard error of the e_{max} index for vessels permeabilised using this technique, was $1/6^{th}$ of that associated with the fixed time method.

4.8.4.2 β-escin produces a fully permeabilised preparation at concentrations between 50-200μM

The two indices of permeabilisation; the e_{max} index and the ET-1 index, both suggested that β-escin consistently produced a fully permeabilised Wistar rat small pulmonary artery at concentrations of between 50 and 200μM. Both indices also suggested that

whilst some individual vessels could be fully permeabilised by 25μM β-escin, this concentration could not be relied upon to do so consistently.

Additionally the e_{max} index was found to be a reliable means of identifying the presence of unpermeabilised SMCs. This index was statistically proven effective for protocols where no sensitising agonists were involved and was also considered to be a useful indicator for protocols involving sensitising agonists. Hence the e_{max} index was adopted as a quality control measure at the end of all future permeabilisation experiments.

4.8.4.3 50μM is the optimum concentration of β-escin for the permeabilisation of the Wistar rat small pulmonary artery

The ET-1 signal transduction index suggested that their was no significant difference in the ET-1 CCRC response of vessels permeabilised with β-escin concentrations between 25-100μM. However as the e_{max} permeabilisation index suggested a concentration of 25μM could not be relied upon to permeabilise the preparation, it was not considered as a viable candidate. Although not significantly different, the mean efficacy and potency of the ET-1 response, following permeabilisation with 50μM β-escin was nearly two-fold greater than that observed in the 100μM β-escin group and qualitatively, the responses appeared more consistent. Hence based on these findings, 50μM was selected as the optimum concentration for β-escin permeabilisation in the Wistar rat small pulmonary artery.

4.8.5 Study 5) "Knocking out" the SR

4.8.5.1 The MI solution adequately buffers agonist induced Ca^{2+} store release

All previous permeabilisation studies to date have relied upon the calcium ionophore, A 23187 to permeabilise and therefore, negate the SR. However the findings of study 3 had suggested that the combined presence of 5mM ATP and 10mM EGTA in the MI

solutions adequately depleted the SR and buffered any residual Ca^{2+} release, without the need for pharmacological intervention. This hypothesis was confirmed in study 5 where it was shown that a single dose addition of 30μM IP_3 did not alter tone in permeabilised vessels bathed in the 10mM EGTA based MI solution, regardless of the presence or absence of additional pharmacological intervention. The IP_3 was active and was shown to be capable of mobilising SR Ca^{2+}, as the un-buffered response in the 50μM EGTA based MI solution demonstrated. However even this response was substantially smaller than expected, further corroborating suspicions that exogenous 5mM ATP was causing chronic Ca^{2+} store discharge. The advantage of avoiding the "belt and braces" approach of negating the SR with a Ca^{2+} ionophore, was that the permeabilised preparation would not be exposed to the ionophores' vehicle, DMSO, which has been found to have detrimental effects on the contractile responses of intact small pulmonary arteries (unpublished observations). This effect was also witnessed in the current series of studies. For example, unpermeabilised vessels bathed in a 5mM ATP MI solution and treated with thapsigargin (which is dissolved in DMSO) showed a decrease in the potency of their response to ET-1. As a result of these findings the SR received no additional pharmacological intervention in future studies.

4.9 The permeabilisation protocol

Based on the combined findings of the protocol development studies, a permeabilisation protocol was formulated which was found to be appropriate for use in both the control and chronically hypoxic Wistar rat small pulmonary artery. The protocol was also found to be applicable to the mouse 1st generation intra-lobar (C-class) pulmonary artery, however 10μM β-escin was the optimum concentration of the permeabilising agent in this vessel (Fig 4.18).

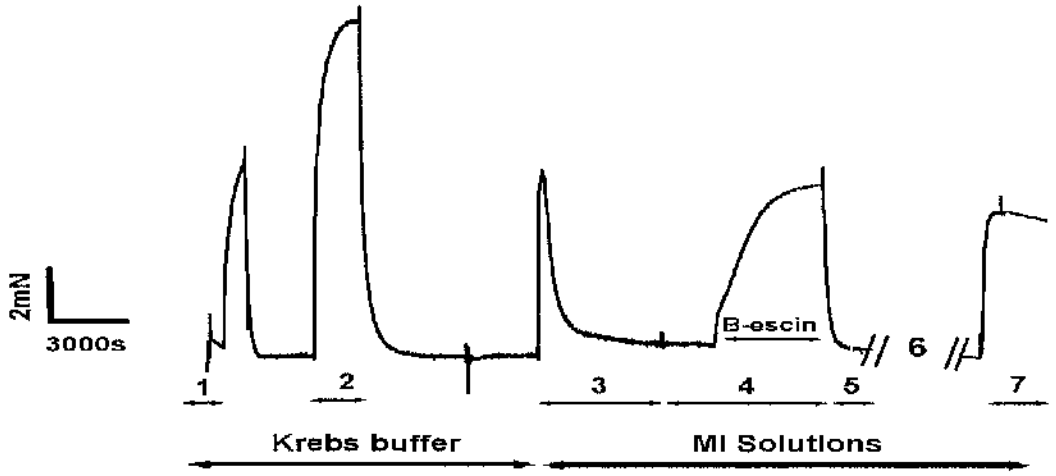


Figure 4.18 The permeabilisation protocol and the e_{max} index of permeabilisation

Extracts of an experimental trace showing the protocol used to permeabilise and confirm permeabilisation, in rat and mouse intra-pulmonary arteries. 1) Vessel set up and normalisation – as described in the general methods. 2) Tension standard response (using a nominally iso-osmotic120mM KCl solution). 3) Introduction of the MI solution (pCa 9.9) and equilibration of response). 4) Permeabilisation (pCa 5.7 MI solution + β-escin (rat; 50μM, mouse; 10μM). 5) Washout and equilibration (pCa 9.9 MI solution). 6) Specific sensitivity protocol under study (Ca^{2+} CCRCs or agonist studies at pCa 6.64). 7) The e_{max} permeabilisation index (confirmation of full permeabilisation).

Chapter 5

Results Part II

ii. Calcium Sensitivity Studies in the Control and Chronically Hypoxic Wistar rat

5.1 Introduction

5.1.1 The Chronically hypoxic rat model of pulmonary arterial hypertension

Hypoxia has been defined as a reduction in the oxygen supply to the respiring cells. In the pulmonary circulation, when alveolar PO_2 levels drop below ~70mmHg, a unique contractile phenomenon known as hypoxic pulmonary vasoconstriction (HPV) occurs (Von Euler & Liljestrand, 1947;Kato & Staub, 1966;Nagasaka *et al.*, 1984) (See chapter 1). If the exposure to alveolar hypoxia is chronic, such as during a prolonged high altitude sojourn, HPV is accompanied by a vascular remodelling of the small pulmonary arteries (SPAs) resulting in the development of secondary pulmonary hypertension (SPH) (Hislop & Reid, 1976;Smith & Heath, 1977;Herget *et al.*, 1978;Meyrick & Reid, 1978;Meyrick & Reid, 1980b;Meyrick & Reid, 1980a;Caldwell & Blatteis, 1983). The mechanisms behind the changes are still not fully elucidated but are believed to be mediated by a series of influences in addition to sustained HPV, including an imbalance of vasoconstrictor/vasodilator factors and altered reactivity of the vasculature to these factors (Shimoda *et al.*, 2000). The molecular and cellular adaptations that underlie these changes have been found to share similarities with some of the changes that occur in pulmonary arterial hypertension (PAH) (see chapter 1). For example, remodelled intra-lobar pulmonary arteries isolated from the chronically hypoxic (CH) rat have been reported to demonstrate morphological changes such as substantial intimal proliferation, medial hypertrophy and adventitial thickening, which remain even after three days of recovery in normoxia (Meyrick & Reid, 1980a;Meyrick & Reid, 1980b). Chronic hypoxia has also been reported to attenuate endothelium dependant NO relaxation in these vessels (Rodman, 1992;Carville *et al.*, 1993) and increase the constrictor response of endogenous agonists such as ET-1 and 5-HT (Eddahibi *et al.*, 1993;MacLean *et al.*, 1995;Eddahibi *et al.*, 1997;McCulloch *et al.*, 1998). However intimal proliferation

associated with SPH is polyclonal, whereas in PAH proliferation tends to be monoclonal (Lee *et al.*, 1998) and not all studies agree that chronic hypoxia causes intimal proliferation (Smith & Heath, 1977). Additionally, there are conflicting reports regarding the aforementioned functional changes, some of which, appear to be inconsistent with those occurring in PAH. (Bonnet *et al.*, 2001a;Sauzeau *et al.*, 2003a).

Although the chronically hypoxic rodent is not a perfect model of PAH, it has been widely used to elucidate the mechanisms involved in the aetiology of this disease (Rabinovitch *et al.*, 1988;Suggett & Barer, 1988;McCulloch *et al.*, 1998;MacLean & Morecroft, 2001) and its use has undoubtedly contributed to the development of new effective therapies for PAH such as the ET-1 antagonist bosentan (Tracleer).

5.1.2 Chronic hypoxia and elevated basal tone

One of the hallmarks of hypoxic PH is the increase in vascular tone. The increase in tone is initiated by HPV immediately upon exposure to hypoxia, the sub-acute phase of which is thought to be mediated by the Rho kinase (ROK) pathway (Wang *et al.*, 2001;Wang *et al.*, 2003). However following exposure to chronic hypoxia, elevated basal tone remains long after the hypoxic challenge is withdrawn and there is substantial evidence to suggest that it causes the modulation of; receptor expression, ion channel activity and signal transduction pathways (Rodman, 1992;Eddahibi *et al.*, 1993;MacLean *et al.*, 1996b;Shimoda *et al.*, 1999;Chassagne *et al.*, 2000;Bonnet *et al.*, 2001a;Keegan *et al.*, 2001;Sauzeau *et al.*, 2003b). The effects of these changes may contribute to an increased resting $[Ca^{2+}]_{cyt}$, which has been reported to be elevated in PASMCs from both chronically hypoxic rats and human PAH patients (Shimoda *et al.*, 1999;Yuan *et al.*, 1998a). However, it has also been suggested that increases in calcium sensitivity (CaS) may accompany the elevated $[Ca^{2+}]_{cyt}$ in PH occurring secondary to chronic hypoxia and

that responses to agonists may be augmented by this increase in CaS (Shimoda *et al.*, 2000;Nagaoka *et al.*, 2004b).

5.1.3 Chronic hypoxia, calcium sensitisation and Rho kinase

In PASMCs obtained from rat intra-lobar pulmonary arteries, CaS was reported to be augmented following exposure to chronic hypoxia, based on the observation that pharmacological blockade of voltage gated channels significantly reduced maximum tension induced by ET-1 in control but not hypoxic animals (Shimoda *et al.*, 2000). More recently, studies have implicated the ROK pathway with increased CaS in response to agonist stimulation. The small GTPase Rho A and its effector ROK have been demonstrated to cause increased CaS in a variety of smooth muscle preparations including PASMCs (Janssen *et al.*, 2001a;Janssen *et al.*, 2001b). Endogenous agonists such as ET-1 and PE which exert their influence through G-protein linked receptors have been shown to activate this pathway, resulting in increased CaS in systemic arteries (Miao *et al.*, 2002;Wingard *et al.*, 2003). Activated ROK phosphorylates the myosin-binding sub-unit of MLCP and/or CPI-17 resulting in the inactivation of MLCP (Kimura *et al.*, 1996;Feng *et al.*, 1999;Eto *et al.*, 2001). Thus ROK modulates CaS by inhibition of MLCP (see chapter 1).

Recent reports from *in vivo* studies have also suggested a role for the ROK pathway in the development of PH secondary to chronic hypoxia. In mice, subcutaneous treatment with the ROK inhibitor Y27632 (30mg/kg/day) during exposure to chronic hypoxia attenuated RVP, right ventricular hypertrophy and remodelling of the distal pulmonary vasculature (Fagan *et al.*, 2004). In other studies, pulmonary arterial pressure (PAP) and pulmonary vascular resistance (PVR) were virtually normalised in the chronically hypoxic rat, by subsequent acute intravenous or oral treatment with Y27632 (Nagaoka *et al.*, 2004a;Nagaoka *et al.*, 2004b). Fasudil, another selective inhibitor of ROK has also

been reported to substantially improve survival in the monocrotaline induced model of PH in the rat (Abe *et al.*, 2004). These findings suggest a central role for the ROK mediated change CaS in the development of PH.

In contrast however, CaS has been reported to be attenuated in PASMCs isolated from rat main (extra-lobar) pulmonary arteries and in arterial ring preparations following exposure of the animal to chronic hypoxia (Bonnet *et al.*, 2001a). These findings were based on the attenuation of the contractile response to ET-1 and angiotensin II following chronic hypoxia, despite no change in the amount of Ca^{2+} mobilised by these agonists as determined by indo 1 fluorescence. Consistent with these findings, attenuated contractile responses to ET-1, NA and the thromboxane A2 analogue U46619 have been reported in the main pulmonary artery of the chronically hypoxic rat (Bialecki *et al.*, 1998a;Bialecki *et al.*, 1998b;Sauzeau *et al.*, 2003a). The attenuation was found to be associated with an inhibition of ROK mediated CaS, which was demonstrated by performing Ca^{2+} CCRCs in the presence and absence of the ROK inhibitor Y27632 and GTPγS in permeabilised strips of main pulmonary artery from control and chronically hypoxic rats. These findings were supported by RT PCR and Western blot analysis which showed RhoA mRNA and RhoA expression to be reduced by 80% in the main pulmonary artery of rats following 2 weeks chronic hypoxia (Sauzeau *et al.*, 2003a). The chronic hypoxia induced down regulation of Rho A was however prevented by sildenafil, indicating a role for the NO/cyclic GMP pathway in the alteration of RhoA/ROK signalling in the main pulmonary artery of the chronically hypoxic rat (Sauzeau *et al.*, 2003a;Sauzeau *et al.*, 2003b). Interestingly, lungs taken from an *in vivo* mouse study which demonstrated increased ROK activity following exposure to chronic hypoxia, found that treatment with Y27632 caused an increase in expression of endothelial NOS (NOS III) protein. A number of studies in systemic vascular preparations have reported that the NO/cGMP mediated pathway and the ROK pathway which both terminate in modulation of MLCP

activity, are antagonistic to each other (Bolz *et al.*, 2003;Budzyn *et al.*, 2004). However the *in vivo* mouse study also found that conversely, Y27632 reduced NOS III protein in control lungs (Fagan *et al.*, 2004) suggesting that the relationship between the ROK and NO pathways may be more complex than first thought.

5.1.4 Aims

The background account suggests that changes in CaS occur in PH secondary to chronic hypoxia. However, the nature of these changes remains controversial. The aim of this chapter was therefore to determine the role of CaS and the influence of the ROK pathway in the regulation of tone in the isolated small pulmonary artery (SPA) of the control and chronically hypoxic Wistar rat.

5.2 Methods

5.2.1 Set up

Isolated small pulmonary arteries (D-class) from chronically hypoxic and control Wistar rats, were set up on the myograph and normalised as described in chapter 2. The right ventricular/total ventricular ratio (RV/TV) was used to assess the development of PH (see chapter 2). Subsequent to determination of the vessels maximum response to a nominally iso-osmotic 120mM KCl solution, the following experiments were performed. During the performance of the cumulative concentration response curves (CCRCs) each response was allowed to fully stabilise before the next solution exchange. During solution exchange, vessels were washed twice with the following solution to minimise the possibility of dilution by the previous solution.

5.2.2 Study 1) The effect of chronic hypoxia on basal Ca^{2+} sensitivity in the Wistar rat small pulmonary artery

To determine if CaS was modulated following exposure to chronic hypoxia, Ca^{2+} CCRCs were performed in permeabilised and unpermeabilised vessels. Unpermeabilised vessels were studied in order to gain insight into how much the change in CaS influenced the response to extra-cellular Ca^{2+} in these vessels. Small pulmonary arteries from animals exposed to chronic hypoxia will be referred to as chronically hypoxic vessels.

5.2.2.1 Ca^{2+} CCRCs in unpermeabilised small pulmonary arteries

Age matched control (AM) and chronically hypoxic (CH) vessels were challenged with the following mock intra-cellular (MI) solutions; pCa; 6.64, 5.70, 5.40, 5.10, 4.55, 4.00, 3.50, 3.00, 2.50, 2.00, 1.50 in a sequential manner.

5.2.2.2 Ca^{2+} CCRCs in 50μM β-escin permeabilised small pulmonary arteries

Age matched and chronically hypoxic vessels were permeabilised using the method described in the protocol development. Following permeabilisation the vessels were washed in pCa 9.9 MI solution and allowed to return to baseline. Vessels were then exposed to the following mock intra-cellular (MI) solutions; pCa; 6.64, 6.17, 5.70, 4.55, 4.00, 2.50 in a sequential manner.

5.2.3 Study 2) The role of Rho kinase in Ca^{2+} sensitivity modulation in the chronically hypoxic Wistar rat small pulmonary artery

5.2.3.1 Ca^{2+} CCRCs in the presence and absence of the ROK inhibitor Y27632 in permeabilised, age matched and chronically hypoxic small pulmonary arteries

Age matched and chronically hypoxic vessels were permeabilised and then allowed to equilibrate as in study 1. During this recovery period some of the vessels from both groups were incubated with 10μM Y27632 (45 mins). A Ca^{2+} CCRC was then performed as in study 5.2.2.2.

5.2.4 Study 3) Confirming the suspected constitutive activity of Rho kinase in age matched, Wistar control rat small pulmonary arteries

5.2.4.1 Single dose addition of the ROK inhibitor Y27632 to a sub-maximal raised tone in permeabilised, age matched small pulmonary arteries in the presence of 5mM ATP

Age matched vessels were permeabilised as previously described, then allowed to return to baseline in a pCa 9.9 MI solution. Vessels were then exposed to a pCa 6.64 MI solution to create a sub-maximal raised pre-tone (~25% of Fi_{max}). One of each of the paired vessels was then treated with a single dose of 10μM Y27632 whilst the remaining vessel received a single dose of DMSO equivalent to the concentration present in the

Y27632 dose. The vessels were then observed until either any response had fully stabilised or for 45 mins, whichever was the shorter.

5.2.4.2 Single dose addition of the ROK inhibitor Y27632 (10μM) to a sub-maximal raised tone in unpermeabilised, age matched small pulmonary arteries in the absence of 5mM ATP

The response of age matched control vessels to a nominally iso-osmotic 120mM KCl solution was determined. Following washout and equilibration the vessels were incubated for 10mins with 2μM thapsigargin and then exposed to a 5mM ATP based pCa 9.9 MI solution to drain the SR. The vessels were then placed in a ØATP based pCa 9.9 MI solution, allowed to stabilise and then exposed to a ØATP pCa 4.55 – 4.00 MI solution in order to create a stable raised tone that was ~ 25% of the maximum tension standard response. Following the attainment of a stable raised pre-tone, one of each pair of vessels was treated with a single dose of 10μM Y27632 whilst the other vessel of the pair received a single dose of DMSO equivalent to the concentration present in the Y27632 dose. The vessels were then observed until either any response had fully stabilised or for 45 minutes, whichever was the shorter.

5.2.5 Quality control assessment

Following the chosen experimental procedures, all permeabilised vessels were challenged with the e_{max} index of permeabilisation, to determine the presence of any significant population of unpermeabilised SMCs (see chapter 4) Vessels with an Fe_{max} response which was more than 3% greater than their Fi_{max} response were discarded from the study. In vessels partaking in the Ca^{2+} CCRCs this was determined from the CCRC responses.

5.3 Results

5.3.1 Study 1) The effect of chronic hypoxia on Ca^{2+} sensitivity in the Wistar rat small pulmonary artery

5.3.1.1 Ca^{2+} CCRCs in unpermeabilised vessels

Neither the efficacy nor the potency of the relationship between extra-cellular Ca^{2+} concentration and force production was significantly altered by exposure to 2weeks chronic hypoxia (n=7, P>0.05, Mann Whitney test). However, chronically hypoxic vessels appeared less sensitive to extra-cellular Ca^{2+} at low concentrations and more sensitive at mid to high concentrations causing an increase in the slope of the relationship between the [Ca^{2+}] of the MI solution and force (Slope: AM; 0.7 ± 0.1 vs CH 1.0 ± 0.1, n=7, P<0.05, Mann Whitney test) (Fig 5.1)

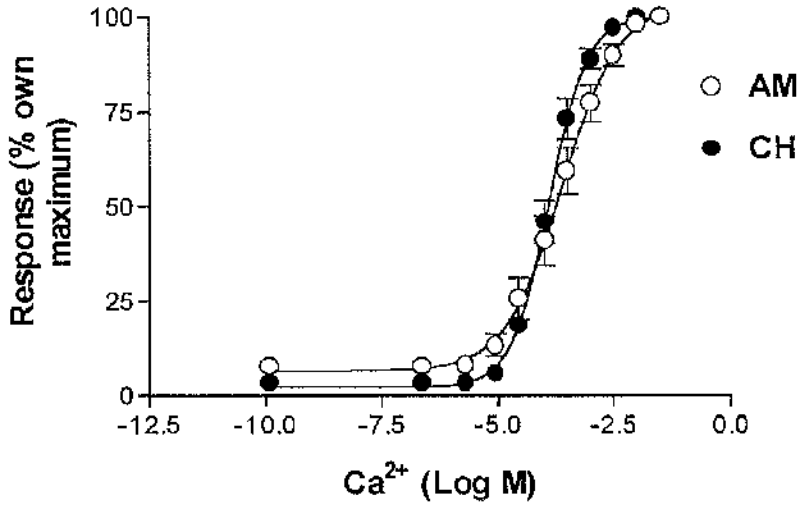


Figure 5.1 Ca^{2+} CCRCs in unpermeabilised age matched control and chronically hypoxic Wistar rat, small pulmonary arteries

Graph showing Ca^{2+} CCRCs in unpermeabilised age matched (AM) (White circles - O) and chronically hypoxic (CH) (Black circles - ●) small pulmonary arteries (potency and efficacy P>0.05, Hill slope P<0.05, n=7, Mann Whitney test).

5.3.1.2 Ca^{2+} CCRCs in 50μM β-escin permeabilised vessels.

As shown in Fig 5.2, chronic hypoxia was associated with a small but significant decrease CaS (pEC_{50}: AM; 6.33 ± 0.02, n=11 vs CH; 6.24 ± 0.02, n=10, $P<0.05$, Mann Whitney test) which was most pronounced in the lower quartile of the response curve (Response at pCa 6.64: AM; 22 ± 1%, of Fi_{max}, n=11 vs CH; 14 ± 2%, n=10, $P<0.005$, Mann Whitney test). Consistent with these findings, chronically hypoxic vessels also demonstrated a small but significant increase in the slope of the relationship between cytosolic [Ca^{2+}] and force (Slope: AM; 1.57 ± 0.08, n=11 vs CH; 1.79 ± 0.1, n=10, $P<0.05$, Mann Whitney test).

The vessels from the age matched and chronically hypoxic rats used in both experiments, were of comparable internal diameter and replicated transmural pressure ($P>0.05$, unpaired t-tests). Furthermore, there was no difference in the magnitude of the maximal responses of the age matched and chronically hypoxic vessel (Unpermeabilised vessels: AM; 2.81 ± 0.33mN vs CH; 4.53 ± 1.24mN, n=7) (Permeabilised vessels: AM; 1.85 ± 0.2mN, n=11 vs CH; 2.20 ± 0.3mN, n=10) ($P>0.05$, unpaired t-tests). The c_{max} index of permeabilisation confirmed the vessels used in the permeabilisation study were fully permeabilised (Fe_{max} was <0.5 ± 0.5% greater than Fi_{max} in both age matched and chronically hypoxic vessels, n=6, $P>0.05$, Mann Whitney test). The RV/TV was significantly larger in the chronically hypoxic vessel group relative to the age matched control vessel group and was consistent with the development of PH ($P<0.0001$, unpaired t-test).

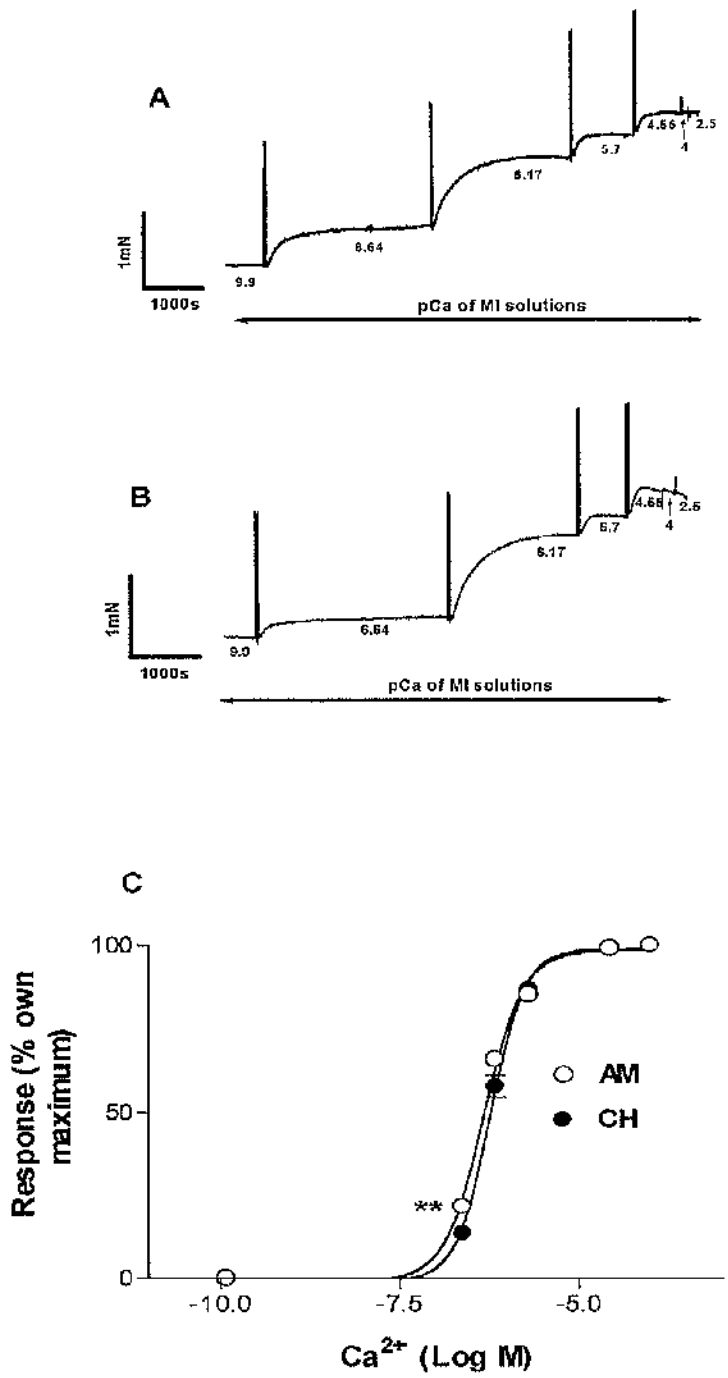


Figure 5.2 The effect of chronic hypoxia on constitutive Ca^{2+} sensitivity in the Wistar rat, small pulmonary artery

A) Experimental trace of a Ca^{2+} CCRC in a permeabilised age matched (AM) control vessel B) Experimental trace of a Ca^{2+} CCRC in a permeabilised vessel following exposure to 2weeks chronic hypoxia (CH) C) Graph showing Ca^{2+} CCRCs in permeabilised AM (White circles - O) and CH (Black circles - ●) (** response at pCa 6.64, n=10-11, P<0.005, Mann Whitney test).

5.3.2 Study 2) The role of Rho kinase in Ca^{2+} sensitivity modulation in the chronically hypoxic Wistar rat small pulmonary artery.

5.3.2.1 Ca^{2+} CCRCs in the presence and absence of the ROK inhibitor Y27632 in permeabilised, age matched and chronically hypoxic small pulmonary arteries

Incubation with 10μM Y27632 caused an attenuation of CaS in age matched control vessels. However, this was only significant over the lower quartile of the response (pEC_{50}: AM; 6.36 ± 0.03 vs AM+Y; 6.30 ± 0.03, P>0.05) (Slope: AM; 1.75 ± 015 vs AM+Y; 2.07 ± 0.22, P>0.05) (Response at pCa 6.64: AM; 22 ± 2%, of Fi_{max} vs AM + Y27632; 11 ± 1%, P<0.01) (All n=5, Mann Whitney tests). The chronically hypoxic vessel response was not significantly altered by the Y27632 compound (pEC_{50}: CH; 6.30 ± 0.03 vs CH+Y; 6.28 ± 0.02, P>0.05) (Slope: CH; 2.15 ± 0.21 vs CH+Y; 2.11 ± 0.18, P>0.05) (Response at pCa 6.64: CH; 12 ± 3% of Fi_{max} vs CH + Y27632; 10 ± 2%, P>0.05) (All n=5, Mann Whitney tests). Comparison of the three attenuated pCa 6.64 responses (CH, CH + Y27632 and AM + Y27632) found no difference between them (n=5, P>0.05, Kruskal Wallis test) (Fig 5.3).

The vessels from the four groups were of comparable internal diameter and replicated transmural pressure (P>0.05, unpaired ANOVA). Furthermore, there was no difference in the magnitude of the maximal responses of any of the groups (AM; 2.00 ± 0.34mN, vs AM+Y; 1.84 ± 0.31mN, CH; 1.65 ± 0.42mN, CH + Y; 1.82 ± 0.21, n=5, P>0.05, unpaired ANOVA). The e_{max} index of permeabilisation confirmed that all vessels used in the study were fully permeabilised (Fe_{max} was <1 ± 1% greater than Fi_{max} in all groups, n=5, P>0.05, Kruskal Wallis test). The RV/TV was significantly larger in the chronically hypoxic vessels consistent with the development of PH (P<0.0001, unpaired t-test).

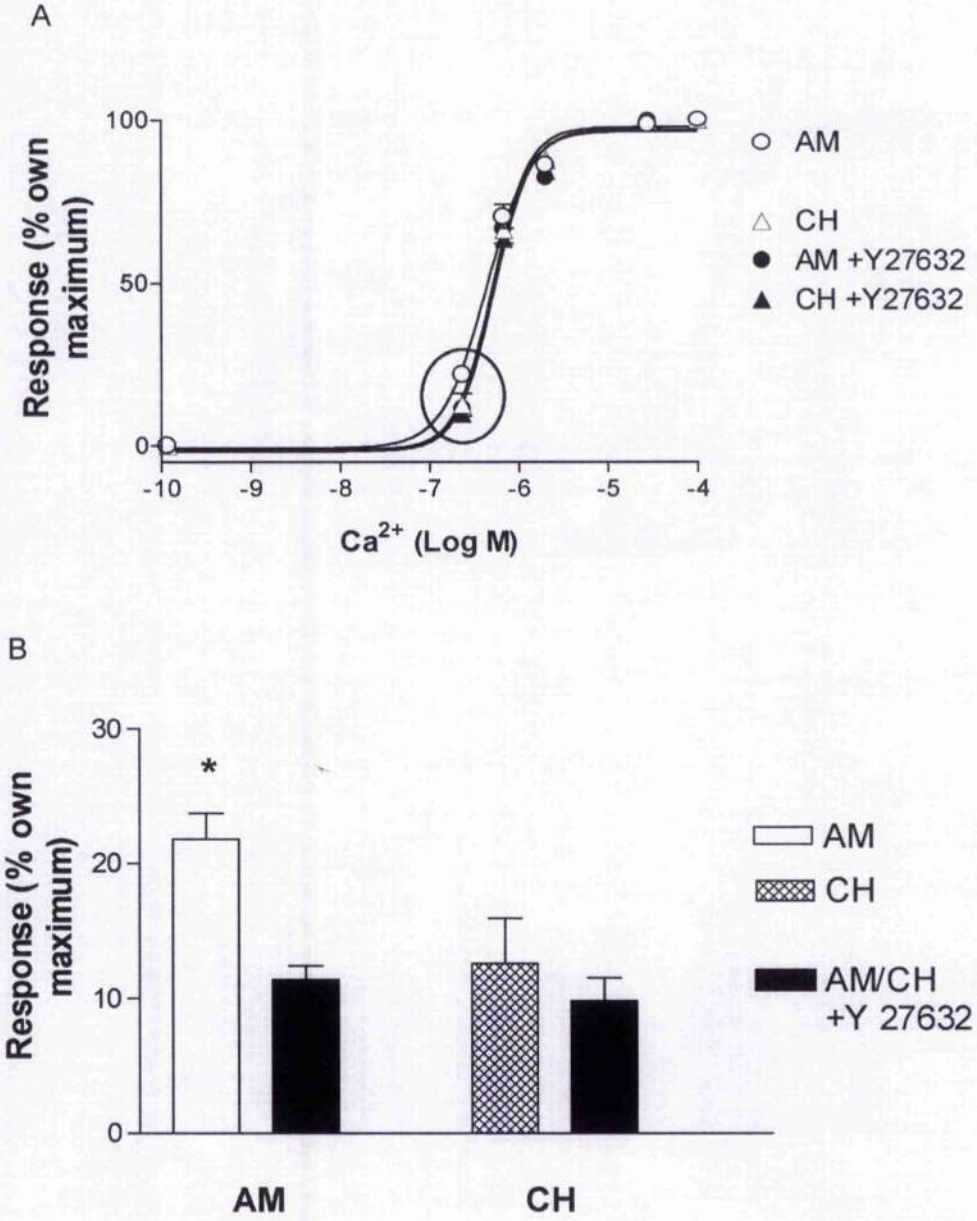


Figure 5.3 The effect of Rho kinase inhibition on constitutive Ca^{2+} sensitivity in small pulmonary arteries from control and chronically hypoxic Wistar rats

A) Ca^{2+} CCRCs in age matched (AM) and chronically hypoxic (CH) vessels in the presence and absence of the Rho kinase inhibitor Y27632. B) Bar graph showing the response of AM and CH vessels in the presence and absence of Y27632 at pCa 6.64. (All groups n=5, *P<0.01, Mann Whitney test).

5.3.3 Study 3) Confirming the suspected constitutive activity of Rho kinase in age matched, Wistar control rat small pulmonary arteries

The results of the previous study suggested that ROK was constitutively active in age matched control vessels as had previously been noted during the protocol development studies. However, it had previously only been possible to make an indirect comparison of the effects of exogenous 5mM ATP on this pathway. A stable raised tone in the presence of 5mM ATP was now achievable following permeabilisation. Hence, it was now possible to compare the effects of exogenous 5mM ATP on ROK.

5.3.3.1 The effect of a single dose addition of the ROK inhibitor Y27632 on a sub-maximal raised tone in 50μM β-escin permeabilised, age matched small pulmonary arteries in the presence of 5mM ATP

A single dose of 10μM Y27632 caused a significant decrease in the pCa 6.64 pre-tone (P/T; 0.49 ± 0.12mN vs AM + Y; 0.23 ± 0.06, n=6, $P<0.05$, paired t-test) which was absent in the time control (T/C) vessels (P/T; 0.31 ± 0.10 vs AM T/C; 0.30 ± 0.09, n=6, $P>0.05$, paired t-test). When expressed as a percentage of the pre-tone, there was a highly significant difference of 51 ± 7% between vessels exposed to the single dose of 10μM Y27632 and the time control vessels (AM +Y; -53 ± 6% vs AM T/C; -2 ± 5%, n=6, $P<0.005$, Mann Whitney test) (Fig 5.4). This equated to a decrease that was 11 ± 1% of Fi_{max}.

The vessels from the two groups were of comparable internal diameter and replicated transmural pressure ($P>0.05$, unpaired t-test). The mean pre-tone was not significantly different between the groups whether expressed in mN (P/T: AM T/C 0.31 ± 0.10mN vs AM +Y; 0.49 ± 0.12mN, n=6, $P>0.05$, unpaired t-test) or as a percentage of Fi_{max} (AM T/C; 21 ± 2% of Fi_{max} vs AM + Y; 24 ± 2%, n=6, $P>0.05$, Mann Whitney test). The e_{max} index of permeabilisation suggested all vessels used in the study were fully permeabilised

(Fc_{max} was 1 ± 1% greater than Fi_{max} in both groups, n=6, P>0.05, Wilcoxon matched pairs test).

5.3.3.2 The effect of a single dose addition of the ROK inhibitor Y27632 on sub-maximal raised tone unpermeabilised, age matched small pulmonary arteries in the absence of 5mM ATP

A single dose addition of 10μM Y27632 caused a significant decrease in pCa 4.55-4.00 pre-tone (P/T; 2.57 ± 0.95mN vs AM + Y; 1.72 ± 0.91, n=5, P<0.05, paired t-test) which was absent in the time control (T/C) vessels (P/T; 2.62 ± 2.21 vs AM T/C; 2.62 ± 2.21, n=3, P>0.05, paired t-test). When expressed as a percentage of the pre-tone, there was a significant mean difference of 47 ± 16% between vessels exposed to the 10μM Y27632 bolus and the time control vessels (AM +Y; -47 ± 12%, n=5 vs AM T/C; -0.2 ± 0.2%, n=3, P<0.05, Mann Whitney test) (Fig 5.4). This equated to a decrease that was 14 ± 4 % of the 120mM KCl tension standard.

The vessels from the two groups were of comparable internal diameter and replicated transmural pressure (P>0.05, unpaired t-test). The mean pre-tone (expressed as a percentage of the maximum response to iso-osmotic 120mM KCl) was not significantly different between the groups (AM T/C 35 ± 9%, n=3 vs AM +Y; 30 ± 7%, n=5, P>0.05, Mann Whitney test).

5.3.3.3 Comparison of the effect of a single addition of 10μM Y27632 to permeabilised and unpermeabilised small pulmonary arteries with a sub-maximally raised pre-tone

The reduction in tone caused by the single dose addition of 10μM Y27632 to either permeabilised vessels (P/V) in the presence of 5mM ATP or unpermeabilised vessels (U/V) in the absence of ATP was of a comparable magnitude when expressed either as a percentage of the reduction in pre-tone (P/V; -51 ± 7% of P/T, n=6 vs U/V; -47 ± 16%,

n=5) (Fig 5.4) or as a percentage of the vessels own maximum response (P/V; 11 ± 1% of $\mathbf{Fi}_{max}$, n=6 vs U/V; 14 ± 4% of the 120mM KCl tension standard, n=5).

The vessels from the two groups were of comparable internal diameter and replicated transmural pressure (P>0.05, unpaired t-test). The mean pre-tones (expressed as a percentage of $\mathbf{Fi}_{max}$ in P/V or the 120mM iso-osmotic KCl tension standard in U/V) were also of comparable magnitudes (P/T: P/V; 24 ± 2%, n=6 vs U/V; 30 ± 7%, n=5).

The mean internal diameter and replicated transmural pressure of the vessels used in this study was 285 ± 9μm and 13.4 ± 0.3mmHg respectively (n=75vessels/42 rats). The mean RV/TV ratio was follows: Age matched controls; 0.232 ± 0.004, n=27. Chronically hypoxic rats; 0.348 ± 0.009, n=15.

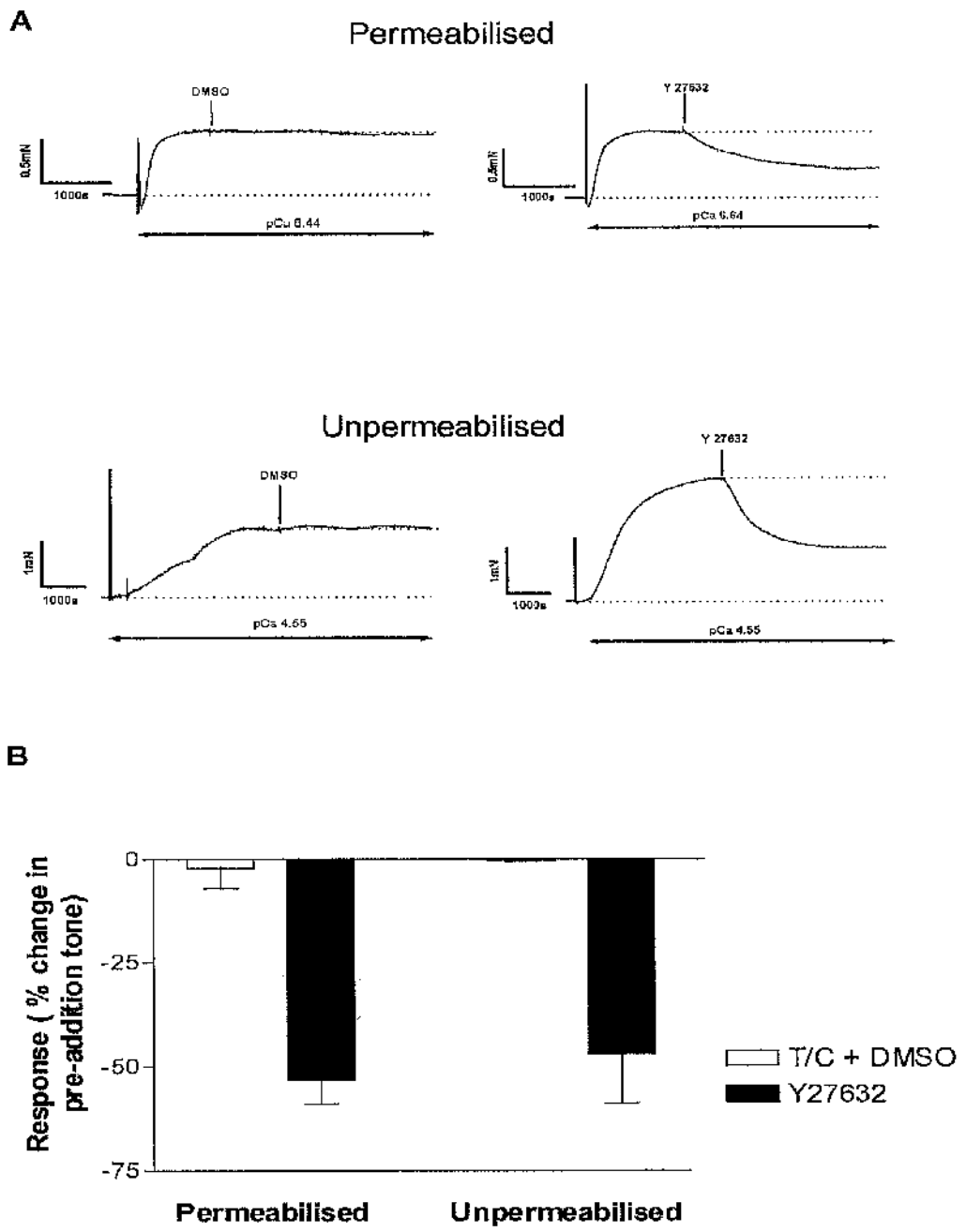


Figure 5.4 The constitutive activity of Rho kinase is not altered by exogenous 5mM ATP

A) Experimental traces showing the effect of a single dose addition of the Rho kinase inhibitor Y27632 in permeabilised age matched vessels in the presence of 5mM ATP and unpermeabilised age matched vessels in the absence of exogenous ATP. Time control (T/C) responses treated with the vehicle DMSO are on the left for comparison. B) Bar chart showing the relative reduction in pre-tone caused by Y27632 or the vehicle DMSO; in permeabilised vessels in the presence of 5mM ATP (n=6) and unpermeabilised vessels in the absence of exogenous ATP (n=5).

5.4 Discussion

5.4.1 Unpermeabilised chronically hypoxic Wistar rat small pulmonary arteries do not show an obvious change in their sensitivity to extra-cellular Ca^{2+}

The Ca^{2+} CCRCs performed in unpermeabilised vessels did not suggest a substantial difference in the response to extra-cellular [Ca^{2+}], although the greater slope of the relationship between the [Ca^{2+}] of the MI solution and force suggested that chronically hypoxic vessels may have been slightly less sensitive to extra-cellular Ca^{2+} at concentrations up 10μM. Although this could have been due to a reduction in sarcolemmal Ca^{2+} conductance, this has not been reported in these vessels, in fact Ca^{2+} conductance has been reported to be enhanced in PAECs following exposure to chronic hypoxia (Fantozzi *et al.*, 2003).

5.4.2 Ca^{2+} sensitivity is attenuated in the β-escin permeabilised chronically hypoxic Wistar rat small pulmonary artery in the absence of a functional endothelium

In permeabilised small pulmonary arteries, chronic hypoxia was associated with a small but significant decrease in CaS which was most pronounced in the lower quartile of the response. At a $[Ca^{2+}]_{cyt}$ of 230nM, which lies within the normal range for G-protein agonist induced tonic contraction, exposure to 2weeks chronic hypoxia reduced basal CaS by just over 40% relative to control vessels. $[Ca^{2+}]_{cyt}$ during the tonic phase of ET-1 induced contraction in smooth muscle cells has been reported at ~ 200nM (Schroeder *et al.*, 2000). Incubation with the ROK inhibitor Y27632 reduced the CaS of control vessels to levels similar to that observed in the chronically hypoxic vessels, but did not further attenuate CaS in the hypoxic vessels. These findings suggest that in β-escin permeabilised vessels, ROK is constitutively active in control vessels but that its activity

may be reduced in rats exposed to 2weeks chronic hypoxia. The data are consistent with the reported loss of the CaS response to GTPγS in the rat, permeabilised main (extra-lobar) pulmonary artery following 2weeks chronic hypoxia (Sauzeau *et al.*, 2003a). The same group also found that Rho A expression was attenuated by 80% in these vessels and that Rho A expression in whole lung preparations (indicative of intra-lobar pulmonary arteries) was decreased by 30%. Non linear regression found the effects of chronic hypoxia and ROK inhibition were most pronounced between ~30-300nM suggesting physiological relevance.

5.4.3 Rho kinase is constitutively active in the control Wistar rat small pulmonary artery

The constitutive activity of ROK was further corroborated in both permeabilised and unpermeabilised vessels by the addition of a single dose of Y27632 to vessels with a sub-maximally raised tone. In both conditions, inhibition of ROK caused a 50% reduction in the pre-constricted tone, which was entirely consistent with the 50% attenuation caused by ROK inhibition during the permeabilised control vessel Ca^{2+} CCRC at a $[Ca^{2+}]_{cyt}$ of 230nM. Constitutive activity of the ROK pathway has also been reported in resistance arteries from hamster gracilis muscle (Bolz *et al.*, 2003) and in rat small mesenteric arteries, where it is thought to be involved in maintaining basal tone (VanBavel *et al.*, 2001). However, the concentration of Y27632 used (10μM), has been reported to cause partial inhibition of PKC_{δ} (Eto *et al.*, 2001) and therefore the possibility of atypical PKC involvement in constitutive CaS cannot be ruled out.

5.4.4 Exogenous 5mM ATP does not modulate Rho kinase activity in the Wistar rat small pulmonary artery

As the response in the permeabilised vessels was performed in the presence of exogenous 5mM ATP and the response in the unpermeabilised vessels in the absence of exogenous ATP, the comparable results also suggested that ROK mediated CaS was not noticeably modified by the presence 5mM ATP in the MI solution. This was a question, which had been posed during the protocol development but could not be satisfactorily answered with the initial experiments described in chapter 4.

5.4.5 Chronic hypoxia changes the activity of the Rho kinase and nitric oxide/cGMP pathways

Whilst the findings are generally consistent with those previously described in the only other permeabilisation study, they are in direct opposition to the *in vivo* evidence. One common thread that links the previous *in vivo* and *in vitro* permeabilisation studies is the relationship that has been reported between ROK, NOS III and CaS. Inhibition of ROK has been shown to augment NOS III in endothelial cells (Bivalacqua *et al.*, 2004). Thus it is possible that the *in vivo* effects of Y27632 were not simply due to its effects on the ROK sensitisation pathway in smooth muscle cells, but also by preventing ROK located within endothelial cells from inhibiting NOS III. This would be consistent with a report that NOS III protein was substantially increased in hypoxic mouse lungs which had been treated with Y27632 (Fagan *et al.*, 2004) and that down regulation of NOS III in human PAECS exposed to hypoxia was prevented by ROK inhibition (Takemoto *et al.*, 2002). Interestingly Sauzeau *et al.*, (2003a) reported that oral treatment with sildenafil a PDE 5 inhibitor, which prevents cGMP (NO 2nd messenger) breakdown, substantially attenuated right ventricular hypertrophy and prevented vascular remodelling in rats exposed to 2weeks chronic hypoxia, but that subsequent administration of Y27632 had no effect. Studies have now shown that NO causes relaxation by reducing CaS as well as by

reducing $[Ca^{2+}]_{cyt}$ and that in systemic resistance arteries the decrease in CaS may be the dominant mechanism (Bolz *et al.*, 2003;Soloviov *et al.*, 2004). This pathway exerts its influence on MLCP and there is evidence that in smooth muscle the NO/cGMP and ROK pathways antagonise one another by their opposing actions on MLCP (Budzyn *et al.*, 2004;Bolz *et al.*, 2003). It seems reasonable given the finding that acute hypoxia inhibits MLCP through ROK (Wang *et al.*, 2003) to hypothesise that in chronic hypoxia, CaS would increase in smooth muscle cells as decreased activity of the NO/cGMP pathway would leave the constitutive activity and/or hypoxic stimulation of ROK unopposed. However decreased NO/PKG signalling has been shown to result in a strong decrease in RhoA mRNA in both rat and human systemic arteries (Sauzeau *et al.*, 2003b). Hence, whilst hypoxia may increase the activity of ROK in smooth muscle cells by decreasing NO/cGMP antagonism, it may paradoxically decrease Rho A expression.

The finding by Sauzeau et al., (2003a) that Rho A expression was also substantially down-regulated in rat pulmonary arteries following 2weeks chronic hypoxia, may explain the discrepancies between the *in vivo* dosing studies and the *in vitro* permeabilisation study. For example, Y27632 could prevent the increased activation of ROK and hence prevent an increase in CaS during the onset of hypoxia, but would paradoxically also have no effect in untreated fully adapted chronically hypoxic animals if subsequent negative feedback had down regulated Rho A expression to a point which prevented significant ROK activation. Unfortunately the immediate normalisation of PVR following intravenous administration of Y27632 in chronically hypoxic rats after their return to normoxia reported by Nagaoka *et al.*, (2004b), suggested the ROK pathway was still strongly involved in the elevated vasomotor tone even after 3-4weeks. In fact the magnitude of the response reported by Nagaoka *et al.*, (2004b) suggest that ROK mediated Ca^{2+} sensitisation is entirely responsible for the elevated PVR and hence PAP of chronic hypoxia induced PH in the rat. However one variable that may require further

consideration and could provide a possible explanation for the discrepancies is the length of exposure to chronic hypoxia. Interestingly, all of the studies which have shown a decrease in CaS in rats, have used a 2 week exposure to chronic hypoxia (Sauzeau *et al.*, 2003b;Sauzeau *et al.*, 2003a;Bonnet *et al.*, 2001a) where as those studies suggesting an increase in CaS have used a 3-4 week exposure period (McMurtry *et al.*, 2003;Nagaoka *et al.*, 2004a;Nagaoka *et al.*, 2004b). The exposure period to hypoxia has previously been reported to modulate the sensitivity of response to ET-1, with a decrease in potency occurring after 2weeks exposure to chronic hypoxia, but returning towards normal following 3-4 weeks exposure (Bialecki *et al.*, 1998a;Bialecki *et al.*, 1998b).

5.4.6 How does the loss of endothelial function affect the interpretation of the present study?

It was also considered that the loss of the endothelium may explain the discrepancy between the similar results of the present and the previous *in vitro* permeabilisation study and the diametrically opposite responses of the *in vivo* study by Nagaoka et al., (2004). The permeabilisation studies almost certainly result in the destruction of the endothelium (Laher *et al.*, 1995) and therefore only report on the constitutive activity of the sensitising pathways. However, the ability of GTPγS to increase CaS in the permeabilised control vessels but not in those exposed to 2weeks chronic hypoxia (Sauzeau *et al.*, 2003a) is contrary to the loss of the endothelium or some endothelium derived activating factor being responsible for the discrepancy. In conjunction with the RT PCR reports, this supports the down regulation of Rho A rather than a functional uncoupling of the mechanism due to the permeabilisation procedure or exposure to chronic hypoxia.

Interestingly, it was observed during the protocol development, that the 1μM ET-1 response was not changed in contol and chronically hypoxic vessels following permeabilisation, and both were compatible. In fact, ET-1 was still capable of increasing

CaS to some extent following incubation with Y27632, suggesting ET-1 does not exert its effects on CaS solely through the ROK pathway. These findings are consistent with reports that ET-1 is staurosporine sensitive (Bialecki *et al.*, 1998a;Bialecki *et al.*, 1998b) and therefore its effect on CaS may well be, at least partly mediated through PKC. It was also reported, that although the maximum response to ET-1 in unpermeabilised vessels was attenuated following 2weeks chronic hypoxia, the staurosporine sensitive component of the response was substantially augmented (Bialecki *et al.*, 1998a). Hence in the Wistar rat small pulmonary artery, whilst chronic hypoxia may attenuate the constitutive activity of ROK it does not necessarily prevent agonists from increasing CaS.

5.5 Conclusion

The findings of the present study suggest that in the Wistar rat small pulmonary artery, a basal sensitisation of the contractile apparatus exists in the absence of any external influence and that it is mediated by constitutive activity of ROK. 2weeks chronic hypoxia was found to attenuate the basal CaS, but did not prevent subsequent agonist induced Ca^{2+} sensitisation, which may have been mediated by an alternative pathway (e.g. PKC). Further studies in control and chronically hypoxic small pulmonary arteries in the presence of GTPγS, and the analysis of RhoA expression, would determine if the potential for agonist induced sensitisation was attenuated in this pathway and confirm whether the attenuated CaS was associated with down regulation of Rho A expression. Additionally, performing; 1) agonist CCRCs at 230nM Ca^{2+}, and 2) Ca^{2+} CCRCs in the presence of an optimal concentration of the agonist, in permeabilised control and chronically hypoxic vessels would determine if chronic hypoxia modulates the contractile response to these agonists through changes in the CaS component of the response.

Chapter 6

Results Part II

iii. The role of Calcium Sensitivity in the 5-HT hypothesis of Pulmonary Arterial Hypertension

6.1 Introduction

6.1.1 Chronic hypoxia causes increased 5-HT mediated contraction in the Wistar rat small pulmonary artery

Serotonin (5-HT) has been widely reported as a pulmonary vasoconstrictor and co-mitogen and a number of studies have implicated it with the remodelling and vasoconstriction associated with pulmonary hypertension (PH) secondary to chronic hypoxia (CH) (MacLean *et al.*, 1996b;Eddahibi *et al.*, 1997;Keegan *et al.*, 2001;Murdoch *et al.*, 2003). A review of the postulated role of 5-HT in the development of pulmonary arterial hypertension (PAH) was presented in the general introduction (chapter 1).

5-HT induced vasoconstriction in the control Wistar rat, small pulmonary artery (SPA) is of low efficacy and potency and is mediated through the 5-HT_{2A} receptor, although the 5-HT_{1B} receptor is present. However, in the chronically hypoxic rat, there is a marked increase in the contractile response to 5-HT and this is at least partly due to an increase in 5-HT_{1B} receptor mediated vasoconstriction (MacLean *et al.*, 1996b). It has been demonstrated however that in control rats, responses to 5 carboxamidotryptamine (5-CT), a non selective 5-HT_1 receptor agonist, can be potentiated by elevated basal tone induced by ET-1 activation of the Gq-coupled receptor and further potentiated by NOS III inhibition using L-NAME. The responses of control vessels were so augmented by these conditions that they where not significantly different from responses in chronically hypoxic vessels. However these conditions did not further augment the increased constriction induced by chronic hypoxia (MacLean & Morecroft, 2001). The phenomenon was reported to be due synergistic interactions between the Gq-coupled receptors (i.e. stimulated by ET-1) and Gi-coupled receptors (i.e. stimulated by 5-HT/5-CT), however the synergy was not thought to be simply the result of an elevation of intra-cellular Ca^{2+}. ET-1 is known to increase CaS in these vessels (Evans *et al.*, 1999) and

inhibition of the NO/cGMP pathway has been shown to augment the ability of Rho kinase to increase CaS (Sauzeau *et al.*, 2003a). Hence, it raises the question of whether the reported synergy in the control rat and the augmented response in the chronically hypoxic rat, are linked to an ability of 5-HT to increase CaS. 5-HT has been shown to increase CaS in ovine, porcine, bovine, rat and human systemic arteries through activation of Rho kinase (ROK) (Akopov *et al.*, 1998;Shimokawa *et al.*, 1999;Weber & Webb, 2001;Kandabashi *et al.*, 2002;Nishikawa *et al.*, 2003) and this response has also been shown to be increased in some pathological states such as systemic hypertension (Weber & Webb, 2001). Although 5-HT has not yet been demonstrated to increase CaS in any pulmonary vessels its mitogenic action has been shown to be blocked by 5-$HT_{1B/1D}$ antagonists and the ROK inhibitor Y27632 clearly suggesting this pathway can be activated by its presence (Liu *et al.*, 2004).

6.1.2 The 5-HT transporter (5-HTT) and its role in PH

The 5-HT transporter (5-HTT) has also been linked to the aetiology of PAH, although its role is still controversial (see chapter1). In summary, both an increase (Eddahibi *et al.*, 1999) and decrease (Jeffery *et al.*, 2000;Hoshikawa *et al.*, 2003;MacLean *et al.*, 2004) in 5-HTT expression have been reported following chronic hypoxia. In transgenic mice that over-express the 5-HTT, RVP was elevated three-fold but that there was no evidence of right ventricular hypertrophy or vascular remodelling. However following 2weeks chronic hypoxia, both right ventricular hypertrophy and vascular remodelling were significantly greater than in hypoxic controls despite 5-HTT expression being significantly reduced (MacLean *et al.*, 2004). These findings suggested that an increase in 5-HTT expression/activity might augment vasoconstriction prior to the onset of vascular remodelling. If this was so it could provide valuable insight into the aetiology of PAH. Conversely however, it was observed that 5-HTT over-expression substantially

reduced the potency but not efficacy of the contractile response to 5-HT in the small pulmonary artery of the mouse (unpublished observations). Hence *in vitro,* 5-HTT over-expression reduced the contractile activity to 5-HT, presumably by reducing local 5-HT concentrations around the 5-HT receptors, but *in vivo* appeared to increase overall contractile activity. This raised the possibility that over-expression of the 5-HTT could somehow result in an increase CaS.

6.1.3 Aims

Presently there are no reports of the influence of 5-HT receptor activation or the effect of the 5-HTT on CaS in the pulmonary circulation. The aims of this study were therefore two-fold: 1) To determine if 5-HT receptor activation can modulate CaS in the Wistar rat small pulmonary artery. 2) To determine if over-expression of the 5-HTT causes an increase in the CaS of the C57BL/6×CBA mouse small pulmonary artery.

6.2 Methods

6.2.1 Set up

Small pulmonary arteries (D-class vessels) from chronically hypoxic (CH) and age matched (AM) control Wistar rats and 1st generation intra-lobar small pulmonary arteries (C-class vessels) from *C57BL/6×CBA* transgenic (TG) and wild type (WT) control mice were harvested, set up on the myograph and normalised as described in the general methods. Permeabilisation studies were performed at 24^{0}C, whilst general pharmacology experiments were performed at 37^{0}C (see chapter 2). Rat and mouse vessels were permeabilised with 50μM and 10μM β-escin respectively (see chapter 4). The right ventricular/total ventricular ratio (RV/TV) was used to assess the development of PH (see chapter 2). Subsequent to determination of the vessels maximum response to a nominally iso-osmotic 120mM KCl solution (permeabilisation studies) or a 50mM hyper-osmotic KCl addition (general pharmacology), the following experiments were performed.

6.2.2 Study 1) The effect of 5-HT receptor agonists on Ca^{2+} sensitivity in the age matched control and chronically hypoxic Wistar rat, permeabilised small pulmonary artery at sub-maximal tone (pCa 6.64)

Permeabilised age matched control and chronically hypoxic vessels were sub-maximally constricted in a pCa 6.64 MI solution. When this pre-agonist addition, tone (pre-tone) had stabilised, a single dose of 100μM 5-HT, 5-CT, αMe 5-HT or CP 94253 was added and the response observed until it had stabilised. A second dose of 320μM 5-HT, 5-CT, αMe 5-HT or CP 94253 was then added and the response observed until it had stabilised. 5-CT is a non-selective 5-HT_1 receptor agonist, whilst CP 94253 is a selective 5-HT_{1B} receptor agonist. αMe 5-HT is a selective 5-HT_2 receptor agonist. Two concentrations of

each agonist were used as bi phasic responses were observed during initial experiments (Fig 6.1).

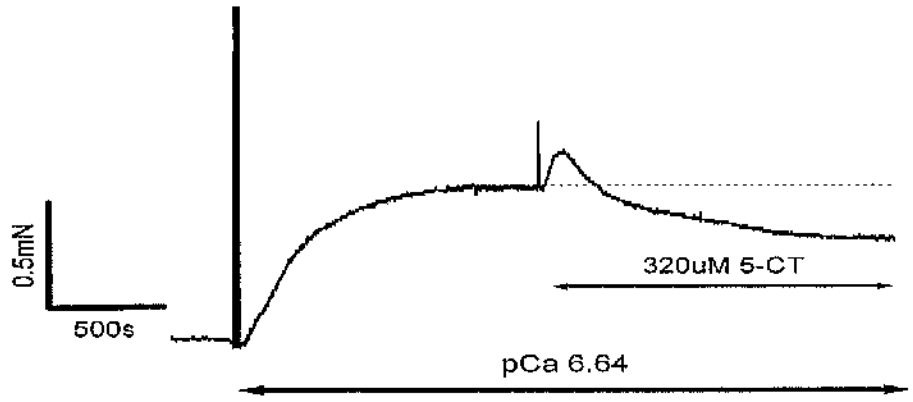


Figure 6.1 Experimental trace showing a bi-phasic response to a single dose of 5CT

This exceptional response implied that 5-HT and 5-CT could activate more than one 5-HT receptor subtype. Because of this, preliminary CCRCs to 5-HT and 5-CT were performed at pCa 6.64 (not shown). These suggested that the maximum constrictor action to 5-HT/5-CT occurred at 100μM and the maximum dilator action at 320μM.

6.2.3 Study 2) Effect of over-expression of the 5-HT transporter on Ca^{2+} sensitivity in the C57BL/6×CBA mouse intra-lobar pulmonary artery

6.2.3.1 Permeabilisation studies

Permeabilised wild type (WT) and transgenic (TG) vessels were exposed to the following stepwise increases in free Ca^{2+} concentration pCa; 9.9, 7.12, 6.64, 6.16, 5.7, 4.55, 4.0, 2.5. Each increase was performed by a solution exchange, where the previous MI solution was drained and the vessel washed twice in the following solution. The response was then allowed to stabilise before the next solution exchange was performed.

6.2.3.2 General pharmacology studies

Following the determination of the response to 50mM KCl, cumulative concentration response curves (CCRCs) to 5-HT (1nM – 0.1mM) were performed in the presence and absence of the 5-HTT inhibitor citalopram (0.1μM) in unpermeabilised wild type and transgenic vessels. Vessels not pre-treated with citalopram were then thoroughly washed and allowed to return to baseline once more before a CCRC to ET-1 (1pM – 1μM) was performed.

6.3 Results

6.3.1 Study 1) Effect of 5-HT agonists on Ca^{2+} sensitivity in permeabilised age matched control and chronic hypoxic Wistar rat small pulmonary arteries

6.3.1.1 Effect of 5-HT on Ca^{2+} sensitivity at pCa 6.64 in age matched control vessels

5-HT, α me 5-HT and 5-CT all failed to significantly modulate CaS at either 100μM or 320μM doses (n=6, all groups, $P>0.05$, paired t-test). However 3/6 vessels challenged with 5-HT and 3/6 vessels challenged with 5-CT did demonstrate a limited modulation of CaS. In these vessels 5-HT and 5-CT, caused a small increase in tone at 100μM which was then either sustained (1/6 vessels) or followed by a slightly larger decrease in tone at 320μM (2/6 vessels) (Fig 6.2).

100μM CP 94253 did not significantly modulate CaS, however 320μM CP 94253 caused a substantial reduction in pre-tone (P/T) of 65 ± 11% (P/T; 0.43 ± 0.09mN vs 320μM; 0.16 ± 0.05mN, n=6), which was significant and observed in all vessels tested ($P<0.01$, paired t-test) (Fig 6.3 and fig 6.4). This equated to 18 ± 4% of $\mathbf{Fi}_{max}$.

6.3.1.2 Effect of 5-HT on Ca^{2+} sensitivity at pCa 6.64 in chronically hypoxic vessels

2 weeks chronic hypoxia did not significantly alter any of the responses observed from that observed in control vessels (n=5, all groups, $P>0.05$, unpaired t-tests). 320μM CP 94253 caused a reduction of the pre-tone (P/T) by 45 ± 8% (P/T; 0.40 ± 0.09mN vs 320μM; 0.23 ± 0.06mN, n=5, $P<0.05$, paired t-test) (Fig 6.3). This equated to 9 ± 2% of $\mathbf{Fi}_{max}$.

Comparison of the age matched control and chronically hypoxic, CP 94253 responses when standardised to their paired $\mathbf{Fi}_{max}$ suggested the chronically hypoxic response was attenuated relative to the control response, however this was not significant (AM; 18 ± 4% of $\mathbf{Fi}_{max}$ n=6 vs CH; 9 ± 2%, n=5, P>0.05, unpaired t-test) (Fig 6.4). The response was compared in this manner so that the change in CaS could be expressed against a constant rather than the pCa 6.64 pre-tone, which itself can be modulated by hypoxia as was shown in chapter 5.

Mean pre-tone was not significantly different between the any of the age matched control groups (n=6, P>0.05, unpaired ANOVA) or the chronically hypoxic groups (n=5, P>0.05, unpaired ANOVA) either expressed in mN or as a percentage of $\mathbf{Fi}_{max}$. However, the pre-tone of the age matched control groups was significantly larger than the pCa 6.64 pre-tone of the chronically hypoxic groups (AM; 27 ± 2% n=24/6 vs CH; 21 ± 1%, n=20/5, P<0.05, unpaired t-test) consistent with the reduction in constitutive CaS reported in chapter 5. Vessels form all groups were of comparable internal diameter and replicated transmural pressure (n=5-6, P>0.05, unpaired ANOVA). The mean vessel size and replicated transmural pressure was 268 ± 5μm and 13.4 ± 0.2mmHg respectively (n=44 vessels/11rats). In completely unresponsive vessels, exposure to 1μM ET-1 caused a significant, 51 ± 5% increase in mean pre-tone (n=8, P<0.05, paired t-test) suggesting the integrity of the signal transduction system was preserved in both age matched and chronically hypoxic non-responsive vessels. The e_{max} index of permeabilisation, suggested all the vessels were fully permeabilised ($\mathbf{Fe}_{max}$; 1 ± 1% greater than $\mathbf{Fi}_{max}$, all groups n=5-6, P>0.05, paired t-test). The RV/TV ratio was significantly larger in the chronically hypoxic group consistent with the development of PH (AM; 0.233 ± 0.006 vs CH; 0.363 ± 0.013, n=5-6, P<0.0001, unpaired t-test).

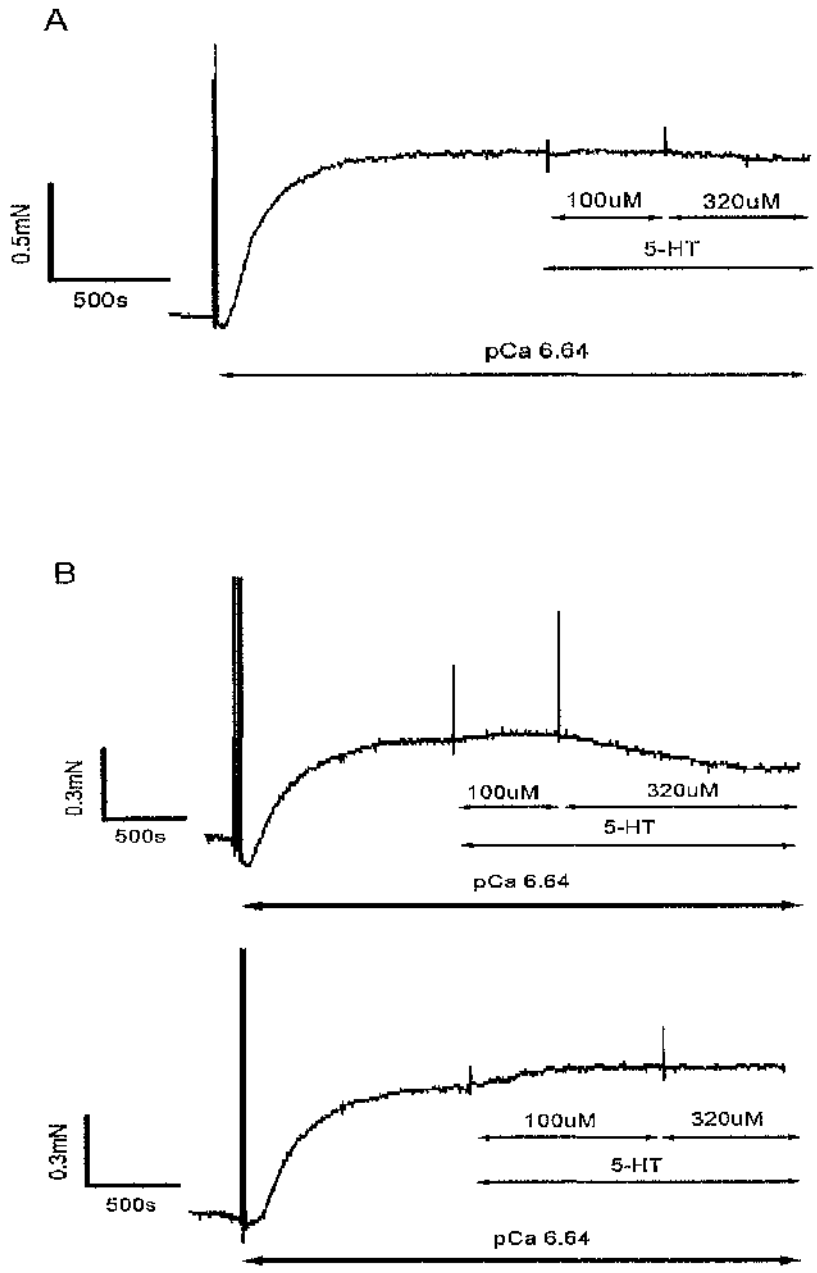


Figure 6.2 Experimental traces showing the effect of 5-HT on Ca^{2+} sensitivity in the Wistar rat, small pulmonary artery

A) The typical response to 5-HT, 5CT or αMe 5-HT in age matched control and chronically hypoxic vessels. B) Some individual vessels demonstrated the ability to modulate CaS in either a bi-phasic or mono-phasic manner to 5-HT or 5-CT but not αMe 5-HT.

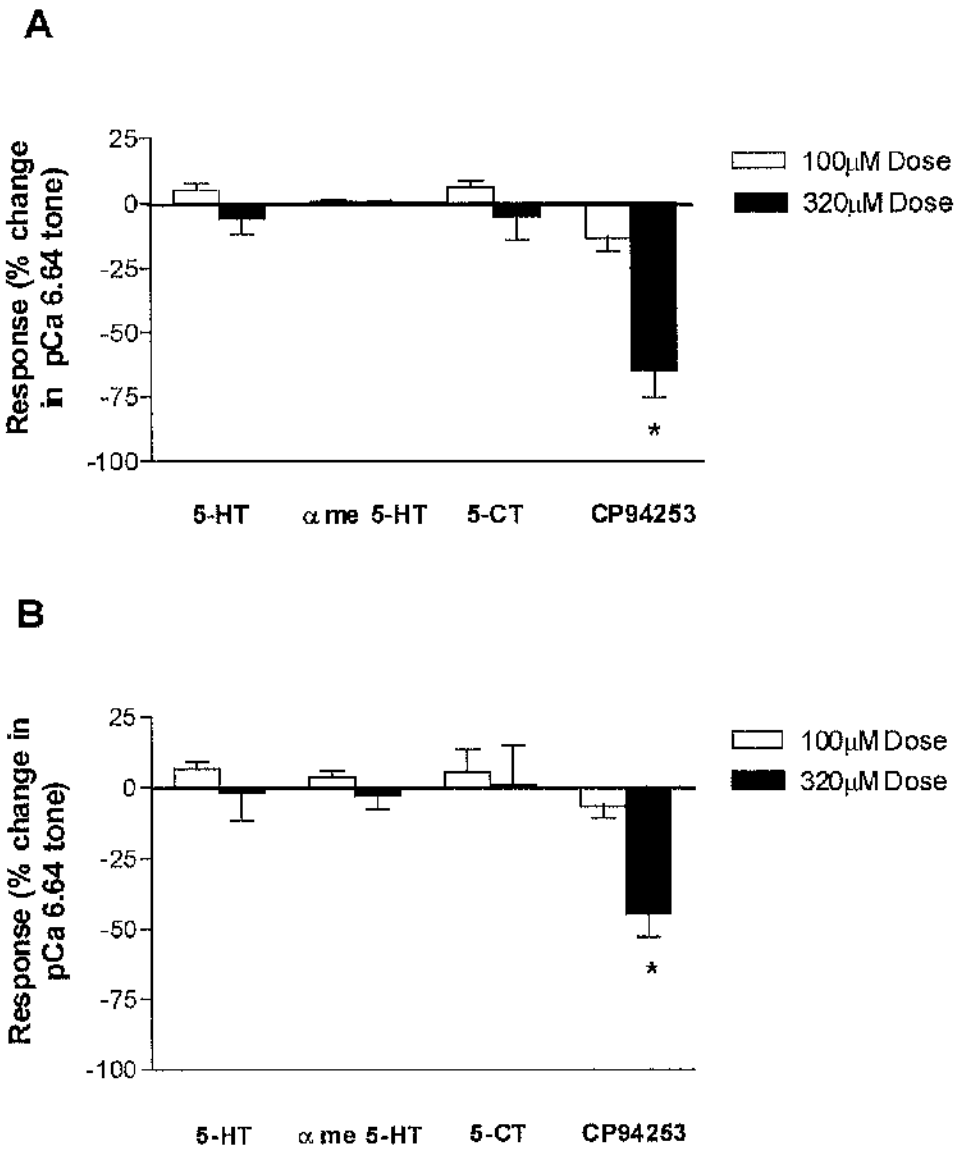


Figure 6.3 The effect of 5-HT agonists on Ca^{2+} sensitivity in the age matched control and chronically hypoxic Wistar rat small pulmonary artery

The effect of single dose additions of 5-HT, αMe 5-HT, 5-CT and CP 94253 on CaS at pCa 6.64 in the A) age matched control rat SPA (n=6, **P*<0.01, paired t-test) and B) chronically hypoxic rat SPA (n=5, **P*<0.05, paired t-test).

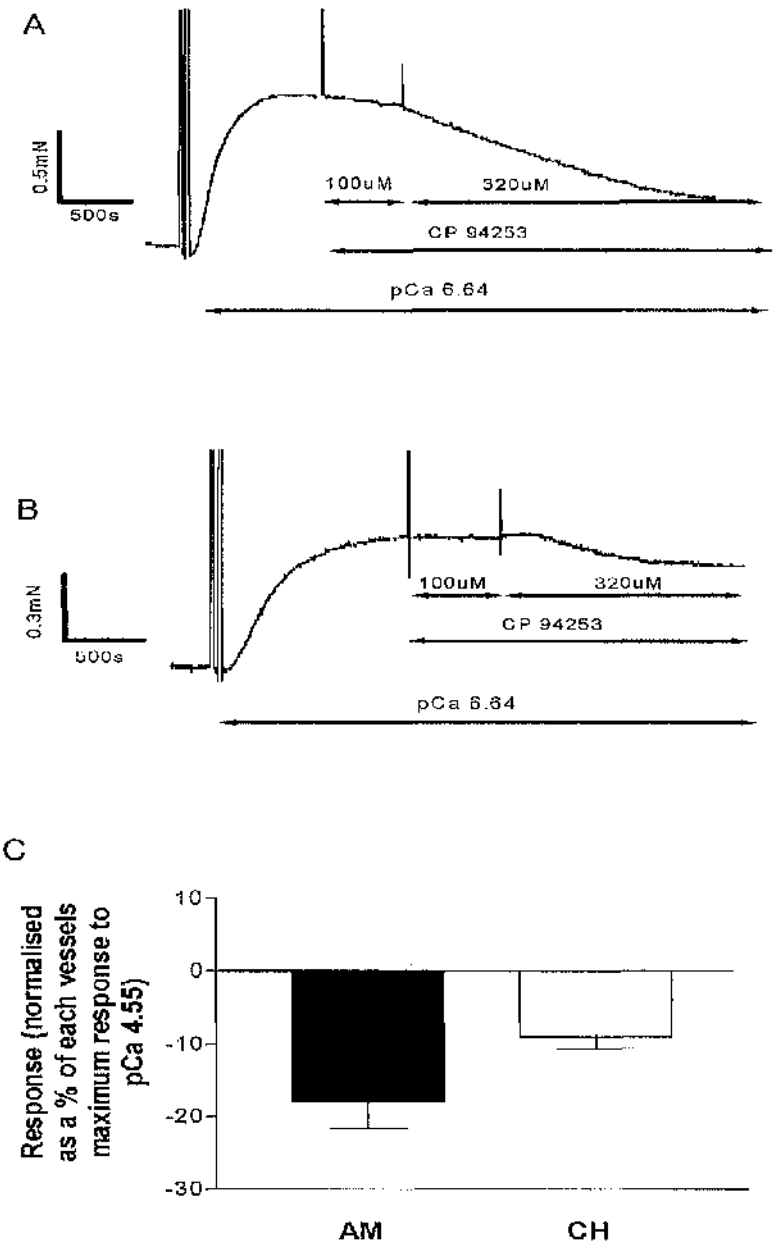


Figure 6.4 The effect of the selective 5-HT$_{1B}$ receptor agonist CP 94253 on Ca^{2+} sensitivity in the Wistar rat small pulmonary artery

A) Experimental trace showing a strong response to CP 94253 in an age matched (AM) control vessel B) An apparently attenuated response to CP 94253 in a chronically hypoxic (CH) vessel C) Bar graph comparing the desensitisation causes by 320μM CP 94253 in AM and CH vessels, normalised as a percentage Fi_{max} (pCa 4.55 response). The mean attenuation was not significant (n=5-6, *P*>0.05, unpaired t-test)

6.3.2 Study 2) Effect of over-expression of the 5-HT transporter on Ca^{2+} sensitivity in the C57BL/6×CBA mouse intra-lobar pulmonary artery

6.3.2.1 Permeabilisation studies

Ca^{2+} sensitivity was not significantly different between wild type (WT) and transgenic (TG) mice which over express the 5-HTT at any point on the Ca^{2+} CCRC curve (pEC_{50} values, WT; 6.17 ± 0.02, n=9 vs TG; 6.15 ± 0.02, n=8, P>0.05, unpaired t-test) (Fig 6.5).

The vessels from the two groups were of comparable internal diameter and replicated transmural pressure (n=8-9, P>0.05, unpaired t-tests). The e_{max} index of permeabilisation, suggested all the vessels were fully permeabilised (Fe_{max}; <1 ± 1% greater than Fi_{max}, n=8-9, P>0.05, paired t-test). The RV/TV ratio was not significantly different between the wild type and transgenic groups (WT; 0.240 ± 0.004, n=9 vs TG; 0.242 ± 0.008, n=8, P>0.05, unpaired t-test) and was not indicative of PH, however all transgenic animals were confirmed to over-express 5-HTT by PCR of tail tips.

6.3.2.2 General pharmacology studies

Over-expression of the 5-HTT caused a marked reduction in the potency (pEC_{50}: WT; 7.16 ± 0.08, n=5 vs TG; 5.66 ± 0.05, n=4, P<0.0001, unpaired t-test) but not the efficacy (n=4-5, P>0.05 unpaired t-test) of the 5-HT response when compared to wild type controls. This effect was substantially but not entirely reversed by incubation with 0.1μM citalopram (pEC_{50}: TG; 5.66 ± 0.05, n=4 vs TG +0.1μM Cit; 6.71 ± 0.07, n=5, P<0.0001 unpaired t-test) (pEC50: WT control; 7.16 ± 0.08 vs TG +0.1μM Cit; 6.71 ± 0.07, n=5, P<0.005 unpaired t-test), however citalopram had no effect whatsoever on the wild type response (n=5, P>0.05, unpaired t-test) (Fig 6.6).

The response to ET-1 was not significantly changed with regard to either potency or efficacy by over-expression of the 5-HTT (n=4-5, P>0.05 unpaired t-tests) (Fig 6.6).

RV/TV data was not obtained as hearts were used in alternative studies, however 5-HTT over-expression was confirmed in transgenic animals by PCR of tail tips.

Mean internal diameter and replicated transmural pressure, were not significantly different between any of the groups (n=4-5, $P>0.05$, unpaired ANOVA). The mean vessel size and replicated transmural pressure of the mouse vessels used in the permeabilisation and pharmacology studies was 527 ± 13μm and 13.9 ± 0.2mmHg respectively (n=36 vessels/27 mice).

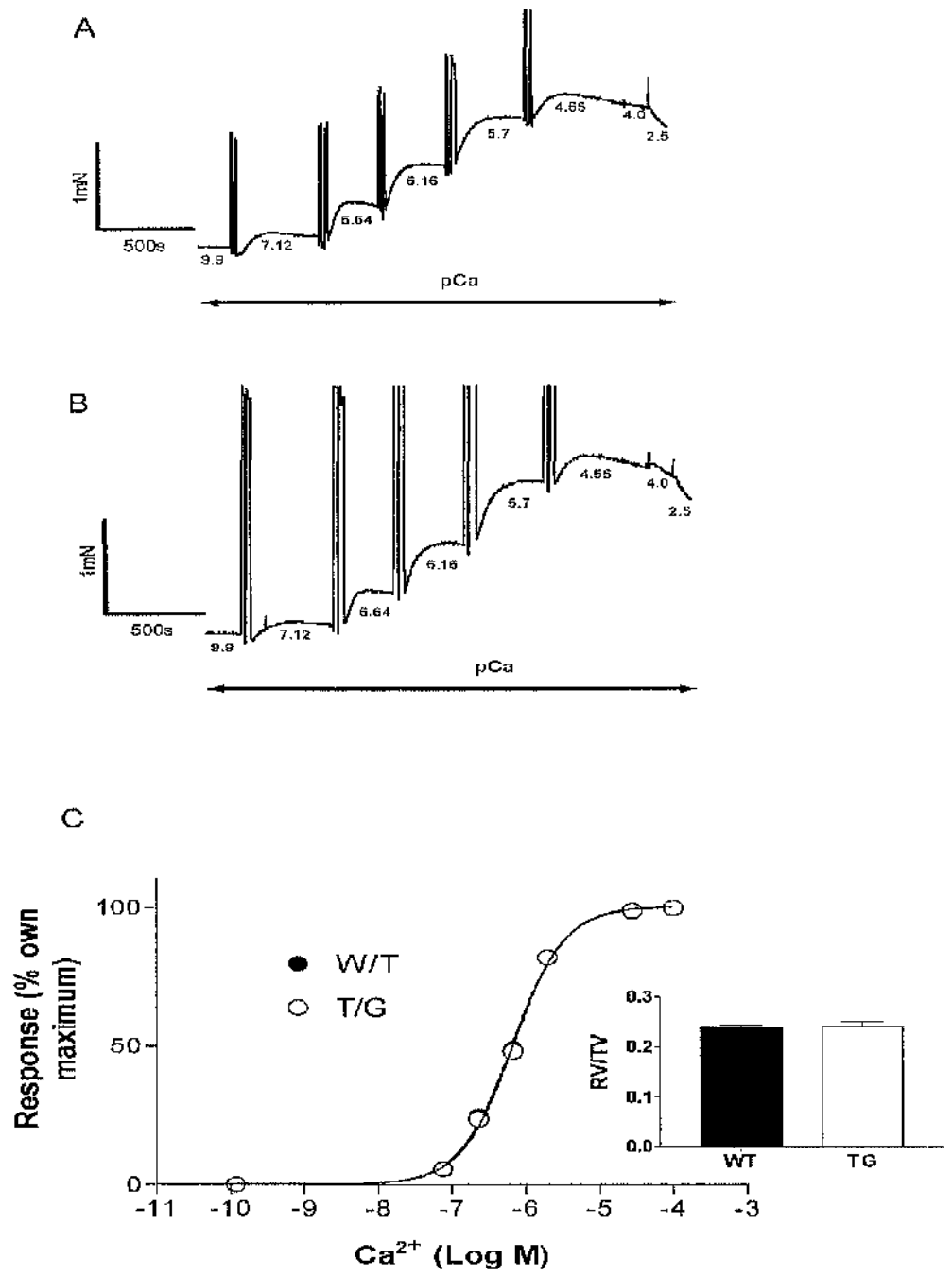


Figure 6.5 The effect of 5-HTT over-expression on Ca^{2+} sensitivity in the permeabilised C57BL/6×CBA mouse intra-lobar pulmonary artery

Experimental traces of Ca^{2+} CCRCs performed in A) Wild type (WT) control mice and B) transgenic (TG) mice over-expressing the 5-HTT. C) Graph comparing the Ca^{2+} CCRCs for permeabilised WT and TG mouse intra-lobar pulmonary arteries (n=8-9, P>0.05, unpaired t-test). Inset shows the right ventricular/total ventricular (RV/TV) ratio (n=8-9, P>0.05 unpaired t-test).

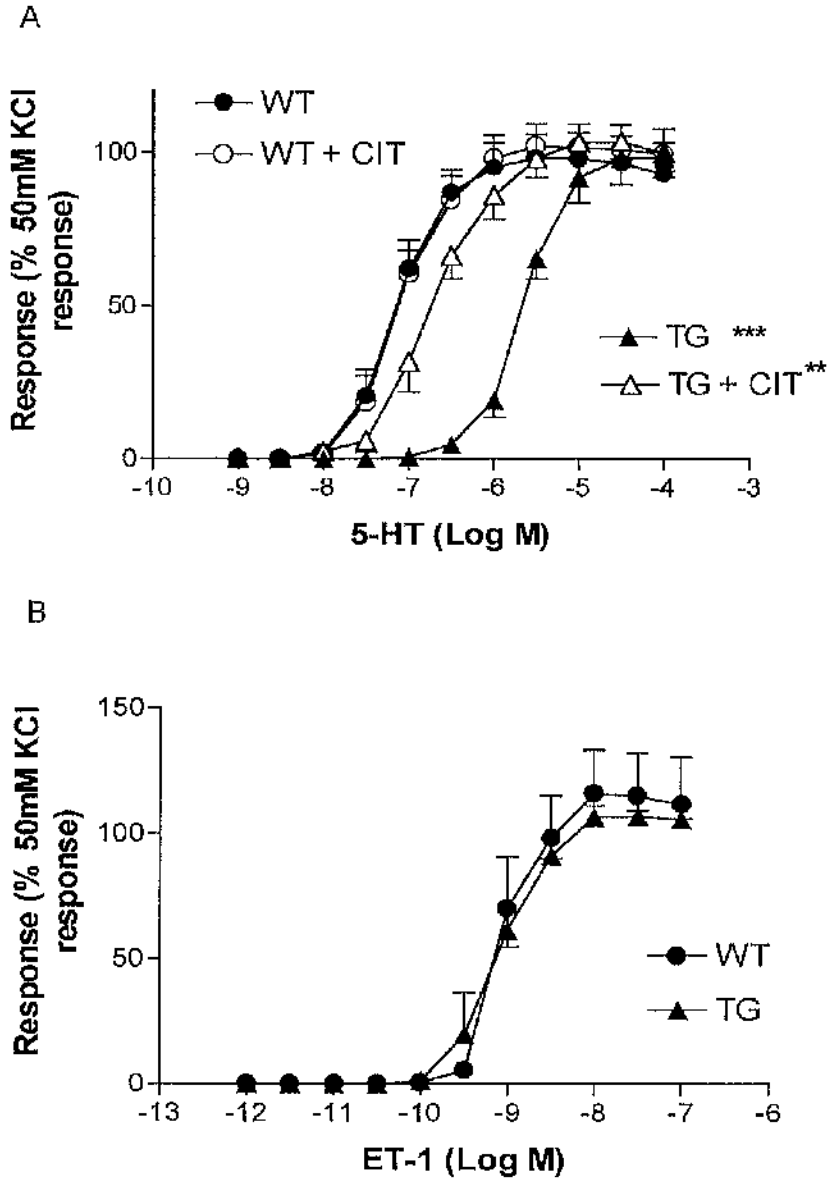


Figure 6.6 The effect of 5-HTT over-expression on 5-HT and ET-1 pharmacology in the isolated unpermeabilised C57BL/6×CBA mouse intra-lobar pulmonary artery

A) 5-HT CCRCs in the wild type (WT) and transgenic (TG) mouse in the presence and absence of 0.1uM citalopram (n=4-5,**P<0.005, ***P<0.0001, unpaired t-test) B) ET-1 CCRCs in the WT and TG mouse (n=4-5, P>0.05, unpaired t-test).

6.4 Discussion

6.4.1 The effect of 5-HT agonists on CaS in the normal and chronically hypoxic Wistar rat small pulmonary artery

6.4.1.1 5-HT does not modulate CaS in the rat small pulmonary artery

The data suggest that in β-ecin permeabilised, normal or chronically hypoxic Wistar rat, small pulmonary arteries, 5-HT does not significantly affect CaS. The findings were therefore not consistent with increased CaS mediating the enhanced contractile response to 5-HT in the unpermeabilised chronically hypoxic rat small pulmonary artery. However 5-HT did modulate CaS to a limited extent at very high concentrations (100-320μM) in some individual vessels (Fig 6.2). These responses were therefore examined using selective and non-selective 5-HT receptor agonists in an attempt to separate the individual components of the small bi-phasic response and hence determine if two or more response signals were opposing one another. In a disease state such as PAH, it is possible that selective expression of a single receptor subtype could occur thus favouring one of the prospective components (MacLean *et al.*, 1996b;Keegan *et al.*, 2001). This could unmask an enhanced ability to modulate CaS, as might have occurred in the previously reported synergistic responses between 5-CT and ET-1 in unpermeabilised vessels (MacLean & Morecroft, 2001).

6.4.1.2 Activation of the $5\text{-HT}_{1B/D}$ receptor decreases CaS in the rat small pulmonary artery

The mono and bi-phasic responses observed in a small number of vessels challenged with either 5-HT or the non-selective 5-HT_1/5-HT_7 receptor agonist 5-CT, were indicative of an ability to both increase and decrease CaS in these vessels through either the 5-HT_1 or 5-HT_7 receptors. Interestingly, vessels from the same animal tended to behave in a similar manner to 5-HT and 5-CT, but showed no response whatsoever to the 5-HT_2

receptor agonist αMe 5-HT suggesting 5-HT_2 receptors have no influence CaS even at supra maximal concentration. The findings suggest the responses were therefore limited to the $5HT_{1B}$, 5-HT_{1D} and/or 5-HT_7 receptors. As the 5-HT_7 receptor has not yet been identified in the rat pulmonary artery it was considered that the response was most likely mediated through the 5-$HT_{1B/1D}$ receptor. The selective 5-HT_{1B} receptor agonist CP 94253 did not influence CaS at concentrations less than 100μM but caused a substantial decrease at 320μM ($P<0.01$), in all vessels tested, reducing tone to a level similar to that reported by the action of the ROK inhibitor Y 27632 in chapter 5.

In vessels which had been exposed to 2weeks chronic hypoxia, the effect of CP 94253 appeared to be attenuated by ~ 50% when the responses were normalised to their paired Fi_{max} response but this was not significant ($P>0.05$). Chronic hypoxia is reported to increase expression of the 5-HT_{1B} in the rat (MacLean *et al.*, 1996b) and therefore one may expect its ability to decrease CaS to be augmented under these conditions. However, the findings of chapter 5 (and the comparative levels of pre-tone in the present study) suggest that chronic hypoxia down-regulates constitutive CaS in β-escin permeabilised, rat small pulmonary arteries. Therefore if the pathway which mediates the CaS response to CP 94253 was down-regulated by exposure to 2weeks chronic hypoxia, the potential of the 5-HT_{1B} to cause desensitisation might have been limited despite its increased expression. Although CP 94253 is a specific 5-HT_{1B} receptor agonist, at the concentrations required to cause the reported desensitisation response its specificity would be doubtful, and its action can therefore only be narrowed to either the 5-HT_{1B} or 5-HT_{1D} receptor.

Due to time constraints, the present study did not determine which receptor subtype was responsible for the small increase in CaS that was observed in some vessels. However, when considered with the augmented desensitisation observed in the presence of CP

94253, it did raise the possibility that 5-HT and 5-CT were capable of substantial modulation of CaS at very high concentrations, but that 2 opposing actions mediated through different receptor subtypes may typically resulted in no net change of CaS. This would require the use of specific $5HT_{1B}$ and $5\text{-}HT_{1D}$ antagonists in the presence of 5-HT or 5-CT to determine if a significant increase in CaS can be unmasked under these conditions.

6.4.2 The effect of over-expression of the 5-HTT on CaS in the C57BL/6×CBA mouse small pulmonary artery

Over-expression of the 5-HTT had no effect on the Ca^{2+} CCRCs in permeabilised mouse vessels suggesting basal CaS was completely unaffected. This was consistent with the results of the ET-1 general pharmacology experiment in unpermeabilised mouse vessels, which found the response to be unchanged by 5-HTT over-expression. In contrast, the potency of the 5-HT CCRC contractile response in unpermeabilised vessels was attenuated some thirty-fold by over-expression of the 5-HTT. This attenuation was substantially although not entirely, reversed by citalopram. Citalopram has only trivial effects on CaS (unpublished study, not shown) and therefore its effect was believed to be due to its action as a selective serotonin re-uptake inhibitor (Wanstall *et al.*, 2003). Hence it is hypothesised that 5-HTT over-expression caused a localised reduction in 5-HT concentration around the 5-HT cell surface receptors, which was virtually reversed by citalopram. The findings of this study are therefore contrary to over-expression of the 5-HTT causing and elevation in PAP through an increase in CaS of the contractile apparatus. Furthermore over-expression of 5-HTT did not cause an increase in the overall contractile responsiveness to 5-HT or ET-1 and therefore the enhanced PAP associated with 5-HTT over-expression cannot be explained by the findings of this study.

6.4.3 Conclusion

The findings of this study are not consistent with 5-HT mediating a change in CaS in the control or chronically hypoxic rat, or 5-HTT over-expression modulating CaS in the mouse. However, selective activation of the 5-HT_{1B} receptor demonstrated that CaS can be modulated by selective 5-HT agonists.

Chapter 7

General conclusion

7.1 Conclusion

7.1.1 Re-statement of aims

The initial aims of this thesis were: 1) To determine the role of human urotensin II (hU-II) in the regulation of pulmonary vascular tone and whether hU-II could be involved in the aetiology of pulmonary arterial hypertension (PAH). 2) To determine the role of Ca^{2+} sensitivity (CaS) in the regulation of pulmonary vascular tone and to determine whether modulations in CaS could be involved in the aetiology of PAH. The two areas chosen for study where pulmonary hypertension (PH) secondary to chronic hypoxia and the 5-HT system.

7.1.2 hU-II and the regulation of pulmonary arterial tone

hU-II was found to be a potent but low efficacy vasoconstrictor of normal rat, extra-lobar and 1st generation intra-lobar pulmonary arteries but had no action in any mouse or human vessels tested. The constrictor response was generally not augmented in rat models of PAH. The findings of this thesis are therefore not consistent with a role for hU-II in the actiology of PAH.

7.1.3 Ca^{2+} sensitivity and the regulation of pulmonary arterial tone

A permeabilisation protocol was successfully developed, which permitted the study of the phenomenon of Ca^{2+} sensitisation in the Wistar rat small pulmonary artery. The protocol consistently yielded fully permeabilised small pulmonary arteries with intact 2^{nd} messenger systems. Furthermore the maximum contractile response of the permeabilised preparations was typically seven-fold greater than previously had been reported (Evans *et al.*, 1999).

Rho kinase was found to be constitutively active in normal rat small pulmonary arteries resulting in an enhanced level of CaS in these vessels, which was subsequently attenuated following 2weeks chronic hypoxia. This finding suggested that changes in constitutive CaS occur in the chronically hypoxic rat model of PAH and warrant further investigation, especially with regard to the exposure period to hypoxia.

5-HT did not modulate CaS in the normal or chronically hypoxic rat small pulmonary artery. Therefore the reported enhanced contractile responses to 5-HT following 2weeks exposure to chronic hypoxia are not mediated through an increase in CaS. However activation of the $5\text{-HT}_{1B/D}$ receptor using the selective agonist CP did significantly attenuate CaS suggesting further investigation using selective antagonists is warranted. Over-expression of the 5-HT transporter (5-HTT) was not found to modulate CaS in the mouse small pulmonary artery. Pharmacological studies in the mouse, suggested the effect of the 5-HTT on the 5-HT vasoconstrictor profile, was primarily mediated by its impact on local 5-HT concentrations around the cell surface receptors.

Appendix

Health and Safety assessment

Questionnaire on the Use of Human Tissues in Research

Name of Research Group Pulmonary vascular

Group Leader Prof. MR MacLean

Senior Technician Mrs Lynn Laughlin

Laboratory Location Lab 501 WMB

1 Does your laboratory carry out experiments with human tissues? Yes

1 resected human lung: pulmonary arteries/airways
2 human abdominal arteries

2 Do you have any stored human tissue? Yes

3 Do you have any plans to work with human tissues? Yes

If you have answered Yes to any of the above questions, please answer also the remaining questions. Otherwise this section of the form can be disregarded.

4 Has everyone in your group read the new guidelines and received appropriate instruction in the use, storage and disposal of human tissues? Yes

5 If the answer to Q4 is No, then please indicate when you plan to carry out the training and have the forms confirming that this has taken place completed.

Date.....

6 Please indicate specifically where you have human tissues stored and the nature of the tissue stored.

Liquid Nitrogen storage: pulmonary arteries

7 Please indicate how you intend to dispose of tissue once your investigations are complete. Tissue is double-bagged and transported to the Biohazard bin at the Western Infirmary Glasgow for incineration. A full record is kept in the laboratory.

Full details of the type of tissue, the method of disposal and the date of disposal must be kept by the research group leader and should be forwarded to Paul Phillips.

8 Please provide documentary evidence (photocopies) that the appropriate ethical permissions have been obtained.

Attached:
- West Ethics Committee approval (00/37) for inguinal hernia biopsies (for provision of abdominal arteries)
- West Ethics committee approved patient consent form for use of resected lung tissue.

Completed by: ..... **Date:** 8/03/01

H:\Docs\my info\Human tissue questionnaire FEB 01.doc

West Glasgow Hospitals ethics committee approval

West Glasgow Hospitals

PART OF THE NORTH GLASGOW UNIVERSITY HOSPITALS NHS TRUST

WEST ETHICS COMMITTEE
Western Infirmary
Dumbarton Road
Glasgow G11 6NT

Direct Line: 211 6238
Fax: 211 1920

Our Ref: AHT

Your Ref:

Please reply to: Mrs A H Torrie
SECRETARY - WEST ETHICS COMMITTEE

18 April, 2000

Dr Colin Berry
MRC Clinical Training Fellow
Department of Medicine
Western Infirmary

Dear Colin,

00/37(2) Dr C Berry et al - **An investigation of the vasopressor effect of urotensin compared with other known vasoconstrictor molecules in human resistance arteries in healthy volunteers.**

The Committee at the meeting held today ratified Chairman's approval but wished to draw to your attention that the protocol submitted contains an error i.e. that the biopsies will not be gluteal, they are in fact inguinal hernia biopsies. The amendment should come back to the secretary for filing. This study now has full and unqualfied Ethics Committee approval.

Please note that the approval contained in this letter is valid for all sites which form part of the North Glasgow Trust. If however, this research is to be carried out at sites within the North Glasgow Trust other than the one covered by this letter, then a covering letter signed by the person responsible for the research on that site, should be sent listing names, titles and addresses of all collaborating researchers. A copy of this approval letter should be passed to them.

It should be noted that although Ethics Committee approval has been granted, Trust Management approval is still required. This should be obtained through the Research and Development Office at Gartnavel General Hospital (tel: 211 0115).

Kind regards.

Yours sincerely,

Andrea H Torrie
SECRETARY - WEST ETHICS COMMITTEE

Patient consent form

West Glasgow Hospitals

DEPARTMENT OF CARDIOTHORACIC SURGERY

CONSENT FORM FOR PERMISSION TO USE RESECTED LUNG TISSUE FOR RESEARCH PURPOSES IN PATIENTS UNDERGOING LUNG OPERATIONS

As it will have been explained to you, you are about to undergo an operation to remove all, or part of, your lung. Doctors in the Pathology Department will perform a number of tests on the tissue that is removed during the operation. This will allow your surgeon to give you a diagnosis and prognosis and guide you if any other treatment is required. Once they have completed their examination, the tissue is normally discarded.

Because we are constantly engaged in research ourselves and in collaboration with others, we are seeking your permission to use some of this tissue that is normally discarded. This would be used in a number of ongoing projects designed to investigate cancer and in detailing the anatomy & physiology of the blood vessels within the lung.

If you wish your resected tissues to be used in this way, the following guarantees will be given:

- No tissue will be removed apart from what is deemed necessary for your operation as decided by your surgeon.
- Sampling of tissue will not prejudice in any way the results of your operation or affect the ability of the pathologist to produce an accurate report.
- Samples used for research will be removed from tissue that would normally be discarded.
- In cases of cancer research, small samples may be frozen and stored to allow tests to be done at a future date.

Should you not wish tissues to be removed, this will not affect your treatment in any way.

Details of the current research projects and the project leaders are to be found below. The ultimate responsibility rests however with your surgeon to whom all queries should be addressed.

The pharmacology of endogenous vasoactive factors in human pulmonary arteries.
Supervisor: Professor M MacLean, University of Glasgow

Molecular markers of chemosensitivity in non-small cell lung cancer.
Supervisor: Professor R Brown, Beatson Institute

The physiology of human pulmonary vasculature.
Supervisor: Dr. AJ Peacock, Western Infirmary, Glasgow.

These projects have been approved by the Research & Ethics Committee for North Glasgow Hospitals University Trust.

CONSENT

I,.....consent to the use of any tissues removed from me during my lung operation to be used for the purposes of research. I have read the notes above and understand them. I appreciate that under certain circumstance it may be necessary to store some tissue samples for analysis at a later date.

Patient signature: ..... Date: .....

Surgeon's name: .....

Surgeon's signature:..... Date: .....

Guidelines for the use of human tissue

Use of Human Tissue –Prof.M MacLean 's Lab

1- All tissue that enters the lab must be logged in the Human Tissue Log Book;

Date
#Age
#Organ
#Disease Type
#Hospital of origin
#Reference number

2- Date and name of person who collected tissue must be recorded on sheet on wall.

3- Photocopy of concent form must be placed in folder in drawer that contains Log book.

4- Gloves must be worn at all times,however they must be removed before handling other equipment.

5- When using the microscope the handles must be covered in nesco film for adjustment.

6- Work area must be covered in benchcote,all instruments,tissues cottonbuds must be contained on benchkote.

7- All waste must be placed in the yellow waste bags which should be taped next to work area.The waste should be taken that day to the Hospitial for disposal and the person responsible sign the disposal sheet.

8- All instruments used must be decontaminated for 45-60minutes but no longer in Virkon Soln 10% in Water.A designated tub has been labelled for this use.Plastic containers used for transport must be soaked in the same soln overnight before being placed in wash tub.

9- Lab worker who do not follow above guide lines will not be allowed to work with Human tissue.

Bibliography

Aaronson, P. I., Robertson, T. P., & Ward, J. P. (2002). Endothelium-derived mediators and hypoxic pulmonary vasoconstriction. *Respir.Physiol Neurobiol.* **132**, 107-120.

Abacioglu, N., Ercan, Z. S., Kanzik, L., Zengil, H., Demiryurek, T., & Turker, R. K. (1987). Endothelium-dependent relaxing effect of histamine on the isolated guinea-pig main pulmonary artery strips. *Agents Actions* **22**, 30-35.

Abe, K., Shimokawa, H., Morikawa, K., Uwatoku, T., Oi, K., Matsumoto, Y., Hattori, T., Nakashima, Y., Kaibuchi, K., Sueishi, K., & Takeshit, A. (2004). Long-term treatment with a Rho-kinase inhibitor improves monocrotaline-induced fatal pulmonary hypertension in rats. *Circ.Res.* **94**, 385-393.

Affolter, J. T., Newby, D. E., Wilkinson, I. B., Winter, M. J., Balment, R. J., & Webb, D. J. (2002). No effect on central or peripheral blood pressure of systemic urotensin II infusion in humans. *Br.J.Clin.Pharmacol.* **54**, 617-621.

Akopov, S. E., Zhang, L., & Pearce, W. J. (1997). Physiological variations in ovine cerebrovascular Ca^{2+} sensitivity. *Am.J.Physiol* **272**, H2271-H2281.

Akopov, S. E., Zhang, L., & Pearce, W. J. (1998). Regulation of Ca^{2+} sensitization by PKC and rho proteins in ovine cerebral arteries: effects of artery size and age. *Am.J.Physiol* **275**, H930-H939.

Allbritton, N. L., Oancea, E., Kuhn, M. A., & Meyer, T. (1994). Source of nuclear Ca^{2+} signals. *Proc.Natl.Acad.Sci.U.S.A* **91**, 12458-12462.

Ames, R. S., Sarau, H. M., Chambers, J. K., Willette, R. N., Aiyar, N. V., Romanic, A. M., Louden, C. S., Foley, J. J., Sauermelch, C. F., Coatney, R. W., Ao, Z., Disa, J., Holmes, S. D., Stadel, J. M., Martin, J. D., Liu, W. S., Glover, G. I., Wilson, S., McNulty, D. E., Ellis, C. E., Elshourbagy, N. A., Shabon, U., Trill, J. J., Hay, D. W., Douglas, S. A., & . (1999). Human urotensin-II is a potent vasoconstrictor and agonist for the orphan receptor GPR14. *Nature* **401**, 282-286.

Arner, A. & Hellstrand, P. (1985). Effects of Ca^{2+}calcium and substrate on force-velocity relation and energy turnover in skinned smooth muscle of the guinea-pig. *J.Physiol* **360**, 347-365.

Arner, A. & Pfitzer, G. (1999). Regulation of cross-bridge cycling by Ca^{2+} in smooth muscle. *Rev.Physiol Biochem.Pharmacol.* **134**, 63-146.

Barnard, J. W., Wilson, P. S., Moore, T. M., Thompson, W. J., & Taylor, A. E. (1993). Effect of nitric oxide and cyclooxygenase products on vascular resistance in dog and rat lungs. *J.Appl.Physiol* **74**, 2940-2948.

Barnes, P. J., Baraniuk, J. N., & Belvisi, M. G. (1991). Neuropeptides in the respiratory tract. Part I. *Am.Rev.Respir.Dis.* **144**, 1187-1198.

Barnes, P. J. & Liu, S. F. (1995). Regulation of pulmonary vascular tone. *Pharmacol.Rev.* **47**, 87-131.

Behm, D. J., Doe, C. P., Johns, D. G., Maniscalco, K., Stankus, G. P., Wibberley, A., Willette, R. N., & Douglas, S. A. (2004). Urotensin-II: a novel systemic hypertensive factor in the cat. *Naunyn Schmiedebergs Arch.Pharmacol.* **369**, 274-280.

Bennett, R. T., Jones, R. D., Morice, A. H., Smith, C. F., & Cowen, M. E. (2004). Vasoconstrictive effects of endothelin-1, endothelin-3, and urotensin II in isolated perfused human lungs and isolated human pulmonary arteries. *Thorax* **59**, 401-407.

Bennie, R. E., Packer, C. S., Powell, D. R., Jin, N., & Rhoades, R. A. (1991). Biphasic contractile response of pulmonary artery to hypoxia. *Am.J.Physiol* **261**, L156-L163.

Bern, H. A. & Lederis, K. (1969). A reference preparation for the study of active substances in the caudal neurosecretory system of teleosts. *J.Endocrinol.* **45**, Suppl-Suxii.

Bern, H. A., Pearson, D., Larson, B. A., & Nishioka, R. S. (1985). Neurohormones from fish tails: the caudal neurosecretory system. I. "Urophysiology" and the caudal neurosecretory system of fishes. *Recent Prog.Horm.Res.* **41**, 533-552.

Berne and Levy (1998). *Physiology*, 4th ed. Mosby.

Berridge, M. J. (1993). Inositol trisphosphate and Ca^{2+} signalling. *Nature* **361**, 315-325.

Bhargava, A., Kumar, A., Yuan, N., Gewitz, M. H., & Mathew, R. (1999). Monocrotaline induces interleukin-6 mRNA expression in rat lungs. *Heart Dis.* **1**, 126-132.

Bialecki, R. A., Fisher, C. S., Murdoch, W. W., & Barthlow, H. G. (1998a). Chronic hypoxia increases staurosporine sensitivity of pulmonary artery smooth muscle to endothelin-1. *Pulm.Pharmacol.Ther.* **11**, 159-163.

Bialecki, R. A., Fisher, C. S., Murdoch, W. W., Barthlow, H. G., Stow, R. B., Mallamaci, M., & Rumsey, W. (1998b). Hypoxic exposure time dependently modulates endothelin-induced contraction of pulmonary artery smooth muscle. *Am.J.Physiol* **274**, L552-L559.

Birnbaumer, L., Zhu, X., Jiang, M., Boulay, G., Peyton, M., Vannier, B., Brown, D., Platano, D., Sadeghi, H., Stefani, E., & Birnbaumer, M. (1996). On the molecular basis and regulation of cellular capacitative Ca^{2+} entry: roles for Trp proteins. *Proc.Natl.Acad.Sci.U.S.A* **93**, 15195-15202.

Bivalacqua, T. J., Champion, H. C., Usta, M. F., Cellek, S., Chitaley, K., Webb, R. C., Lewis, R. L., Mills, T. M., Hellstrom, W. J., & Kadowitz, P. J. (2004). RhoA/Rho-kinase suppresses endothelial nitric oxide synthase in the penis: a mechanism for diabetes-associated erectile dysfunction. *Proc.Natl.Acad.Sci.U.S.A* **101**, 9121-9126.

Blaustein, M. P. (1993). Physiological effects of endogenous ouabain: control of intracellular Ca^{2+} stores and cell responsiveness. *Am.J.Physiol* **264**, C1367-C1387.

Blaustein, M. P. & Lederer, W. J. (1999). Sodium/calcium exchange: its physiological implications. *Physiol Rev.* **79**, 763-854.

Bloom and Fawcett (1975). *A textbook of histology*, 10th ed. Saunders.

Boe, J., Boe, M. A., Simonsson, B. G., & Stahl, E. (1980). In vitro effects of parasympathetic agonists and atropine on human segmental pulmonary arteries. *Lung* **157**, 65-70.

Boe, J. & Simonsson, B. G. (1980). Adrenergic receptors and sympathetic agents in isolated human pulmonary arteries. *Eur.J.Respir.Dis.* **61**, 195-202.

Boe, J. & Simonsson, B. G. (1981). Effects of angiotensin II and bradykinin on isolated human pulmonary arteries. *Eur.J.Respir.Dis.* **62**, 95-101.

Bogdanov, Y. D., Wildman, S. S., Clements, M. P., King, B. F., & Burnstock, G. (1998). Molecular cloning and characterization of rat $P2Y_4$ nucleotide receptor. *Br.J.Pharmacol.* **124**, 428-430.

Bohm, F. & Pernow, J. (2002). Urotensin II evokes potent vasoconstriction in humans in vivo. *Br.J.Pharmacol.* **135**, 25-27.

Bolz, S. S., Vogel, L., Sollinger, D., Derwand, R., de Wit, C., Loirand, G., & Pohl, U. (2003). Nitric oxide-induced decrease in Ca^{2+} sensitivity of resistance arteries is attributable to activation of the myosin light chain phosphatase and antagonized by the RhoA/Rho kinase pathway. *Circulation* **107**, 3081-3087.

Bonnet, S., Belus, A., Hyvelin, J. M., Roux, E., Marthan, R., & Savineau, J. P. (2001a). Effect of chronic hypoxia on agonist-induced tone and Ca^{2+} signaling in rat pulmonary artery. *Am.J.Physiol Lung Cell Mol.Physiol* **281**, L193-L201.

Bonnet, S., Hyvelin, J. M., Bonnet, P., Marthan, R., & Savineau, J. P. (2001b). Chronic hypoxia-induced spontaneous and rhythmic contractions in the rat main pulmonary artery. *Am.J.Physiol Lung Cell Mol.Physiol* **281**, L183-L192.

Bortner, C. D. & Cidlowski, J. A. (1999). Caspase independent/dependent regulation of K^+, cell shrinkage, and mitochondrial membrane potential during lymphocyte apoptosis. *J.Biol.Chem.* **274**, 21953-21962.

Bottrill, F. E., Douglas, S. A., Hiley, C. R., & White, R. (2000). Human urotensin-II is an endothelium-dependent vasodilator in rat small arteries. *Br.J.Pharmacol.* **130**, 1865-1870.

Boulay, G., Brown, D. M., Qin, N., Jiang, M., Dietrich, A., Zhu, M. X., Chen, Z., Birnbaumer, M., Mikoshiba, K., & Birnbaumer, L. (1999). Modulation of Ca^{2+} entry by polypeptides of the inositol 1,4, 5-trisphosphate receptor (IP_3R) that bind transient receptor potential (TRP): evidence for roles of TRP and IP_3R in store depletion-activated Ca^{2+} entry. *Proc.Natl.Acad.Sci.U.S.A* **96**, 14955-14960.

Bradley, D. E., McNary, W. F., & el Bermani, A. W. (1970). The distribution of acetylcholinesterase and catecholamine containg nerves in the rat lung. *Anat.Rec.* **167**, 205-207.

Brkovic, A., Hattenberger, A., Kostenis, E., Klabunde, T., Flohr, S., Kurz, M., Bourgault, S., & Fournier, A. (2003). Functional and binding characterizations of urotensin II-related peptides in human and rat urotensin II-receptor assay. *J.Pharmacol.Exp.Ther.* **306**, 1200-1209.

Budzyn, K., Marley, P. D., & Sobey, C. G. (2004). Chronic mevastatin modulates receptor-dependent vascular contraction in eNOS-deficient mice. *Am.J.Physiol Regul.Integr.Comp Physiol* **287**, R342-R348.

Buzzard, C. J., Pfister, S. L., & Campbell, W. B. (1993). Endothelium-dependent contractions in rabbit pulmonary artery are mediated by thromboxane A2. *Circ.Res.* **72**, 1023-1034.

Caldwell, R. W. & Blatteis, C. M. (1983). Effect of chronic hypoxia on angiotensin-induced pulmonary vasoconstriction and converting enzyme activity in the rat. *Proc.Soc.Exp.Biol.Med.* **172**, 346-350.

Carville, C., Raffestin, B., Eddahibi, S., Blouquit, Y., & Adnot, S. (1993). Loss of endothelium-dependent relaxation in proximal pulmonary arteries from rats exposed to chronic hypoxia: effects of in vivo and in vitro supplementation with L-arginine. *J.Cardiovasc.Pharmacol.* **22**, 889-896.

Cech, S. (1969). Adrenergic innervation of blood vessels in the lung of some mammals. *Acta Anat.(Basel)* **74**, 169-182.

Cech, S. (1973). Cholinesterase-containing nerve fibres on blood vessels in lungs of some laboratory mammals. *Z Zellforsch.Mikrosk.Anat.* **140**, 91-100.

Celermajer, D. S., Dollery, C., Burch, M., & Deanfield, J. E. (1994). Role of endothelium in the maintenance of low pulmonary vascular tone in normal children. *Circulation* **89**, 2041-2044.

Chartrel, N., Leprince, J., Dujardin, C., Chatenet, D., Tollemer, H., Baroncini, M., Balment, R. J., Beauvillain, J. C., & Vaudry, H. (2004). Biochemical characterization and immunohistochemical localization of urotensin II in the human brainstem and spinal cord. *J.Neurochem.* **91**, 110-118.

Chassagne, C., Eddahibi, S., Adamy, C., Rideau, D., Marotte, F., Dubois-Rande, J. L., Adnot, S., Samuel, J. L., & Teiger, E. (2000). Modulation of angiotensin II receptor expression during development and regression of hypoxic pulmonary hypertension. *Am.J.Respir.Cell Mol.Biol.* **22**, 323-332.

Cherry, P. D., Furchgott, R. F., Zawadzki, J. V., & Jothianandan, D. (1982). Role of endothelial cells in relaxation of isolated arteries by bradykinin. *Proc.Natl.Acad.Sci.U.S.A* **79**, 2106-2110.

Cherry, P. D. & Gillis, C. N. (1987). Evidence for the role of endothelium-derived relaxing factor in acetylcholine-induced vasodilatation in the intact lung. *J.Pharmacol.Exp.Ther.* **241**, 516-520.

Chootip, K., Ness, K. F., Wang, Y., Gurney, A. M., & Kennedy, C. (2002). Regional variation in P2 receptor expression in the rat pulmonary arterial circulation. *Br.J.Pharmacol.* **137**, 637-646.

Christman, B. W., McPherson, C. D., Newman, J. H., King, G. A., Bernard, G. R., Groves, B. M., & Loyd, J. E. (1992). An imbalance between the excretion of thromboxane and prostacyclin metabolites in pulmonary hypertension. *N.Engl.J.Med.* **327**, 70-75.

Communi, D., Robaye, B., & Boeynaems, J. M. (1999). Pharmacological characterization of the human P2Y11 receptor. *Br.J.Pharmacol.* **128**, 1199-1206.

Conlon, J. M., O'Harte, F., Smith, D. D., Tonon, M. C., & Vaudry, H. (1992). Isolation and primary structure of urotensin II from the brain of a tetrapod, the frog Rana ridibunda. *Biochem.Biophys.Res.Commun.* **188**, 578-583.

Conti, M. A. & Adelstein, R. S. (1981). The relationship between calmodulin binding and phosphorylation of smooth muscle myosin kinase by the catalytic subunit of 3':5' cAMP-dependent protein kinase. *J.Biol.Chem.* **256**, 3178-3181.

Cool, C. D., Rai, P. R., Yeager, M. E., Hernandez-Saavedra, D., Serls, A. E., Bull, T. M., Geraci, M. W., Brown, K. K., Routes, J. M., Tuder, R. M., & Voelkel, N. F. (2003). Expression of human herpesvirus 8 in primary pulmonary hypertension. *N.Engl.J.Med.* **349**, 1113-1122.

Coulouarn, Y., Fernex, C., Jegou, S., Henderson, C. E., Vaudry, H., & Lihrmann, I. (2001). Specific expression of the urotensin II gene in sacral motoneurons of developing rat spinal cord. *Mech.Dev.* **101**, 187-190.

Coulouarn, Y., Jegou, S., Tostivint, H., Vaudry, H., & Lihrmann, I. (1999). Cloning, sequence analysis and tissue distribution of the mouse and rat urotensin II precursors. *FEBS Lett.* **457**, 28-32.

Coulouarn, Y., Lihrmann, I., Jegou, S., Anouar, Y., Tostivint, H., Beauvillain, J. C., Conlon, J. M., Bern, H. A., & Vaudry, H. (1998). Cloning of the cDNA encoding the urotensin II precursor in frog and human reveals intense expression of the urotensin II gene in motoneurons of the spinal cord. *Proc.Natl.Acad.Sci.U.S.A* **95**, 15803-15808.

Cowan, K. N., Heilbut, A., Humpl, T., Lam, C., Ito, S., & Rabinovitch, M. (2000). Complete reversal of fatal pulmonary hypertension in rats by a serine elastase inhibitor. *Nat.Med.* **6**, 698-702.

Cowan, K. N., Jones, P. L., & Rabinovitch, M. (1999). Regression of hypertrophied rat pulmonary arteries in organ culture is associated with suppression of proteolytic activity, inhibition of tenascin-C, and smooth muscle cell apoptosis. *Circ.Res.* **84**, 1223-1233.

Cruickshank, S. F., Baxter, L. M., & Drummond, R. M. (2003). The Cl^- channel blocker niflumic acid releases Ca^{2+} from an intracellular store in rat pulmonary artery smooth muscle cells. *Br.J.Pharmacol.* **140**, 1442-1450.

D'Alonzo, G. E., Barst, R. J., Ayres, S. M., Bergofsky, E. H., Brundage, B. H., Detre, K. M., Fishman, A. P., Goldring, R. M., Groves, B. M., & Kernis, J. T. (1991). Survival in patients with primary pulmonary hypertension. Results from a national prospective registry. *Ann.Intern.Med.* **115**, 343-349.

Daly and Hebb (1966). *Pulmonary and bronchial vascular system* William and Wilkins.

Davidson, D. & Eldemerdash, A. (1991). Endothelium-derived relaxing factor: evidence that it regulates pulmonary vascular resistance in the isolated neonatal guinea pig lung. *Pediatr.Res.* **29**, 538-542.

Dawson, C. A., Grimm, D. J., & Linehan, J. H. (1979). Lung inflation and longitudinal distribution of pulmonary vascular resistance during hypoxia. *J.Appl.Physiol* **47**, 532-536.

Demiryurek, A. T., Wadsworth, R. M., & Kane, K. A. (1991). Effects of hypoxia on isolated intrapulmonary arteries from the sheep. *Pulm.Pharmacol.* **4**, 158-164.

Dempsey, E. C., Frid, M. G., Aldashev, A. A., Das, M., & Stenmark, K. R. (1997). Heterogeneity in the proliferative response of bovine pulmonary artery smooth muscle cells to mitogens and hypoxia: importance of protein kinase C. *Can.J.Physiol Pharmacol.* **75**, 936-944.

Deuchar, G. A., Stirrat, S., Herold, N., Morecroft, I., Dempsie, Y., Nilsen, M., Hicks, M. N., & MacLean, M. R. (2005). Effects of human urotensin II in the rat, rabbit and human pulmonary circulation: efects of chronic hypoxia. *Submitted for publication 2005.*

Dipp, M., Nye, P. C., & Evans, A. M. (2001). Hypoxic release of Ca^{2+} from the sarcoplasmic reticulum of pulmonary artery smooth muscle. *Am.J.Physiol Lung Cell Mol.Physiol* **281**, L318-L325.

Docherty, J. R. (1988). No evidence for more than one type of α_1-adrenoreceptor in rabbit pulmonary artery. *J.Auton.Pharmacol.* **8**, 327-332.

Douglas, S. A. & Ohlstein, E. H. (2000). Human urotensin-II, the most potent mammalian vasoconstrictor identified to date, as a therapeutic target for the management of cardiovascular disease. *Trends Cardiovasc.Med.* **10**, 229-237.

Douglas, S. A., Sulpizio, A. C., Piercy, V., Sarau, H. M., Ames, R. S., Aiyar, N. V., Ohlstein, E. H., & Willette, R. N. (2000). Differential vasoconstrictor activity of human urotensin-II in vascular tissue isolated from the rat, mouse, dog, pig, marmoset and cynomolgus monkey. *Br.J.Pharmacol.* **131**, 1262-1274.

Douglas, S. A., Tayara, L., Ohlstein, E. H., Halawa, N., & Giaid, A. (2002). Congestive heart failure and expression of myocardial urotensin II. *Lancet* **359**, 1990-1997.

Dschietzig, T., Bartsch, C., Pregla, R., Zurbrugg, H. R., Armbruster, F. P., Richter, C., Laule, M., Romeyke, E., Neubert, C., Voelter, W., Baumann, G., & Stangl, K. (2002). Plasma levels and cardiovascular gene expression of urotensin-II in human heart failure. *Regul.Pept.* **110**, 33-38.

Dubyak, G. R. & el Moatassim, C. (1993). Signal transduction via P2-purinergic receptors for extracellular ATP and other nucleotides. *Am.J.Physiol* **265**, C577-C606.

Duke, H. N. (1954). The site of action of anoxia on the pulmonary blood vessels of the cat. *J.Physiol* **125**, 373-382.

Eddahibi, S., Adnot, S., Frisdal, E., Levame, M., Hamon, M., & Raffestin, B. (2001a). Dexfenfluramine-associated changes in 5-hydroxytryptamine transporter expression and development of hypoxic pulmonary hypertension in rats. *J.Pharmacol.Exp.Ther.* **297**, 148-154.

Eddahibi, S., Fabre, V., Boni, C., Martres, M. P., Raffestin, B., Hamon, M., & Adnot, S. (1999). Induction of serotonin transporter by hypoxia in pulmonary vascular smooth muscle cells. Relationship with the mitogenic action of serotonin. *Circ.Res.* **84**, 329-336.

Eddahibi, S., Hanoun, N., Lanfumey, L., Lesch, K. P., Raffestin, B., Hamon, M., & Adnot, S. (2000). Attenuated hypoxic pulmonary hypertension in mice lacking the 5-hydroxytryptamine transporter gene. *J.Clin.Invest* **105**, 1555-1562.

Eddahibi, S., Humbert, M., Fadel, E., Raffestin, B., Darmon, M., Capron, F., Simonneau, G., Dartevelle, P., Hamon, M., & Adnot, S. (2001b). Serotonin transporter overexpression is responsible for pulmonary artery smooth muscle hyperplasia in primary pulmonary hypertension. *J.Clin.Invest* **108**, 1141-1150.

Eddahibi, S., Raffestin, B., Pham, I., Launay, J. M., Aegerter, P., Sitbon, M., & Adnot, S. (1997). Treatment with 5-HT potentiates development of pulmonary hypertension in chronically hypoxic rats. *Am.J.Physiol* **272**, H1173-H1181.

Eddahibi, S., Springall, D., Mannan, M., Carville, C., Chabrier, P. E., Levame, M., Raffestin, B., Polak, J., & Adnot, S. (1993). Dilator effect of endothelins in pulmonary circulation: changes associated with chronic hypoxia. *Am.J.Physiol* **265**, L571-L580.

Ekhterae, D., Platoshyn, O., Krick, S., Yu, Y., McDaniel, S. S., & Yuan, J. X. (2001). Bcl-2 decreases voltage-gated K^+ channel activity and enhances survival in vascular smooth muscle cells. *Am.J.Physiol Cell Physiol* **281**, C157-C165.

Emery, C. J., Bee, D., & Barer, G. R. (1981). Mechanical properties and reactivity of vessels in isolated perfused lungs of chronically hypoxic rats. *Clin.Sci.(Lond)* **61**, 569-580.

Eto, M., Kitazawa, T., Yazawa, M., Mukai, H., Ono, Y., & Brautigan, D. L. (2001). Histamine-induced vasoconstriction involves phosphorylation of a specific inhibitor protein for myosin phosphatase by protein kinase C α and δ isoforms. *J.Biol.Chem.* **276**, 29072-29078.

Eto, M., Kozai, T., Cosentino, F., Joch, H., & Luscher, T. F. (2002). Statin prevents tissue factor expression in human endothelial cells: role of Rho/Rho-kinase and Akt pathways. *Circulation* **105**, 1756-1759.

Evans, A. M., Cobban, H. J., & Nixon, G. F. (1999). ET(A) receptors are the primary mediators of myofilament Ca^{2+} sensitization induced by ET-1 in rat pulmonary artery smooth muscle: a tyrosine kinase independent pathway. *Br.J.Pharmacol.* **127**, 153-160.

Fagan, K. A., Oka, M., Bauer, N. R., Gebb, S. A., Ivy, D. D., Morris, K. G., & McMurtry, I. F. (2004). Attenuation of acute hypoxic pulmonary vasoconstriction and hypoxic pulmonary hypertension in mice by inhibition of Rho-kinase. *Am.J.Physiol Lung Cell Mol.Physiol* **287**, L656-L664.

Fantozzi, I., Zhang, S., Platoshyn, O., Remillard, C. V., Cowling, R. T., & Yuan, J. X. (2003). Hypoxia increases AP-1 binding activity by enhancing capacitative Ca^{2+} entry in human pulmonary artery endothelial cells. *Am.J.Physiol Lung Cell Mol.Physiol* **285**, L1233-L1245.

Feng, J., Ito, M., Kureishi, Y., Ichikawa, K., Amano, M., Isaka, N., Okawa, K., Iwamatsu, A., Kaibuchi, K., Hartshorne, D. J., & Nakano, T. (1999). Rho-associated kinase of chicken gizzard smooth muscle. *J.Biol.Chem.* **274**, 3744-3752.

Fineman, J. R., Heymann, M. A., & Soifer, S. J. (1991). N omega-nitro-L-arginine attenuates endothelium-dependent pulmonary vasodilation in lambs. *Am.J.Physiol* **260**, H1299-H1306.

Finlay, M., Barer, G. R., & Suggett, A. J. (1986). Quantitative changes in the rat pulmonary vasculature in chronic hypoxia--relation to haemodynamic changes. *Q.J.Exp.Physiol* **71**, 151-163.

Fishman and Hecht (1969). *The pulmonary circulation and interstitial space* The University of Chicago press.

Fishman, A. P. (1961). Respiratory gases in the regulation of the pulmonary circulation. *Physiol Rev.* **41**, 214-280.

Fishman, A. P. (1976). Hypoxia on the pulmonary circulation. How and where it acts. *Circ.Res.* **38**, 221-231.

Fishman, A. P. (1998). Etiology and pathogenesis of primary pulmonary hypertension: a perspective. *Chest* **114**, 242S-247S.

Fishman, A. P. (1999). Aminorex to fen/phen: an epidemic foretold. *Circulation* **99**, 156-161.

Frangos, J. A., Eskin, S. G., McIntire, L. V., & Ives, C. L. (1985). Flow effects on prostacyclin production by cultured human endothelial cells. *Science* **227**, 1477-1479.

Frank, H., Mlczoch, J., Huber, K., Schuster, E., Gurtner, H. P., & Kneussl, M. (1997). The effect of anticoagulant therapy in primary and anorectic drug-induced pulmonary hypertension. *Chest* **112**, 714-721.

Frid, M. G., Aldashev, A. A., Dempsey, E. C., & Stenmark, K. R. (1997). Smooth muscle cells isolated from discrete compartments of the mature vascular media exhibit unique phenotypes and distinct growth capabilities. *Circ.Res.* **81**, 940-952.

Fritts, H. W., Jr., Harris, P., Clauss, R. H., Odell, J. E., & Cournarnd, A. (1958). The effect of acetylcholine on the human pulmonary circulation under normal and hypoxic conditions. *J.Clin.Invest* **37**, 99-110.

Fu, X., Gong, M. C., Jia, T., Somlyo, A. V., & Somlyo, A. P. (1998). The effects of the Rho-kinase inhibitor Y-27632 on arachidonic acid-, GTPγS-, and phorbol ester-induced Ca^{2+}-sensitization of smooth muscle. *FEBS Lett.* **440**, 183-187.

Fujita, A., Takeuchi, T., Nakajima, H., Nishio, H., & Hata, F. (1995). Involvement of heterotrimeric GTP-binding protein and rho protein, but not protein kinase C, in agonist-induced Ca^{2+} sensitization of skinned muscle of guinea pig vas deferens. *J.Pharmacol.Exp.Ther.* **274**, 555-561.

Furchgott, R. F., Cherry, P. D., Zawadzki, J. V., & Jothianandan, D. (1984). Endothelial cells as mediators of vasodilation of arteries. *J.Cardiovasc.Pharmacol.* **6 Suppl 2**, S336-S343.

Furchgott, R. F. & Vanhoutte, P. M. (1989). Endothelium-derived relaxing and contracting factors. *FASEB J.* **3**, 2007-2018.

Furchgott, R. F. & Zawadzki, J. V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* **288**, 373-376.

Gailly, P., Gong, M. C., Somlyo, A. V., & Somlyo, A. P. (1997). Possible role of atypical protein kinase C activated by arachidonic acid in Ca^{2+} sensitization of rabbit smooth muscle. *J.Physiol* **500 (Pt 1)**, 95-109.

Ganitkevich, V. Y. & Isenberg, G. (1993). Membrane potential modulates inositol 1,4,5-trisphosphate-mediated Ca^{2+} transients in guinea-pig coronary myocytes. *J.Physiol* **470**, 35-44.

Garattini, S., Mennini, T., & Samanin, R. (1989). Reduction of food intake by manipulation of central serotonin. Current experimental results. *Br.J.Psychiatry Suppl* 41-51.

Gardiner, S. M., March, J. E., Kemp, P. A., & Bennett, T. (2004). Bolus injection of human UII in conscious rats evokes a biphasic haemodynamic response. *Br.J.Pharmacol.* **143**, 422-430.

Gardiner, S. M., March, J. E., Kemp, P. A., Davenport, A. P., & Bennett, T. (2001). Depressor and regionally-selective vasodilator effects of human and rat urotensin II in conscious rats. *Br.J.Pharmacol.* **132**, 1625-1629.

Gartlon, J., Parker, F., Harrison, D. C., Douglas, S. A., Ashmeade, T. E., Riley, G. J., Hughes, Z. A., Taylor, S. G., Munton, R. P., Hagan, J. J., Hunter, J. A., & Jones, D. N. (2001). Central effects of urotensin-II following ICV administration in rats. *Psychopharmacology (Berl)* **155**, 426-433.

Gelband, C. H. & Gelband, H. (1997). Ca^{2+} release from intracellular stores is an initial step in hypoxic pulmonary vasoconstriction of rat pulmonary artery resistance vessels. *Circulation* **96**, 3647-3654.

Geraci, M. W., Moore, M., Gesell, T., Yeager, M. E., Alger, L., Golpon, H., Gao, B., Loyd, J. E., Tuder, R. M., & Voelkel, N. F. (2001). Gene expression patterns in the lungs of patients with primary pulmonary hypertension: a gene microarray analysis. *Circ.Res.* **88**, 555-562.

Ghisdal, P., Vandenberg, G., & Morel, N. (2003). Rho-dependent kinase is involved in agonist-activated Ca^{2+} entry in rat arteries. *J.Physiol* **551**, 855-867.

Giaid, A. (1998). Nitric oxide and endothelin-1 in pulmonary hypertension. *Chest* **114**, 208S-212S.

Giaid, A. & Saleh, D. (1995). Reduced expression of endothelial nitric oxide synthase in the lungs of patients with pulmonary hypertension. *N.Engl.J.Med.* **333**, 214-221.

Giaid, A., Yanagisawa, M., Langleben, D., Michel, R. P., Levy, R., Shennib, H., Kimura, S., Masaki, T., Duguid, W. P., & Stewart, D. J. (1993). Expression of endothelin-1 in the lungs of patients with pulmonary hypertension. *N.Engl.J.Med.* **328**, 1732-1739.

Gibson, A. (1987). Complex effects of Gillichthys urotensin II on rat aortic strips. *Br.J.Pharmacol.* **91**, 205-212.

Gibson, A., Wallace, P., & Bern, H. A. (1986). Cardiovascular effects of urotensin II in anesthetized and pithed rats. *Gen.Comp Endocrinol.* **64**, 435-439.

Girgis, R. E., Li, D., Zhan, X., Garcia, J. G., Tuder, R. M., Hassoun, P. M., & Johns, R. A. (2003). Attenuation of chronic hypoxic pulmonary hypertension by simvastatin. *Am.J.Physiol Heart Circ.Physiol* **285**, H938-H945.

Glusa, E. & Richter, M. (1993). Endothelium-dependent relaxation of porcine pulmonary arteries via 5-HT_{1C}-like receptors. *Naunyn Schmiedebergs Arch.Pharmacol.* **347**, 471-477.

Golovina, V. A., Platoshyn, O., Bailey, C. L., Wang, J., Limsuwan, A., Sweeney, M., Rubin, L. J., & Yuan, J. X. (2001). Upregulated TRP and enhanced capacitative Ca^{2+} entry in human pulmonary artery myocytes during proliferation. *Am.J.Physiol Heart Circ.Physiol* **280**, H1746-H1755.

Greenberg, B., Rhoden, K., & Barnes, P. J. (1987). Endothelium-dependent relaxation of human pulmonary arteries. *Am.J.Physiol* **252**, H434-H438.

Gurney, A. M. & Allam, M. (1995). Inhibition of Ca^{2+} release from the sarcoplasmic reticulum of rabbit aorta by hydralazine. *Br.J.Pharmacol.* **114**, 238-244.

Hampl, V. (2000). [Nitric oxide and regulation of pulmonary vessels]. *Cesk.Fysiol.* **49**, 22-29.

Harden, T. K., Boyer, J. L., & Nicholas, R. A. (1995). P2-purinergic receptors: subtype-associated signaling responses and structure. *Annu.Rev.Pharmacol.Toxicol.* **35**, 541-579.

Harder, D. R., Madden, J. A., & Dawson, C. (1985). A membrane electrical mechanism for hypoxic vasoconstriction of small pulmonary arteries from cat. *Chest* **88**, 233S-235S.

Hardingham, G. E., Chawla, S., Johnson, C. M., & Bading, H. (1997). Distinct functions of nuclear and cytoplasmic Ca^{2+} in the control of gene expression. *Nature* **385**, 260-265.

Hauge, A. (1968a). Conditions governing the pressor response to ventilation hypoxia in isolated perfused rat lungs. *Acta Physiol Scand.* **72**, 33-44.

Hauge, A. (1968b). Role of histamine in hypoxic pulmonary hypertension in the rat. I. Blockade or potentiation of endogenous amines, kinins, and ATP. *Circ.Res.* **22**, 371-383.

Hauge, A. & Melmon, K. L. (1968). Role of histamine in hypoxic pulmonary hypertension in the rat. II. Depletion of histamine, serotonin, and catecholamines. *Circ.Res.* **22**, 385-392.

Hay, D. W., Luttmann, M. A., & Douglas, S. A. (2000). Human urotensin-II is a potent spasmogen of primate airway smooth muscle. *Br.J.Pharmacol.* **131**, 10-12.

Herget, J., Suggett, A. J., Leach, E., & Barer, G. R. (1978). Resolution of pulmonary hypertension and other features induced by chronic hypoxia in rats during complete and intermittent normoxia. *Thorax* **33**, 468-473.

Herve, P., Drouet, L., Dosquet, C., Launay, J. M., Rain, B., Simonneau, G., Caen, J., & Duroux, P. (1990). Primary pulmonary hypertension in a patient with a familial platelet storage pool disease: role of serotonin. *Am.J.Med.* **89**, 117-120.

Herve, P., Launay, J. M., Scrobohaci, M. L., Brenot, F., Simonneau, G., Petitpretz, P., Poubeau, P., Cerrina, J., Duroux, P., & Drouet, L. (1995). Increased plasma serotonin in primary pulmonary hypertension. *Am.J.Med.* **99**, 249-254.

Hill, N. S. & Ou, L. C. (1984). The role of pulmonary vascular responses to chronic hypoxia in the development of chronic mountain sickness in rats. *Respir.Physiol* **58**, 171-185.

Hillier, C., Berry, C., Petrie, M. C., O'Dwyer, P. J., Hamilton, C., Brown, A., & McMurray, J. (2001). Effects of urotensin II in human arteries and veins of varying caliber. *Circulation* **103**, 1378-1381.

Himpens, B., Kitazawa, T., & Somlyo, A. P. (1990). Agonist-dependent modulation of Ca^{2+} sensitivity in rabbit pulmonary artery smooth muscle. *Pflugers Arch.* **417**, 21-28.

Hishikawa, K., Nakaki, T., Marumo, T., Hayashi, M., Suzuki, H., Kato, R., & Saruta, T. (1994). Pressure promotes DNA synthesis in rat cultured vascular smooth muscle cells. *J.Clin.Invest* **93**, 1975-1980.

Hislop, A. & Reid, L. (1976). New findings in pulmonary arteries of rats with hypoxia-induced pulmonary hypertension. *Br.J.Exp.Pathol.* **57**, 542-554.

Holden, W. E. & McCall, E. (1984). Hypoxia-induced contractions of porcine pulmonary artery strips depend on intact endothelium. *Exp.Lung Res.* **7**, 101-112.

Horiuti, K. (1986). Some properties of the contractile system and sarcoplasmic reticulum of skinned slow fibres from Xenopus muscle. *J.Physiol* **373**, 1-23.

Hoshikawa, Y., Nana-Sinkam, P., Moore, M. D., Sotto-Santiago, S., Phang, T., Keith, R. L., Morris, K. G., Kondo, T., Tuder, R. M., Voelkel, N. F., & Geraci, M. W. (2003). Hypoxia induces different genes in the lungs of rats compared with mice. *Physiol Genomics* **12**, 209-219.

Hussain, M. B. & Marshall, I. (1997). Characterization of α_1-adrenoceptor subtypes mediating contractions to phenylephrine in rat thoracic aorta, mesenteric artery and pulmonary artery. *Br.J.Pharmacol.* **122**, 849-858.

Hyman, A. L. & Kadowitz, P. J. (1979). Pulmonary vasodilator activity of prostacyclin (PGI_2) in the cat. *Circ.Res.* **45**, 404-409.

Hyman, A. L. & Kadowitz, P. J. (1988). Tone-dependent responses to acetylcholine in the feline pulmonary vascular bed. *J.Appl.Physiol* **64**, 2002-2009.

Hyman, A. L. & Kadowitz, P. J. (1989). Influence of tone on responses to acetylcholine in the rabbit pulmonary vascular bed. *J.Appl.Physiol* **67**, 1388-1394.

Hyman, A. L., Lippton, H. L., & Kadowitz, P. J. (1986). Nature of α_1 and postjunctional α_2 adrenoceptors in the pulmonary vascular bed. *Fed.Proc.* **45**, 2336-2340.

Hyman, A. L., Lippton, H. L., & Kadowitz, P. J. (1990). Analysis of pulmonary vascular responses in cats to sympathetic nerve stimulation under elevated tone conditions. Evidence that neuronally released norepinephrine acts on α_1,α_2, and β_2-adrenoceptors. *Circ.Res.* **67**, 862-870.

Hyman, A. L., Nandiwada, P., Knight, D. S., & Kadowitz, P. J. (1981). Pulmonary vasodilator responses to catecholamines and sympathetic nerve stimulation in the cat. Evidence that vascular β_2 adrenoreceptors are innervated. *Circ.Res.* **48**, 407-415.

Ignarro, L. J., Byrns, R. E., Buga, G. M., & Wood, K. S. (1987). Mechanisms of endothelium-dependent vascular smooth muscle relaxation elicited by bradykinin and VIP. *Am.J.Physiol* **253**, H1074-H1082.

Iizuka, K., Dobashi, K., Yoshii, A., Horie, T., Suzuki, H., Nakazawa, T., & Mori, M. (1997). Receptor-dependent G protein-mediated Ca^{2+} sensitization in canine airway smooth muscle. *Cell Calcium* **22**, 21-30.

Iizuka, K., Ikebe, M., Somlyo, A. V., & Somlyo, A. P. (1994). Introduction of high molecular weight (IgG) proteins into receptor coupled, permeabilized smooth muscle. *Cell Calcium* **16**, 431-445.

Iizuka, K., Yoshii, A., Samizo, K., Tsukagoshi, H., Ishizuka, T., Dobashi, K., Nakazawa, T., & Mori, M. (1999). A major role for the rho-associated coiled coil forming protein kinase in G-protein-mediated Ca^{2+} sensitization through inhibition of myosin phosphatase in rabbit trachea. *Br.J.Pharmacol.* **128**, 925-933.

Ingram, R. H., Jr., Szidon, J. P., & Fishman, A. P. (1970). Response of the main pulmonary artery of dogs to neuronally released versus blood-borne norepinephrine. *Circ.Res.* **26**, 249-262.

Ingram, R. H., Szidon, J. P., Skalak, R., & Fishman, A. P. (1968). Effects of sympathetic nerve stimulation on the pulmonary arterial tree of the isolated lobe perfused in situ. *Circ.Res.* **22**, 801-815.

Inoue, T. & Kannan, M. S. (1988). Nonadrenergic and noncholinergic excitatory neurotransmission in rat intrapulmonary artery. *Am.J.Physiol* **254**, H1142-H1148.

Itoh, H., Itoh, Y., Rivier, J., & Lederis, K. (1987). Contraction of major artery segments of rat by fish neuropeptide urotensin II. *Am.J.Physiol* **252**, R361-R366.

Itoh, H., McMaster, D., & Lederis, K. (1988). Functional receptors for fish neuropeptide urotensin II in major rat arteries. *Eur.J.Pharmacol.* **149**, 61-66.

Itoh, T., Kajikuri, J., & Kuriyama, H. (1992). Characteristic features of noradrenaline-induced Ca^{2+} mobilization and tension in arterial smooth muscle of the rabbit. *J.Physiol* **457**, 297-314.

Janssen, L. J., Lu-Chao, H., & Netherton, S. (2001a). Excitation-contraction coupling in pulmonary vascular smooth muscle involves tyrosine kinase and Rho kinase. *Am.J.Physiol Lung Cell Mol.Physiol* **280**, L666-L674.

Janssen, L. J., Premji, M., Netherton, S., Coruzzi, J., Lu-Chao, H., & Cox, P. G. (2001b). Vasoconstrictor actions of isoprostanes via tyrosine kinase and Rho kinase in human and canine pulmonary vascular smooth muscles. *Br.J.Pharmacol.* **132**, 127-134.

Jeffery, T. K., Bryan-Lluka, L. J., & Wanstall, J. C. (2000). Specific uptake of 5-hydroxytryptamine is reduced in lungs from hypoxic pulmonary hypertensive rats. *Eur.J.Pharmacol.* **396**, 137-140.

Jin, N., Packer, C. S., & Rhoades, R. A. (1992). Pulmonary arterial hypoxic contraction: signal transduction. *Am.J.Physiol* **263**, L73-L78.

Jones, P. L., Cowan, K. N., & Rabinovitch, M. (1997). Tenascin-C, proliferation and subendothelial fibronectin in progressive pulmonary vascular disease. *Am.J.Pathol.* **150**, 1349-1360.

Kadowitz, P. J. & Hyman, A. L. (1973). Effect of sympathetic nerve stimulation on pulmonary vascular resistance in the dog. *Circ.Res.* **32**, 221-227.

Kadowitz, P. J., Joiner, P. D., & Hyman, A. L. (1973). Differential effects of phentolamine and bretylium on pulmonary vascular responses to norepinephrine and nerve stimulation. *Proc.Soc.Exp.Biol.Med.* **144**, 172-176.

Kai, Y. (1969). [Study on the distribution of symatheic nerves in the lung using the Falck-Hillarp's fluorescent method]. *Bull.Chest Dis.Res.Inst.Kyoto Univ* **2**, 225-245.

Kamm, K. E. & Stull, J. T. (1985). The function of myosin and myosin light chain kinase phosphorylation in smooth muscle. *Annu.Rev.Pharmacol.Toxicol.* **25**, 593-620.

Kandabashi, T., Shimokawa, H., Mukai, Y., Matoba, T., Kunihiro, I., Morikawa, K., Ito, M., Takahashi, S., Kaibuchi, K., & Takeshita, A. (2002). Involvement of rho-kinase in agonists-induced contractions of arteriosclerotic human arteries. *Arterioscler.Thromb.Vasc.Biol.* **22**, 243-248.

Karaki, H., Sato, K., Ozaki, H., & Murakami, K. (1988). Effects of sodium nitroprusside on cytosolic Ca^{2+} level in vascular smooth muscle. *Eur.J.Pharmacol.* **156**, 259-266.

Karaki, H., Urakawa, N., & Kutsky, P. (1984). Potassium-induced contraction in smooth muscle. *Nippon Heikatsukin.Gakkai Zasshi* **20**, 427-444.

Kato, M. & Staub, N. C. (1966). Response of small pulmonary arteries to unilobar hypoxia and hypercapnia. *Circ.Res.* **19**, 426-440.

Kay, J. M., Smith, P., & Heath, D. (1971). Aminorex and the pulmonary circulation. *Thorax* **26**, 262-270.

Keegan, A., Morecroft, I., Smillie, D., Hicks, M. N., & MacLean, M. R. (2001). Contribution of the 5-HT_{1B} receptor to hypoxia-induced pulmonary hypertension: converging evidence using 5-HT_{1B}-receptor knockout mice and the 5-$HT_{1B/1D}$-receptor antagonist GR127935. *Circ.Res.* **89**, 1231-1239.

Kent, R. L., Sheldon, R. J., & Harakal, C. (1983). The effects of hyperosmolar solutions on isolated vascular smooth muscle examined with verapamil. *Pharmacology* **26**, 157-163.

Kerrick, W. G. & Hoar, P. E. (1981). Inhibition of smooth muscle tension by cyclic AMP-dependent protein kinase. *Nature* **292**, 253-255.

Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., Yamamori, B., Feng, J., Nakano, T., Okawa, K., Iwamatsu, A., & Kaibuchi, K. (1996). Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science* **273**, 245-248.

Kirchengast, M. & Munter, K. (1999). Endothelin-1 and endothelin receptor antagonists in cardiovascular remodeling. *Proc.Soc.Exp.Biol.Med.* **221**, 312-325.

Kitazawa, T., Eto, M., Woodsome, T. P., & Brautigan, D. L. (2000). Agonists trigger G protein-mediated activation of the CPI-17 inhibitor phosphoprotein of myosin light chain phosphatase to enhance vascular smooth muscle contractility. *J.Biol.Chem.* **275**, 9897-9900.

Kitazawa, T., Kobayashi, S., Horiuti, K., Somlyo, A. V., & Somlyo, A. P. (1989). Receptor-coupled, permeabilized smooth muscle. Role of the phosphatidylinositol cascade, G-proteins, and modulation of the contractile response to Ca^{2+}. *J.Biol.Chem.* **264**, 5339-5342.

Kitazawa, T., Polzin, A. N., & Eto, M. (2004). CPI-17-deficient smooth muscle of chicken. *J.Physiol* **557**, 515-528.

Kobayashi, S., Kitazawa, T., Somlyo, A. V., & Somlyo, A. P. (1989). Cytosolic heparin inhibits muscarinic and α-adrenergic Ca^{2+} release in smooth muscle. Physiological role of IP_3 in pharmacomechanical coupling. *J.Biol.Chem.* **264**, 17997-18004.

Komori, K. & Vanhoutte, P. M. (1990). Endothelium-derived hyperpolarizing factor. *Blood Vessels* **27**, 238-245.

Konishi, M. & Watanabe, M. (1995). Molecular size-dependent leakage of intracellular molecules from frog skeletal muscle fibers permeabilized with β-escin. *Pflugers Arch.* **429**, 598-600.

Kovitz, K. L., Aleskowitch, T. D., Sylvester, J. T., & Flavahan, N. A. (1993). Endothelium-derived contracting and relaxing factors contribute to hypoxic responses of pulmonary arteries. *Am.J.Physiol* **265**, H1139-H1148.

Krick, S., Platoshyn, O., Sweeney, M., Kim, H., & Yuan, J. X. (2001). Activation of K^+ channels induces apoptosis in vascular smooth muscle cells. *Am.J.Physiol Cell Physiol* **280**, C970-C979.

Krick, S., Platoshyn, O., Sweeney, M., McDaniel, S. S., Zhang, S., Rubin, L. J., & Yuan, J. X. (2002). Nitric oxide induces apoptosis by activating K^+ channels in pulmonary vascular smooth muscle cells. *Am.J.Physiol Heart Circ.Physiol* **282**, H184-H193.

Kukkonen, J. P., Lund, P. E., & Akerman, K. E. (2001). 2-aminoethoxydiphenyl borate reveals heterogeneity in receptor-activated Ca^{2+} discharge and store-operated Ca^{2+} influx. *Cell Calcium* **30**, 117-129.

Labarrere, P., Chatenet, D., Leprince, J., Marionneau, C., Loirand, G., Tonon, M. C., Dubessy, C., Scalbert, E., Pfeiffer, B., Renard, P., Calas, B., Pacaud, P., & Vaudry, H. (2003). Structure-activity relationships of human urotensin II and related analogues on rat aortic ring contraction. *J.Enzyme Inhib.Med.Chem.* **18**, 77-88.

Laffi, G., Marra, F., Gresele, P., Romagnoli, P., Palermo, A., Bartolini, O., Simoni, A., Orlandi, L., Selli, M. L., & Nenci, G. G. (1992). Evidence for a storage pool defect in platelets from cirrhotic patients with defective aggregation. *Gastroenterology* **103**, 641-646.

Laher, I., Thorin-Trescases, N., Ding, A., Laporte, R., & Osol, G. (1995). α-Toxin perfusion: a new method for selective impairment of endothelial function in isolated vessels or intact vascular beds. *Can.J.Physiol Pharmacol.* **73**, 1669-1673.

Lapp, H., Boerrigter, G., Costello-Boerrigter, L. C., Jaekel, K., Scheffold, T., Krakau, I., Schramm, M., Guelker, H., & Stasch, J. P. (2004). Elevated plasma human urotensin-II-like immunoreactivity in ischemic cardiomyopathy. *Int.J.Cardiol.* **94**, 93-97.

Laufs, U., Endres, M., Custodis, F., Gertz, K., Nickenig, G., Liao, J. K., & Bohm, M. (2000). Suppression of endothelial nitric oxide production after withdrawal of statin treatment is mediated by negative feedback regulation of rho GTPase gene transcription. *Circulation* **102**, 3104-3110.

Launikonis, B. S. & Stephenson, D. G. (1999). Effects of β-escin and saponin on the transverse-tubular system and sarcoplasmic reticulum membranes of rat and toad skeletal muscle. *Pflugers Arch.* **437**, 955-965.

Leach, R. M., Hill, H. M., Snetkov, V. A., Robertson, T. P., & Ward, J. P. (2001). Divergent roles of glycolysis and the mitochondrial electron transport chain in hypoxic pulmonary vasoconstriction of the rat: identity of the hypoxic sensor. *J.Physiol* **536**, 211-224.

Leach, R. M., Robertson, T. P., Twort, C. H., & Ward, J. P. (1994). Hypoxic vasoconstriction in rat pulmonary and mesenteric arteries. *Am.J.Physiol* **266**, L223-L231.

Leach, R. M. & Treacher, D. F. (1995). Clinical aspects of hypoxic pulmonary vasoconstriction. *Exp.Physiol* **80**, 865-875.

Lee, P., Langevitz, P., Alderdice, C. A., Aubrey, M., Baer, P. A., Baron, M., Buskila, D., Dutz, J. P., Khostanteen, I., & Piper, S. (1992). Mortality in systemic sclerosis (scleroderma). *Q.J.Med.* **82**, 139-148.

Lee, S. D., Shroyer, K. R., Markham, N. E., Cool, C. D., Voelkel, N. F., & Tuder, R. M. (1998). Monoclonal endothelial cell proliferation is present in primary but not secondary pulmonary hypertension. *J.Clin.Invest* **101**, 927-934.

Lee, S. L., Dunn, J., Yu, F. S., & Fanburg, B. L. (1989). Serotonin uptake and configurational change of bovine pulmonary artery smooth muscle cells in culture. *J.Cell Physiol* **138**, 145-153.

Lee, S. L., Wang, W. W., & Fanburg, B. L. (1997). Association of Tyr phosphorylation of GTPase-activating protein with mitogenic action of serotonin. *Am.J.Physiol* **272**, C223-C230.

Lee, S. L., Wang, W. W., Lanzillo, J. J., & Fanburg, B. L. (1994a). Regulation of serotonin-induced DNA synthesis of bovine pulmonary artery smooth muscle cells. *Am.J.Physiol* **266**, L53-L60.

Lee, S. L., Wang, W. W., Lanzillo, J. J., & Fanburg, B. L. (1994b). Serotonin produces both hyperplasia and hypertrophy of bovine pulmonary artery smooth muscle cells in culture. *Am.J.Physiol* **266**, L46-L52.

Lee, S. L., Wang, W. W., Moore, B. J., & Fanburg, B. L. (1991). Dual effect of serotonin on growth of bovine pulmonary artery smooth muscle cells in culture. *Circ.Res.* **68**, 1362-1368.

Lee, Y. H., Park, B. G., Ahn, D. S., & Kang, B. S. (1996). Characteristics of Ca^{2+} release mechanisms from an intracellular Ca^{2+} store in rabbit coronary artery. *Yonsei Med.J.* **37**, 38-46.

Lesch, K. P., Balling, U., Gross, J., Strauss, K., Wolozin, B. L., Murphy, D. L., & Riederer, P. (1994). Organization of the human serotonin transporter gene. *J.Neural Transm.Gen.Sect.* **95**, 157-162.

Lim, M., Honisett, S., Sparkes, C. D., Komesaroff, P., Kompa, A., & Krum, H. (2004). Differential effect of urotensin II on vascular tone in normal subjects and patients with chronic heart failure. *Circulation* **109**, 1212-1214.

Lindberg, F. & Andersson, K. E. (1988). Vasodilator responses to α-human-atrial natriuretic peptide in isolated omental and pulmonary arteries from rabbit and man. *Acta Physiol Scand.* **134**, 391-397.

Lippton, H. L., Nandiwada, P. A., Hyman, A. L., & Kadowitz, P. J. (1984). Influence of cyclooxygenase blockade on responses to isoproterenol, bradykinin and nitroglycerin in the feline pulmonary vascular bed. *Prostaglandins* **28**, 253-270.

Liu, H. & Lee, S. S. (1999). Cardiopulmonary dysfunction in cirrhosis. *J.Gastroenterol.Hepatol.* **14**, 600-608.

Liu, Q., Pong, S. S., Zeng, Z., Zhang, Q., Howard, A. D., Williams, D. L., Jr., Davidoff, M., Wang, R., Austin, C. P., McDonald, T. P., Bai, C., George, S. R., Evans, J. F., & Caskey, C. T. (1999). Identification of urotensin II as the endogenous ligand for the orphan G-protein-coupled receptor GPR14. *Biochem.Biophys.Res.Commun.* **266**, 174-178.

Liu, S. F., Crawley, D. E., Evans, T. W., & Barnes, P. J. (1991). Endogenous nitric oxide modulates adrenergic neural vasoconstriction in guinea-pig pulmonary artery. *Br.J.Pharmacol.* **104**, 565-569.

Liu, S. F., Crawley, D. E., Evans, T. W., & Barnes, P. J. (1992a). Endothelium-dependent nonadrenergic, noncholinergic neural relaxation in guinea pig pulmonary artery. *J.Pharmacol.Exp.Ther.* **260**, 541-548.

Liu, S. F., Crawley, D. E., Rohde, J. A., Evans, T. W., & Barnes, P. J. (1992b). Role of nitric oxide and guanosine 3',5'-cyclic monophosphate in mediating nonadrenergic, noncholinergic relaxation in guinea-pig pulmonary arteries. *Br.J.Pharmacol.* **107**, 861-866.

Liu, S. F., McCormack, D. G., Evans, T. W., & Barnes, P. J. (1989). Characterization and distribution of P2-purinoceptor subtypes in rat pulmonary vessels. *J.Pharmacol.Exp.Ther.* **251**, 1204-1210.

Liu, Y., Suzuki, Y. J., Day, R. M., & Fanburg, B. L. (2004). Rho kinase-induced nuclear translocation of ERK1/ERK2 in smooth muscle cell mitogenesis caused by serotonin. *Circ.Res.* **95**, 579-586.

Loyd, J. E., Butler, M. G., Foroud, T. M., Conneally, P. M., Phillips, J. A., III, & Newman, J. H. (1995). Genetic anticipation and abnormal gender ratio at birth in familial primary pulmonary hypertension. *Am.J.Respir.Crit Care Med.* **152**, 93-97.

Machado, R. D., Pauciulo, M. W., Thomson, J. R., Lane, K. B., Morgan, N. V., Wheeler, L., Phillips, J. A., III, Newman, J., Williams, D., Galie, N., Manes, A., McNeil, K.,

Yacoub, M., Mikhail, G., Rogers, P., Corris, P., Humbert, M., Donnai, D., Martensson, G., Tranebjaerg, L., Loyd, J. E., Trembath, R. C., & Nichols, W. C. (2001). BMPR2 haploinsufficiency as the inherited molecular mechanism for primary pulmonary hypertension. *Am.J.Hum.Genet.* **68**, 92-102.

Macintyre, P. D., Bhargava, B., Hogg, K. J., Gemmill, J. D., & Hillis, W. S. (1992). The effect of i.v. sumatriptan, a selective 5-HT1-receptor agonist on central haemodynamics and the coronary circulation. *Br.J.Clin.Pharmacol.* **34**, 541-546.

MacLean, M. R. (1999). Pulmonary hypertension, anorexigens and 5-HT: pharmacological synergism in action? *Trends Pharmacol.Sci.* **20**, 490-495.

MacLean, M. R., Alexander, D., Stirrat, A., Gallagher, M., Douglas, S. A., Ohlstein, E. H., Morecroft, I., & Polland, K. (2000a). Contractile responses to human urotensin-II in rat and human pulmonary arteries: effect of endothelial factors and chronic hypoxia in the rat. *Br.J.Pharmacol.* **130**, 201-204.

MacLean, M. R., Clayton, R. A., Templeton, A. G., & Morecroft, I. (1996a). Evidence for 5-HT1-like receptor-mediated vasoconstriction in human pulmonary artery. *Br.J.Pharmacol.* **119**, 277-282.

MacLean, M. R., Deuchar, G. A., Hicks, M. N., Morecroft, I., Shen, S., Sheward, J., Colston, J., Loughlin, L., Nilsen, M., Dempsie, Y., & Harmar, A. (2004). Overexpression of the 5-hydroxytryptamine transporter gene: effect on pulmonary hemodynamics and hypoxia-induced pulmonary hypertension. *Circulation* **109**, 2150-2155.

MacLean, M. R., Herve, P., Eddahibi, S., & Adnot, S. (2000b). 5-hydroxytryptamine and the pulmonary circulation: receptors, transporters and relevance to pulmonary arterial hypertension. *Br.J.Pharmacol.* **131**, 161-168.

MacLean, M. R., McCulloch, K. M., & Baird, M. (1995). Effects of pulmonary hypertension on vasoconstrictor responses to endothelin-1 and sarafotoxin S6C and on inherent tone in rat pulmonary arteries. *J.Cardiovasc.Pharmacol.* **26**, 822-830.

MacLean, M. R. & Morecroft, I. (2001). Increased contractile response to 5-hydroxytryptamine1-receptor stimulation in pulmonary arteries from chronic hypoxic rats: role of pharmacological synergy. *Br.J.Pharmacol.* **134**, 614-620.

MacLean, M. R., Sweeney, G., Baird, M., McCulloch, K. M., Houslay, M., & Morecroft, I. (1996b). 5-Hydroxytryptamine receptors mediating vasoconstriction in pulmonary arteries from control and pulmonary hypertensive rats. *Br.J.Pharmacol.* **119**, 917-930.

Madden, J. A., Dawson, C. A., & Harder, D. R. (1985). Hypoxia-induced activation in small isolated pulmonary arteries from the cat. *J.Appl.Physiol* **59**, 113-118.

Maggi, C. A., Patacchini, R., Perretti, F., Tramontana, M., Manzini, S., Geppetti, P., & Santicioli, P. (1990). Sensory nerves, vascular endothelium and neurogenic relaxation of the guinea-pig isolated pulmonary artery. *Naunyn Schmiedebergs Arch.Pharmacol.* **342**, 78-84.

Maguire, J. J. (2003). Discovering orphan receptor function using human in vitro pharmacology. *Curr.Opin.Pharmacol.* **3**, 135-139.

Maguire, J. J. & Davenport, A. P. (2002). Is urotensin-II the new endothelin? *Br.J.Pharmacol.* **137**, 579-588.

Maguire, J. J., Kuc, R. E., & Davenport, A. P. (2000). Orphan-receptor ligand human urotensin II: receptor localization in human tissues and comparison of vasoconstrictor responses with endothelin-1. *Br.J.Pharmacol.* **131**, 441-446.

Maguire, J. J., Kuc, R. E., Wiley, K. E., Kleinz, M. J., & Davenport, A. P. (2004). Cellular distribution of immunoreactive urotensin-II in human tissues with evidence of increased expression in atherosclerosis and a greater constrictor response of small compared to large coronary arteries. *Peptides* **25**, 1767-1774.

Mandegar, M., Fung, Y. C., Huang, W., Remillard, C. V., Rubin, L. J., & Yuan, J. X. (2004). Cellular and molecular mechanisms of pulmonary vascular remodeling: role in the development of pulmonary hypertension. *Microvasc.Res.* **68**, 75-103.

Marshall, C. & Marshall, B. E. (1983). Influence of perfusate P_{O2} on hypoxic pulmonary vasoconstriction in rats. *Circ.Res.* **52**, 691-696.

Marshall, C. & Marshall, B. E. (1992). Hypoxic pulmonary vasoconstriction is not endothelium dependent. *Proc.Soc.Exp.Biol.Med.* **201**, 267-270.

Mathas, S., Hinz, M., Anagnostopoulos, I., Krappmann, D., Lietz, A., Jundt, F., Bommert, K., Mechta-Grigoriou, F., Stein, H., Dorken, B., & Scheidereit, C. (2002). Aberrantly expressed c-Jun and JunB are a hallmark of Hodgkin lymphoma cells, stimulate proliferation and synergize with NF-kappa B. *EMBO J.* **21**, 4104-4113.

Matsui, T., Amano, M., Yamamoto, T., Chihara, K., Nakafuku, M., Ito, M., Nakano, T., Okawa, K., Iwamatsu, A., & Kaibuchi, K. (1996). Rho-associated kinase, a novel serine/threonine kinase, as a putative target for small GTP binding protein Rho. *EMBO J.* **15**, 2208-2216.

Matsushita, M., Shichiri, M., Imai, T., Iwashina, M., Tanaka, H., Takasu, N., & Hirata, Y. (2001). Co-expression of urotensin II and its receptor (GPR14) in human cardiovascular and renal tissues. *J.Hypertens.* **19**, 2185-2190.

McCulloch, K. M., Docherty, C., & MacLean, M. R. (1998). Endothelin receptors mediating contraction of rat and human pulmonary resistance arteries: effect of chronic hypoxia in the rat. *Br.J.Pharmacol.* **123**, 1621-1630.

McKay, K. O., Black, J. L., & Armour, C. L. (1991). The mechanism of action of endothelin in human lung. *Br.J.Pharmacol.* **102**, 422-428.

McLean, A. S. (1986). Computed tomography scanning in acute pulmonary problems. *Anaesth.Intensive Care* **14**, 84-86.

McMahon, T. J., Hood, J. S., Bellan, J. A., & Kadowitz, P. J. (1991). N omega-nitro-L-arginine methyl ester selectively inhibits pulmonary vasodilator responses to acetylcholine and bradykinin. *J.Appl.Physiol* **71**, 2026-2031.

McMahon, T. J., Hood, J. S., & Kadowitz, P. J. (1992). Pulmonary vasodilator response to vagal stimulation is blocked by N omega-nitro-L-arginine methyl ester in the cat. *Circ.Res.* **70**, 364-369.

McMurtry, I. F., Bauer, N. R., Fagan, K. A., Nagaoka, T., Gebb, S. A., & Oka, M. (2003). Hypoxia and Rho/Rho-kinase signaling. Lung development versus hypoxic pulmonary hypertension. *Adv.Exp.Med.Biol.* **543**, 127-137.

McMurtry, I. F., Morris, K. G., & Petrun, M. D. (1980). Blunted hypoxic vasoconstriction in lungs from short-term high-altitude rats. *Am.J.Physiol* **238**, H849-H857.

McTavish, D. & Heel, R. C. (1992). Dexfenfluramine. A review of its pharmacological properties and therapeutic potential in obesity. *Drugs* **43**, 713-733.

Meyrick, B. & Reid, L. (1978). The effect of continued hypoxia on rat pulmonary arterial circulation. An ultrastructural study. *Lab Invest* **38**, 188-200.

Meyrick, B. & Reid, L. (1980a). Endothelial and subintimal changes in rat hilar pulmonary artery during recovery from hypoxia. A quantitative ultrastructural study. *Lab Invest* **42**, 603-615.

Meyrick, B. & Reid, L. (1980b). Hypoxia-induced structural changes in the media and adventitia of the rat hilar pulmonary artery and their regression. *Am.J.Pathol.* **100**, 151-178.

Meyrick, B. & Reid, L. (1983). Pulmonary hypertension. Anatomic and physiologic correlates. *Clin.Chest Med.* **4**, 199-217.

Miao, L., Dai, Y., & Zhang, J. (2002). Mechanism of RhoA/Rho kinase activation in endothelin-1- induced contraction in rabbit basilar artery. *Am.J.Physiol Heart Circ.Physiol* **283**, H983-H989.

Michelakis, E. D., McMurtry, M. S., Wu, X. C., Dyck, J. R., Moudgil, R., Hopkins, T. A., Lopaschuk, G. D., Puttagunta, L., Waite, R., & Archer, S. L. (2002). Dichloroacetate, a metabolic modulator, prevents and reverses chronic hypoxic pulmonary hypertension in rats: role of increased expression and activity of voltage-gated potassium channels. *Circulation* **105**, 244-250.

Miller, J. R., Silver, P. J., & Stull, J. T. (1983). The role of myosin light chain kinase phosphorylation in β-adrenergic relaxation of tracheal smooth muscle. *Mol.Pharmacol.* **24**, 235-242.

Moncada, S., Palmer, R. M., & Higgs, E. A. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol.Rev.* **43**, 109-142.

Morecroft, I., Heeley, R. P., Prentice, H. M., Kirk, A., & MacLean, M. R. (1999). 5-hydroxytryptamine receptors mediating contraction in human small muscular pulmonary arteries: importance of the 5-HT_{1B} receptor. *Br.J.Pharmacol.* **128**, 730-734.

Morgan, J. P. & Morgan, K. G. (1984). Alteration of cytoplasmic ionized Ca^{2+} levels in smooth muscle by vasodilators in the ferret. *J.Physiol* **357**, 539-551.

Mori, M., Sugo, T., Abe, M., Shimomura, Y., Kurihara, M., Kitada, C., Kikuchi, K., Shintani, Y., Kurokawa, T., Onda, H., Nishimura, O., & Fujino, M. (1999). Urotensin II is the endogenous ligand of a G-protein-coupled orphan receptor, SENR (GPR14). *Biochem.Biophys.Res.Commun.* **265**, 123-129.

Morrell, N. W., Yang, X., Upton, P. D., Jourdan, K. B., Morgan, N., Sheares, K. K., & Trembath, R. C. (2001). Altered growth responses of pulmonary artery smooth muscle cells from patients with primary pulmonary hypertension to transforming growth factor-β1 and bone morphogenetic proteins. *Circulation* **104**, 790-795.

Mulvany, M. J. & Halpern, W. (1977). Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circ.Res.* **41**, 19-26.

Murdoch, R., Morecroft, I., & MacLean, M. R. (2003). 5-HT moduline: an endogenous inhibitor of 5-$HT_{1B/1D}$-mediated contraction in pulmonary arteries. *Br.J.Pharmacol.* **138**, 795-800.

Murray, P. A., Lodato, R. F., & Michael, J. R. (1986). Neural antagonists modulate pulmonary vascular pressure-flow plots in conscious dogs. *J.Appl.Physiol* **60**, 1900-1907.

Murray, T. R., Chen, L., Marshall, B. E., & Macarak, E. J. (1990). Hypoxic contraction of cultured pulmonary vascular smooth muscle cells. *Am.J.Respir.Cell Mol.Biol.* **3**, 457-465.

Nagaoka, T., Fagan, K. A., Gebb, S. A., Morris, K. G., Suzuki, T., Shimokawa, H., McMurtry, I. F., & Oka, M. (2004a). Inhaled Rho Kinase Inhibitors are Potent and Selective Vasodilators in Rat Pulmonary Hypertension. *Am.J.Respir.Crit Care Med.*

Nagaoka, T., Morio, Y., Casanova, N., Bauer, N., Gebb, S., McMurtry, I., & Oka, M. (2004b). Rho/Rho kinase signaling mediates increased basal pulmonary vascular tone in chronically hypoxic rats. *Am.J.Physiol Lung Cell Mol.Physiol* **287**, L665-L672.

Nagasaka, Y., Bhattacharya, J., Nanjo, S., Gropper, M. A., & Staub, N. C. (1984). Micropuncture measurement of lung microvascular pressure profile during hypoxia in cats. *Circ.Res.* **54**, 90-95.

Namiki, A., Hirata, Y., Ishikawa, M., Moroi, M., Aikawa, J., & Machii, K. (1992). Endothelin-1- and endothelin-3-induced vasorelaxation via common generation of endothelium-derived nitric oxide. *Life Sci.* **50**, 677-682.

Nandiwada, P. A., Kadowitz, P. J., Said, S. I., Mojarad, M., & Hyman, A. L. (1985). Pulmonary vasodilator responses to vasoactive intestinal peptide in the cat. *J.Appl.Physiol* **58**, 1723-1728.

Nathan, C. (1992). Nitric oxide as a secretory product of mammalian cells. *FASEB J.* **6**, 3051-3064.

Neely, C. F., Haile, D., & Matot, I. (1993). Tone-dependent responses of 5-hydroxytryptamine in the feline pulmonary vascular bed are mediated by two different 5-hydroxytryptamine receptors. *J.Pharmacol.Exp.Ther.* **264**, 1315-1326.

Neild, T. O., McLean, J. R., & Mee, R. B. (1986). An in vitro study of the pharmacological and electrophysiological properties and the adrenergic innervation of small pulmonary arteries from children with pulmonary hypertension. *Res.Commun.Chem.Pathol.Pharmacol.* **53**, 265-268.

Nelson, M. T., Patlak, J. B., Worley, J. F., & Standen, N. B. (1990). Ca^{2+} channels, K^+ channels, and voltage dependence of arterial smooth muscle tone. *Am.J.Physiol* **259**, C3-18.

Newby, D. E. & Jalan, R. (2000). Urotensin II: Better than somatostatin for portal hypertension? *Hepatology* **31**, 1201-1202.

Newman, J. H., Wheeler, L., Lane, K. B., Loyd, E., Gaddipati, R., Phillips, J. A., III, & Loyd, J. E. (2001). Mutation in the gene for bone morphogenetic protein receptor II as a cause of primary pulmonary hypertension in a large kindred. *N.Engl.J.Med.* **345**, 319-324.

Niiro, N., Koga, Y., & Ikebe, M. (2003). Agonist-induced changes in the phosphorylation of the myosin- binding subunit of myosin light chain phosphatase and CPI-17, two regulatory factors of myosin light chain phosphatase, in smooth muscle. *Biochem.J.* **369**, 117-128.

Nishikawa, Y., Doi, M., Koji, T., Watanabe, M., Kimura, S., Kawasaki, S., Ogawa, A., & Sasaki, K. (2003). The role of rho and rho-dependent kinase in serotonin-induced contraction observed in bovine middle cerebral artery. *Tohoku J.Exp.Med.* **201**, 239-249.

Nishimura, T., Vaszar, L. T., Faul, J. L., Zhao, G., Berry, G. J., Shi, L., Qiu, D., Benson, G., Pearl, R. G., & Kao, P. N. (2003). Simvastatin rescues rats from fatal pulmonary hypertension by inducing apoptosis of neointimal smooth muscle cells. *Circulation* **108**, 1640-1645.

Nishiwaki, K., Nyhan, D. P., Stuart, R. S., Desai, P. M., Peterson, W. P., Rock, P., Pribble, C. G., & Murray, P. A. (1993). Pulmonary vascular α_1-adrenoreceptor activity in conscious dogs after left lung autotransplantation. *J.Appl.Physiol* **74**, 733-741.

Norel, X., Walch, L., Costantino, M., Labat, C., Gorenne, I., Dulmet, E., Rossi, F., & Brink, C. (1996). M1 and M3 muscarinic receptors in human pulmonary arteries. *Br.J.Pharmacol.* **119**, 149-157.

Nothacker, H. P., Wang, Z., McNeill, A. M., Saito, Y., Merten, S., O'Dowd, B., Duckles, S. P., & Civelli, O. (1999). Identification of the natural ligand of an orphan G-protein-coupled receptor involved in the regulation of vasoconstriction. *Nat.Cell Biol.* **1**, 383-385.

Ogata, M., Ohe, M., Shirato, K., & Takishima, T. (1993). Effects of a combination therapy of anticoagulant and vasodilator on the long-term prognosis of primary pulmonary hypertension. *Jpn.Circ.J.* **57**, 63-69.

Oka, M., Hasunuma, K., Webb, S. A., Stelzner, T. J., Rodman, D. M., & McMurtry, I. F. (1993). EDRF suppresses an unidentified vasoconstrictor mechanism in hypertensive rat lungs. *Am.J.Physiol* **264**, L587-L597.

Okpaka, D. T. (1972). A dual action of histamine on guinea pig lung vessels. *Br.J.Pharmacol.* 311-321.

Pace, J. B. (1971). Sympathetic control of pulmonary vascular impedance in anesthetized dogs. *Circ.Res.* **29**, 555-568.

Pang, L. M., O'Brodovich, H. M., Mellins, R. B., & Stalcup, S. A. (1982). Bradykinin-induced increase in pulmonary vascular permeability in hypoxic sheep. *J.Appl.Physiol* **52**, 370-375.

Parekh, A. B. & Penner, R. (1997). Store depletion and Ca^{2+} influx. *Physiol Rev.* **77**, 901-930.

Park, M. K., Zakheim, R. M., Mattioli, L., & Sunderson, J. (1977). Altered reactivity of rat pulmonary arterial smooth muscle to vasoactive agents in hypoxia. *Proc.Soc.Exp.Biol.Med.* **155**, 274-277.

Partanen, M., Laitinen, A., Hervonen, A., Toivanen, M., & Laitinen, L. A. (1982). Catecholamine- and acetylcholinesterase-containing nerves in human lower respiratory tract. *Histochemistry* **76**, 175-188.

Paysant, J., Rupin, A., Simonet, S., Fabiani, J. N., & Verbeuren, T. J. (2001). Comparison of the contractile responses of human coronary bypass grafts and monkey arteries to human urotensin-II. *Fundam.Clin.Pharmacol.* **15**, 227-231.

Peacock, A. J. (1999). Primary pulmonary hypertension. *Thorax* **54**, 1107-1118.

Pearson, D., Shively, J. E., Clark, B. R., Geschwind, I. I., Barkley, M., Nishioka, R. S., & Bern, H. A. (1980). Urotensin II: a somatostatin-like peptide in the caudal neurosecretory system of fishes. *Proc.Natl.Acad.Sci.U.S.A* **77**, 5021-5024.

Pediani, J. D., McGrath, J. C., & Wilson, S. M. (1999). P2Y receptor-mediated Ca^{2+} signalling in cultured rat aortic smooth muscle cells. *Br.J.Pharmacol.* **126**, 1660-1666.

Pepke-Zaba, J., Higenbottam, T. W., Dinh-Xuan, A. T., Stone, D., & Wallwork, J. (1991). Inhaled nitric oxide as a cause of selective pulmonary vasodilatation in pulmonary hypertension. *Lancet* **338**, 1173-1174.

Persson, M. G., Gustafsson, L. E., Wiklund, N. P., Moncada, S., & Hedqvist, P. (1990). Endogenous nitric oxide as a probable modulator of pulmonary circulation and hypoxic pressor response in vivo. *Acta Physiol Scand.* **140**, 449-457.

Pfitzer, G. (2001). Invited review: regulation of myosin phosphorylation in smooth muscle. *J.Appl.Physiol* **91**, 497-503.

Piene, H. (1976). The influence of pulmonary blood flow rate on vascular input impedance and hydraulic power in the sympathetically and noradrenaline stimulated cat lung. *Acta Physiol Scand.* **98**, 44-53.

Pitt, B. R., Weng, W., Steve, A. R., Blakely, R. D., Reynolds, I., & Davies, P. (1994). Serotonin increases DNA synthesis in rat proximal and distal pulmonary vascular smooth muscle cells in culture. *Am.J.Physiol* **266**, L178-L186.

Rabinovitch, M., Mullen, M., Rosenberg, H. C., Maruyama, K., O'Brodovich, H., & Olley, P. M. (1988). Angiotensin II prevents hypoxic pulmonary hypertension and vascular changes in rat. *Am.J.Physiol* **254**, H500-H508.

Rembold, C. M. (1992). Regulation of contraction and relaxation in arterial smooth muscle. *Hypertension* **20**, 129-137.

Remillard, C. V. & Yuan, J. X. (2004). Activation of K^+ channels: an essential pathway in programmed cell death. *Am.J.Physiol Lung Cell Mol.Physiol* **286**, L49-L67.

Rich et al (2003). The Pathology of Pulmonary Arterial Hypertension. *WHO symposium on pulmonary arterial hypertension 2003*.

Rich, S., Dantzker, D. R., Ayres, S. M., Bergofsky, E. H., Brundage, B. H., Detre, K. M., Fishman, A. P., Goldring, R. M., Groves, B. M., & Koerner, S. K. (1987). Primary pulmonary hypertension. A national prospective study. *Ann.Intern.Med.* **107**, 216-223.

Rich, S. & McLaughlin, V. V. (2003). Endothelin receptor blockers in cardiovascular disease. *Circulation* **108**, 2184-2190.

Robbins, I. M., Barst, R. J., Rubin, L. J., Gaine, S. P., Price, P. V., Morrow, J. D., & Christman, B. W. (2001). Increased levels of prostaglandin D(2) suggest macrophage activation in patients with primary pulmonary hypertension. *Chest* **120**, 1639-1644.

Robertson, T. P., Aaronson, P. I., & Ward, J. P. (1995). Hypoxic vasoconstriction and intracellular Ca^{2+} in pulmonary arteries: evidence for PKC-independent Ca^{2+} sensitization. *Am.J.Physiol* **268**, H301-H307.

Robertson, T. P., Ward, J. P., & Aaronson, P. I. (2001). Hypoxia induces the release of a pulmonary-selective, Ca^{2+}-sensitising, vasoconstrictor from the perfused rat lung. *Cardiovasc.Res.* **50**, 145-150.

Robin, E. D. (1982). Some basic and clinical challenges in the pulmonary circulation. *Chest* **81**, 357-363.

Rodman, D. M. (1992). Chronic hypoxia selectively augments rat pulmonary artery Ca^{2+} and K^+ channel-mediated relaxation. *Am.J.Physiol* **263**, L88-L94.

Rodman, D. M., Yamaguchi, T., O'Brien, R. F., & McMurtry, I. F. (1989). Hypoxic contraction of isolated rat pulmonary artery. *J.Pharmacol.Exp.Ther.* **248**, 952-959.

Roos, S. D., Weir, E. K., & Reeves, J. T. (1976). Meclofenamate does not reduce chronic hypoxic pulmonary vasoconstriction. *Experientia* **32**, 195-196.

Rothman, R. B., Ayestas, M. A., Dersch, C. M., & Baumann, M. H. (1999). Aminorex, fenfluramine, and chlorphentermine are serotonin transporter substrates. Implications for primary pulmonary hypertension. *Circulation* **100**, 869-875.

Rubin, L. J. (1997). Primary pulmonary hypertension. *N.Engl.J.Med.* **336**, 111-117.

Rubin, L. J., Badesch, D. B., Barst, R. J., Galie, N., Black, C. M., Keogh, A., Pulido, T., Frost, A., Roux, S., Leconte, I., Landzberg, M., & Simonneau, G. (2002). Bosentan therapy for pulmonary arterial hypertension. *N.Engl.J.Med.* **346**, 896-903.

Rubin, L. J., Groves, B. M., Reeves, J. T., Frosolono, M., Handel, F., & Cato, A. E. (1982). Prostacyclin-induced acute pulmonary vasodilation in primary pulmonary hypertension. *Circulation* **66**, 334-338.

Rubin, L. J. & Roux, S. (2002). Bosentan: a dual endothelin receptor antagonist. *Expert.Opin.Investig.Drugs* **11**, 991-1002.

Rubino, A., Ziabary, L., & Burnstock, G. (1999). Regulation of vascular tone by UTP and UDP in isolated rat intrapulmonary arteries. *Eur.J.Pharmacol.* **370**, 139-143.

Ruf, A. & Morgenstern, E. (1995). Ultrastructural aspects of platelet adhesion on subendothelial structures. *Semin.Thromb.Hemost.* **21**, 119-122.

Russell, F. D., Kearns, P., Toth, I., & Molenaar, P. (2004). Urotensin-II-converting enzyme activity of furin and trypsin in human cells in vitro. *J.Pharmacol.Exp.Ther.* **310**, 209-214.

Russell, F. D., Meyers, D., Galbraith, A. J., Bett, N., Toth, I., Kearns, P., & Molenaar, P. (2003). Elevated plasma levels of human urotensin-II immunoreactivity in congestive heart failure. *Am.J.Physiol Heart Circ.Physiol* **285**, H1576-H1581.

Russell, F. D., Molenaar, P., & O'Brien, D. M. (2001). Cardiostimulant effects of urotensin-II in human heart in vitro. *Br.J.Pharmacol.* **132**, 5-9.

Saetrum, O. O., Nothacker, H., Ehlert, F. J., & Krause, D. N. (2000). Human urotensin II mediates vasoconstriction via an increase in inositol phosphates. *Eur.J.Pharmacol.* **406**, 265-271.

Sartore, S., Chiavegato, A., Faggin, E., Franch, R., Puato, M., Ausoni, S., & Pauletto, P. (2001). Contribution of adventitial fibroblasts to neointima formation and vascular remodeling: from innocent bystander to active participant. *Circ.Res.* **89**, 1111-1121.

Sato, K., Ozaki, H., & Karaki, H. (1988). Changes in cytosolic Ca^{2+} level in vascular smooth muscle strip measured simultaneously with contraction using fluorescent Ca^{2+} indicator fura 2. *J.Pharmacol.Exp.Ther.* **246**, 294-300.

Sauzeau, V., Le Jeune, H., Cario-Toumaniantz, C., Smolenski, A., Lohmann, S. M., Bertoglio, J., Chardin, P., Pacaud, P., & Loirand, G. (2000a). Cyclic GMP-dependent protein kinase signaling pathway inhibits RhoA-induced Ca^{2+} sensitization of contraction in vascular smooth muscle. *J.Biol.Chem.* **275**, 21722-21729.

Sauzeau, V., Le Jeune, H., Cario-Toumaniantz, C., Vaillant, N., Gadeau, A. P., Desgranges, C., Scalbert, E., Chardin, P., Pacaud, P., & Loirand, G. (2000b). P2Y(1), $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors are coupled to Rho and Rho kinase activation in vascular myocytes. *Am.J.Physiol Heart Circ.Physiol* **278**, H1751-H1761.

Sauzeau, V., Le Mellionnec, E., Bertoglio, J., Scalbert, E., Pacaud, P., & Loirand, G. (2001). Human urotensin II-induced contraction and arterial smooth muscle cell proliferation are mediated by RhoA and Rho-kinase. *Circ.Res.* **88**, 1102-1104.

Sauzeau, V., Rolli-Derkinderen, M., Lehoux, S., Loirand, G., & Pacaud, P. (2003a). Sildenafil prevents change in RhoA expression induced by chronic hypoxia in rat pulmonary artery. *Circ.Res.* **93**, 630-637.

Sauzeau, V., Rolli-Derkinderen, M., Marionneau, C., Loirand, G., & Pacaud, P. (2003b). RhoA expression is controlled by nitric oxide through cGMP-dependent protein kinase activation. *J.Biol.Chem.* **278**, 9472-9480.

Savineau, J. P. & Marthan, R. (1994). Activation properties of chemically skinned fibres from human isolated bronchial smooth muscle. *J.Physiol* **474**, 433-438.

Schroeder, A. C., Imig, J. D., LeBlanc, E. A., Pham, B. T., Pollock, D. M., & Inscho, E. W. (2000). Endothelin-mediated Ca^{2+} signaling in preglomerular smooth muscle cells. *Hypertension* **35**, 280-286.

Seghaye, M. C., Duchateau, J., Bruniaux, J., Demontoux, S., Detruit, H., Bosson, C., Lecronier, G., Mokhfi, E., Serraf, A., & Planche, C. (1997). Endogenous nitric oxide

production and atrial natriuretic peptide biological activity in infants undergoing cardiac operations. *Crit Care Med.* **25**, 1063-1070.

Seguchi, H., Nishimura, J., Toyofuku, K., Kobayashi, S., Kumazawa, J., & Kanaide, H. (1996). The mechanism of relaxation induced by atrial natriuretic peptide in the porcine renal artery. *Br.J.Pharmacol.* **118**, 343-351.

Seiler, K. U., Wassermann, O., & Wensky, H. (1976). On the role of serotonin in the pathogenesis of pulmonary hypertension induced by anorectic drugs; an experimental study in the isolated perfused rat lung, II. Fenfluramine, mazindol, mefenorex, phentermine and R 800. *Clin.Exp.Pharmacol.Physiol* **3**, 323-330.

Sheedy, W., Thompson, J. S., & Morice, A. H. (1996). A comparison of pathophysiological changes during hypobaric and normobaric hypoxia in rats. *Respiration* **63**, 217-222.

Shenouda, A., Douglas, S. A., Ohlstein, E. H., & Giaid, A. (2002). Localization of urotensin-II immunoreactivity in normal human kidneys and renal carcinoma. *J.Histochem.Cytochem.* **50**, 885-889.

Shimoda, L. A., Sham, J. S., & Sylvester, J. T. (2000). Altered pulmonary vasoreactivity in the chronically hypoxic lung. *Physiol Res.* **49**, 549-560.

Shimoda, L. A., Sylvester, J. T., & Sham, J. S. (1999). Chronic hypoxia alters effects of endothelin and angiotensin on K^+ currents in pulmonary arterial myocytes. *Am.J.Physiol* **277**, L431-L439.

Shimokawa, H., Seto, M., Katsumata, N., Amano, M., Kozai, T., Yamawaki, T., Kuwata, K., Kandabashi, T., Egashira, K., Ikegaki, I., Asano, T., Kaibuchi, K., & Takeshita, A. (1999). Rho-kinase-mediated pathway induces enhanced myosin light chain phosphorylations in a swine model of coronary artery spasm. *Cardiovasc.Res.* **43**, 1029-1039.

Shin, H. M., Je, H. D., Gallant, C., Tao, T. C., Hartshorne, D. J., Ito, M., & Morgan, K. G. (2002). Differential association and localization of myosin phosphatase subunits during agonist-induced signal transduction in smooth muscle. *Circ.Res.* **90**, 546-553.

Singh, S. & Evans, T. W. (1997). Nitric oxide, the biological mediator of the decade: fact or fiction? *Eur.Respir.J.* **10**, 699-707.

Smith, P. & Heath, D. (1977). Ultrastructure of hypoxic hypertensive pulmonary vascular disease. *J.Pathol.* **121**, 93-100.

Soloviev, A., Lehen'kyi, V., Zelensky, S., & Hellstrand, P. (2004). Nitric oxide relaxes rat tail artery smooth muscle by cyclic GMP-independent decrease in Ca^{2+} sensitivity of myofilaments. *Cell Calcium* **36**, 165-173.

Somlyo, A. P. & Himpens, B. (1989). Cell Ca^{2+} and its regulation in smooth muscle. *FASEB J.* **3**, 2266-2276.

Somlyo, A. P. & Somlyo, A. V. (1994). Smooth muscle: excitation-contraction coupling, contractile regulation, and the cross-bridge cycle. *Alcohol Clin.Exp.Res.* **18**, 138-143.

Somlyo, A. P. & Somlyo, A. V. (2003). Ca^{2+} sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. *Physiol Rev.* **83**, 1325-1358.

Stamler, J. S., Loh, E., Roddy, M. A., Currie, K. E., & Creager, M. A. (1994). Nitric oxide regulates basal systemic and pulmonary vascular resistance in healthy humans. *Circulation* **89**, 2035-2040.

Steudel, W., Ichinose, F., Huang, P. L., Hurford, W. E., Jones, R. C., Bevan, J. A., Fishman, M. C., & Zapol, W. M. (1997). Pulmonary vasoconstriction and hypertension in mice with targeted disruption of the endothelial nitric oxide synthase (NOS III) gene. *Circ.Res.* **81**, 34-41.

Stewart, D. J., Levy, R. D., Cernacek, P., & Langleben, D. (1991). Increased plasma endothelin-1 in pulmonary hypertension: marker or mediator of disease? *Ann.Intern.Med.* **114**, 464-469.

Stirrat, A., Gallagher, M., Douglas, S. A., Ohlstein, E. H., Berry, C., Kirk, A., Richardson, M., & MacLean, M. R. (2001). Potent vasodilator responses to human urotensin-II in human pulmonary and abdominal resistance arteries. *Am.J.Physiol Heart Circ.Physiol* **280**, H925-H928.

Stull, J. T., Hsu, L. C., Tansey, M. G., & Kamm, K. E. (1990). Myosin light chain kinase phosphorylation in tracheal smooth muscle. *J.Biol.Chem.* **265**, 16683-16690.

Suggett, A. J. & Barer, G. R. (1988). Experimental prevention of hypoxic pulmonary hypertension in animals by drugs. *Eur.Heart J.* **9 Suppl J**, 13-18.

Suhara, H., Sawa, Y., Fukushima, N., Kagisaki, K., Yokoyama, C., Tanabe, T., Ohtake, S., & Matsuda, H. (2002). Gene transfer of human prostacyclin synthase into the liver is effective for the treatment of pulmonary hypertension in rats. *J.Thorac.Cardiovasc.Surg.* **123**, 855-861.

Surks, H. K., Mochizuki, N., Kasai, Y., Georgescu, S. P., Tang, K. M., Ito, M., Lincoln, T. M., & Mendelsohn, M. E. (1999). Regulation of myosin phosphatase by a specific interaction with cGMP- dependent protein kinase Iα. *Science* **286**, 1583-1587.

Sward, K., Dreja, K., Susnjar, M., Hellstrand, P., Hartshorne, D. J., & Walsh, M. P. (2000). Inhibition of Rho-associated kinase blocks agonist-induced Ca^{2+} sensitization of myosin phosphorylation and force in guinea-pig ileum. *J.Physiol* **522 Pt 1**, 33-49.

Szarek, J. L., Bailly, D. A., Stewart, N. L., & Gruetter, C. A. (1992). Histamine H1-receptors mediate endothelium-dependent relaxation of rat isolated pulmonary arteries. *Pulm.Pharmacol.* **5**, 67-74.

Takahashi, K., Totsune, K., Murakami, O., Arihara, Z., Noshiro, T., Hayashi, Y., & Shibahara, S. (2003). Expression of urotensin II and its receptor in adrenal tumors and stimulation of proliferation of cultured tumor cells by urotensin II. *Peptides* **24**, 301-306.

Takahashi, K., Totsune, K., Murakami, O., & Shibahara, S. (2001). Expression of urotensin II and urotensin II receptor mRNAs in various human tumor cell lines and secretion of urotensin II-like immunoreactivity by SW-13 adrenocortical carcinoma cells. *Peptides* **22**, 1175-1179.

Takaya, J., Teraguchi, M., Nogi, S., Ikemoto, Y., & Kobayashi, Y. (1998). Relation between plasma nitrate and mean pulmonary arterial pressure in ventricular septal defect. *Arch.Dis.Child* **79**, 498-501.

Takemoto, M., Sun, J., Hiroki, J., Shimokawa, H., & Liao, J. K. (2002). Rho-kinase mediates hypoxia-induced downregulation of endothelial nitric oxide synthase. *Circulation* **106**, 57-62.

Tansey, M. G., Luby-Phelps, K., Kamm, K. E., & Stull, J. T. (1994). Ca^{2+}-dependent phosphorylation of myosin light chain kinase decreases the Ca^{2+} sensitivity of light chain phosphorylation within smooth muscle cells. *J.Biol.Chem.* **269**, 9912-9920.

Taraseviciene-Stewart, L., Kasahara, Y., Alger, L., Hirth, P., Mc, M. G., Waltenberger, J., Voelkel, N. F., & Tuder, R. M. (2001). Inhibition of the VEGF receptor 2 combined with chronic hypoxia causes cell death-dependent pulmonary endothelial cell proliferation and severe pulmonary hypertension. *FASEB J.* **15**, 427-438.

Thanassoulis, G., Huyhn, T., & Giaid, A. (2004). Urotensin II and cardiovascular diseases. *Peptides* **25**, 1789-1794.

Todorovich-Hunter, L., Dodo, H., Ye, C., McCready, L., Keeley, F. W., & Rabinovitch, M. (1992). Increased pulmonary artery elastolytic activity in adult rats with

monocrotaline-induced progressive hypertensive pulmonary vascular disease compared with infant rats with nonprogressive disease. *Am.Rev.Respir.Dis.* **146**, 213-223.

Tortora (1995). *Principles of human anatomy*, 7th ed. HarperCollins.

Totsune, K., Takahashi, K., Arihara, Z., Sone, M., Ito, S., & Murakami, O. (2003). Increased plasma urotensin II levels in patients with diabetes mellitus. *Clin.Sci.(Lond)* **104**, 1-5.

Totsune, K., Takahashi, K., Arihara, Z., Sone, M., Murakami, O., Ito, S., Kikuya, M., Ohkubo, T., Hashimoto, J., & Imai, Y. (2004). Elevated plasma levels of immunoreactive urotensin II and its increased urinary excretion in patients with Type 2 diabetes mellitus: association with progress of diabetic nephropathy. *Peptides* **25**, 1809-1814.

Totsune, K., Takahashi, K., Arihara, Z., Sone, M., Satoh, F., Ito, S., Kimura, Y., Sasano, H., & Murakami, O. (2001). Role of urotensin II in patients on dialysis. *Lancet* **358**, 810-811.

Tucker, A., Weir, E. K., Reeves, J. T., & Grover, R. F. (1976). Failure of histamine antagonists to prevent hypoxic pulmonary vasoconstriction in dogs. *J.Appl.Physiol* **40**, 496-500.

Tuder, R. M., Cool, C. D., Geraci, M. W., Wang, J., Abman, S. H., Wright, L., Badesch, D., & Voelkel, N. F. (1999). Prostacyclin synthase expression is decreased in lungs from patients with severe pulmonary hypertension. *Am.J.Respir.Crit Care Med.* **159**, 1925-1932.

Uehata, M., Ishizaki, T., Satoh, H., Ono, T., Kawahara, T., Morishita, T., Tamakawa, H., Yamagami, K., Inui, J., Mackawa, M., & Narumiya, S. (1997). Ca^{2+} sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature* **389**, 990-994.

Van Riper, D. A., Weaver, B. A., Stull, J. T., & Rembold, C. M. (1995). Myosin light chain kinase phosphorylation in swine carotid artery contraction and relaxation. *Am.J.Physiol* **268**, H2466-H2475.

VanBavel, E., van der Meulen, E. T., & Spaan, J. A. (2001). Role of Rho-associated protein kinase in tone and Ca^{2+} sensitivity of cannulated rat mesenteric small arteries. *Exp.Physiol* **86**, 585-592.

Voelkel, N. F. (1997). Appetite suppressants and pulmonary hypertension. *Thorax* **52 Suppl 3**, S63-S67.

Voelkel, N. F. & Tuder, R. M. (1995). Cellular and molecular mechanisms in the pathogenesis of severe pulmonary hypertension. *Eur.Respir.J.* **8**, 2129-2138.

Voelkel, N. F., Tuder, R. M., Wade, K., Hoper, M., Lepley, R. A., Goulet, J. L., Koller, B. H., & Fitzpatrick, F. (1996). Inhibition of 5-lipoxygenase-activating protein (FLAP) reduces pulmonary vascular reactivity and pulmonary hypertension in hypoxic rats. *J.Clin.Invest* **97**, 2491-2498.

Von Euler, U. S. & Liljestrand, G. (1947). Observations on the pulmonary arterial blood pressure of the cat. *Acta.Phys.Scand.* 301-320.

Wang, Y., Coe, Y., Toyoda, O., & Coceani, F. (1995). Involvement of endothelin-1 in hypoxic pulmonary vasoconstriction in the lamb. *J.Physiol* **482 (Pt 2)**, 421-434.

Wang, Z., Jin, N., Ganguli, S., Swartz, D. R., Li, L., & Rhoades, R. A. (2001). Rho-kinase activation is involved in hypoxia-induced pulmonary vasoconstriction. *Am.J.Respir.Cell Mol.Biol.* **25**, 628-635.

Wang, Z., Lanner, M. C., Jin, N., Swartz, D., Li, L., & Rhoades, R. A. (2003). Hypoxia inhibits myosin phosphatase in pulmonary arterial smooth muscle cells: role of Rho-kinase. *Am.J.Respir.Cell Mol.Biol.* **29**, 465-471.

Wanstall, J. C., Fiore, S. A., Gambino, A., & Chess-Williams, R. (2003). Potentiation of 5-hydroxytryptamine (5-HT) responses by a 5-HT uptake inhibitor in pulmonary and systemic vessels: effects of exposing rats to hypoxia. *Naunyn Schmiedebergs Arch.Pharmacol.* **368**, 520-527.

Wanstall, J. C., Kaye, J. A., & Gambino, A. (1997). The in vitro pulmonary vascular effects of FK409 (nitric oxide donor): a study in normotensive and pulmonary hypertensive rats. *Br.J.Pharmacol.* **121**, 280-286.

Ward, J. P., Knock, G. A., Snetkov, V. A., & Aaronson, P. I. (2004). Protein kinases in vascular smooth muscle tone-role in the pulmonary vasculature and hypoxic pulmonary vasoconstriction. *Pharmacol.Ther.* **104**, 207-231.

Weber, D. S. & Webb, R. C. (2001). Enhanced relaxation to the rho-kinase inhibitor Y-27632 in mesenteric arteries from mineralocorticoid hypertensive rats. *Pharmacology* **63**, 129-133.

Weir, E. K., McMurtry, I. F., Tucker, A., Reeves, J. T., & Grover, R. F. (1976a). Prostaglandin synthetase inhibitors do not decrease hypoxic pulmonary vasoconstriction. *J.Appl.Physiol* **41**, 714-718.

Weir, E. K., Reeve, H. L., Huang, J. M., Michelakis, E., Nelson, D. P., Hampl, V., & Archer, S. L. (1996). Anorexic agents aminorex, fenfluramine, and dexfenfluramine inhibit potassium current in rat pulmonary vascular smooth muscle and cause pulmonary vasoconstriction. *Circulation* **94**, 2216-2220.

Weir, E. K., Seavy, J., Mlczoch, J., Genton, E., & Reeves, J. T. (1976b). Platelets are not essential for the pulmonary vascular pressor response to hypoxia. *J.Lab Clin.Med.* **88**, 412-416.

West, J. (1995). *Respiratory Physiology*.

Wilkinson, I. B., Affolter, J. T., de Haas, S. L., Pellegrini, M. P., Boyd, J., Winter, M. J., Balment, R. J., & Webb, D. J. (2002). High plasma concentrations of human urotensin II do not alter local or systemic hemodynamics in man. *Cardiovasc.Res.* **53**, 341-347.

Wingard, C. J., Husain, S., Williams, J., & James, S. (2003). RhoA-Rho kinase mediates synergistic ET-1 and phenylephrine contraction of rat corpus cavernosum. *Am.J.Physiol Regul.Integr.Comp Physiol* **285**, R1145-R1152.

Wood, P. (1958). Pulmonary hypertension with special reference to the vasoconstrictive factor. *Br.Heart J.* **20**, 557-570.

Yeager, M. E., Halley, G. R., Golpon, H. A., Voelkel, N. F., & Tuder, R. M. (2001). Microsatellite instability of endothelial cell growth and apoptosis genes within plexiform lesions in primary pulmonary hypertension. *Circ.Res.* **88**, E2-E11.

Yoshida, M., Suzuki, A., & Itoh, T. (1994). Mechanisms of vasoconstriction induced by endothelin-1 in smooth muscle of rabbit mesenteric artery. *J.Physiol* **477 (Pt 2)**, 253-265.

Yoshii, A., Iizuka, K., Dobashi, K., Horie, T., Harada, T., Nakazawa, T., & Mori, M. (1999). Relaxation of contracted rabbit tracheal and human bronchial smooth muscle by Y-27632 through inhibition of Ca^{2+} sensitization. *Am.J.Respir.Cell Mol.Biol.* **20**, 1190-1200.

Yuan, J. X., Aldinger, A. M., Juhaszova, M., Wang, J., Conte, J. V., Jr., Gaine, S. P., Orens, J. B., & Rubin, L. J. (1998a). Dysfunctional voltage-gated K^+ channels in pulmonary artery smooth muscle cells of patients with primary pulmonary hypertension. *Circulation* **98**, 1400-1406.

Yuan, X. J. (1995). Voltage-gated K^+ currents regulate resting membrane potential and $[Ca^{2+}]_i$ in pulmonary arterial myocytes. *Circ.Res.* **77**, 370-378.

Yuan, X. J., Tod, M. L., Rubin, L. J., & Blaustein, M. P. (1990). Contrasting effects of hypoxia on tension in rat pulmonary and mesenteric arteries. *Am.J.Physiol* **259**, H281-H289.

Yuan, X. J., Wang, J., Juhaszova, M., Gaine, S. P., & Rubin, L. J. (1998b). Attenuated K^+ channel gene transcription in primary pulmonary hypertension. *Lancet* **351**, 726-727.

Zaidi, S. H., You, X. M., Ciura, S., Husain, M., & Rabinovitch, M. (2002). Overexpression of the serine elastase inhibitor elafin protects transgenic mice from hypoxic pulmonary hypertension. *Circulation* **105**, 516-521.

Zeng, W., Mak, D. O., Li, Q., Shin, D. M., Foskett, J. K., & Muallem, S. (2003). A new mode of Ca^{2+} signaling by G protein-coupled receptors: gating of IP_3 receptor Ca^{2+} release channels by $G_{\beta\gamma}$. *Curr.Biol.* **13**, 872-876.

Zhang, F. & Morice, A. H. (1994). Effect of levcromakalim on hypoxia, KCl and prostaglandin $F_2\alpha$-induced contractions in isolated rat pulmonary artery. *J.Pharmacol.Exp.Ther.* **271**, 326-333.

Zhang, S., Fantozzi, I., Tigno, D. D., Yi, E. S., Platoshyn, O., Thistlethwaite, P. A., Kriett, J. M., Yung, G., Rubin, L. J., & Yuan, J. X. (2003). Bone morphogenetic proteins induce apoptosis in human pulmonary vascular smooth muscle cells. *Am.J.Physiol Lung Cell Mol.Physiol* **285**, L740-L754.

Zhang, Y., Li, J., Cao, J., Chen, J., Yang, J., Zhang, Z., Du, J., & Tang, C. (2002). Effect of chronic hypoxia on contents of urotensin II and its functional receptors in rat myocardium. *Heart Vessels* **16**, 64-68.

Zhu, X., Jiang, M., Peyton, M., Boulay, G., Hurst, R., Stefani, E., & Birnbaumer, L. (1996). trp, a novel mammalian gene family essential for agonist-activated capacitative Ca^{2+} entry. *Cell* **85**, 661-671.