Interactions between potassium channels and serotonin in pulmonary arterial hypertension

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Abstract

Pulmonary arterial hypertension (PAH) is a progressive disease which results from increases in mean pulmonary artery pressure and pulmonary vascular resistance. If untreated it leads to right ventricular failure and death. 5-Hydroxytryptamine (5-HT) has been implicated in the disease process and is thought to promote both vasoconstriction and remodelling of the pulmonary vasculature. The activity of potassium ion (K^+) channels plays a major role in influencing pulmonary artery tone by regulating resting membrane potential, intracellular Ca^{2+} concentration and contraction of vascular smooth muscle. This study aimed to investigate possible interactions between 5-HT and K^+ channels in the development of PAH in the mouse.

The actions of 5-HT and a range of K^+ channel blockers were investigated on isolated intralobar pulmonary arteries (IPA) from wild type (WT) mice and mice over-expressing the serotonin transporter (5HTT), which spontaneously develop PAH. Both 5-HT and linopirdine, a KCNQ K^+ channel inhibitor, were found to induce contraction of IPA, but were more potent in IPA from WT mice than 5-HTT+ mice. The 5-HT induced vasoconstriction was found to involve influx of Ca^{2+} from the extracellular space, Ca^{2+} release from the sacroplasmic reticulum and a rho kinase–dependent increase in the sensitivity of the contractile machinery of pulmonary artery smooth muscle cells (PASMC) to intracellular Ca^{2+}. Ca^{2+} entered the cell via both voltage operated calcium channels (VOCC), activated by membrane depolarisation, and a separate Ca^{2+} entry pathway, the latter appearing to contribute more in 5-HTT+ mice. The effects of linopirdine were shown to be due entirely to the entry of Ca^{2+} through VOCC in both WT and 5-HTT+ mice IPA. The difference in vasoconstrictor potency between WT and 5HTT+ mice was not seen with any other K^+ channel blocker, suggesting a selective loss of KCNQ channels and/or VOCC in PAH resulting from 5HTT over expression.

KCNQ channel activity was further investigated using the KCNQ channel openers, flupirtine and retigabine. These agents were more potent in dilating IPA from WT mice compared to 5-HTT+ mice, consistent with the loss of expression or activity of KCNQ channels in 5-HTT+ mice. Despite this, orally administered flupirtine was shown to

reverse two indices of established PAH in the 5HTT+ mice; right ventricular pressure and right ventricular hypertrophy. This action of flupirtine was also seen in chronic hypoxic mice, where it prevented the development of PAH.

In conclusion, this study provides evidence of an interaction between KCNQ channels and the 5-HT system in the development of PAH. By showing that a KCNQ channel opener can attenuate PAH, both in the developing and established disease situation, this study proposes a new potential therapeutic target in the treatment of PAH.

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List of abbreviations

2-ABP	2-Aminoethyl diphenylborinate
4-AP	4-Aminopyridine
5-HT	5-hydroxytryptamine
5-HTT	5-hydroxytryptamine transporter
5-HTT+	Mice overexpressing 5-hydroxytryptamine transporter
5-HTTLPR	5-HT transporter gene
ABC	ATP-binding cassette
AC	Adenylate cyclase
ACh	Acetylcholine
ADP	Adenosine diphosphate
AKAPs	4-3-3 Proteins, and A-Kinase Anchoring proteins
ANOVA	One-way analysis of variance
ATP	Adenosine 5' triphosphate
BK_{Ca}	Large conductance calcium activated potassium channels
BMPR-2	Bone morphogenetic protein type 2 receptor
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
$[Ca^{2+}]_{ext.}$	Extracellular calcium
$[Ca^{2+}]_i$	Intracellular calcium
CaM	Calmodulin
cAMP	Cyclic 3', 5' adenosine monophosphate
cGMP	Guanosine 3', 5' monophosphate
Cl^-	Chloride ion
CMC	Carboxymethylcellulose
CO	Cardiac output
COX	Cyclo-oxygenase
DAG	Diacylglycerol
DM	Dissociation medium
DMSO	Dimethylsulphoxide
EDHF	Endothelium-derived hyperpolarising factor

EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
E_K	Equilibrium potential for potassium
*E*m	Resting membrane potential
ERK	Extracellular signal-regulated protein kinase
ET-1	Endothelin-1
ETA	Endothelin A receptor
ETB	Endothelin B receptor
FPAH	Familial pulmonary arterial hypertension
GAP	GTPase-activating protein
GIRK	G-protein regulated potassium channel
GPCR	G-protein-coupled receptor
GTP	Guanine 5' triphosphate
GAP	GTPase-activating protein
HEPES	4-2-hydroxyethyl-1-piperazineethanesulphonic acid
HR	Heart rate
HPV	Hypoxic pulmonary vasoconstriction
I_{Ca}	Calcium current
IK_{Ca}	Intermediate conductance calcium activated potassium channels
I_K	Potassium current
I_{KA}	'A' type potassium current
I_{KDR}	Delayed rectifier potassium current
I_{KN}	Non inactivating potassium current
I_{KV}	Voltage gated potassium current
IC	Internal circumference
IPA	Intralobar Pulmonary Artery
IP_3	Inositol trisphosphatase
IP_3R	Inositol trisphosphatase receptor
IPAH	Idiopathic pulmonary arterial hypertension
K^+	Potassium ion
$[K^+]_{ext.}$	Extracellular potassium

$[K^+]_i$	Intracellular potassium
K_{ATP}	ATP-sensitive K^+ channel
K_{Ca}	Calcium activated potassium channel
KChAPs	Potassium channel-associated protein K_V channel-interacting proteins
KChIPs	Voltage gated potassium channel-interacting proteins
KCNQ	Voltage gated potassium- 7 subfamily channel
K_{IR}	Inwardly rectifying potassium channel
K_V	Voltage gated potassium channel
M	Muscarinic
MAO	Monoamine oxidase
MAPK	Mitogen activated protein
MLC	Myosin light chain
MLCK	Myosin light chain kinase
mRVP	Mean right ventricular pressure
NA	Noradrenaline
Na^+	Sodium ion
NANC	Non-adrenergic non-cholinergic system
NO	Nitric oxide
PAH	Pulmonary arterial hypertension
PAP	Pulmonary artery pressure
PASMC	Pulmonary artery smooth muscle cells
PE	Phenylephrine
PH	Pulmonary hypertension
PDE	Phosphodiesterase
PGE	Prostaglandin E (PGE)
PGF2α	Prostaglandin F2α
PGI_2	Prostacyclin
PI	Phosphatidylinositol
PIP_2	Phosphotidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C

PLC	Phospholipase C
pCO_2	Partial pressure of carbon dioxide
pO_2	Partial pressure of oxygen
PSS	Physiological salt solution
PVR	Pulmonary vascular resistance
ROCC	Receptor operated calcium channel
RV/LV+S	Ratio of right ventricle to left ventricle plus septum
RVH	Right ventricular hypertrophy
RV	Right ventricle
LV	Left ventricle
LV+S	Left ventricle plus septum
RVP	Right ventricular pressure
SEM	Standard error of the mean
SK_{Ca}	Small conductance calcium activated potassium channels
SMC	Smooth muscle cell
SOCC	Store operated calcium channel
TEA	Tetraethylammonium
TM	Transmembrane
Tph	Tryptophan hydroxylase
TALK	Two-pore domain alkaline-activated potassium channel
TASK	Two-pore domain acid-sensitive potassium channel
THIK	Two pore domain halothane inhibited potassium channel
TRAAK	TWIK-1 related arachidonic acid-stimulated potassium channel
TRESK	TWIK-related spinal cord potassium channel
TREK	TWIK-1 related potassium channel
TWIK	Tandem pore weakly inward-rectifying potassium channel
VIP	Vasoactive interstitial peptide
VOCC	Voltage operated calcium channel
WT	Wild type

Chapter 1

Introduction

There has been a great amount of research into the pulmonary circulation in the past 50 years. There is now a greater understanding of the mechanisms responsible for controlling pulmonary vasomotor tone and vascular growth, and this has led to new approaches for the treatment of pulmonary circulation disorders.

1.1 Anatomy of the pulmonary circulation

The pulmonary circulation transmits virtually the entire cardiac output from the right side of the heart to the left side (figure 1.1). The pulmonary artery (PA) leaves the right ventricle and bifurcates before entering the lungs. The left branch extends into the hilum of the left lung, and here it divides in two, with a branch extending into each lobe. The right PA also divides into two branches, one branch enters the middle and lower lobe and the second branch enters the upper lobe of the right lung. The pulmonary arteries then run parallel to the bronchial tree until the terminal alveoli are reached. At this stage the arterioles become capillaries, which form a mesh like framework around individual alveoli, to facilitate gaseous transfer. Thereafter venules emerge, and these vessels become veins. Four main pulmonary veins leave the lungs, and these convey the oxygenated blood into the left atrium of the heart, (Berne & Levy, 1998). There are seventeen orders of pulmonary arterial vessels (Singhal *et. al .,* 1973).

The pulmonary circulation is composed of arteries, capillaries and veins. PAs have walls made up of three layers; tunica intima, tunica media and tunica adventitia (figure 1.2). The intimal layer is composed of a flat sheet of endothelial cells, above a layer of connective tissue. The medial layer is bound by the internal and external laminae and provides the vessel with mechanical strength and the ability to contract. Within the medial layer, smooth muscle cells (SMCs) are found embedded within a matrix of elastin and collagen fibres. The adventitial layer is composed of connective tissue, and holds the vessel in place. The adventitia of the normal PA contains a range of fibroblast subpopulations (Stenmark *et. al.,* 2002).

Within the pulmonary arterial system the small muscular arteries have a much thinner muscular media, with less elastin, and fewer SMCs than would be found in comparable systemic vessels (Berne & Levy, 1998). The vessels arising immediately from the main pulmonary arteries, down to arteries of 1 mm internal diameter, are referred to as elastic arteries. This is due to their elastic structure, composed of a thin intima, thick adventitia and media layer composed of elastic laminae layers and SMCs. Arteries of internal diameters 100 μm – 1 mm are mostly muscular, having ~ six SMC layers, bound by distinct internal and external laminae (Brenner *et. al.,* 1935; Heath & Edwards, 1958). Distally, towards the apex of the lungs, arteries become less muscular (Meyrick *et. al.,* 1983). Beyond the terminal bronchioles, within the respiratory acinus, arteries are partially muscularised as the vascular SMC layer is reduced. For example, pulmonary arteries that have internal diameters of > 100 μm have single elastic laminae, with few SMCs (Heath & Edwards, 1958). The intra-acinar arteries have no SMCs present (Demello *et. al.,* 1997). The pulmonary vascular system has a large network of 'sheet-like' capillaries. They are in close connection to the alveoli, and hence the surface area for gas exchange is very vast at 90-126m^2 in adults (Levick, 2000).

1.2 Function of the pulmonary circulation

The main function of the pulmonary circulation is gas exchange. Carbon dioxide from venous blood diffuses through the interstitial space and alveoli walls into the alveoli, and oxygen is taken up from the alveoli into the PA in the opposite direction. The pulmonary circulation receives the entire cardiac output, with effectively five litres passing through this system per minute (Berne & Levy, 1998). An important factor to take into consideration is the ventilation to perfusion ratio, which is essentially a measurement of the efficiency of gas exchange (Berne & Levy, 1998). When the pulmonary system is functioning efficiently the ventilation to perfusion ratio is approx. 0.8 in man, which occurs when ventilation is four litres per minute and the cardiac output is five litres per minute (Levick, 2000). The pulmonary circulation must accommodate each stroke volume whilst maintaining a low arterial pulmonary pressure.

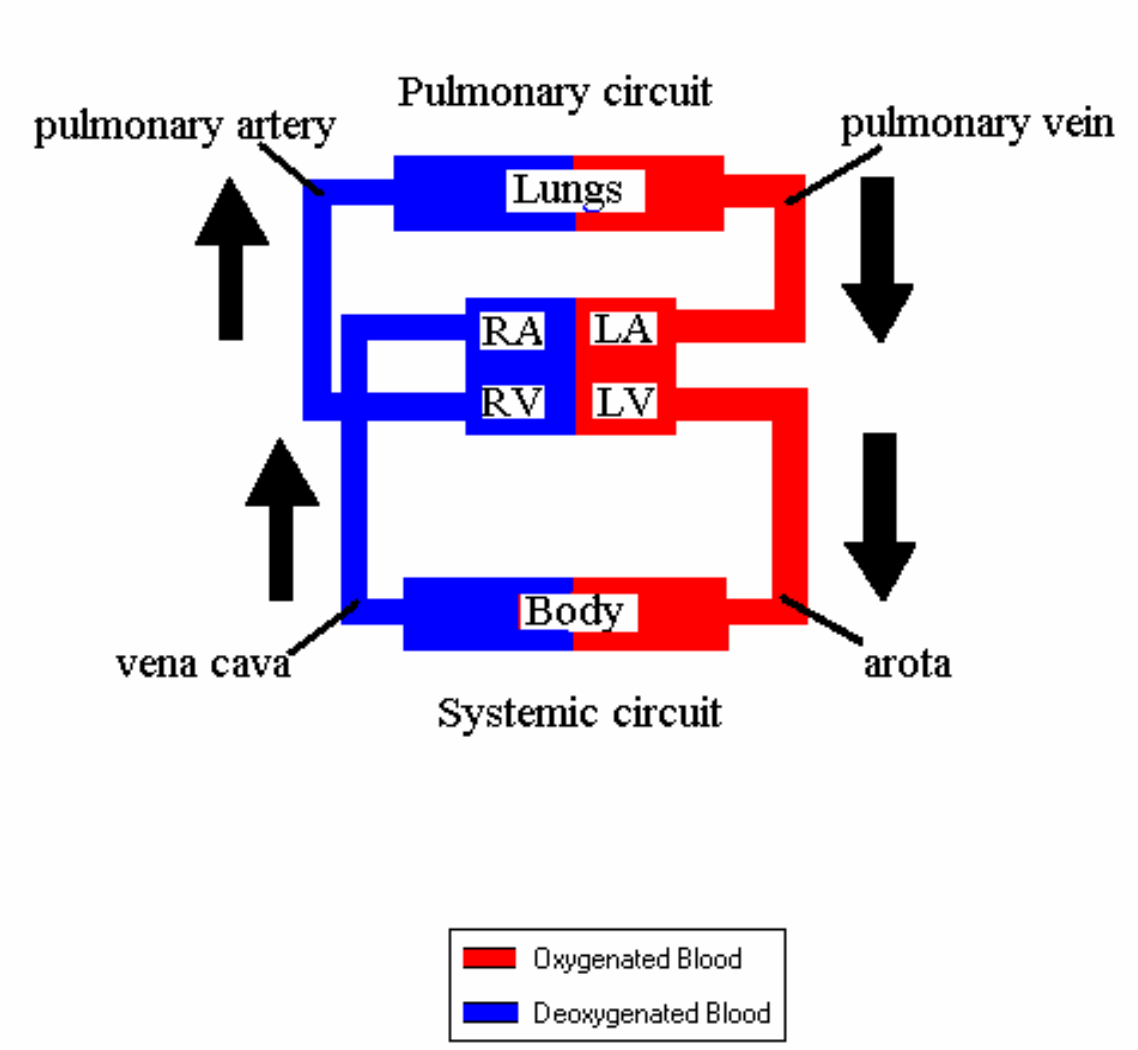


Figure 1.1 Diagram of the circulation system.

RA: right atrium, LA: left atrium, RV: right ventricle, LV: left ventricle

Deoxygenated blood flows from the right ventricle of the heart into the PA. It passes to the pulmonary capillaries, upon which carbon dioxide is offloaded and oxygen is taken up into the blood. The oxygenated blood then flow into the left side of the heart, and is subsequently pumped through to the systemic circuit.

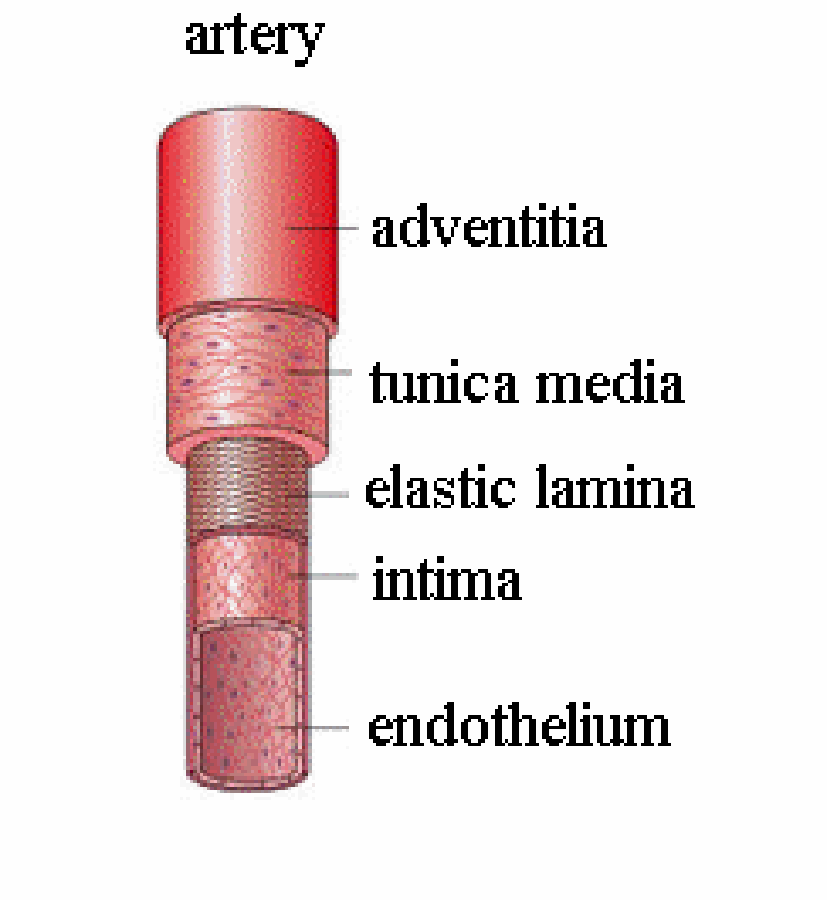


Figure 1.2 Illustration of the structure of a pulmonary artery.

This picture illustrates the position of the layers of cells which make up the pulmonary artery wall.

The pulmonary circulation is also involved in non-respiratory functions. The endothelium and blood act as metabolic filters, converting hormones and other signalling molecules between active and inactive states. For example, angiotensin converting enzyme, found on pulmonary endothelium cells, is involved in the production of angiotensin II and inactivates bradykinin (Johnson & Erdos, 1997; Stewart, 1976; Cushman *et. al.,* 1971). The lung is also the major site of 5-HT removal from the bloodstream via uptake by the transporters on lung cells (Alabaster & Bakhle 1970). Endothelial cells, SMCs, platelets and fibroblasts all have 5-HT transporters (5-HTTs) on their plasma membranes (Strum & Junod, 1972; Bryan-Lluka *et. al.,* 1995; Welsh *et. al.,* 2004).

1.3 Physiology of the pulmonary circulation

The normal pulmonary circulation is known to be a high flow, low resistance circuit, which is very different to that of the systemic circulation. The total blood volume in the pulmonary circulation is approximately 10 % of the total blood circulating in the body. The low resistance ensures that the entire cardiac output can be handled by the lung circulation without causing the right ventricle to do excessive work (Berne & Levy, 1998).

The mean pulmonary arterial pressure in the resting normal adult is 15 mmHg, whereas in the systemic circuit the mean arterial pressure is 100 mmHg (Morgan, 2004). Mean pulmonary arterial pressure (MPAP) can be calculated from the measured values of diastolic pulmonary pressure (DPAP) and systolic pulmonary pressure (SPAP) using equation 1.

(equation 1) $$MPAP = DPAP + (SPAP - DPAP)/3$$

The flow of blood through the pulmonary circulation is influenced by the difference in pressure between the pulmonary arteries and the pulmonary veins. The pressure difference is referred to as the perfusion pressure. Also affecting the blood flow in the pulmonary circuit is the resistance imposed by the blood vessels. In the systemic system most resistance to blood flow lies within the pre-capillary muscular arterioles (~ 70 %), however in the pulmonary system the resistance is more equally distributed between arteries, capillaries and veins (Lumb, 2000). Levick, (2000) suggests that the pressure is divided

between the vessels in the following fashion; arterial vessel 30 %, microvasculature 50 % and veins 20 %. As the pre-capillary segment contributes to the majority of pulmonary vascular resistance it follows that a small change in tone in this area can lead to large rises in pulmonary arterial pressure, as can be seen using Poiseuille's law for steady flow (see equation 2 where L is vascular length, η is blood viscosity, r is vessel radius).

(equation 2) $$PVR = 8\eta L/ \pi r^4$$

1.3.1 Regulation of pulmonary blood flow

As the entire CO flows past the alveoli, perfusion greatly exceeds nutritional needs. This means that metabolic factors exert no influence on flow. The metabolic needs of the bronchi are met via a second distinct blood flow, an independent systemic bronchial circulation (Berne & Levy, 1998). The pulmonary circulation is under the control of passive and active factors.

1.3.1.1 Passive factors regulating the control of pulmonary blood flow

These are the factors that induce a change in the pulmonary vessel resistance, independent of the active contraction of SMCs. Blood flow through the lungs is unevenly distributed in a normal upright individual. Gravity has an effect on blood distribution, as lungs are located above and below the point where the PA leaves the heart. At the apex of the lung the pressure is lower relative to that at the base. This is due to the hydrostatic pressure on blood being pumped through this circuit (Morgan, 2004). Above the heart, as the pressure on the blood in the pulmonary arterial system is low, the pressure of air in the alveoli will have an impact on perfusion. As interstitial pressure is more negative at the apex, the relative alveolar pressure will be greatest at this level. This can cause the individual alveoli to be fully distended, which can lead to compression of the capillaries, as these are thin and soft walled. The variance of pressure in the pulmonary vascular system within the lung is best described by dividing the lung into three distinct regions/ zones:

- Zone 1- (Apex) Alveolar pressure ≥ Arterial pressure > Venous pressure

  Alveolar pressure exceeds both arterial pressure and venous pressure, which results in capillaries being relatively compressed. Therefore in zone 1 blood flow will be low or non-existent.

- Zone 2- (Heart level) Arterial pressure > Alveolar pressure > Venous pressure

Arterial pressure exceeds alveolar pressure and blood flows progressively along the pulmonary vascular system depending on arterial-alveolar pressure difference. Blood flow through capillaries will behave as a starling resistor when alveolar pressure exceeds capillary pressure. The starling resistor phenomenon describes how flow through a collapsible tube surrounded by a pressure chamber is determined by the upstream pressure minus the pressure chamber, as opposed to the downstream pressure, when chamber pressure exceeds downstream pressure (Berne & Levy, 1998). As long as capillary pressure is higher than alveolar pressure at the entrance and lower than alveolar pressure at the end of the capillaries, the capillaries are open and blood will flow, but when alveolar pressure is higher than the PA pressure, the capillaries will be completely closed.

- Zone 3- (Base) Arterial pressure > Venous pressure > Alveolar pressure

As blood flows towards the bottom of the lung because of gravity, venous pressure increases. Blood flow is continuous and all vessels are open because both arterial and venous pressures exceed alveolar pressure. (Morgan, 2004; Berne & Levy, 1998).

1.3.1.2 Active regulation of pulmonary blood flow

Whilst the pulmonary blood flow is mainly controlled normally by the passive factors described above, active factors can affect vascular resistance by acting to constrict or relax pulmonary artery smooth muscle cells (PASMCs). Active factors include autonomic nerves, humoral factors, the endothelium and respiratory gases.

The pulmonary vasculature is innervated by components of the autonomic nervous system; sympathetic (adrenergic) and parasympathetic (cholinergic) nerves (Hebb, 1969), and a third, non-adrenergic non-cholinergic system (NANC) has also been identified (Kobayashi & Amenta, 1994). However, the extent of innervation is regional and species specific (Kobayashi & Amenta, 1994; Barnes & Liu, 1995). Species variation can be seen from differences in sympathetic innervation; sheep, guinea pig and canine pulmonary arteries are richly innervated whereas calf and swine PA have sparse innervation (Hebb, 1969); however there is a complete lack of innervation within the intralobar vessels of rat (McLean

et. al., 1985) while human intrapulmonary arteries have been shown to be densely innervated. The neurotransmitter released from the sympathetic nerve fibres serving the pulmonary circulation is noradrenaline (NA). Species variation also arises from the receptors for this catecholamine within the pulmonary vasculature (Kobayashi & Amenta, 1994; Barnes & Liu, 1995). α- and β-adrenoceptors mediate NA responses, with α-receptors mediating vasoconstriction and β-receptor stimulation resulting in vasodilation. It has been suggested that sympathetic activation leads to an increase in vascular resistance and a decrease in vascular compliance, and hence an increase in pulmonary arterial pressure (Barnes & Liu, 1995), resulting mainly from α-receptor stimulation (Ingram *et. al.,* 1968; Hyman, 1986). Vasodilation upon β-receptor stimulation plays a lesser role in pulmonary vascular tone regulation as it only occurs upon α-receptor blockade in the feline vascular bed (Hyman *et. al.,* 1981). When β-receptors are inhibited the level of constriction in response to sympathetic stimulation is increased (Hyman *et. al.,* 1986).

The parasympathetic influence is less than that of the sympathetic system (Barnes & Liu, 1995), although there is both species and regional variation in this system within the pulmonary vasculature too. Regional variation is seen in mouse where intralobar arteries have no parasympathetic innervation, while extralobar arteries and large pulmonary veins from these animals are severed by this part of the autonomic nervous system (Cech, 1973). Parasympathetic innervation of the pulmonary vascular system has been shown in many animal groups, including rabbit, dog and human (Cech, 1973; Fillenz, 1970), however in rat the parasympathetic innervation only serves the pulmonary vein (Bradley *et. al.,* 1970). Upon stimulation of the parasympathetic nervous system, acetylcholine (ACh) is released (Kobayashi & Amenta, 1994). This neurotransmitter has been found to induce both vasodilation and vasoconstriction of the pulmonary circulation. In the human pulmonary system the response to ACh is vasodilation (Nandiwada *et. al.,* 1983), however in the rabbit pulmonary vasculature ACh induces vasoconstriction (Sada *et. al.,* 1987). This difference in ACh response within the pulmonary vasculature has been shown to be the result of varying levels of pre-existing tone. The constrictor response is generated under normal physiological conditions while relaxation is the response seen under conditions of increased tone (Barnes & Liu, 1995). It has been shown that in human isolated pulmonary arteries

where tone was induced, ACh caused endothelium-dependent relaxation (Greenberg *et. al.,* 1987; Thom *et. al.,* 1987). However, at basal tone or after removal of endothelium, ACh induced contraction in human pulmonary arteries (Norel *et. al.,* 1996). Muscarinic (M) receptors on endothelial and SMCs mediate the ACh response (Altiere *et. al.,* 1994), and it has been shown that species variation exists in the muscarinic receptor types that mediate ACh responses. M3 receptors are thought to be the main receptors involved in the contraction of human and rabbit isolated PAs in response to ACh (Norel *et. al.,* 1996; Altiere *et. al.,* 1994), whereas both M1 and M2 receptors have been proposed to be involved in the ACh induced increases in pulmonary vascular resistance in canine pulmonary circulation (El-Kashef *et. al*., 1991). With regards to ACh induced relaxation responses, M3 receptors have been implicated in rat and rabbit pulmonary arteries (MacCormack *et. al.,* 1988; Altiere *et. al.,* 1994), while in human isolated pulmonary arteries this response has been linked to both M3 and M1 receptors (Norel *et. al.,* 1996). The parasympathetic nervous system is thought to have a minor influence on pulmonary vascular tone, as blockade of these fibres does not result in an increase in arterial pressures (Murray *et. al.,* 1986).

NANC control has been found within the pulmonary circuit. This is thought to be manifestations of neurotransmission in sympathetic, parasympathetic and sensory nerves (Barnes & Liu 1995). NANC autonomic innervation has been demonstrated to mediate relaxation of pulmonary arteries (Liu *et. al.,* 1992; Scott *et. al.,* 1996; Gumusel *et. al.,* 2001). Numerous compounds have been proposed as NANC neurotransmitters, including substance P, adenosine trisphosphate (ATP), calcitonin gene related peptide, nitric oxide (NO) and adenosine (Kobayashi & Amenta 1994; Barnes & Liu 1995). It has been shown that NANC relaxation exists in human and rat pulmonary arteries, where it is partly mediated through NO (Scott *et. al.,* 1996; Gumusel *et. al.,* 2001). In guinea pig pulmonary arteries a study showed that NANC relaxation is mediated predominantly through NO (Scott & McCormack 1999), although Liu *et. al.,* (1991; 1992) suggest that different mechanisms are involved in mediating NANC relaxation in main and branch PA of guinea pig. In the branch PA, this group propose that NANC relaxation is mediated by ATP, whereas in the main PA it is likely to be mediated by release of peptides such as calcitonin

gene related peptide. It is thought that NANC vasodilator nerves represent the major neural vasodilator mechanism within pulmonary vessels, therefore any alteration in this system could result in vasoconstriction (Barnes & Liu, 1995).

There are a number of endogenous agents that alter vascular tone by relaxing or contracting the SMCs within the vessel wall. The action of these endogenous agents can vary between species and depend on the level of pre-existing tone. These humoral factors are secreted from the endothelium, the lung parenchyma and the rest of the circulation and have various effects, mediated by receptors on either endothelial cells or SMCs. Within the normoxic pulmonary circulation the balance between these vasodilators and vasoconstrictors is shifted towards vasodilation and low basal tone. Humoral factors that in general induce vasoconstriction include: angiotensin II (Morrell *et. al.,* 1995), 5-hydroxytryptamine (5-HT) (MacLean *et. al.,* 1994; 1996), thromboxane A_2 (Buzzard *et. al.,* 1993), neuropeptide Y (Obara *et. al.,* 1989; Martling *et. al.,* 1990). Vasodilation has been shown to occur in response to many vasoactive mediators, for example atrial natriuretic peptide (Lindberg & Andersson 1988), vasoactive intestinal peptide (Nandiwada *et. al.,* 1985; Martling *et. al.,* 1990), prostacyclin (PGI_2) (Hyman & Kadowitz 1979) and NO (Cremona *et. al.,* 1999; Ferrario *et. al.,* 1996), bradykinin (Lippton *et. al.,* 1984), histamine (Abcioglu *et. al.,* 1987), substance P (Archer *et. al.,* 1986a), prostaglandin E (PGE) (Jourdan *et. al.,* 1997). In some cases humoral factors can induce both constriction and relaxation of the pulmonary vasculature, suggesting species variation. For example ATP has a constrictor action on adult cat pulmonary vascular beds (Lippton *et. al.,* 1992), however in fetal sheep pulmonary vasculature it induces relaxation (Konduri *et. al.,* 1992a).

1.3.1.3 Endothelial cell control of pulmonary blood flow

Endothelial cells influence the tone of SMCs via generation, metabolism and degradation of many vasoactive substances. The importance of the endothelium in regulating tone in the lung circulation was highlighted with the discovery that the endothelium released PGI_2 and thromboxane A2 (Gossage *et. al.,* 1994; Prins *et. al.,* 1994). Another major finding was that PGI_2 was released by endothelial cells in response to shear stress within pulmonary vessels (Frangos *et. al.,* 1985). In addition, PGI_2 was found to be an important hormone for

the pulmonary vasodilation that occurs upon birth (Cassin *et. al.,* 1993). Endothelial cells are directly exposed to the shearing forces of blood flow. In response they synthesise and release vasoactive substances, which act to allow local control of flow (Busse & Fleming, 2003). By releasing mediators in a paracrine manner, endothelial cells can influence local vasodilation or vasoconstriction of the pulmonary circulation. The release of these mediators can be induced by other vasoactive substances, for example, acetylcholine acts on endothelial cells to induce the release of NO, which in turn relaxes smooth muscle (McMahon *et. al.,* 1992; Gambone *et. al.,* 1997). Another relaxing factor arising from endothelial cells in response to ACh is endothelium-derived hyperpolarising factor (EDHF) (Chen *et. al.,* 1988). NO and PGI_2 have been well characterised. NO is synthesised by NO synthase (NOS) and is continuously released from endothelial cells. NO acts to promote increased levels of cyclic guanosine monophosphate (cGMP), a second messenger within SMCs. PGI_2 is synthesised by cyclo-oxygenase (COX) and causes vasodilation via the second messenger cyclic adenosine monophosphate (cAMP). cGMP and cAMP mediate part of the response to NO and PGI_2, by causing hyperpolarisation of the SMC, and hence induce vasodilation (Feletou & Vanhouttte, 2000). The exact pathway by which EDHF mediates vasodilation is unknown, however it acts to induce NO- and PGI_2 -independent vasodilation. EDHF has been shown to induce relaxation by hyperpolarising the SMC membrane by opening K^+ channels (Garland *et. al.,* 1995). Several reports indicate that the K^+ channels involved in the EDHF response are sensitive to the combination of charybdotoxin, which inhibits large conductance calcium (Ca^{2+}) activated K^+ channels (BK_{Ca}), and apamin, which blocks small conductance Ca^{2+} activated K^+ channels (SK_{Ca}) (Nelson & Quayle, 1995; Cook & Quast, 1990). The K^+ channels that mediate EDHF responses differ depending on the vascular bed and species studied. For example, in rat PA, Ca^{2+} activated K^+ channels (K_{Ca}) mediate EDHF (Karamsetty *et. al.,* 2001), whereas in the canine PA preparation, ATP activated K^+ channels have been shown to mediate EDHF responses (Gambone *et. al.,* 1997).

The endothelium also has a metabolic function and thereby regulates the amount of vasoactive substances circulating in the blood stream. This is brought about by enzymes on the endothelial cell membrane acting on substances within the blood (Kobayashi & Amenta

1994), some of which are discussed above. By transporting hormones/ mediators into the endothelial cytoplasm, vasoactive components can be removed from the blood stream, for example 5-HT and NA are removed by the endothelium via endothelial uptake mechanisms (Alabaster & Bakhle, 1970). The endothelial cell layer behaves as a structural barrier between the bloodstream and the underlying SMCs. An example of the importance of this barrier can be seen from the fact that bradykinin induces endothelium cell dependent vasodilation in the pulmonary circulation, however when the endothelium is removed this agonist induces constriction (Altura & Chand, 1981). Any damage or dysfunction of the endothelial layer in the pulmonary circulation may therefore have profound effects on vascular tone. Hence the endothelium is of extreme importance in the maintenance of low basal tone via modulation of nervous, humoral and mechanical influences

1.3.1.4 Hypoxic control of pulmonary blood flow

The composition of, and particularly the partial pressure of oxygen within inspired air can affect tone of the pulmonary circulation. Hypoxia (condition where the partial pressure of oxygen level is low), induces vasoconstriction within the pulmonary circulation (Fishman, 1961), whilst causing vasodilation in most systemic vascular beds. The pulmonary arterial system responds to hypoxia in a distinct manner, which is known as hypoxic pulmonary vasoconstriction (HPV). Before birth HPV has a physiological role in diverting blood flow from the lungs to the systemic circulation. The function of HPV after birth is to match ventilation to perfusion, and it does this by changing the vascular resistance in reaction to changes in local oxygen content. HPV can be defined as the rapid, reversible increase in pulmonary vascular resistance. Acute ventilatory hypoxia causes a rapid increase in PVR, which plateaus within twenty minutes, and is then maintained. Upon return to normoxia PVR falls promptly to normal physiological levels (Ward & Aaronson, 1999).

As HPV occurs in isolated lungs (free of nerve innervation and blood), isolated arteries and PASMCs, it is thought that the process is intrinsic to the PASMCs (Archer, 2001, Madden *et. al.,* 1992). The HPV response varies in relation to the diameter of the vessel involved; small intrapulmonary resistance arteries (IPAs) are thought to be the main site of HPV (McCulloch *et. al.,* 2000). This allows for regional control of blood flow without leading to

elevation of the entire pulmonary vascular resistance and hence doesn't cause the consequent increase in right heart work (Archer, 2001). The exact mechanisms underlying HPV remain unresolved. It is well known that an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) is involved in the HPV response, however, the mechanisms by which this rise in Ca^{2+} arises is debated. One hypothesis for HPV induced increase in $[Ca^{2+}]_i$, is that Ca^{2+} entry is mediated primarily via voltage dependent L-type channels (McMurtry *et. al.,* 1976), as a result of hypoxia-induced inhibition of voltage-gated K^+ channels (K_V channels) and resultant depolarisation of the cell membrane. However, studies have shown that blockers of L-type Ca^{2+} channels only result in a minimal inhibition of the HPV response (Ward & Aaronson 1999; Ward *et., al.,* 2005). Other studies have suggested a role for Ca^{2+} from intracellular stores in the HPV response (Jabr *et. al.,* 1997; Liu *et. al.,* 2001). It has also been suggested that mitochondria play a role in the HPV response, with mitochondria responding to alterations in oxygen tension, resulting in the generation of a signal that modulates redox-sensitive K^+ channels, hence controlling membrane potential and therefore Ca^{2+} influx (Archer & Michelakis, 2002).

1.4 Pulmonary artery smooth muscle contraction

Contraction of PASMCs can be initiated by mechanical, electrical, and chemical stimuli. PASMC contraction is initiated by a rise in free $[Ca^{2+}]_i$ (Hirano *et. al.,* 1991), which can arise from flux of Ca^{2+} into the cells via Ca^{2+} channels or from intracellular stores such as the endoplasmic reticulum. Calmodulin (CaM) is an intracellular Ca^{2+}-binding protein, which binds to Ca^{2+} as the concentration of Ca^{2+} rises within the cell. Ca^{2+}-CaM activates myosin light chain kinase (MLCK). MLCK phosphorylates myosin light chain (MLC) in the presence of ATP, thereby activating cross-bridge cycling and contraction (Hartshorne *et. al.,* 1987). Phosphorylation of MLC is reversed by myosin phosphatase (MLCP) (Ito *et. al.,* 1991). MLCK and MLCP exert a balancing effect, allowing contraction and relaxation to occur. Many drugs that induce SMC contraction and relaxation do so via G-protein coupled receptors (GPCRs).

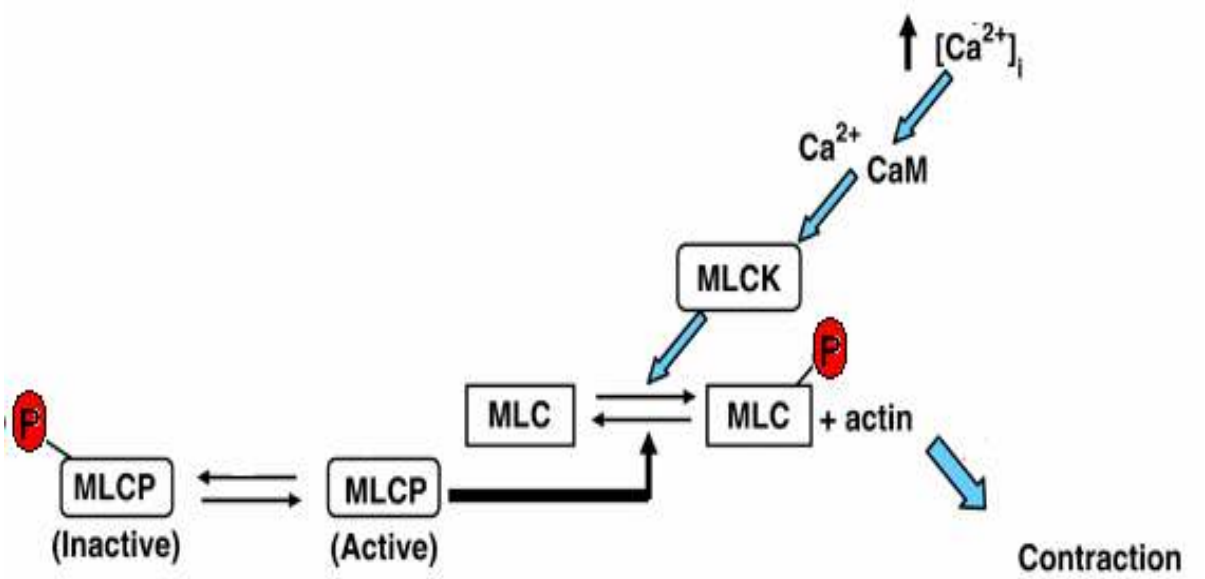


Figure 1.3 Mechanism of SMC contraction.

The level of free $[Ca^{2+}]_i$ is a major determinant of SMC contraction. Ca^{2+} binds and forms a complex with calmodulin (CaM). CaM activates MLCK, which subsequently phosphorylates MLC. This stimulates myosin ATPase activity and promotes crossbridge cycling with actin filaments. The formation of these crossbridges underlies SMC cell contraction. Phosphorylation of MLC is reversed by MLCP. Figure adapted from Nahorski, (2006).

Revised Clinical Classification of Pulmonary Hypertension
(World Health Organisation World Symposium on PAH Venice 2003)

1. **Pulmonary Arterial Hypertension (PAH)**
 1.1 Idiopathic (IPAH)
 1.2 Familial (FPAH)
 1.3 Associated with:
  1.3.1 Collagen Vascular Disease
  1.3.2 Congenital systemic to pulmonary shunts
  1.3.3 Portal hypertension
  1.3.4 HIV infection
  1.3.5 Drugs & toxins
  1.3.6 Other (Thyroid disorders, Glycogen Storage Disease, Gaucher's Disease, Hereditary Hemorrhagic Telangiectasia, Hemoglobinopathies, Myeloproliferative Disorders, Splenectomy)
 1.4 Associated with significant venous or capillary involvement
  1.4.1 Pulmonary veno-occlusive disease
  1.4.2 Pulmonary capillary hemangiomatosis
 1.5 Persistent pulmonary hypertension of the Newborn

2 **Pulmonary Venous Hypertension**
 2.1 Left-sided atrial or ventricular heart disease
 2.2 Left-sided valvular heart disease

3. **Pulmonary Hypertension associated with hypoxemia**
 3.1 Chronic obstructive pulmonary disease
 3.2 Interstitial lung disease
 3.3 Sleep-disordered breathing
 3.4 Alveolar hypoventilation disorders
 3.5 Chronic exposure to high altitude
 3.6 Developmental abnormalities

4. **Pulmonary Hypertension due to chronic thrombotic and/or embolic disease**
 4.1 Thromboembolic obstruction of proximal pulmonary arteries
 4.2 Thromboembolic obstruction of distal pulmonary arteries
 4.3 Non-thrombotic pulmonary embolism (tumor, parasites, foreign material)

5. **Miscellaneous**
Sarcoidosis, Histiocytosis X, Lymphangiomatosis, Compression of pulmonary vessels (adenopathy, tumor, fibrosing mediastinitis)

Table 1.1 Revised Clinical Classification of Pulmonary Hypertension.

This classification identifies different categories of pulmonary

1.5 Pulmonary Arterial Hypertension

Pulmonary arterial hypertension (PAH) represents Group 1 within the Pulmonary Hypertension (PH) World Health Organisation clinical classification system (see table 1.1). It is a disease which occurs when pulmonary arterial pressure (PAP) increases above 25 mmHg at rest or 30 mmHg during exericise (Barst *et. al.,* 2004). Dresdale, Michtom and Schultz, (1954) were the first to use the term primary pulmonary hypertension, and they began to investigate how to treat it. They treated PPH patients with a pulmonary vasodilator drug, tolazoline, which gave relieve to the condition. Harris *et. al*. (1957), investigated the potential of the vasodilator drug, acetylcholine. This drug was given via the intravascular route, and degraded naturally during first pass of the lungs. Harris *et. al*. (1957) showed that under normoxic conditions, acetylcholine had no effect. On the other hand, during conditions of hypoxia, acetylcholine caused vasodilation (Fishman, 1976).

1.5.1 Genetic Basis of pulmonary arterial hypertension

PAH has been shown to occur in families, hence the term *familial* PAH (FPAH), which accounts for 6% of PAH cases (Rich *et. al.,* 1987). The inheritance pattern is autosomal dominant, with a female to male ratio of 2:1 (Loyd *et. al.,* 1984). Within FPAH there is genetic anticipation, in that the disease occurs at younger ages (Gaine & Rubin, 1998). A marker for the disease was found on chromosome 2q31-32 (Morse *et. al.,* 1997). This led to the finding of a mutation in the bone morphogenetic protein type 2 receptor (BMPR2) gene, in association with FPAH (Lane *et. al.,* 2000). Another possible modulating influence may be changes in the gene encoding the 5-HTT. 5-HTT is coded by a single gene on chromosome 17q11.2 and a variant in the promoter region has been shown (Lesch *et. al.,* 1996). A polymorphism with long (L) and short (S) forms affect the expression of the 5-HTT, with the L allele encoding for increased expression of the transporter (Eddahibi *et. al.,* 2001). Genetic predisposition does not automatically lead to FPAH. Less then 20% of individuals with a BMPR2 mutation go on to develop the disease. Moreover, most individuals who develop the disease do not have an identified genetic mutation (Thomson *et. al.,* 2000). PAH can occur secondary to other events and these may include exposure to exogenous stimuli, for example hypoxia (Chan & Loscalzo, 2008).

1.5.3 Epidemiology of pulmonary arterial hypertension

PAH is a progressive disease that cannot be cured. The mean age of developing PAH is 36, however it can develop at any age (Rich *et. al.,* 1987) and is independent of race (Rich *et. al.,* 1987).

PAH has an estimated prevalence of 30 - 50 cases per million (Peacock, 2003). PAH patients may develop some of the following symptoms: breathlessness, dizziness, fainting, chest pain, palpitations and increased lethargy. As these symptoms are non-specific, i.e., they may be caused by conditions other than PAH, diagnosis is extremely difficult. Right heart catheterisation is essential to confirm diagnosis and determine prognosis (Gaine, 2000). This investigation measures mean pulmonary arterial pressure, right atrial pressure and cardiac output. There are four functional classes for PAH in table 1.2 with class I being the least severe and class IV being the most advanced.

The prognosis for patients with PAH is poor, with the median length of survival after diagnosis being 2.8 years (Gaine *et. al.,* 1998). The current drug therapy for PAH depends on the underlying disease (see table 1.1), and treatment is dictated by severity of disease and symptoms. Available drugs used to treat PAH fall into one of four pharmacological classes, PGI_2 analogues, endothelin receptor antagonists, phosphodiesterase type V inhibitors and Ca^{2+} channel blockers (Takaoka, 2007). The rationale for the use of diuretics is provided because of their ability to reduce blood volume, and therefore blood pressure, which would reduce right ventricular pre-load (Baumhakel *et. al.,* 2005). For PAH patients whose lungs are still vasoreactive, i.e. when their lungs still have the ability to respond to local mediators, targeted drug treatment can be provided. Ca^{2+} channels blockers have been used in the treatment of PAH, however in clinical trials it was shown that only 10 – 25 % of patients with PAH responded to treatment with Ca^{2+} channel blockers (Rich *et. al.,* 1992; Sitbon *et. al.,* 1998). PGI_2 has also been used in the treatment of PAH. This prostanoid has been shown to vasodilate pulmonary vessels, inhibit platelet aggregation and inhibit PASMC proliferation. As PGI_2 is very unstable, more stable analogues have been designed for the treatment of PAH, and include epoprostenol. Epoprostenol has been shown to improve long term survival for patients with PAH (McLaughlin *et. al.,* 2002; Kim *et. al.,*

2003). Epoprostenol was the first treatment approved by the Food and Drug Administration for IPAH, and is suitable for patients with advanced disease (Patients with class IV symptoms – see table 1.2) and for those failing other treatments (O'Callaghan *et. al.,* 2007). A third type of drug therapy includes the use of ET receptor anatgonists, as ET has been shown to induce pulmonary vasoconstriction, and proliferation of fibroblasts and SMCs. ET acts on two receptors on the PA, action on ET-A receptors results in vasoconstriction and proliferation of PASMCs and action on ET-B receptors on endothelial cells results in an increase of NO and PGI_2 production. An increase in circulating ET and ET-A receptors have been shown in patients with PAH (Stewart *et. al.,* 1991). Bosentan is an ET receptor antagonist with a higher affinity for ET-A relative to ET-B (Clozel *et. al.,* 1994), and has been shown to be beneficial in patients with PAH (Rubin *et. al.,* 2002). Another strategy which is being used to treat PAH, is the NO induced vasodilation pathway. This mediator induces vasodilation of the underlying PASMC in part by increasing the levels of cGMP, a second messenger in SMCs. Increased cGMP levels results in PASMC relaxation. cGMP is degradated by the enzyme phosphodiesterase 5 (PDE5), and by inhibiting the action of PDE5, the action of cGMP can be prolonged. Sildenafil is a PDE5 inhibitor and is currently being used to treat patients with PAH. As PDE5 expression is higher in pulmonary vessels relative to other organs (Corbin *et. al.,* 2005), sildenafil induces selective pulmonary vasodilations without undesirable systemic effects (Ghofrani *et. al.,* 2006). In the Sildenafil Use in Pulmonary Hypertension trial, sildenafil was shown to improve exercise capacity, symptoms and heamodynamics in patients with PAH (Galie *et. al.,* 2005).

New York Health Association/ World Health Organisation Classification of functional status of patients with pulmonary hypertension:

I Patients with pulmonary hypertension in whom there is no limitation of usual physical activity; ordinary physical activity does not cause increased dyspnoea, fatigue, chest pain or pre-syncope.

II Patients with pulmonary hypertension who have mild limitation of physical activity. There is no discomfort at rest, but normal physical activity causes increased dyspnoea, fatigue, chest pain or pre-syncope.

III Patients with pulmonary hypertension who have a marked limitation of physical activity. There is no discomfort at rest, but less than ordinary activity causes increased dyspnoea, fatigue, chest pain or pre-syncope.

IV Patients with pulmonary hypertension who are unable to perform any physical activity and who may have signs of right ventricular failure at rest. Dyspnoea and/or fatigue may be present at rest and symptoms are increased by almost any physical activity.

Table 1.2 New York Heart Association/ World Health Organisation Classification of functional status of patients with pulmonary hypertension.

This classification has been used to determine treatment for patients with pulmonary hypertension. (Table taken from Barst *et. al.,* 2004)

1.5.4 Pathobiology of pulmonary arterial hypertension

PAH has multifactorial pathology. Vascular wall remodelling and vasoconstriction are seen in the PAH disease process (Mandegar *et. al.,* 2004), with pulmonary vasoconstriction believed to be an early characteristic of the disease (Wood, 1958). The primary pathological changes in the vasculature lead to elevation in PVR and PAP. An increase in resistance will result in increased pulmonary pressure. In PAH the increase in PAP leads to an increase in right ventricular after load which is the pressure against which the right ventricle has to work in order to push the blood into the pulmonary circulation. If this continues, over time it leads to right ventricular hypertrophy and reduced contractility. This leads to right heart failure in PAH patients (Mandegar *et. al.,* 2004). Cellular pathway abnormalities have been described that have a major impact during the development of PAH. These include impaired endothelial cell function, dysfunction or altered expression of K^+ channels in PASMCs, altered 5-HTT regulation in PASMCs, oxidant stress and enhanced matrix production (Rubin *et. al.,* 2004). Evidence shows that PAH is associated with a change in both the rates of cell proliferation and apoptosis, which results in thickened and sometimes obstructed pulmonary arteries (Humbert *et. al.,* 2004), and these two processes can involve all cells that make up the vascular walls of the PAs including PASMCs, endothelial cells, fibroblasts and connective tissue fibres. In PAH, pulmonary vascular resistance is increased (Voelkel *et. al.,* 1997) due to vasoconstriction, remodelling and thrombosis.

1.5.4.1 Vasoconstriction

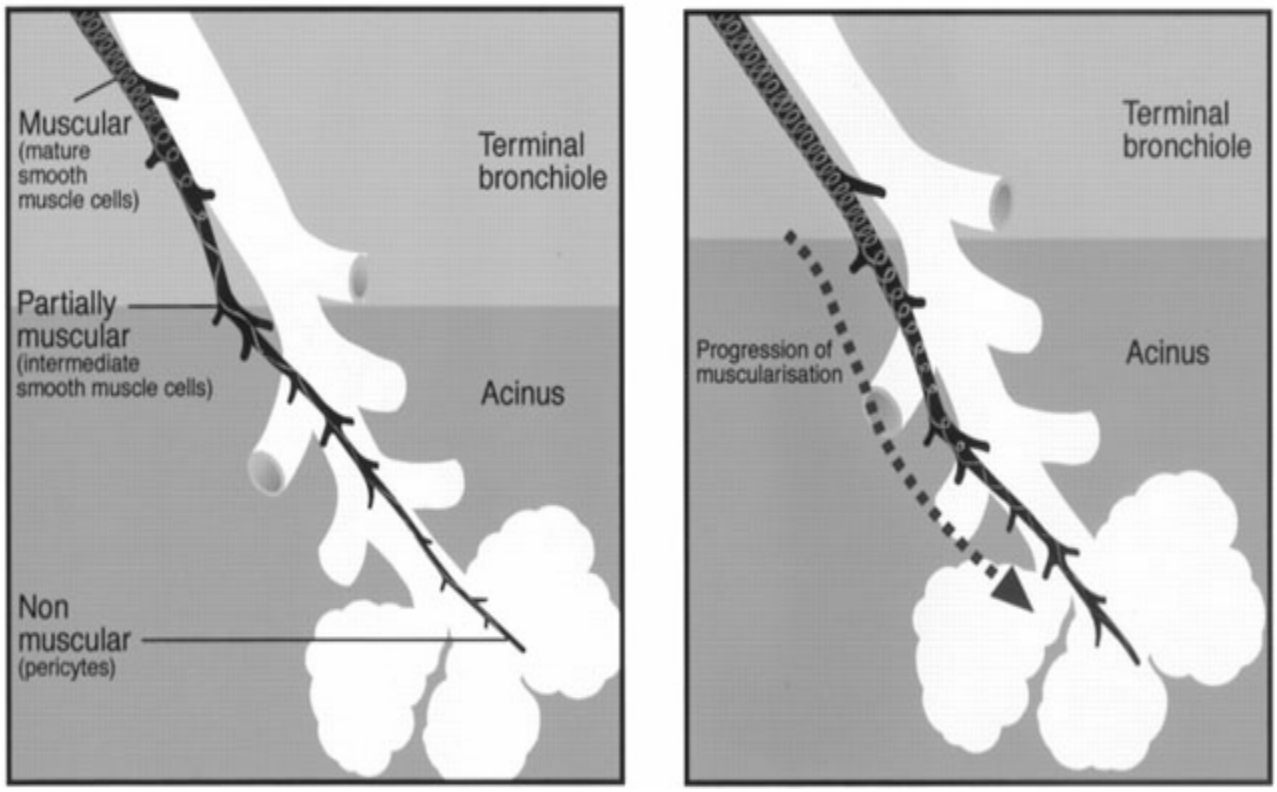
Vasoconstriction is thought to occur early during PAH. The pulmonary vessels constrict and narrow, causing an increase in pulmonary blood pressure and resistance to flow. Dysfunction of PA endothelial cells plays a critical role in the development of PAH. In PAH there is a reduction in the endothelial production of PGI_2 and NO and an increase in the amount of ET produced, resulting in vasoconstriction (Humbert *et. al.,* 2004). The humoral factors that potentiate pulmonary hypertension are generally vasoconstrictors, such as ET (Eddahibi *et. al.,* 1995).

1.5.4.2 Remodelling

Remodelling of blood vessels involves all layers within the blood vessel wall and is complicated by cellular heterogeneity within each compartment of the blood vessel wall (Jeffery *et. al.,* 2002). Within the arteries, there is intimal thickening and fibrosis, medial hypertrophy and adventitial changes (Olschewski *et. al.,* 2001).

The earliest structural change seen in PAH is the muscularisation of the terminal portion of the pulmonary arterial circulation (see figure 1.4). The extension of PASMCs into small peripheral, normally non-muscularised pulmonary arterioles is a common PAH feature (Humbert *et. al.,* 2004). This is due to hyperplasia of SMCs extending distally in a layer internal to the original internal elastic lamina (Heath *et. al.,* 1987), and differentiation of precursor cells into SMCs (Meyrick & Reid, 1980a), and is termed neomuscularisation (figure 1.4). The proximal muscular arteries are subjected to higher intraluminal pressure secondary to the vasoconstriction and remodelling of peripheral arteries, which results in a reduction in vessel lumen diameter due to the hypertrophy of PASMCs (Hislop *et. al.,* 1976). Also seen in PAH is a layer of myofibroblasts and extracellular matrix formation between the endothelium and the internal elastic lamina, known as the neointima (Humbert *et. al.,* 2004). Another remodelling feature is adventitial hypertrophy (Chazova *et. al.,* 1995). In PAH, adventitial thickening with increased extracellular matrix deposition is prominent in the small, muscular PAs (Stenmark *et. al.,* 1997).

These three forms of remodelling can lead to the formation of plexiform lesions within the lungs (figure 1.5 & figure 1.6). These are a mass of disorganised vessels with endothelial cells, SMCs, myofibroblasts, and macrophages. Plexiform lesions are mainly made up of proliferating endothelial cells. These lesions can occlude the lumen of small PAs completely (Tuder *et. al.,* 1994). Plexiform lesions are relatively typical, and they are also seen in other forms of PH (Lee *et. al.,* 1998).



(A) **(B)**

Figure 1.4 Diagrams of pulmonary arteries in the lung.

A. The normal situation of uneven distribution of PASMC phenotypes and non-muscular pre-capillary arterioles.

B. In the pulmonary hypertensive lung there is progression of muscularisation into the non-muscular terminal parts of the arterial tree. (Figure from MacLean *et. al.,* 2000).

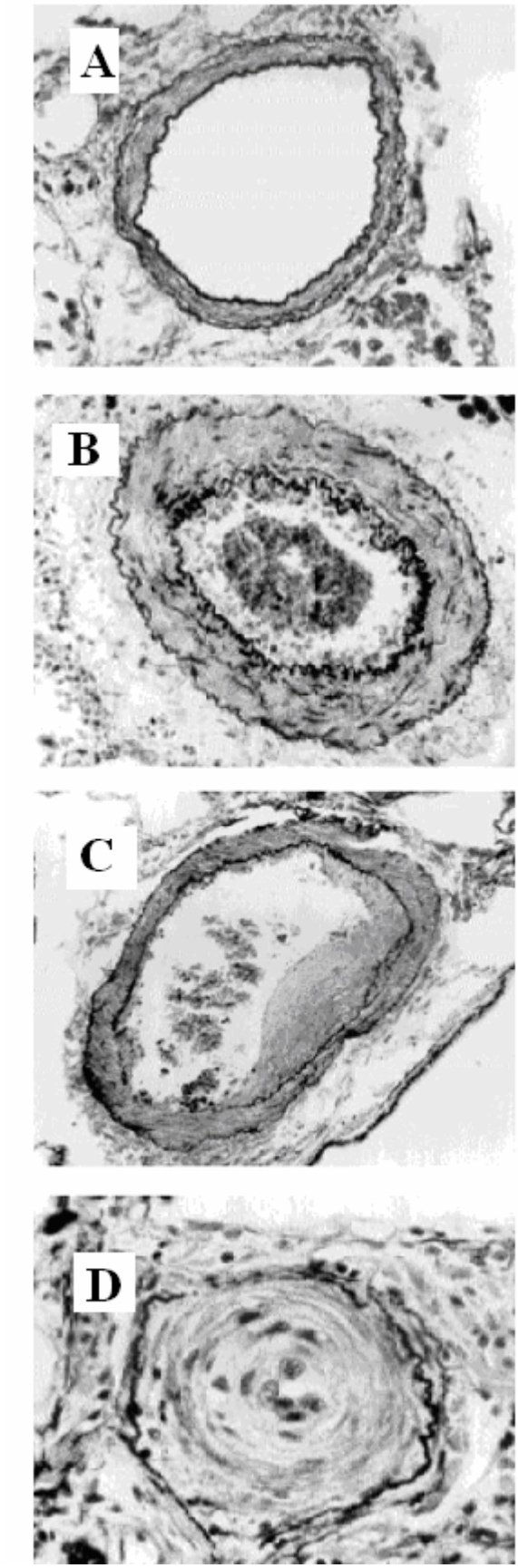


Figure 1.5 Images of remodelling of small muscular PA.
Image taken from NIH Publication No 96- 3291 (1996)

A. Normal small PA
B. PA wall becomes more muscularised
C. Fibrous tissue lines the inner wall of the small PA
D. Fibrous tissue builds up around the inner wall of the PA, leading to narrowing of the PA

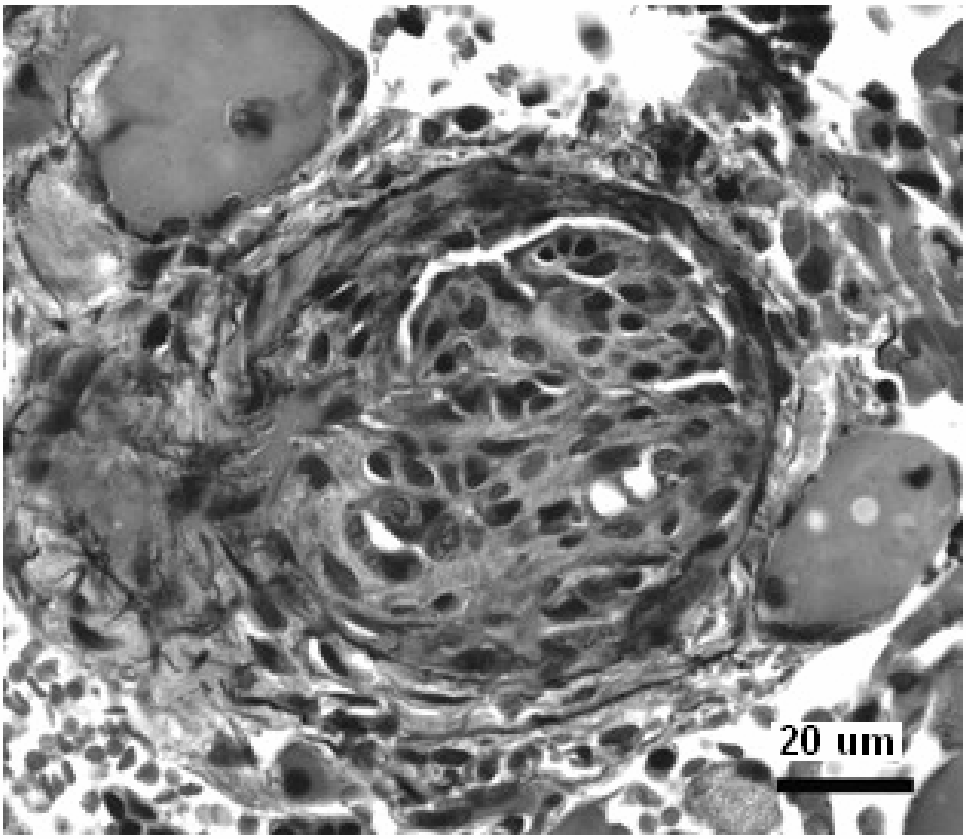


Figure 1.6 Image of a plexiform lesion.

There is a tuft of capillaries forming a web that is present in the lumen of a dilated artery. (Zabka *et. al.,* 2006)

1.6 The 5-HT hypothesis of PAH

During the period 1967 - 1973 an unexplained epidemic of PAH occurred in Europe. This was the scale of a ten-fold increase in PAH occurrence. It was caused by the sale of an over the counter appetite suppressant, aminorex. This amphetamine-like drug was brought onto the market in 1965 and removed after the outbreak of PAH (Fishman, 1999). In the 1980s a second outbreak of PAH happened in France, and this also occurred due to anorexigen prescription. The drug in question this time was fenfluramine (Douglas *et. al.,* 1981). Investigations involving fenfluramine derivatives (DL-fenfluramine and dexfenfluramine) have also shown that use of these drugs resulted in pulmonary hypertension (Brenot *et. al.,* 1993). Fenfluramines like aminorex, are congeners of the amphetamines. Another strategy in the treatment of obesity was to prescribe fenfluramine and phentermine (which is a constitutional isomer of methamphetamine) in combination, resulting in equivalent weight loss, fewer adverse effects, and better appetite control (Weintraub *et. al.,* 1984), however cases of pulmonary hypertension were found in response to this treatment (Mark *et. al.,* 1997). A multicentre study was carried out called the International Primary Pulmonary Hypertension study, and it reported a strong association between prior use of anorexigens and PAH (Abenhaim *et. al.,* 1996). It was stated that the risk for obese persons who use anorexic agents for more than three months would be more than 30 times higher than for non-users (Abenhaim *et. al.,* 1996).

Aminorex, fenfluramine, dexfenfluramine, and phentermine belong to a class of amphetamine-like drugs that interact with monoamine systems in the brain. Fenfluramine increases synaptic cleft concentrations of 5-HT through the inhibition of neuronal reuptake (Mennini *et. al.* 1985) and potentiation of 5-HT release from stores in rat brain tissue (Costa *et. al.,* 1971). Fenfluramines also cause an elevation in plasma 5-HT levels by inducing 5-HT release from neurones and platelets, and inhibiting reuptake of 5-HT by the 5-HTT (Herve *et. al.,* 1995). Dexfenfluramine both inhibits the 5-HTT (Semple *et. al.,* 1996) and increases exocytotic release of 5-HT (Gobbi *et. al.,* 1993). Aminorex has similar actions, and induces release of 5-HT from platelets, inhibits 5-HT reuptake and inhibits 5-HT metabolism by monoamine oxidase (MAO), collectively resulting in an elevation in

plasma 5-HT levels (Humbert, *et. al.,* 2004). Phentermine has been shown to increase the 5-HT levels by inhibiting 5-HT degradation via MAO (Ulus *et. al.,* 2000).

5-HT is synthesised by the intestinal enterochromaffin cells, and is actively loaded into platelets where it is stored. 5-HT circulates mainly as a reserve pool stored in platelets and minimally in plasma with up to 98 % of 5-HT in the body found within platelets. Free 5-HT in the plasma is rapidly metabolised in the liver and lung (Hart & Block, 1989). Another reason that the pulmonary vascular bed is not exposed to high levels of 5-HT is because of the position of the lungs, with the lung being a secondary filter, downstream from the liver. 5-HT is released from pulmonary neuroendocrine cells and neuroepithelial bodies within the airways. Airway hypoxia induces secretion of 5-HT from neuroendocrine cells and neuroepithelial bodies into the bloodstream (Fu *et. al.,* 2002), which may contribute to secondary PAH (Johnson & Geiorgieff, 1989). The 5-HT hypothesis of PAH is strengthened by reports linking increased plasma 5-HT and PAH. Cases have been identified in which patients have increased plasma 5-HT levels caused by a platelet storage disease, and who also have PAH (Herve *et. al.,* 1990), and cases have been described where patients have PAH associated with elevated serum 5-HT levels (Herve *et. al.,* 1995). The normal plasma concentration of 5-HT is 1 – 2 nM, however this can be elevated to 30 nM in conditions of PH (Herve *et. al.,* 1990; 1995; Anderson *et. al.,* 1987).

A number of animal models of PAH support the 5-HT hypothesis of PAH. The fawn-hooded rat has a genetic deficit in 5-HT platelet storage which is characterised by the deficient uptake of 5-HT into platelets (Aulakh *et. al.,* 1994). Fawn-hooded rats develop PAH from the age of four weeks and this can be accelerated by exposure to altitude (Marsboom & Janssens 2004). Mild hypoxia exposure causes the development of PAH in fawn-hooded rats (Sato *et. al.,* 1992). Chronic hypoxic rat models are frequently used to investigate PAH. In conditions of chronic hypoxia, PASMC proliferation and a sustained elevation in PAP occurs (Eddahibi *et. al.,* 1998). In chronic hypoxic mice, structural remodelling occurs within the vessels of the lung; muscularisation has been found in arterioles, which previously were non-muscular, with vessels which had this muscularisation prior to hypoxia having an increased muscular layer (Wohrley *et. al.,*

1995). The result of structural changes within the vasculature leads to an increase in resistance of these vessels and an increase in right ventricular workload. Therefore this model represents PAH in that it is characterized by vascular proliferation and remodelling. PAH develops following reduction of atmospheric pressure or following reduction of the partial oxygen tension at atmospheric pressure (Marsboom & Janssens 2004), and this stimulus has been used to study PAH. Mice lacking the 5-HTT gene (5-HTT-/- mice) have been exposed to chronic hypoxia. 5-HTT transports 5-HT into the PASMC from the bloodstream (see section 1.6.1.1). In the chronic hypoxic 5-HTT-/- mouse, 5-HT blood concentration was also lower in the 5-HTT-/- mice relative to controls. This result was expected since platelet 5-HT contributes largely to whole blood 5-HT. The main finding of the study was that mice deficient in 5-HTT developed less PAH than controls when exposed to hypoxia suggesting that 5-HTT is important in the development of PAH (Eddahibi *et. al.* 2000). Mice over-expressing 5-HTTs have been generated and used to study PAH (MacLean *et. al.,* 2004). Transgenic mice with 5-HTT over-expression occurring selectively in PASMCs have been shown to spontaneously develop PAH (Guignabert *et. al.,* 2006).

1.6.1 5-HT pharmacology

5-HT (also known as serotonin) was first discovered in 1948, when it was isolated from blood serum (Rapport *et. al.,* 1948a). In 1949, the chemical structure of this compound was found to be 5-hydroxytryptamine (Rapport *et. al.,* 1949). 5-HT is found in high concentrations within the intestine, blood and the CNS. 5-HT is synthesised (figure 1.7) in a two step process from the amino acid precursor, tryptophan, which is converted to 5-hydroxytryptophan via the enzyme tryptophan hydroxylase (Tph). There are two genes which encode tryptophan hydroxylase, *Tph1* and *Tph2*, with *Tph1* catalysing the rate-limiting step in the synthesis of 5-HT in the periphery (Walther & Bader, 2003). Recently it was shown that *Tph1* and peripheral 5-HT are critical to the development of hypoxia induced PAH (Morecroft *et. al.,* 2007). The proposed role of 5-HT in the PAH disease process is further strengthened by the findings that expression of the *Tph1* gene is increased in lungs and pulmonary endothelial cells from patients with idiopathic PAH

(Eddahibi *et. al.,* 2006). 5-Hydroxytryptophan is decarboxylated by L-aromatic acid decarboxylase to form 5-HT. Metabolism of 5-HT occurs via oxidative deamination, catalysed by MAO, the product of which is then converted to 5-hydroxyindoleacetic acid via aldehyde dehydrogenase. 5-HT is preferentially metabolised by type A monoamine oxidase (Weyler *et. al.,* 1990).

1.6.1.1 5-HT transporter

A 5-HT transporter (known as 5-HTT/ SERT) has been identified and cloned (Hoffman *et. al.,* 1991; Ramamoorthy *et. al.,* 1993). The 5-HTT protein is composed of ~ 630 amino acids, which form twelve transmembrane (TM) domains with both the amino and carboxyl termini on the intracellular side of the membrane (Ni & Watts, 2006). 5-HTT is present in many tissues including platelets and nerve endings, and is also abundantly expressed in the lung, where it is predominantly located on PASMCs (Ramamoorthy *et. al.,* 1993). 5-HTT is sodium (Na^+) dependant, and it acts to remove 5-HT from the extracellular environment and transport it into the cell, where it can be metabolised or repackaged into vesicles (Ni & Watts, 2006). A model has been proposed for the transport of 5-HT via the 5-HTT: one sodium ion (Na^+) binds to the extracellular portion of the protein, then $5\text{-}HT^+$ binds to the transporter and this is followed by one chloride (Cl^-) ion (Nelson & Rudnick, 1979). The driving force for this co-transport of Na^+, Cl^- and 5-HT into the cell arises from the Na^+ concentration gradient across the cell membrane. The Na^+ concentration gradient arises from the action of Na^+/ K^+ ATPase, which acts to pump K^+ into the cell and Na^+ out of the cell against their respective concentration gradients. The binding of Na^+, Cl^- and 5-HT results in a conformational change of the 5-HTT protein which causes it to move from facing extracellularly to facing intracellularly upon which $5\text{-}HT^+$, Na^+ and Cl^- are released into the cytoplasm (Nelson & Rudnick, 1979). K^+ within the cytoplasm then bind to the 5-HTT, which induces the protein to return to its original orientation, and the K^+ is then released into the extracellular environment (Nelson & Rudnick, 1979; Ni & Watts, 2006). The result is an overall decrease in the concentration of 5-HT within the extracellular environment, hence reduced activation of 5-HT receptors.

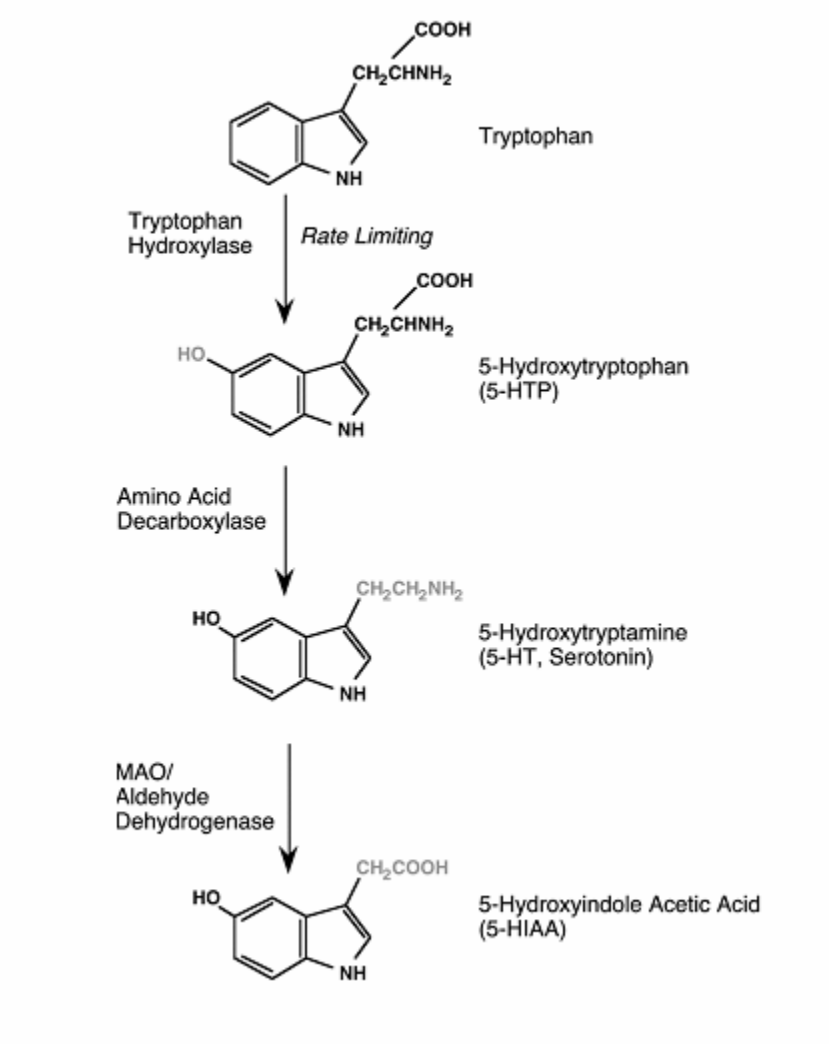


Figure 1.7 5-HT synthesis and metabolism

5-HT is synthesised from dietary tryptophan. Tryptophan is converted to 5-hydroxytryptophan by the enzyme tryprophan hydroxylase (*Tph*). 5-hydroxytryptophan is then converted to 5-HT by a non-specific decarboxylase. Degradation of 5-HT to 5-hydroxyindoleacetic acid occurs via the enzyme MAO-A

1.6.1.2 5-HT receptors and expression in pulmonary arteries

The first suggestion that there was more than one 5-HT receptor came from work on guinea pig ilieum, as it was found that only part of the 5-HT induced contractile response was blocked by morphine (Gaddum & Picarelli, 1957). Continued research and molecular biology techniques have to date identified at least seven classes of 5-HT receptor according to their structural, operational, and transduction pathways (Alexander *et. al.,* 2006). Alternative splicing occurs within the 5-HT super family of receptors resulting in the isoforms of the 5-HT2A, 5-HT2C, 5-HT4 and 5-HT7 receptors (Guest *et. al.,* 2000; Canton *et. al.,* 1996; Gerald *et. al.,* 1995). Molecular diversity also arises from RNA editing, which occurs within the 5-HT2C family of channels (Burns *et. al.,* 1997). 5-HT receptors belong to the GPCR superfamily, with the exception of the 5-HT3 receptor, which is a ligand-gated ion channel (Pauwels, 2000). GPCR receptors have seven transmembrane α-helices, with an extracellular N terminus and intracellular C terminus (Kroeze *et. al.,* 2003). Upon binding of 5-HT to the extracellular portion of the receptor, G-proteins are activated, which initiate second messenger signalling pathways (figure 1.8). G-proteins consist of alpha (α), beta (β), and gamma (γ) subunits. G-proteins are classified by their alpha subunit, which have been divided into four families, known as Gαs, Gαi, Gαq/11, and Gα12/13. These groups differ primarily in effector recognition, but share a similar mechanism of activation (Neves *et. al.,* 2002). 5-HT acts on various receptors and therefore has different effects within cells according to the signalling/ effector pathway activated. 5-HT1 and 5-HT5 receptors are linked to inhibitory G-proteins (Gαi), 5-HT4, 6, 7 are linked to stimulatory G-proteins (Gαs) (Alexander *et. al.,* 2006; Noda *et. al.,* 2004), and 5-HT2 receptors couple preferentially to Gαq proteins (Hoyer *et. al.,* 2002).

The 5-HT1 receptor family has been subdivided into 5-HT1A, 1B, 1D, 1E & 1F (Alexander *et. al.,* 2006). In the past it was thought that the 5-HT1B receptor was exclusively expressed in rodent (hamster, mouse, and rat) tissues, whereas the closely related 5-HT1D receptor was believed to be expressed in other species (humans, cows, dogs, and guinea pigs), which lead to suggestion that the 5-HT1B and 5-HT1D receptors were species homologues (Hoyer & Middlemiss, 1989). It has since been shown that that 5-HT1B and 5-HT1D receptors are not pharmacologically identical, with selective pharmacological

agents being used to distinguish between them (Price *et. al.,* 1997). Subsequent studies have now shown that the 5-HT1B and 5-HT1D receptors are separate gene products which are not exclusively expressed in rodent or non rodent mammals (Hamblin *et. al.,* 1992). In cases where 5-HT1B and 5-HT1D receptors could not be distinguished, the receptor was referred to as the 5-HT1B/1D receptor. The major signalling pathway of the 5-HT1 receptor family is the adenylate cyclase (AC)/ cAMP system (Sumner *et. al.,* 1992; Alexander *et. al.,* 2006) (diagram 1.8). 5-HT1 GPCRs are linked to Gαi proteins that inhibit AC, which would otherwise catalyse the conversion of ATP to cAMP. 5-HT1 receptors have been shown to couple to differential signalling cascades, depending on the cell line the receptor is expressed in. Some of the 5-HT1 receptors have also been linked to phosphatidylinositol (PI) hydrolysis through activation of phospholipase C (PLC) (Hoyer *et. al.,* 1994). This hydrolysis gives rise to the second messengers diacylglycerol (DAG) which activates protein kinase C (PKC) (Nishizuka, 1988), and inositol trisphosphatase (IP_3) which acts to increase $[Ca^{2+}]_i$ (Berridge, 1993).

Within the 5-HT2 family there are three subfamilies, 5-HT2A, 2B & 2C (Alexander *et. al.,* 2006). The 5-HT2A receptor has 45% amino acid sequence homology with the 5-HT2B receptor (Hamblin *et. al.,* 1992). Signalling transduction from 5-HT2 receptors is mediated via the action of Gαq G-proteins. Gαq proteins couple to PLCβ effector pathway. Therefore 5-HT2 receptor activation results in PI hydrolysis through activation of PLCβ giving rise to IP_3 and DAG (Hoyer *et. al.,* 1994; Alexander *et. al.,* 2006).

To date several 5-HT receptors have been found in PAs. In rabbit PAs, mRNA for the following receptors has been identified: 5-HT1A, 5-HT1B, 5-HT1D, 5-HT3A, 5-HT3B, 5-HT4, 5-HT6 and 5-HT7. The expression of 5-HT1A was found at a much lower level than that of the other receptors (Molderings *et. al.,* 2006). 5-HT1B/1D, 5-HT2A, 5-HT2B, 5-HT4 and 5-HT7 receptor mRNA has been found in porcine PAs. A similar profile of receptors was also found in cultured human PASMCs with the exception of 5-HT4 (Ullmer *et. al.,* 1995). In PAs, 5-HT1B/1D and 5-HT2A receptors have been shown to mediate the contractile response to 5-HT in functional and binding studies (MacLean *et. al.,* 1996; Morecroft *et. al.,* 1999).

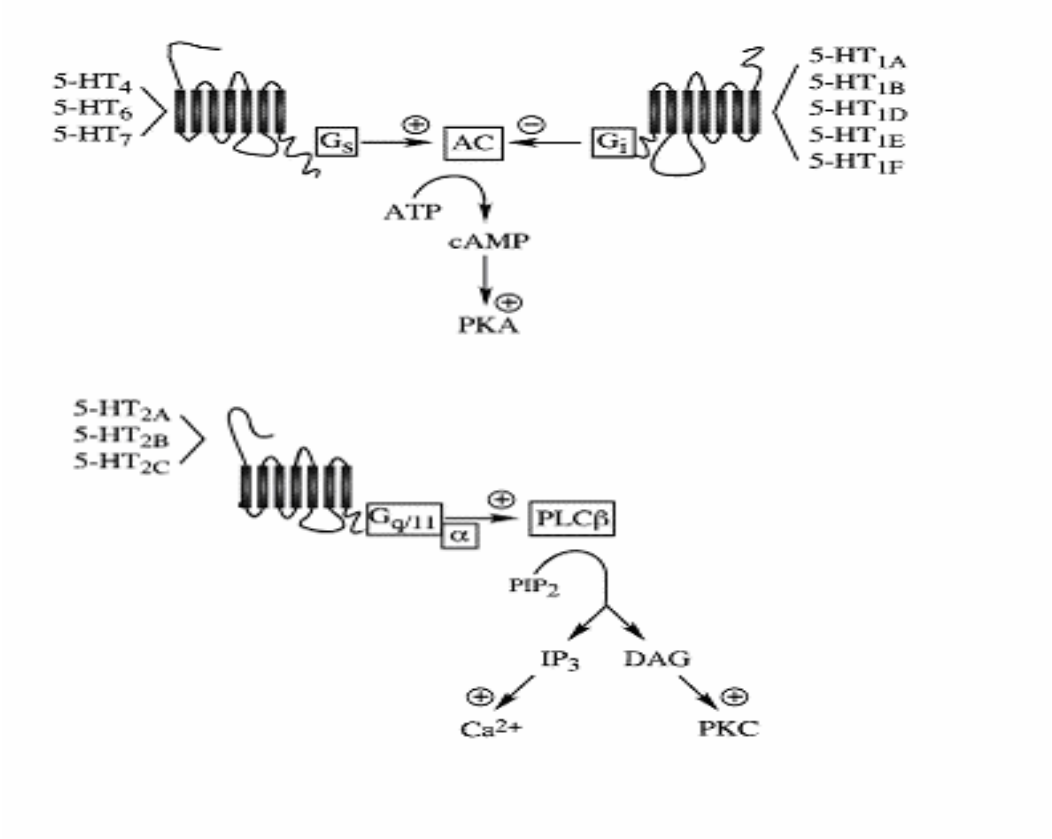


Figure 1.8 Signalling cascades of the 5-HT G-protein coupled receptors.

Signalling cascades of the G-protein coupled 5-HT receptors. 5-HT1 receptors typically inhibit AC through the G*αi* family of G-proteins, whereas 5-HT4, 5-HT6, and 5-HT7 receptors typically stimulate AC through G*αs* family G-proteins. Activation of AC results in increased production of cAMP, leading to activation of protein kinase A (PKA). 5-HT2 receptors activate PLC-β through G*αq* family G-proteins, resulting in accumulation of PIP_2 to IP_3 and DAG. Generation of IP_3 results in elevation $[Ca^{2+}]_i$, whereas DAG activates PKC. Figure adapted from Raymond *et. al.,* (2001).

1.6.2 5-HT and PAH

5-HT has a dual effect on pulmonary vasculature. 5-HT has been shown to induce PA vasoconstriction and PA remodelling, two important characteristics of PAH (figure 1.9).

1.6.2.1 5-HT and pulmonary vasoconstriction

5-HT is known to have a pressor effect on the lung vasculature. Pharmacological studies have been carried out to investigate the identity of receptors involved in the 5-HT-induced contraction of pulmonary arteries. There is a species difference in the mechanism by which 5-HT causes this effect. In rat, mouse, cow and dog pulmonary arteries, 5-HT causes vasoconstriction via the 5-HT2A receptor (Chand *et. al.,* 1980; MacLean *et. al.,* 1994 & 1996; Witzenrath *et. al.,* 2006). In comparative studies using human large pulmonary arteries, 5-HT was found to act via the 5-HT2A and 5-HT1B receptors (Morecroft *et. al.,* 1999).

The specificity of 5-HT receptor agonists and antagonists has been used to identify the receptors involved in 5-HT responses. 5-HT and sumatriptan (a 5-HT1B/1D agonist), caused vasoconstriction of human small muscular pulmonary arteries. The response to 5-HT was inhibited by ketanserin (a selective 5-HT2A antagonist) at concentrations of 5-HT which were considered pathological (> 0.1 μM), however GR55562 (5-HT1B/1D-selective antagonist) inhibited 5-HT-induced contractions at all concentrations of 5-HT tested. SB-224289 (a selective antagonist for 5-HT1B receptors) and BRL15572 (a selective antagonist for 5-HT1D receptors) were tested on the sumatriptan-induced vasoconstriction, and only SB-224289 inhibited the response indicating the importance of 5-HT1B receptors in mediating vasoconstriction in human small muscular arteries (Morecroft *et. al.,* 1999). Drugs that block 5-HT1B receptors could prove to be beneficial in the treatment of PAH. As it is the 5-HT2A receptor that is important for mediating vasoconstriction effects in the systemic system (Saxena & *Villalon,* 1990), 5-HT1B antagonists would not cause systemic hypotension (Herve *et. al.,* 1995; MacLean *et. al.,* 2000).

An uncovering of receptor participation in the 5-HT induced vasoconstriction response has been shown to occur in the presence of tone in the PA. In bovine PAs, sumatriptan failed to

induce vasoconstriction under basal conditions. However, when thromboxane-mimetic U46619 was added to the preparation to induce tone, sumatriptan induced a potent vasoconstriction. In the absence of tone, 5-HT responses were shown to be mediated by 5-HT2A receptors whereas in the presence of tone, 5-HT activated 5-HT1D/1B receptors to induce the vasoconstriction response (MacLean *et. al.,* 1994). In chronic hypoxic rats, whose endogenous pulmonary arterial tone was increased relative to control rats (in both large and resistance arteries), 5-HT induced contraction was mediated by both 5-HT2A receptors and 5-HT1B receptors (Maclean *et. al.,* 1996). The involvement of the 5-HT1 receptors in the presence of tone can be seen as an example of pharmacological synergism. This phenomenon describes a situation in which a greater than additive effect (synergism) results from the amplification of signal transduction signals resulting from crosstalk between two GPCRs, and could lead to the unmasking of receptors that are otherwise 'silent'. For example in rat small PAs, increased vascular tone via either G*αq*-coupled receptor activation or elevated KCl potentiated G*αi*-coupled 5-HT1 receptor-induced vasoconstriction (MacLean & Morecroft, 2001).

1.6.2.2 5-HT and pulmonary vascular remodelling

5-HT is a known mitogen for SMCs isolated from bovine, porcine and rat aorta and it is a potent inducer of rat and bovine PASMC proliferation (Lee *et. al.,* 1991, Eddahibi *et. al.,* 1999b, Pitt *et. al.,* 1994). Proliferation of PASMCs has been shown to be an important factor in PA remodelling seen in PAH, and 5-HTT over-expression has been shown to be associated with PASMC hyperplasia in patients with PAH (Eddahibi *et. al.,* 2001). The mechanism by which 5-HT induces PA hyperplasia involves the transport of 5-HT into PASMCs. Evidence for this was provided using the 5-HTT blockers, fluoxetine and paroxetine. These drugs inhibit both 5-HT uptake and 5-HT induced SMC proliferation at similar concentrations, which suggests that 5-HT uptake and 5-HT-induced PASMC proliferation are related (Eddahibi *et. al.,* 1999b). The proliferation response of PASMC to 5-HT was blocked by drugs which block 5-HT transport, but not by antagonists of 5-HT receptors, in bovine vessels (Lee *et. al.,* 1991). These results indicate that 5-HT needs to be taken into the PASMC to induce its mitogenic effect (see figure 1.10).

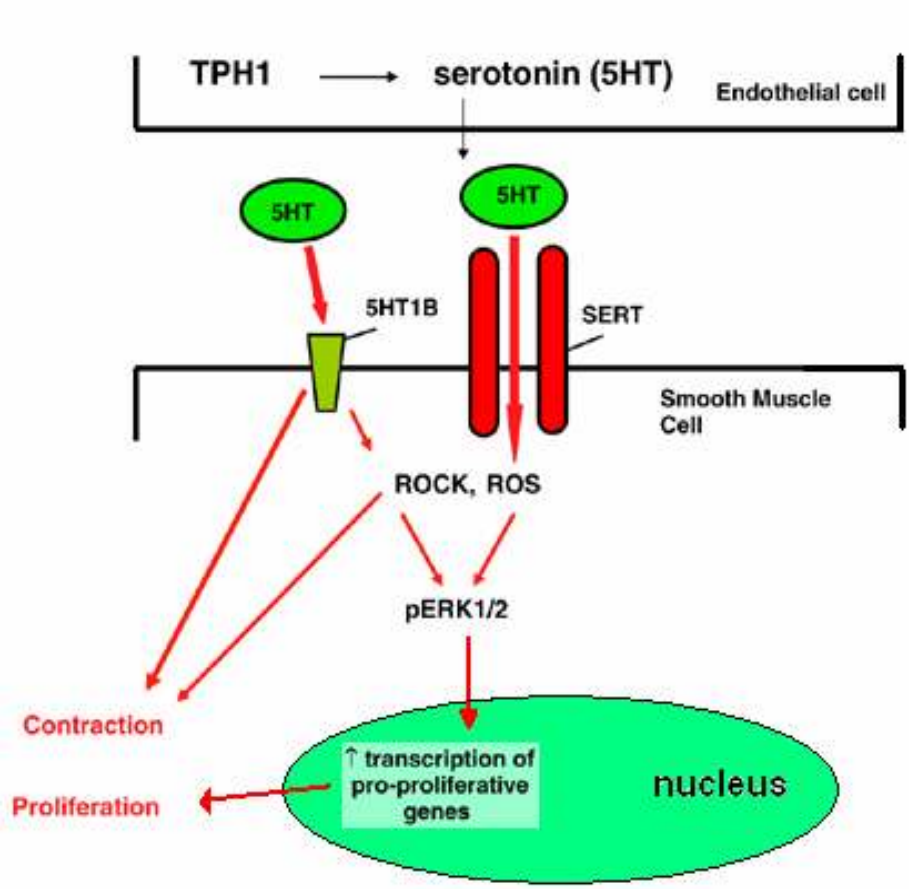


Figure 1.9 Diagram showing the effects of 5-HT on a PASMC.

5-HT is synthesized in the PA endothelial cells by *Tph1*. 5-HT can then influence PASMC proliferation and/or contraction via activity at the 5-HTT and 5-HT receptors (particularly the 5-HT1B receptor in humans). Figure adapted from Dempsie & MacLean, (2008)

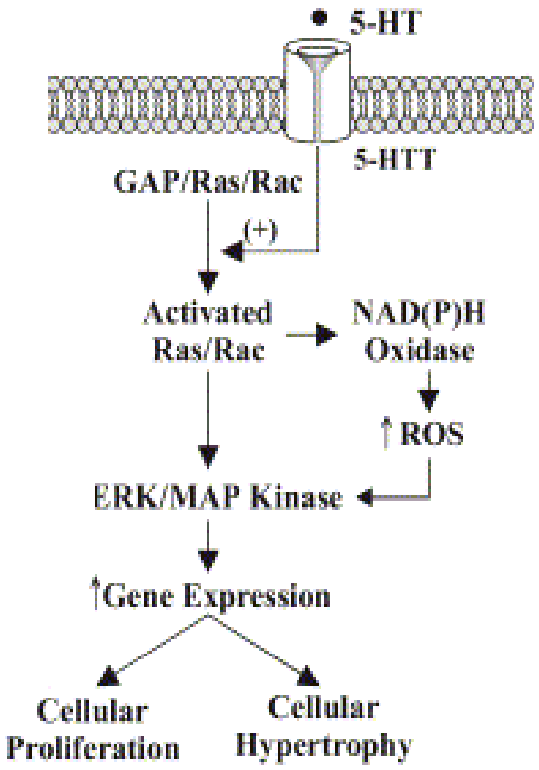


Figure 1.10 Diagram illustrating the proposed role of 5-HTT in the development of vascular medial hypertrophy.

5-HT interacts with 5-HTT on the cell membrane, which leads to activation of GTPase-activating protein (GAP) and/or Ras and/or Rac. The ensuing signal leads to the formation and release of ROS, which in turn activates extracellular signal-regulated kinase (ERK)-1 or ERK-2 and mitogen-activated protein (MAP) kinases. This results in upregulation of the expression of genes involved in both cellular hypertrophy and proliferation. Figure adapted from Mandegar *et. al.,* (2004)

The proposed intracellular signalling response is 5-HT entry via 5-HTT, which results in activation of GTPase-activating protein (GAP) and/or Ras and/or Rac. These intracellular molecules induce the formation and release of reactive oxygen species (ROS), which in turn activates extracellular signal-regulated kinase (ERK)-1 or ERK-2 and mitogen-activated protein (MAP) kinases (Lee *et. al.,* 1999). The signalling cascade result is upregulation of the expression of genes involved in both cellular hypertrophy and proliferation (Mandegar *et. al.,* 2004).

1.6.4 The role of PASMC membrane potential in 5-HT uptake

The transport of 5-HT into PASMC via 5-HTT is coupled directly to the electrochemical potential associated with the co-transported ions. The removal of 5-HT from the plasma has been shown to be closely linked to the Na^+/ K^+ ATPase by studies in which removal of Na^+ from the extracellular environment inhibited the uptake of 5-HT, and similar removal of K^+ inhibited 5-HT uptake by about one third (Dubilei, 1976). The action of Na^+/ K^+ ATPase maintains the ionic gradients of K^+ and Na^+ across the membrane, which in turn helps to determine the membrane potential of the PASMC.

1.7 K^+ and membrane potential of PASMCs

The resting membrane potential arises from the movement of ions across the PASMC membrane. Partly due to the electrogenic nature of the Na^+/K^+ ATPase, pumping three Na^+ out of the cell for every two K^+ it brings into the cell, the membrane potential is negative at rest. Na^+/K^+ ATPase action results in a high $[K^+]_i$. This results in a net electrochemical gradient being established that favours the passive movement of K^+ out of the cell (Al-Habori, 1994). The resting membrane is more permeable to the movement of K^+ relative to any other ions, and therefore the resting membrane potential arises partly from K^+ ion efflux along its electrochemical gradient (Nelson & Quayle, 1995), and this drives the membrane potential towards the Nernstian K^+ equilibrium potential for K^+ (E_K = ~ 80 mV) (Gurney *et. al*., 2002). However, the outward movement of K^+ ions is opposed by a non-specific leakage conductance, the distinct permeability of the membrane to other ions such as Ca^{2+}, Na^+ and Cl^- results in the resting membrane potential being more depolarised (positive) than E_K. The actual membrane potential of PASMCs is approximately –50 mV

to –60 mV, found in both intact and isolated preparations of PASMCs (Casteels *et. al.,* 1977; Suzuki & Twarog 1982; Clapp & Gurney, 1992). There is universal consensus that the resting potential of PASMCs is determined mainly by a non-inactivating K^+ conductance, I_{KN} (Gurney *et. al.,* 2002; 2003). However, there is debate as to the specific K^+ channels that transmit the current which maintains resting membrane potential in PASMCs.

The link between vessel tone and K^+ channel activity is the membrane potential. As K^+ channels set the membrane potential in SMCs, they control the possibility of voltage operated Ca^{2+} channels (VOCC) being open, and hence regulate Ca^{2+} entry into the cell (Nelson *et. al.,* 1990). Depolarisation of the membrane potential opens VOCCs, increasing Ca^{2+} entry, which leads to vasoconstriction (Nelson & Quayle, 1995). It has been suggested that reduced K^+ current, leading to a depolarised PASMC membrane, in turn gives rise to increased Ca^{2+} within the cytoplasm and this may play a critical role in stimulating PASMC contraction and proliferation (Platoshyn *et. al.,* 2000).

1.7.1 K^+ channels – general features

K^+ ion channels are membrane-spanning proteins with three functional properties: (1) a central pore through which ions flow along their electrochemical gradient, (2) a selectivity filter that determines what types of ion are transmitted through the pore, and (3) a gating structure that controls when the pore opens and closes therefore dictating whether permeation occurs. There are a number of K^+ channels found in PASMCs. Each specific K^+ channel is assigned to a family by structure, with all K^+ channels being related members of a single protein family. They consist of a primary pore-forming α-subunit, often associated with accessory or regulatory β-subunits. A common feature between all K^+ channel α-subunits is that they contain a "P loop" domain. This is an amino acid segment between two trans-membrane helices that dips into the membrane without fully crossing it. K^+ channel P-loops have a consensus amino acid sequence (Thr-Val-Gly-Tyr-Gly) that has been called the K^+ channel ''signature sequence''(Doyle *et. al.,* 1998). This sequence has been proposed to correspond to the selectivity filter of the aqueous pore forming region of the channel protein (Doyle *et. al.,* 1998). K^+ channels are tetrameric and therefore four

copies of the filter motif come together to form the pore (Sansom *et. al.,* 2002). The pore forming α-subunits may form homomeric or heteromeric channels between subfamilies. There are more than seventy different genes encoding K^+ channel α-subunits in the human genome. The criterion for the sub-grouping of K^+ channels is the number of TM regions possessed by the α-subunits.

1.7.1.1 Families of K^+ channels

K^+ channels have been sub-grouped into three main groups based on their structural properties: 2TM, 4TM and 6TM K^+ channels (figure 1.11 and 1.12). While all these channels are selective for K^+ over other ions, they exhibit distinct biophysical properties.

1.7.1.1.1 Two transmembrane domain (2TM) K^+ channels

The 2TM channels are also know as the inwardly rectifying K^+ (K_{IR}) channel family. Each subunit contains a single pore domain between two membrane spanning regions (S1 and S2). Both the N and C termini of these subunits are located intracellularly. This K_{IR} family of channels consists of 7 subfamilies (K_{IR}1-7), which can be further subdivided. K_{IR}3 channels are also known as G-protein regulated K^+ channels (GIRK) and K_{IR}6 channels are also known as ATP-sensitive K^+ channels (K_{ATP}) (Alexander *et. al.,* 2006).

Approximately two thirds of the K_{IR} channel is found within the cell cytoplasm, and this domain forms a cytoplasmic pore (Nishida & MacKinnon, 2002). This is the section of the channel which is crucial for modulation by intracellular gating compounds (Yang *et. al.,* 1995). Inward rectification refers to the ability of an ion channel to allow greater influx than efflux of ions. K_{IR} channels conduct K^+ ions from outside to inside the cell more readily than in the opposite direction (Doupnik *et. al.,* 1995). Inward rectification is induced by cytoplasmic ions such as polyamines and magnesium ions. These ions plug the pore upon depolarisation and thereby inhibit the outward flow of K^+ (Vandenberg, 1987; Matsuda *et. al.,* 1987; Lopatin *et. al.,* 1994). Within this sub-group of K^+ channels, they are differentiated from one another by their individual strength of rectification and gating. GIRK channels (K_{IR}3) are strongly rectifying and activated by Gβγ. K_{ATP} (K_{IR}6.2) channels are weakly rectifying and gated by the ATP/ adenosine diphosphate (ADP) ratio

(Bichet *et. al.,* 2003). K_{ATP} channels link the cell membrane potential to the metabolic status of the cell. At physiological levels of intracellular ATP these K_{ATP} channels are inhibited, however, when ATP levels within the cell are reduced the K_{ATP} channels open. K_{ATP} channels are bound to a protein sulfonylarea receptor of the ATP-binding cassette (ABC) family and they are inhibited by sulfonylurea drugs (Demolombe & Escande, 1996). Another feature of K_{IR} channels is that they can be regulated by phosphotidylinositol 4,5-bisphosphate (PIP_2) (Huang *et. al.,* 1998). K_{IR} channel opening requires PIP_2 binding to basic cytoplasmic domains, whereas depletion of PIP_2 seems to close the channel (Huang *et. al.,* 1998; Shyng *et. al.,* 2000). pH has also been shown to play a role in the regulation of some K_{IR} channels, decreasing internal pH increased the sensitivity of $K_{IR}1.1$ channels to extracellular K^+ by altering the properties of the selectivity filter (Dahlmann *et. al.,* 2004).

1.7.1.1.2 Four transmembrane domain (4TM) K^+ channels

Each of the subunits of these channels contains two rather than one pore-forming sequence. These channels contain two copies of the selectivity filter motif in one polypeptide chain, two chains merging to form the intact channel (Sansom *et. al.,* 2002). 4TM channels have an extended extracellular loop between transmembrane segment S1 and the pore-forming segment P1 and a short cytoplasmic N-terminus (Salinas *et. al.,* 1999). This family includes seven different members: TWIK (tandem pore weakly inward-rectifying K^+ channel), TREK (TWIK-1 related K^+ channel), TRAAK (TWIK-1 related arachidonic acid-stimulated K^+ channel), TASK (two-pore domain acid-sensitive K^+ channel), TALK (two-pore domain alkaline-activated K^+ channel), THIK (two-pore domain halothane inhibited K^+ channel) and TRESK (TWIK-related spinal cord K^+ channel) (Alexander *et. al.,* 2006). 4TM domain K^+ channels are also named using an alternative nomenclature as KCNKx, where x is a number indicating the order in which each member was identified. There are 17 members of this family, according to the Human Genome Organization Nomenclature Committee database.

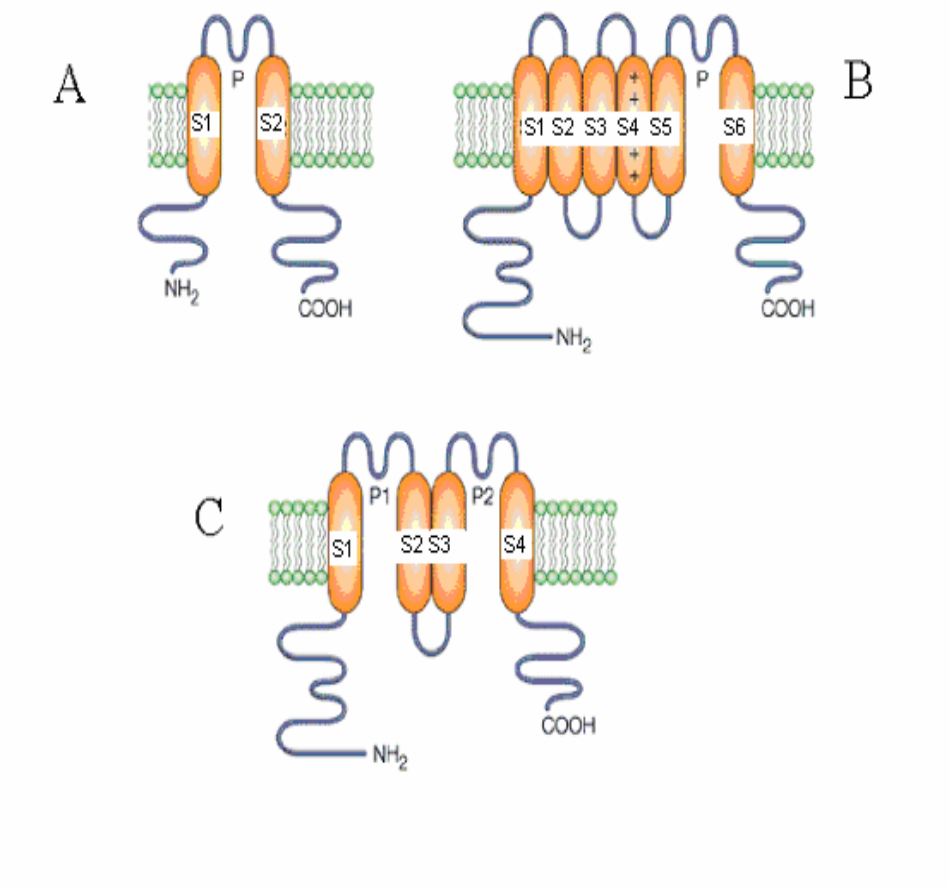


Figure 1.11 Diagram showing the α-subunits of each family of K^+ channel.

A) 2TM/P channels

B) 6TM/P channels

C) 4TM/2P channels

Figure adapted from Choe, (2002)

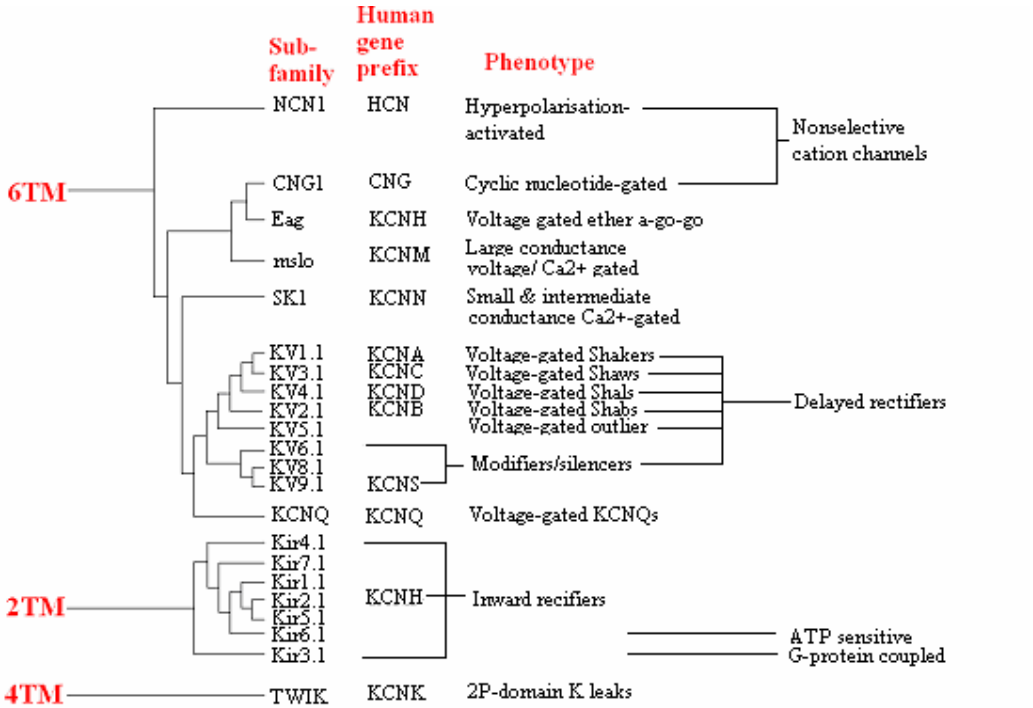


Figure 1.12 Phylogenetic tree of K^+ channels.

The tree shown consists of only one representative member of each subfamily. The branching shown is expected to preserve evolutionary relationships. Figure adapted from Hille, (2001).

4TM K^+ channels are voltage independent, remaining open at all membrane potentials. In this way they transmit leak K^+ (background) currents, open at potentials where K_V channels are closed. For this reason these K^+ channels are suggested to be responsible for mediating the background K^+ ion current that is thought to set the resting membrane potential in several cells (Lesage *et. al.,* 1996).

TREK and TRAAK channel activity is mechanosensitive and activated by arachidonic acid (Patel *et. al.,* 1998; Lesage *et. al.,* 1996; Maingret *et. al.,* 1999). TREK channels are also opened in response to heat, mild intracellular acidosis, and inhalation anaesthetics (Patel *et. al.,* 1998; 1999; Maingret *et. al.,* 2000). TASK channels are sensitive to external pH variation and inhibited by mild external acidosis (Durprat *et. al.,* 1997; Leonoudakis *et. al.,* 1998). TWIK-1 is a weakly inward rectifying K^+ channel that is stimulated by protein kinase C (PKC) and inhibited by mild internal acidification (Lesage *et. al.,* 1997). TALK channels are also pH sensitive, being inhibited by acidic conditions and activated by alkaline conditions (Johansson, 2003). THIK channels have been shown to transmit current that is activated by arachidonic acid and inhibited by the volatile anaesthetic, halothane (Rajan *et. al.,* 2000).

1.7.1.1.3 Six transmembrane domain (6TM) K^+ channels

This is the main family of voltage gated K^+ (K_V) channels. Both the N and C termini of these subunits are located intracellularly. Subfamilies of the α-subunits of K_V channels arise from diverse gene coding. These include K_V1- K_V6, K_V8 and K_V9 (Coetzee *et. al.,* 1999), and each subfamily can be subdivided again ($K_{VX..X}$). Accessory subunits attached to the α-subunits increases the diversity of K_V channels and alters their biophysical and pharmacological properties. These include auxiliary β-subunits, K^+ channel-associated proteins (KChAP), K_V channel-interacting proteins (KChIPs), 14-3-3 Proteins, and A-Kinase Anchoring proteins (AKAPs). The K_V7 sub-family of the K_V family of K^+ channels are also known as the KCNQ subfamily. Another group of 6TM channels are known as K_{Ca} channels (BK_{Ca}, SK_{Ca} and IK_{Ca}). BK_{Ca} channels have 7TM domains but are classified alongside the 6TMs. SK_{Ca} and intermediate conductance K_{Ca} channels (IK_{Ca}) are voltage independent so classed separately from K_V channels. K_V10, 11 and 12 make up the 'EAG' subset of channels. For the purposes of this study K_V1-4, 5-6, 8-12 will be referred to as K_V channels and K_V7 channels will be referred to as KCNQ channels.

K_V channels were named originally when they were identified in the fruit fly *Drosphila* melanogaster, and the first one identified was *Shaker* (Tempel *et. al.,*1987; Papazian *et. al.,* 1987). Following this channel, related channels were identified from *Drosphila* also, and these were *Shab, Shaw* and *Shal*, based on homology to *Shaker*. (Butler *et. al.,* 1989; Wei *et. al.,* 1990). *Shaker, Shab, Shaw* and *Shal* channels are also found in mammals, and are named accordingly, with Shaker's mammalian counterpart being named K_V1, (*Shab* = K_V2, *Shal* = K_V3, *Shaw* = K_V4). The membrane potential determines whether the channel is open, and therefore provides a means for the membrane voltage to feedback onto itself. K_V channels are activated by depolarisation, but exhibit a wide diversity of activation and inactivation kinetics. When a voltage gated channel opens, charged amino acids move through the membrane electrical field, coupling electrical work to the opening process (Sigworth, 1994). Ongoing depolarisation causes some K_V channels to open and then close, a process known as inactivation. K_V channels show inactivation with sustained depolarisation, with the rate of inactivation varying between subfamilies (Standen & Quayle 1998). Certain K_V channels inactivate slowly and these types are known as delayed rectifiers, and carry what is known as delayed rectifier K^+ current (I_{KDR}). I_{KDR} exhibits little inactivation over a 100 – 200 ms depolarisation (Mathie *et. al.,* 1998). Other K_V channels show rapid inactivation and carry transient outward current, known as the "A" current (I_{KA}). Fast inactivation, which occurs on the order of milliseconds to tens of milliseconds, is also referred to as N-type inactivation (Rasmusson *et. al.,* 1998). Fast inactivation of the K_V channel occurs via a 'ball and chain' mechanism. K_V channels that undergo fast inactivation have an 'inactivation ball' that precedes the S1 domain of each α-subunit. This 'inactivation ball' is approximately 20 amino acids in length, found at the N-terminal of the channel protein, and is connected to S1 by approximately 30 amino acids (also referred to as the chain region). The function of the region is to plug the pore, and it does so by binding to the internal mouth of the channel upon activation (Hoshi *et. al.,* 1990). It has been shown that only one 'inactivation ball' is enough to inactivate the channel (even though there are four 'inactivation balls' within the channel unit).

KCNQ channels are encoded by the genes $K_V7.1$- $K_V7.5$. These genes were shown to encode K^+ channel subunits with the '*shaker*-like' motif of 6TM domains and a single P-loop, but they have distinct properties (Robbins, 2001). K^+ currents transmitted when different KCNQ are heterologously expressed are activated at negative membrane

potentials (below - 60 mV), and are outwardly rectifying with little or no inactivation (Robbins, 2001).

There are five subfamilies of K_{Ca} channels (K_{Ca}1-5), which have also been subdivided on the principle of channel conductance and pharmacology. The subfamilies include BK_{Ca}, with single channel conductance of ~100 – 300 pS. These channels are encoded by K_{Ca}1.1, also known as *Slo* or *Slo*1 (Marty, 1981). IK_{Ca} with single channel conductance of ~25 – 100 pS is encoded by the K_{Ca}3.1 gene (Ishii *et. al*., 1997; Joiner *et. al*., 1997)) and SK_{Ca} with single channel conductance of ~2 – 25 pS is encoded by the K_{Ca}2.1, 2.2, 2.3 genes (Park 1994; Kohler *et. al*., 1996). Compared with the Kv channels, the α-subunit of the BK_{Ca} channel has an extra hydrophobic domain that leads to 7TM domains and an extracellular N-terminus (Wallner *et. al.,* 1996). The N-terminus acts as a binding domain for the modulatory transmembrane β-subunit. There is a regulatory domain located C-terminal to the pore-forming TM domain, which is known as the RCK domain (regulates conductance of K^+). The position of this ancillary domain allows it to exert control over the transmission of ions through the channel pore (Sansom *et. al.,* 2002). It has been suggested that upon Ca^{2+} binding to the channel, there is a transition in RCK that results in the S2 segment helices being pulled out so as to open the channel (Sansom *et. al.,* 2002). The C-terminus determines the Ca^{2+} sensitivity of the channel (Wei *et. al.,* 1994). A series of highly conserved negatively charged residues are present in the C-terminus, which are known to have the Ca^{2+} binding sites and are called the "Ca^{2+} bowl" (Schreiber & Salkoff, 1997). IK_{Ca} and SK_{Ca} channels are exclusively gated by Ca^{2+} and exhibit no voltage sensitivity. IK_{Ca} channels are bound to CaM via a binding domain (CaMBD) and the channel opens when Ca^{2+} binds to calmodulin (Sansom *et. al.,* 2002).

1.7.2 K^+ channels found in PASMCs

There is much diversity of K^+ channel expression and function in the pulmonary arterial vasculature. K^+ channels found in PASMCs include four different classes, each class distinguished by specific physiological, kinetic and pharmacological properties. These include:

- K_V & KCNQ channels
- K_{Ca} channels

- K_{ATP} channels
- K_{2P} channels

1.7.2.1 K_V & KCNQ channels

To date PAs have been found to express five α-subunit genes from the *Shaker* family (K_V1.1- K_V1.5), one α-subunit gene from the *Shab* family (K_V2.1), one α-subunit gene from the *Shaw* family (K_V3.1), one α-subunit gene from the *Shal* family (K_V4.2), a K_V channel modulatory subunit (K_V9.3), and three β-subunits K_Vβ1.1, K_Vβ2 and K_Vβ3 (Patel *et. al.,* 1997; Osipenko 2000; Yuan 1998; Hulme *et. al.,* 1999; Platoshyn *et. al.,* 2001; Archer 1998). Activation of K_V causes an increase in K^+ efflux, (i.e. K^+ current), which leads to PASMC hyperpolarisation. This induces the closure of VOCCs, causing reduced Ca^{2+} current (I_{Ca}) flow into the cell, and results in vasodilation.

KCNQ1 and the regulatory subunit K_V3.4 have been shown to be present in lung (Teng *et. al.,* 2003). More recently, mRNA expression of KCNQ1, 4, 5 was identified in rat PASMCs, with KCNQ4 expression greater than KCNQ1 or KCNQ5 (Joshi *et. al.,* 2008(submitted)). These channels can form homo-multimers or co-assemble functionally with other subfamilies or β subunits to form hetero-multimeric channels (Kubisch *et. al.,* 1999; Lerche *et. al.,* 2000). Joshi *et. al.,* 2006, suggest a functional role for KCNQ channels in the regulation of resting membrane potential of PASMCs.

It has been postulated that K_V1.5 and K_V2.1 channels play a major role in controlling resting membrane potential in PASMCs (Yuan, 1995; Archer *et. al.,* 1998; Platoshyn *et. al.,* 2000). Evidence supporting the role of K_V channels includes pharmacological studies, such as when K_V current (I_{KV}) was inhibited by 4-aminopyridine (4-AP), the resulting membrane depolarisation opened VOCCs, causing Ca^{2+} influx and an increase in the $[Ca^{2+}]_i$ within the PASMC (Yuan, 1995; Platoshyn *et. al.,* 2000). Using anti K_V1.5 antibodies, Archer *et. al.,* (1998) demonstrated K^+ current (I_K) inhibition, and selective inhibition of the increase in $[Ca^{2+}]_i$ in response to 4-AP in rat PA. Anti K_V2.1 induced an increase in resting tension and wiped out 4-AP induced contraction in rat pulmonary rings also (Archer et. al. 1998). It has also been suggested that NO promotes the opening of K_V channels in PASMCs, the resulting membrane hyperpolarisation causing a decrease in $[Ca^{2+}]_i$ within the PASMC cytoplasm (Yuan *et. al.,* 1996).

However, this role of K_V channels in regulating the resting membrane potential of PASMCs has been questioned, since the voltage dependence of these channels does not make them suitable candidates for setting resting membrane potential over the physiological range (Gurney *et. al.,* 2002). The role of KCNQ channels in the maintenance of the resting membrane potential of PASMCs is now receiving more attention, as these channels are open at the resting membrane potential (Robbins *et. al.,* 2001). Further evidence suggesting the involvement of KCNQ channels arises from the fact that KCNQ channel blockers appear to inhibit the background K^+ conductance in mice and rat PASMCs and cause vasoconstriction (Gurney *et. al.,* 2005; Joshi *et. al.,* 2006).

1.7.2.2 Ca^{2+} activated K^+ channels (K_{Ca})

BK_{Ca} are found in high density and ubiquitously in vascular SMCs (Kume *et. al.,* 1992) and have been implicated in the regulation of vascular tone (Hasunuma *et. al.,* 1991). Activation of BK_{Ca} channels causes an increase in K^+ current out of the cell, leading to hyperpolarisation of PASMCs, and subsequent vasodilation. Activation provides a negative feedback mechanism to limit further Ca^{2+} entry and hence regulate contraction, because the opening and closing of BK_{Ca} channels is maintained not only by the membrane potential of the PASMC, but also by $[Ca^{2+}]_i$. Therefore upon arterial constriction and corresponding increase in $[Ca^{2+}]_i$, BK_{Ca} channels are activated, promoting hyperpolarisation, and subsequent closure of VOCC. Numerous studies have shown that K_{Ca} channel activity increases as the cell membrane depolarises and the $[Ca^{2+}]_i$ increases (Nelson & Quayle, 1995; Brayden, 1996).

1.7.2.3 K^+ channels inhibited by intracellular ATP (K_{ATP})

Although these channels are present in PASMCs, when ATP levels in the cytosol are within normal limits, the contribution that this family of channels make to the movement of K^+ is minor (Clapp & Gurney, 1992). In PASMCs, the main role of K_{ATP} channels is to hyperpolarise the membrane in response to changes in the metabolic state of the cell. They also have a role in mediating the hyperpolarisation response to vasodilators acting as K^+ channel openers (Gurney, 2004). Examples of vasodilators that activate K_{ATP} include endogenous mediators such as vasoactive peptide (VIP) and PGI_2 (Brayden, 2002).

1.7.2.4 Two-pore domain K^+ channels (K_{2P})

Mammalian lung expresses mRNA for several types of 4TM K^+ channels, including TASK1, TASK2, TASK3, TWIK1, and TWIK2 (Lesage *et. al.,* 2000; Kim *et. al.,* 2000). TASK1 and TASK2 were found to be present in the vessels in both the PASMC and the endothelial cell layers (Gardener *et. al*., 2004). This group concluded that TASK1 channels were important in the regulation of vascular tone. There is evidence that TASK1 and TASK2 have an important role in transmitting the K^+ current that sets the resting membrane potential (E_m) in PASMCs (Gurney *et. al.,* 2003; Gonczi *et. al.,* 2006).

1.8 K^+ channels and PAH

K^+ channels are thought to play a role in the molecular mechanisms of PAH. Down-regulation of K_V channel genes has been found in PAH patient lungs (Geraci *et. al.,* 2001) and down-regulation of K^+ channel expression and inhibited K^+ channel function has been observed in PASMCs from patients with IPAH (Yuan *et. al.,* 1998). K^+ channel dysfunction could be related to some of the contributing factors in the development of PAH.

PASMCs from human and experimental hypoxic PAH animals are deficient in K^+ channels, resulting in a depolarised PASMC membrane and the activation of VOCC channels (Yuan *et. al.,* 1998; Sweeney & Yuan, 2000; Platoshyn *et. al.,* 2001). Oral dichloroacetate which has been shown to increase I_K (Rozanski *et. al.,* 1998) was shown to restore the expression and function of K_V channels, decreased remodelling and PVR in rats with hypoxic pulmonary hypertension (Michelakis *et. al.,* 2002).

In PASMC, inhibition of K_V channels is associated with cell proliferation, the resultant depolarisation induced increase in cytoplasmic Ca^{2+} allowing for progression through the cell cycle (Platoshyn *et. al.,* 2000). Altered K^+ channel activity was shown to result in cell proliferation and increased tone, which are two important factors that contribute to the development of PAH.

1.8.1 5-HT and K^+ channels in PAH

Evidence shows that the decreased expression/ function of K^+ channels in PASMCs may be involved in the pathogenesis of PAH and anorexigen-induced PH (Weir *et. al.,* 1996; Yuan *et. al.,* 1998). Anorexigens inhibit both the expression and function of K_V channels in PASMCs (Wang *et. al.,* 1998; Perchenet *et. al.,* 2001). This may be due to the increase in plasma 5-HT resulting from the inhibition of 5-HTT activity. It has been shown that 5-HT can inhibit $K_V1.5$ currents and increase the membrane potential of PASMC (Cogolludo *et. al.,* 2006). These results implicate both 5-HT and K^+ in the disease process of PAH.

The uptake of 5-HT in PASMCs is determined by the 5-HTT, which is linked to the electrochemical gradient of Na^+. This suggests that the uptake of 5-HT may be voltage dependent. If this is the case, then the resting membrane potential of PASMCs becomes important in the proliferating action of 5-HT within the PASMCs. As K^+ channels are responsible for the resting potential, any dysfunction in these K^+ channels may disrupt the membrane potential and hence disrupt the function of the 5-HTT. Alternatively, a rise in the plasma 5-HT concentration could inhibit K^+ channel activity and give rise to membrane depolarisation, which would exacerbate the 5-HT induced vasoconstriction by causing Ca^{2+} entry.

1.9 Study Aims

The hypothesis under investigation in this study is that 5-HT and K^+ channels can interact and that this may be an important contributor to the development of PAH. There could be a synergistic interaction between 5-HT and K^+ channels, whereby 5-HT promotes depolarisation, which in turn enhances 5-HT action. It is therefore proposed that the inhibition or down-regulation of K^+ channels contributes to increased vascular tone in PAH, and the effect is potentiated by 5-HT. 5-HT receptors and transporters are recognised targets for FPAH. This study will examine the modifying factors that may influence these proteins as therapeutic targets.

Chapter 2

Materials and methods

2.1 Animal models

Animal models are an essential method for investigating animal physiology and pharmacology. They have provided investigators with the means of identifying and developing novel therapeutic strategies.

2.1.1 The 5-HTT+ mouse model of pulmonary hypertension

The C57BL/6_CBA wild-type strain used to generate the 5-HTT+ mouse (MacLean *et. al.,* 2004). The transgene was a 500-kb yeast artificial chromosome (YAC35D8) containing the h5-HTT gene flanked by 150 kb of 5' and 300 kb of 3' sequence, with the "short" allele of the 5-HT transporter gene (5-HTTLPR) in the promoter region and the 10-repeat allele of the variable number tandem repeat in intron 2 (Shen *et. al.,* 2000). The 5-HTT+ mice (female) were used at 5-6 month, with weights of 25 - 40 g. Female wild type (WT) mice were also used at 5-6 months of age, with weights of 25-40 g. Mice were bred in the central biological services research facility, University of Glasgow.

2.1.2 Hypoxic Mouse model

WT mice were maintained in a hypobaric hypoxic environment to induce PAH. As atmospheric pressure decreases, the partial pressures of the gaseous components decrease, and hence the partial pressure of inspired O_2 decreases. However, the percentage of the gaseous components of air remains constant (O_2 ~21 %, N_2 ~78 % and CO_2 ~1 %). The environment was controlled by withdrawing air from the chamber via a vacuum pump, until the pressure within the chamber was equivalent to ~0.5 atmosphere. The chamber was continuously flushed with room air to maintain conditions of low humidity and CO_2. Mice were kept in this environment for a period of 14 days.

2.1.2.1 Maintenance of animals

The hypobaric chamber was kept in an animal holding room, which was set up to maintain the temperature at approximately 21 ˚C, humidity at 55 % and gave 20 changes of filtered air per hour. A 12 hour on, 12 hour off light cycle was maintained. An animal cage was placed in the chamber, with food and water dispensers facing the rear of the cage which allows the animals to be monitored.

2.1.2.2 Hypobaric chamber design and components

A picture of the chamber used is shown in figure 2.1. The chamber holds two standard mouse cages, with up to six mice in each individual cage. The chamber was constructed from transparent highly resistant Perspex, with a door that can be removed. Air was passed into the chamber through a valve on the top right hand of the door and it was continuously removed via a pump. The pressure within the chamber was constantly displayed on a gauge. The pressure was maintained by the inlet air valve. Opening the valve allowed more air to circulate into the chamber and hence increased the pressure to atmospheric pressure; closing the valve stopped air entering the chamber and therefore decreased air pressure within the chamber. The air temperature within the chamber was similar to that outside the chamber and was monitored daily via an internal and external thermometer.

The animals were placed in the chamber, the chamber door was locked into place and the pump was turned on. When the inlet valve was closed slightly, this tightly sealed the chamber door. By adjusting the inlet valve, chamber air pressure was reduced gradually. The pump at the back of the cage continuously pumped a fixed volume of air out of the cage. By adjusting the inlet valve, and hence the volume of air entering the chamber, a vacuum condition could be created. The desired pressure to induce chronic hypoxia in mice is 550 mbar. The chamber air pressure was reduced in a series of steps of 50 mbar over a two day period. The chamber conditions were checked daily and records were made of pressure, temperature (inside and outside the chamber) and humidity. Checks were also made to ensure animals had enough food and water. Daily checks were also important to ensure the animals were not showing any signs of distress. Food and water was also maintained daily throughout the study. Animal cages were changed every three days in order to provide clean and fresh bedding for the animals.

2.1.3 Hemodynamic measurements

Mice were anaesthetised with 3 % isoflurane in oxygen. Maintenance of anaesthesia was via 1 – 1.5 % isoflurane in oxygen. The level of anaesthesia was constantly monitored throughout the procedure by absence of hind limb withdrawal and tail

withdrawal reflexes. Under maintenance anaesthesia, a portion of skin inferior to the rib cage was removed. A hypodermic (25 gauge) needle attached to a transducer was used to measure right ventricular pressure. The needle was attached to a pressure transducer (Elcomatic E751A) connected to a MP100 data acquisition system (BIOPAC Systems Inc, Santa Barbra). The results obtained were analysed using Acquiknowledge 3.5 software.

The needle was placed onto the skin above the diaphragm and advanced under the rib cage into the abdomen, it was then slowly advanced into the right ventricle (RV). The position of the needle was confirmed by the waveform of the pressure tracing recorded and displayed on a computer screen. To record left ventricular pressure, the needle was further advanced into the left ventricle (LV). Once this procedure was completed and recordings of pressure were made, the animal was killed by cervical dislocation. The heart and lung tissue were subsequently removed.

2.14 Determination of heart weights

The chambers of the heart were opened and excess blood was blotted out. The heart was then weighed. The atria and large blood vessels were then dissected from the ventricular mass, which was separated into the RV wall and the LV, including the septum. These tissues were weighed separately, and the ratio of right ventricular (RV) free wall weight to left ventricle plus septum (LV + S) was used as an index of right ventricular hypertrophy.

2.2 In-vitro pharmacological studies

2.2.1 Wire myography

Wire myography was introduced by Mulvany & Halpern in 1976, and is now an established technique used to investigate small vessel contractility and relaxation. Measurements of muscle contraction can be made from small vessels and other tubular tissues with diameters of 60 – 400 μm.

2.2.2 Equipment

The wire myographs used were DMT610M units, purchased from Danish Myo-technology Ltd., Aarhus, Denmark (figure 2.3). The setup had four individual organ

chamber baths, each capable of having one vessel mounted in it. Each individual myograph chamber had two stainless steel jaws to hold the vessel in place, and had individually controlled gas flow and suction (for removing solution and washing tissue). Myograph chamber temperature (± 0.1 ˚C), was controlled electronically at 37.5 ˚C ± 0.1 ˚C, via internal heating units. The temperature and tension for each chamber was displayed digitally on the base unit (figure 2.3). The entire setup was connected to a computer, via a analogue to digital converter. The output from the transducer (measuring force) from each chamber was recorded via a programme called Myodaq20 and analysis of the recordings was carried out using a computer programme called Myodata (MyoDaq software, Danish Myotech, Aarhus, Denmark).

2.2.3 Tissue preparation - Dissection of intralobar pulmonary arteries (IPAs)

Mice were killed with an overdose of sodium pentobarbitone (200 mg/ kg intraperitoneally). Lungs were rapidly removed and placed in ice cold physiological salt solution (PSS). PSS used consisting of (in mM): NaC1 119, NaHCO3 25, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 0.6, $CaC1_2$ 2.5, glucose 11.1, [pH 7.4].

The larger left lobe of the lungs was identified, removed and mounted on a dissection plate. The lobe was placed with the visceral surface facing up from the dish, and the parietal side lying inferiorly. During dissection, the lobe was periodically washed with fresh PSS, to prevent the tissue drying out. A dissecting microscope was used to identify the airway. An incision was made into the opening of the airway, this incision carried on down the airway length, cutting from the large proximal airway along the bronchial tree to the distal bronchiole. When the airway was dissected out, the IPA was found immediately underneath. The IPA (internal diameter ~ 200 - 300 μm) was carefully dissected from the lobe and surrounding connective tissue removed (figure 2.3). The vessel was kept on ice cold PSS before being mounted on the myography setup.

Figure 2.1 Picture of hypobaric chamber used to generate hypoxic mice.

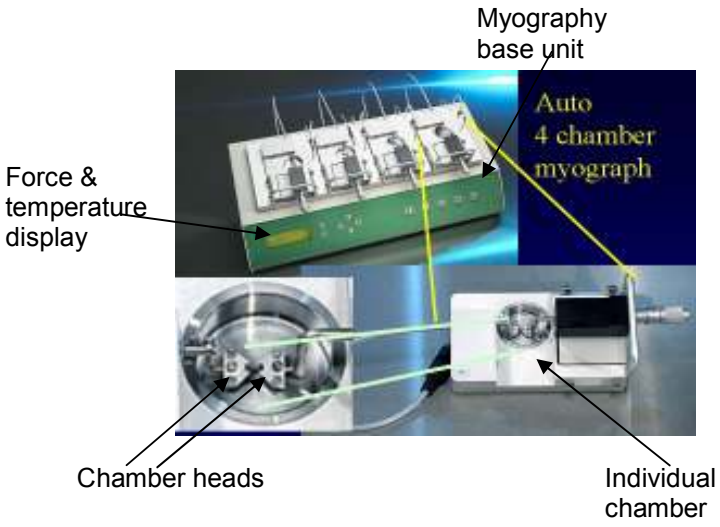


Figure 2.2 Picture of wire myograph used to measure vessel constriction.

Image from www.adinstruments.com/products/hardware/research/product/DMT610M

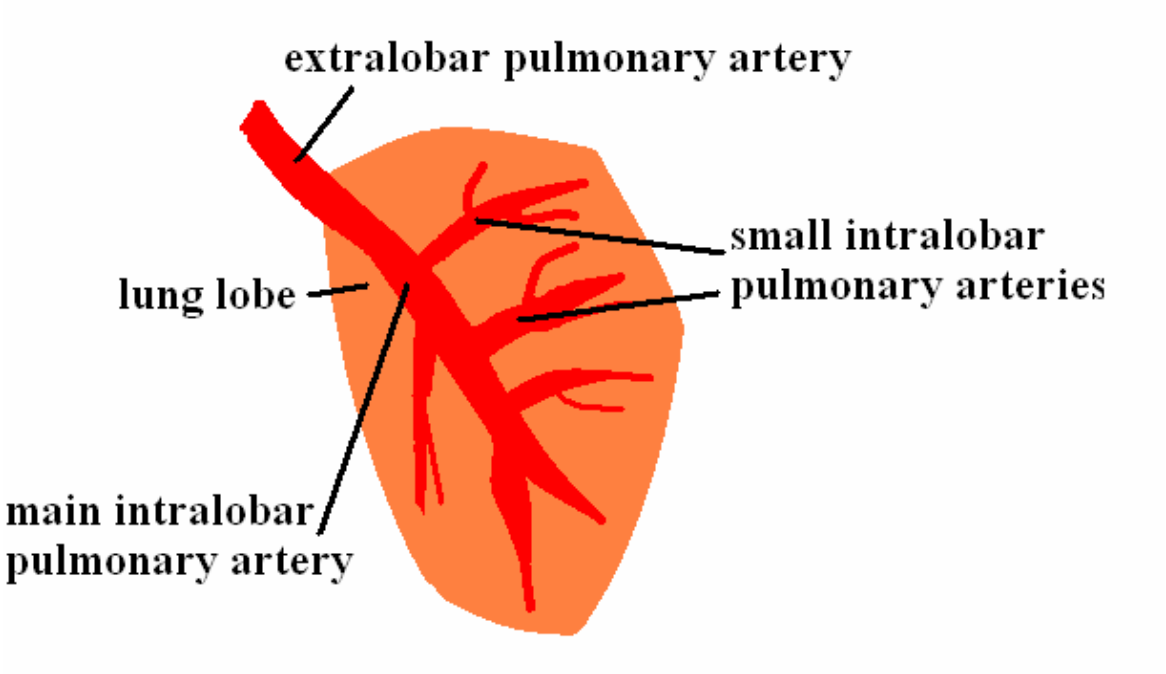


Figure 2.3 Diagram showing location of main intralobar pulmonary artery which was used in myography experiments.

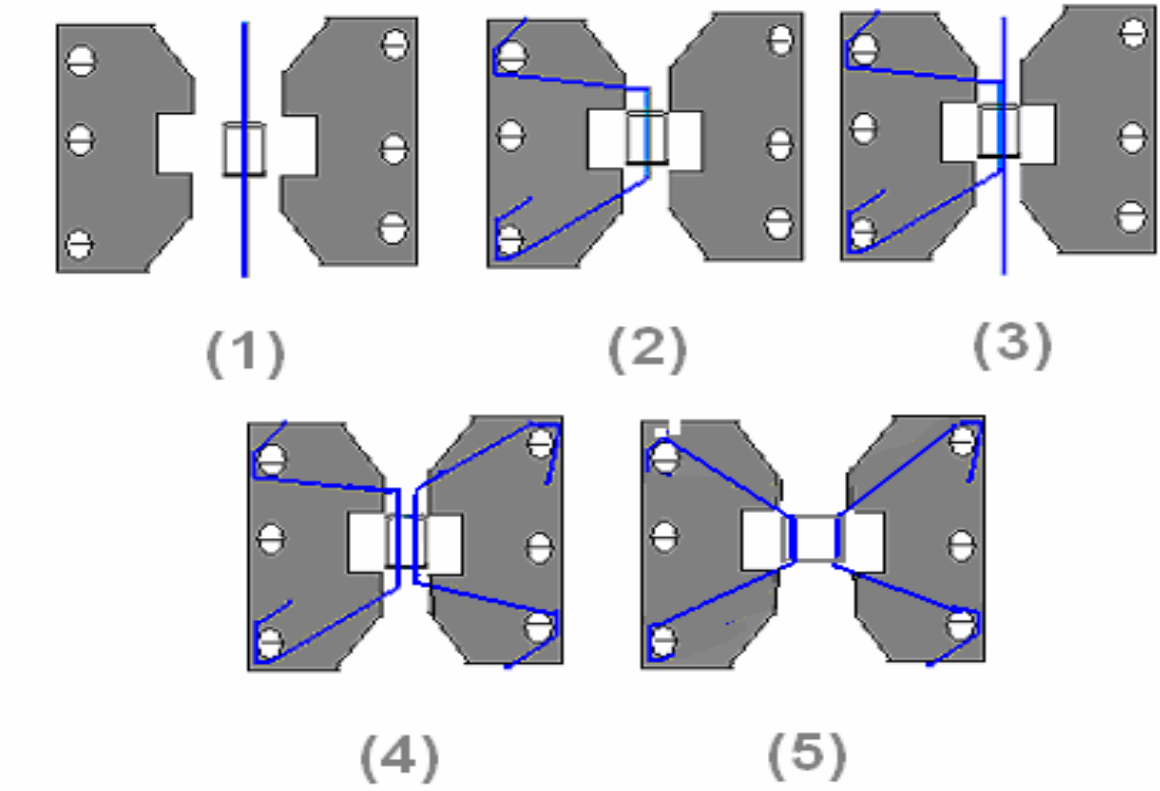


Figure 2.4 Diagram showing the artery mounting procedure.

(1 & 2) Vessel cannulated with wire is transferred to the myography chamber, and mounted to myography chamber head.

(3 & 4) Vessel is cannulated with second wire, which is the mounted to the second myography chamber head.

(5) Vessel is stretched during the normalisation procedure (section 2.2.4)

2.2.4 Myograph mounting procedure

All vessels underwent the same mounting procedure (figure 2.4). Each myograph chamber was filled with 5 ml Krebs and bubbled with 16 % O_2 / 5 % CO_2 (balance N_2). Prior to each experiment, a bag was filled from a combination of gas cylinders with this gas mixture to mimic *in vivo* partial pressure of oxygen (pO_2), and this was pumped into each chamber.

In order to carry out time-matched control experiments, each vessel dissected was cut to provide two sections of ~ 2 mm each. Each section of artery had a 40 μm diameter stainless steel wire placed through its lumen. This was then placed in the myograph chamber and was secured in place by adjusting the adjacent heads of the myograph so that they met trapping the wire between them. Wire at each side of the vessel was manipulated under the screws of one of the jaws, in a clockwise fashion, and the screws secured. Excess wire was removed (figure 2.4 (1 & 2)). The second head of the myograph was moved back from the first, and a second wire was carefully inserted through the vessel lumen (figure 2.4 (3)). Again the two myograph heads were adjusted so they met and the second wire was mounted in an identical fashion on the second head. Finally the two heads were separated slightly, without stretching the vessel, and the wires were adjusted until they were on the same plane. When the heads were slightly opened this provided the zero level of tension for set-up measurements. This is shown in figure 2.4 (1 – 5).

2.2.5 Transmural pressure applied to vessels and normalisation

The internal circumference (IC) of a vessel when under no active tone but distended by a transmural pressure of 100 mmHg is termed IC_{100}. For systemic preparations the figure of 0.9 x L_{100} is chosen as maximum active tension development is optimal at this point. Under these conditions the equivalent pressure of the vessel is between 60 - 70 mmHg. The pulmonary vasculature is normally under the conditions of low resistance and low pressure, and therefore must be set up under conditions which replicate this.

Normoxic mice IPAs were set up to mimic *in-vivo* transmural pressure at ~16 mmHg. The hypoxic mouse lung circulation experiences a higher transmural pressure, (30

mmHg), to that of the normoxic lung, and so different conditions were used by normalising the vessels to a transmural pressure of ~ 30 mmHg.

Mulvany and Halpern developed the original normalisation procedure in 1977. Normalisation allows for vessels to exhibit a resting transmural pressure, based on the Law of Laplace. The first measurement necessary for normalisation of each vessel was the ICi (internal circumference for given amount of stretch (i) applied to the vessel) (see equation 2.1). ICi was calculated using the micrometer readings, and the IC value when no stretch was applied (IC_0). For two wires with a 40 μm diameter, IC_0 would be equal to 205.6 μm. In equation 2.1, point 'X_O' refers to the point at which the two 40 μm wires were just touching, and point 'X_i' being the point to which the vessel is stretched. The difference between the two micrometer readings at the two points is equivalent to the distance between the two wires.

(equation 2.1) $IC_i = (IC_0) + 2(X_i - X_O)$

Arteries were incrementally stretched using the micrometer and passive force (F) was displayed on the baseunit. Wall tension was calculated by dividing the passive force (F) (which is measured and displayed on the base unit), by twice the segment length, as the vessel has both an upper and lower wall, and is therefore, for vessels 2 mm in length was equal to Force / (2 x 2).

The effective pressure (Pi) is an estimate of the pressure necessary to extend a vessel to the measured internal circumference, and was determined using the Laplace equation 2.2. Rearranging equation 2.2 gives equation 2.3.

equation 2.2 $Pi = \text{Wall tension} / (IC_i / 2\pi)$

equation 2.3 $Pi = (2\pi) \times \text{Wall tension} / IC_i$

Equation 2.4 shows equation 2.3 substituting the values for wall tension and IC_i:

equation 2.4 $Pi = (2\pi) \times F / 2 \times L\ (205.6 + (Xi - Xo))$

2.2.6 Protocol for myography experiments

Vessels were allowed to equilibrate for 30 min after mounting in a myograph chamber prior to the beginning of an experiment. During this period the chambers were heated to 37 ˚C and bubbled with the O_2 / CO_2/ N_2 gas mixture. The appropriate tension was applied to each vessel, (dissected to 2mm in length), as described in section 2.2.5.

50 mM KCl was added to each chamber in order to stimulate and check the viability of the vessel. Upon reaching a maximal response, the chamber was washed out six times using bicarbonate PSS, and the vessels were allowed to return to baseline tension for 30 minutes. The vessels were then stimulated again with KCl and if the vessel contracted ≥ 1 mN, the vessel was considered viable and the response to the second 50 mM KCl application was used as a contractile reference for the subsequent experiment. Again, each vessel was washed, allowed to return to baseline tension and left for 30 minutes to equilibrate, before any drug was added.

When performing a vasoconstrictor concentration response experiment, for 5-HT and K^+ channel blocking agents, a suitable time period was left between each dose until contraction began. From this point each subsequent dose was added when contraction of the vessel had stabilised. In vasodilator concentration response curve experiments, (responses to KCNQ channel openers) each vessel was pre-constricted to ~ 70 % of its maximum response (response to 50 mM KCl), using phenylepherine (PE), (10 – 100 nM). Once the vessels started to relax, subsequent doses of agonist were added when the response had levelled off.

2.2.7 Data Analysis of pharmacological studies

Maximum contractile responses to agonists were calculated as a percentage of the contraction produced by KCl (50 mM) and were expressed as the means ± standard error of the mean (SEM). In the case where relaxation was measured, data were expressed as a percentage of the response relative to PE-induced pre-contraction in the vessels and were also expressed as the means ± SEM.

2.2.7.1 Measurement of agonist potency

The potency of a drug is commonly quantified as the EC50, the concentration that leads to 50 % maximal response. The pEC50, equal to the – log of the EC50 is used as standard to compare drug potencies. A computer statistics programme, GraphPad Prism, (for Windows) (version 4.03), was used to measure pEC50 values. pEC50 values were measured after constructing individual concentration response curves for each drug and the mean ± SEM calculated for each group of vessels.

2.2.7.2 Statistical analysis of pharmacological studies

When comparing two groups of data, Student's t-test was the statistical method chosen to investigate statistical difference. One way analysis of variance (ANOVA), followed by an appropriate ad hoc post test, was used to investigate statistical differences between three or more groups of data. $P< 0.05$ was deemed to indicate statistical significance.

2.3 Flupirtine dosing study

Animal dosing studies provide a means of investigating the actions of drugs *in vivo*, and have become an established, though highly regulated practice.

In this dosing study, the WT and 5-HTT+ mice described in section 2.1 were used. Each mouse was given a unique identification number, which was used throughout the study to identify the animal during *in vivo* experiments and tissues which were removed from the animal subsequent to culling. Animals were housed in a manner that would allow each animal to be identified by means of ear tag and colour.

Prior to commencing and during the dosing study, each animal was weighed. Body weight measurement allowed the health of the animal to be identified. These measurements were also used to calculate the amount of drug/ vehicle that each mouse should receive. Mice were kept in groups of 5 per cage and kept in either a hypoxic chamber or control environment. Each cage contained mice from the same group, i.e. WT and 5-HTT+ mice were housed in separate cages. The mice were observed daily.
The procedure used to dose the animals involved the oral gavage technique. A 1 ml syringe containing the solution to be administered was connected to a small diameter

straight gavage tube. Animals were manually restrained, and the gavage tube was inserted through the mouth into the oesophagus. The solution was then delivered directly into the stomach. Care was taken to ensure that the tube did not enter the trachea, or cause any damage to the oesophagus.

Flupirtine is not readily soluble in water, so was prepared as a suspension in 1 % Carboxymethylcellulose (CMC). The concentration of flupirtine used was 30mg/ kg/ day. The mixture was then homogenised. The vehicle and flupirtine suspensions were frozen until required.

2.3.1 Dosing protocol

Oral doses of flupirtine were administered as it has been shown that flupirtine is almost completely absorbed from the gastro-intestinal tract in experimental animals (Obermeier *et. al.,* 1985). *In vivo* studies to date have used doses of 15 mg/ kg/ day in mice (Diamantis *et. al.,* 1987); 40 mg/ kg in rats (Nickel, 1987) and 8, 16, 25 and 50 mg/ kg in rats (Sofia *et. al.,* 1987). Mackie *et. al.,* (2008) dosed rats with 0.01 – 3.0 mg/ kg flupirtine to show that flupirtine produced significant dose-dependent decreases in mean arterial pressure and mesenteric vascular resistance. Sofia *et. al.,* (1987) found that the oral ED_{50} for analgesic activity of flupirtine in mice was 27.4 mg/ kg after one test day, and this level rose to 33.8 mg/ kg after nineteen test day. Based on the data obtained over this dose range, it was decided that 30 mg/ kg/ day would not be toxic to the mice and would be an appropriate dose. The plasma half life of flupirtine in rats and dogs is 2.5 – 3.5 hrs (Schuster *et. al.,* 1998).

A total of 60 female mice were used; 40 WT mice (section 2.1.1) and 20 5-HTT+ mice. Half of the WT mice were subject to 2 weeks hypoxia (section 2.2.3), with the other half being age matched controls. From each group, 10 mice were dosed with flupirtine and 10 were dosed with vehicle (CMC) once per day for 14 consecutive days. Dosing occurred at the same time of day throughout the study.

2.4 Membrane potential measurement

2.4.1 Isolation of PASMCs

PASMCs were isolated from intralobar pulmonary arteries. This allowed the cell membrane potential of individual cells to be measured, as in the intact vessel they are coupled electrically to other PASMCs and are under the influence of endothelial cells.

In order to dissociate PASMCs an appropriate digesting protocol was constructed. A dissociation medium (DM), of the following composition was used (mM): NaCl 110; KCl 5; NaH_2PO_4 0.5; $NaHCO_3$ 10; HEPES 10; phenol red 0.03; taurine 10; EDTA 0.5; $CaCl_2$ 0.16; $MgCl_2$ 2; glucose 10. This medium was bubbled with air and the pH adjusted to 7.0 using NaOH. Into this solution the following enzymes were added and the final solution was mixed by vortexing:

- 1 mg/ ml collagenase type XI
- 0.45 mg/ ml protease (type XXIV bacterial, EC no.232-642-4)
- 2 mg/ ml bovine serum albumin (fatty acid and globulin free)
- 2 mg/ ml trypsin inhibitor

When the solution was fully mixed, five or six strips of intralobar pulmonary artery were added. The solution containing the vessels was then incubated at 37 °C for 8 min (the mixture was titrated gently after 4 min in incubation). The strips of artery were removed from the solution and washed in 0.5 ml enzyme-free DM. Using a glass Pasteur pipette with wide fire-polished tip (~ 2 mm), the artery strips were transferred to an eppendorf tube containing 0.5 ml DM. Subsequently, isolation of the smooth muscle cells was carried out by gentle titration with the Pasteur pipette. Examination of the cells was carried out with a microscope and the solution of PASMCs was titrated to dislodge PASMCs from the vessel tissue as required. The final solution containing freshly isolated PASMCs was stored at 4 °C until use. This protocol was carried out daily.

Initial attempts to isolate PASMCs from 5-HTT+ mice using the above procedure were unsuccessful. Success was obtained using the same procedure but with the concentrations of collagenase and protease doubled, and the initial incubation time at 37 °C extended to 20 min.

2.4.2 Electrophysiology

The patch clamp technique was used to measure membrane potential. This method allows ion channel behaviour to be investigated by monitoring the movement of ions as an electrical current. Patch clamp is mostly used to voltage clamp cells and record current flow, however in this study, the current clamp mode was used to measure membrane potential under conditions of zero current flow.

The original voltage-clamp technique was developed by Cole, (1949) and Hodgkin, Huxley, (1952), who worked on squid giant axon. Their version of voltage-clamp controlled the membrane potential using wires inserted into and along the length of the axon. This type of approach was limited to large cylindrical preparations. Subsequent developments led to the introduction of glass intracellular microelectrodes. These were hard glass capillaries which were melted and pulled to a fine tip, much less than 1 μm in diameter. The advantage of this technique was that the tip of the microelectrode could penetrate the plasma membrane, and the membrane resealed around the tip of the electrode as it entered the cell. This type of voltage clamp is used to make measurements from medium sized cells, such as neurons, and can be used to record membrane potential from smooth muscle in intact arteries.

The patch-clamp technique was invented by Neher & Sakmann, (1976) and further developed by Hamill *et. al.,* (1981). This technique allows small fragile cells, such as smooth muscle cells, to be voltage clamped. In this procedure the tip of a fire-polished glass micropipette (~ 1 μm in diameter) is brought onto the cell surface and a small amount of suction is applied to the back of the patch pipette (figure 2.7). This enables a high resistance seal (>10 GΩ), referred to as a 'giga seal', to form between the pipette and the cell membrane. Depending on the nature of the study there are a number of patch clamp configurations, however in this study the whole cell mode was used (figure 2.6).

2.4.2.1 Patch-clamp protocols

A few drops of cells were allowed to settle for ~ 10 min on the glass bottom of an experimental chamber, placed on an inverted microscope in a patch clamp set-up. When the cells had adhered to the surface they were superfused with PSS (section 2.4)

at a rate of 0.5 ml/ min at room temperature, and the solution was bubbled with 20% O_2/ 5% CO_2.

The patch pipette, contained an internal solution that mimicked the internal environment of the cells, composed of (mM): 130 KCl, 1 $MgCl_2$, 1 EGTA, 20 HEPES, 0.5 Na_2GTP; pH adjusted to 7.2 with 1M KOH. The patch pipette was attached to a pipette holder. Positive pressure was applied to the patch pipette via a 2 ml syringe attached by tubing to the pipette holder via a side arm. This prevented any debris from attaching to the pipette as it entered the bathing solution and was positioned near a cell using a micromanipulator. The amplifier was set to voltage clamp mode and an oscilloscope displayed the voltage and current outputs. As the patch pipette entered the bathing solution a junction potential was formed, which was cancelled out manually using a pipette offset control. A hyperpolarising step of 10 mV (from -80 mV to -90 mV) and 30 ms duration, repeated every 350 ms, was applied to the circuit. This resulted in a rectangular waveform being displayed on the current trace. Using Ohms Law pipette resistance was calculated: pipette resistances of 3-5 MΩ were found to be the most suitable for recording membrane potential from mice PASMCs. Viewing through the microscope, and using the micromanipulator, the tip of the patch pipette was moved until it was just above the cell. As the patch pipette was brought slowly onto the surface of the cell membrane, the amplitude of the current trace reduced. Negative pressure was applied to the patch pipette via gentle suction, using the 2 ml syringe. This resulted in further reduction in the current amplitude, leaving only small transient currents at the onset and offset of the voltage step, caused by pipette capacitance, which were cancelled by adjusting the fast and slow capacitance compensation dials on the control box. This indicated the formation of a giga seal.

Subsequent to giga seal formation, the membrane under the pipette tip was ruptured by applying further negative pressure to the patch pipette, to form a low resistance pathway between the cytosol and the pipette. This is the whole-cell mode of the patch-clamp technique. Successful entry into the cytosol was indicated by large sharp current transients at the onset and offset of the applied voltage step, due to charging of the cell capacitance.

In order to measure membrane potential, the amplifier was switched to current-clamp mode with current set at zero. Recording from the cell was started immediately after seal formation, and the membrane potential was continuously recorded throughout the experiment. Each cell from which recordings were made was allowed to equilibrate, until a steady membrane potential was recorded, which was seen as a steady trace on the computer monitor. This was recorded as the resting cell membrane potential.

Experiments were also carried out to investigate the membrane potential response to 5-HT. After recording resting membrane potential 5-HT was then added to the local environment of the cell via a gravity fed perfusion pipette positioned close to the cell. Membrane potential was measured in the presence of 5-HT. Initially 1 μM 5-HT was added, and any change in membrane potential was measured. The membrane potential was allowed to stabilise. 10 μM 5-HT was subsequently added to the cell's local environment, and membrane potential was measured until a steady trace was shown on the computer monitor.

2.4.2.2 Pipettes & Pipette solution

Recording pipettes were made from borosilicate glass capillaries (Harvard Apparatus Ltd). Pipettes were pulled in two stages using a vertical multi-stage pipette puller (Narishige, Japan, model PB-7). The tips of the pipettes were fire polished to produce a smooth and clean tip (aids seal formation), and the shank of the pipette was then lightly coated with beeswax. The beeswax produced a hydrophobic coating of the shank, which reduces pipette capacitance and noise in the recording. Recording pipettes were back filled with enough internal solution (~ 5 μl) to contact a Ag/ AgCl electrode wire in the pipette holder, which connected the cell to the patch-clamp amplifier. Any air bubbles were removed by gently flicking the pipette. In order to complete an electrical circuit, an Ag/ AgCl pellet, which was connected to ground, was placed in the bathing solution containing the PASMCs and formed a reference electrode.

2.4.2.4 Sources of error

During current flow a potential difference was created between the electrodes, known as a junction potential. This can lead to incorrect recordings. The use of a Ag/ AgCl wire in the pipette holder and Ag/AgCl pellet in the bath stabilises this junction potential. Ag/ AgCl dissociates into Ag^+ and Cl^- ions when electrons flow through a circuit.

When electrons (and therefore current) flow in the opposite direction, the reverse occurs and the ions recombine. This allowed the charge in the solution near the electrodes to be constant. As these ions are continually in transition, a potential difference is created between the bathing (or pipette) solution and the electrode, which at equilibrium is known as the half cell potential. This was kept stable for each Ag/ AgCl electrode by keeping the Cl^- ion concentration constant in each experiment. These potential differences remained constant during the experiments and were compensated for at the beginning of each experiment.

Junction potentials arise between two different solutions when the two solutions are a) in contact, b) composed of ions at different concentrations and c) contain ions of different mobilities. A liquid-liquid junction potential occurred when the recording pipette was first inserted into the bathing solution. This was due to the pipette solution and the bath solution having different Na^+ and K^+ activities. The pipette offset control was used to zero the output when the pipette was in the bathing solution. Upon the formation of a giga-seal with the cell membrane, the pipette solution was no longer in direct contact with the bathing solution, and hence the liquid junction potential disappeared, but the compensating offset remained. This was not corrected for in the current clamp experiments carried out in this study, but gave rise to a recording error of less than 2 mV.

2.4.2.5 Data analysis of current clamp studies

Membrane potentials were recorded and analysed using WinWCP software (Whole cell program, WCP, Strathclyde Electrophysiology Software, John Dempster 2005). Data is expressed as the means ± SEM of *n* cells. These were compared using Student's t-test. $P< 0.05$ was deemed to indicate statistical significance.

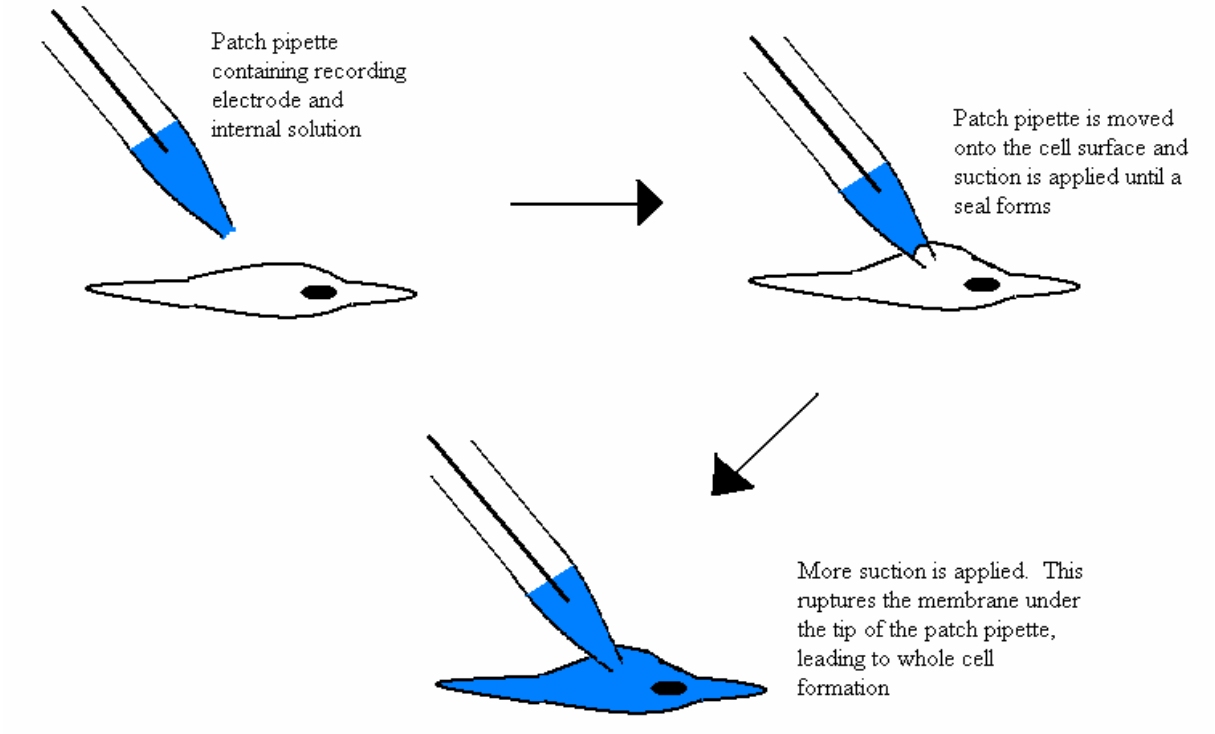


Figure 2.6 Diagram showing the Whole Cell Patch Clamp technique.

Patch pipette was brought to the PASMC surface. Light suction was applied to the back of the patch pipette in order to form seal with the cell membrane. Once a tight seal was achieved, further suction was applied to the pipette, which ruptured the cell membrane, resulting in whole cell formation.

2.5 Drugs and solutions

PSS used in myography and patch clamp experiments is shown in table 1. Internal solution used in patch clamp experiments is shown in table 2. A list of all drugs and reagents used as part of this study is shown in table 3. Table 4 indicates the solvents in which drugs were dissolved.

	Concentration (mM)		
Salt	**5.9mM K+ PSS**	**20 mM K+ PSS**	**50 mM K+ PSS**
NaCl	119	99	69
KCl	4.7	18.8	48.8
$MgSO_4.7H_2O$	0.6	0.6	0.6
KH_2PO_4	1.2	1.2	1.2
$CaCl_2$	2.5	2.5	2.5
$NaHCO_3$	25	25	25
Glucose	11.1	11.1	11.1

Table 2.1 PSS solutions. Unless otherwise stated in each chapter results section, experiments were carried out using 5.9 mM K^+ PSS.

Salt	**Concentration (mM)**
KCl	130
$MgCl_2$	1
EGTA	1
HEPES	20
Na_2GTP	0.5

Table 2.2 Internal pipette solution.

Compound	Supplier
4-aminopyridine	Sigma
5-hydoxytryptamine	Sigma
bovine serum albumin (BSA) (fatty acid and globulin free)	Sigma
$CaCl_2$	Sigma
capsaicin	Tocris
collagenase (type XI)	Sigma
ethylenediaminetetraacetic acid (EDTA)	Sigma
ethylene glycol tetraacetic acid (EGTA)	Sigma
flupirtine	Sigma
glucose	Sigma
glibenclamide	Sigma
HEPES	Sigma
KCl	Sigma
HEPES	Sigma
KH_2PO_4	Sigma
ketanserin tartrate	Tocris
linopirdine	Tocris
magnesium chloride	Sigma
$MgCl_2$	Sigma
$MgSO_4.7H_2O$	Sigma
NaCl	Sigma
$NaHCO_3$	Sigma
NaH_2PO_4	Sigma
NaOH	Sigma
protease (type XXIV bacterial, EC no.232-642-4)	Sigma
retigabine	Astra Zeneca
phenol red	Sigma
taurine	Sigma
tetraethylammonium	Tocris
trypsin inhibitor	Sigma
zinc chloride	Tocris

Table 2.3 Drugs & reagents.

Compound	Solvent
4-aminopyridine	PSS
2-aminoethyl diphenylborinate	DMSO
5-hydoxytryptamine	PSS
capsaicin	DMSO
flupirtine	CMC
glibenclamide	DMSO
ketanserin tartrate	PSS
linopirdine	PSS
magnesium chloride	PSS
retigabine	DMSO
tetraethylammonium	PSS
zinc chloride	PSS

Table 2.4 List of solvents used to dissolve drugs.

PSS = 5.9 mM K^+ PSS

DMSO = Dimethylsulphoxide

CMC = Carboxymethylcellulose

Chapter 3

Characterisation of K^+ channels mediating resting tone in PASMCs and the effects of K^+ channel blockers on 5-HT induced contraction

3.0 Introduction

PA tone and SMC contraction depend largely on the $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ is influenced by several signalling pathways, and by the membrane potential of the SMC. It is a well recognised phenomenon that depolarisation of SMC membrane results in the opening of VOCCs, through which Ca^{2+} enters the cell and induces contraction. Changes in the resting membrane potential have been described in animal models of pulmonary vasculature disease. For example, PASMCs were depolarised during PAH caused by chronic exposure to hypoxic conditions (Post *et. al.,* 1992, Archer *et. al.,* 1993, Osipenko *et. al.,* 1997, Yuan *et. al.,* 1993). The changes found in the disease state may be due to an alteration in the expression or activity of K^+ channels that maintain the K^+ efflux at rest (Smirnov *et. al.,* 1994, Osipenko *et. al.,* 1998).

A number of studies have described K_V channels in mediating the resting potential in PASMCs (Yuan *et. al.,* 2005, Archer *et. al.,* 1998). $K_V1.5$, $K_V2.1$ and $K_V9.3$ have been proposed as the K^+ channel subunits of most importance in regulating membrane potential (Archer *et. al.,* 1998; Patel *et. al.,* 1997). In contrast, another study has indicated that classical delayed rectifier K_V channels are unlikely to mediate resting K^+ conductance, as they have a very low probability of opening at resting membrane potential (Gurney *et. al.,* 2006; Gurney *et. al.,* 2002). KCNQ channels were recently implicated in playing a role in the maintenance of resting K^+ conductance in SMCs within the pulmonary vasculature (Joshi *et. al.,* 2006; 2008(submitted)). These channels are proposed to be involved as they have a low voltage threshold for activation, show little or no inactivation during sustained depolarisation and are outwardly rectifying (Robbins, 2001). When the KCNQ channel blockers, linopirdine and XE991, were applied to rat PA it was found that these drugs were potent vasoconstrictors. The constriction was found to be due to an action on the underlying PASMCs and a functional role for KCNQ channels in regulating the resting membrane potential was suggested (Joshi *et. al.,* 2006). Two-pore domain K^+ channels are also candidates for mediating resting potential, as their biophysical properties make them likely candidates for involvement as background conductance pathways (Lesage & Lazdunski, 2000). There are several members of the TASK family, but TASK1 appears to be of major significance in rabbit and rat PASMCs (Gurney *et. al.,* 2003; Gardener *et. al.,* 2004) while TASK2 may play a lesser role in mediating resting K^+ conductance (Gonczi *et.*

al., 2006). The activation of BK_{Ca} and K_{ATP} channels can also affect membrane potential. However, these channels appear to be silent at the resting potential and their main role in the pulmonary vasculature seems to be to dilate arteries in response to a change in the metabolic state of the cell (Clapp & Gurney, 1991; 1992). BK_{Ca} channels were shown not to be necessary to maintain the resting membrane potential in rabbit PASMCs (Osipenko *et. al.,* 1997). I_K flowing through BK_{Ca} channels has been identified in rabbit PASMCs (Clapp & Gurney, 1991; Vandier & Bonnet, 1996), and this current was shown to result from a high $[Ca^{2+}]_i$ and depolarisation of the cell membrane. The Ca^{2+}-activated K^+ current serves as a negative feedback mechanism in regulating the degree of membrane depolarisation and hence vascular tone (Brayden & Nelson, 1992). Contributions of K_{ATP} channels to resting membrane potential of rabbit PASMCs have been reported (Clapp & Gurney, 1992). Within the intact pulmonary circulation, K_{ATP} channels can contribute to reducing basal vascular tone, but do so primarily at the conduit artery level rather than in resistance segments of the cat PA tree (Shirai *et. al.,* 2001). At normal physiological conditions, with intracellular ATP concentration in the millimolar range, nearly all K_{ATP} channels are closed (Nelson & Quayle, 1995). This would explain why the addition of glibenclamide (a K_{ATP} channel inhibitor) had no effect on membrane potential or $[Ca^{2+}]_i$ (Yuan *et. al.,* 1995). Therefore, the role of K_{ATP} channels may be modulatory in times of metabolic stress.

K_V channels transmit currents, which can be subdivided into rapid inactivating currents (I_{KA}) and delayed rectifier currents (I_{KDR}) and non inactivating currents (I_{KN}). Depending on the alpha-subunits composing K_V channels different biophysical properties are conveyed that resemble I_{KA} or I_{KDR} channels found in native cells. It is been proposed that expression of certain alpha subunits give rise to currents which behave in a classical delayed rectifier manner, these subunits are K_V1.1, 1.2, 2.1, 2.2, 3.1 and 3.2 (Mathie *et. al.,* 1998). Expression of the alpha subunits K_V1.4, 3.4, 4.1, 4.2 and 4.3 results in the transmission of currents that correspond to I_{KA} type currents (Mathie *et. al.,* 1998). The main pharmacological agents used to block K_V channels are tetraethylammonium ions (TEA) and 4-AP, however these classical K_V channel blockers are not specific (table 3.1). TEA acts on two separate sites on the K_V channel, one site located on the external side and one located on the internal face of the channel (Mathie *et. al.*, 1998). The external TEA binding site is located on the carboxyl end of the pore region of the alpha-subunit, and it has a cysteine residue located here which

confers sensitivity to TEA (Kavanaugh *et. al.,* 1991, 1992). The alpha-subunits that have this residue include K_V1.1, 1.6, 2.1, 2.2, 3.1-3.4, making channels composed with these subunits, more sensitive to external TEA blockade. In the case of K_V1.1, for TEA to bind it requires all of the four alpha subunits to contribute to the binding process, therefore heteromeric channels may show varying degrees of TEA blockade (Kavanaugh *et. al.*, 1992; Mathie *et. al.*, 1998). TEA (10 mM) has been shown to depolarise the membrane of rabbit PASMCs (Casteels *et. al.,* 1977), and 1 mM TEA depolarised and contracted rat PASMCs (Suzuki & Twarog, 1982). Within this concentration range (1 – 10 mM) TEA inhibits a number of K_V channels, including K_V1, 2 and 3 (table 3.1). Using TEA to identify K_V channels involved in maintaining the resting membrane potential is complicated further as TEA has also been shown to block K_{Ca} channels; ≤ 10 mM TEA has been shown to be a blocker of BK_{Ca} channels in VSMCs (Nelson & Quayle, 1995). TEA also has an effect on KCNQ channels (Robbins, 2001).

Most of the evidence supporting the role of delayed rectifier channels in resting potential regulation involved the use of 4-AP. For example, 4-AP sensitive K_V channels that are active under resting conditions have been suggested to be the major contributors to the regulation of resting membrane potential in rat PASMCs (Yuan *et. al.,* 1995). This drug is a blocker of K_V channels, and it has been shown to reduce the resting potential in PASMCs and increase tension in the intact PA (Hara *et. al.,* 1980; Osipenko *et. al.,* 1997; Doi *et. al.,* 2000). Hara *et. al.,* (1980) showed that 10 mM 4-AP depolarised the cell membrane of guinea pig PA and induced contraction. Using mice PASMCs, Yuan *et. al.,* (1995) presented evidence that 0.3 -10 mM 4-AP depolarised myocytes in a dose-dependent manner and furthermore 10 mM 4-AP induced an increase in $[Ca^{2+}]_i$. 4-AP acts by blocking the channel pore (Bouchard & Fedida, 1995) and the specific K_V channels blocked by this drug are determined by the concentration used. The concentrations of 4-AP that block various K_V channels are presented in table 3.1. The concentration of 4-AP which Yuan *et. al.*, (1995) showed to depolarise mice PASMCs (0.3 - 10 mM) indicates that K_V 1, 2 and 3 were blocked (table 3.1). At 5 - 10 mM 4-AP, the concentration shown to increase $[Ca^{2+}]_i$ (Yuan *et. al.,* 1995; Doi *et. al.,* 2000) K_V 1, 2 and 3 channels would be inhibited. Like TEA, 4-AP has been seen to affect both I_{KA} and I_{KDR} currents; however it has little effect on KCNQ currents. Another drug that has been shown to have an effect on K_V channels is capsaicin.

Capsaicin is a drug with several effects. It has been shown that the main site of action of capsaicin is vanilloid receptors on sensory nerve fibres (Oh *et. al.,* 1996; Wardle *et. al.*, 1996; Wood *et. al.,* 1997). However, it has also been shown to cause pronounced inhibition of I_{KDR} in PASMCs (Osipenko *et. al.,* 2000). Dubois, (1982) carried out a study in which capsaicin blocked the fast but not the slowly inactivating outward K^+ current in the frog node of Ranvier. This inhibitory action of capsaicin was also studied using rat pituitary cells; it was shown to produce a time dependent block of fast-activating, fast-inactivating K^+ current and slowly-activating, slowly-inactivating K^+ current (Kehl, 1994), and this block of K^+ current by capsaicin was due to open channel blockade. The concentration of capsaicin required to block K_V1 channels is 100 μM (Fischer *et. al.,* 2001).

KCNQ channels transmit currents which have similar biophysical properties which are similar to I_{KN} (Robbins, 2001; Evans *et. al.,* 1996), and it has been suggested that KCNQ channels may transmit a component of this current (Joshi *et. al.,* 2006). Linopirdine inhibits KCNQ channels with the following potency profile KCNQ2 = KCNQ3 > KCNQ1 > KCNQ5 > KCNQ4 (Robbins, 2001). KCNQ currents are inhibited by 10 μM linopirdine in SMCs, resulting in systemic arterial contraction (Mackie *et. al.,* 2008) however another group found that linopirdine did not affect the systemic circulation, however, it did contract rat PA (Joshi *et. al.,* 2008(submitted)). TASK channels have been implicated in conducting I_{KN} (Gurney *et. al.,* 2003). I_K transmitted by 2-pore domain channels, such as TASK K^+ channels, is blocked by zinc ions (Zn^{2+}). The sensitivity of TASK channels has been debated, with some authors suggesting that TASK1 channels are more sensitive to Zn^{2+} than TASK3 channels (Leonoudakis *et. al.,* 1998; Kim *et. al.,* 2000; Cooper *et. al.,* 2004). This was disputed by another study, which showed the reverse; that TASK3 is more sensitive to Zn^{2+}, with TASK1 channels being relatively insensitive to the ion (Clarke *et. al.,* 2004). However it is generally agreed that TASK2 channels are insensitive to Zn^{2+} blockade. TASK1 and TASK2 channels are expressed in PASMCs and the TASK current in PASMCs was substantially inhibited by 200 μM Zn^{2+} (Gurney *et. al.,* 2003; Gardener *et. al*., 2004), therefore Zn^{2+} was used to distinguish between these two channel types for the purposes of this study. Zn^{2+} has also been shown to inhibit certain K_V channels (Bardoni *et. al.,* 1994), therefore any potential rise in PA basal tone in response to Zn^{2+} application could also be attributable to K_V channel blockade.

ATP activated K^+ channels are sensitive to glibenclamide, with maximal inhibition at around 1 μM (Clapp & Gurney 1991; Clapp *et. al.,* 1993). K_{ATP} channels are hetero-octomers composed of two disparate subunits. Four of the subunits belong to the K_{IR} superfamily of K^+ channels. It is the other four subunits, known as SUR subunits, which contain sites that have high affinity for sulfonylurea compounds such as glibenclamide (Bryan *et. al.,* 2004).

The K^+ channel blockers discussed above were used in the following experiments to investigate the classes of K^+ channels involved in regulating the resting membrane potential in mouse PA. Most studies to date have focused on other species of PA and therefore the following experiments would allow mouse PA to be compared to other animals in response to the K^+ channel blockers used. The effects of K^+ channel blockers were investigated on WT and 5-HTT+ mice IPAs. The aim of these experiments was to investigate if the classes of K^+ channels involved in maintaining the low tone of WT mouse IPA was similar to those in the PAH model, the 5-HTT+ mouse. As mentioned previously, K^+ channel down regulation has been shown in other models of PAH. For example, a loss of K_V current has been observed in rat PASMCs that had chronic hypoxic PAH induced (Sweeney & Yuan, 2000). The experiments carried out as part of this study could potentially identify if this down regulation occurs in 5-HTT+ mice IPAs, in which the cause of PAH is known.

It has been shown that 5-HT can inhibit K^+ channels in a number of cell systems. For example, 5-HT was found to inhibit TASK1 like K^+ currents and cause depolarisation in neurons (Czirjak *et. al.,* 2001; Talley *et. al.,* 2000). TASK1 and TASK3 conductances are inhibited by a wide variety of $G\alpha_q$-coupled receptors (Talley *et. al.,* 2000) and more specifically by 5-HT2A receptors (Hopwood & Trapp, 2005). In a recent study by Cogolludo *et. al.,* (2006), it was indicated that 5-HT2A receptor activation inhibits K_V currents in rat PASMCs and human $K_V1.5$ channels. In the third set of experiments described in this chapter, 5-HT contractile responses were investigated in the presence of K^+ channel blockers in order to investigate any links between K^+ channels and 5-HT, thereby potentially identifying possible interactions between K^+ channels and 5-HT in mouse PA. By repeating these experiments using IPAs from 5-HTT+ mice, this would highlight if any interactions between 5-HT and K^+ channels seen in WT vessels were altered in the PAH model.

K^+ channel blockers	Channel of action in PASMC	Concentration required to inhibit channel
TEA (extracellular)	1) Kv1x, a)1.1 b)1.2 c)1.3 d)1.6 2) Kv2x, 3) Kv3X 4) BK_{Ca} 5) KCNQ (incomplete)	1) a) 0.5 mM [χ] b) > 10 mM [χ] c) 10- 50 mM [χ] d) 1.7 – 7 mM [χ] 2) 4-10 mM [χ α] 3) 0.09-0.3 mM [χ α] 4) 1 mM [β] 5) 30 mM [γ]
4-AP	1) Kv1x, a)1.1 b)1.2 c)1.3 d)1.4 e)1.5 f)1.6 g)1.7 2) Kv2x 3) Kv3x 4) Kv4.1 (4.2 and 4.3 blocked incompletely)	1) a) 0.16 - 1.1 mM [χ α] b) 0.2 - 0.8 mM [χ α] c) 0.2 - 1.5 mM [χ α] d) 0.7 - 13 mM [χ α] e) < 0.1 – 0.4 mM [χ α] f) 0.3-1.5 mM [χ α] g) 0.25 mM [χ] 2) 0.5- 4.5 mM [χ α] 3) 0.02 – 1.2 mM [χ α] 4) 9 mM [χ] (2 – 10 mM)
Capsaicin	1) K_V1.1, 1.2, 1.3, 1.5 2) K_V3.1	1) 100 μM* 1 – 3 μM [θ]
Glibenclamide	K_{ATP}	10 μM [β, λ]
Zinc chloride	1) TASK1 2) K_V1.1, 1.4, 1.5	1)175 μM [ν] 2) ≥ 200 μM [τ]
Linopirdine	KCNQ	10 μM [o π]

Table 3.1 Pharmacology of K^+ channel blockers.

[χ] Coetzee *et. al.,* 1999; [α] Mathie *et. al.,* 1998; [β] Yildiz *et. al.,* 2006; [γ] Suh & Hille, 2007; * Grissmer *et. al.,* 1994; [θ] Lo *et. al.,* 1995; [λ] Lopez-Valverde *et. al.,* 2005; [ν] Leonoudakis *et. al.,* 1998; [τ] Harrison *et. al.,* 1993; [o] Joshi *et. al.,* 2006; [π] Mackie *et. al.,* 2008.

3.2 Results

3.2.1 Effect of 50 mM KCl in WT and 5-HTT+ mice IPAs

Contractions in WT and 5-HTT+ mice IPA were measured relative to the contraction induced by 50 mM KCl (figure 3.1). The contraction to 50 mM KCl in WT tissues was 1.7 ± 0.13 mN (n = 12) and in 5-HTT+ vessels the contraction induced was 1.7 ± 0.12 mN (n = 12). There was no significant difference between these values.

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3.2.2 Effects of K^+ channel blockers in WT and 5-HTT+ mice IPAs.

Increasing concentrations of TEA (1 nM – 1 M), were applied to vessels under resting tone. The resulting contractions were measured and concentration response curves constructed. In IPAs from WT and 5-HTT+ mice, the blocker had very little effect on resting tone within the concentration range that has been shown to inhibit K^+ channels (figure 3.2). From the concentration response curves it can be seen that TEA only began to have an effect on resting tone at concentrations above 10 mM. The pEC50 value of the response in WT mice IPAs was 1.5 ± 0.1, and in and 5-HTT+ vessels the pEC50 value was 1.0 ± 0.03 and there was no statistically significant difference between these values.

4-AP produced concentration dependent constriction in mice IPA vessels (figure 3.3), however contraction was not seen until a concentration of 3.5 mM was reached in the bathing solution. The maximum response was not reached at the highest concentration of 4-AP tested (10 mM). In response to 10 mM 4-AP, the contraction induced in WT vessels was 25.3 ± 7.0 % (n = 6) of the response induced by 50 mM KCl, whereas in 5-HTT+ vessels 10 mM 4-AP induced a much greater contraction of 75.0 ± 8 % (n = 5) ($P < 0.001$), but overall the sensitivity to 4-AP was similar in the vessels from both types of mice.

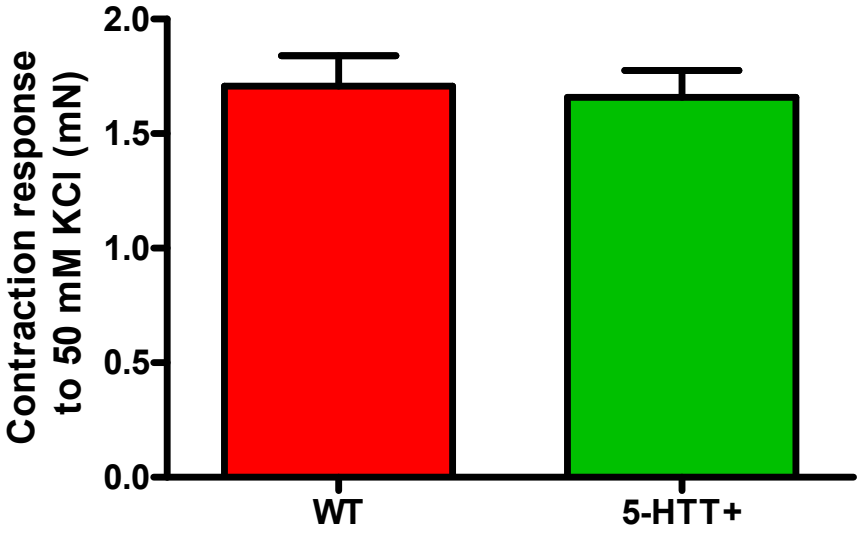


Figure 3.1 Effect of 50 mM KCl in WT and 5-HTT+ mice IPAs.

Concentration obtained to KCl (50 mM). Data are expressed as force induced in mN. Columns represents the mean ± SEM (n = 12).

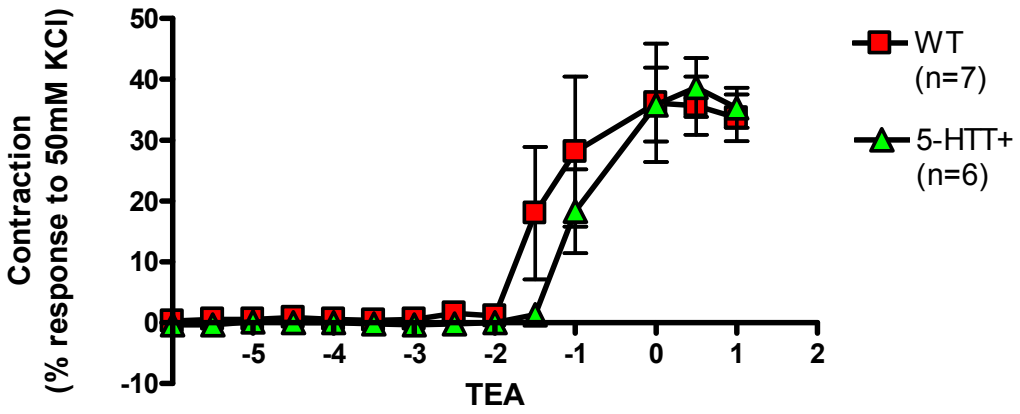


Figure 3.2 TEA induced contractions in WT and 5-HTT+ mice IPAs.

Concentration response curve obtained to TEA (1 μM – 1 M). Data are expressed as a percentage of the reference contraction to 50 mM KCl in each vessel. Each point represents the mean ± SEM (n = 6 – 7).

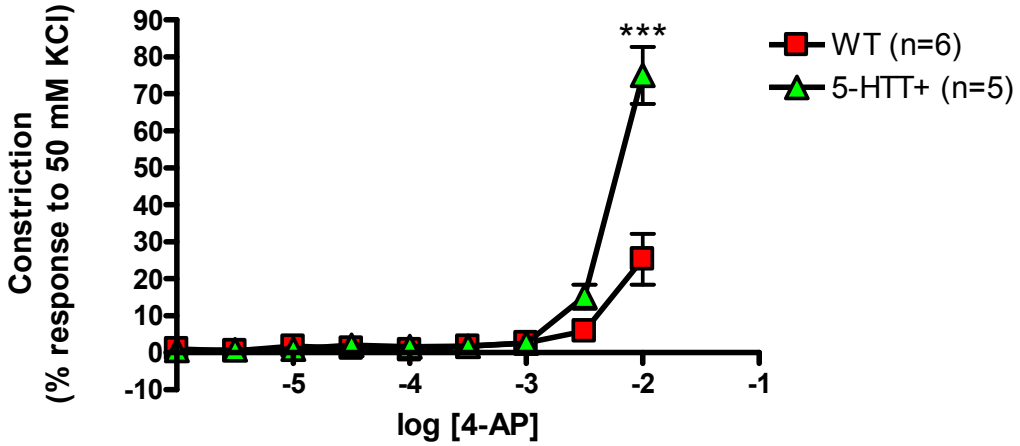


Figure 3.3 4-AP induced contractions in WT and 5-HTT+ mice IPAs.

Concentration response curve obtained to 4-AP (1 μM – 10 mM). Data are expressed as a percentage of the reference contraction to 50 mM KCl in each vessel. Each point represents the mean ± SEM (n = 5 - 6). *** = $P < 0.001$ indicates difference from WT value.

WT and 5-HTT+ mice IPAs failed to constrict in response to glibenclamide at concentrations where this drug has been shown to block K_{ATP} channels. At 10 μM glibenclamide, there was no change in the tension of WT or 5-HTT+ mice IPAs (figure 3.4).

Capsaicin induced very little vasoconstriction in WT and 5-HTT+ mice IPAs, (figure 3.5). At the highest concentration of tested (100 μM), which has been shown to block K_V1 channels (Grissmer *et. al.,* 1994), the amplitude of contraction in WT mice IPA was only 2.2 ± 3 % (n = 5) of the contraction induced by 50 mM KCl and 0.12 ± 0.1 % (n = 8) in 5-HTT+ mice. There was no significant difference between these responses.

At the concentration required to inhibit TASK channels (100 – 500 μM), $ZnCl_2$ had no effect on resting tone in WT or 5-HTT+ mice IPAs. Vasoconstriction in both types of vessel occurred at concentrations of 1 mM and above (figure 3.6). The maximal contraction was not reached even at the highest concentration tested (350 mM).

In mice IPAs, linopirdine produced concentration dependent constriction over the range of concentrations that inhibits KCNQ channels (< 10 μM) (Coetzee *et. al.,* 1999) (figure 3.7). The maximum response to linopirdine in WT vessels was reached at 3.5 μM. However, linopirdine relaxed WT vessels at concentrations above 10 μM, as previously described (Joshi *et. al.,* 2006). There was a significant difference ($P < 0.05$) in the Emax for PA constriction between WT and 5-HTT+ mice. The maximum contractile response to linopirdine in WT mice IPAs was measured at approximately 3.5 μM with an Emax value of 56 ± 9 %, (n = 7) of the contraction induced by 50 mM KCl. In the case of the 5-HTT+ vessels the Emax was 27 ± 2 %, (n = 8), measured at 100 μM. The difference in the linopirdine response between the vessels from the two animals is also shown by a significant difference between the pEC50 values, which were 7 ± 0.2 for WT vessels and 5 ± 0.1 for 5-HTT+ vessels ($P < 0.001$). In WT mice IPAs, linopirdine produced relaxation at 100 μM, whereas in 5-HTT+ vessels relaxation was not seen until 300 μM.

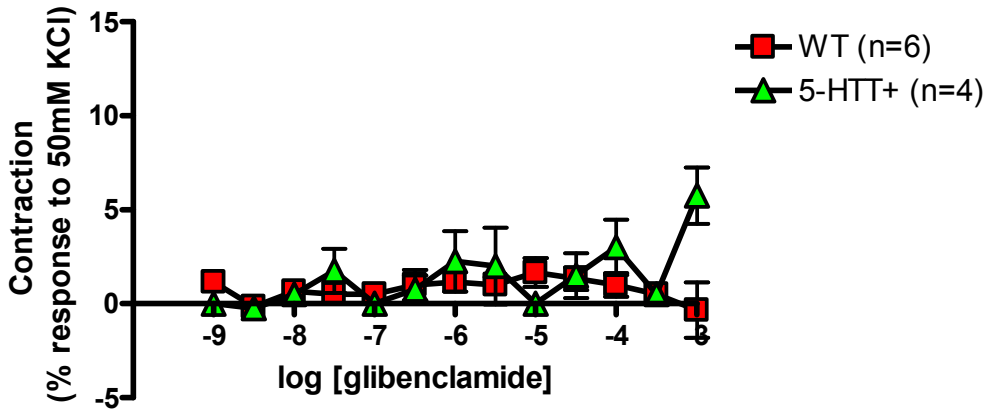


Figure 3.4 Glibenclamide induced contractions in WT and 5-HTT+ mice IPAs.
Concentration response curve obtained glibenclamide (1 nM – 1 mM). Data are expressed as a percentage of the reference contraction to 50 mM KCl in each vessel. Each point represents the mean ± SEM (n = 4 - 6).

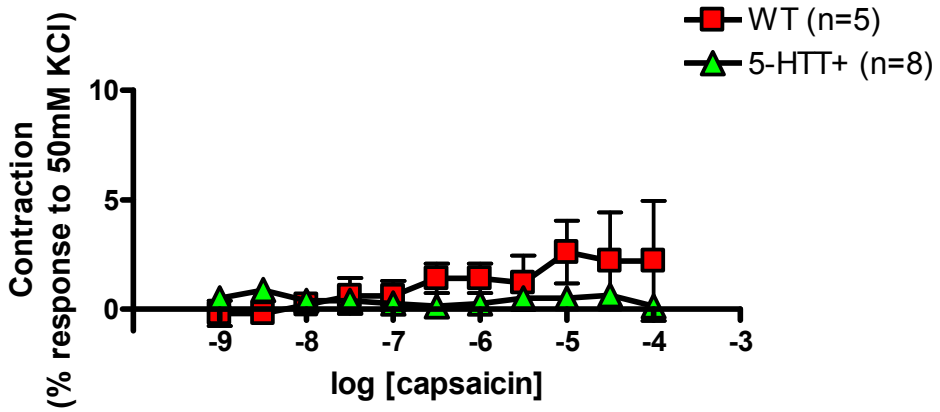


Figure 3.5 Capsaicin induced contractions in WT and 5-HTT+ mice IPAs.
Concentration response curve obtained to capsaicin (1 nM – 100 μM). Data are expressed as a percentage of the reference contraction to 50 mM KCl in each vessel. Each point represents the mean ± SEM (n = 5 - 8).

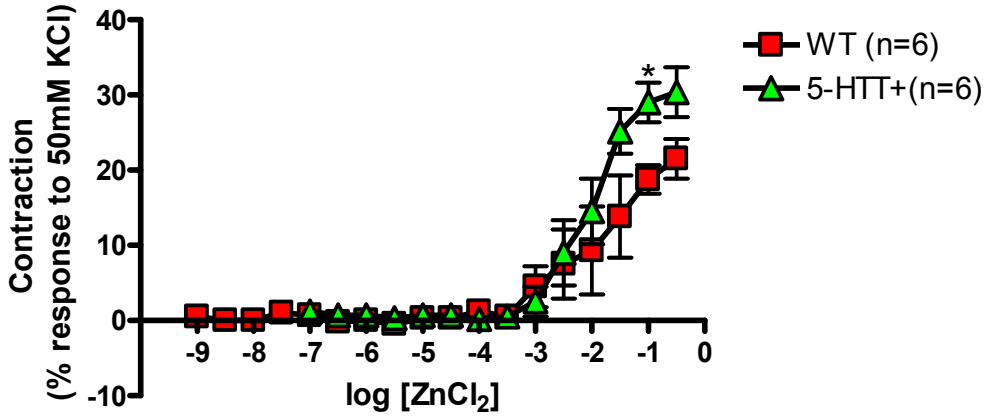


Figure 3.6 $ZnCl_2$ induced contractions in WT and 5-HTT+ mice IPAs.

Concentration response curve obtained to $ZnCl_2$ (1 nM – 350 mM). Data are expressed as a percentage of the reference contraction to 50 mM KCl in each vessel. Each point represents the mean ± SEM (n = 6). * = P < 0.05 indicates difference from WT value.

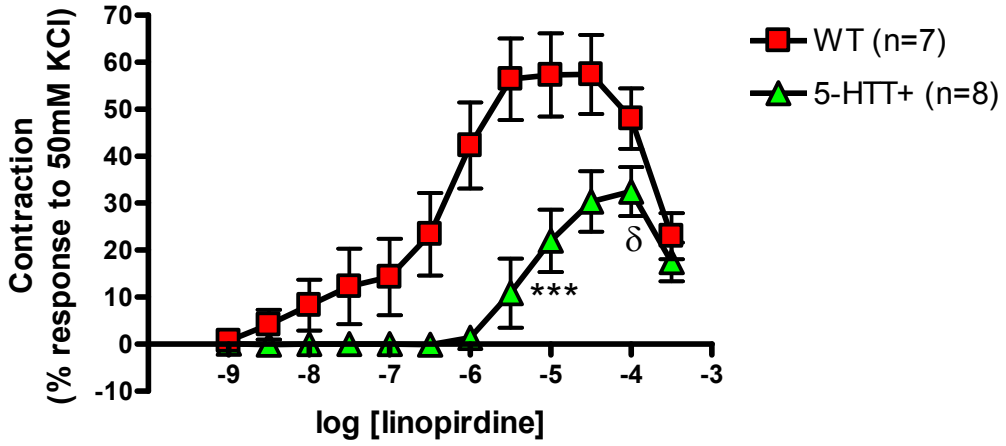


Figure 3.7 Linopirdine induced contractions in WT and 5-HTT+ mice IPAs.

Concentration response curve obtained to Linopirdine (1 nM – 350 μM). Data are expressed as a percentage of the reference contraction to 50 mM KCl in each vessel. Each point represents the mean ± SEM (n = 7 – 8). *** = P < 0.001 indicates difference from WT pEC50. δ = P < 0.05 indicates difference from WT Emax.

3.2.3 Effects of K^+ channel blockers on 5-HT induced constriction

Having established the effects of K^+ channel blockers on resting tone, the next step was to determine if they had an effect on the constrictor response to 5-HT. 5-HT has been shown to induce contraction in rat PAs over a 1 nM to 350 μM concentration range (MacLean *et. al.,* 1996b). The 5-HT-induced concentration dependent contraction responses found in WT mice IPAs were also seen in 5-HTT+ mice IPAs. 5-HT elicited contraction in IPA vessels from WT mice more potently than in 5-HTT+ mice (figure 3.8). 5-HT began to induce contraction in WT mice IPAs at 35 nM – 100 nM, whereas in 5-HTT+ vessels, the first contraction to 5-HT occurred at 1 μM. The pEC50 values were significantly different between the WT and 5-HTT+ vessels, with values of 7 ± 0.1 (n = 11) and 5 ± 0.1 (n = 6) respectively ($P < 0.001$). The maximum response induced by 5-HT did not differ between the vessels; the Emax value in WT vessels was 98 ± 7 % of that induced by 50 mM KCl, and 86 ± 8 % for 5-HTT+ vessels.

In experiments investigating the effects of K^+ channel blockers on 5-HT induced contraction, tissue matched IPA were used. In each experiment one segment of IPA was pre-treated with a K^+ channel blocker. A segment of IPA from the same mouse lung was treated with vehicle. The sensitivity of WT and 5-HTT+ mice IPAs to 5-HT was unaffected by the presence of 10 mM TEA (figure 3.9). This was evident in WT vessels as pEC50 values were the same for both control responses and responses in the presence of TEA, which were 7 ± 0.1 (n = 6 – 7). A similar pattern was seen between 5-HTT+ control vessels and 5-HTT+ vessels pre-treated with TEA; pEC50 values were 5.3 ± 0.1 (n = 6) compared to 5.6 ± 0.1 (n = 6), respectively. The original 5-HT potency difference between WT and 5-HTT+ mice IPAs remained in the presence of TEA, as pEC50 values remained different ($P < 0.001$).

In the presence of 3 mM 4-AP, the 5-HT induced contraction response remained unchanged in WT mice IPAs (figure 3.10). 5-HT was equally as potent in the control environment as when in the presence of 4-AP in contracting WT vessels; the pEC50 value was 7 ± 0.1 (n = 5 – 7) in both environments. In 5-HTT+ mice IPAs there was significant difference between the potency of 5-HT in control environment and when 4-AP was present. In 5-HTT+ vessels, the 5-HT concentration response curve was shifted to the left in the presence of 4-AP, resulting in a pEC50 value of 5.8 ± 0.1 (n = 7) compared with 5.4 ± 0.1 (n = 7) in the control environment ($P < 0.01$).

$ZnCl_2$ did not affect the potency of 5-HT in WT or 5-HTT+ vessels (figure 3.11). In the case of WT mice IPAs, 5-HT induced contraction had a pEC50 value of 7 ± 0.1 (n = 6) and 7 ± 0.04 (n = 6) in the presence and absence of 300 μM $ZnCl_2$, respectively. The pEC50 value for 5-HT acting on 5-HTT+ vessels was unchanged by the presence $ZnCl_2$ in the bathing solution, at 5 ± 0.1 (n = 6).

Pre-incubation of IPAs with 100 nM linopirdine did not alter the potency of 5-HT in WT or 5-HTT+ mice. This KCNQ channel blocker failed to change the pEC50 value in WT or 5-HTT+ mice IPAs (figure 3.12). The pEC50 value for 5-HT induced contraction in WT mice IPAs in the presence of 100 nM linopirdine was 7 ± 0.2 (n = 7) compared with 7 ± 0.1 (n = 7) in the control environment. In 5-HTT+ mice vessels, the pEC50 value for 5-HT induced contraction was 5. ± 0.1 (n = 7 - 8) in the presence and absence of linopirdine.

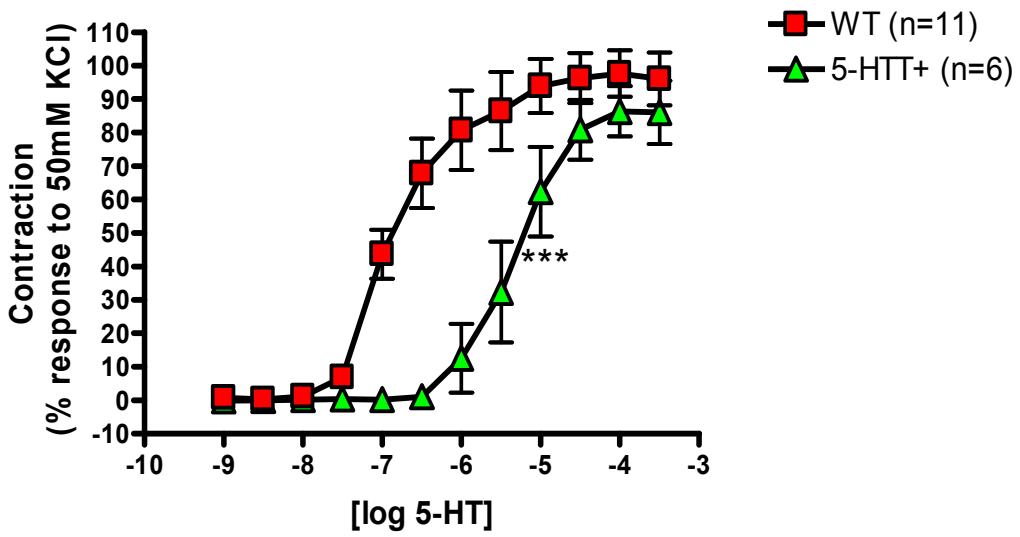


Figure 3.8 5-HT-induced contractions in WT and 5-HTT+ mice IPAs.

Concentration response curve obtained to 5-HT (1nM – 350 μM). Data are expressed as a percentage of the reference contraction to 50 mM KCl in each vessel. Each point represents the mean ± SEM (n = 6 – 11). *** = $P < 0.001$ indicates difference from WT pEC50.

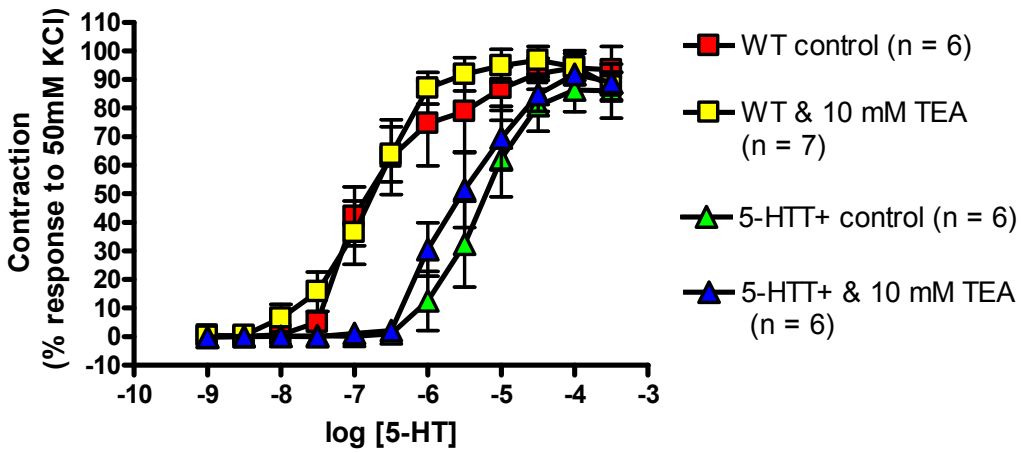


Figure 3.9 Lack of effect of 10 mM TEA on 5-HT-induced contraction on IPA from WT and 5-HTT+ mice.

Concentration response curve obtained to 5-HT, (1 nM – 350 μM). Data are expressed as a percentage of the reference contraction to 50 mM KCl in each vessel. Each point represents the mean ± SEM (n = 6 – 7).

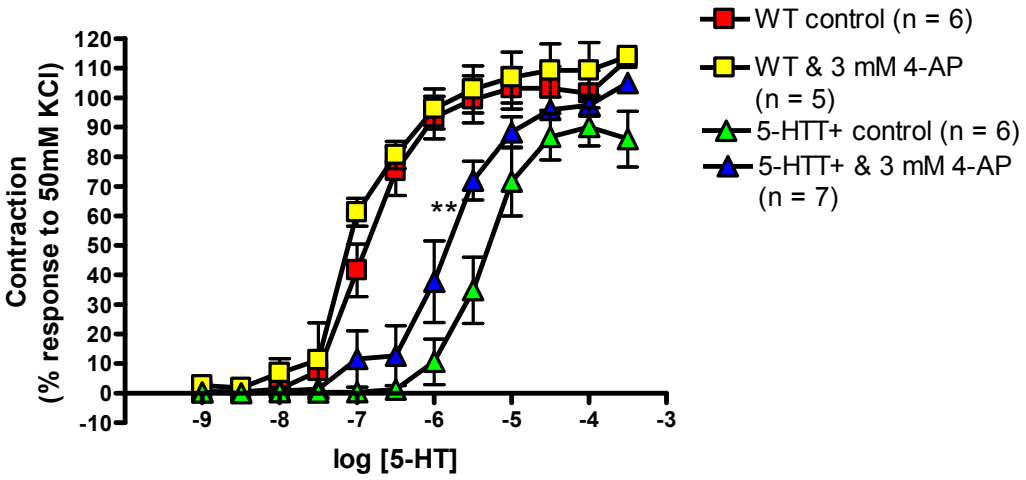


Figure 3.10 Effect of 3 mM 4-AP on 5-HT-induced contraction on IPA from WT and 5-HTT+ mice.

Concentration response curve obtained to 5-HT (1 nM – 350 μM). Data are expressed as a percentage of the reference contraction to 50 mM KCl in each vessel. Each point represents the mean ± SEM (n = 5 – 7). ** = $P < 0.01$ indicates a significant difference of the 5-HTT+ & 4-AP pEC50 from 5-HTT+ control value.

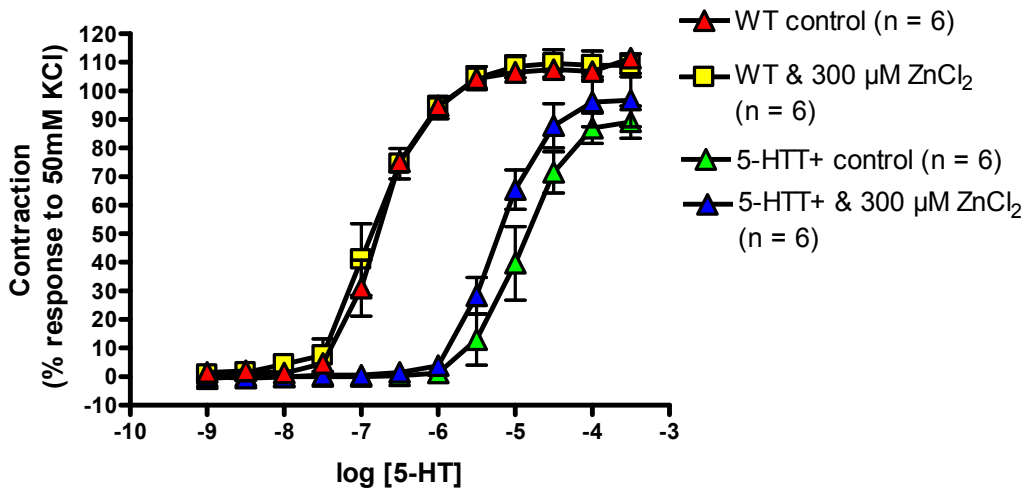


Figure 3.11 Lack of effect of 300 nM $ZnCl_2$ on 5-HT-induced contraction on IPA from WT and 5-HTT+ mice.

Concentration response curve obtained to 5-HT, (1 nM – 350 µM). Data are expressed as a percentage of the reference contraction to 50 mM KCl in each vessel. Each point represents the mean ± SEM (n = 6).

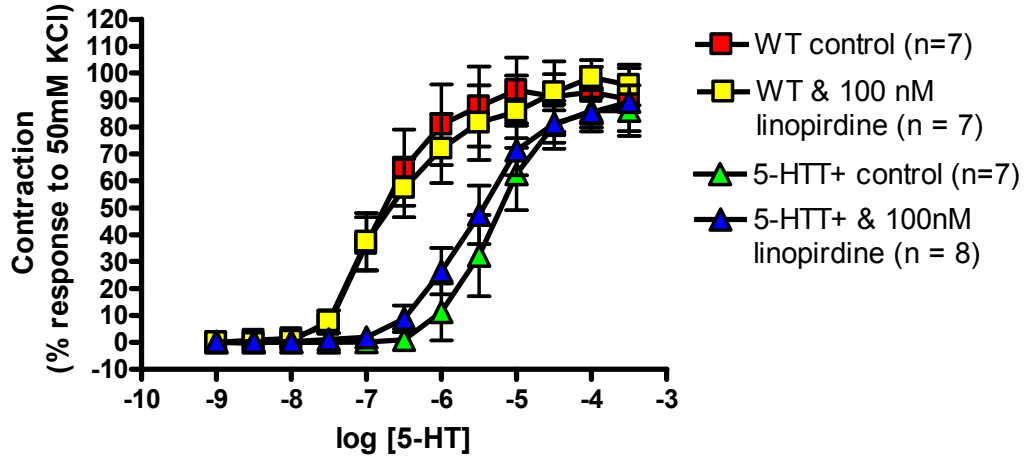


Figure 3.12 Lack of effect of 100 nM Linopirdine, on 5-HT-induced contraction on IPA from WT and 5-HTT+ mice.

Concentration response curve obtained to 5-HT (1 nM – 350 µM). Data are expressed as a percentage of the reference contraction to 50mM KCl in each vessel. Each point represents the mean ± SEM (n = 7 – 8).

3.3 Discussion

The effects of K^+ channel inhibitors were tested on baseline tone in intralobar PA from WT and 5-HTT+ mice in order to identify potential K^+ channels that might be important in regulating resting tone in these vessels. The response to these K^+ channel inhibitors were measured relative to the contraction induced by 50 mM KCl in the vessels. The response to KCl was similar in both WT and 5-HTT+ IPA. TEA, 4-AP, capsaicin, glibenclamide and Zn^{2+} ions all failed to change the resting tone in these vessels, within the concentration ranges that are specific to the blockade of K^+ channels. However, linopirdine, a blocker of KCNQ channels, did increase tone in these vessels within the concentration range known to inhibit KCNQ channels (< 10 μM) (Coetzee *et. al.,* 1999).

There is overlap of K^+ channels that are blocked by some of the K^+ channel inhibitors used in this study. For example TEA, 4-AP and capsaicin have all been shown to block K_V1 channels. In these experiments, none of these blockers affected resting tone until very high concentrations were tested. TEA inhibits $K_V1.1$, 1.2, 1.3 and 1.6, with $K_V1.1$ channels the most sensitive, being blocked at 0.5 mM TEA (table 3.1) (Coetzee *et. al.,* 1999). Between 4 – 10 mM TEA, K_V2 channels are also inhibited, while a much lower concentration is required to inhibit K_V3 channels, ~ 0.1 – 0.3 mM (Coetzee *et. al.,* 1999). BK_{Ca} channels are also inhibited by 1 mM TEA (Yildiz *et. al.,* 2006). In this study, TEA failed to cause constriction of mice IPAs until a concentration of 35 - 100 mM. This is similar to what has been reported in rat PA, were 10 mM TEA had little effect on basal tone (Archer *et. al.,* 1996). The resting potential was similarly found to be insensitive to TEA in rabbit and rat PASMCs (Archer *et. al.,* 1996; Evans *et. al.,* 1996). At concentrations of 35 – 100 mM, the action of TEA may not be specific to blockade of K^+ channels, and therefore constriction at high TEA concentrations could be due to an action distinct from K^+ channel blockade altogether. At concentrations of > 10 mM, the osmolarity of the Krebs solution bathing the IPA vessels would change, which may lead to hyperosmostic stress and affect the way the vessels respond to TEA. Osmolarity of the bathing solution has been shown to affect responses to agonists in other studies. For example, in rat tail artery, the response to the constrictor agonist PE was nearly doubled in a hyperosmotic environment relative to that in an isosmotic medium (Rocha *et. al.,* 1995).

4-AP inhibits K_V1 channels within the very low mM range (see table 3.1). $K_V1.5$ is the most sensitive, being blocked by < 0.1- 0.4 mM 4-AP (Coetzee *et. al.,* 1999). 4-AP also inhibits K_V2, K_V3 and K_V4, with K_V3 channels, (blocked at 0.02 – 1.2 mM), being the most sensitive of the three groups (Coetzee *et. al.,* 1999). 4-AP had no effect on the resting tone of mice IPAs until the concentration of 4-AP within the bathing solution reached 3.5 mM. This concentration is well above the concentration required to block most K_V channels, with the exception of K_V 4.1. In addition, 4-AP has been shown to be much less potent in blocking heteromeric $K_V1.4$/ K_V 1.5 channels, with an IC_{50} value of 7 – 8 mM (Po *et. al.,* 1993). The maximum contraction was not necessarily reached with the maximum concentration of 4-AP tested (10 mM), but at 10 mM, contraction was greater in WT mice IPA relative to 5-HTT+ mice IPA. If the contractile effect of 4-AP reflects inhibition of K^+ channels, the channels involved are likely to be Kv4.1 or heteromeric $K_V1.4$/ K_V 1.5 channels, and the expression of these may be altered in the 5-HTT+ mouse. However due to the non-specific nature of 4-AP at these high concentrations, the observed effects may be unrelated to an action on K^+ channels. As with TEA, osmotic changes may also become relevant at these concentrations. It has previously been found that hypertensive pulmonary vessels show an increased sensitivity to 4-AP (Osipenko *et. al.,* 1998; Priest *et. al.,* 1998), which suggests that 4-AP sensitive channels may make a greater contribution to the resting membrane potential in PAH. Osipenko *et. al.* (1998) showed that 1 mM 4-AP induced a greater contraction in the hypertensive rat PA relative to control rat vessels when the PAH was induced by chronic hypoxia. The results shown in this chapter were conducted using a different model of PAH, the 5-HTT+ mouse model. If the contractile effect of 10 mM 4-AP on 5-HTT+ mice vessels does reflect inhibition of K_V channels, then the results suggests that any change in K^+ channel expression that develops in the 5HTT+ mouse is different from that seen in the chronic hypoxic mouse.

The ability of capsaicin to induce vasoconstriction was revealed in canine arteries (Toda *et. al.,* 1972). Capsaicin induces contraction in canine mesenteric arteries with an IC_{50} value of 3 μM (Porszasa *et. al.,* 2002). K_V currents are blocked by capsaicin in rat pituitary cells (Kehl, 1994). At 100 μM capsaicin inhibits the K_V1 family of voltage gated K^+ channels, and this concentration has been shown to block K_V1 channels in rabbit PASMCs (Osipenko *et. al.,* 2000). In this study, however, capsaicin failed to induce a change in tone in IPAs from WT or 5-HTT+ mice.

Taken together, the results from the experiments with TEA, 4-AP and capsaicin imply that the K^+ channels underlying the resting membrane potential of mouse IPA are not of the K_V1 type, because these drugs failed to consistently alter the resting tone within the concentration ranges known to inhibit the K_V1 channels. It is therefore most unlikely that these channels are open at rest. Using the results from the TEA and 4-AP experiments, the same rationale can be used to suggest that the K_V2 subfamily of K_V channels are not involved in maintaining the low resting tension in mouse IPA.

The contribution of K_{ATP} channels to resting tone in mice IPAs was investigated using glibenclamide. In this study glibenclamide failed to alter IPA tone from WT and 5-HTT+ mice. This would suggest that K_{ATP} channels are not involved in the regulation of tone in these vessels. This is consistent with the proposal that this subset of K^+ channels is not open under basal conditions (Clapp & Gurney, 1992).

TASK channels were investigated in WT and 5-HTT+ mice IPAs using $ZnCl_2$. $ZnCl_2$ failed to constrict mice IPAs until the millimolar range. This may have been due to an action of Zn^{2+} independent of its effect on K channels, as Zn^{2+} can block VOCCs (Busselberg *et. al.,* 1994). Therefore the possibility that this effect counteracted any constriction must be considered. However data discussed below suggests that this explanation is unlikely. Thus the lack of effect of Zn^{2+} until mM concentrations argues against a substantial involvement of TASK channels in regulating the resting membrane potential of PASMCs in mice IPA.

This study provides evidence of a potential role for KCNQ channels in the regulation of low resting tone in mice IPAs. In WT mice IPAs, linopirdine induced contraction at a concentration of ~ 10 nM. Linopirdine is considered to be a selective blocker of KCNQ channels (Jentsch, 2002; Robbins, 2001). Thus it seems likely that KCNQ channels were open at rest and contributing to the resting membrane potential in the WT mice PASMCs. Linopirdine was less effective in 5-HTT+ vessels, but again it induced an increase in tone < 10 μM. This concentration corresponds to KCNQ channels being open at rest even in 5-HTT+ mice PASMCs, so they may contribute to regulating the membrane potential in these vessels also. This is consistent with previous studies; for example KCNQ channels have been proposed as the K^+ channels setting the resting

membrane potential in neurons (Oliver *et. al.,* 2003). A paper under review suggests that KCNQ channels are major regulators of resting membrane potential in PASMCs (Joshi *et. al.,* 2008(submitted)).

The reduced activity of linopirdine in 5-HTT+ mice IPAs is interesting. In the diseased state, there may be down regulation of KCNQ channels or altered activity of KCNQ channels (e.g. β-subunit regulation). A loss of KCNQ channel expression in 5-HTT+ mice PASMCs might be expected to lead to depolarisation of these cells, if KCNQ channels are important in the regulation of low resting tone. It could be the case that linopirdine was more potent in WT vessels relative to 5-HTT+ mice IPAs due to 5-HTT+ PASMCs being more hyperpolarised than those in WT vessels. If the latter was the case then a higher concentration of linopirdine may be required to depolarise the 5-HTT+ mice PASMCs sufficiently to reach the threshold of contraction. This is because a greater proportion of K^+ channels would need to be blocked in order for the membrane to reach a level of depolarisation at which VOCCs would open. It is interesting that the differential response to linopirdine in WT and 5-HTT+ vessels resembles the differential sensitivity to 5-HT. Therefore, another possible explanation for reduced sensitivity to linopirdine in 5-HTT+ mice IPAs relative to WT vessels could be that linopirdine could be interacting with 5-HT receptors, the expression of which is altered in 5-HTT+ mice, with an increased expression of 5-HT2A receptors (Dempsie *et. al.,* 2008(submitted)).

5-HT was more potent in WT mice IPAs relative to 5-HTT+ mice. This has also been shown in a recent publication, which also showed that this reduction in 5-HT sensitivity in 5-HTT+ mice was reversed by a 5-HTT+ inhibitor (Dempsie *et. al.,* 2008(submitted)). This indicates that the reduction in potency of 5-HT is due to the over-expressed 5-HTT removing 5-HT from receptor sites in 5-HTT+ mice.

Experiments were conducted in this study to investigate possible inhibition of K^+ channels by 5-HT in WT and 5-HTT+ mice IPAs, as this has been suggested to occur in PA from rats (Cogolludo *et. al.,* 2006). Due to the increased number of 5-HTT in 5-HTT+ mice IPAs (which act to remove 5-HT from solution) a higher concentration of 5-HT is required to act on the 5-HT receptors to initiate contraction, relative to WT

vessels. Of all the K+ channel inhibitors tested only 4-AP altered the 5-HT response, and this potentiation of the 5-HT response was only found in 5-HTT+ mice, (with the 5-HT concentration response curve shifted to the left). This may be due to a synergistic action between 5-HT and 4-AP, leading to a greater effect of these drugs in combination than what is found when they are acting in isolation. At a concentration of 3 mM 4-AP, most of the K_V1, 2 and 3 channels should be blocked (table 3.1). This inhibition of K_V may induce some depolarisation of the underlying PASMCs, but not enough depolarisation to activate VOCC sufficiently to evoke contraction. Chapter four presents results indicating that a proportion of the 5-HT induced increase in $[Ca^{2+}]_i$ is not voltage dependent. Therefore with 5-HT acting to increase $[Ca^{2+}]_i$ within PASMCs (by voltage independent mechanisms), and 4-AP depolarising the membrane, these agents would collectively result in a potentiation of the 5-HT response in 5-HTT+ vessels. The results suggest a potentially small contribution of K_V channels in maintaining resting membrane potential of PASMCs in 5-HTT+ vessels, but not enough to allow membrane potential to depolarise sufficiently to evoke contraction when 4-AP when is applied on its own. Thus it is possible that in the PASMCs of 5-HTT+ vessels there is a greater contribution of K_V channels to the resting potential than in WT vessels. In this way, the nature of the K^+ channels regulating membrane potential in PAH caused by 5-HTT over-expression may show a similar, but smaller, shift to that observed in PAH induced by chronic hypoxia (Osipenko *et. al.,* 1998).

5-HT induced contraction of WT and 5-HTT+ mice IPA was not inhibited by any of the K^+ channel blockers tested (TEA, 4-AP, $ZnCl_2$ and linopirdine). This result could be analysed in two different ways. Firstly, it may be suggested that as the K^+ channel blockers did not inhibit the 5-HT induced response, then 5-HT was not inducing its effect via inhibition of the K^+ channel being blocked by one of the drugs above. However, it may be argued, that if 5-HT was acting to inhibit the K^+ channel under investigation (i.e. acting in the same manner as TEA, 4-AP, $ZnCl_2$ or linopirdine) , then addition of the K^+ channel blocker would not alter the response to 5-HT. Further investigation is necessary to investigate if 5-HT inhibits K^+ channels in mice IPA.

3.4 Conclusion

The experiments carried out in this part of the project suggest that of all the K_V channels investigated, only members of the KCNQ subfamily are open at rest and play an important role in maintaining the resting tone within mouse IPA, with a potentially minor role for 4-AP sensitive channels also involved in the 5-HTT+ mice vessels. A further finding was that the potency of linopirdine inducing contraction was reduced in the 5-HTT+ mouse model of PAH. The linopirdine potency difference observed between the two vessel types mirrored that seen with 5-HT, with the WT vessels being more sensitive to both linopirdine and 5-HT. Despite the apparent contribution of KCNQ channels to resting tone, linopirdine did not alter the sensitivity of mice IPAs to 5-HT in either WT or 5-HTT+ mice, implying that a change in activity of KCNQ channels was not involved in the mechanism leading to 5-HT induced constriction.

Experiments investigating potential links between 5-HT contraction and K^+ channel blockade indicated that 4-AP was the only blocker that affected the 5-HT induced contraction, and only in 5-HTT+ mice IPAs. This result could suggest a potential role for 4-AP sensitive K_V channels in the 5-HT response.

The chapters that follow aim to investigate the mechanisms underlying the difference in potency of 5-HT and linopirdine between WT and 5-HTT+ mice IPAs.

Chapter 4

Mechanisms of action underlying vasoconstriction induced by 5-HT and linopirdine

4.1 Introduction

Vasoconstriction mediated by 5-HT is induced via an increase in the concentration of free Ca^{2+} within the cytoplasm (Yuan *et. al.,* 1997). The mechanism by which 5-HT increases $[Ca^{2+}]_i$ and hence induces vasoconstriction in WT and 5-HTT+ mice IPA was investigated in this chapter.

The free $[Ca^{2+}]_i$ in muscle and other cells at rest is approximately 20-50 nM (Martonosi & Pikula, 2003). A large concentration gradient exists between the extracellular medium, the sarcoplasmic reticulum (SR) and the cytoplasm. In smooth muscle the free $[Ca^{2+}]_i$ is approximately 10,000 times lower than that present in the extracellular space, which is in the millimolar range (Marin *et. al.* 1999), whereas in the SR the Ca^{2+} concentration is approximately 0.1 - 2.0 mM (Martonosi & Pikula, 2003). The large gradients are maintained by three main mechanisms; Ca^{2+} entry from the extracellular space, exchange of Ca^{2+} between the cytoplasm and the intracellular stores and Ca^{2+} extrusion (Karaki *et. al.,* 1997). Factors that determine the extent of Ca^{2+} entry include the magnitude of the membrane potential, the transmembrane Ca^{2+} gradient and Ca^{2+} channel activity. Ca^{2+} enters the cytoplasm through Ca^{2+} channels located in the plasma and SR membranes. VOCC opening is regulated by membrane potential. VOCCs transmit Ca^{2+} into SMCs when the membrane is depolarised. The VOCC channels involved in regulating Ca^{2+} influx in PASMCs are L-type channels (McMurtry *et. al.,* 1976). A group of drugs known as dihydropyridines (e.g. nifedipine, nicardipine) block these channels directly and are used as vasodilators (Spah *et. al.,*1988; Turlapaty *et. al.,* 1989). Contractile responses to 5-HT are partially inhibited by VOCC blockers in PA from human, canine and sheep tissue (Drummond & Wadsworth, 1994; Mikkelsen *et. al.,* 1983; Wilson *et. al*., 2005). However, in IPA derived from rat lungs, nicardipine did not significantly reduce the increase in free $[Ca^{2+}]_i$ observed in response to 5-HT (Guibert *et. al.,* 2003). As part of the 5-HT induced response in PA involves VOCC in the above tissues, then it may be the case that 5-HT induces membrane depolarisation of PASMCs, causing the membrane potential to move towards a potential at which VOCCs are open, resulting in Ca^{2+} influx (McMurtry *et .al,.* 1976), and hence SMC contraction. 5-HT was shown previously to induce membrane depolarisation, for example, in rat PASMCs (Cogolludo *et. al.,* 2006). 5-HT induced contraction of isolated rat PASMCs was proposed to be due to 5-HT acting on 5-HT2A receptors to

reduce K^+ current through K^+ channels and cause depolarisation (Cogolludo *et. al.,* 2006).

Contraction of PASMCs can also occur via a mechanism known as pharmacomechanical coupling, which depends neither on changes in membrane potential (although changes may occur) nor, consequently, on Ca^{2+} entry via VOCCs. Pharmacomechanical coupling results in a rise in free $[Ca^{2+}]_i$ induced by a combination of Ca^{2+} release from intracellular stores and Ca^{2+} entry through non voltage operated channels. One major (non voltage dependent) route by which Ca^{2+} can cross the membrane is via store operated Ca^{2+} channels (SOCCs). These are defined as plasma membrane Ca^{2+} channels that are opened in response to a decrease in the concentration of Ca^{2+} within the intracellular stores, (Putney, 1993). Activation of SOCCs allows Ca^{2+} entry into the cytoplasm and the re-loading of intracellular Ca^{2+} stores (Berridge 1995). This is also known as capacitative Ca^{2+} entry (CCE) or depletion activated entry (Putney 1999, 2001). A second important non voltage dependent route by which Ca^{2+} can enter the cell is via receptor-operated Ca^{2+} channels (ROCCs) which are activated by agonists acting on a range of GPCRs. ROCCs provide a variety of pathways by which Ca^{2+} can be delivered into the cytoplasm and the SR in order to initiate and/or maintain specific types of intracellular Ca^{2+} signals (Barritt *et. al.,* 1999). In the SR membrane of SMCs there are two types of Ca^{2+} channel that play a role in releasing Ca^{2+} from the stores. The IP_3 receptor (IP_3R) is activated by IP_3 and provides the main mechanism by which the activation of GPCRs causes a rise in $[Ca^{2+}]_i$. 5-HT receptor activation results in IP_3 production and Wilson *et. al.* (2005) showed that activation of IP_3 receptors is critical to the initiation of cytosolic Ca^{2+} rises in response to 5-HT.

Although $[Ca^{2+}]_i$ is the primary regulator of SMC contraction, it has been demonstrated that this Ca^{2+}-calmodulin dependent MLC kinase pathway does not solely account for the mechanisms of agonist induced contraction. Ca^{2+} sensitisation, (an increase in force at a constant $[Ca^{2+}]_i$, accounts for another important mechanism in contraction of SMCs (Somlyo & Somlyo, 1994). Small G protein RhoA and its downstream effector Rho kinase were demonstrated to play important roles in mediating the Ca^{2+} sensitivity of SMC contraction through the inhibition of MLCP (Somlyo & Somlyo, 1994). As stated previously MLCP plays a major role in the control of MLC dephosphorylation and hence the level of contraction of SMCs. The drug Y-27632 inhibits Rho kinase and

therefore promotes relaxation (Chiba & Misawa, 2004). Agonists and second messengers modify the MLCK/ MLCP activity ratio independently of the level of $[Ca^{2+}]_i$, giving rise to changes in contractile forces even in the presence of a constant $[Ca^{2+}]_i$. It was shown previously that 5-HT induced contraction is dependent on rho-kinase activation (Rodat-Despoix *et. al.,* 2008).

The potency of the linopirdine induced contraction of IPA was altered in the same manner as the potency of 5-HT induced contraction in 5-HTT+ mice. In this chapter it was investigated whether or not linopirdine behaved similarly to 5-HT due to the two drugs acting via the same pathways. The main aim of the work in this chapter was, however, to determine the mechanisms by which 5-HT induced contraction of mouse IPA. The second aim was to determine if the mechanism by which 5-HT induced contraction differed between the WT mouse and the mouse model of PAH, the 5-HTT+ mouse. The role of extracellular Ca^{2+} ($[Ca^{2+}]_{ext.}$) in the 5-HT induced response was examined to determine if it was a pre-requisite for contraction in WT and 5-HTT+ mice IPA. The roles of different Ca^{2+} entry pathways were investigated in the 5-HT induced responses; SOCC entry, IP_3 induced Ca^{2+} release and VOCC entry. The role of increased Ca^{2+} sensitivity in response to 5-HT was investigated by blocking rho kinase. The role of the PASMC membrane potential in response to 5-HT was determined both by measuring the effect of 5-HT on membrane potential in isolated PASMCs and examining how the contractile response of IPA was affected by conditions that depolarise PASMC. It was also investigated if depolarising the membrane potential could remove the potency differences of 5-HT and linopirdine between WT and 5-HTT+ mice.

4.2 Methods

Methods are described in chapter two, using the following protocols.

4.2.1 Effect of ketanserin on contractile responses to 5-HT and linopirdine

Linopirdine was the only K^+ channel blocker that was more potent in causing contraction of WT mice IPA relative to IPA from 5-HTT+ mice. It was therefore important to distinguish if the differing level of potency of linopirdine between the

vessel types was due to linopirdine acting as a 5-HT receptor agonist. Therefore the linopirdine induced contractions were investigated in the presence of the 5-HT2A receptor antagonist, ketanserin (10 nM).

4.2.2 Resting membrane potential measurement

Experiments were conducted to investigate if 5-HT induced contraction was the result of 5-HT changing the membrane potential of PASMCs in IPAs from WT and 5-HTT+ mice. 5-HT was added to the local environment of the cell at 1 μM and subsequently 10 μM, with the resting potential continuously recorded. Recordings were made until the membrane potential was stable.

4.2.3 Influence of extracellular K^+ concentration ($[K^+]_{ext.}$) on contraction responses to 5-HT and linopirdine

In PASMCs the contractile response is induced by depolarisation of the membrane and/ or by pharmacomechanical coupling, which does not depend on depolarisation (Casteels *et. al.,* 1977). Raising $[K^+]_{ext.}$ shifts the K^+ equilibrium potential and causes membrane depolarisation. Subsequently VOCCs are opened, leading to Ca^{2+} entry and an increase in free $[Ca^{2+}]_i$, resulting in vasoconstriction. The ability to change membrane potential by changing $[K^+]_{ext}$ was used to investigate the method by which 5-HT and linopirdine induced their contractile responses in IPA from WT and 5-HTT+ mice. Myography experiments were conducted in the presence of varying $[K^+]_{ext.}$ in the bathing PSS.

4.2.4 Influence of $[Ca^{2+}]_{ext.}$ and VOCC on contractile responses to 5-HT and linopirdine

To role of $[Ca^{2+}]_{ext.}$ in the contractile response to 5-HT in WT and 5-HTT+ mice was investigated by removing $[Ca^{2+}]_{ext.}$ from the bathing solution. Experiments were conducted in which 5-HT was added to IPAs in control bathing PSS, which were then washed with Ca^{2+} free PSS. The response to 5-HT was then measured in the absence of $[Ca^{2+}]_{ext.}$ in the bathing PSS.

To investigate if constriction induced by 5-HT and linopirdine was mediated by Ca^{2+} influx through VOCCs, the L-type Ca^{2+} channel blocker nifedipine was used.

Contractions to 5-HT and linopirdine were investigated in the presence of 1 μM nifedipine in WT and 5-HTT+ mouse IPA.

4.2.5 Effect of SOCC and store release inhibitors on contractile responses to 5-HT

To investigate the role of SOCCs in the contractile response to 5-HT in mouse IPA, vessels from WT and 5-HTT+ mice were tested with increasing concentrations of 5-HT in the presence 50 μM $NiCl_2$ and 75 μM 2-APB.

4.2.6 Influence of Rho kinase on contractile responses to 5-HT

To investigate the role of rho kinase in the 5-HT induced contraction of IPA from WT and 5-HTT+ mice, increasing concentrations of 5-HT were applied in the presence of 10 μM Y-27632.

4.3 Results

4.3.1 Influence of ketanserin on linopirdine-induced contraction

Ketanserin (10 nM) did not inhibit the linopirdine induced contraction in IPA from WT or 5-HTT+ mice (figure 4.1). The sensitivity of WT mice IPAs to linopirdine remained the same in the absence and presence of ketanserin: pEC50 values in WT control and WT treated tissues were 6 ± 0.1 (n = 8). The pEC50 values in the 5-HTT+ control and 5-HTT+ treated vessels were 6 ± 0.2 (n = 9 - 10).

4.3.2 Membrane potential measurement

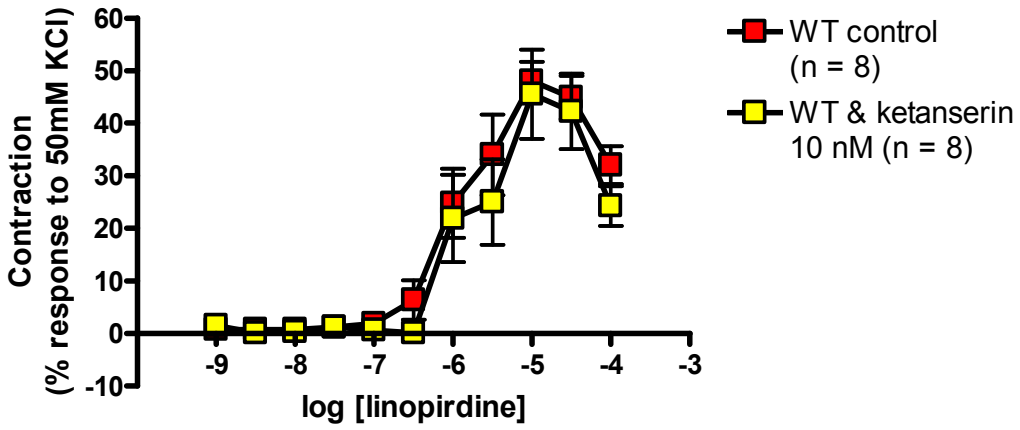
The resting membrane potentials of PASMCs are shown in figure 4.2 for WT and 5-HTT+ mice cells. There was no statistically significant difference between the resting membrane potentials of PASMCs isolated from WT and 5-HTT+ mice. The absolute resting membrane potentials measured for WT mice PASMCs in this study was - 42 ± 4 mV (n = 12). The cell capacitance and input resistance calculated for these cells were 21 ± 2 pF and 6 ± 2 GΩ, respectively. PASMCs from 5-HTT+ mice had a resting membrane potential of -42 ± 3 mV (n = 14), the cell capacitance and input resistance were 18 ± 3 pF and 4 ± 1 GΩ, respectively.

5-HT (1 μM and 10 μM) was added directly to the area around the PASMC that was patch clamped. In the PASMCs from WT mice, 5-HT significantly depolarised the membrane potential by 4 ± 1 mV (n = 7) at 1 μM ($P < 0.01$) and by 7 ± 1 mV (n = 7) at 10 μM ($P < 0.001$) (figure 4.2). Six of the seven cells responded with >3 mV depolarisation to 1 μM 5-HT, and all seven cells depolarised in response to 10 μM 5-HT.

The response to 1 μM 5-HT in the cells isolated from the 5-HTT+ mice tissue was a small but measurable depolarisation in four of the six recordings, however there was little overall change in membrane potential. At 1 μM, 5-HT depolarised the membrane potential by 1 ± 0.3 mV (n = 6), with only one of the six cells patched clamped depolarising by > 1 mV. The effect of 10 μM 5-HT on the 5-HTT+ cells was highly variable, with four of the six recordings showing a hyperpolarising response. On average the response was depolarisation of 1 ± 2 mV (n = 6).

PASMCs from WT tissue depolarised more than cells from 5-HTT+ vessels in response to 1 μM 5-HT ($P < 0.01$) and to 10 μM 5-HT ($P < 0.05$) (figure 4.2). The depolarising action of 5-HT was not always reversible when washing the cells with PPS.

A)



B)

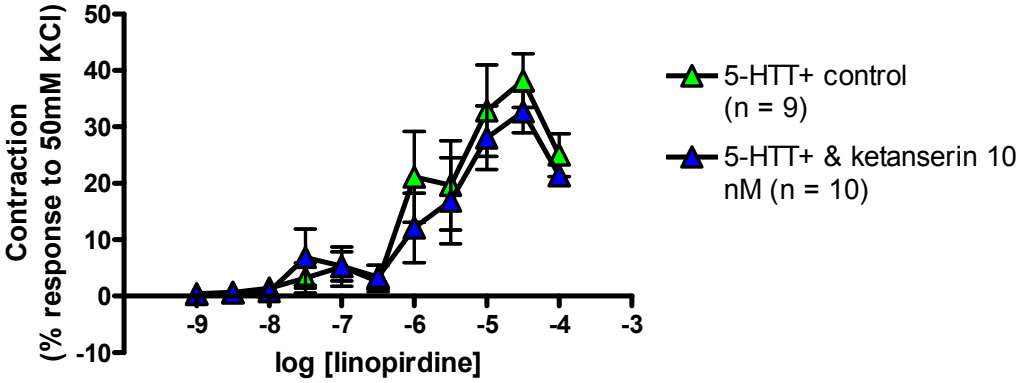
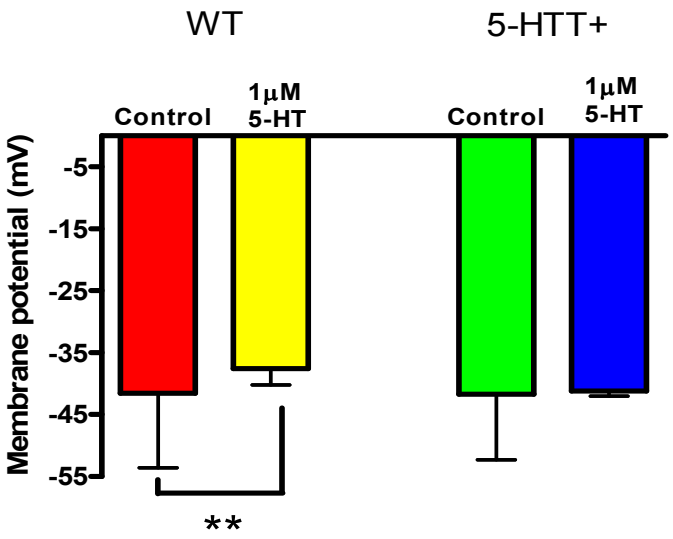


Figure 4.1 Lack of effect of 10 nM ketanserin on linopirdine induced contraction on IPA from WT and 5-HTT+.

Concentration response curve obtained to linopirdine (1 nM – 100 μM) in A) WT mice IPAs and B) 5-HTT+ mice IPAs. Data are expressed as a percentage of the reference contraction to 50 mM KCl in each vessel. Each point represents the mean ± SEM (n = 8 – 10).

A)



B)

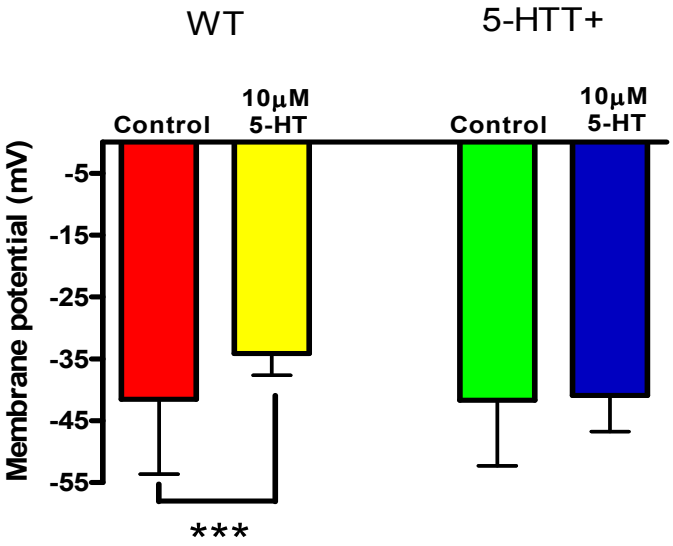


Figure 4.2 Effect of 5-HT on resting membrane potential of PASMCs.

Mean values for the resting membrane potential and the 5-HT induced depolarisation in WT mice IPA smooth muscle (n = 7) and 5-HTT + mice IPA smooth muscle (n = 6) in response to A) 1 μM 5-HT and B) 10 μM 5-HT. Data are the mean ± SEM. (** = $P < 0.01$, *** = $P < 0.001$ indicates difference from control value..

4.3.3 Contraction of IPA to 5-HT and linopirdine in the presence of various $[K^+]_{ext}$

4.3.3.1 5-HT and varying $[K^+]_{ext.}$

IPA vessels from WT mice were significantly more sensitive to 5-HT in the presence of 20 mM $[K^+]_{ext.}$ relative to control concentrations (figure 4.3). The pEC50 value of the 5-HT induced responses from WT vessels in the control environment were measured as 7 ± 1 (n = 7) and in the presence of 20 mM $[K^+]_{ext.}$ the pEC50 value was 7.6 ± 0.1 (n = 6) ($P < 0.001$). However, in the presence of 50 mM $[K^+]_{ext.}$ the maximum amplitude of the 5-HT concentration effect curve was significantly reduced compared to that measured in the control environment, with the maximum response being 106 ± 3 % of 50 mM KCl induced contraction (n = 7) in the presence of 5 mM $[K^+]_{ext.}$ and 41 ± 3 %, (n = 4) in the presence of 50 mM $[K^+]_{ext.,}$ ($P < 0.001$).

In the presence of 20 mM $[K^+]_{ext.}$ IPA vessels from 5-HTT+ mice were significantly more sensitive to 5-HT relative to control concentrations, with the pEC50 value of the 5-HT induced response in the control environment measured as 5.7 ± 0.1 (n = 7), were as in presence of 20 mM $[K^+]_{ext.}$ the pEC50 value was 6 ± 0.1 (n = 6) ($P < 0.05$). The maximum response to 5-HT was reduced under conditions of 50 mM $[K^+]_{ext.}$ The Emax of the 5-HT-induced response was 97 ± 3 % of 50 mM KCl induced contraction (n = 7) in the presence of 5 mM $[K^+]_{ext.}$, however in the presence of 50 mM $[K^+]_{ext.}$ the Emax value was 46 ± 4 % (n = 6) ($P < 0.001$).

Increasing the concentration of $[K^+]_{ext.}$ to 20 mM in the PSS bathing WT tissue did not mimic the effect of 5-HTT over expression in terms of the 5-HT-induced response. When the $[K^+]_{ext.}$ was increased to 50 mM in the PSS bathing WT IPAs, the effect on the 5-HT induced concentration response curve mimicked the effect of 5-HTT over expression to some extent, in that it shifted the curve to the right. The pEC50 value of the 5-HT response in the presence of 50 mM $[K^+]_{ext.}$ also remained significantly different to that of the 5-HTT+ control response ($P < 0.001$). Therefore even a depolarisation of approximately 30 mV at 50 mM $[K^+]_{ext.}$, which by itself caused a large contraction, was not able to mimic shift in the concentration response curve seen in 5-HTT+ mice.

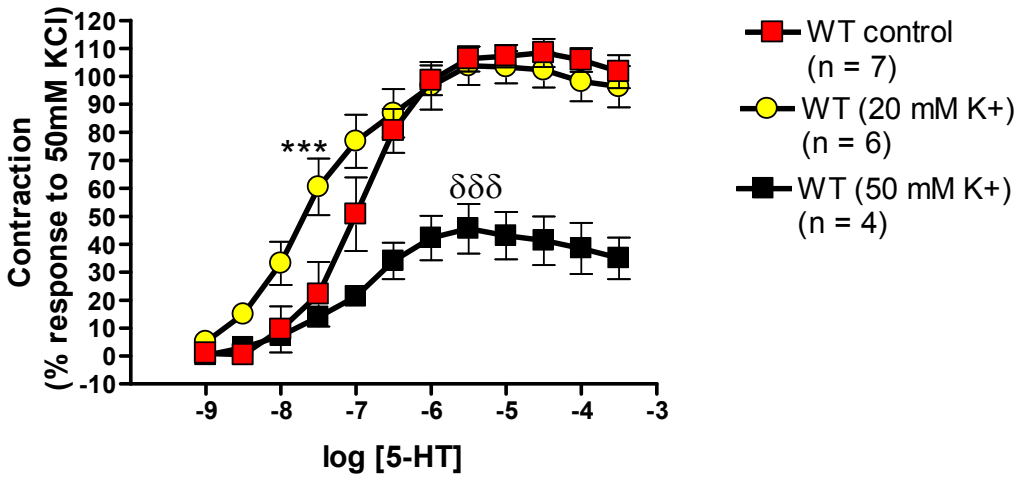
4.3.3.2 Linopirdine and varying $[K^+]_{ext.}$

In the presence of 20 mM $[K+]_{ext.}$, linopirdine induced contractions responses in WT mice IPAs was unaffected relative to the responses measured in the presence of control PSS (figure 4.4). By depolarising the SMC membrane of WT vessels with 50 mM $[K^+]_{ext.}$, linopirdine failed to induce tension, with vasorelaxation of the vessels occurring at approximately 10 μM linopirdine. Under conditions of 20 mM $[K^+]_{ext.}$, the sensitivity to linopirdine was increased in 5-HTT+ mouse IPA, shown by a shift of the concentration response curve to the left and an increased amplitude ($P < 0.001$). In the presence of 50 mM $[K^+]_{ext.}$, the IPA vessels from 5-HTT+ mice failed to contract in response to linopirdine application and exhibited vasorelaxation at ~ 10 μM.

4.3.4 Effect of 5-HT in the absence of $[Ca^{2+}]_{ext.}$

To verify whether or not $[Ca^{2+}]_{ext.}$ was a pre-requisite for 5-HT-induced contractions in WT and 5-HTT+ mouse IPA, the level of contraction to 10 μM 5-HT was recorded in the presence and absence of $[Ca^{2+}]_{ext.}$ (figure 4.5). In WT mice IPAs, the contraction induced by 5-HT was 90 ± 8 % (n = 3) of the response to 50 mM KCl. This contraction was abolished when Ca^{2+} was removed from the bathing solution (4.3 ± 3 %, $P < 0.001$). The response to 5-HT in vessels from 5-HTT+ mice was 61 ± 5 % of the response induced by 50 mM KCl (n = 3) and this response was also abolished in the absence of $[Ca^{2+}]_{ext.}$ (4.3 ± 3 %, $P < 0.001$). A significant difference between the potency of 10 μM 5-HT in the vessels from WT mice and those from 5-HTT+ mice remained when $[Ca^{2+}]_{ext.}$ was present ($P < 0.05$). Since contraction only occurred when $[Ca^{2+}]_{ext.}$ was present, the results indicate that the Ca^{2+} involved in the 5-HT contractile response enters the cell from the extracellular environment.

A)



B)

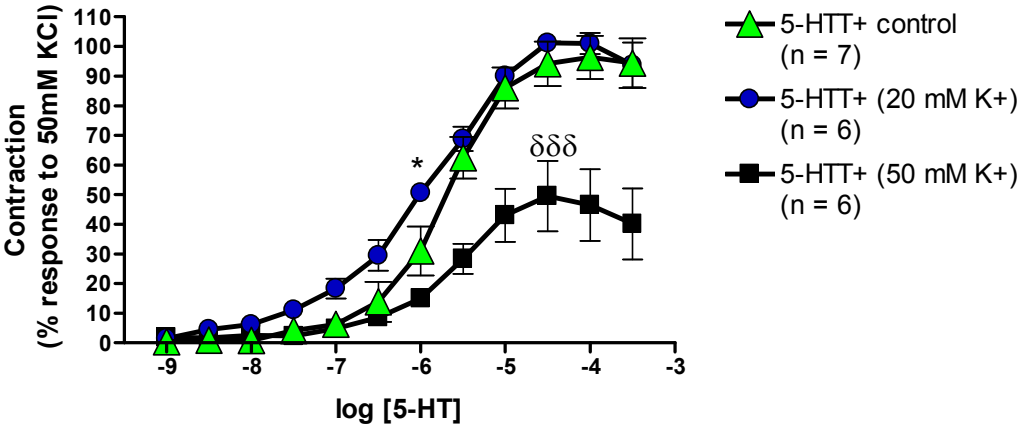
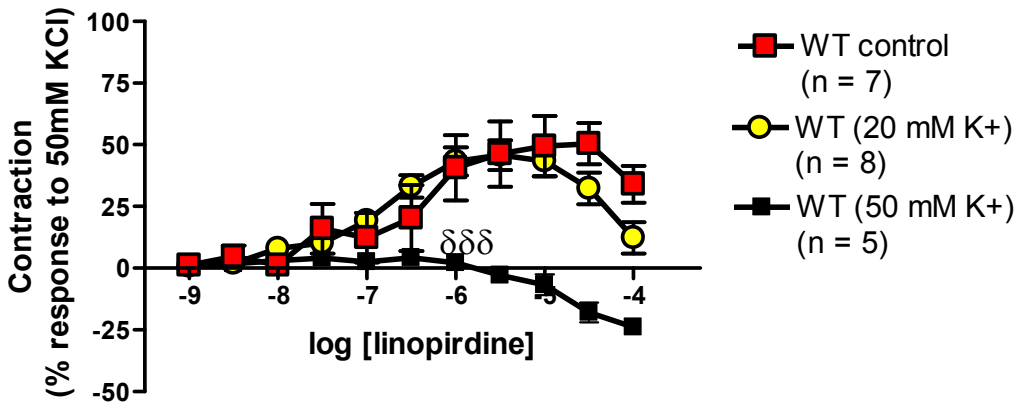


Figure 4.3 Effect of 20 mM and 50 mM $[K^+]_{ext}$ on 5-HT induced contraction.
Concentration response curve obtained to 5-HT (1 nM – 100 μM) in different $[K^+]_{ext}$. in A) WT mice IPAs and B) 5-HTT+ mice IPAs. Data are expressed as a percentage of the reference contraction to 50 mM KCl in each vessel. Each point represents the mean ± SEM (n = 8 – 10). *** = $P < 0.001$, ** = $P < 0.01$ indicates difference from control pEC50. δδδ = $P < 0.05$ indicates difference from control Emax.

A)



B)

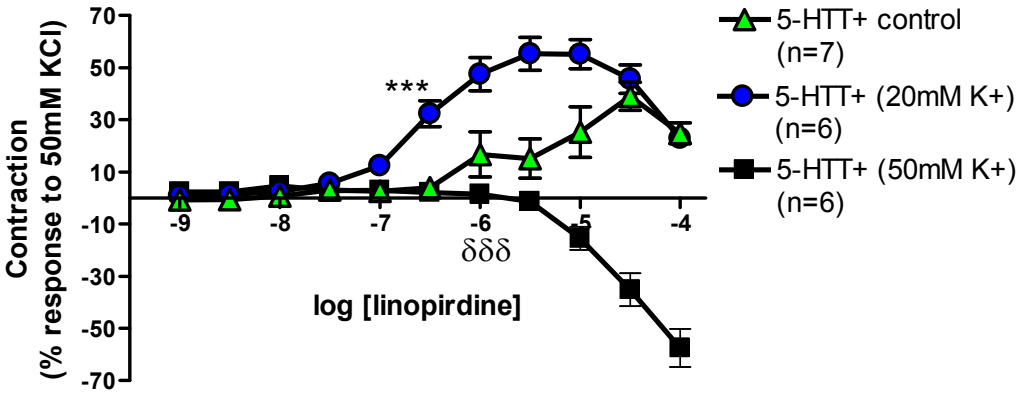


Figure 4.4 Effect of 20 mM and 50 mM $[K^+]_{ext}$ on linopirdine induced contraction.

Concentration response curve obtained to linopirdine (1 nM – 100 μM) in different $[K+]_{ext}$. in A) WT mice IPAs and B) 5-HTT+ mice IPAs. Data are expressed as a percentage of the reference contraction to 50 mM KCl in each vessel. Each point represents the mean ± SEM (n = 8 – 10). *** = $P < 0.001$ indicates difference from control pEC50. δδδ = $P < 0.05$ indicates difference from control Emax.

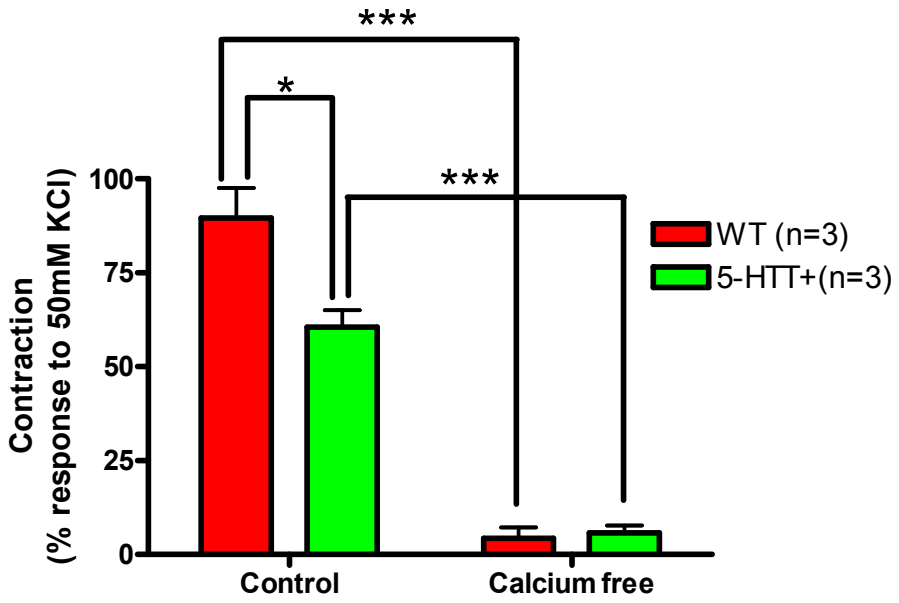


Figure 4.5 Contractile responses of IPAs to 5-HT in the presence and absence of $[Ca^{2+}]_{ext.}$.

Contractile responses to 10 μM 5-HT are compared in normal medium and after the removal of $[Ca^{2+}]_{ext.}$, in WT and 5-HTT+ mice IPA. Data presented as % of 50 mM KCl induced response. Each bar represents mean ± SEM (n = 3) (* = $P < 0.05$, *** = $P < 0.001$).

4.3.5 Effect of nifedipine on 5-HT and linopirdine induced contraction

Increasing concentrations of 5-HT (1 nM – 100 μM) were applied to mice IPAs in the presence of 1 μM nifedipine. DMSO was the vehicle for nifedipine, and therefore the concentration response curve constructed for 5-HT in the presence of nifedipine was compared to that recorded from control experiments where vessels were exposed to DMSO (control responses).

Nifedipine partially inhibited the maximum response to 5-HT in WT vessels (figure 4.6 A). In WT mice IPAs the maximum response to 5-HT in the presence of DMSO was 98 ± 7 % (n = 6) of the response induced by 50 mM KCl, however in the presence of nifedipine, the maximum 5-HT response was partially inhibited to 64 ± 5 % (n = 7) ($P < 0.001$). Nifedipine had a similar action on the maximum response of 5-HT in 5-HTT+ vessels. In 5-HTT mice IPAs the maximum response to 5-HT in the presence of DMSO was 86 ± 5 % (n = 6) of the response induced by 50 mM KCl, which was significantly reduced in the presence of nifedipine to 59 ± 2 % (n = 6) ($P < 0.001$).

Increasing concentrations of linopirdine (1 nM – 350 μM) were applied in the presence of 1 μM nifedipine and the resulting contractions measured and concentration response curve constructed. Again, control experiments were carried out using the vehicle DMSO as with the above experiments.

Nifedipine completely obliterated the linopirdine induced contraction seen in WT vessels (figure 4.6 B). In WT vessels the maximum response to linopirdine in the presence of DMSO was 40 ± 6 % (n = 7) of the response induced by 50 mM KCl, and this was reduced to 4 ± 2 % (n = 8) in the presence of 1 μM nifedipine, which was a significant reduction ($P < 0.001$). IPAs from 5-HTT+ mice also failed to contract in response to linopirdine in the presence of nifedipine. The maximum response to linopirdine in 5-HTT+ mice IPAs was 38 ± 2 % (n = 6) of the response induced by 50 mM KCl, which was reduced to 2 ± 1 % (n = 6) ($P < 0.001$) in the presence of nifedipine.

4.3.6 Influence of SOCC and store release inhibitors on 5-HT induced contraction

$NiCl_2$ (50 μM) significantly reduced the maximum response to 5-HT in WT mice IPAs from 119 ± 6 % (n = 6) of the response induced by 50 mM KCl to 95 ± 16 % (n = 6) ($P < 0.01$). These results are shown in figure 4.7. $NiCl_2$ did not significantly reduce the maximum response to 5-HT in 5-HTT+ mice vessels, which in the control environment was 106 ± 4 % (n = 6) of the response induced by 50 mM KCl and 101 ± 7 % (n = 8) in the presence of $NiCl_2$. However, $NiCl_2$ did reduce sensitivity to 5-HT in 5-HTT+ tissue: the pEC50 values were 5.9 ± 0.04 (n = 8) and 5.7 ± 0.5 (n = 8) in the absence and presence of $NiCl_2$ respectively ($P < 0.001$).

The maximum contraction induced by 5-HT in WT mice IPAs was significantly reduced in the presence of 2-APB (75 μM) (figure 4.8). The WT control 5-HT maximum response was 101 ± 2 % (n = 5) of the contraction induced by 50 mM KCl, which was reduced to 44 ± 3 % (n = 6) ($P < 0.001$). The sensitivity to 5-HT was also reduced in WT vessels when in the presence of 2-APB: pEC50 values in control and treated IPAs were 6.9 ± 0.05, and 6.6 ± 0.1, respectively ($P < 0.05$).

2-APB significantly reduced the maximum level of contraction induced by 5-HT in 5-HTT+ mice IPAs. In control 5-HTT+ vessels, 5-HT induced a maximum contraction of 112 ± 9 % of the contraction induced by 50 mM KCl. However, the presence of 2-APB reduced the amplitude of the response to 66 ± 13 % ($P < 0.05$). 2-APB had a much larger inhibitory effect on 5-HT induced contraction in WT mice IPAs relative to that of $NiCl_2$, (which only reduced the maximum response induced by 5-HT, and had no effect on 5-HT sensitivity).

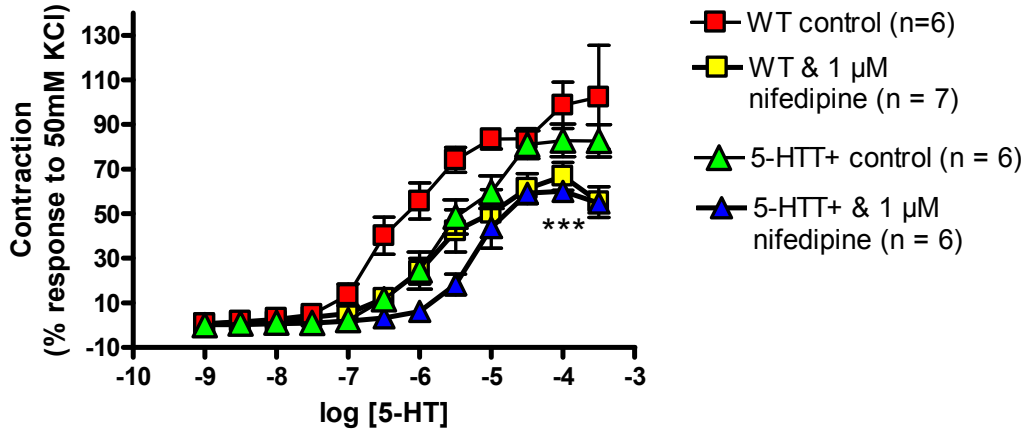
4.3.7 Influence of Y-27632 on 5-HT induced contractions

Y-27632 (10 μM) reduced both the sensitivity of 5-HT in WT mouse IPA vessels and the amplitude of the maximum contraction that 5-HT induced (figure 4.9). The pEC50 value of the control 5-HT response in WT vessels was 6.8 ± 0.1 % (n = 4), while the pEC50 value of the response in the presence of Y-27632 was 6.2 ± 0.2 (n = 5) ($P < 0.05$). The maximum 5-HT contraction induced in control WT mice IPAs was 108 ± 4

% of the contraction induced by 50 mM KCl, which was reduced to 41 ± 4 % in WT treated tissue ($P < 0.001$).

Y-27632 had a similar influence on the 5-HT induced response in 5-HTT+ mice IPAs, reducing both the sensitivity and amplitude of the maximum response (figure 4.8). The pEC50 values of the control and treated 5-HTT+ vessels were 5.7 ± 0.1 (n = 5) to 5.2 ± 0.1 (n = 5), respectively ($P < 0.01$). Y-27632 also reduced the maximum 5-HT induced response in 5-HTT+ tissue from 108 ± 4 %, (n = 5) of the contraction induced by 50 mM KCl to 33 ± 2 %, (n = 5) ($P < 0.001$).

A.



B.

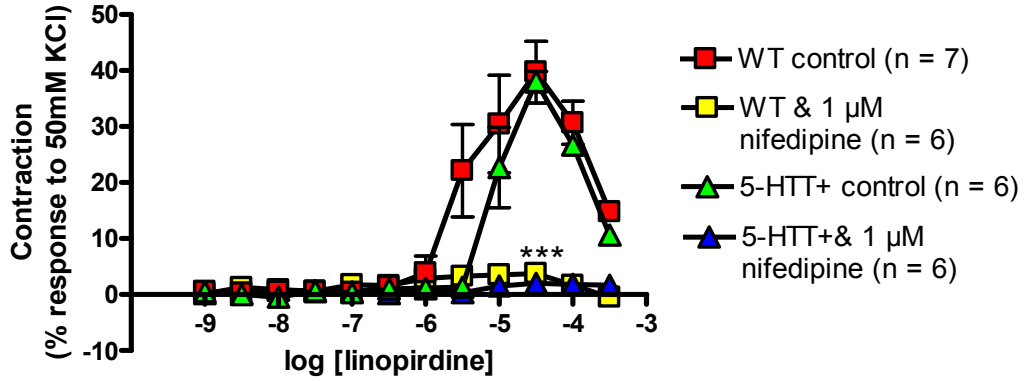


Figure 4.6 Effect of nifedipine on 5-HT and linopirdine-induced contraction in WT and 5-HTT+ mice IPA.

Concentration response curves obtained to A) 5-HT (1 nM – 100 µM) and B) linopirdine (1 nM – 350 µM) in the presence of 1 µM nifedipine in WT and 5-HTT+ mice IPAs. Data are expressed as a percentage of the reference contraction to 50 mM KCl in each vessel. Each point represents the mean ± SEM (n = 6 – 7). *** = $P < 0.001$ indicates a difference of both WT (& nifedipine) and 5-HTT+ (& nifedipine) Emax from control values.

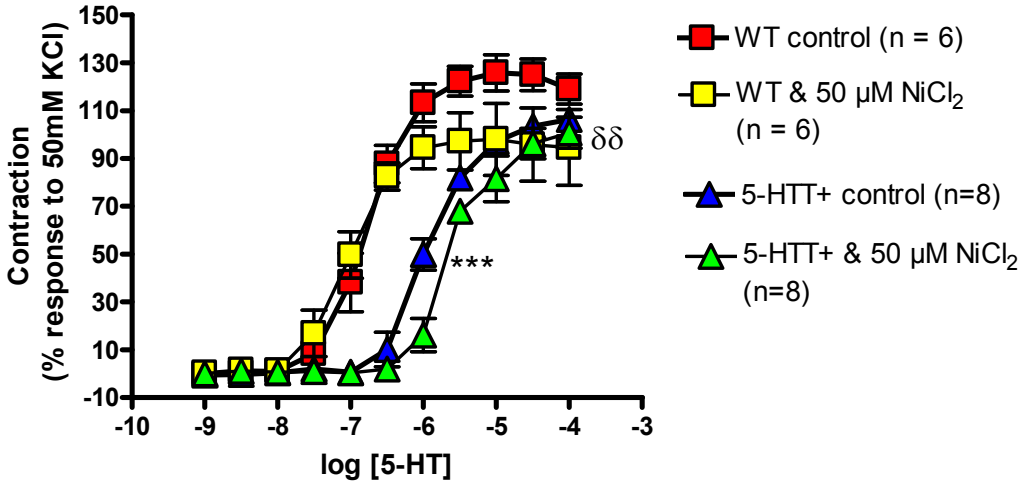


Figure 4.7 Effect of $NiCl_2$ on 5-HT-induced contraction in WT and 5-HTT+ mice IPA.

Concentration response curve obtained to 5-HT (1 nM – 100 μM) in WT and 5-HTT+ mice IPAs in the presence of 50 μM $NiCl_2$. Data are expressed as a percentage of the reference contraction to 50 mM KCl in each vessel. Each point represents the mean ± SEM (n = 6 – 8). *** = $P < 0.001$ indicates a difference of 5-HTT+ (& $NiCl_2$) pEC50 value from 5-HTT+ control value. δδ = $P < 0.01$ indicates difference of WT (& $NiCl_2$) Emax value from WT control value.

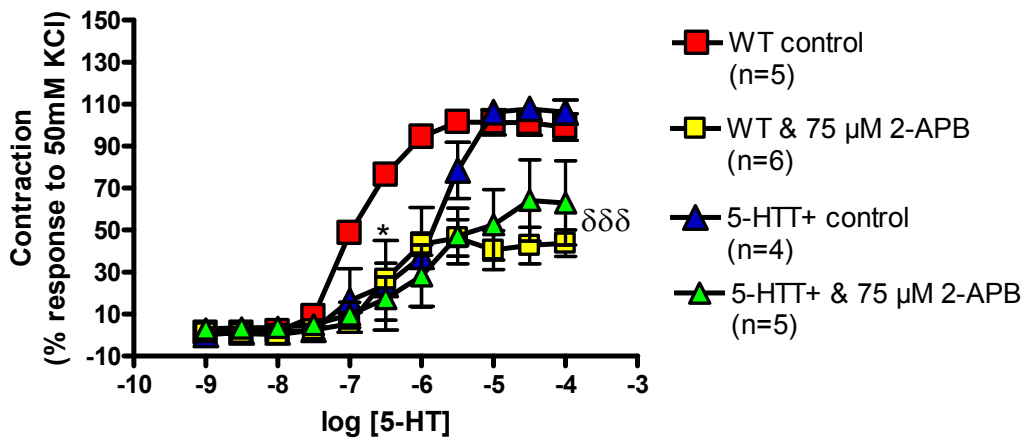


Figure 4.8 Effect of 2-APB on 5-HT-induced contraction in WT and 5-HTT+ mice IPA.

Concentration response curve obtained to 5-HT (1 nM – 100 µM) in WT and 5-HTT+ mice IPAs in the presence of A) 50 µM $NiCl_2$ and B) 75 µM 2-APB. Data are expressed as a percentage of the reference contraction to 50 mM KCl in each vessel. Each point represents the mean ± SEM (n = 4 – 8). * = $P < 0.05$ indicates a difference of WT (& 2-APB) pEC50 value from WT control value. δδδ = $P < 0.001$ indicates difference of both WT (& 2-APB) and 5-HTT+ (& 2-APB) Emax values from control values.

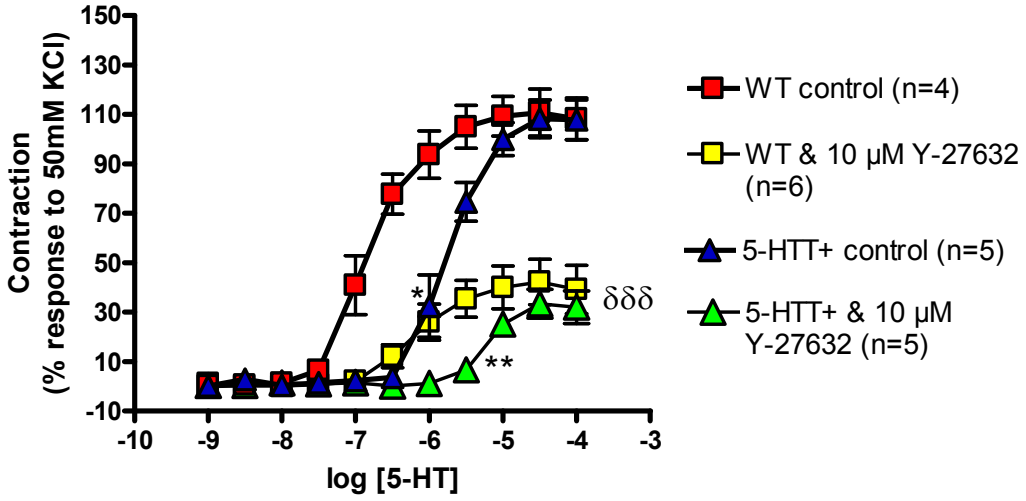


Figure 4.9 Effect of Y-27632 (10 µM) on 5-HT and contraction in IPA from WT and 5-HTT+ mice.

Concentration response curve obtained to 5-HT (1 nM – 100 µM) in WT and 5-HTT+ mice IPAs the presence of 10 µM Y-27632. Data are expressed as a percentage of the reference contraction to 50 mM KCl in each vessel. Each point represents the mean ± SEM (n = 4 – 6). ** = $P < 0.01$, * = $P < 0.05$ indicates a difference of both WT (& Y-27632) and 5-HTT+ (& Y-27632) from control pEC50 values. δδδ = $P < 0.001$ indicates difference of both WT (& Y-27632) and 5-HTT+ (& Y-27632) Emax values from control values.

4.4 Discussion

The results show that $[Ca^{2+}]_{ext.}$ is required for 5-HT induced contraction in WT and 5-HTT+ mice IPAs. However, only a proportion of this Ca^{2+} enters the PASMCs via VOCC channels, (5-HT was found to cause only a small membrane depolarisation in WT cells). In WT mice IPAs, the SOCC channel inhibitors influenced 5-HT induced contraction, with $NiCl_2$ reducing the overall maximum contraction and 2-APB reducing the sensitivity to 5-HT and reducing the maximum response induced. As nifedipine only partially affected the 5-HT response and $NiCl_2$ only inhibiting 5-HT contraction at larger concentrations, there appears be a component of Ca^{2+} influx that is not through VOCC or SOCC.

Overall the mean membrane potential of WT vessel PASMCs was - 42 mV. This is similar to what has been reported previously, with values of - 46 mV being reported in rat PASMCs (Cogolludo *et. al.,* 2006) and - 40 mV in human PASMCs (Olschewski *et. al.,* 2006). In the present study there was no significant difference between the membrane potential of the PASMCs isolated from WT vessels and those isolated from 5-HTT+ vessels. The finding that 5-HTT+ mice PASMCs had the same resting membrane potential and input resistance as WT mice PASMCs suggests that there was no difference in the resting ion conductances. It has been suggested that as part of PAH disease, the membrane potential of PASMCs are depolarised relative to control cells. Compared with normal human PASMCs, idiopathic PAH human PASMC had a depolarised membrane potential (Bonnet *et. al.,* 2007). The PAH found in 5HTT+ mice does not appear to involve such a change.

5-HT (1 μM) induced a small membrane depolarisation of PASMCs from WT mice, with a depolarisation of ~ 7 mV observed at 10 μM 5-HT. In chapter three, the 5-HT concentration response curve shows that at 1 μM 5-HT, the contraction induced in WT vessels was ~ 80 % of the contraction induced by 50 mM KCl, and at 10 μM 5-HT the contraction induced was ~ 95 %. Consistent with the discussion above, the large contraction response to 1 μM and 10 μM 5-HT in WT mice IPAs (due to increase in $[Ca^{2+}]_i$) occurs via a non voltage dependent Ca^{2+} entry pathway, with only a small proportion arising through VOCC. PASMCs from 5-HTT+ mice IPAs failed to significantly depolarise to the concentrations of 5-HT tested. 5-HTT+ vessels

responded to 1 μM 5-HT with a contraction of ~ 10 % of that induced by 50 mM KCl, and contracted ~ 65 % in response to 10 μM 5-HT. Therefore the 5-HT induced contraction in 5-HTT+ mice IPAs is not due to depolarisation of the PASMC membrane, and subsequent opening of VOCC.

At low 5-HT concentrations, contraction was increased in WT and 5-HTT+ mice IPAs in the presence of 20 mM $[K^+]_{ext}$. This increase in sensitivity to 5-HT may be due the depolarisation induced by 20 mM $[K^+]_{ext}$. The depolarisation induced by 20 mM $[K^+]_{ext}$. itself would increase the amount of Ca^{2+} influx and add to Ca^{2+} entry in response to 5-HT.

The fact that 5-HT induced contraction is not solely due to electromechanical coupling (i.e. directly related to membrane depolarisation) has also been shown in previous studies. Rabbit PAs contracted to 1 μM 5-HT; however this concentration did not depolarise the rabbit PASMCs enough to induce the contraction observed (Buryi *et. al.,* 1991). Development of contraction in rabbit PA was connected to activation of ROCCs (Buryi *et. al.,* 1992). The same study went on to show that 5-HT induced contraction has a voltage sensitive component, as it was abolished by membrane hyperpolarisation. However, in rat PASMCs, Cogolludo *et. al.* (2006) presented results that show 10 μM 5-HT significantly depolarised the cells. Therefore, it may be the case that the voltage sensitive and voltage insensitive components of the 5-HT induced contraction are species dependent.

To further characterise the mechanisms of contraction in WT and 5-HTT+ mice IPAs, experiments were carried out to investigate the source of Ca^{2+} involved in the response to 5-HT. 5-HT was applied to IPAs in the presence of control PSS (which contained Ca^{2+}). The vessels were then washed with Ca^{2+}-free PSS, and 5-HT was reapplied. 5-HT acts to induce contraction by increasing $[Ca^{2+}]_i$ in PASMCs (Wilson *et. al.,* 2005). As prolonged elevations of $[Ca^{2+}]_i$ are toxic to the cell, Ca^{2+} pumps are rapidly activated in an attempt to limit the Ca^{2+} signal. A proportion of the $[Ca^{2+}]_i$ is extruded across the cell membrane and the rest is taken up by intracellular stores including the SR. Overall a net loss of Ca^{2+} from the cell occurs which leads to depletion of intracellular Ca^{2+} stores (Petersen, 1996). These stores are then refilled via CCE (Putney, 1990). In order to re-fill the stores Ca^{2+} must be present in the extracellular medium. Following Ca^{2+}

free PSS washout of the initial contraction induced by 5-HT (in the control PSS), reapplication of 5-HT did not result in contraction of the tissues. By washing the vessels with Ca^{2+} free PSS, the intracellular stores were unable to re-fill. This indicates that 5-HT induced contraction is dependent on Ca^{2+} being present in the extracellular environment. The results from the experiments using nifedipine indicate that a proportion of the Ca^{2+} influx involved in the 5-HT induced contraction in WT and 5-HTT+ mice IPAs enters the cell via L-type VOCC. This result has been seen previously in rat PA (Cogolludo *et. al.,* 2006). $NiCl_2$ reduced the maximum 5-HT induced response in WT mice, however at lower concentrations of 5-HT (< 1 μM), the 5-HT induced response was unaffected. This suggests that at low concentrations of 5-HT, the responses are independent of SOCC Ca^{2+} entry, however, SOCCs may be involved in responses to higher 5-HT concentrations in WT mice. The sensitivity of 5-HTT+ mice IPAs to 5-HT was reduced in the presence of $NiCl_2$, suggesting that SOCCs may play a more important role in the 5-HT response in this mouse model. The inhibitory effects of $NiCl_2$ have been observed in the pulmonary vasculature of other animal also. $NiCl_2$ inhibited SOCC Ca^{2+} entry in PASMCs isolated from rat main PA and IPA (McElroy *et. al.,* 2008; Ng and Gurney, 2001) and in canine PASMCs, $NiCl_2$ reduced the increase in $[Ca^{2+}]_i$ induced by 10 μM 5-HT (Wilson *et. al.,* 2005). The different effects of $NiCl_2$ between WT and 5-HTT+ mice IPA may be the result of a change in SOCC subunits, differences in accessory protein composition, or alterations in SOCC regulation.

2-APB reduced the maximum 5-HT induced contraction in WT mice IPAs, an effect that was mirrored in 5-HTT+ vessels. In addition to inhibiting SOCC, 2-APB is an inhibitor of $InsP_3$ induced Ca^{2+} release (Maruyama *et. al.,* 1997), and does so over a range of 1 – 100 μM (Bootman *et. al,.* 2002). This is consistent with the concentration of 2-APB used the present study, and therefore suggests a role for $InsP_3$ induced Ca^{2+} release in the response to 5-HT in mouse IPA. This result provides further insight into the results obtained from experiments in which 5-HT responses were tested in the absence of extracellular Ca^{2+}. In the presence of 2-APB, the difference in 5-HT potency between WT and 5-HTT+ mice was abolished, suggesting that there may be a loss of IP_3 induced constriction in 5-HTT+ mice PASMCs. However the inhibition of Ca^{2+} signalling, (and hence contraction) by 2-APB may not necessarily indicate a role for IP_3 receptors, since 2-APB can cause an unnoticed depletion of intracellular Ca^{2+} stores, and it can affect other cellular processes, such as mitochondrial morphology, which may

indirectly alter cellular Ca^{2+} signalling (Peppiatt *et. al.,* 2003). Therefore further experiments are needed to investigate how 2-APB partially inhibited the 5-HT induced response.

5-HT may activate the rho kinase pathway. Rho kinase activation can increase the sensitivity of the smooth muscle contractile apparatus to Ca^{2+} (Fukata *et. al.,* 2001), and if 5-HT did activate this pathway, it would amplify the effect of Ca^{2+} entering across the membrane. The observation that the rho kinase inhibitor, Y-27632, partially inhibited the 5-HT-induced contraction, suggests that 5-HT receptors couple to the Rho-kinase pathway. In this study Y-27632 greatly reduced the response to 5-HT in WT and 5-HTT+ vessels in a similar manner.

With regards to linopirdine induced contraction, ketanserin (an inhibitor of 5-HT2A receptors) did not alter the linopirdine induced response in either vessel type. These experiments confirm that in WT and 5-HTT+ mice IPAs, linopirdine does not constrict vessels via the 5-HT2A receptor. This study further demonstrates that linopirdine induced contraction of IPAs is mediated via Ca^{2+} entry entirely through VOCC, as nifedipine completely inhibited the linopirdine response, in both tissue types.

To further investigate the method of linopirdine induced contraction, IPA vessels from WT and 5-HTT+ mice were treated with increasing concentrations of linopirdine in the presence of different $[K^+]_{ext}$. Linopirdine induces PA contraction by blocking KCNQ channels and depolarising PASMCs (Joshi *et. al.,* 2006). Moreover, the open probability of KCNQ channels is increased at depolarised potentials (Robbins, 2001). Therefore it would be expected that depolarising the PASMCs by increasing $[K^+]_{ext}$. the effect of blocking KCNQ channels with linopirdine would be enhanced. An increase in the contractile effect of linopirdine would also be expected as depolarisation of the membrane would lead to opening VOCC channels and subsequent Ca^{2+} entry. Surprisingly an increased potency to linopirdine was not seen in the WT tissue in the depolarised conditions. This may have been due to KCNQ channels being maximally open under control conditions, and depolarising the PASMC membrane did not result in any more KCNQ channels being open and hence no further inhibition by linopirdine. Interestingly, the response to linopirdine in 5HTT+ vessels was enhanced by depolarisation with 20 mM $[K^+]_{ext}$. It is possible that in 5-HTT+ vessels there is

reduced activity of KCNQ channels at rest, and upon depolarisation of the PASMC membrane the activity of KCNQ channels was increased, leading to a greater proportion of active KCNQ channels available for block by linopirdine.

4.5 Conclusion

Overall, the main mechanism of 5-HT induced contraction and hence increase of $[Ca^{2+}]_i$ in PASMCs from WT and 5-HTT+ mice IPA involves Ca^{2+} entry from the extracellular environment, and from the SR, amplified by rho kinase activation and subsequent increased sensitivity to Ca^{2+}. Both voltage dependent and voltage independent routes of Ca^{2+} entry are involved in the 5-HT contractile response in these vessels. Part of this Ca^{2+} enters via VOCC in the plasma membrane of PASMCs, and at higher concentrations, some of the Ca^{2+} involved in the contraction response enters the cell via SOCC channels. The results also indicate a greater contribution of IP_3 induced constriction in WT mouse IPA relative to 5-HTT+ vessels.

Linopirdine induced contraction is due to Ca^{2+} entry via VOCCs in WT and 5-HTT+ mice IPAs. However the difference in potency between the vessels may be due to decreased activity of KCNQ channels in the 5-HTT+ mice IPAs.

Chapter 5

Effects of the KCNQ channel openers, flupirtine and retigabine, on pulmonary arteries in PAH

5.1 Introduction

To date 5 genes of the K_V7 family of channels, KCNQ1-5 ($K_V7.1$-7.5) have been reported (Robbins, 2001). Most research to date has been on drugs modulating these channels in the central nervous system, where they have been shown to mediate the M-current (Brown and Adams 1980; Main *et. al.,* 2000). KCNQ channels have also been described as functioning in cardiac cells (Snyders, 1999; Nerbonne, 2000) and the auditory pathways (Neyroud *et. al.,* 2001). KCNQ current is of particular interest as it is a non-inactivating, voltage dependent K^+ current which is involved in regulating neuronal excitability (Brown, 1988) and hence is involved in stabilising neuronal membrane potential.

Pharmacology data was provided in the previous chapters to suggest a role for KCNQ channels in IPA vessels from WT and 5-HTT+ mice. Other studies have investigated the function of KCNQ channels in the vasculature: Joshi *et. al.,* (2006) induced contraction of IPA from rats using the KCNQ blocking agent linopirdine and its more potent analogue, XE991; Yeung & Greenwood (2005) showed that linopirdine and XE991 increased the spontaneous contractile activity of murine portal vein; the KCNQ channel opener retigabine has also been shown to relax pre-contracted murine aortic segments (Yeung *et. al.,* 2007). The above evidence reinforces the suggestion that KCNQ channels have a functional role in the vasculature. Myography data (shown in previous chapters) additionally shows that the response of mice IPA to linopirdine is reduced in the 5-HTT+ vessels, suggesting that there is altered expression of KCNQ channels in PAH.

In addition to drugs that block KCNQ channels, drugs have also been developed that open these channels. The KCNQ channel activator flupirtine is used in Europe as a non-opioid analgesic and in several human trials has proven to be essentially free of cardiovascular side effects (Friedel & Fitton, 1993; Hummel *et. al.,* 1991; Herrmann *et. al.,* 1987). Flupirtine is a less potent analogue of retigabine. Clinical trials have been carried out investigating the use of retigabine in the treatment of patients with epilepsy (Sachdeo *et. al.,* 2005). The critical action of retigabine is to increase potassium current near resting potential, reducing excitability, and this explains at least in part, the anticonvulsant effect of retigabine (Rogawski, 2006). Therefore in cells expressing

KCNQ channels it would cause hyperpolarisation and reduce the opening of VOCC channels. In smooth muscle cells this would reduce contraction. Retigabine selectively activates KCNQ2-5 (Tatulian *et. al.,* 2001) and flupirtine, like retigabine, has no effect on KCNQ1 channels at < 100 μM (Schenzer *et. al.,* 2005; Wuttke *et. al.,* 2005).

If KCNQ channel activation does relax IPA vessels, then KCNQ channel openers could potentially be effective at dilating pulmonary arteries and reducing pulmonary arterial pressure in PAH. To investigate this possibility, the effects of KCNQ channel openers were investigated on pre-constricted IPA. In addition, their effects on two mouse models of PAH were investigated *in vivo*. The effect of flupirtine was investigated on pulmonary vascular haemodynamics and indices of PAH, in 5HTT+ mice and chronic-hypoxic mice, a well characterised model of PAH (Paddenberg *et. al.,* 2007; Raoul *et. al.,* 2007; Fresquet *et. al.,* 2006).

It has been shown that by deleting the 5-HTT gene or pharmacologically inhibiting 5-HTT activity, mice were protected against hypoxia-induced PH (Eddahibi *et. al.,* 2000; Guignabert *et. al.,* 2005), suggesting that 5-HTT+ is integral to the development of PAH. 5-HTT+ mice exhibit spontaneous PAH in the absence of other external environmental stimuli (Guignabert *et. al.,* 2006). In these 5-HTT+ mice, right ventricular systolic pressure was found to be increased and the degree of right ventricular hypertrophy and distal pulmonary vessel muscularisation was more severe in 5HTT+ mice than in wild-type mice following exposure to hypoxia. With regards to hypoxia-induced PAH, a proposed mechanism for this process is the direct action of hypoxia on certain specific genes within PASMC, such as hypoxia-inducible factor. Mice deficient in hypoxia-inducible factor-1 exhibit decreased development of hypoxic PH with reduced muscularisation of distal pulmonary vessels (Yu *et. al.,* 1999). By investigating both these PAH models, a broader role of KCNQ channels can be determined in the overall process of PAH.

As part of this study mice were subject to hypobaric oxygen for two weeks or maintained in a normoxic environment, with age matched siblings used for comparison. Within each group, half were administered flupirtine. The effect of flupirtine on the development of PAH in 5-HTT+ mice and chronic hypoxic mice was investigated by administration of oral flupirtine on a daily basis over a period of two weeks.

5.2 Methods

5.2.1 Myography

Myography experiments were performed as in chapter 2. IPA vessels were preconstricted with phenylephrine (10 – 100 nmol/ L) and cumulative application of increasing concentrations of flupirtine or retigabine (1 nM - 0.1 mM) were applied. Concentration-response curves were constructed from the relaxation responses measured at each concentration of KCNQ opener. Both drug stocks were prepared by dissolving in DMSO, so control experiments were carried out in parallel on matched vessels, using DMSO at the concentration reached around the vessels exposed to drugs. Relaxation was measured as % phenylephrine induced tone.

5.2.2 *In-vivo* experiments

In vivo experiments to investigate the effects of flupirtine on PAH were carried out as described in chapter 2, using the WT, WT hypoxic and 5-HTT+ mice. Mice were treated orally with either flupirtine (30 mg/ kg/ day) or vehicle (1% carboxymethylcellulose) alone on each of the fourteen experimental days. In each mice group measurements were made of right ventricular hypertrophy (RVP) and *in vivo* mean right ventricular pressure (mRVP) as described in chapter 2. These are parameters of pulmonary hypertension and are widely used to investigate this disease (Van Suylen *et. al.,* 1998). IPA vessels were dissected from the experimental mice, and contraction to 5-HT was measured *in vitro*. The purpose was to determine if 5-HT-induced contraction was altered after the mice were treated with the KCNQ channel opener flupirtine.

5.3 Results

5.3.1 Responses of WT & 5-HTT+ IPAs to flupirtine and retigabine

PE (10 – 100 nM) induced a sustained vasoconstriction in vessels from WT and 5-HTT+ mice. The level of pre-constriction by PE was similar in both vessel types at ~ 70 % of that induced by 50 mM KCl. Cumulative addition of DMSO resulted in dilation of IPA from WT and 5-HTT+ mice which had been pre-constricted with PE. .

Flupirtine induced substantial vasodilation in WT mice IPA vessels pre-constricted with PE (n = 6), and did so in a concentration dependent manner (figure 5.1A). Flupirtine began to relax WT vessels at concentrations below 1 μM and induced approximately 50 % relaxation of the PE induced tone at 35 μM in WT mice. However, flupirtine was not an effective vasodilator in 5HTT+ vessels until above 10 μM (n = 5). Flupirtine induced a relaxation of approximately 50 % at a concentration of 35 μM in 5-HTT+ mice also. Retigabine also relaxed IPA vessels pre-contracted with PE, and as with flupirtine, it was more effective in WT than 5HTT+ mice (figure 5.1B). Retigabine strongly relaxed IPAs from WT mice, producing an effect below 1 μM and inducing approximately 50 % relaxation of PE induced tone at 10 μM (n = 6). In 5-HTT+ mice IPA, retigabine did not induce vasodilation until the concentration was 35 μM in the bathing solution (n = 5), and at this concentration the relaxation induced was approximately 55 %.

5.3.2 *In- vivo* studies

The measured indices of PAH were not altered in normoxic WT mice following flupirtine treatment. There was no significant change in mRVP or RV/ (LV + S) ratio (ratio used to as an indicator of right ventricular hypertrophy) (figure 5.2 & 5.3). Heart rate was unaffected by either flupirtine treatment or hypoxia (figure 5.4).

Chronic hypoxia exposure led to changes in the indices of PAH in mice. Hypoxic mice had an increase of ~ 50 % in mRVP relative to normoxic animals (figure 5.2). Hypoxic mice exhibited right ventricular hypertrophy relative to WT controls (figure 5.3). WT hypoxic mRVP was 22 ± 2 mmHg (n = 7, $P < 0.01$ relative to normoxic WT). RV/ (LV + S) ratio in hypoxic mice was 0.3 ± 0.01 (n = 7), however normoxic WT mice had a RV/ (LV + S) ratio of 0.2 ± 0.01 (n = 6, $P < 0.001$). Flupirtine treatment resulted in reduced mRVP and right ventricular hypertrophy measurements compared to that of the control group of mice receiving vehicle (figure 5.2 & 5.3). The mRVP measured in hypoxic mice treated with flupirtine was 15 ± 1 mmHg (n = 9, $P < 0.01$ relative to vehicle dosed mice) and RV/ (LV + S) ratio was 0.2 ± 0.02 (n = 7, $P < 0.05$ relative to vehicle dosed mice). The values of mRVP and RV/ (LV + S) in the hypoxic animals treated with flupirtine did not differ significantly from the WT normoxic animals, indicating that flupirtine inhibited PAH.

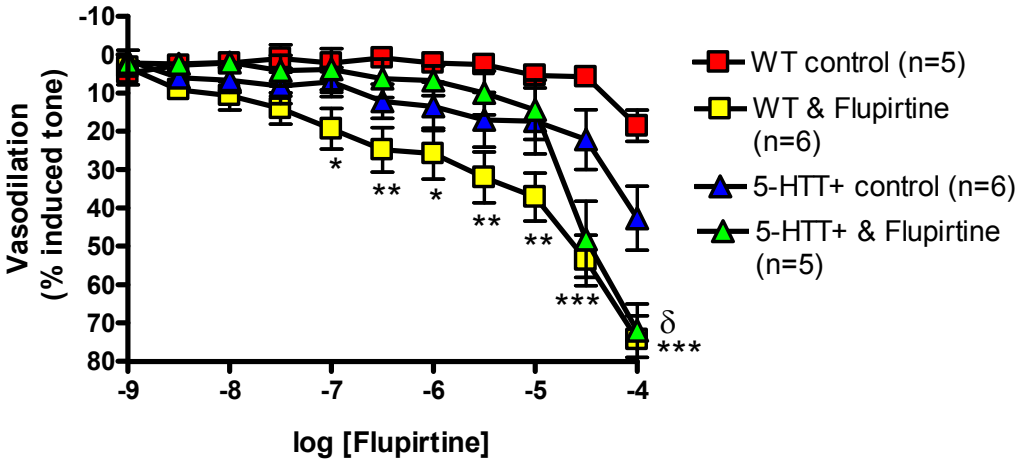
Figure 5.2 shows that 5-HTT+ mice demonstrated markedly elevated mRVP when compared to WT mice (30 ± 3 mmHg (n = 6) compared to 13 ± 1 mmHg (n = 8) respectively ($P < 0.001$), which has been previously reported (MacLean *et. al.,* 2004). Treatment with flupirtine markedly reversed this elevation in mRVP to levels which were not significantly different from that measured in normoxic WT mice, with mRVP in flupirtine treated 5-HTT+ mice measured as 17 ± 8 mmHg (n = 8, $P < 0.001$) compared to vehicle treated 5-HTT+ mice (figure 5.2). Right ventricular hypertrophy was observed in the 5-HTT+ mice compared with WT mice and this was also attenuated by flupirtine (figure 5.3). RV/ (LV + S) ratio was 0.23 ± 0.004 (n = 6) in 5-HTT+ mice dosed with flupirtine relative to 0.25 ± 0.01 (n = 8) in 5-HTT+ mice dosed with vehicle ($P < 0.05$). The RV/ (LV + S) ratio in flupirtine treated 5-HTT+ mice did not significantly differ from the WT mice vehicle treated value, which was 0.22 ± 0.005 (n = 6).

5.3.2 *In-vitro* functional studies of vessels from drug-treated mice

Normoxic WT vessels responded to 5-HT in an identical matter to that reported in previous chapters. The amplitude of the 5-HT response was increased by ~ 70 % in hypoxic IPA from chronic hypoxic mice (n = 6) relative to the response in normoxic mice (n = 6, $P < 0.001$) (figure 5.5).

The response to 5-HT did not differ between vessels derived from normoxic WT mice which had been dosed with flupirtine or vehicle (figure 5.5). Flupirtine treatment did not alter pEC50 values for the 5-HT responses in WT normoxic mice (7 ± 0.1, n = 6 - 7). Flupirtine also had no influence on 5-HT-induced contraction in 5-HTT+ mice IPAs, the pEC50 values of the 5-HT-induced responses were 6 ± 0.1 (n = 7) for both treatment groups. Prior treatment with flupirtine significantly reduced the contractile responses of 5-HT in the IPA vessels from chronic hypoxic WT mice. The maximum 5-HT response was reduced from 174 % ± 14 (n = 6) of the response to 50 mM KCl, to 126 ± 3.0 (n = 7) ($P < 0.01$).

A)



B)

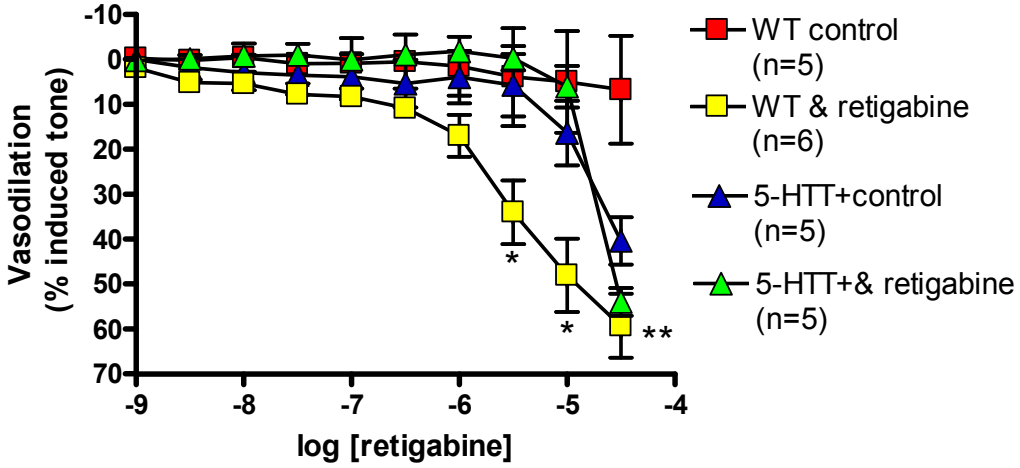


Figure 5.1 Flupirtine and retigabine induced relaxation in WT and 5-HTT+ mice IPAs.

Concentration response curves obtained to A) flupirtine (10 nM –10 mM) and B) retigabine (10 nM – 3.5 mM/ 10 mM) in WT and 5-HTT+ mice. Control (DMSO) data are also shown and illustrate the fall in vascular tone in vessels set up in parallel with those to which flupirtine and retigabine were added. Data are expressed as a percentage of the response to PE induced preconstriction and each point represents the mean ± SEM (n = 5 – 6). *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$ indicates value significantly greater than corresponding value in vehicle treated WT vessels. δ = $P < 0.05$ indicates value significantly greater than corresponding value in vehicle treated 5-HTT+ vessels.

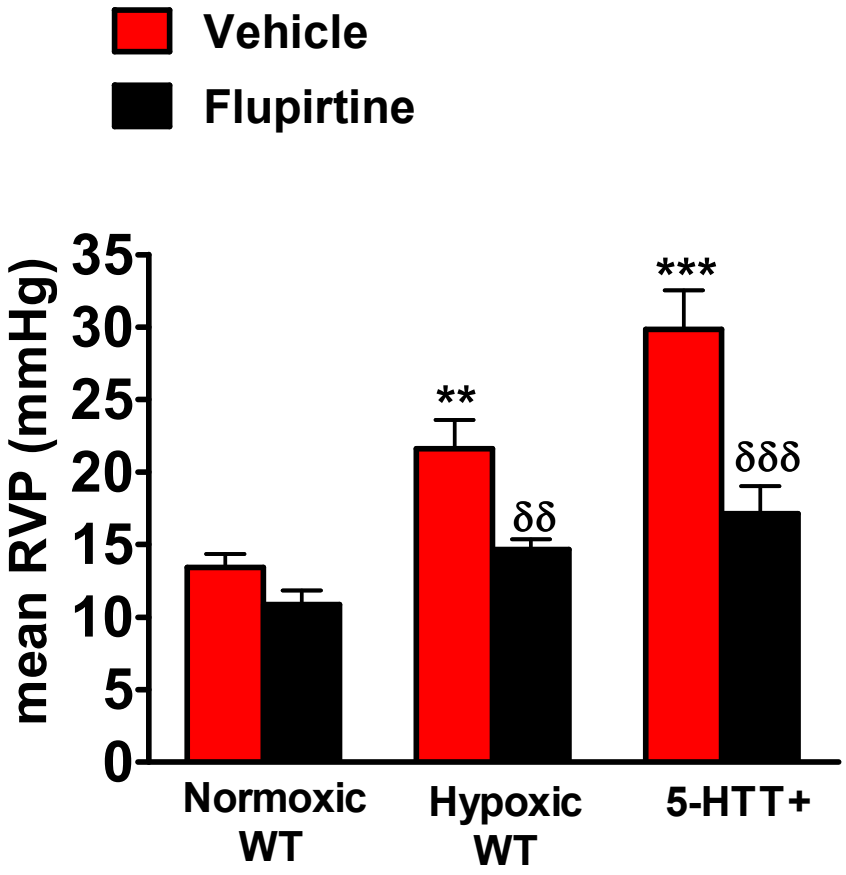


Figure 5.2 Effect of flupirtine on mRVP.

mRVP in normoxic WT, hypoxic WT and 5-HTT+ mice treated with vehicle (■) and flupirtine ■ (n = 6 to 8 mice in each group). * Value significantly greater than corresponding value in normoxic WT mice (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). δ Value significantly less than corresponding value in vehicle treated mice (δ $P < 0.05$, δδ $P < 0.01$, δδδ $P < 0.001$). Data are expressed as mean ± S.E.M.

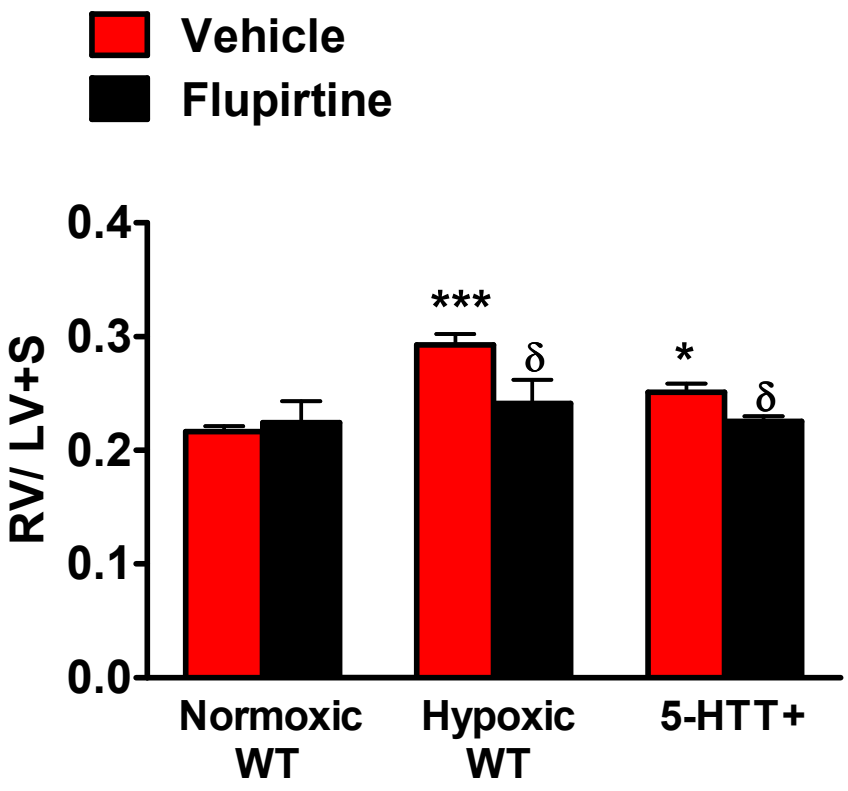


Figure 5.3 Effect of flupirtine on RV/ LV + S.

RV/ LV + S in normoxic WT, hypoxic WT and 5-HTT+ mice treated with vehicle (■) and flupirtine ■ (n = 6 to 8 mice in each group). * Value significantly greater than corresponding value in normoxic WT mice (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). δ Value significantly less than corresponding value in vehicle treated mice (δ $P < 0.05$, δδ $P < 0.01$, δδδ $P < 0.001$). Data are expressed as mean ± S.E.M.

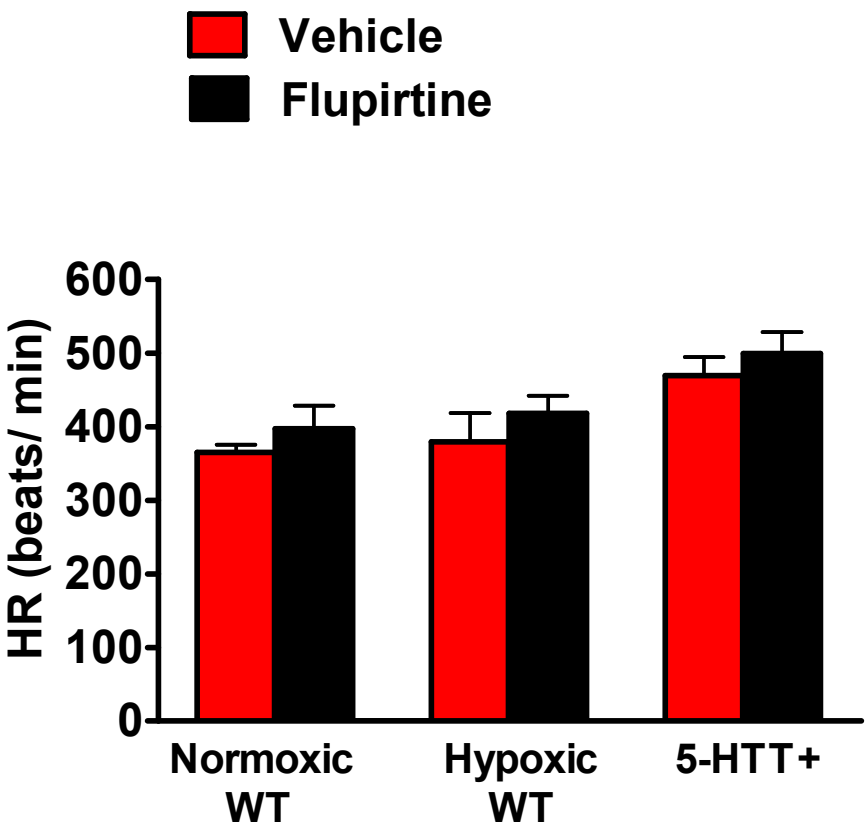


Figure 5.4 Effect of flupirtine on heart rate.

Heart rate in normoxic WT, hypoxic WT and 5-HTT+ mice treated with vehicle (■) and flupirtine ■ (n = 6 to 8 mice in each group). Data are expressed as mean ± S.E.M.

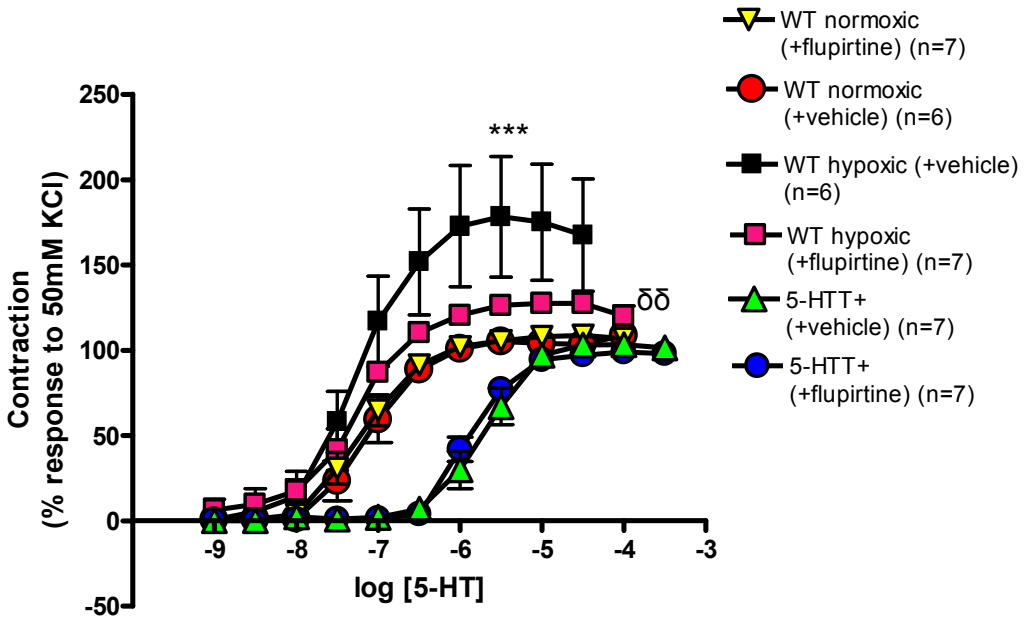


Figure 5.5 5-HT-induced contraction in normoxic and hypoxic WT and 5-HTT+ mice IPAs from vehicle and flupirtine dosed animals.

Concentration response curve obtained to 5-HT (1 nM – 100/ 350 μM) in normoxic, hypoxic WT and 5-HTT+ mice IPAs from vehicle and flupirtine dosed animals. Data are expressed as a percentage of reference contraction to 50 mM KCl in each vessel. Each point represents the mean ± S.E.M. (n = 6 – 7). *** = $P < 0.001$ indicates WT hypoxic (vehicle treated) Emax significantly greater than WT normoxic value. δδ = $P < 0.01$ indicates WT hypoxic (flupirtine treated) Emax significantly lower than corresponding value in vehicle treated WT hypoxic vessels.

5.4 Discussion

The KCNQ channel openers, flupirtine and retigabine, dilated IPA vessels from WT and 5-HTT+ mice. Flupirtine reduced the indices of PAH measured in this study in two models of PAH, the 5-HTT+ mouse model and the chronic hypoxic mouse model.

In previous chapters it was found that linopirdine induced vasoconstriction of mice IPA. The results shown in this chapter are consistent with these observations. KCNQ channel openers, flupiritine and retigabine, relaxed mice IPA. Flupirtine and retigabine dilated mice IPA vessels, a result which has been also witnessed in mouse systemic arteries (Yeung *et. al.,* 2007) and rat pulmonary arteries (Joshi *et. al.,* 2008(submitted)). Vasodilation induced by flupirtine and retigabine in mice aortic segments was reversed with a KCNQ inhibitor (XE991), which indicates that the vasodilatory effects by these agents are mediated by increased flux of K^+ through KCNQ channels (Yeung *et. al.,* 2007). Retigabine was more potent than flupirtine in inducing vasodilation of WT mice IPA. This is similar to their order of potency at opening KCNQ channels in other tissues such as mice aorta (Yeung *et. al.,* 2007). These results indicate that KCNQ channels are found in mice IPA and are important for regulating tone in mouse PA. In addition, the relaxant effects of retigabine and flupirtine in PA suggests that the KCNQ channels present and active are KCNQ2-5, as these are the channels that are selectively activated by retigabine and flupirtine (Tatulian *et. al.,* 2001; Pereta *et. al.,* 2005). The experiments conducted as part of this study show that KCNQ2-5 channel activators are more potent in WT mice IPA relative to 5-HTT+ vessels. This finding is compatible with the results obtained with the KCNQ channel inhibitor, linopirdine (see chapter 3), which was more potent in WT vessels. Overall, these results suggest a reduction in the number or activity of KCNQ2-5 channels in 5-HTT+ mice PA relative to WT vessels.

As flupirtine was found to be an effective vasodilator in mice IPA, its actions on PAH indices (mRVP and right ventricular hypertrophy) were investigated. Flupirtine treatment was found to prevent the increase in mRVP and right ventricular hypertrophy that was observed in vehicle treated chronic hypoxic mice. Moreover, the measurements of mRVP and right ventricular hypertrophy in chronic hypoxic mice subsequent to flupirtine treatment were not significantly different from measurements in normoxic controls. Therefore flupirtine prevented the development of PAH caused by

hypoxia. These results suggest that KCNQ channels may be involved in the development of hypoxic PAH in the mice model. Flupirtine also reduced mRVP and right ventricular hypertrophy in 5-HTT+ mice. Flupirtine treatment attenuated these indices of PAH in 5-HTT+ mice to levels which were not significantly different those measured in normoxic WT mice. These findings in 5-HTT+ mice (which have established PAH), indicate that flupirtine treatment could be effective in reversing PAH, as well as in the prevention of the condition.

The vasodilatory response of 5-HTT+ mice IPAs to flupirtine application was reduced compared to WT vessels, however, flupirtine was still able to reverse PAH in the 5-HTT+ mice. If the reduced relaxation to flupirtine in the 5-HTT+ mice IPAs was due to a reduction in activity and/ or expression of KCNQ channels, it may be the case that the level of KCNQ channel activity was sufficient for flupirtine to act upon to reverse PAH. As flupirtine is an activator of KCNQ channels, it would cause hyperpolarisation of PASMCs; therefore it could prevent the depolarisation needed for contraction (which occurs in PAH). By reducing the level of vasoconstriction, flupirtine treatment may reduce the overall rise in PVR and PAP which contribute to PAH. However, it may also be the case that chronic flupirtine effects differ from acute effects, with flupirtine having a greater effect in the flupirtine dosed 5-HTT+ mice relative to the effects on the WT animal. To establish if this is the case, experiments could be conducted on IPAs from flupirtine dosed 5-HTT+ mice to investigate if the vasodilatory response to acute flupirtine application recovered to that of the WT.

Chronic hypoxic mice IPA vessels exhibited an increased level of response to 5-HT relative to WT normoxic IPAs. The fact that the hypoxic model responded greater to contractile agents has been shown previously (MacLean *et. al.,* 1996; McCulloch *et. al.,* 1998). Chronic hypoxia has been shown to inhibit I_{KN} in PASMCs (Osipenko *et. al.,* 1998). Inhibition of I_K results in membrane depolarisation and opening of VOCC, which results in an increase in $[Ca^{2+}]_i$ and vasoconstriction. Increased contractile responses to 5-HT in IPAs derived from chronic hypoxic mice, may have been the result of pharmacological synergism between 5-HT receptors (as described in chapter 1). Prior dosing of the chronic hypoxic mice with flupirtine prevented the increased contractile response to 5-HT significantly. This may be due to flupirtine preventing vasoconstriction of PASMCs, and hence preventing the tone necessary for the

'unmasking' of 5-HT receptors (MacLean, 1999). 5-HT responses were unchanged in IPA derived from 5-HTT+ mice which were dosed with flupirtine. The results from chapter 4 show that 5-HTT+ mice IPA SMCs are not depolarised relative to WT vessel SMCs, and therefore these vessels do not have the same pre existing tone as seen in chronic hypoxic mice.

Retigabine relaxes mice aorta segments (Yueng *et. al.,* 2007), and hyperpolarise mice VSM (Yueng *et. al.,* 2008), which is compatible with the vasodilatory effects shown here in mice IPA. The results presented in this chapter also show that retigabine was more potent than flupirtine, which would suggest retigabine may be more effective at reducing the indices of PAH than flupirtine.

5.5 Conclusion

Collectively the data in this chapter show that despite loss of sensitivity to the vasodilator action of flupirtine in the 5-HTT+ mouse model of PAH, the drug can prevent PAH from developing in chronic hypoxic mice and reverse established PAH in the 5-HTT+ model of PAH. Therefore KCNQ channels may be a potential therapeutic target in the treatment of PAH. Flupirtine has been shown to act on the systemic circuit attenuating MAP in rats (Mackie *et. al.,* 2008), however it had no effect on MAP in rabbits (Yoro *et. al.,* 2008) and despite published findings in rats, others found no effect of retigabine on systemic pressure in this species (Joshi *et. al.,* 2008(submitted)). Overall, drugs that activate KCNQ channels may be of benefit in the treatment of PAH.

Chapter 6

General discussion

6.1 General discussion

Elevated levels of circulating 5-HT have been reported under several conditions that lead to the development of PAH (MacLean, 2000), such as airway hypoxia and appetite suppressant induced PAH. Plasma 5-HT is increased in patients with PAH (Herve *et. al.,* 1995). Secretion of large amounts of 5-HT from neuroendocrine cells and neuroepithelial bodies within the lung occurs in response to airway hypoxia and increased local 5-HT may contribute to secondary PAH (Johnson & Georgieff, 1989).

It has been shown that 5-HT induces a vasoconstrictor effect in human and other animal PAs, and in PAH this vasoconstriction is more intense (Wanstall & O'Donnell, 1990; MacLean & Morecroft, 2001). This was also observed the present study, with increased vasoconstrictor responses to 5-HT seen in IPA vessels from chronic hypoxic mice. However, this was not observed in vessels from the 5-HTT+ mouse model of PAH. Mice over-expressing the 5-HTT had a reduced sensitivity to 5-HT-induced contraction relative to control vessels. 5-HTT over-expression in PASMCs is a characteristic of human PAH, and studies indicate that 5-HT internalisation by PASMCs plays a prominent role in the pathogenesis of human PAH (Eddahibi *et. al.,* 1999).

Sustained pulmonary vasoconstriction contributes to the elevated pulmonary vascular resistance observed in PAH, and therefore understanding the precise mechanisms of contraction is important in identifying potential therapeutic targets. Vasoconstriction occurs in PAs due to an increase in $[Ca^{2+}]$ within the PASMC cytoplasm. This rise in $[Ca^{2+}]_i$ sets in motion a series of events which results in cross-bridge formation between the myosin heads and the actin filaments and hence, SMC contraction. The present study investigated what brought about this increase in $[Ca^{2+}]_i$ to cause vasoconstriction in WT and 5-HTT+ mice IPA. It was important to first characterise the physiological IPA vasoconstriction in normal mice and compare this to the PAH model to identify potential differences that may occur with the disease. The results show that vasoconstriction of normal mouse IPA vessels in response to 5-HT involves both influx of Ca^{2+} from the extracellular space and release from the SR, as well as rho kinase activity. Ca^{2+} influx in response to 5-HT enters the cell via both VOCC channels and a separate non voltage dependent Ca^{2+} entry pathway. Similar results have been described

in canine IPA; where removal of extracellular Ca^{2+} inhibited the response to 5-HT, SOCC inhibition reduced the 5-HT response and VOCC channel inhibition also partially inhibited the 5-HT response (Wilson *et. al.,* 2005). A critical pathway in the 5-HT induced increase in $[Ca^{2+}]_i$ in canine IPA is the release of Ca^{2+} from the SR, via IP_3 receptor activation (Wilson *et. al.,* 2005). In the present study however, mouse IPA contraction in response to 5-HT involves a partial influence of the sacroplasmic reticulum IP_3 receptor Ca^{2+} release pathway, but Ca^{2+} release from this pathway alone was not enough to induce contraction of mouse IPA. Extracellular Ca^{2+} is necessary for contraction in response to 5-HT in other animals; for example in rat IPA, as removal of extracellular Ca^{2+} inhibited 5-HT induced contraction (Rodat-Despoix *et. al.,* 2008). In rat IPA, the influx of Ca^{2+} through VOCC contributes to Ca^{2+} entry, however, it is not responsible for the entire increase in $[Ca^{2+}]_i$ (Rodat-Despoix *et. al.,* 2008), like the findings in this project for mouse IPA. Another similarity with rat IPA was that in mouse IPA, rho kinase played a major role in the development of vascular tone, which was the case under both physiological and pathological conditions. The involvement of intracellular components in generating a greater response to Ca^{2+} within the PASMC cytoplasm may indicate why Ca^{2+} channel inhibitors alone are not enough to reverse the increase in vascular resistance caused by 5-HT. Ca^{2+} entry via a pathway independent of VOCC, in response to 5-HT application in WT mice, occurred at higher concentrations of 5-HT. However, in 5-HTT+ mice IPAs, this pathway was active at lower concentrations of 5-HT also. Further studies would be needed to identify the precise nature of the pathway involved in the WT and 5-HTT+ mice, as there are a large number of possible candidates, including the large transient receptor potential channel (TRP) superfamily (Snetkov *et. al.,* 2003), of which there are six subfamilies (Alexander *et. al.,* 2006). This highlights a potential therapeutic pathway which could be further investigated in the search for treatments for PAH.

This project investigated the role of K^+ channels in the regulation of resting tone in WT and the 5-HTT over-expression model of PAH. Certain studies in the literature suggest an important role for K_V channels in the development of PAH (Remillard *et. al.,* 2007; Yuan *et. al.,* 1998), however in the present study K_V channels did not have a major influence on resting tone in WT or 5-HTT+ mice IPAs. The K_{Ca} and K_V channel blockers TEA and 4-AP failed to induce contraction of mouse IPA until high concentrations, where they are likely to have non-specific effects. 4-AP at 1 mM, 5

mM and 10 mM has been shown to cause measurable contraction of rat PA rings (Archer *et. al.,* 1998). However, in WT and 5-HTT+ mice IPAs, 4-AP failed to contract the vessels by more than 20 % (of the response induced by 50 mM KCl) until 10 mM. Contraction of mouse PA with 10 mM 4-AP has been shown previously (Xu *et. al.,* 2008). At 10 mM, 4-AP induced a greater response in 5-HTT+ mice IPAs than in WT mice IPAs, however at this concentration, 4-AP action is not specific to the blockade of K_V channels (Doggrell *et. al.,* 1999). Therefore it cannot be confirmed that the differential response to 4-AP is due to a difference in K_V channel expression or function between the normal mouse vessels and vessels from the 5-HTT+ mouse. Externally applied TEA causes a relatively high-affinity blockade of the BK_{Ca} channel with a dissociation constant of 150 - 300 μM, and at higher concentrations of TEA (5-20 mM) it also inhibits delayed rectifier K_V channels (Brayden & Nelson, 1992; Peng *et. al.,* 1996). In this project TEA induced contraction of WT mouse IPA at ~ 35 mM, and in 5-HTT+ mice IPA at ~ 100 mM. At these concentrations the actions of TEA are non-specific. Therefore the results from this study do not support a major role for these channels in determining the resting potential and resting tone of IPAs in WT or 5-HTT+ mice. Glibenclamide and capsaicin failed to contract IPA from WT or 5-HTT+ mice, therefore the channels blocked by these agents (K_{ATP} and K_V channels respectively) do not play a major role in mediating the resting K^+ conductance in the mice IPAs. At rest, K_{ATP} channels may all be inhibited by intracellular ATP (Nelson & Quayle, 1995), therefore any potential blocking action of glibenclamide may already be being carried out by the intracellular ATP levels. Zn^{2+} only began to contract IPA vessels from WT and 5-HTT+ mice at concentrations well above that necessary to inhibit TASK K^+ channels, with initial contractions being measured at only 1 – 10 mM. This suggests that TASK K^+ currents are not the major determinants of the resting membrane potential of the SMCs in these arteries either.

The roles of K_V, K_{Ca}, TASK and KCNQ channels were also investigated in the 5-HT induced vasoconstriction response. Inhibiting K_V and K_{Ca} channels with 4-AP or TEA did not block 5-HT induced contraction of IPA vessels from WT or 5-HTT+ mice. In rat IPA, it has been suggested that 5-HT-induced contraction is mediated at least in part by K_V inhibition, because activation of the 5-HT2A receptor was shown to inhibit K_V current in rat PASMCs (Cogolludo *et. al.,* 2006). However, it has also been shown that in isolated perfused rat lungs, prior treatment with 4-AP did not reduce 5-HT induced

vasoconstriction (Belohlavkova *et. al.,* 2001). $ZnCl_2$ and linopirdine also failed to inhibit the 5-HT contractile response in WT or 5-HTT+ mice IPAs. As suggested in chapter 3, care must be taken when analysing these results, and further investigation is required to investigate if 5-HT induces contraction in these vessels via mechanism that involve the inhibition of K^+ channels. .

One subset of K^+ channels on which 5-HTT over-expression had an effect was KCNQ channels, shown by reduced responses to the KCNQ channel blocker, linopirdine, in IPA from 5-HTT+ mice. It was further shown that this was specifically an action on KCNQ channels, because flupirtine and retigabine (KCNQ channels openers) were also more potent in WT mice relative to mice over-expressing the 5-HTT. Linopirdine was acting on K^+ channels to induce contraction, and not acting as a 5-HT agonist, because its effect was not blocked by ketanserin, a 5-HT2A receptor inhibitor. In addition, linopirdine caused PASMC depolarisation and the subsequent opening of VOCCs. The latter action was shown using the drug nifedipine. Nifedipine blocks VOCCs, and when present, linopirdine failed to contract WT or 5-HTT+ mice IPA. Further evidence that linopirdine was inducing contraction via an action on K^+ channels was provided by depolarising the PASMCs using a high $[K^+]_{ext.}$ in the bathing PSS. Increasing $[K^+]_{ext.}$ reduced the driving force on K^+ efflux from PASMCs, therefore reducing the effectiveness of agents that act by altering the activity of K^+ channels. Depolarising the PASMCs with elevated $[K^+]_{ext.}$ reduced the contractile effect of linopirdine, and when vessels were exposed to an extracellular concentration of 50 mM KCl, linopirdine failed to cause contraction of the IPA vessels altogether. To clarify if the action of linopirdine was due to KCNQ channel inhibition, flupirtine and retigabine were used. Flupirtine and retigabine relaxed mice IPAs, and were more potent in WT IPAs relative to those from 5-HTT+ mice, which is consistent with results of linopirdine addition. It may be the case that KCNQ channels are downregulated in PAH, or that there activity is reduced. The proposal of KCNQ channel activity is effected in PAH was investigated further.

As flupirtine relaxed IPAs from the 5-HTT+ model of PAH, this identified KCNQ channels as a potential target for the treatment of PAH. The influence of flupirtine was investigated on pulmonary vascular haemodynamics and indices of PAH in the 5-HTT+ mouse model, which has established PAH (transgenic mice with 5-HTT over-expression

occurring selectively in SMCs spontaneously develop PAH (Guignabert *et. al.,* 2006)) and the rat hypoxic-model of PAH, which develop hypoxia induced PAH over fourteen days exposure to hypobaric oxygen (Keegan *et. al.,* 2001). Flupirtine reduced mRVP and right ventricular hypertrophy in 5-HTT+ mice and chronic hypoxic mice, an effect which was not observed in WT mice. As a KCNQ channel opener, flupirtine could have prevented the depolarisation of PASMCs and contraction of IPAs which occurs in PAH. The effects of flupirtine collectively suggest that this KCNQ channel opener inhibited and attenuated PAH, in the developing and established disease. The pharmacology of these results suggest that the KCNQ channels involved are KCNQ2-5, as retigabine and flupirtine are selective activators of these channels (Tatulian *et. al.,* 2001). Consistent with this pharmacology is expression of KCNQ channels observed in a recent study which has indicated that KCNQ4 and KCNQ5 channels are expressed in rat PASMC, with KCNQ4 expression being greater than that of KCNQ5 (Joshi *et. al.,* 2008(submitted)).

Experiments have been carried out in systemic vessels investigating the action of flupirtine, which was shown to induce concentration dependent vasodilation of rat mesenteric arteries (Mackie *et. al.,* 2008). In vivo application of flupirtine has also been shown to produce a concentration dependent decrease in mean arterial pressure and mean arterial resistance (Mackie *et. al.,* 2008). The expression of KCNQ channels in arterial vessels has also been described. In rat mesenteric artery, KCNQ1, KCNQ4 and KCNQ5 have been identified (Mackie *et. al.,* 2008). KCNQ4 and KCNQ5 expression in mouse carotid and femoral arteries has also been shown (Yeung et. al., 2007). Although KCNQ channel subunits have been identified in different tissues, homomeric/ heteromeric KCNQ channels may be expressed PAs that are different to homomeric/ heteromeric KCNQ channels expressed in other tissues. The exact composite of KCNQ channels in the pulmonary vasculature may confer different biophysical properties relative to that of KCNQ channels in the systemic vasculature, which could be targeted in the treatment of PAH. Consistent with this suggestion is the findings that linopirdine did not constrict mesenteric arteries at concentrations causing maximal constriction of PAs (Yeung & Greenwood, 2007; Mackie *et. al.,* 2008; Joshi *et. al.,* 2008(submitted)). Thus flupirtine may be effective in PAH by providing selective dilation of PAs, but its impact may be limited due to the potential systemic side effects. Nevertheless, KCNQ channels may represent a novel therapeutic target for the treatment of PAH if PAs are

shown to have a unique set of KCNQ channels or to express the channels at a sufficiently higher level than the systemic circulation to allow some selectivity of action of KCNQ opener drugs.

Overall, this study has shown an interaction between KCNQ channels and the 5-HT system in the PAH disease process. Further to this finding, KCNQ channels openers have been demonstrated to attenuate PAH in both developing and established PAH.

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