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ABSTRACT

In the pulmonary artery smooth muscle cells of rat, Kv7 channels are proposed to make an important contribution to the resting membrane potential and the regulation of intrapulmonary artery (IPA) tone. Although Kv7 channels are proposed to mediate cAMP and cGMP linked vasorelaxation; the physiological modulations of their activity in pulmonary artery are not investigated. A recent study (Chadha et al., 2012a) showed that Kv7.1 activators induced relaxation in various vascular beds. However, a later study (Tsvetkov et al., 2017) showed that this relaxation was not mediated via activation of Kv7.1 channels.

The aims of this study are to determine whether Kv7.1 proteins are expressed in rat IPA and if so, whether they are functional, and to investigate whether Kv7 channels contribute to cAMP and cGMP induced vasorelaxation. The aim is also to determine whether Kv7 channels have similar contributions in regulating conduit and small artery tone.

The effect of pharmacological agents on vessel tension was measured by using myography. Meanwhile, the expression of Kv7 protein was validated using Western blots, and both pharmacological and siRNA knockdown approaches were used to investigate Kv7 channel function.

Kv7.4 and Kv7.1 channels are identified in IPA with no detection of Kv7.5 channels. In the absence of pre-tone, Kv7.1 blocker (HMR1556) had no effect on basal tone, and application of Kv7.1 activators (RL-3 and ML277) produced marked relaxation of pre-constricted arteries, but their relaxant effects were impaired by HMR1556. Linopirdine and XE991 produced profound contraction in IPA and significantly shifted the concentration-response curve of isoprenaline and forskolin to the right without affecting the maximum response, but their effect on treprostinil induced relaxation was opposite. Pan Kv7 blockers significantly impaired the maximum response induced by glyceryl trinitrate, sodium nitroprusside and carbachol. They markedly reduced the EC50 of sildenafil without impairing the maximum response. In conduit vessels, linopirdine, sildenafil and treprostinil produced larger responses than in small vessels; however, Kv7 activators produced similar responses in different size vessels.

In summary, these data show that Kv7.1 is likely to be functionally active in IPA. Data support a major role for Kv7 in cGMP, not cAMP-mediated dilation. Treprostinil acted more like the cGMP agents than cAMP in the presence of pan Kv7 blockers.
DECLARATION

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DEDICATION

Dedicated to the memory of my great father (Fadhil Saleem Qoja), who always believed in me and encouraged me to do my PhD. Sadly, you are gone, but your eternal memory and your belief in me has made this challenging journey possible. I am always proud and lucky that you were my dad.
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### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>[Ca(^{2+})](_i)</td>
<td>Intracellular Ca(^{2+}) concentration</td>
</tr>
<tr>
<td>[Cl(^{-})](_i)</td>
<td>Intracellular Cl(^{-})-concentration</td>
</tr>
<tr>
<td>[K(^{+})](_i)</td>
<td>Intracellular K(^{+}) concentration</td>
</tr>
<tr>
<td>[Na(^{+})](_i)</td>
<td>Intracellular Na(^{+}) concentration</td>
</tr>
<tr>
<td>4-AP</td>
<td>4-aminopyridine</td>
</tr>
<tr>
<td>AC</td>
<td>Adenylyl cyclase</td>
</tr>
<tr>
<td>ANP</td>
<td>Arterial natriuretic peptide</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AVP</td>
<td>Arginine vasopressin</td>
</tr>
<tr>
<td>BK(_{Ca})</td>
<td>Large conductance calcium-dependent K(^{+}) channels</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine mono-phosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine mono-phosphate</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calcitonin gene-related peptide</td>
</tr>
<tr>
<td>CICR</td>
<td>Ca(^{2+})-induced Ca(^{2+}) release</td>
</tr>
<tr>
<td>CNP</td>
<td>C-type natriuretic peptide</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>ECs</td>
<td>Endothelial cells</td>
</tr>
<tr>
<td>EDRF</td>
<td>Endothelium-derived relaxing factor</td>
</tr>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>EPAC</td>
<td>Exchange protein activated by cAMP</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<td>ET-1</td>
<td>Endothelin-1</td>
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<td>G-protein-coupled-receptors</td>
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<td>Description</td>
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<tr>
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</tr>
<tr>
<td>GTN</td>
<td>Glyceryl trinitrate</td>
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<td>Guanosine tri-phosphate</td>
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<tr>
<td>Gβγ</td>
<td>G-protein βγ</td>
</tr>
<tr>
<td>HEK</td>
<td>Human embryonic kidney</td>
</tr>
<tr>
<td>HEPES</td>
<td>2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid</td>
</tr>
<tr>
<td>HPV</td>
<td>Hypoxic pulmonary vasoconstriction</td>
</tr>
<tr>
<td>HT</td>
<td>Hypertension</td>
</tr>
<tr>
<td>IKCa</td>
<td>Intermediate-conductance calcium-activated K⁺ channels</td>
</tr>
<tr>
<td>IKN</td>
<td>Non-inactivating current</td>
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<tr>
<td>IKs</td>
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</tr>
<tr>
<td>IP₃R</td>
<td>Inositol 1,4,5-trisphosphate receptors</td>
</tr>
<tr>
<td>IPA</td>
<td>Intra-pulmonary artery</td>
</tr>
<tr>
<td>K⁺</td>
<td>Potassium ion</td>
</tr>
<tr>
<td>K₂P</td>
<td>2-pore-domain K⁺ channel</td>
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<tr>
<td>KₘATP</td>
<td>ATP-sensitive K⁺ channels</td>
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<td>KᵣCa</td>
<td>Ca²⁺-activated K⁺ channel</td>
</tr>
<tr>
<td>KᵣIR</td>
<td>Inwardly-rectifying K⁺ channels</td>
</tr>
<tr>
<td>Kv</td>
<td>Voltage-gated K⁺ channel</td>
</tr>
<tr>
<td>MLCK</td>
<td>Myosin light chain kinase</td>
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<tr>
<td>MLCP</td>
<td>Myosin light-chain phosphatase</td>
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<td>MPA</td>
<td>Main pulmonary artery</td>
</tr>
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<td>Sodium ion</td>
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<td>Na⁺/Ca²⁺ exchanger</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>PAECs</td>
<td>Pulmonary arterial endothelial cells</td>
</tr>
<tr>
<td>PAH</td>
<td>Pulmonary arterial hypertension</td>
</tr>
<tr>
<td>PAs</td>
<td>Pulmonary arteries</td>
</tr>
<tr>
<td>PASMC</td>
<td>Pulmonary artery smooth muscle cell</td>
</tr>
<tr>
<td>PDE5</td>
<td>Phosphodiesterase-5</td>
</tr>
<tr>
<td>PE</td>
<td>Phenylephrine</td>
</tr>
<tr>
<td>PGF₂α</td>
<td>prostaglandin F₂alpha</td>
</tr>
<tr>
<td>PGI₂</td>
<td>Prostaglandin I₂</td>
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### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>PIP&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PKG</td>
<td>Protein kinase G</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>P-loop</td>
<td>Pore loop</td>
</tr>
<tr>
<td>PMCA</td>
<td>Plasma membrane Ca&lt;sup&gt;2+&lt;/sup&gt;-Mg&lt;sup&gt;2+&lt;/sup&gt;-ATPase</td>
</tr>
<tr>
<td>PSS</td>
<td>Physiological salt solution</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>RMP</td>
<td>Resting membrane potential</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>ROC</td>
<td>Receptor-operated Ca&lt;sup&gt;2+&lt;/sup&gt; channel</td>
</tr>
<tr>
<td>ROCE</td>
<td>Receptor-operated Ca&lt;sup&gt;2+&lt;/sup&gt; entry</td>
</tr>
<tr>
<td>RyR</td>
<td>Ryanodine receptor</td>
</tr>
<tr>
<td>SERCA</td>
<td>Sarcoplasmic/endoplasmic reticulum Ca&lt;sup&gt;2+&lt;/sup&gt;-ATPase</td>
</tr>
<tr>
<td>sGC</td>
<td>Soluble guanylate cyclase</td>
</tr>
<tr>
<td>SHR</td>
<td>Spontaneously hypertensive rat</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SK&lt;sub&gt;Ca&lt;/sub&gt;</td>
<td>Small conductance calcium-activated potassium channel</td>
</tr>
<tr>
<td>SMCs</td>
<td>Smooth muscle cells</td>
</tr>
<tr>
<td>SNP</td>
<td>Sodium nitroprusside</td>
</tr>
<tr>
<td>SOC</td>
<td>Store-operated Ca&lt;sup&gt;2+&lt;/sup&gt; channel</td>
</tr>
<tr>
<td>SOCE</td>
<td>Store-operated Ca&lt;sup&gt;2+&lt;/sup&gt; entry</td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
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<tr>
<td>TASK</td>
<td>TWIK-related acid-sensitive K&lt;sup&gt;+&lt;/sup&gt; channel</td>
</tr>
<tr>
<td>TEA</td>
<td>Tetraethylammonium</td>
</tr>
<tr>
<td>THIK</td>
<td>Tandem pore domain halothane inhibited K&lt;sup&gt;+&lt;/sup&gt; channel</td>
</tr>
<tr>
<td>TMDs</td>
<td>Transmembrane domains</td>
</tr>
<tr>
<td>TRAAK</td>
<td>TWIK-related arachidonic acid activated K&lt;sup&gt;+&lt;/sup&gt; channel</td>
</tr>
<tr>
<td>TREK</td>
<td>TWIK related K&lt;sup&gt;+&lt;/sup&gt; channel</td>
</tr>
<tr>
<td>TRESK</td>
<td>TWIK-related spinal cord K&lt;sup&gt;+&lt;/sup&gt; channel</td>
</tr>
<tr>
<td>TRPC6</td>
<td>Transient receptor potential 6</td>
</tr>
<tr>
<td>TWIK</td>
<td>Tandem of pore domains in a weak inward rectifier K&lt;sup&gt;+&lt;/sup&gt; channels</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>TXA2</td>
<td>Thromboxane A2</td>
</tr>
<tr>
<td>U46619</td>
<td>9,11-dideoxy-9a,11a-methanoepoxy prostaglandin F2a</td>
</tr>
<tr>
<td>VDCC</td>
<td>Voltage-dependent Ca$^{2+}$ channels</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cell</td>
</tr>
<tr>
<td>ZnPy</td>
<td>Zinc pyrithione</td>
</tr>
<tr>
<td>β-AR</td>
<td>Beta-adrenergic receptor</td>
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CHAPTER 1
GENERAL INTRODUCTION
1.1 Blood circulation in the lung

1.1.1 Structure and function of the pulmonary circulatory system

The lungs facilitate the exchange of gases between the blood and the atmosphere. The atmospheric gases enter the lungs through the trachea, which branches into the bronchi, then increasingly smaller bronchioles (Fishman, 2011; Ince et al., 2018). Blood circulation in the lungs is unique with regards to its function and volume. For instance, the lungs have 2 vascular systems, consisting of the bronchial and pulmonary vessels. The bronchial arteries provide 1% of the oxygenated cardiac output to the lungs and supply oxygenated blood to the pulmonary vessels, as well as the walls of the airways. They do not take part in gaseous exchange (Fishman, 2011; Suresh and Shimoda, 2016). The pulmonary arteries (PAs) supply deoxygenated blood to the lungs for gaseous exchange, which takes place at the alveolar capillary membranes (Burrowes et al., 2005; Townsley, 2012; Walker et al., 2015). The blood circulating in the lungs is characterised by low vascular resistance and a low intravascular arterial pressure. This low arterial pressure prevents the movement of fluid from the pulmonary vascular system into the interstitial spaces, hence allowing the operation of the right ventricle with minimal energy (Naeije and Chesler, 2012). The pulmonary arteries show an average pressure of 12-15 mmHg, which is 1/6th the pressure noted in systemic arteries. The vascular resistance is low partly because of the low intrinsic tone of pulmonary arteries (Fishman, 2011). This low tone reflects the balance between vasodilator and vasoconstrictor influences on the blood vessels (Barnes and Liu, 1995). The main pulmonary artery, originating from the right ventricle divides into the right and the left PAs, which branch further to supply blood to every lobe of the lungs. The intra-pulmonary arteries (IPAs) end in an extensive network of capillaries that offer a large surface area for optimal gaseous exchange (Burrowes et al., 2005; Townsley, 2012) (Figure 1.1). Compared to arteries in the systemic circulation, the pulmonary arteries possess thinner walls with fewer vascular smooth muscle cells (SMCs).
Figure 1.1 Blood circulation in the lung
Illustrates the pulmonary vascular tree as it passes from the heart. Inset illustrates the capillary circulation in the alveolar sac where gas exchange takes place. Pulmonary arteries are blue in colour and pulmonary veins are pink (‘Pulmonary circulation’, 2018).

The pulmonary arteries consist of 3 anatomical layers; the *tunica adventitia*, *tunica media* and *tunica intima* (Figure 1.2). The outermost layer, the *tunica adventitia*, consists of numerous cell types, such as immunomodulatory cells, fibroblasts, *vasa vasorum* endothelial cells (ECs), progenitor cells and adrenergic nerves (Rhodin, 1980; Stenmark et al., 2011). It contains dense fibro-elastic tissues that provide stability to the vascular walls and acts as a biological processing centre for retrieving, integrating, storing and releasing significant regulators of vascular wall functions (Rhodin, 1980; Stenmark et al., 2011). The middle layer, the *tunica media*, consists of many spindle-shaped, SMCs, which are arranged concentrically in layers around the circumference of the vessel to form a low-pitched helix or spiral, interspersed by collagenous fibril bundles, elastic sheets and a network of elastic fibrils. The SMCs regulate the blood vessel diameter and resist blood flow (Rhodin, 1980; Martinez-Lemus, 2012). The innermost vascular layer, the *tunica intima*, consists of a single layer of endothelial cells (ECs) on a thin connective tissue layer. The long axis of the elongated ECs is parallel to the flow of blood (Rhodin, 1980; Resnick et al., 2003).
1.1.2 Regulation of the pulmonary circulation

The low vascular tone of pulmonary arteries is maintained in part by passive factors, like gravity, vascular structure, lung volume, alveolar pressure and the mechanical effect of breathing. Active factors, such as humoral and neural agents, are important for regulating vascular tone (Barnes and Liu, 1995; Fishman, 2011; Suresh and Shimoda, 2016). The endothelium releases many important vasoactive substances, like nitric oxide (NO), prostaglandin I₂ (PGI₂), which is also known as prostacyclin, relaxing factors that lead to pulmonary vasodilation. Contracting factors include endothelin-1 (ET-1) and thromboxane A₂ (TXA₂), which lead to pulmonary vasoconstriction (Barnes and Liu, 1995; Makino et al., 2011; Suresh and Shimoda, 2016). The pulmonary blood vessels are innervated by sympathetic and sensory-motor nerve fibres (El-Bermani et al., 1982; Kummer, 2011; Rothman et al., 2015).

Tone in the pulmonary circulation is also influenced by endothelium-independent local mediators, such as the respiratory gases. In particular, pulmonary vascular tone is affected by hypoxia, which leads to vasoconstriction. This condition diverts the blood flow from less-oxygenated regions to well-oxygenated areas in the

Figure 1.2 Cross section of pulmonary artery
Cross section of the pulmonary artery illustrating the different layers of the vessel: adventitia (tunica adventitia), smooth muscle (tunica media) and endothelium (tunica intima) layers.
lung, in order to optimise the perfusion–ventilation ratio (Sylvester et al., 2012; Jernigan and Resta, 2014). This hypoxic pulmonary vasoconstriction (HPV) is exclusive to the pulmonary circulation, with a decrease in oxygen tension in systemic blood vessels leading to vasodilation (Ward and McMurtry, 2009; Fishman, 2011; Suresh and Shimoda, 2016). HPV is most pronounced in the low-resistance IPAs, as conduit vessels did not respond, or even dilated, during exposure to hypoxic conditions (Yuan et al., 1990; Weir and Archer, 1995). Hypoxia increases the cytoplasmic concentration of calcium ions (Ca$^{2+}$) in pulmonary artery smooth muscle cells (PASMCs) and leads to Ca$^{2+}$ sensitisation of the contractile apparatus (Jernigan et al., 2004; Makino et al., 2011).

1.1.3 The role of the arterial smooth muscle cells in vessel contraction

The contraction of SMCs present in the walls of PA determines the vessel diameter and the blood flow resistance of the pulmonary arterial system (Gurney et al., 2010). Hence, the smooth muscle tone plays a significant role in regulating pulmonary arterial pressure and in the distribution of blood in the lungs. Arteries are relaxed after being isolated from the lungs, but constrict when exposed to vasoactive substances or depolarising stimuli (Casteel et al., 1977a; Somlyo and Somlyo, 1994; Gurney et al., 2010). Excitation-contraction coupling in PASMCs, involves electro-mechanical and pharmaco-mechanical coupling (Somlyo and Somlyo, 1968; Casteel et al., 1977a; 1977b). Electro-mechanical coupling is the development of contraction in response to depolarisation, whereas pharmaco-mechanical coupling is usually activated by a receptor agonist and involves contraction that is independent of changes in membrane potential. In all cases, contraction of the PASMCs is induced by an increase in the intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) (Casteel et al., 1977a; Makino et al., 2011).

The low tone of resting PASMCs is aided by a steady potassium ion (K$^+$) efflux through the plasma membrane, due to K$^+$ channels that are active at the resting potential. The polarisation of the cell membranes closes voltage-gated Ca$^{2+}$ channels, which prevents extracellular Ca$^{2+}$ from entering the cytoplasm and contracting the cells (Gurney et al., 2010).
1.1.4 Endothelial-dependent relaxation mechanism of pulmonary arteries

The vascular endothelium releases numerous vasoactive chemicals, comprising both vasoconstrictor and vasorelaxant products. Some like NO and PGI\textsubscript{2}, play a significant role in controlling and maintaining vascular tone (Félétou and Vanhoutte, 1999; Fleming and Busse, 1999; Tanaka \textit{et al.}, 2004), and regulating cell proliferation (Maeda \textit{et al.}, 2000). There is evidence that impaired NO production can lead to pulmonary hypertension (PH) (Hampl and Herget, 2000). NO is generated by endothelial NO synthase (eNOS), acting on L-arginine. The activity of eNOS increases due to increasing \([\text{Ca}^{2+}]_i\) in the pulmonary arteriolar ECs (PAEC) (Wilkins \textit{et al.}, 2008; Gao, 2017), and the generated NO diffuses to the vascular SMCs, where it activates the soluble guanylate cyclase (sGC) enzyme to convert guanosine tri-phosphate (GTP) to cyclic guanosine mono-phosphate (cGMP) (Moncada and Higgs, 2006). The increase in cGMP concentration leads to a decrease in the \([\text{Ca}^{2+}]_i\) and the myofilament Ca\textsuperscript{2+} sensitivity (Moncada, 1991; Moncada and Higgs, 2006; Gao and Raj, 2006; Wilkins \textit{et al.}, 2008). An increasing \([\text{Ca}^{2+}]_i\) in PAEC also leads to the opening of Ca\textsuperscript{2+}-activated K\textsuperscript{+} (K\textsubscript{Ca}) channels, which increases the K\textsuperscript{+} efflux to the intercellular space between the SMCs and the ECs, leading to membrane hyperpolarisation in the PASMC (Edwards \textit{et al.}, 1998). Endothelium-derived NO is stimulated by different circulating vasoactive agents, like substance P, adenosine triphosphate (ATP), bradykinin and acetylcholine, which bind to G-protein-coupled-receptors (GPCRs) on the endothelial cell surface and cause an increase in the endothelial \([\text{Ca}^{2+}]_i\) (Boulanger and Vanhoutte, 1997; Gao, 2017). For example, activation of muscarinic (M) receptors (M1, M3 or M5) activates the G\textsubscript{q} protein-coupled phospholipase C (PLC), which stimulates the production of inositol 1,4,5-trisphosphate (IP\textsubscript{3}) and diacylglycerol (DAG) (Harvey, 2012). The IP\textsubscript{3} molecules act on IP\textsubscript{3} receptors (IP\textsubscript{3}R) to increase the release of Ca\textsuperscript{2+} from the endoplasmic reticulum (ER) of endothelial cells. Carbachol is a widely used the endothelium-dependent vasodilator that acts by stimulating muscarinic receptors (Bolton \textit{et al.}, 1984; Bolton and Clapp, 1986; Sato \textit{et al.}, 1990). The carbachol-induced relaxation of pulmonary arteries can be attributed entirely to the production of NO (Murata \textit{et al.}, 2002).

PGI\textsubscript{2} is the main vasodilator prostanoid in the pulmonary vasculature and is synthesised by the cyclooxygenase (COX) enzyme, mainly in the ECs (Brannon \textit{et al.},
PGI₂ production in the ECs is important for maintaining the low resistance condition of the pulmonary microcirculatory system (Miller, 2006). COX is the rate-limiting enzyme in PGI₂ production (Fitzpatrick and Soberman, 2001). Thus, COX inhibition increased the pulmonary arterial pressure in many species (Tan et al., 1997). Ataya and Alnuaimat (2016) also observed that the administration of PGI₂ analogues helped in treating pulmonary arterial hypertension (PAH). Some researchers suggested that the hypoxia can stimulate the release of PGI₂ molecules from the ECs (Fredricks et al., 1994). The phospholipase A₂ enzyme, which helps in the release of the arachidonic acid from the cellular membrane, is activated by Ca²⁺ and starts PGI₂ synthesis (Majed and Khalil, 2012). As well as causing vasodilation, EC-derived PGI₂ helps to regulate EC function and maintaining the endothelium health (Zardi et al., 2005). The PGI₂ system can act synergistically with the NO pathway (Gambone et al., 1997; Zellers et al., 2000; Niwano et al., 2003; Majed and Khalil, 2012), thereby improving relaxation of the pulmonary arteries (Zellers et al., 2000).

The vasodilation induced by PGI₂ is mediated after it binds to the IP receptors and nuclear peroxisome proliferator-activated receptors (PPARs) (Li et al., 2012). The binding of PGI₂ to the IP receptors leads to vasodilatation due to the activation of the G-protein-coupled adenylyl cyclase (AC), which increases the intracellular cyclic adenosine mono-phosphate (cAMP) concentration (Narumiya et al., 1999; Majed and Khalil, 2012; Clapp and Gurung, 2015). PGI₂ has been suggested to activate K⁺ channels, including ATP-sensitive K⁺ channels (K_ATP) (Dumas et al., 1997), small conductance SKCa (Dong et al., 1998), voltage-gated (Kᵥ) (Dong et al., 1998), large-conductance Ca²⁺-dependent K⁺ channel (BKCa) (Tanaka et al., 2004), 2-pore-domain K⁺ channels (K₂P) (Olschewski et al., 2006) and the inward-rectifier K⁺ channel (KᵢR) (Orie et al., 2006). These effects may occur in a cAMP-dependent or independent manner and bring about vascular SMC (VSMC) membrane hyperpolarisation, a decrease in Ca²⁺ influx through the L-type voltage-gated Ca²⁺ channels and consequently VSMC relaxation (Niwano et al., 2003; Tanaka et al., 2004; Orie et al., 2006). Also, the binding of the PGI₂ to the nuclear PPARβ/δ receptor causes vasodilatation by activating Ca²⁺-dependent K⁺ channels (Li et al., 2012).
1.1.5 Pulmonary Arterial Hypertension

Pulmonary arterial hypertension (PAH) is a rare, but severe, chronic and progressive disease that affects the functioning of the right heart and can also lead to mortality due to failure of the right heart. PAH is due to a constant increase in the pulmonary vascular resistance, owing to vasoconstriction and remodelling in the tunica adventitia, media, and intima layers of the distal pulmonary arteries, which can result in the loss of the smaller arterioles (Simonneau et al., 2004; Malenfant et al., 2013; Ayon et al., 2016). At a cellular level, PAH leads to vascular tone imbalance, inflammation, proliferation of the pulmonary arterial SMCs and ECs, the presence of thrombosis and resistance to apoptotic cell death (Simonneau et al., 2004; Malenfant et al., 2013). EC dysfunction plays a major role in the development and the progression of the vascular PAH pathology (Ranchoux et al., 2018). In a clinical setting, PAH is diagnosed by an increase in the average resting pulmonary arterial pressure (>25 mmHg) and left atrial pressure (≥15 mmHg) (Badesch et al., 2009; Lan et al., 2018). In the past few years, many researchers have striven to understand the pathophysiology of PAH and to generate novel treatment strategies (Archer et al., 2010). Currently, 5 classes of targeted drugs are available for treating PAH. These are the sGC stimulators, phosphodiesterase-5 (PDE5) inhibitors, prostacyclin receptor agonists, prostacyclin analogues and the endothelin receptor antagonists. Despite these novel drugs, PAH still has a poor prognosis (Lan et al., 2018).

Various changes in many signalling systems have been identified, which may contribute to PAH. For example, K+ channel activity and expression are reduced in PAH (Osipenko et al., 1998; Remillard et al., 2007). In one study, the researchers noted that in-vivo gene transfer of an O2-sensitive K+ channel (Kv1.5) could decrease PH and restore HPV in the chronically-hypoxic rat model of the disease (Pozeg et al., 2003). Wanstall (1996) suggested that the use of drugs that open K+ channels and act pulmonary vasodilators could help in the treatment of PAH. Morecroft et al. (2009) considered the Kv7 K+ channels as a good molecular target for designing new drugs for PAH treatment.
1.2 Pulmonary artery smooth muscle contraction

1.2.1 Calcium ion regulation

The \([\text{Ca}^{2+}]_i\) in the PASMC is vital for regulating vascular tone, pulmonary arterial constriction, migration and cell proliferation (Jernigan and Resta, 2014). Resting \([\text{Ca}^{2+}]_i\) must be maintained at <100 nM, which results in a 10,000-fold lower concentration gradient compared to the external environment, at ~1.8 mM (Hempens et al., 1992). All cells possess complex regulatory mechanisms for maintaining the high \(\text{Ca}^{2+}\) gradient between the intracellular and extracellular environments. Similar gradients exist between the cytoplasm and ER in endothelial cells or sarcoplasmic reticulum (SR) in muscle cells (Mandal et al., 2016). Many coordinating systems help in controlling the \([\text{Ca}^{2+}]_i\): (i) release of \(\text{Ca}^{2+}\) ions from the SR/ER via \(\text{Ca}^{2+}\) release channels; (ii) entry of \(\text{Ca}^{2+}\) ions through receptor-operated \(\text{Ca}^{2+}\) channels (ROC), voltage-dependent \(\text{Ca}^{2+}\) channels (VDCC) and store-operated \(\text{Ca}^{2+}\) channels (SOCs); (iii) \(\text{Ca}^{2+}\) sequestration into the SR/ER through the SR/ER \(\text{Ca}^{2+}-\text{Mg}^{2+}\) ATPase (SERCA); (iv) \(\text{Ca}^{2+}\) extrusion via the plasma membrane through the \(\text{Ca}^{2+}-\text{Mg}^{2+}\) ATPase (PMCA); (v) \(\text{Ca}^{2+}\) transport, by the \(\text{Na}^+/\text{Ca}^{2+}\) exchanger (NCX) in the plasma membrane (Zhang et al., 2005b; Zheng and Wang, 2007); and (vi) release and sequestration of \(\text{Ca}^{2+}\) ions from lysosomes and mitochondria, as illustrated in figure 1.3 (Zhang et al., 2005a; Zhang et al., 2005b; Ayon et al., 2016; Makino et al., 2011).

Mechanical stimuli, receptor stimulation and alveolar hypoxia can all lead to the release of \(\text{Ca}^{2+}\) ions from the SR and entry of \(\text{Ca}^{2+}\) ions through the plasma membrane-based \(\text{Ca}^{2+}\)-permeable channels. \(\text{Ca}^{2+}\) ions are released through \(\text{Ca}^{2+}\) permeable channels, known as ryanodine receptors (RyR) and IP$_3$R, present in the SR membrane. The RyR and the IP$_3$R consist of 3 different subtypes, which are expressed in PASMCs (Yang et al., 2005; Zhang et al., 2005a). The release of \(\text{Ca}^{2+}\) ions from SR plays a vital role in receptor-mediated vasoconstriction and HPV (Wang et al., 2001; Zheng et al., 2005; Kinnear et al., 2008; Connolly et al., 2013). There is evidence that RyR and IP$_3$R are tightly coupled to ion channels in the plasma membrane (Dahan et al., 2012). So that \(\text{Ca}^{2+}\) released from the SR can activate specific population of \(\text{K}^+\) or \(\text{Cl}^-\) channels to hyperpolarise or depolarise the membrane, respectively (Zhang et al., 2005a). The IP$_3$-dependent release of \(\text{Ca}^{2+}\) ions or \(\text{Ca}^{2+}\) influx sarcolemmal channels can activate the RyR on the SR membrane, which induces further \(\text{Ca}^{2+}\) release from the reserve. This is known as \(\text{Ca}^{2+}\)-induced \(\text{Ca}^{2+}\) release (CICR) (Wang et al., 2008; Jernigan and Resta, 2014).
and it amplifies the signal for contraction. Once SR Ca\(^{2+}\) stores have been depleted, they signal to the membrane to activate store-operated Ca\(^{2+}\) influx, in order to refill the store. This transient release of Ca\(^{2+}\) ions from the intracellular reserves is accompanied by a sustained influx of the Ca\(^{2+}\) ions from the extracellular spaces, including the voltage-dependent Ca\(^{2+}\) influx via the L- and T-type channels, and the voltage-independent Ca\(^{2+}\) ion influx through the ROCs and SOCs (Jernigan and Resta, 2014).

In the PASMCs, the L-type VDCCs are responsible for cell proliferation and contraction (Fleischmann et al., 1994; Rodman et al., 2005). The main Ca\(^{2+}\) entry route that leads to contraction is L-type Ca\(^{2+}\) channel as indicated by the powerful vasodilator effects of dihydropyridine Ca\(^{2+}\) antagonists (Clapp and Gurnery, 1991). The L-channels are activated by voltages ≥ −30 mV and are inhibited by dihydropyridine antagonists. The L-type VDCC can also be indirectly activated by non-receptor tyrosine kinases, protein kinase C (PKC), GPCRs and acute hypoxia (Post et al., 1992; Franco-Obregon et al., 2011).
and Lopez-Barneo, 1996; Wijetunge et al., 2000; Luke et al., 2012). Acute hypoxia leads to depolarisation, thus increasing the probability of opening the VDCCs and promoting contraction (Post et al., 1992, 1995).

Extracellular ligands activate GPCRs in the plasma membrane, many of which are coupled to PLC and stimulate the synthesis of the second messengers DAG and IP$_3$. DAG can open ROC, which are cation channels with varying selectivity for Ca$^{2+}$. The subsequent depolarisation and Ca$^{2+}$ influx results in increasing [Ca$^{2+}$]i (Jernigan and Resta, 2014). IP$_3$ activates the SR/ER membrane-based IP$_3$R, which stimulates the release and mobilisation of Ca$^{2+}$ ions into the cytosol. This causes Ca$^{2+}$ ion depletion in SR/ER and triggers store-operated Ca$^{2+}$ entry (SOCE) after SOC activation (Snetkov et al., 2003; Putney, 2009; Jernigan and Resta, 2014; Ayon et al., 2016). Coupling of SOCE to vasoconstriction appears to take place selectively in the intrapulmonary arteries, but not in the smaller arteries from the other vascular systems (Snetkov et al., 2003).

The transient receptor potential 6 (TRPC6) channel is thought to act as a ROC (Bergdahl et al., 2005), to mediate predominately Na$^+$ influx and depolarisation (Lemos et al., 2007; Dietrich and Gudermann, 2014). P2X receptors are another type of ROC, which are gated by ATP and purinergic receptor agonists. P2X receptors in vascular smooth muscle show high Ca$^{2+}$ permeability, so that Ca$^{2+}$ entry through these channels can directly activate contraction (Beech and Bolton, 1989). TRPV1 and TRPV4 are also cation channels that can contribute to PASMC Ca$^{2+}$ influx (Dahan et al., 2012); moreover, a functional linkage between TRPV4 and RyR, has been suggested to increase contraction and [Ca$^{2+}$]i in PASMCs (Dahan et al., 2012). The main SOC is thought to be ORAI, which is a pore-forming subunit for Ca$^{2+}$ release-activated Ca$^{2+}$ channels and forms a Ca$^{2+}$ channel in the plasma membrane when activated by STIM1 in the SR membrane, following SR depletion (Zhang et al., 2005a; Penna et al., 2008; Wang et al., 2008; Ng et al., 2010). TRPC1 may also interact with STIM1 and contribute to SOC (Ng et al., 2010; Makino et al., 2011).

Cellular Ca$^{2+}$ homeostasis is maintained by several proteins, including the PMCA, which exports cytoplasmic Ca$^{2+}$ ions using ATP hydrolysis for energy (Strehler et al., 2007). The PMCA is a plasmalemmal P-type Ca$^{2+}$-ATPase (Makino et al., 2011) that is stimulated in the presence of oxidants like matrix metalloprotease 2 and hydrogen peroxide (Mandal et al., 2003; 2016). SERCA contributes to Ca$^{2+}$ homeostasis
by pumping $Ca^{2+}$ into the SR/ER (Periasamy and Huke, 2001; Periasamy and Kalyanasundaram, 2007). Both SERCA2a and SERCA2b are functionally expressed in PASMCs (Clark et al., 2010; Evans, 2010). $Ca^{2+}$ is also removed from PASMC by NCX (Zhang et al., 2005b; 2007).

1.2.2 Mechanism of smooth muscle cell contraction

When $[Ca^{2+}]_i$ increases beyond the basal level, $Ca^{2+}$ binds to calmodulin (CaM) and forms a $Ca^{2+}$-CaM complex that activates myosin light chain kinase (MLCK), which hydrolyses ATP to phosphorylate the myosin light chains. This leads to myosin interacting with actin filaments to form cycling of cross-bridges and cause muscular contraction (Figure 1.4).

PASMC contraction depends on a balance between the activities of $Ca^{2+}$/CaM-activated MLCK and myosin light chain phosphatase (MLCP). MLCP is regulated by enzymes like PKC, tyrosine kinase and Rho kinases, which may be stimulated by acute hypoxia, GPCR ligands and mechanical stimuli. These enzymes inactivate MLCP, leading to a higher muscle contraction for a specific $[Ca^{2+}]_i$. This phenomenon occurs despite any changes in the PASMC $[Ca^{2+}]_i$ (Luke et al., 2012; Jernigan and Resta, 2014) and allows smooth muscle to generate sustained contraction with minimal energy consumption. Rho kinase directly phosphorylates and inactivates MLCP, causing smooth muscle contraction. It also indirectly inhibits MLCP by activating a MLCP-inhibitor protein, leading to $Ca^{2+}$-sensitisation of the contractile proteins (Oka et al., 2008; Makino et al., 2011).

Phosphorylated myosin light chains are de-phosphorylated by the $Ca^{2+}$-independent myosin light chain phosphatase (MLCP), causing smooth muscle relaxation (Makino et al., 2011).

1.2.3 Influence of membrane potential on smooth muscle contraction

The resting membrane potential of PASMC is a significant regulator of cellular activity and pulmonary vascular tone. The membrane potential (Em) depends on the balance of different ion channel activities in the membrane and the concentration gradients of $K^+$, $Na^+$ and $Cl^-$ ions across the cell membrane. The resting membrane potential (RMP) value of PASMC is between -60 and -40 mV because of a relatively higher permeability to the $K^+$ compared to other ions (Casteels et al., 1977b; Clapp et al., 1993; Osipenko et
The membrane of resting PASMCs is permeable mainly to K⁺ due to the leakage of K⁺ through background K⁺ channels, which remain open at rest. Small changes in K⁺ channel activity can significantly affect the membrane potential (Casteels et al., 1977b; Osipenko et al., 1997). PASMCs maintain a high intracellular K⁺ concentration (140 mM) and low extracellular concentration (~ 5 mM). The Na⁺ gradient is in the opposite direction.

**Figure 1.4 Mechanism of PASMCs contraction.**
The mechanism of contraction of a SMC in response to G-protein-coupled-receptor activation (GPCR) and an increase in the [Ca²⁺]. ROK: Rho kinase; CaM: Calmodulin; MLCK: myosin light chain kinase; MLCP: myosin light chain phosphatase. SERCA pump, SR/ER Ca²⁺-Mg²⁺ ATPase; RyR: ryanodine receptor; PLC: phospholipase C; PIP₂: phosphatidylinositol 4,5-bisphosphate; IP₃R: inositol 1,4,5-trisphosphate receptor; ROC: receptor-operated channel. The figure adapted from Makino et al. (2011).

There are a number of drugs that can activate K⁺ channels and hyperpolarise PASMC. Such drugs relax PASMCs and decrease vascular tone (Clapp et al., 1993; Wanstall, 1997). In contrast, drugs that block K⁺ channels and cause depolarisation, increase pulmonary vascular resistance (Hasunuma et al., 1991) by causing contraction of pulmonary vascular smooth muscles (Nelson and Quayle, 1995; Archer et al., 1996; Gurney, 2005). Although PASMC contract in response to depolarising stimuli, depolarisation does not evoke action potentials (Suzuki and Towarg, 1982; Gurney et
al., 2010). Instead, PASMCs respond in a graded fashion to depolarisation, with longer depolarisation activity, which results in greater Ca$^{2+}$ influx and contraction.

Since the RMP depends on the steady leakage of K$^+$, the K$^+$ channels open at rest must be always open at voltage values near the resting potential, i.e., from -60 to -50 mV (Casteels et al., 1977b; Suzuki and Towarg, 1982). The molecular nature of the K$^+$ channels that lead to the RMP in PASMCs is still subject to debate, and it appears that multiple K$^+$ channels may help in setting this Em value. These include the voltage-gated delayed rectifiers Kv1.5 and Kv2.1 (Archer et al., 2001, Remillard & Yuan, 2004; Moudgil et al., 2006; Platoshyn et al., 2006; Remillard et al., 2007). However, their pharmacological and biophysical properties differ and show different pharmacology from the properties of the resting K$^+$ conductance in that they activate at more positive potentials (Gurney et al., 2002). Voltage-independent K$^+$ channels, like the TASK (TWIK related acid-sensitive K$^+$ channel) channels are expressed in PASMC and do have properties consistent with setting the Em (Gurney et al. 2003; Olschewski et al. 2006; Gurney and Manoury, 2009). TASK channels are inhibited by hypoxia and may explain how hypoxia evokes depolarisation (Olschewski et al. 2006). More recently, voltage-gated Kv7 channels were proposed as regulators of the RMP of various vascular beds including PASMC (Joshi et al., 2006, 2009; Mani et al., 2011; Khanamiri et al., 2013). They present appropriate pharmacological and biophysical properties and also show sensitivity to hypoxia (Sedivy et al., 2014).

1.3 Pharmacology of the pulmonary arterial system

1.3.1 Vasoconstrictors: Phenylephrine and U46619 and their mechanism of action

Phenylephrine (PE) is an $\alpha_1$-adrenoceptor agonist (Hieble et al., 1995). It binds to the $\alpha_1$-receptors present on vascular SMCs and mimics the effects of sympathetic adrenergic nerve activation on blood vessels (Minneman et al., 1994). $\alpha_1$-adrenergic receptors are present on the SMCs of most vessels. Their expression varies depending on the organ and location in the vascular tree, which results in variable response to different adrenoceptor agonists (Minneman and Esbenshade, 1994). The $\alpha_1$-receptor is a G$\alpha_{q/11}$-coupled receptor, which activates smooth muscle contraction by the IP$_3$ and DAG signal transduction pathway (Minneman et al., 1994; Cotecchia, 2010; Brozovich...
et al., 2016). PE is a very strong vasoconstrictor, which can produce a maximal constrictive response in the rat IPA (Gurney and Howarth, 2009).

U46619 (9,11-dideoxy-9a,11a-methanoepoxy prostaglandin F2a) is a thromboxane (TP) receptor agonist and a powerful vasoconstrictor, which is essentially a TXA2 mimetic (Alapati et al., 2007; Ellinsworth et al., 2014). U46619 stimulates contraction via activation of voltage-independent and -dependent Ca\(^{2+}\) entry pathways (Tasaki et al., 2003). After the activation of TP receptors, the contraction induces when it is coupled with either Gaq/11 or, to a large degree, Ga12/13, which induces the biosynthesis of IP\(_3\) via coupling to PLC, and activates distinctive RhoA guanine nucleotide exchange factors that evokes the activation of Rho kinase, resulting in an increase in [Ca\(^{2+}\)]\(_i\) and enhancement of response of the contractile proteins to Ca\(^{2+}\) (Cogolludo et al., 2003; Momotani et al., 2011; Ellinsworth et al., 2014). The phosphorylation condition in the MLCP is altered by Rho kinase by increasing the phosphorylation of MLCP and causing its inactivation, thus inducing contraction (Tsai and Jiang, 2006; Ellinsworth et al., 2014). Moreover, as reported by Cogolludo et al. (2003, 2005), U46619 inactivates the Kv channel and causes a decline in the Kv current. According to Tasaki et al. (2003), contractions stimulated by U46619 are potently reliant on trans-plasmalemmal Ca\(^{2+}\) influx. The contractile responses based on U46619 include PKC and VDCC. On the other hand, as reported by Alapati et al. (2007), the contractile response of the bovine pulmonary artery induced by the U46619, has the involvement of Rho kinase along with a Cl\(^-\) sensitivity increment. However, this does not mediate via activating of VDCC, but possibly by Ca\(^{2+}\) release from the SR and Ca\(^{2+}\) influx through SOC.

### 1.3.2 Vasodilators

Endothelium-derived relaxing factors (EDRF) (i.e., NO and PGI\(_2\)) stimulate cGMP/cAMP synthesis, which further induces hyperpolarisation of the membrane potential in PASMC, causing pulmonary vasodilatation (Makino et al., 2011). The sGC-cGMP pathway was further understood with the discovery of NO as an EDRF molecule (Denninger and Marletta, 1999; Murad, 2006). NO, a gaseous nitrogen signalling molecule, maintains a low arterial pressure in pulmonary blood circulation (Mandal et al., 2016). The sGC plays an important role in the NO signalling as it converts GTP to cGMP (Schmidt et al., 1993; Dasgupta et al., 2015), which then activates various
protein kinases, opens the Ca\(^{2+}\)-gated K\(^+\) channels, reduces the [Ca\(^{2+}\)], further relaxing the SMCs (Lincoln and Cornwell, 1993). NO also induced the relaxation of the SMCs by increasing the SERCA2a glutathionylation (Adachi, 2010). The NO molecules are either produced endogenously in the pulmonary ECs or can be exogenously administered (Schmidt et al., 1993). The exogenous and endogenous NO modulates the vascular tone. NO was seen to modulate the basal pulmonary tone (Aranda et al., 1999). The NO-sGC-cGMP pathway was severely affected in the PAH, which increased the sGC expression but decreased its functionality due to the sGC oxidation, NO bioavailability, and a subsequent loss of the sGC’s haem group (Dias-Junior et al., 2008; Dasgupta et al., 2015).

NO can be exogenously administered by inhalation to produce a selective pulmonary vasodilation process (Aranda and Pearl, 1998; Aranda et al., 1999) since it diffuses from the alveoli to the pulmonary vascular SMCs. NO does not lead to systemic vasodilation as in the pulmonary bloodstream; NO gets inactivated since it binds to the haemoglobin molecules (Patterson et al., 1999). Furthermore, this inhaled NO reduces the PAH and improves the hypoxemia in animals (Pison et al., 1993) or humans (Aranda and Pearl, 1998).

Glyceryl trinitrate (GTN) is an effective vasodilator. Based on a pharmacological perspective, GTN is a prodrug, while NO is its active form (Hashimoto and Kobayashi, 2003; Lange et al., 2009). The mechanism of action used by GTN for activating the sGC is uncertain (Ignarro et al., 1981; Kollau et al., 2005). Several enzymatic reactions are involved in the GTN bioactivation (McGuire et al., 1998; Ferreira and Mochly-Rosen, 2102). Some researchers noted that the mitochondrial aldehyde dehydrogenase-2 enzyme catalysed the conversion of GTN to glyceryl dinitrate and inorganic nitrite, which activated the sGC (Ignarro, 2002; Chen et al., 2005; Beretta et al., 2008; Badejo et al., 2010). GTN is popularly used for treating angina, heart failure and relieving the pulmonary congestion symptoms (Kopman, 1979; Ignarro, 2002; Hashimoto and Kobayashi, 2003; Badejo et al., 2010). The intravenous GTN injections also decrease the systemic and pulmonary arterial pressure and increase the cardiac output (Badejo et al., 2010). Some animal studies indicated that the GTN displayed a considerable vasodilator bioactivity in the pulmonary vascular system, which further increased the vasodilator response and improved the vasoconstrictor tone (Kadowitz et al., 1981; Badejo et al., 2010).
Similar to GTN, sodium nitroprusside (SNP) releases NO, but it is an inorganic compound, (Hussain et al., 2017) that can bind to the sGC, leading to vasodilation and increasing the intracellular cGMP level (Moncada, 1997). SNP was seen to be an effective vasodilator. Like the NO inhalation, SNP acts on cGMP and causes a dose-dependent decrease in the pulmonary arterial pressure and vascular resistance, thus, improving the cardiac output (Patterson et al., 1999). SNP causes vasodilation in the systemic and pulmonary circulatory systems (Aranda et al., 1999; Freitas et al., 2012). Hence, systemic arterial hypotension was seen to be a common side effect of SNP administration (Freitas et al., 2012).

cAMP-dependent dilation:
cAMP is an important physiological secondary messenger in the vascular system and inhibits the proliferation, contraction, and the migration of SMCs (McDaniel et al., 1994; Koyama et al., 2001). β-adrenoceptors are a type of GPCR that signal through the Gαs proteins and stimulate cAMP synthesis by AC isoenzymes (Bylund et al., 1994). Isoprenaline is a non-selective β-adrenergic receptor (β-AR) agonist that stimulates the Gαs/cAMP/PKA pathway and induces vasodilation (Bylund et al., 1994; Mani et al., 2016b). However, there are studies reported that isoproterenol relaxes the vascular SMCs by causing cAMP-dependent protein kinase G (PKG) activation (Jiang et al., 1992; White et al., 2000). Protein kinase A (PKA) phosphorylates several proteins involved in the regulation of smooth muscle tone. Mani et al. (2016b) showed that stimulation of β-ARs by isoprenaline led to the PKA-dependent activation of Kv7.5 channel currents in A7r5 cells. Stott et al. (2015a) reported that isoproterenol increased the open probability of Kv7 channels in renal arterial SMCs of rat, without altering the single-channel conductance. They also found that isoprenaline- and forskolin-induced vasorelaxation was decreased in the presence of a selective Kv7 channel blocker, linopirdine [1,3-dihydro-1-phenyl-3,3-bis(4-pyridinylmethyl)-2H-indol-2-one] (Chadha et al., 2012b; Lee et al., 2015). Forskolin is a direct AC activator, which increases the intracellular cAMP concentration and causes vasorelaxation (Stief et al., 2000; Barman et al., 2003). The vasodilatory effect of cAMP-producing molecules can involve “cross-activation” of PKG by cAMP (Toyoshima et al., 1998; Hill et al., 2016). PKA can also be stimulated by cGMP (Cornwell et al., 1994).
Cross talk between the cAMP and cGMP:

Cross talk between the cAMP and cGMP pathways can also occur at the level of PDEs. There are eleven types of mammalian PDE and many are expressed in pulmonary arteries. PDEs vary in substrate specificity and some are modulated by cyclic nucleotides, giving further opportunities for cross talk. Narayanan et al. (2012) observed that in rat aorta, the forskolin-induced phosphorylation of the IP₃R was inhibited by preventing PKG activation, suggesting that the IP₃R was phosphorylated by PKG. Hill et al. (2016) also noted that BKCa channel activation by forskolin was reduced by a PKG inhibitor, suggesting the involvement of PKG-dependent pathways.

Single-channel studies confirm that the BKCa channels, in arterial smooth muscles can be opened by PKG activation, via phosphorylation of the channel (Robertson et al., 1993). PKG stimulates BKCa activity in pulmonary arteries (Archer et al., 1994), but BKCa channels were opened by PKA in several tissues (Minami et al., 1993), including vascular SMCs (Schubert et al., 1999). Barman et al. (2003) noted that cAMP-dependent vasodilators could activate BKCa channels in PASMC via PKA-independent and PKG-dependent signalling pathways, further demonstrating cross-activation between cAMP and cGMP pathways in PASMCs.

BAY41–2272 is a haem-dependent, NO-independent, sGC enzyme stimulator (Evgenov et al., 2006; Andersson, 2018) that caused a 200-fold increase in the sGC activity (Schmidt et al., 2002; Evgenov et al., 2006). BAY41-2272 did not show any selectivity towards the pulmonary vasculature but decreased blood pressure in many animal models (Dasgupta et al., 2015). BAY 41-2272 administration significantly decreased PAH in animal models (Evgenov et al., 2006). BAY41-2272 treatment also reversed the structural and hemodynamic changes that develop during monocrotaline- and chronic hypoxia-induced experimental PAH (Deruelle et al., 2006; Dumitrascu et al., 2006). Rabbit models suffering from congenital diaphragmatic hernia also showed a lower pulmonary artery pressure when administered antenatal BAY41-2272 (Vuckovic et al., 2016). In vitro studies showed that BAY41-2272 also decreased vascular SMC proliferation (Joshi et al., 2011) and migration, while causing apoptosis in PASMCs (Zhang et al., 2015). It also stimulated the neovessel formation (Pyriochou et al., 2006). Hence, BAY41-2272 has been successfully used for treating heart failure and PH (Shah et al., 2018).
Sildenafil is an effective pulmonary vasodilator, which preserves cGMP levels in the lung by preventing its breakdown and relaxes PASMC (Pauvert et al., 2003; Rich, 2006). Sildenafil also inhibited the vascular remodelling resulting from chronic exposure to hypoxia (Steiner et al., 2005). PDE-5 inhibitors reduced pulmonary arterial resistance and pulmonary arterial blood pressure, and they are approved molecules for treating PAH (Sastry et al., 2002; Wilkins et al., 2008). Not surprisingly, patients with a defective or disturbed NO pathway were found to show less beneficial effects of PDE5 inhibitors compared to people with a normal NO pathway (Andersson, 2018). Sildenafil has anti-proliferative effects on human PASMCs, which are likely to also contribute to its beneficial effects in PAH (Andersson, 2018).

Treprostinil is one of many Food-drug administration (FDA) approved prostacyclin analogues, which is clinically used for treating PAH patients. Treprostinil binds to IP receptors and reduces pulmonary arterial tone in these patients (Asaki et al., 2015). It activates AC and cAMP signalling pathway (Olschewski et al., 2006; Cunningham et al., 2016), and recently, it was reported that treprostinil relaxing action was partly due to release of NO from the endothelium (Fuchikami et al., 2017). Treprostinil shows a higher affinity for IP and EP2 receptors compared to other prostanoid receptors (Clapp et al., 2002; Aronoff et al., 2007; Whittle et al., 2012).

### 1.3.3 Differential sensitivity to vasoactive agents in the pulmonary arterial system

IPA show different responses to vasoactive stimuli, depending on their site and size in the pulmonary system. Resistance and conduit IPA show varied contractile responses to different vasoactive agents (Leach et al., 1989). The resistance vessels were seen to be less responsive to molecules like serotonin, PE, noradrenaline and ET-1 compared to the conduit blood vessels (Leach et al., 1990; 1992; Priest et al., 1997; Gurney and Howarth, 2009).

In contrast, the resistance and conduit arteries displayed a similar sensitivity to prostaglandin F2alpha (PGF2α) induced vasoconstriction (Gurney and Howarth, 2009). Carbachol-induced a larger maximum relaxation in conduit vessels compared to that in the resistance arteries, although these differently-sized vessels displayed similar sensitivity to GTN (Gurney and Howarth, 2009). Conduit and resistance arteries also showed a distinct sensitivity to K⁺-induced contraction, with resistance arteries being
unable to maintain contraction at high [K⁺] (Gurney and Howarth, 2009). Resistance IPAs constrict to response to hypoxia, while the larger conduit IPAs showed no response or even dilation (Shirai et al., 1986; Weir and Archer, 1995; Urena et al., 1996).

There may be several reasons for these differences, but a consistent finding at the cellular level is heterogeneity in K⁺ channels in small and large arteries (McCulloch et al., 2000). A longitudinal heterogeneity was noted in ion channel distribution, from conduit to resistance arteries (McCulloch et al., 2000). It was also seen that Kv1.2 and Kv2.1 channels are expressed at a high level in conduit IPA compared to the resistance vessels, whereas the Kv1.3 channel is expressed only in the resistance IPA (Patel et al., 1997). A variation was noted in the types of K⁺ current and regional K⁺ channel population, in cells isolated from conduit and resistance arteries (Albarwani et al., 1995; Archer et al., 1996). Smirnov et al. (2002) showed that resistance arteries contain a uniform population of cells with pronounced Kv currents, whereas conduit vessels contain an additional cell type with high expression of BKCa. Similar variation was noted in the response between the small IPA and conduit vessels in other animal species like sheep (Kemp et al., 1997) or rabbits (Franco-Obregon and Lopez-Barneo, 1996; Bae et al., 1999). When investigating the action of drugs on pulmonary arteries, it is therefore important to assess them in vessels from different parts of pulmonary arterial tree.

1.4 **Potassium channels**
K⁺ channels play a crucial role in regulating the RMP of different cell types in the body (Gurney et al., 2010) and are a major determinant for contractility of smooth muscle (Nelson et al., 1990). They do this by controlling K⁺ flux across the cell membrane, which generates electrical current across the membrane also in non-excitable cells (Casteel et al., 1977b). K⁺ channel activation causes membrane hyperpolarisation, while their inhibition can lead to depolarisation, VDCC activation, an increase in the [Ca²⁺], and subsequent vasoconstriction (Yuan, 1995). K⁺ channels also act as a target for different control mechanisms within the cell (Choe, 2002) and play an essential role in cell proliferation (Blackiston et al., 2009; Urrego et al., 2014). The K⁺ channel family is the largest family of ion channels; there are >70 known genes that encode the α-subunits.
of K+ channels. Many types of K+ channel can therefore be expressed in the plasma membrane (Hille, 2001; Alexander et al., 2011).

The pore in a K+ channel is mostly formed by co-assembling more than one α-subunit of the same family. The α-subunits, which can be identical or different, result in different K+ channels (homomultimers or heteromultimers) with distinct kinetics, voltage dependence and pharmacological properties (Kaczmarek, 2013). K+ channels are subject to different post-translational modification processes, like phosphorylation (Lang and Shumilina, 2013), glycosylation (Norring et al., 2013) and sumoylation (Benson et al., 2007) all of which can affect their properties. Further diversification of K+ channels results from the co-assembly of α-subunits with β-subunits, which are non-pore forming subunits (Robbins, 2001). The possibility of forming physiologically relevant channels by clustering α-subunits from different families can also increase the diversification of K+ channels (Barhanin et al., 1996; Abbott et al., 1999). Even small changes in the function of K+ channels can result in considerable neurological abnormalities and diseases (Heron et al., 2012), highlighting the importance of these channels for cell function.

1.4.1 Potassium channel architecture and expression in pulmonary artery

A typical K+ channel is composed of four pore-forming α-subunits that are clustered together to form a pore through the plasma membrane, which regulates K+ flux through it (Biggin et al., 2000; Choe, 2002). The basic unit for a K+ channel is two transmembrane domains (TMDs) with a pore loop (P-loop) between them. Channels with this structure are referred to as tandem pore domain channels (2TMD/P) (Robbins, 2001). The P-loop is responsible for K+ selectivity. Every K+ channel consists of four P-loops, which are arranged in a tetrameric fashion and selectively filter K+ (Biggin et al., 2000). K+ channels are classified into different families depending on the number of TMD and P-loops in their α-subunit. Further subfamilies depend on the amino acid sequence homology (Robbins, 2001). At least eight families of α-subunit genes have been identified, each consisting of several individual members (Coetzee et al., 1999; Robbins, 2001). Each K+ channel subfamily consists of different members and is further characterised by distinctive biophysical and pharmacological characteristics (Coetzee et
The classification for the α-subunit based on the topologies of TMD and P-loops is shown in figure 1.5.

**Figure 1.5 Topological structure of the α-subunit of the K⁺ channels**

a) and b) represent the planar membrane topology of one subunit in the voltage-gated K⁺ channels (these channels comprise of 6 TMDs, which consisted a P-loop, i.e., 6TMD/1P), c) represents the Ca²⁺-activated K⁺ channels (consisting of 7 TMDs, and a P-loop, i.e., 7TMD/1P), d) represents the 2-pore domain K⁺ channels (consisting of two 2TMD/1P channel repeats, i.e., 4TMD/2P), e) presents the structure of K₂P channels, (consisting of 2 TMDs, and a P-loop, i.e., 2TMD/1P), and are characterised by an inwardly rectifying K⁺ currents. The voltage sensors, which is present in the TM domain 4 of the Kv and BKCa channels, are indicated by plus signs and the P-loop is indicated by P. The pore-forming α-subunit of the large conductance Ca²⁺-activated K⁺ channels consisted of 2 high-affinity Ca²⁺ binding sites, known as the Ca²⁺ bowl. The information of the figure is gathered from Choe (2002) and Benarroch (2009).

### 1.4.1.1 Six-transmembrane domains and one pore loop (6TMD/1P)

This family of α-subunits is the largest of the K⁺ channel α-subunit families. It includes Kv1-4 (delayed rectifier) K⁺ channels, Kv10-12, KCa2-4 and Kv7.1-7.5 K⁺ channels (Moudgil et al., 2006; Gurney et al., 2010). Kv is the largest class of this family, and functional Kv channels are homo- and/or hetero-tetramers composed of four pore-forming α-subunits and four regulatory β-subunits (Korovkina and England, 2002; Ko et al., 2008). These Kv channels are a diverse superfamily, comprising of members that...
share \( K^+ \) selectivity and an intrinsic voltage-dependent activation (Lee \textit{et al.}, 2005). \( \text{Kv} \) channel subunits have six TMD (S1-S6) and one P-loop between S5-S6 (Jepps \textit{et al.}, 2012). The S4 TMD is the voltage-sensing region of \( \text{Kv} \) channels (Bezanilla, 2000). The S4 TMD voltage sensor consists of a conserved region made of positive Lys or Arg amino acids (Aggarwal and MacKinnon, 1996). These lead to depolarisation-dependent \( \text{Kv} \) channel opening. The COOH terminus of the protein is situated near the P-loop and frequently holds motifs for modulating the channel rather than the direct control of \( K^+ \) flow. The NH\(_2\) terminus participates in the inactivation mechanism for the channel (Robbins, 2001). When the \( \alpha\)-subunit co-assembles with the \( \beta\)-subunits, this causes changes in the characteristics of \( \text{Kv} \) channels (Bähring \textit{et al.}, 2001; Ko \textit{et al.}, 2008). A major difference between the structures of the \( \text{Kv}1-4 \) and other \( \text{Kv} \) channel families is the location of the “cytoplasmic cores”, which refers to an array of various protein domains and motifs lying on the cytoplasmic face of the protein (Barros \textit{et al.}, 2012) (Figure 1.5 a; b).

Pulmonary arterial smooth muscle cells express several genes for \( K^+ \) channels, including \( \text{Kv} \) channels and their subfamilies, along with complementary \( \beta\)-subunits (Archer \textit{et al.}, 1998; Yuan \textit{et al.}, 1998b; Gurney \textit{et al.}, 2010). In PASMCs, an assembly of various \( \alpha\)- and \( \beta\)-subunits form functional \( \text{Kv} \) channels, which generate \( \text{Kv} \) currents that display heterogeneous biophysical properties (Smirnov \textit{et al.}, 2002; Platoshyn \textit{et al.}, 2004). PASMCs express two members of the \( \text{Kv}10-12 \) channel family, i.e., \( \text{Kv}10.1 \) and \( \text{Kv}11.1 \) (Platoshyn \textit{et al.}, 2004); however, studies have not yet investigated their functional role in detail (Gurney \textit{et al.}, 2010).

1.4.1.2 7TMD/1P

The \( \text{BK}_{\text{Ca}} \) is the only member of this family of \( K^+ \) channels. The \( \text{BK}_{\text{Ca}} \) channel is characterised by a high conductance for \( K^+ \), its sensitivity to \([\text{Ca}^{2+}]_i\) and voltage, as well as its ubiquitous expression (Toro \textit{et al.}, 2014). The main difference from the \( \text{Kv} \) channel \( \alpha\)-subunit is that it has an additional S0 TMD, which is attached to the S1 TMD at the NH\(_2\) terminus (Wallner \textit{et al.}, 1996; Yang \textit{et al.}, 2015) (Figure 1.5c), located on the extracellular side (Biggin \textit{et al.}, 2000). The \( \text{BK}_{\text{Ca}} \) channel is formed by four \( \alpha\)-subunits that each consist of seven TMD (Toro \textit{et al.}, 2014). The \( \text{K}_{\text{Ca}} \) channel co-assemble with \( \beta\)-subunits (2TMD/0P) that have a regulatory role, while the \( \text{Ca}^{2+} \) sensitivity motifs in the \( \text{K}_{\text{Ca}} \) \( \alpha\)-subunit are thought to be located on its COOH terminus (Jiang \textit{et al.}, 2001; Robbins, 2001). The \( \text{BK}_{\text{Ca}} \) channel is found in PASMCs (Platoshyn
et al., 2004) and acts as a negative feedback regulator that limits vasoconstriction in response to the elevation of intracellular Ca\(^{2+}\) (Gurney et al., 2010).

1.4.1.3 2TMD/1P

This family represents the simplest architecture of K\(^+\) channel \(\alpha\)-subunits. It is composed of two TMDs and one P-loop (Fig 1.5e). Four of the 2TMD/1P cluster to form a functional K\(^+\) channel (Robbins, 2001). This family of channels is known as K\(_{IR}\), because they preferentially conduct the flux of K\(^+\) in the opposite direction to the normal physiological flux, i.e. inward (Abraham et al., 1999). This family consists of K\(_{IR}1-6\) subfamilies and it has been reported that K\(_{IR}2.1\) and K\(_{IR}2.4\) are functionally active in human PASMCs (Tennant et al., 2006). K\(_{IR}\) channels are blocked by intracellular Mg\(^{2+}\) and Ca\(^{2+}\) and external Ba\(^{2+}\) (Makino et al., 2011).

Some K\(_{IR}\) subfamilies are formed after co-assembly of the sulphonyl urea-binding subunit 2B (SUR2B) with K\(_{IR}6.1\) or K\(_{IR}6.2\) to produce two specific channel families, i.e., K\(_{NDP}\) (which is sensitive to nucleoside diphosphates) and the K\(_{ATP}\) (which is ATP-sensitive) (Beech et al., 1993; Cui et al., 2002; Gurney et al., 2010). Some researchers detected expression of the K\(_{IR}6.1\) and SUR2B in rat and human PASMCs (Clapp and Gurney, 1992; Clapp et al., 1993; Cui et al., 2002).

1.4.1.4 4TMD/2P

This family differs by having two P-loops in a single \(\alpha\)-subunit and needs only two \(\alpha\)-subunits to form a pore (Biggin et al., 2000) (Figure 1.5d). The resulting K\(_{2}\)P channels from these \(\alpha\)-subunits are widely expressed and play a key role in determining background K\(^+\) conductance for cells in which they are found. Fifteen channels belonging to this family have been identified (Lotshaw, 2007; Schmidt et al., 2014).

The name given to K\(_{2}\)P channel subfamilies originated from their functional attributes, such as TWIK (tandem of pore domains in a weak inward rectifier K\(^+\) channel) (Lesage et al., 1996), TREK (TWIK related K\(^+\) channel) (Fink et al., 1996), TASK (Duprat et al., 1997), THIK (tandem pore domain halothane inhibited K\(^+\) channel) (Rajan et al., 2001), TRAAK (TWIK-related arachidonic acid activated K\(^+\) channel) (Fink et al., 1998) and TRESK (TWIK-related spinal cord K\(^+\) channel) (Sano et al., 2003). Of the K\(_{2}\)P family, only TASK-1, TASK-2, TREK-1, TREK-2, TWIK-2 and THIK-1 have
been detected in the pulmonary vasculature (Koh et al., 2001; Gurney et al., 2002, 2003; Gardener et al., 2004).

The K$_2$P channels give rise to a non-inactivating current, called IK$_N$, which is characterised by its lack of time and voltage dependence (Gurney et al., 2003). Also known as ‘leakage’ or ‘background’ channels, they lack a voltage sensing domain and behave independently of the membrane voltage. Many environmental factors, such as the presence of protons (Reyes et al., 1998), arachidonic acid (Maingret et al., 1999), membrane stretch, hypoxia and inhalation of anaesthetics (Patel et al., 1999) affect the currents through K$_2$P channels in PASMCs.

1.4.1.5 β- or non-pore forming subunits
β-subunits, found in some K$^+$ channel families (Biggin et al., 2000), do not directly participate in forming the pore or sensing voltage (Wang et al., 1996b). They vary in the number of TMD forming the protein and in the location of their termini. Some β-subunits play an essential role in determining the activation characteristics of some K$^+$ channels (Uebele et al., 1998; Abbott et al., 1999), while others play a role in regulating channel inactivation (Rettig et al., 1994; Morales et al., 1996) or confer sensitivity to certain drugs (Inagaki et al., 1995). To date, 4 types of β-subunit (Kvβ1–4) have been identified. Out of these, PASMCs express Kvβ1, Kvβ2 and Kvβ3 (Yuan et al., 1998b).

1.4.2 Potassium channels in the regulation of PASMC membrane potential
K$^+$ channels play a role in regulating pulmonary vascular tone (Moudgil et al., 2006; Joshi et al., 2009). They also participate in vascular remodelling as they regulate apoptosis and cell proliferation (Moudgil et al., 2006; Makino et al., 2011). They assist in the maintenance of low vascular tone by polarising the cell membrane and preventing Ca$^{2+}$ influx through VDCC (Joshi et al., 2009). Small changes in K$^+$ channel activity can significantly affect the membrane potential; hence, factors targeting K$^+$ channels can modulate vascular tone (Coppock et al., 2001).

The RMP of PASMCs depends on a non-inactivating K$^+$ conductance, but there is a lack of consensus regarding the molecular nature of the underlying K$^+$ channels. This may be made of many voltage-dependent and -independent constituents (Osipenko et al., 1997; Joshi et al., 2006). Voltage-gated channels of the Kv1 and Kv2 families
(Patel et al., 1997; Archer et al., 1998; Moudgil et al., 2006; Remillard et al., 2007) have been regarded as the main mediators of the RMP but this is disputed by many studies (Osipenko et al., 1998; Gurney and Manoury, 2009). Kv channels were considered as major regulators of the RMP and basal vascular tone, because 4-aminopyridine (4-AP), which inhibits Kv channels induced membrane depolarisation and increased the \([\text{Ca}^{2+}]\) (Sweeney and Yuan, 2000; Platoshyn et al., 2000), however, the effects of 4-AP on RMP and tension occurred at higher concentrations than required to block Kv currents (Evans et al., 1996; Osipenko et al., 1997; Gurney and Manoury, 2009). Numerous researchers investigated the role played by the Kv1.5 and Kv2.1 subunits in the pulmonary arterial system (Moudgil et al., 2006; Remillard et al., 2007). These subunits were seen to set the RMP, so that any loss in the activity could depolarise the SMCs, which induced vasoconstriction and PAH (Archer et al., 2004; Moudgil et al., 2006; Remillard et al., 2007). In one study, Russell et al. (1994) observed that the Kv1.2/Kv1.5 subunits were conducting delayed rectifier K\(^+\) current, which was 4-AP-sensitive. In another study, the researchers investigated the role played by the Kv channels in the establishment of membrane potential by testing the depolarising activity of the anti-Kv1.5 and anti-Kv2.1 antibodies in the resistance PASMCs. It resulted in vasoconstriction, which was similar to the effect of 4-AP, (Archer et al., 2004). Also, in the case of PAH (experimental or human), the loss of Kv1.5 activity led to membrane depolarisation (Yuan et al., 1998a; Moudgil et al., 2006). However, Gurney et al. (2010) stated that this activity did not fit their biophysical properties and many other studies provided evidence against this activity (Osipenko et al., 1998; Archer et al., 2001; Gurney and Manoury, 2009). Channels of Kv1-4 families open within a few milliseconds of membrane depolarisation so respond to and prevent the membrane excitation (Gurney et al., 2010). Gurney et al. (2010) clarified that losing of the Kv channel expression could make the SMCs very excitable, by making them able to sustain membrane depolarisation and maintain the \([\text{Ca}^{2+}]\) entry into the SMCs through the VDCC. Thus, our laboratory has concluded that 4-AP-sensitive Kv channels are unlikely to be a major contributor to RMP and low vascular tone.

It was reported that PASMCs possess hypoxia-inhibited delayed rectifier current, which is not present in the systemic vascular SMCs (Post et al., 1992, 1995; Yuan et al., 1993, 1995), and which could be generated by one or many Kv channels,
like the Kv1.2, Kv1.5, Kv2.1, Kv3.1, and/or Kv9.3 (Archer et al., 1998). An inhibition of the O$_2$-sensitive Kv channels could initiate the HPV (Weir et al., 2005). Several studies indicated that Kv1.5 could be the likely subunit that generated the O$_2$-sensitive Kv currents in the PASMCs (Patel et al., 1997; Archer et al., 1998, 2001). Furthermore, many researchers also stated that the expression and activity of these channels significantly decreased in the hypoxic PAH rats, while the Kv1.5 expression was reduced in idiopathic PAH patients (Yuan et al., 1998a). Loss of the PASMC Kv1.5 and Kv2.1 channels contributed to the PAH pathogenesis as it caused sustained depolarisation that increased the [Ca$^{2+}$]$_i$ and K$^+$ concentration, which further stimulated the cell proliferation and inhibited apoptosis, respectively (Moudgil et al., 2006).

The Kv2.1 subunit in the pulmonary arterial system could exist as a complex with the electrically-silent Kv9.3 subunits. This could shift the threshold for activating the membrane potential nearer to −50 mV (Patel et al., 1997; Hulme et al., 1999). This Kv2.1/Kv9.3 heteromer could cause the O$_2$-sensitive K$^+$ currents in the PASMCs (Moudgil et al., 2006). However, like the Kv1.5, the Kv2.1/Kv9.3 complex would also be closed at the RMP. Gurney et al. (2010) explained that the voltage-dependent channels, having a low activation threshold, compared to the Kv1.5 or Kv2.1 subunits were the major factors responsible for the IK$_N$ and RMP in the PASMCs.

The BK$_{Ca}$ channels are expressed in SMCs and are activated by increasing [Ca$^{2+}$]$_i$ and membrane depolarisation (Korovkina and England, 2002; Lu et al., 2006). These channels remain closed at the RMP during physiological conditions (Gurney et al., 2010; Ayon et al., 2016). An inhibition of the Ca$^{2+}$ ion influx decreases the BK$_{Ca}$ current and increases the IK$_V$, which shows that the [Ca$^{2+}$]$_i$ regulated the BK$_{Ca}$ channels (Cox and Rusch, 2002). The voltage and the Ca$^{2+}$ gating in the BK$_{Ca}$ channels were synergistic; hence, it was concluded that the BK$_{Ca}$ channels led to several coupling changes in the [Ca$^{2+}$]$_i$, which further altered the membrane potential (Makino et al., 2011). Furthermore, the BK$_{Ca}$ channels could be regulated by the EDRF and endothelium-derived hyperpolarizing factor, which indicated that the BK$_{Ca}$ channels played a significant role in the endothelium-dependent vasorelaxation process (Zhao et al., 1997; Kandilci et al., 2008). Researchers observed that acute hypoxia also led to an inhibition of the BK$_{Ca}$ channel’s splice variant (McCartney et al., 2005). Chronic hypoxia-induced pulmonary hypertensive rats showed an increase in their BK$_{Ca}$ expression (Resnik et al., 2006). This was proposed to be a protective mechanism which
counteracted the vasoconstriction observed in the PAH. Some studies also indicated that the BK\textsubscript{Ca} channels in the arterial SMCs could be opened by the PKG activation, which phosphorylated the BK\textsubscript{Ca} channels (Robertson \textit{et al.}, 1993). It was shown that the PKG stimulated the BK\textsubscript{Ca} channel activity in the pulmonary arterial system and NO caused vasodilation through the PKG-mediated BK\textsubscript{Ca} channel activation (Archer \textit{et al.}, 1994), while the PKA opened the BK\textsubscript{Ca} channels (Minami \textit{et al.}, 1993) in many tissues like the vascular SMCs (Schubert \textit{et al.}, 1999). In the PASMCs, NO was caused vasodilation through the PKG-mediated BK\textsubscript{Ca} channel activation (Archer \textit{et al.}, 1994; Peng \textit{et al.}, 1996; Saqueton \textit{et al.}, 1999).

The K\textsubscript{IR} channels, expressed in the PASMCs and PAECs, were believed to prevent the membrane hyperpolarisation, set the RMP, mediate the K\textsuperscript{+}-induced vasodilation and also decrease the loss of the intracellular K\textsuperscript{+} (Tennant \textit{et al.}, 2006; Makino \textit{et al.}, 2011). K\textsubscript{ATP} channels are also expressed in PASMC, but these channels are closed at the RMP under normal physiological conditions (Robertson \textit{et al.}, 1992; Gurney \textit{et al.}, 2010). Consequently, they do not contribute to the RMP and blocking the channels does not evoke depolarisation or vasoconstriction. However, activation of K\textsubscript{ATP} channels evokes vasodilation of PA, by causing PASMC hyperpolarisation (Clapp \textit{et al.}, 1993; Gurney \textit{et al.}, 2002). This can occur as a consequence of metabolic changes or agents that open K\textsubscript{ATP} (Clapp \textit{et al.}, 1993).

The K\textsubscript{2P} family channels activate at all membrane potentials and are generally referred as “leak” or “background” channels. They are well suited to regulating the RMP. PASMCs express several K\textsubscript{2P} channels and there is strong evidence that TASK channels make a major contribution to the RMP (Gurney \textit{et al.}, 2003; Olschewski \textit{et al.}, 2006; Gurney and Manoury, 2009).

TASK-1 channels were shown to mediate the non-inactivating, background K\textsuperscript{+} current that contributes to the RMP in PASMC (Gurney \textit{et al.}, 2003). Their involvement was demonstrated by siRNA-inhibition of TASK-1 expression and by the sensitivity of the non-inactivating current and RMP to pH and anandamide (Gurney \textit{et al.}, 2003; Olschewski \textit{et al.}, 2006). Acid pH evoked depolarisation of both rat and human PASMCs (Gardener \textit{et al.}, 2004). The TASK-1 channel is of a particular interest since this molecule was detectable at the transcription and protein levels in PASMCs of many species (Ayon \textit{et al.}, 2016), including rabbits (Gurney \textit{et al.}, 2003), rats (Gardener \textit{et al.}, 2004; Manoury \textit{et al.}, 2011) and humans (Olschewski \textit{et al.}, 2006). The activation
of TASK-1 channels may be responsible for the hyperpolarising effect of molecules like prostacyclin (Olschewski et al., 2006) and β-adrenoceptor agonist (Bieger et al., 2006).

1.4.3 Differential potassium channel expression in the pulmonary arteries

Arteries from various species or even within the same species express differential ion channel types and densities (Archer, 1996; McCulloch et al., 2000; Smirnov et al., 2002). The α- and β-subunits of Kv channels were seen to be expressed in higher abundance in resistance pulmonary arteries, in comparison to conduit arteries (Archer et al., 1996; Coppock and Tamkun, 2001; Bonnet and Archer, 2007). A single segment of artery was also found to possess different types of SMC (Archer et al., 1996). Differences were identified between conduit and resistance PASMCs in the distribution of BKCa and Kv channels (Smirnov et al., 2002; Archer et al., 2004; Bonnet and Archer, 2007). Electrophysiological studies indicated that resistance PASMCs possess a homogeneous Kv current, while the conduit PASMCs possess a mixture of the Kv and BKCa currents, with BKCa channels generating the majority of the whole-cell current (Hogg et al., 2002). These BKCa channels can be inhibited by tetraethylammonium (TEA); however, 4-AP shows no inhibitory activity against these channels. On the other hand, smaller PASMCs are present in the resistance arteries and possess a higher density of the 4-AP-sensitive Kv channels (Smirnov et al., 2002; Archer et al., 2004). In a separate study conduit and resistance PAs were proposed to both have cells expressing TEA and 4-AP-sensitive current, interpreted as indicating the activities of BKCa and Kv channels (Archer et al., 1996; Moudgil et al., 2006). As TEA ions inhibit some Kv channels as well as BKCa (McCulloch et al., 2000), these results could be explained entirely by Kv channels. In accordance with these results, the small pulmonary arteries contract rapidly in response to 4-AP, but not inhibitors of BKCa (Moudgil et al., 2006). Resistance arteries were found to be highly enriched with 4-AP-sensitive Kv1.5 (Archer et al., 2004; Park et al., 2010).

1.5 Vascular Kv7 channels

Recently, researchers discovered that vascular muscle expresses members of the Kv7 channel family. Every member of this family displays a characteristic function and tissue distribution pattern (Abbott, 2015). Recently, Kv7 channels were also seen to play a vital role in the vascular system (Ohya et al., 2003; Joshi et al., 2006; Yeung et
al., 2007; Mackie et al., 2008), uterine system (McCallum et al., 2009), SMCs and the pulmonary epithelial cells (Greenwood et al., 2009), in various animal species, including humans. There are five α-subunits, Kv7.1–5, which are encoded by the KCNQ 1–5 genes (Robbins, 2001). These α-subunits are either arranged as four homomers or a heteromeric mix to form a functional channel (Jentsch, 2000; Schwake et al., 2003; Greene and Hoshi, 2017). Heteromers form in a few specific combinations; Kv7.3 with Kv7.2 (Wang et al., 1998; Bal et al., 2008); Kv7.5 with Kv7.3 or Kv7.4 (Lerche et al., 2000; Schroeder et al., 2000); and Kv7.1 with Kv7.5 (Oliveras et al., 2014). The heteromerisation of Kv7 isoforms influence the kinetics of K⁺ currents. Complexity is increased by the presence of KCNE regulatory (β) subunits (Abbott and Goldstein, 2001; Schwake et al., 2006). The non-pore forming KCNE1-5 subunits affect ion selectivity, membrane trafficking, channel gating, conductance, regulation and pharmacology (Roura-Ferrer et al., 2009; Abbott, 2015).

There is evidence K⁺ channels from the Kv7 family contribute to the RMP of VSMCs and the regulation of vascular tone (Ohya et al., 2003; Mackie et al., 2008; Joshi et al., 2006, 2009; Ng et al., 2011). Drugs that block Kv7 channels constrict PAs, while drugs that activate them are vasodilators (Joshi et al., 2006, 2009).

In terms of structure, Kv7 channels differ from the other Kv channels in having a long intracellular C-terminus domain (Delmas and Brown, 2005), while possessing a distinctive and conserved A-domain specific to different subunits (Schwake et al., 2003). The C-terminus plays a vital role in channel gating, trafficking, assembly and scaffolding with signalling proteins (Haitin and Attali, 2008). Furthermore, cytosolic regulatory molecules and various accessory proteins (CaM and PIP₂) can interact with the C-terminal region (Soldovieri et al., 2011). Binding of CaM to the C-terminus of Kv7 channels leads to the development of functional channels (Wen and Levitan, 2002) and modulates their trafficking from the ER to the cell membrane (Etxieberria et al., 2008). Soldovieri et al. (2011) showed that CaM plays a vital role in the maturation of Kv7.2/3 channels and plasma membrane expression. The effects of CaM are, however, subunit specific. CaM interacts with low-affinity binding sites and functions as a Ca²⁺ sensor to decrease the currents generated by Kv7.2, Kv7.4 and Kv7.5 channels, but not by Kv7.1 and Kv7.3 channels (Gamper et al., 2005). Additionally, it causes the suppression of neuronal M currents, produced by Kv7.2/7.3 hetero-multimers (Gamper et al., 2005). PIP₂ also affects the open probability of Kv7 channels, stabilising the
channels in their open state (Delmas and Brown, 2005; Povstyan et al., 2017). In a recent study, researchers noted that the presence of both PIP$_2$ and the βγ subunits of G proteins was necessary for the effective functioning of Kv7.4 channels (Povstyan et al., 2017). Furthermore, the CaM-binding site overlaps with the PIP$_2$-binding site, so the two proteins could functionally cooperate, resulting in complex regulation of channel properties (Haitin and Attali, 2008).

### 1.5.1 Biophysical and molecular properties of the Kv7 channels

Kv7 channels possess unique biophysical characteristics, including a negative voltage activation threshold (between –60 and –80 mV), which is near the RMP of PASMC. Importantly, the Kv7.1, Kv7.2, Kv7.3, Kv7.2/7.3 and Kv7.5 channels are all 4-AP-insensitive (Kubisch, 1999; Robbins, 2001; Mackie and Byron, 2008; Khammy et al., 2018), which is consistent with the low sensitivity of the PASMC RMP to 4-AP.

Another property of Kv7 channels that is consistent with a role in controlling RMP is their slow and incomplete inactivation upon membrane depolarisation (Robbins, 2001; Mackie and Byron, 2008; Gurney et al., 2010; Soldovieri et al., 2011). They would, therefore, contribute to IK$_N$ recorded from PASMC (Evans et al., 1996; Osipenko et al., 1997), and responsible for the RMP. While a portion of IK$_N$ is mediated by TASK channels (Gurney et al., 2003), the remaining voltage-dependent current could be mediated by Kv7 channels.

In our laboratory, Joshi et al. (2009) observed that IK$_N$ was inhibited by Kv7 channel blocker linopirdine, but it did not affect the delayed rectifier current. This suggests a selective action on the slow-activating, non-inactivating background K$^+$ conductance that sets the RMP in PASMCs (Joshi et al., 2009). The pan Kv7 channel blockers, linopirdine and XE991 [10,10-bis(4-pyridinylmethyl)-9(10H)-anthracenone], caused maximal depolarisation at a concentration of 10 μM and 1 μM, respectively, similar to the concentrations that blocked M-currents in rat neurones (Lamas et al., 1997; Schnee and Brown, 1998) and heterologously expressed Kv7 channels. However, these concentrations are lower than needed to inhibit a range of other K$^+$ channels (Wladyka and Kunze, 2006). At their maximal concentrations, linopirdine and XE991 led to a ≈40% decrease in IK$_N$ amplitude and the membrane potential (Joshi et al., 2009). This was likely because multiple channels mediate IK$_N$, as reflected in different voltage-dependent and voltage-independent components (Gurney et al., 2003; Gurney
and Joshi, 2006). Gurney et al. (2003) noted that TASK-1 channel inhibition led to a ≈50% decrease in IK$_N$ and membrane potential. Thus TASK-1 and Kv7 channels could work together to regulate the RMP and PASMCs.

Similar to the neuronal M-currents, Kv7 currents recorded for VSMC may be suppressed by the activation of specific GPCRs. Studies on the A7r5 cell line, which is a cultured, aortic rat SMC cell line, found that the vasoconstrictor hormone arginine vasopressin (AVP) suppressed Kv7.5 currents via a PKC-dependent pathway (Brueggemann et al., 2007). Kv7.4 currents were suppressed only if the channels were formed by a complex with Kv7.5 subunits. The complete inhibition of Kv7.5 currents by AVP caused membrane depolarisation and led to action potential firing in A7r5 cells (Brueggemann et al., 2007; Mani et al., 2009). Physiological concentrations of AVP also led to a PKC-dependent suppression of Kv7 currents in mesenteric artery SMCs, and this led to constriction of pressurised mesenteric arteries (Mackie et al., 2008). In addition, the Kv7 channel activators could relax mesenteric arteries that were pre-constricted with AVP, both in-vitro and in-vivo (Mackie et al., 2008; Brueggemann and Byron, 2012).

In another study, Jepps et al. (2011) observed that Kv7.4 channels were mainly responsible for the Kv7 currents that helped in maintaining the SMC RMP and vascular tone of mesenteric arteries and aorta. They found that the ability of Kv7 modulators to modify currents was significantly impaired in SMC isolated from the vessels of hypertensive rats, which showed a ~50% lower Kv7.4 protein expression compared to vessels from normotensive rats (Jepps et al., 2011). Chadha et al. (2012b) proposed that Kv7.4 channels are the major contributor to Kv7 channel-mediated relaxation because knocking down Kv7.4 expression will reduce the currents recorded from SMCs and the response to Kv7 modulators. These studies are consistent with the observations of Joshi et al. (2009), who suggested that the KCNQ4-encoded channels could contribute significantly to the Kv7 currents in PASMCs and the regulation of pulmonary vascular tone.

### 1.5.2 Expression of the Kv7 channel in the vasculature

Initially, the expression of Kv7 channels was believed to be limited to only cardiac myocytes and neurons, but KCNQ gene expression is now known to be ubiquitous (Brown, 2008; Mackie and Byron, 2008). Ohya et al. (2003) provided the first evidence
of the existence of Kv7 channels in VSMCs, noting the expression of KCNQ1 transcripts in murine portal vein myocytes. Later, Kv7.4 and Kv7.5 channels were also found to be expressed in the same myocytes (Yeung et al., 2008).

Several studies have described the expression of KCNQ channel genes in different vascular systems, including carotid, mesenteric, femoral, cerebral and pulmonary arteries as well as the aorta of different species. Studies showing the expression of KCNQ and KCNE genes in vascular tissue are summarised in table 1.1. All systems showed high expression of KCNQ1, KCNQ4 and KCNQ5 mRNA transcripts, while KCNQ2 and 3 transcripts showed lower or no expression (Brueggemann et al., 2007; Yeung et al., 2007; Mackie et al., 2008; Joshi et al., 2009; Chadha et al., 2012b; Jepps et al., 2015; Lee et al., 2015; Haick and Byron, 2016). There is evidence that Kv7.1, Kv7.4 and Kv7.5 subunits may form functional channels in artery SMC, but recent studies suggest that Kv7.4/7.5 heteromers are the dominant molecule present in mesenteric and cerebral arteries (Brueggemann et al., 2013, 2014; Chadha et al., 2014). Rat and human mesenteric arteries showed similar Kv7 channel expression suggesting that rodents provide a good model for studying Kv7 channel function and expression in the regulation of vascular contractility in humans (Ng et al., 2011).

Kv7.1 channels may also be functionally expressed in arteries. Kv7.1 channel blockers, like chromanol 293B [trans-N-[6-Cyano-3,4-dihydro-3-hydroxy-2,2-dimethyl-2H-1-benzopyran-4-yl]-N-methyl-ethanesulfonamide] and L-768,673, were unable to constrict human or rodent arteries (Yeung et al., 2007; Mackie et al., 2008; Joshi et al., 2009; Ng et al., 2011; Chadha et al., 2012a). Thus, Kv7.1 channels are unlikely to contribute to SMC RMP and maintain low resting tone. However, a selective Kv7.1 channel activator induced vasodilation of pre-constricted arteries, showing that these channels could function in VSMCs under certain conditions (Chadha et al., 2012a). While these studies mainly employed systemic arteries, there is preliminary evidence that Kv7.1 channels may play a role in pulmonary arteries (Chadha et al., 2012a). Some researchers reported that the Kv7.1 and Kv7.5 channels co-assemble in skeletal and SMCs (Brown and Passmore, 2009; Oliveras et al., 2014), though the Kv7.1/Kv7.5 heteromeric channels were seen to be retained in the ER (Oliveras et al., 2014).
1.5.3 **Kv7 channel function in pulmonary arteries**

Joshi *et al.* (2006) investigated the role played by the vascular Kv7 channels in the pulmonary arterial system, by determining the concentration-dependent constriction of rat intrapulmonary arteries by Kv7 channel blockers. They showed that the drugs contracted PASMC independently of neuronal or endothelial interference (Joshi *et al.*, 2006). The contraction was shown to depend on the activation of the voltage-sensitive Ca\(^{2+}\) channels (Joshi *et al.*, 2006), due to membrane depolarisation in the pulmonary arterial myocytes (Joshi *et al.*, 2009). As shown in Table 1.1, rat pulmonary arteries express the KCNQ1, 4 and 5 genes, though the functional role played by the specific Kv7 subunits in the PASMCs is not yet clear. High Kv7.4 expression could indicate a significant role for the subunit (Joshi *et al.*, 2009; Gurney *et al.*, 2010), especially as Kv7.4 expression was reduced in models of PAH and that correlated with altered responsiveness to Kv7 modulators (Morecroft *et al.*, 2009; Sedivy *et al.*, 2014). Researchers investigated the PAH-affected mouse (Morecroft *et al.*, 2009) and rat models (Sedivy *et al.*, 2014), and noted that the flupirtine treatment could decrease the PAH-related symptoms, like cardiac hypertrophy, an increased right ventricular pressure (Morecroft *et al.*, 2009) and an increase in the pulmonary vascular resistance (Sedivy *et al.*, 2014).

1.5.4 **Regulation of vascular Kv7 channels**

Kv7 channels may function as the endpoints for Gas-linked receptor agonists (Chadha *et al.*, 2012b; Khanamiri *et al.*, 2013; Chadha *et al.*, 2014; Stott *et al.*, 2015b). The activation of adrenoceptors with isoprenaline relaxed renal arteries in a linopirdine-sensitive manner (Chadha *et al.*, 2012b). This suggests that β-receptors couple to Kv7 channels. Linopirdine also inhibited relaxation to forskolin, which directly activates AC (Chadha *et al.*, 2012b). Furthermore, β-receptor stimulation could increase Kv7 currents in renal arterial myocytes, and Kv7.4 channels could be significantly involve in β-adrenoceptor mediated vasodilation (Chadha *et al.*, 2012b). This finding is supported by the effects of adenosine on rat coronary arteries, which evoked PKA-mediated relaxation that was reduced by Kv7 blockers (Khanamiri *et al.*, 2013). Mani *et al.* (2016b) compared the sensitivities of over-expressed human Kv7.4, Kv7.5 and Kv7.4/7.5 channels to stimulation of the endogenous β-adrenergic pathway in A7r5 VSMCs. They found that Kv7.4 channels were insensitive to isoproterenol stimulation.
(Mani et al., 2016b), but Kv7.5 currents, endogenously expressed in the cells or resulting from over-expression of human Kv7.5, were significantly increased in response to isoproterenol treatment. This effect was attributed to increasing cAMP levels and PKA activation (Mani et al., 2016b). In contrast to the findings of Chadha et al. (2012b), this study, therefore, concluded that Kv7.5 channels are the mediators of β-adrenergic dilation. It should be noted, however, that Kv currents stimulated by β-AR stimulation are inhibited by 4-AP, suggesting that they are not mediated by Kv7 channels (Cole et al., 1996; Alejandro et al., 1998).

PKA is not the only downstream effector of the β-AR/cAMP pathway. Stott et al. (2016) showed that exchange protein activated by cAMP (EPAC) contributes to βAR-mediated vasorelaxation. Pre-constricted rat renal artery and mesenteric artery segments could partially relax in response to an EPAC-specific activator, while an EPAC inhibitor prevented this effect. Kv7 channel blockers also inhibited the vasorelaxant effects of the EPAC activator. Patch clamp studies showed that the EPAC activator increased linopirdine-sensitive currents in renal and mesenteric artery myocytes (Stott et al., 2016). These results indicate that Kv7 channels could contribute to β-AR/ cAMP vasodilation mediated through EPAC signal transduction in mesenteric arteries, although this was not the case in renal arteries (Stott et al., 2016).

Stott et al. (2015a) proposed a third mechanism, by which β-AR activation could lead to Kv7 channel activation, involving G-protein βγ (Gβγ) subunits functioning as the signalling intermediate. This was based on their finding that inhibitors of Gβγ abolished Kv7 currents and evidence that Kv7.4 and Gβγ subunits co-localise in renal artery myocytes. On the other hand, Povstyan et al. (2017) suggested that Gβγ may stimulate Kv7 channel activity by increasing the channel’s sensitivity to PIP2.

The calcitonin gene-related peptide (CGRP) could act as another Kv7 channel stimulating vasodilator in middle cerebral arteries. CGRP acts on Gas-coupled CGRP receptors in VSMCs (Bol et al., 2012; Byron and Brueggemann, 2017), elevates cAMP levels and stimulates PKA. Chadha et al. (2014) found that the CGRP-induced dilation of middle cerebral artery was inhibited by Kv7 blockers and KCNQ4 knockdown with siRNA. As they also found that co-localisation of Kv7.4 and Kv7.5 proteins, they concluded that heteromeric Kv7.4/Kv7.5 channels contribute to CGRP-stimulated vasodilation.
<table>
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<td>mKCNE1–5</td>
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<td>Femoral artery</td>
<td>mKCNQ1, mKCNQ4</td>
<td>mKCNE1, mKCNE4–5</td>
<td>Yeung et al. (2007)</td>
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<tr>
<td>Thoracic aorta</td>
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<td>mKCNE1, mKCNE3–5, rKCNE2–4</td>
<td>Yeung et al. (2007); Jepps et al. (2015)</td>
</tr>
<tr>
<td>Pulmonary artery</td>
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<td>-</td>
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<tr>
<td>Basilar artery</td>
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<td>-</td>
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</tr>
<tr>
<td>Middle cerebral artery</td>
<td>rKCNQ1, rKCNQ4–5</td>
<td>rKCNE1–4</td>
<td>Zhong et al. (2010); Jepps et al. (2015)</td>
</tr>
<tr>
<td>Renal artery</td>
<td>rKCNQ1, rKCNQ3–5</td>
<td>rKCNE2–4</td>
<td>Chadha et al. (2012b); Jepps et al. (2015)</td>
</tr>
<tr>
<td>Coronary artery</td>
<td>mKCNQ1, mKCNQ4, rKCNQ1, rKCNQ4–5, pKCNQ1, pKCNQ4–5</td>
<td>rKCNE2–5</td>
<td>Khanamiri et al. (2013); Lee et al. (2015); Morales-Cano et al. (2015) Hedegaard et al. (2014)</td>
</tr>
<tr>
<td>Gracilis artery</td>
<td>rKCNQ1, rKCNQ3–5</td>
<td>-</td>
<td>Zavaritskaya et al. (2013)</td>
</tr>
<tr>
<td>Penile artery</td>
<td>rKCNQ3, rKCNQ4, rKCNQ5</td>
<td></td>
<td>Jepps et al. (2016)</td>
</tr>
</tbody>
</table>

Abbreviations: h= human; m = murine; p= porcine; r= rat.
Stott et al. (2015b) also proposed that Kv7 channels are an important component of the natriuretic peptide-dependent vasodilation in rat aorta, due to cGMP-linked activation of the channels. Guanylate cyclase activation by NO or stimulation of the membrane-bound natriuretic peptide receptor both increase cytoplasmic cGMP levels. Stott et al. (2015b) used sodium nitroprusside (SNP), a NO donor, and either a C-type or atrial natriuretic peptide to stimulate cGMP synthesis and evoke cGMP-dependent relaxations. These relaxations were inhibited by linopirdine, but not inhibited by the Kv7.1-specific inhibitor HMR1556 \([N-\{(3R,4S)-3,4-Dihydro-3-hydroxy-2,2\text{-dimethyl-6-(4,4,4-trifluorobutoxy)-2H-1-benzopyran-4-yl}\}-N\text{-methylmetanesulfonamide}\] or other \(K^+\) channel blockers (Stott and Greenwood, 2015; Stott et al., 2015b). A recent study showed that linopirdine reduced endothelium-dependent dilation of coronary arteries (Goodwill et al., 2016). Perhaps that reflects inhibition of cGMP-dependent activation of the channels.

1.5.5 **Pharmacology of Kv7 channels**

Flupirtine \([N-2\text{-amino-6-}[(4\text{-fluorophenyl})\text{methyl}]\text{amino}-3\text{-pyridinyl}][\text{carbamate}]\) and retigabine \([\text{ethyl }2\text{-amino-4-}[(4\text{-fluorophenyl})\text{methyl}]\text{amino}[\text{phenyl}]\text{carbamate}]\) are two of the most characterised Kv7 channel activators (Schenzer et al., 2005; Bentzen et al., 2006; Morecroft et al., 2009). They activate the Kv7.2-Kv7.5 subtypes by interacting with molecular residues present at the S5 and S6 domains of the channel, where tryptophan present at S5 is an important residue. Flupirtine and retigabine cannot activate Kv7.1 channels since they do not contain tryptophan in their S5 domain (Schenzer et al., 2005; Wuttke et al., 2005; Bentzen et al., 2006). These drug molecules are used clinically. Flupirtine has been a popular analgesic drug in several European countries since 1984, and retigabine was approved by the FDA in 2010 for use as an adjuvant drug for the treatment of epilepsy in adults (Harris and Murphy, 2011; Grunnet et al., 2014).

Several compounds have been developed with differential selectivity for subtypes of Kv7 channels. When a low concentration of the Kv7 channel modulator was used, they showed selectivity for Kv7 channels compared to other vascular \(K^+\) channels, such as linopirdine and XE991 (Lamas et al., 1997; Schnee and Brown, 1998). On the other hand, some of Kv7 channel activators, like celecoxib and retigabine, can inhibit L-type VGCC, at the concentrations required for activating Kv7 channels (Brueggemann...
et al., 2009; Mani et al., 2011). This complicates studies with Kv7 activators because Ca\(^{2+}\) channel inhibition also causes vasodilation (Clapp and Gurney, 1991). Based on this drawback care should be taken when interpreting the effects of the retigabine.

Retigabine showed a 100-fold higher potency for activating Kv7 channels in comparison to the gamma-aminobutyric acid type A (GABA\(_A\)) receptors (Rundfeldt and Netze, 2000). It has high selectivity for Kv7 channels compared with other vascular K\(^+\) channels, so when used at low concentration affects only Kv7 channels. The analgesic effects of flupirtine were initially believed to result from its GABA\(_A\) receptor-potentiating effects and N-methyl-D-aspartate (NMDA) receptor blocking (Kornhuber et al., 1999). It is now thought that the analgesic activity depends on its capacity to increase Kv7-mediated neuronal M currents within its therapeutic range (2-6 μM) (Martire et al., 2004). At these concentrations, flupirtine increased the Kv7 currents without decreasing the L-type Ca\(^{2+}\) current in A7r5 cells, (Brueggemann et al., 2009). For that reason, its vasodilatory effect most likely results from Kv7 channel activation.

In recent years there has been a steady introduction of new Kv7 modulator, with varying Kv7 subtype selectivity. S-1 [(S)-N-[1-(3-morpholin-4-yl-phenyl)-ethyl]-3-phenyl-acrylamide] and BMS-204352 [(3S)-3-(5-Chloro-2-methoxyphenyl)-3-fluoro-1,3-dihydro-6-(trifluoromethyl)-2H-Indol-2-one] are drug molecules that show higher selectivity for activating Kv7.4 and Kv7.5 channels, compared to Kv7.2 or Kv7.3 channels (Bentzen et al., 2006). S-1 also inhibits Kv7.1 channels and at higher concentrations can activate Kv7.2-7.5 channels, as it binds to the same tryptophan residue as retigabine (Bentzen et al., 2006). Other novel Kv7 channel activators including ICA-069673 [N-(2-Chloro-5-pyrimidinyl)-3,4-difluorobenzamide] and ML213 [N-(2,4,6-Trimethylphenyl)-bicyclo[2.2.1]heptane-2-carboxamide]. ML213 was initially identified as a selective Kv7.2 and Kv7.4 channel activator (Brueggemann et al., 2014a), but it can also increase the maximal conductance of Kv7.5 channels, as well as Kv7.4/7.5 channels (Brueggemann et al., 2014a). ICA-069673 robustly activates Kv7.4 channels with a lesser effect on Kv7.5 channels (Brueggemann et al., 2014a). Heteromeric Kv7.4/7.5 channels were seen to display an intermediate response to ICA-069673 (Brueggemann et al., 2014a). These results suggest that ML213 and ICA-069673 may bind to different sites on Kv7 channels, making them selective for Kv7.4, Kv7.5, and Kv7.4/7.5 channel subtypes (Brueggemann et al., 2014a).
Kv7 channels are also modulated by fenamate and other non-steroidal anti-inflammatory drugs. Meclofenamic acid is a Kv7.2/3 channel activator (Peretz et al., 2005). While diclofenac [2-[(2,6-Dichlorophenyl)amino]benzeneacetic acid] is the only drug to date that can differentiate between Kv7.4 and Kv7.5 channels. Diclofenac enhanced Kv7.4 channel activators, show an intermediate effect on heteromeric Kv7.4/7.5 channels and blocked Kv7.5 channels (Brueggemann et al., 2011a).

Celecoxib increased Kv7 channel currents in airway SMCs and VSMCs (Brueggemann et al., 2011b; Mani et al., 2011). It activates homomeric channels consisting of Kv7.2, Kv7.3, Kv7.4 or Kv7.5 subunits, along with heteromeric Kv7.2/Kv7.3 or Kv7.3/Kv7.5 channels. However, it inhibited Kv7.1 and Kv7.1/KCNE1 channels (Brueggemann et al., 2009; Du et al., 2011).

ICA-27243 [N-(6-chloropyridin-3-yl)-3,4-difluorobenzamide] is a 20-fold more potent activator of heteromeric Kv7.2/7.3 channels compared to Kv7.4 channels, but is a weak activator of heteromeric Kv7.3/7.5 channels and does not activate Kv7.3 channels in neurons (Padilla et al., 2009; Blom et al., 2010). It binds to the voltage sensor domain in S1-S4, rather than the pore domain in S5-S6 segment (Padilla et al., 2009). SMB-1 is another activator that shows high selectivity for Kv7.4 channels but is also a Kv7.2 channel inhibitor (Blom et al., 2014).

RL-3 [5-(2-Fluorophenyl)-1,3-dihydro-3-(1H-indol-3-ylmethyl)-1-methyl-2H-1,4-benzodiazepin-2-one] is a benzodiazepine that activates the Kv7.1 channel; however, its activity is significantly weakened when Kv7.1 is co-expressed with the KCNE1 subunit (Salata et al., 1998). This could be due to competition of RL-3 with the KCNE1 subunit for the same interaction site on the KCNQ1 subunit (Salata et al., 1998; Seebohm et al., 2003).

Zinc Pyrithione (ZnPy) was proposed as a potent activator of Kv7 channels, which was ineffective against Kv and KCa channels (Xiong et al., 2007). The structures of ZnPy and retigabine differ remarkably; hence they were proposed to interact at different sites in the Kv7 channel and they differ in their subunit selectivities (Xiong et al., 2008; Eid and Gurney, 2018). ZnPy activates Kv7.1, Kv7.4 and Kv7.5 subtypes, but not Kv7.3 (Xiong et al., 2008). More recently ZnPy was shown to activate Kv7 channels by acting as a Zn2+ ionophore and increasing Zn2+ flux across cell membrane. As a consequence, it interferes with the regulation of Kv7 channels by PIP2 (Gamper et al., 2005). Our laboratory recently found that it also activates BKCa channels in
PASMCs (Eid and Gurney, 2018). This lack of selectivity limits its usefulness for investigating Kv7 channel function in the arterial system (Eid and Gurney, 2018).

During the evaluation of Kv7 channel blockers, XE991 and linopirdine emerged as popular chemical molecules, which are often employed as channel markers (Jentsch, 2000; Robbins, 2001). Linopirdine block Kv7.2 and/or Kv7.3 channels by directly interacting with them (Lamas et al., 1997). Both drug molecules showed a 20-fold lower potency at inhibiting neuronal delayed rectifier, A-type, BKCa currents and Kv10–Kv12 currents, compared with M-currents. They also showed a 100-fold lower potency in inhibiting Kv1.2, Kv2.1 and Kv4.3 channels (Lamas et al., 1997; Schnee and Brown, 1998; Wang et al., 1998; Wladyka and Kunze, 2006; Haick and Byron, 2016). However, when used at concentrations required for blocking Kv7 channels, they could affect other recombinant K+ channels (Wang et al., 1998, 2000).

Linopirdine and XE991 have been widely used as selective Kv7 channel blockers at ≤10 μM concentrations (Schnee and Brown, 1998; Wladyka and Kunze, 2006), but it was recently observed that 10 μM XE991 inhibited recombinant Kv1.2/Kv1.5 and Kv2.1/Kv9.3 channels (Zhong et al., 2010). As these channels have been proposed to play a vital role in the IPAs, these off-target effects are a potential complication when interpreting results of the drug. Zhong et al. (2010) observed that it is better to use linopirdine rather than XE991 since it did not inhibit Kv2.1/Kv9.3 channels.

Chromanol 293B and its potent analogue, HMR1556, are specific Kv7.1 channel inhibitors (Bleich et al., 1997; Thomas et al., 2003). Chromanol inhibits Kv7.1 with an IC50 of 6.9 μM, while HMR1556 displayed an IC50 of 74 nM (Lerche et al., 2007; Towart et al., 2009). Chromanol 293B activity is dependent on the β- subunits present in the Kv7.1 channel. For example, the presence of the KCNE3 subunit led to a 30-fold increase in Kv7.1 channel inhibitor potency compared to the KCNE1 subunit (Bett et al., 2006). Additionally, chromanol can act as a Kv1.5 channel inhibitor (Yang et al., 2000; Lerche et al., 2007).

Pharmacological studies have uncovered a range of different Kv7.1 blockers, like L-768,673 [2-(2,4-trifluoromethyl)-N-(2-oxo-5-phenyl-1-(2,2,2-trifluoroethyl)-2,3-dihydro-1H-benzo(e)(1,4)diazepin-3-yl)acetamide], BMS-208782 [(S)-4-(3-Butyl-1,2,4-oxadiazol-5-yl) -N-(2,2- dimethylcyclopentyl methyl) benzamide] and JNJ 303 [2-(4-Chlorophenoxy)-2-methyl-N-[5-[(methylsulfonyl) amino]tricycle [ 3.3.1.13,7] dec-2-
yl]-propanamide] (Towart et al., 2009). Selective blockers of other Kv7 subtypes are not yet commercially available, although a Kv7 targeted toxin (SsTx toxin) purified from the venom of the Chinese red head centipede has recently become available. SsTx blocks KCNQ1,2,4 and 5 with IC values around 2.5 µM (Luo et al., 2018).

1.5.6 Vascular KCNQ channel-associated channelopathies

The physiological significance of Kv7 channels was demonstrated by showing that mutations in the KCNQ1-4 genes are responsible for several diseases. These include long QT syndrome (Wang et al., 1996a; Jespersen et al., 2007; Peroz et al., 2008), non-syndromic autosomal dominant deafness (Kubisch, 1999; Kharkovets et al., 2000) and benign familial neonatal convulsions (Biervert et al., 1998). Studies also showed that single-nucleotide polymorphisms in the KCNQ1 gene were related to an increased probability of developing type-2 diabetes (Unoki et al., 2008). Researchers have also observed a link between Kv7 channel dysfunction and the pathogenesis of diseases like hypertension and other disorders of smooth muscle (Chadha et al., 2012b; Zavaritskaya et al., 2013; Stott et al., 2015b; Sedivy et al., 2015). However, the role played by Kv7 channels in these pathologies is not completely understood. Decreased Kv7 channel activity could lead to increased vascular resistance (Jepps et al., 2011; Chadha et al., 2012b; Khanamiri et al., 2013; Stott et al., 2015b). Hypertensive rats showed significantly decreased Kv7 channel activity in their aortae, renal and mesenteric arteries (Jepps et al., 2011; Khanamiri et al., 2013). The expression of Kv7.4 channels, in particular, was significantly decreased in renal arteries (Chadha et al., 2012b), mesenteric arteries (Jepps et al., 2011; Zavaritskaya et al., 2013), left coronary artery (Khanamiri et al., 2013) and aortae (Stott et al., 2015b) of spontaneously hypertensive rats (SHRs). Decreased Kv7.4 expression was also noted in the mesenteric arteries of mice with angiotensin II-induced hypertension (Jepps et al., 2011). Decreased Kv7.4 expression could trigger impaired isoproterenol relaxation and decrease the ability of the arteries to regulate total peripheral resistance (Jepps et al., 2011; Chadha et al., 2012b). Myocytes with few Kv7 channels would likely be depolarised, thereby increasing vasoconstriction, vascular resistance and blood pressure. A decrease in Kv7.4 channel expression in the SHR models might also weaken their vasodilatory cGMP pathway (Stott et al., 2015b).
Kv7 channels have also been implicated in the regulation of renin release from the kidney. Renin is secreted in response to renal hypoperfusion or β-adrenergic stimulation (Peti-Peterdi and Harris, 2010). A decrease in Kv7.4 channel expression and function in the SHR could, by reducing the vasodilatory response to β-adrenergic stimuli, cause renal artery stenosis or renal hypoperfusion (Chadha et al., 2012b), followed by the activation of the renin-angiotensin-aldosterone system and further vasoconstriction (Chadha et al., 2012b; Fosmo and Skraastad, 2017).

Impaired Kv7 channel function may be responsible for coronary vascular dysfunction in diabetic patients and could affect the body’s defence mechanism against hypoxia (Fosmo and Skraastad, 2017). Studies observed that Kv7 channel expression, activity and function in left coronary arteries had significantly decreased when mice were subjected to hyperglycaemia (Liu et al., 2001; Li et al., 2003; Bubolz et al., 2005). Bubolz et al. (2005), reported that hyperglycaemia also affected the vasodilatory activity of the left coronary artery in response to forskolin.

Kv7 channels have also been implicated in the aetiology of PAH. Decreased Kv7.4 expression was linked to the development of PAH in several mouse models and administering flupirtine was able to prevent PAH development and reverse the disease in an established model (Morecroft et al., 2009; Sedivy et al., 2015). Sedivy et al. (2015) observed that Kv7.4 expression in the rat pulmonary arteries was significantly reduced when they were subjected to a hypoxic environment for 3-5 days. Though Kv7.4 expression was reduced, flupirtine could still induce relaxation of the pulmonary vessels in perfused lungs of hypoxic rats, indicating that sufficient Kv7 channel remained to act as a target to dilate the arteries. This is opposite to that noted in the systemic circulatory system (Haick and Byron 2016). This increases the potential for using Kv7 channel activator as a therapeutic option for the treatment of PAH.

Kv7 channels may play an important role in many other vascular diseases, like erectile dysfunction (Jepps et al., 2016). KCNQ3, KCNQ4 and the KCNQ5 genes were down-regulated, while KCNQ1 expression was up-regulated, in the corpus cavernosum from the heart-failure prone, SHR. However, KCNQ1–5 expression was unaffected in the penile arteries of the same model (Jepps et al., 2016).
1.6 Research Aims and Hypothesis

It is evident that different members of the Kv7 channel family are expressed in arterial smooth muscle cells of different vascular beds of rodents and humans. In fact, many studies have shown that members of the Kv7 family are involved in regulating vascular tone, acting as an end-target for the cAMP and cGMP signalling pathways. Controversy remains regarding the nature of the Kv7 channel subtypes involved. For example, Kv7.4 and Kv7.5 channels have separately been proposed as the main subunit mediating SMC Kv7 current and as the target for cAMP. There is also disagreement over whether relaxation in response to Kv7.1 activator drugs is mediated via the activation of Kv7.1 channels or not. These discrepancies may reflect variation in Kv7 subunit expression in different vascular beds and/or in different sections of the vascular bed. Little is known about which Kv7 subunits participate in the regulation of pulmonary artery tone and how they may be regulated. Therefore, this study aimed to test the hypothesis that Kv7 channels mediate pulmonary vasodilation resulting from activation of the cAMP and cGMP signalling pathways. A second aim was to determine if activation of Kv7.1 channels can give rise to relaxation of constricted pulmonary arteries. As arteries from different parts of the pulmonary circulation have different pharmacological properties, a further aim was to test the hypothesis that different sized IPAs respond differently to Kv7 modulators. To address these hypotheses normal male rats were used and several techniques were employed.

- The effects of Kv7 channel modulators on pulmonary artery tone were studied using pin and wire myography.
- Organ culture of pulmonary arteries was optimised to enable siRNA transfection of intact vessels.
- siRNA knockdown of Kv7 channel subunits was employed, along with myography, to assess their involvement in artery responses to Kv7 channel modulators.
- Western blotting (WB) techniques were used to assess the expression of the Kv7 proteins.

It is expected that the results of this study will contribute to the further understanding of the roles of Kv7 channels in the regulation of pulmonary vascular tone and the physiological pathways that employ Kv7 channels as the end target to mediate changes in vascular tone.
CHAPTER 2
MATERIALS AND METHODS
2.1 Dissection and tissue preparation

For all experiments normal male Sprague Dawley rats (280-360 g) were used, and they were euthanized by cervical dislocation under Schedule 1 of the Animals (Scientific Procedures) Act 1986 that conformed to the Guide for the Care and Use of Laboratory Animals. The lungs were rapidly removed and placed into a cold physiological salt solution (PSS) that composed of (mM): 122 NaCl, 5 KCl, 10 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid (HEPES), 0.5 KH$_2$PO$_4$, 0.5 Na$_2$HPO$_4$, 1 MgCl$_2$, 5 glucose, and 1.8 CaCl$_2$, where the pH was adjusted to 7.4 with 3M NaOH. When necessary, the whole heart and brain were removed and snap frozen for Western blotting purposes, the conduit IPA (outer diameter ~900-1100μm) and small IPA (outer diameter ~300-450μm) were dissected and cut into rings of approximately 2mm in length through the use of micro-scaled Petri dishes and mounted in a vessel myograph for isometric tension measurement. Some of the vessels were incubated at 37°C for up to 3 days to evaluate the preserved contractile function and the knockdown of KCNQ1,4 and -5 subtypes. The average number of IPA segments used from each rat were four to eight vessel segments depending on the availability of myograph. During the dissection, the PSS was kept cool (~5 °C) by constantly being replaced at an approximate 5 minutes interval. The dissection tools were sterilized with 70% ethanol and rinsed thoroughly with sterilised double distilled water, while the PSS was sterilized by filtering it through a 0.2 μm filter prior to the incubation process.

2.2 Myography

Myography is designed to measure the isometric tension changes of the mounted blood vessel that had responded to the applied pharmacological agents. This measurement technique was initially developed by Mulvany et al. (1976) in the study of large vessels but was later adapted in the analysis of vessels with smaller circumferences. In our myography experiments, the IPA constriction that was produced from the contraction of PASMCs had led to detection of force from the force transducer in the chamber unit. Meanwhile, the larger vessels were mounted on stainless steel pins (Figure 2.1A), the smaller vessels on the other hand, had been mounted on two 40 μm diameter stainless steel wires, where the vessel lumen was mounted as a ring preparation by threading it over the two parallel stainless-steel wires and secured to the jaws of the myograph. (Figure 2.1b) (DMT, Model 610, Denmark). One of the supports (jaws) is attached to a
precision micrometre, that allows the manual control of vessel circumference and stretch, while the other is attached to a force transducer (that is sensitive to a 0.01 mN tension variation) for measurements of force/tension development (Figure 2.1C). The changes in the tension of mounted artery were recorded continually in a computer by using LAbChart® 7 software (v7.3 - Windows version) that is in line with the PowerLab acquisition system (ADInstruments, U.K.).

The myograph temperature was set at 37°C (the actual reading was ~36.5 °C) and air bubbles with a constant rate were introduced in the chambers to aerate the PSS as well as to ensure a homogenous drug mixture of the solution. The PSS and 80 mM of the KPSS were subsequently warmed to 37 °C in a water bath prior to being added into the chambers. The KPSS had been prepared by the equimolar substitution of KCl for NaCl.

After the mounting of the vessels, a 15 minutes allowance was then given for the vessels to warm up. A basal tension of 4 mN was applied to detect the optimal response of the stimulated vessels, which were allowed to equilibrate for about 40 minutes before the start of the experiments. These were washed thrice for every 15 minutes during the equilibration stage. The optimal basal tension was determined in pilot experiments; both conduit and small IPAs showed the largest response to 50 mM KCl when the applied basal tension was either 4 or 5 mN. Accordingly, 4 mN tension was selected as the optimal basal tension in all experiments.

At the beginning of each experiment, the mounted vessels were tested with 50 mM of KCl for 5 minutes and then washed until the vessel tension had returned to the initial baseline. This test was repeated thrice to ensure a reproducible response. For experiments that gauged the response of IPA towards the 10 mM KCl, the solution was added directly into the chamber 5 minutes prior to washing and the tests were only conducted after the third application of the 50 mM KCl. Meanwhile, the constrictive responses of the vessels towards the corresponding 10 and 50 mM KCl were measured at a 5 minutes interval upon the addition of KCl, and the contraction responses to the 10 mM KCl was measured as a percentage of the contraction resulted from the addition of 50 mM KCl.
Figure 2.1 Schematic diagram illustrating the pin and wire myograph

A. Shows the mounting pin for conduit vessel. B. Shows the mounting jaws of the small vessels. The myograph system consists of both fixed and moveable jaws. C. An illustration of the wire myograph system. A small artery segment is mounted over the two parallel stainless-steel wires and is secured to the opposite jaw, which is then followed by an applied tension from the micropositioner that is connected to the moveable jaw. The fixed jaw is then connected to a force transducer that detects the changes of force exerted by the mounted vessel as a response to the applied KCl or drugs. The output of the force transducer is connected to a myo-interface and then to a computer by an amplifier (Power Lab, AD instrument) so that a continuous measurement of the isometric tension changes can be conducted through the use of a data acquisition software (LABChart® 7 software v7.3). All of the chambers in the myography system had been subjected to a controlled air via air pipe and the suction of chamber content via vacuum pipe.
2.3 Experimental procedure for studying the effects of Kv7 modulators

2.3.1 Procedures for studying the effects of Kv7 blockers
The response to the Kv7 blockers was tested following the washout and the return of vessels tension to the baseline. This involved evaluating the effect of 0.01 - 30 μM of linopirdine and XE991 on conduit and small IPA (only linopirdine). The response towards the 0.1- 1 μM HMR1556 was tested in the absence and presence of a pre-constrictor. The pre-tone was induced by adding 1 μM of the phenylephrine or a 20, 40 or 80 nM of the U46619. The drugs were added in a cumulative manner of up to a 20 minutes interval if there had been no response or if the response had demonstrated a steady state. The contraction responses toward the Kv7 blockers were then measured as a percentage of contraction induced by the addition of 50 mM KCl, as KCl was used as a standard reference for contractile agents.

2.3.2 Procedures for studying IPA responses to Kv7 activators
Following the test with 50 mM KCl and washout to return the vessels tension to the baseline, further tests were then conducted on the conduit IPA response towards flupirtine and retigabine (Kv7.2-7.5 activators), ICA069673 (Kv7.2/ Kv7.3, Kv7.3/ Kv7.5 and Kv7.4 activators), RL-3 and ML277 (Kv7.1 activators). The effects of flupirtine and RL-3 were also evaluated on the small vessels. These were done by pre-constricting the conduit and small vessels with 1 μM of phenylephrine or 30 nM of U46619 (only for small vessels) and once the constriction tone had reached a steady-state, the vessels were tested with different concentration levels of a Kv7 activator in a cumulative manner. To ensure that the data obtained from the use of Kv7 activators were due to their relaxant effect rather than the loss of phenylephrine-induced tone during the experiment and/or the effect of DMSO, time control experiments for flupirtine were run in parallel by adding same volume of vehicle containing equivalent volume of DMSO.

The increasing concentration levels of flupirtine (0.1 to 200 μM), retigabine (0.01 to 200 μM) or ICA069673 (0.01 to 100 μM) were added in a cumulative manner of up to a 20 minutes interval if there had been no response or if the response had
reached a steady state. Similar to the abovementioned scenario, the increasing concentration levels of RL-3 (0.1 to 5 μM) or ML277 (0.1 to 10 μM) were also applied in a cumulative manner to the constricted IPA. Under this circumstance, the relaxation was measured as a percentage of the induced constriction just prior to the addition of a Kv7 activator.

To test if the RL-3 mediated relaxation in the pulmonary vasculature had been mediated through the activation of Kv7.1 K⁺ channels, the RL-3 effects on artery segments were subsequently examined with the presence of either HMR1556 (0.1, 0.3, 1 or 10 μM), ±293B (1 or 10 μM) or -293B (1 μM). For the same purpose, 1 μM of the HMR1556 was applied prior to the addition of 1 μM phenylephrine and the increasing concentration level of ML277. As a way to negatively control the effect of HMR1556 on the relaxation of RL-3 and ML277, the effects of the Kv7.1 blockers were tested on the relaxation responses towards retigabine. This was achieved by pre-constricting the vessels with 1 μM of phenylephrine with the presence of either 10 μM of HMR1556, ±293B or 1-10 μM -293B and was again tested with different concentration levels of retigabine in a cumulative manner. The same process was also repeated for testing the relaxing effect of ICA069673 (0.01 to 100 μM) in the presence of 1 μM HMR1556.

As a way of testing, if the Kv7 activators-induced vasodilation had been achieved by the activating K⁺ channels, the response to RL-3, ML277, flupirtine and ICA06973 were also studied in vessels pre-constricted with 80 mM of KPSS. According to Brayden (2002), drugs that activate K_{ATP} channels would result in vasodilation. As a way of eliminating the K_{ATP} K⁺ channels’ role in mediating the relaxing effect of RL-3, the K_{v7.1} activator was subsequently tested with the presence of 1 μM glibenclamide, the K_{ATP} channel blocker. Likewise, the same analysis was also conducted on the effect of HMR1556 (1 μM) on the relaxation induced by the K_{ATP} channel activator, levcromakalim (0.01 μM to 10 μM).
2.4 Procedures for studying the involvement of Kv7 channels as end targets in physiological vasodilator pathways.

2.4.1 Procedures for studying the role of Kv7 potassium channels in cAMP-mediated vasodilation

Following the reproducible response to the 50 mM KCl and the washout for returning the tension to its initial baseline, constriction of the vessels was induced with 1 µM phenylephrine. Once the induced constriction had reached a steady state, the vessels were once again tested with the cumulative addition of isoprenaline (10 nM to 1 µM), forskolin (10 nM to 1 µM) and treprostinil (0.1 nM to 1 µM) at increasing concentration levels. The experiments were conducted in the absence and presence of different Kv7 blockers, for instance, 100 nM of iberiotoxin, being a BKCa channel blocker, which was solely added or combined with XE991 (only for isoprenaline). The isoprenaline relaxing effects were also tested with the presence of either 100 µM of gallein, a Gβγ subunits inhibitor or Rp-8-Br-cAMPs, a PKA inhibitor, while the relaxing effect of treprostinil was analysed in conduit and small vessels, where the tension had been induced by 30 nM of U46619.

2.4.2 Procedures for studying the involvement of Kv7 potassium channels in cGMP-mediated vasodilation

After conducting the test with 50 mM KCl and the return of the vessels’ tone to baseline from the washout, constriction of the IPA segments was further induced with 1 µM phenylephrine. Once the tone had reached a steady-state, the vessels were further tested with the increasing concentration levels of either SNP (0.1 nM to 3 µM), GTN (0.1 nM to 3 µM) or BAY41-2272 (1 nM to 3 µM) that were added in a cumulative manner and in the absence or presence of 1 µM HMR1556, linopirdine or XE991. Similarly, the relaxing effect of carbachol (1 nM to 30 µM), A23187 (0.3 nM to 1 µM) and sildenafil (0.1 nM to 10 µM) were also analysed in the absence and presence of 1 µM Kv7 blockers, while in the case of sildenafil, the relaxing effect was tested in conduit and small vessels, where the pre-tone had been induced by a 30 nM of the U46619.
2.5 Organ culture of IPA.

After dissection, some IPA segments were incubated for three days in HEPES- or bicarbonate-buffered PSS, or in the medium with the absence or presence of 0.5 or 1 % of antibiotic mixture (10000 units penicillin, 10 mg streptomycin and 25 µg amphotericin B per ml). The medium was advanced Dulbecco’s modified Eagle medium (DMEM)/F-12, which contained 10% bovine serum albumin (BSA). For the organ culturing purpose, the vessel segments cultured in a 4-well culture plate, containing 1 ml of the respective PSS or medium, and the plates containing HEPES-buffered PSS were sealed by parafilm unless it is specified. The plates were kept at 37°C in an incubator that has a humidified atmosphere of 5% CO₂. Each day, the incubated artery segment would be transferred to the next well that had contained the same pre-warmed PSS or medium. The forceps that were used for transporting the vessel segments were sterilised with 70% ethanol and rinsed with sterilised double distilled water.

As it was reported that from the commencement of small interfering RNA (siRNA) transfection of ex-vivo tissues until the knockdown of the target protein had required a 2 – 4 days culturing of isolated vessels (Lin et al., 2004; Yu et al., 2004). Chadha et al. (2012b) incubated renal artery for 3 days to achieve knockdown of Kv7.4 protein. Therefore, the vessels were subjected to a 3-day incubation period.

2.6 Optimising siRNA transfection.

Prior to conducting the transfection procedure, the RNase decontamination solution (RNaseZap™) was used to remove any possible source of contamination with RNase so as to prevent the enzymatic reactions. In the transfection process, the procedure had utilised the Eppendorf tubes and RNase free tips.

2.6.1 Reverse permeabilisation

The permeabilisation technique was used to transfect the ex vivo IPA with the fluorescence- labelled siRNA (Silencer® FAM Labelled Negative Control) and dsRNA (BLOCK-iT™ Alexa Fluor™ Red Fluorescent Control). The isolated IPA segments were then exposed to different consecutive solutions with a temperature of around 4°C, where the experiments were conducted in a cold room. The vessels were placed in three
successive solutions that had contained the following (mM): (1) 10 EGTA, 120 KCl, 5 Na$_2$ATP, 20 HEPES, 2 MgCl$_2$ and 100 nM FAM labelled siRNA or labelled dsRNA (pH 6.8; 30 minutes); (2) 120 KCl, 5 Na$_2$ATP, 20 HEPES, 2 MgCl$_2$ and 100 nM FAM labelled siRNA or labelled dsRNA (pH 6.8; 60 minutes); and (3) 120 KCl, 5 Na$_2$ATP, 20 HEPES, 10 MgCl$_2$ and 100 nM FAM labelled siRNA or labelled dsRNA (pH 6.8; 30 minutes). Following that, the IPA segments were subsequently placed in a fourth solution with the following (mM) of 120 NaCl, 5 KCl, 5 Na$_2$ATP, 10 HEPES, 10 MgCl$_2$ and 5.6 glucose of an adjusted pH of 7.1 at 4 °C and the gradual increase (0.001 to 0.01 to 0.1 to 1 mM) of calcium concentration at a 15 minutes time interval. The control vessels were either not exposed to the labelled-siRNA or -dsRNA, or left exposed for the same duration as reverse permeabilisation but without undergoing the same procedure before being incubated in 12-well plates for 24 hours at 37°C and 5% CO$_2$ in the DMEM/F-12 that contained 10% of BSA and 1% of the antibiotic mixture.

The same procedure was repeated but with the addition of a transfecting reagent. 12 µl of each lipofectamine and N-TER were added with the 100nM labelled siRNA or dsRNA as stated by the manufacturing procedure, and instead of medium, deionised double distilled water was used in the preparation of the mixture before being added into solution numbers 1, 2 and 3.

The incubated IPA segments were subsequently flash-frozen in OCT by immersing in a Dewar of liquid nitrogen-cooled isopentane and were cut into 10 µm thickness sections to assess the uptake of the labelled siRNA or dsRNA. Since the FAM-labelled siRNA would emit a green fluorescence under fluorescence microscopy, an emission filter with a peak of 525 nm was used in the fluorescence microscope. However, the vessels which had been transfected with the labelled dsRNA emitted a red signal under fluorescence microscopy and were monitored at 593 nm. The same procedure was then repeated for assessing the uptake of the fluorescence labelled dsRNA of the transfected IPA segments when using another transfection protocol.
2.6.2 Procedure for Lipofectamine® RNA interference (RNAi) MAX transfection.

The lipofectamine RNAiMAX is a cationic lipid-based transfecting reagent for delivering siRNA into the cells, which had shown high efficacy in delivering siRNA into the neural, human mesenchymal and human embryonic stem cells (Tian et al., 2007; Zhao et al., 2008; Kamaci et al., 2011). Apart from requiring a low concentration of siRNA in providing maximal viability in the transfected cells and having a lower cytotoxic effect when compared to the other transfecting reagents (Kamaci et al., 2011), the lipofectamine RNAiMAX is also used for transfecting hard to transfect cells such as the mesenchymal stem cells (De Becker et al., 2007).

Although the lipofectamine RNAiMAX is not commonly used for transfecting intact arteries with a small interference RNA (siRNA), since it has demonstrated a high efficacy of siRNA delivery with minimal cytotoxic effect, we have therefore tried transfecting the PASMCs of ex vivo conduit IPA with either KCNQ1, 4 or 5 siRNA to knockdown the respective protein of the treated vessels.

The transfection mixture of the labelled dsRNA was prepared strictly in accordance to the manufacturer’s instruction, but with manipulation of the reagent volume and the concentration level of the fluorescent dsRNA. The preparation of the transfection mixtures are shown in Table 2.1.

The vessels were then placed in the desired concentration level of the transfection mixture and incubated for 24 hours at 37°C with 5% CO₂. To assess the transfection efficiency, the incubated IPA segments were then flash-frozen in the OCT, cut into a transverse section of 10 µm thickness and analysed under the fluorescence microscope.

To knockdown a certain Kv7 protein subtype, the same transfection procedure was repeated with KCNQ1, 4 or 5 siRNA, where the vessels were subjected to a 3-day incubation period. On the second day, the vessel segments were transferred to a freshly prepared and pre-warmed transfection mixture and were placed in the advanced DMEM/F12 that had contained 10% BSA and 1% antibiotic for 24 hours on the third day before being used for myography experiments.
# MATERIALS AND METHODS

## Table 2.1 RNAi transfection protocol

<table>
<thead>
<tr>
<th>Steps</th>
<th>Procedural details</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Component</td>
</tr>
<tr>
<td>1. Dilution of lipofectamine\textsuperscript{®} RNAiMAX reagent in the medium (Opti-MEM\textsuperscript{®})</td>
<td>Medium</td>
</tr>
<tr>
<td></td>
<td>Lipofectamine</td>
</tr>
<tr>
<td>2. Dilution of the labelled dsRNA in the medium (Opti-MEM\textsuperscript{®})</td>
<td>Medium</td>
</tr>
<tr>
<td></td>
<td>Labelled-dsRNA (20 µM)</td>
</tr>
<tr>
<td>3. Addition of the diluted dsRNA to the diluted lipofectamine</td>
<td>Diluted labelled dsRNA</td>
</tr>
<tr>
<td></td>
<td>Diluted lipofectamine</td>
</tr>
<tr>
<td>4. Incubation</td>
<td>The mixture is incubated for five minutes at room temperature</td>
</tr>
<tr>
<td>5. Addition of the dsRNA-lipofectamine complex to the well that had contained the required volume of advanced DMEM/F12</td>
<td>dsRNA-lipofectamine complex/well</td>
</tr>
<tr>
<td></td>
<td>Advanced DMEM/F12 that consisted of 10% BSA and 1% antibiotic</td>
</tr>
<tr>
<td></td>
<td>Final concentration of the dsRNA/well (nM)</td>
</tr>
<tr>
<td></td>
<td>Final volume of the lipofectamine/well (µl)</td>
</tr>
</tbody>
</table>
2.6.3 Procedure for N-TER™ oligo transfection.

N-TER is a nanoparticle siRNA transfection reagent, which is based on a peptide transfection reagent that is responsible for delivering siRNA into the cells. The N-TER system allows the conjugation of siRNA into the nanoparticle surface through N-TER peptide and is designed for in vitro application. The superiority of the N-TER reagent as compared to the lipid-based siRNA transfection reagents are shown in its ability of not only transfecting hard to transfect cells but also result in the quicker, less toxic and a more stable delivery of the siRNA (Morris et al., 1997; Simeoni et al., 2003). For this reason, we have decided to use the N-TER Nanoparticle siRNA Transfection System to deliver the KCNQ1, 4 or 5 siRNA into the PASMCs of intact vessels to knockdown the corresponding Kv7.1, 4 or 5 K⁺ channels. Although we have adhered to the procedures set by Sigma-Aldrich in the preparation of the labelled-dsRNA or KCNQ-siRNA nanoparticle formation solution (NFS), we have; however, manipulated the reagent volume and the concentration level of the labelled-dsRNA or KCNQ siRNA in the final mixture. The NFS preparation procedure had comprised preparing the labelled-dsRNA dilution (Table 2.2), N-TER dilution (Table 2.3) and the preparation of NFS with the labelled dsRNA by controlling the addition of the content from the corresponding tubes of 1-A, 2-A and 3-A to tubes 1-B, 2-B and 3-B (Table 2.4). The resulted mixtures were subsequently incubated for 15 to 20 minutes at room temperature to allow the formation of the N-TER peptide/labelled dsRNA NFS. After that, the NFS were diluted in the advanced DMEM/F12 so that the desired concentration level of the labelled dsRNA is achieved in the final transfection mixture, as shown in Table 2.4.
- MATERIALS AND METHODS -

Table 2.2 Preparation of the labelled-dsRNA dilution

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Tube 1-A (µl)</th>
<th>Tube 2-A (µl)</th>
<th>Tube 3-A control (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 µM dsRNA</td>
<td>7.5</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Dilution buffer</td>
<td>42.5</td>
<td>40</td>
<td>50</td>
</tr>
<tr>
<td>Final volume</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

Table 2.3 Preparation of N-TER dilution

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Tube 1-B (µl)</th>
<th>Tube 2-B (µl)</th>
<th>Tube 3-B control (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-TER</td>
<td>15</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Water</td>
<td>35</td>
<td>34</td>
<td>34</td>
</tr>
<tr>
<td>Final volume</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

Table 2.4 Dilution of NFS in the advanced DMEM/F12.

<table>
<thead>
<tr>
<th>Steps</th>
<th>The resulted NFS from mixing tube</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1-A and 1-B</td>
</tr>
<tr>
<td>NFS of the labelled and controlled dsRNA(µl)</td>
<td>16.7</td>
</tr>
<tr>
<td>Advanced DMEM/F12 that had contained 10% BSA and 1% antibiotic (µl)</td>
<td>983.3</td>
</tr>
<tr>
<td>The final concentration of the labelled dsRNA/well (nM)</td>
<td>25</td>
</tr>
<tr>
<td>The final volume of the N-TER/well (µl)</td>
<td>3</td>
</tr>
</tbody>
</table>

The vessels were then incubated in the transfection mixture at 37°C and 5% CO₂ for 1 day and flash-frozen in the OCT for assessing transfection efficiency.

To knockdown the Kv7.1, 4 or 5 protein, the same transfection procedure was repeated for KCNQ1, 4 or 5 siRNA on the first and second day of culturing, where the vessels were subjected to a 3-day incubation period. To achieve the knockdown of Kv7 proteins, similar procedural steps were followed as in lipofectamine RNAiMAX transfection protocol.
2.7 Western blotting

2.7.1 Sample preparation

The expression of the Kv7 protein subtypes in the IPA, brain, heart and whole lung was analysed through the WB technique. The protein samples were obtained from either freshly isolated or flash frozen tissues that had been stored at – 80 ºC. The tissues were then placed into a chilled RIPA lysis buffer (1 mM Ethylenediamintetraacetic acid (EDTA), 25 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40 (v/v) 0.5% sodium deoxycholate (w/v), 1% SDS (w/v), 1 mM PMSF and 1x complete, Mini, EDTA-free Protease inhibitor Cocktail (Roche, UK) and ground by using a glass tapered tissue grinder that was chilled on ice during tissue disruption. After almost all the tissue samples had been ground, they were then centrifuged at 12000 RPM for 15 minutes at 4 ºC to separate the protein extracts from the unbroken cells and cellular debris. The supernatant was aspirated and placed in a chilled and freshly labelled Eppendorf tube.

After the protein concentration in each of the sample had been quantified by using the bicinchoninic acid (BCA) protein assay with NANO DROP 1000 spectrophotometer, the samples were then frozen at -80 ºC for later use.

2.7.2 Electrophoresis

The samples were then diluted with a 4:1 ratio of the loading and 5X Laemmli buffer with an equal amount of proteins loaded into each of the wells. Prior to loading the samples into the gel, the proteins had been denatured by boiling them for 5 minutes at 95 ºC. A 10 % of SDS-PAGE was used for each of the experiment that is freshly prepared, and depending on their molecular weight; the proteins were separated by electrophoresis. The 10 wells gel were then assembled in the tank of the electrophoresis and were filled to a specified level by the running buffer, which was then followed by the manual setup of the Mini-PROTEIN® 3 Cell electrophoresis apparatus (Bio-Rad). After the loading of samples and marker (Precision Plus Protein™ Standard-Dual Colour), into the wells, the electrophoresis was carried out at a constant electric current of 84 mA for around 2 hours. Once the electrophoresis process had been completed, the gel was then removed and kept in the blotting buffer before the start of the western blotting process.
2.7.3 Protein detection

After the electrophoresis process, the separated proteins of the samples were transferred to the nitrocellulose and polyvinylidene-fluoride (PVDF) membrane through the use of a Trans-blot® cell apparatus (Bio-rad). The transfer sandwich of the gel and the membrane was subsequently set up in the trans-blot system as described in the user manual and was followed by blotting from the transfer device that had operated for 1 hour at 110V. Following the transfer, the membrane was subjected to a three 5 minutes wash along with the 0.1% Tween-20 Tris-buffered saline (TTBS) and then blocked at room temperature for 1 hour by a blocking solution (5% dried milk powder TTBS). After this point, the membrane was again subjected to a three 5 minutes wash and incubated overnight at 4 ºC in an appropriate dilution of the primary antibody (Table 2.5) and once more undergo a three 10 minutes wash under the TBST before being incubated at room temperature with the relevant secondary antibody that was diluted in 1% milk powder-TTBS (Table 2.5). After undergoing the incubation, the membrane was once more subjected to a three 10 minutes wash with the TBST and incubated in the SuperSignal™ chemiluminescent substrate, as stated in the manufacturer’s instructions. The membrane was then submitted to an imaging analysis through the use of ChemiDoc™ + image lab software (version 6.0.0) that was provided by Bio-Rad.

The band quantification was determined by using the image lab software (6.0.1) that was provided by Bio-Rad.

<table>
<thead>
<tr>
<th>Antibody type</th>
<th>Source</th>
<th>Dilution ratio</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-KCNQ1</td>
<td>Mouse-Monoclonal</td>
<td>1:500</td>
<td>NeuroMab</td>
</tr>
<tr>
<td>Anti-KCNQ4</td>
<td>Mouse-Monoclonal</td>
<td>1:500</td>
<td>NeuroMab</td>
</tr>
<tr>
<td>Anti-KCNQ5</td>
<td>Rabbit-Polyclonal</td>
<td>1:500</td>
<td>Alomone labs</td>
</tr>
<tr>
<td>Anti-α-Tubulin</td>
<td>Mouse-Monoclonal</td>
<td>1:2000</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>antibody</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-Mouse IgG/HRP</td>
<td>Donkey-Polyclonal</td>
<td>1:3000 unless</td>
<td>Jackson ImmunoResearch</td>
</tr>
<tr>
<td></td>
<td></td>
<td>otherwise</td>
<td></td>
</tr>
<tr>
<td>Anti-Rabbit IgG/HRP</td>
<td>Goat-Polyclonal</td>
<td>1:3000 unless</td>
<td>Dako</td>
</tr>
<tr>
<td></td>
<td></td>
<td>otherwise</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.5 Types and concentration of antibodies used for western blot.
2.8 Analysis of data

The responses to the added KCl were measured 5 minutes after the addition of KCl to the bath, where in this case, the response of the vessels to the third application of 50 mM KCl was measured and taken as a standard reference for studying the responses of vessels to other vasoconstrictors. The responses to phenylephrine, U46619, linopirdine and XE991 were measured as a percentage of the last KCl response of the experiment, while the relaxation response to Kv7 activators and the other vasorelaxant chemicals were measured as a percentage of the induced constriction just prior to the addition of the relaxing agent.

The response values were used to plot the log$_{10}$ concentration-response curves from which the EC$_{50}$ and maximum response were extracted by fitting the data to the Hill equation (equation 1). GraphPad Prism® software 7.04 for Windows (GraphPad Software, San Diego, California USA) was employed to plot data and fit curves unless stated otherwise.

\[
R = R_{\text{min}} + (R_{\text{max}}-R_{\text{min}})/(1+10^{(\text{LogEC}_{50}-X)/K})
\]  

(1)

where R is the response, R$_{\text{min}}$ is the minimum response, R$_{\text{max}}$ is the maximum response, X is the concentration and K is the Hill slope. pEC$_{50}$ was used for statistical calculations in studying the response differences. If a maximum response was not reached, then the EC$_{50a}$ was measured as the concentration of a chemical that resulted in 50% inhibition of the vasoconstrictor-induced tone.

The data are expressed as mean ± s.e.m of n number of animals. Statistical analyses were performed by GraphPad Prism® 7.04. Comparisons were made using the paired and unpaired Student’s t-test or one-way analysis of variance (ANOVA). Two-way ANOVA was also used when it is appropriate. Prior to the analysis of the results, each data set was tested for normal distribution by using the D’Agostino-Pearson omnibus normality test and/or Shapiro-Wilk normality test. If the data had followed a normal distribution, then a t-test was used. However, if the data set was not normally distributed or the sample number was low, a Mann-Whitney test was used.

ANOVA was used when comparing responses between more than two normally distributed data sets. The ordinary one-way ANOVA along with the Holm-Sidak’s multiple comparisons test was used for paired data. Otherwise, the nonparametric Kruskal-Wallis and Dunn's multiple comparisons test was used. Unpaired nonparametric datasets employed the Friedman and Dunn's multiple comparisons test.
Two-way ANOVA was used when there were two independent variables. Differences were considered as significant if \( P < 0.05 \).

### 2.9 Drugs and chemical reagents

The HMR1556, racemic chromanol 293B, (−)-[3R,4S]-chromanol 293B, ICA069673, RL-3 (L-364,373), ML277, Bay41-2272, gallein and iberiotoxin were purchased from Tocris Cookson (Bristol, UK), while phenylephrine hydrochloride, XE991 dihydrochloride, nifedipine, flupirtine maleate, retigabine, isoprenaline hydrochloride, SNP, forskolin, glibenclamide, nifedipine, levrcromakalim and carbachol had been obtained from Sigma-Aldrich (Poole, UK). As for U46619 (9,11-Dideoxy-9\( \alpha \),11\( \alpha \)-methanoepoxy prostaglandin \( \text{F}_2\alpha \), 9,11-Dideoxy-9\( \alpha \),11\( \alpha \)-methanoepoxy prosta5Z,13E-dien-1-oic acid) and Rp-8-Br-cAMPs (PKA inhibitor), these had been purchased from Enzo Life Sciences (Farmingdale, NY, USA) and BIOLOG Life Sciences (Bremen, Germany), respectively. The A23187, on the other hand was purchased from CALBIOCHEM (La Jolla, CA). GTN was purchased from David Bull laboratory (Victoria, Australia). The stock solutions (10mM) had been prepared either in water, DMSO or ethanol according to the manufacturers’ instruction and stored as aliquots at -20 °C, while the light-sensitive chemicals are stored in a light-resistant container. All pharmacological agents were diluted to the working concentrations in PSS as a way of maintaining a <0.1% vehicle volume in the final bath volume.

The advanced DMEM/F-12, Opti-MEM™ Reduced Serum Medium, the BSA were purchased from the Thermo Fisher SCIENTIFIC, Gibco brand (Grand Island, NY, USA). Also, Lipofectamine™ RNAiMAX Transfection Reagent and BLOCK-iT™ Alexa Fluor™ Red Fluorescent Control were purchased from the Thermo Fisher SCIENTIFIC, but Invitrogen brand (Carlsbad, CA, USA), while Penicillin-Streptomycin-Amphotericin B Mixture and N-TER™ Nanoparticle siRNA Transfection System were acquired from Lonza (Portsmouth, NH, USA) and SIGMA-ALDRICH (St. Louis, MO, USA), respectively.
CHAPTER 3
PHARMACOLOGICAL DISSECTION OF Kv7 CHANNEL
3.1 Background

Vessel size affects the sensitivity of the pulmonary arteries to pharmacological agents (Leach et al., 1989). Varying expression of receptors, depending on location in the vascular tree, results in variable responses to different agonists (Chootip et al., 2002). Variation in the distribution of electrophysiologically distinct PASMCs between conduit and resistance PA of rat resulted in different responses to NO and hypoxia (Archer et al., 1996). In particular, the K⁺ currents recorded from PASMCs showed heterogeneity along the PA tree both in rat (Archer et al., 1996; Smirnov et al., 2002) and rabbit (McCulloch et al., 2000). PASMCs in conduit vessels are enriched with BKCa channels, while myocytes in resistance PA are enriched with delayed rectifier K⁺ channels (Archer et al., 1996; Michelakis et al., 1997). Joshi et al. (2006, 2009) tested the effects of Kv7 channel modulators on vessels with external diameters ranging between 200 and 400 µm, but is not known if their effects vary in vessels of different diameters. Therefore, we investigated the difference in response between conduit (~900-1100 µm) and small (~300-450 µm) pulmonary arteries to drugs that modulate Kv7 activity.

Most studies investigated the role of Kv7.4 and/or Kv7.5 K⁺ channels in the vasculature (Brueggemann et al., 2007, 2014a; Jepps et al., 2011) and employed non-selective Kv7 blockers and activators. In pulmonary artery, pan Kv7 blockers (linopirdine and XE991) and activators of Kv7.2-Kv7.5 (flupirtine and retigabine) were all found to modulate pulmonary artery tone (Joshi et al., 2006, 2009). However, the KCNQ1 gene, which encodes Kv7.1 channel, has been shown to be consistently expressed in arterial smooth muscle cells of rodents and humans (Yeung et al., 2007; Joshi et al., 2009; Zhong et al., 2010; Ng et al., 2011), but, the role and functional impact of Kv7.1 channel remains unclear. RL-3, an activator of Kv7.1 K⁺ channel was shown to induce vasodilation in rat pulmonary and systemic arteries (Chadha et al., 2012a), but, the selectivity of RL-3 for Kv7.1 channel in evolving this effect was not investigated. Therefore, we investigated the functional role of Kv7.1 channels in conduit and small intrapulmonary artery, using Kv7.1 selective activators and blockers.

Some of the therapeutically effective drugs for PAH act by enhancing cyclic nucleotide signalling. Treprostinil is a prostacyclin analogue acts that by increasing the cAMP level in cells expressing IP receptors, and it induces vasodilation in the pulmonary arteries (Whittle et al., 2012). Sildenafil is a PDE5 inhibitor that induces vascular smooth muscle relaxation by inhibiting cGMP hydrolysis (Ballard et al., 1998;
Pauvert et al., 2003). In this way, it potentiates NO-stimulated vasodilation, which involves the activation of sGC to stimulate cGMP production. There is evidence that Kv7 K\(^+\) channels are involved in both cAMP and cGMP-dependent vasorelaxation. Blockers of Kv7 channels inhibited dilation of rat renal artery mediated by activation of the cAMP pathway (Chadha et al., 2012b; Mani et al., 2016b) and dilation of penile artery mediated by activation of the cGMP pathway (Stott et al., 2015b; Jepps et al., 2016). Chadha et al. (2012b) suggested that Kv7.4 K\(^+\) channels act as end target channels in the renal artery to mediate vasodilation post cAMP-PKA pathway activation, but Mani et al. (2016b) reported that the Kv7.5 channel is the main downstream target that of cAMP/PKA activation. The cGMP pathway has been proposed to activate Kv7 K\(^+\) channel as a downstream target to induce vasorelaxation (Stott et al., 2015b; Jepps et al., 2016). Kv7 channels could, therefore, be a common downstream end target for mediating cAMP and/or cGMP vasodilation in pulmonary arteries. If this is the case, interfering with the function of these channels in the pulmonary artery will impair the cAMP and/or cGMP signalling pathway. We investigated if Kv7 acts as a downstream target for cyclic nucleotides in pulmonary artery.

The aims of the experiments reported in this chapter were to:

1. Investigate the effect of IPA size on the response of Kv7 channel modulators.
2. Understand the functional role of Kv7.1 channel in pulmonary artery.
3. Probe the role of Kv7 channels as end target for cyclic nucleotide signalling in IPA.
3.2 Results

3.2.1 Responses of intrapulmonary arteries to vasoconstrictors

Raising the extracellular K⁺ concentration induces constriction of pulmonary artery by depolarising SMCs (Casteels et al., 1977a), which causes the influx of Ca²⁺ through voltage-gated Ca²⁺ channels. Adding KCl to rat pulmonary arteries induces constriction above 10 mM, with the maximum response at 50 mM (Gurney and Howarth, 2009). Figure 3.1A shows contractile responses of small and conduit IPAs to 10 and 50 mM KCl. The average response of small vessels to 50 mM KCl was significantly larger in amplitude than the constriction of conduit vessels, as shown in figure 3.2A. However, both conduit and small vessels showed negligible responses to 10 mM KCl.

The α₁-adrenoceptor agonist, phenylephrine, induces the maximum constrictive response in rat IPA at 1 μM (Gurney and Howarth, 2009). Conduit and small IPAs both contracted in response to 1 μM phenylephrine (Figure 3.1B), but the amplitude of constriction was significantly larger in conduit vessels than small vessels (Figure 3.2B). The response to phenylephrine amounted to 90 ± 4 % (n=10) of the response to 50 mM KCl in conduit vessels and 32 ± 5% (n=9) of the response to 50 mM KCl in small vessels.

The TP agonist, U46619, constricts pulmonary arteries with an EC₅₀ around 54 nM and maximum effect above 300 nM (McIntyre et al., 1996; Jeffery and Wanstall, 2001). The constriction induced by 30 nM U46619 was similar in amplitude in conduit and small arteries (Figure 3.1C and 3.2C), amounting to 75 ± 7% (n=4) of the response to 50 mM KCl in conduit vessels and 56 ± 10% (n=4) of the response to 50 mM KCl in small vessels. The amplitudes of contraction of conduit arteries to phenylephrine and U46619 were not significantly different. However, small vessels produced a more robust response to U46619 than phenylephrine (P= 0.0034, unpaired t-test).
Figure 3.1 Responses of pulmonary artery to vasoconstrictors.
Representative traces of tension generated upon application of 50 mM or 10 mM KCl (A), 1 µM phenylephrine (PE) (B) and 30 nM U46619 (C) of conduit (left-hand panels) and small (right-hand panels) intrapulmonary arteries. Calibration bars are 1 mN vertical and 5 min horizontal.
Figure 3.2 Influence of artery size on the contractile responses of pulmonary artery.
Summary of contractile responses to 50 mM or 10 mM KCl (A), 1 µM PE (B) and 30 nM U46619 (C) of conduit and small intrapulmonary arteries. Response to KCl and PE were measured 5 min and 30 min after exposure, respectively, while the peak response to U46619 was measured. Data plotted as mean ± s.e.m of experiments on vessels from n animals. * P<0.05, ***P<0.001 conduit vessels versus small vessels.
3.2.2 Responses of conduit and small intrapulmonary arteries to Kv7 channel blockers

To compare vasoconstrictive responses of conduit and small IPA to linopirdine, blood vessels were challenged with linopirdine at concentrations ranging from 10 nM to 30 μM, added cumulatively. Linopirdine induced constriction in a concentration-dependent manner in both conduit and small IPA, but the amplitude of constriction was seen to be greater in conduit vessels (Figure 3.3A). Figure 3.3B compares the relationship between linopirdine concentration and artery contraction in small and conduit vessels. Responses to linopirdine are measured as a percentage of the contraction induced by adding 50 mM KCl, to account for differences in contractility between the vessels to linopirdine. The maximum constriction was produced at around 10 μM linopirdine, but the mean maximum constriction was significantly larger in conduit (56 ± 7 %, n=5) than small (20 ± 3 %, n=5, P=0.0012, unpaired t-test) vessels. However, similar mean pEC50 was seen, i.e. in small arteries, it was 5.7 ± 0.2 (EC50=2.04 μM) and 5.4 ± 0.1 (EC50=3.72 μM) in conduit arteries.

To investigate the impact of blocking Kv7.1 K+ channels on the basal tone of IPA, vessels were challenged with the Kv7.1 blocker (HMR1556) at concentrations ranging from 0.1 to 1 μM, added cumulatively. HMR1556 did not affect basal tension in the absence of pre-tone. HMR1556 was tested on four separate arteries by incubating the mounted artery segments in contact with 1 µM HMR1556 for 1 hour or longer, but no response was detected. Since raising the tone of mesenteric and pulmonary arteries with a vasoconstrictor was found to enhance responses to Kv7 blockers (Chadha et al., 2012a), the effect of applying “pre-tone” on the response to HMR1556 was tested. Tone was raised by adding 1 µM phenylephrine or U46619 at 20, 40 or 80 nM. In these conditions, HMR1556 caused an additional contraction, as presented in figure 3.4A. When phenylephrine was present, HMR1556 evoked a concentration-dependent increase in tension. However, when the vessels were stimulated with U46619, HMR1556 contracted arteries by 5-15 % of constriction induced by 50 mM KCl, but the amplitude of the response did not increase with increasing HMR1556 concentration (Figure 3.4B). In fact, when 80 nM U46619 was employed, there was a decrease in response to HMR1556 with increasing concentration. Although U46619 revealed a contractile response to HMR1556, the effect was similar at all three U46619 concentrations.
Figure 3.3 Linopirdine constriction of conduit and small IPA.
A. Representative traces showing concentration-dependent contraction induced by linopirdine in small (upper panel) and conduit (lower panel) arteries. Calibration bars are 1 mN vertical and 20 min horizontal. B. Concentration-response relationship for constriction induced by linopirdine in conduit and small vessels. Constriction is plotted as percentage of the constriction induced by 50 mM KCl. Each point is plotted as the mean ± s.e.m of experiment on vessels from 5 animals.
Figure 3.4 HMR1556 caused some constriction in IPA in the presence of pre-tone. 
A. Representative traces showing concentration-dependent contraction induced by HMR1556 in arteries exposed to 1 μM phenylephrine or different concentration of U46619 as indicated. Calibration bars are 1 mN vertical and 10 min horizontal. B. Concentration-dependence of contraction induced by HMR1556 in arteries preconstricted with phenylephrine or U46619. Constriction responses of vessels to HMR1556 were normalised against constriction induced by 50 mM KCl. Each point is plotted as the mean ± s.e.m of vessels from n animals number, as indicated.
3.2.3 Response of intrapulmonary artery to Kv7 channel activators

3.2.3.1 Flupirtine relaxation in different sized vessels

To test whether the vasodilator response of flupirtine is different between conduit and small IPA segments, the vessels were pre-constricted with 30 nM U46619 or 1 μM phenylephrine and then were challenged with flupirtine over a concentration range of 0.1 μM to 200 μM, added cumulatively. The data obtained with phenylephrine (n=2) or U46619 (n=3) as the constrictor were combined. Flupirtine reduced the constriction in a concentration-dependent manner in both conduit and small IPA, as illustrated in figure 3.5A. The concentration-response relationships in conduit and small arteries are compared in figure 3.5B. The relaxation, induced at the highest concentration of flupirtine tested appeared to approach a maximum, reaching 83 ± 5 % (n=5) in conduit and 71 ± 9 % (n=5) in small vessels. As a clear maximum level was not achieved, the EC$_{50a}$ was measured as the concentration of flupirtine that inhibited the vasoconstrictor-induced tone by 50%. The mean pEC$_{50a}$ was similar for conduit and small vessels, being 4.71 ± 0.1 (EC$_{50a}$ =20 μM) and 4.56 ± 0.14 (EC$_{50a}$ =31 μM), respectively. To ensure that the obtained data for flupirtine were not affected by the use of two constrictors, we calculated the pEC$_{50a}$ and maximum response reached at 200 μM of flupirtine. The pEC$_{50a}$ and maximum response obtained by flupirtine were 4.39 and 67 %, respectively, when phenylephrine used and were 4.74 and 75 %, respectively, when the U46619 used.
Figure 3.5 Relaxation induced by flupirtine was similar in conduit and small vessels.
A. Representative traces showing concentration-dependent relaxation induced by flupirtine in conduit and small arteries pre-constricted with 30 nM U46619. Calibration bars are 1 mN vertical and 20 min horizontal. B, Log concentration-response curves were for flupirtine-induced relaxation of conduit and small arteries. Vessels were constricted with PE (n=2) or U46619 (n=3) and the data combined. Relaxation was measured as percentage of the U46619 or phenylephrine-induced constriction just before the addition of flupirtine. Each point is plotted as the mean ± s.e.m of vessels from 5 animals.
3.2.3.2 Vasodilation of pulmonary arteries to Kv7.1 activators

The Kv7.1 K+ channel activator, RL-3, relaxes mesenteric artery (Chadha et al., 2012a). To test whether Kv7.1 K+ channels can be activated to dilate the pulmonary vasculature, artery segments were pre-constricted with 30 nM U46619 (n= 3 to 4) or 1 μM phenylephrine (n=2) and then challenged with RL-3 at a concentration range of 0.01 μM to 5 μM, added cumulatively. RL-3 reduced the tension developed in the presence of vasoconstrictor in a concentration-dependent manner, in both conduit and small IPA. The data obtained with phenylephrine and U46619 were combined to produce the concentration-response relationships shown in figure 3.6. The maximum relaxation induced by RL-3 was similar in both types of vessel, reaching 93 ± 4 % (n=6) in conduit and 91 ± 10 % (n=5) in small arteries. The mean pEC50 was also similar for conduit and small vessels, being 6.34 ± 0.06 (EC50 =0.45 μM) and 6.25 ± 0.12 (EC50 =0.56 μM), respectively. As in the previous section, to check that the obtained data for RL-3 were not affected by the use of two constrictors, we calculated the pEC50 and maximum relaxation reached at 5 μM of RL-3. The results showed that the pEC50 and maximum relaxation for both conditions were similar.

![Figure 3.6](image.png)

**Figure 3.6** Artery size does not affect relaxation response to RL-3 in pulmonary vessels.

Log concentration-response curves were for RL-3-induced relaxation of conduit and small arteries constricted with PE or U46619. Relaxation was measured as percentage of the induced constriction just prior to the addition of RL-3. Data plotted as mean ± s.e.m of n number of vessels from different rats.
To further investigate the functionality of Kv7.1 K⁺ channels in the conduit pulmonary artery, the effect of ML277 (Kv7.1 activator) is assessed. Figure 3.7A shows relaxation responses to ML277 in the absence (upper panel) and presence (lower panel) of 1 μM HMR1556 (Kv7.1 inhibitor). Arteries were constricted with 1 μM phenylephrine in the absence or presence of 1 μM HMR1556, and then challenged with ML277 at 0.01 μM to 10 μM, added cumulatively. ML277 reduced the phenylephrine-stimulated constriction in a concentration-dependent manner, with or without HMR1556 present.

The effect of HMR1556 on the concentration-response relationship for ML277-induced relaxation is shown in figure 3.7B. HMR1556 shifted the ML277 log concentration-response curve to the right without affecting the maximum relaxation, which was 84 ± 3 % (n=8) in control conditions and 78 ± 4 % (n=8) in the presence of HMR1556. The mean pEC₅₀ was significantly reduced by HMR1556 from 6.74 ± 0.06 (EC₅₀ =0.18 μM, n=8) in its absence to 6.43 ± 0.07 (EC₅₀ =0.37 μM, n=8; p=0.005, unpaired two-tailed t-test) in its presence.
Figure 3.7 HMR1556 inhibits ML277-induced relaxation in conduit pulmonary artery.
A, Representative traces showing concentration-dependent relaxation induced by ML277 in conduit arteries pre-constricted with 1 µM phenylephrine in the absence (upper trace) or presence (lower trace) of 1 µM HMR1556. Calibration bars are 1 mN vertical and 20 min horizontal. B, Log concentration response curve for ML277-induced relaxation of arteries in the absence or presence of 1 µM HMR1556. Relaxation was measured as percentage of the phenylephrine-induced constriction just prior to the addition of ML277. Data plotted as mean ± s.e.m of vessels from 8 animals.
3.2.3.3 Evidence for potassium channel involvement in relaxation to Kv7 activators

The effects of the drugs were also tested on arteries that were constricted by replacing the PSS with KPSS containing 80 mM K\(^+\) (Figure 3.8A and 3.9A, lower panel). If the effects of the Kv7.1 activators were due to the activation of Kv7 channels, they should be prevented by depleting the trans-membrane K\(^+\) concentration gradient (Casteels et al., 1977a). The experiments were carried out on the conduit arteries by comparing the relaxation responses to RL-3 and ML277 when arteries were contracted by phenylephrine (1 μM) or high concentration of K\(^+\) (80 mM KPSS). RL-3 (0.1, 0.5 and 1 μM) (figure 3.8A) or ML277 (0.03, 0.3 and 1 μM) (figure 3.9A), were added cumulatively to vessels contracted with either stimulant, and the resulted relaxation was quantified as a percentage. Figure 3.8 B and 3.9 B, compares the concentration-relaxation response curves for RL-3 and ML277, respectively, when arteries were contracted by phenylephrine or high concentration of K\(^+\).

RL-3 and ML277 reduced the constriction activated by phenylephrine in a concentration-dependent manner, reaching over 70% relaxation at 1 μM. Below 0.3 μM, neither Kv7.1 activator caused relaxation of K\(^+\)-activated vessels. At higher concentrations, both Kv7.1 activators caused relaxation of vessels pre-constricted with K\(^+\), but the responses were small in comparison to those evoked in phenylephrine-constricted vessels. Thus, although depleting the K\(^+\) gradient reduced relaxations to RL-3 and ML277, it did not abolish them.
Figure 3.8 Relaxation to RL-3 was not abolished by depleting the K⁺ gradient.  
A. Representative traces demonstrating concentration-dependent relaxation induced by RL-3 in conduit arteries pre-constricted with 1 μM phenylephrine (upper panel) or 80 mM KPSS (lower panel). Calibration bars are 1 mN vertical and 20 min horizontal. B, relaxation response induced by (0.1, 0.5 and 1 μM) RL-3 added cumulatively to arteries pre-constricted with 1 μM phenylephrine or 80 mM KPSS. Relaxation was measured as percentage of phenylephrine or KPSS induced constriction just prior to the addition of RL-3. Data plotted as mean ± s.e.m of 5 experiments. ** P<0.01, **** P<0.0001 vessel pre-constricted with KPSS versus vessel pre-constricted with phenylephrine, (Two-way ANOVA followed by Sidak multiple comparisons test).
Figure 3.9 Relaxation to ML277 was not voided by depleting the K⁺ gradient.  
A. Representative traces showing concentration-dependent relaxation induced by ML277 in conduit arteries pre-constricted with PE (1 µM, upper panel) or KPSS (80 mM, lower panel). Calibration bars are 1 mN vertical and 20 min horizontal.  
B, relaxation response induced by 0.03, 0.3 and 1 µM ML277 added cumulatively to arteries constricted with phenylephrine or 80 K⁺. Relaxation was measured as percentage of PE or K⁺ induced constriction just prior to the addition of ML277. Data plotted as mean ± s.e.m of vessels dissected from 5 rats. **** P<0.0001 vessel pre-constricted with K⁺ versus vessel pre-constricted with PE, (Two-way ANOVA followed by Sidak multiple comparisons test).
Then for further investigating the extent of K⁺ channel involvement in the relaxing effect of Kv7 channel activators, we tested RL-3 and flupirtine mediated vasodilation in conduit and small arteries, and over a wider range of concentrations. RL-3 concentration-response curves when conduit and small arteries were contracted by U46619 compared with high K⁺ contracted vessels are shown in figure 3.10 A. Responses to RL-3 were smaller in K⁺-constricted conduit arteries across the entire RL-3 concentration range. In small arteries, relaxation was significantly smaller in K⁺-contracted arteries, except at the highest RL-3 concentration, where it approached the maximum relaxation measured in U46619-contracted arteries.

Relaxation responses to flupirtine, a pan-Kv7 activator, were also compared in conduit and small arteries when contraction was evoked by U46619 or K⁺. The relaxing effect of flupirtine was significantly reduced in pulmonary arteries pre-constricted with 80 mM K⁺ compared with U46619, except at the highest concentration (200 µM) of flupirtine (figure 3.10 B).
Figure 3.10 RL-3 and flupirtine relax arteries less effectively when contracted by high K⁺ concentration.

Summary of relaxation response induced by 0.1-3 μM RL-3 (A), or 10-200 μM flupirtine (B), added cumulatively to conduit or small pulmonary arteries pre-constricted with 30 nM U46619 or 80 mM K⁺. Relaxation was measured as percentage of the induced constriction measured just before the addition of RL-3 or flupirtine. Data plotted as mean ± s.e.m. Number of experiments (n) as indicated above. * P<0.05, ** P<0.01, *** P<0.001 compared with conduit vessel pre-constricted with U46619. # P<0.05, ## P<0.01, ### P<0.001 compared with small vessel pre-constricted with U46619. Two-way ANOVA followed by Sidak for multiple comparisons test.
3.2.3.4 Effects of Kv7.1 blockers of relaxation to Kv7 activators

3.2.3.4.1 HMR1556 antagonism of relaxation induced by Kv7.1 activators

To test whether RL-3 mediated relaxation in the pulmonary vasculature is mediated through activation of Kv7.1 K+ channels, its effects on conduit artery segments pre-constricted with 1 μM phenylephrine were tested in the absence or presence of HMR1556 (0.1, 0.3, 1 or 10 μM). Vessels were challenged with RL-3 at a concentration range of 0.01 μM to 5 μM, added cumulatively. RL-3 inhibited the constriction induced by phenylephrine in a concentration-dependent manner, in the absence or presence of HMR1556, as shown in figure 3.11A. The sensitivity to RL-3 was, however, reduced in the presence of HMR1556 (figure 3.11B). HMR1556 shifted the concentration-response curve of RL-3 to the right in a concentration-dependent manner, with little effect on the maximum response, which was 95 ± 3 % (n= 12) in control conditions and 84 ± 14 % (n=8) in the presence of 10 μM HMR1556. The mean pEC$_{50}$ was significantly reduced (P<0.0001, unpaired two-tailed t-test) by HMR1556, being 6.01 ± 0.14 (EC$_{50}$ =0.97 μM) in its presence and 6.59 ± 0.05 (EC$_{50}$ =0.26 μM) in its absence.

The rightward shift in the log concentration-response curve produced by HMR1556 is compatible with a competitive antagonist action. To test if HMR1556 acts as a competitive inhibitor to RL-3, Schild analysis (Arunlakshana and Schild, 1959; Wylie and Chen, 2007) was applied, as shown in figure 3.12. The log (concentration ratio -1) was plotted against the log of the HMR1556 concentration, where the concentration ratio is the RL-3 EC$_{50}$ at each HMR1556 concentration divided by the EC$_{50}$ measured in the absence of the HMR1556. The Schild plot yielded a straight line with slope equals to 0.18 and x-axis intercept at -7.46.
- PHARMACOLOGICAL DISSECTION OF Kv7 CHANNEL -

A

PE  0.01  0.05  0.1  0.5  1  5 (μM)

0.1 μM PE HMR1566

0.3 μM PE HMR1566

1 μM PE HMR1566

10 μM PE HMR1566

[RL-3]
Figure 3.11 HMR1556 shifts the RL-3 concentration-relaxation curve to the right.  
A, Representative traces showing concentration-dependent relaxation induced by RL-3 in conduit arteries constricted with 1 μM phenylephrine in the absence or presence of different concentration of HMR1556. Calibration bars are 1 mN vertical and 20 min horizontal.  
B, Log concentration-response curves for RL-3-induced relaxation of arteries in the absence or presence of HMR1556 at 0.1, 0.3, 1 and 10 μM. Relaxation was measured as percentage of the phenylephrine-induced constriction measured just before the addition of RL-3. Data plotted as mean ± s.e.m of vessels from n rats.

Figure 3.12 Schild analysis of HMR1556 antagonism of RL-3-induced vasodilation. 
Schild plot performed with data derived from HMR1556 concentration-response curve in figure 3.11. The concentration ratio (CR) is the ratio of RL-3 EC₅₀ values in the presence and absence of HMR1556. Line is best fit to the data by linear regression with slope = 0.18, x-intercept = -7.464, R² = 0.93.
3.2.3.4.2 Effect of chromanol 293B on RL-3-induced relaxation

The ability of the Kv7.1 channel blocker, chromanol 293B, to antagonise the dilator effect of Kv7.1 activators was also tested. Both the racemates (±293B) and the (-)-[3R, 4S] enantiomer (-293B) of chromanol 293B were tested, as the latter is 7-fold more potent at inhibiting Kv7.1 channels (Yang et al., 2000). Conduit vessels were pre-constricted with 1 μM phenylephrine in the absence or presence of 1 or 10 μM ±293B or 1 μM -293B. They were then challenged with RL-3 at concentrations between 0.01 μM and 5 μM, added cumulatively. As before, RL-3 reduced the phenylephrine constriction in a concentration-dependent manner. As illustrated in figure 3.13A, it remained effective in the presence of chromanol 293B, whether present as the racemate or enantiomer. The concentration-response relationship for the RL-3 relaxation in the absence or presence of chromanol is shown in figure 3.13B. The maximum relaxation induced by RL-3 was 90 ± 5 % (n= 6) in the presence of 1 μM ± 293B, 94 ± 6 % (n= 6) in the presence of 10 μM of ±293B and 97 ± 6 % (n= 9) in the presence of 1 μM of -293B. These values are similar to the maximum relaxation induced by RL-3 in the absence of any blocker. Similarly, in the presence of 1 or 10 μM ±293B the pEC$_{50}$ was 6.71 ± 0.1 (EC$_{50}$ =0.20 μM) and 6.71 ± 0.1 (EC$_{50}$ =0.19 μM), respectively. In the presence of 1 μM -293B the pEC$_{50}$ was 6.73 ± 0.09 (EC$_{50}$ =0.19 μM). These values are all similar to the pEC$_{50}$ of the RL-3 relaxation measured in the absence of any blocker, which was in this series of experiments and it was 6.59 ± 0.05 (EC$_{50}$ =0.26 μM, n=12).
Figure 3.13 Chromanol 293B has no effect on relaxation induced by RL-3.

A, Representative traces showing concentration-dependent relaxation induced by RL-3 in phenylephrine (1μM) constricted conduit arteries in the presence of 1 μM ±293B or -293B. Calibration bars are 1 mN vertical and 20 min horizontal. B, Log concentration-response curve for RL-3-induced relaxation of conduit arteries in the absence or presence of 1 or 10 μM ±293B or 1 μM -293B. Relaxation was measured as percentage of the phenylephrine-induced constriction just prior to the addition of RL-3. Data plotted as mean ± s.e.m of vessels from n animals.
3.2.3.4.3 Relaxation of conduit intrapulmonary arteries to retigabine

Retigabine is a Kv7 activator that does not activate Kv7.1 channels (Wuttke et al., 2005), so its relaxing effect on pulmonary artery should be resistant to block by Kv7.1 inhibitors. As a negative control for the effect of HMR1556 on relaxation to RL-3 and ML277, the effects of Kv7.1 blockers were tested on relaxation responses to retigabine. Vessels were pre-constricted with 1 μM phenylephrine in the absence or presence of 10 μM HMR1556 or ±293B, or 1-10 μM -293B. They were then challenged with retigabine at concentrations of 0.01 μM to 200 μM, added cumulatively. Retigabine reduced the constriction evoked by phenylephrine in a concentration-dependent manner in the absence or presence of Kv7.1 channel blockers, as shown in figure 3.14A. The concentration-response relationships for retigabine-induced relaxation are compared in the different conditions in figure 3.14B. Neither HMR1556, nor chromanol 293B inhibited the relaxation response to retigabine in pulmonary arteries.

Retigabine relaxed vessels with a maximum response of 95 ± 3 % (n=9) inhibition of the phenylephrine-induced tone and pEC_{50} =5.30 ± 0.06 (EC_{50} =5 μM). In the presence of 10 μM HMR1556 these values were unchanged 97 ± 3 % (n=7) at maximum and pEC_{50} =5.43 ± 0.08 (EC_{50} =3.7 μM). At 10 μM, ±293B also had no effect, the maximum relaxation being 93 ± 3 %, (n=3) and pEC_{50} =5.34 ± 0.07 (EC_{50} =4.5 μM). In the presence of 1 μM -293B, the maximum relaxation (96 ± 4 %, n=4) and pEC_{50} (5.55 ± 0.1, EC_{50} =2.8 μM) were also unchanged relative to retigabine in the absence of blockers. At 10 μM -293B, the retigabine concentration-response curve appeared to be shifted towards lower concentrations. The maximum relaxation (102 ± 3 %, n=4) was not changed, but, the pEC_{50}, at 5.67 ± 0.09 (EC_{50} = 2.15 μM), was significantly increased in comparison with the control (P<0.05, one-way ANOVA, followed by a Dunnett’s multiple comparisons test).
Figure 3.14 Effect of Kv7.1 channel blockers on retigabine-evoked relaxation.
A, Representative traces demonstrating concentration-dependent relaxation evoked by retigabine. Conduit arteries constricted with 1 µM phenylephrine in the absence or presence of 10 µM HMR1556, ±293B or -293B (1 or 10 µM). Calibration bars are 1 mN vertical and 20 min horizontal. B, Log concentration-response curve for retigabine-induced relaxation of conduit arteries in the absence or presence of HMR1556, ±293B or -293B at the concentration stated. Relaxation was measured as percentage of the phenylephrine-evoked constriction just prior to the addition of retigabine. Data plotted as mean ± s.e.m of arteries from n number of rats.
3.2.3.5 Do \(K_{\text{ATP}}\) channels contribute to the effects of Kv7.1 modulators?

Drugs that activate \(K_{\text{ATP}}\) channels cause vasodilation (Brayden, 2002). To exclude a role of \(K_{\text{ATP}}\) \(K^+\) channels in mediating the relaxing effect of RL-3, the Kv7.1 activator was tested in the presence of the \(K_{\text{ATP}}\) channel blocker, glibenclamide. As shown in figure 3.15A, RL-3 retained its ability to relax conduit IPAs that were pre-constricted with 1 \(\mu\)M phenylephrine in the presence of 1 \(\mu\)M glibenclamide. Rather than inhibiting the relaxation induced by RL-3, glibenclamide appeared to enhance relaxation, shifting the RL-3 concentration-response curve to the left. The maximum relaxation induced by RL-3 in the presence of 1 \(\mu\)M of glibenclamide was 103 ± 4 % (n=6), similar to the maximum relaxation measured in the absence of glibenclamide, in this series of experiments. In the presence of 1 \(\mu\)M glibenclamide, the pEC\(_{50}\) was 6.86 ± 0.06 (EC\(_{50}\) = 0.14 \(\mu\)M, n=6), which is not significantly different from the pEC\(_{50}\) in control conditions.

The effect of HMR1556 (1 \(\mu\)M) on the relaxation induced by the \(K_{\text{ATP}}\) channel activator, levocromakalim, was also tested. As shown in figure 3.15B, vessels pre-constricted with 1 \(\mu\)M phenylephrine with or without HMR1556, relaxed in response to levocromakalim (0.01 \(\mu\)M to 10 \(\mu\)M), applied cumulatively. Levocromakalim produced a similar, concentration-dependent relaxation in the absence and presence of HMR1556. The maximum relaxation was 93 ± 3 % (n=5) under control conditions and 92 ± 4 % (n=4) in the presence of 1 \(\mu\)M HMR1556. The mean levocromakalim pEC\(_{50}\) in the absence of HMR1556 was 7.37 ± 0.52 (EC\(_{50}\) = 42.6 nM), compared with 7.34 ± 0.57 (EC\(_{50}\) =45.9 nM) in its presence. Thus, HMR1556 had no effect on the relaxation mediated by the activation of \(K_{\text{ATP}}\) channels.
Figure 3.15 Effects of RL-3 and HMR1556 on pulmonary arteries are independent of KATP potassium channels.

A. Log concentration-response curves were for RL-3-induced relaxation of arteries in the absence or presence of 1 µM glibenclamide. B. Log concentration-response curves were for levcromakalim-induced relaxation of arteries in the absence or presence of 1 µM HMR1556. Relaxation was measured as percentage of the phenylephrine-induced constriction measured just before the addition of RL-3 in A, and levcromakalim in B. Data plotted as mean ± s.e.m of vessels from n animals.
3.2.3.6 Vascular action of ICA069673

ICA069673 was developed as a Kv7 K\(^+\) channel activator with selectivity for heteromeric Kv7.2/ Kv7.3 over Kv7.3/ Kv7.5 (Amato et al., 2011), but at micromolar concentrations, it also activates homomeric and heteromeric Kv7.4 channels (Brueggemann et al., 2014a). It has little effect on homomeric Kv7.5 channels or cardiac Kv7.1/KCNE1 channels (Amato et al., 2011; Brueggemann et al., 2014a). To further examine the subtypes of Kv7 K\(^+\) channel that induces relaxation in IPA, testing of ICA069673 was done in arteries that were pre-constricted with 1 μM phenylephrine with or without 1 μM HMR1556. Cumulative addition of ICA069673 was done at concentration ranging from 0.01 μM to 100 μM. It decreased the constriction caused by phenylephrine in a concentration-dependent manner in the absence or presence HMR1556, as demonstrated in figure 3.16A. The ICA069673 concentration-response relationship is shown in figure 3.16B. HMR1556 did not affect the relaxation response to ICA069673 in pulmonary arteries. ICA069673 produced a maximum relaxation of 104 ± 4 % (n=4) and pEC\(_{50}\) =5.22 ± 0.05 (EC\(_{50}\) =6 μM). In the presence of 1 μM HMR1556 these values were unchanged, at 101 ± 6 % (n=4) maximum relaxation and pEC\(_{50}\) = 5.2 ± 0.09 (EC\(_{50}\) =6.3 μM).

To test whether ICA069673 induced vasodilation by activating K\(^+\) channels, its relaxing effects were compared between vessels pre-constricted with 1 μM phenylephrine or 80 mM K\(^+\). As demonstrated in figure 3.17A, the constricted vessels were compared against rising concentrations of ICA069673 (0.1, 3 and 30 μM), which were added cumulatively. As presented in figure 3.17B, the relaxation induced by ICA069673 was almost abolished by depleting the transmembrane K\(^+\) gradient. As noted above, in phenylephrine-constricted arteries ICA069673 evoked relaxation at concentrations above 0.1 μM and caused almost complete relaxation at 30 μM. In contrast, when arteries were constricted with 80 mM K\(^+\), ICA069673 had no effect at concentrations below 30 μM and the relaxation at 30 μM was no more than 10%. Therefore, the relaxations induced by ICA069673 were entirely impaired by depleting the K\(^+\) gradient.
Figure 3.16 Relaxation of pulmonary arteries by ICA069673.
A. Representative traces showing concentration-dependent relaxation induced by ICA069673 in conduit arteries pre-constricted with phenylephrine (1 µM) in the absence (upper panel) or presence (lower panel) of HMR1556 (1 µM). Calibration bars are 1 mN vertical and 20 min horizontal. B. Log concentration-response curve for ICA069673-evoked relaxation of arteries in the absence or presence of HMR1556. Relaxation was measured as percentage of the phenylephrine-induced constriction just before the addition of ICA069673. Data plotted as mean ± s.e.m of vessels from 4 animals.
Figure 3.17 Relaxation to ICA069673 depends on the transmembrane K⁺ gradient.

A. Representative traces showing concentration-dependent relaxation of conduit arteries by ICA069673. Vessels were pre-constricted with 1 µM phenylephrine or 80 mM KPSS. Calibration bars are 1 mN vertical and 10 min horizontal.

B. Relaxation responses induced by ICA069673 (0.1, 3 and 30 µM), added cumulatively to arteries constricted with 1 µM phenylephrine or 80 mM K⁺. Relaxation was measured as percentage of the phenylephrine or K⁺-induced constriction, just prior to the addition of ICA069673. Data plotted as mean ± s.e.m of vessels from 4 animals. **** P<0.0001 compared with vessels pre-constricted with 80 K⁺. Two-way ANOVA followed by Sidak multiple comparisons test.
3.2.4 Kv7 potassium channels in cAMP-mediated vasodilation

3.2.4.1 Kv7 potassium channels in mediating β-adrenoceptor stimulating vasorelaxation

To determine if the Kv7 K+ channels are involved in β-adrenoceptor mediated vasorelaxation in the pulmonary vasculature, the ability of Kv7 antagonist to interfere with it was examined. Conduit pulmonary arteries were constricted with phenylephrine and challenged with β-adrenoceptor agonist, isoprenaline, at a concentration range of 10 nM to 1 μM, added cumulatively. As demonstrated in figure 3.18A, isoprenaline decreased the phenylephrine-stimulated constriction in a concentration-dependent manner with or without drugs blocking the Kv7 or BKCa K+ channels added alone or in combination. Figure 3.18B shows the concentration-response relationship for isoprenaline-induced relaxation. Kv7 blockers did not abolish the relaxation response to isoprenaline; however, linopirdine and XE991 shifted isoprenaline concentration-response curve to the right without affecting the maximum response induced by isoprenaline.

Isoprenaline relaxed arteries with pEC50= 8.08 ± 0.1 (EC50= 8 nM) and maximum response of 98 ± 4 % (n=8) above 100 nM. None of the K+ channel blockers altered the maximum response to isoprenaline, which ranged from 88 % in the presence of 5 μM linopirdine and 1 μM HMR1556 to 99 % in the presence of iberiotoxin. The isoprenaline concentration-response relationship was, however, shifted to the higher concentration when it was applied in the presence of linopirdine (1 or 5 μM) or XE991 (1 μM). Linopirdine reduced the pEC50 to 7.73 ± 0.09 (n=7, P=0.031 with ANOVA followed by Dunnett’s multiple comparison test) at 1 μM and to 7.63 ± 0.05 (n=4, P=0.016) at 5 μM. Similar values were obtained at 1 and 5 μM linopirdine, implying that the maximum inhibition had been reached. XE991 reduced the pEC50 to 7.53 ± 0.07 (n=11, P<0.0001), comparable to that measured in the presence of linopirdine. HMR1556 also appeared to shift the isoprenaline concentration-response curve to higher concentration, but the pEC50 measured in the presence of HMR1556 (7.89 ± 0.14, n=5) did not differ significantly from the control.

Stimulation of the cAMP pathway in rat pulmonary artery smooth muscle has been reported to activate BKCa channels (Barman et al., 2003). Despite that, the selective BKCa channel blocker, iberiotoxin (100 nM), did not affect the isoprenaline concentration-response curve (Figure 3.18B) which retained a pEC50 of 8.07 ± 0.08 (n=4). When iberiotoxin was added with XE991 (1 μM), the isoprenaline pEC50 was 7.38 ± 0.04 (n=4, P=0.0001) close to the value obtained when only XE991 was present.
- PHARMACOLOGICAL DISSECTION OF Kv7 CHANNEL -

A

[Graph showing PE, PE + Ibtx, PE + XE991, PE + Ibtx + XE991 with concentration axis labeled as [isoprenaline], (M)]
Figure 3.18 Relaxation induced by isoprenaline was inhibited by pan Kv7 potassium channel blockers.

A, Representative traces demonstrating concentration-dependent relaxation produced by isoprenaline in conduit arteries pre-constricted with 1 µM phenylephrine in the absence or presence of 1 µM XE991, 100 nM iberiotoxin or 100 nM iberiotoxin plus 1 µM XE991. Calibration bars are 1 mN vertical and 5 min horizontal. B, Log concentration-response curves were for isoprenaline-induced relaxation of arteries in the presence or absence of 1 or 5 µM linopirdine, 1 µM XE991, 1 µM HMR1556, 100 nM iberiotoxin or 100 nM iberiotoxin combined with 1 µM XE991. Arteries were constricted with 1 µM phenylephrine. Relaxation was measured as percentage of the phenylephrine-induced constriction just before the addition of isoprenaline. Data plotted as mean ± s.e.m of vessels from n number of animals.
3.2.4.2  Kv7 potassium channels in cAMP-dependent dilation of pulmonary artery
Forskolin is a direct activator of adenyl cyclase and therefore, bypasses β-AR (Stief et al., 2000; Barman et al., 2003). We, therefore, tested the effects of Kv7 channel blockers on forskolin-mediated vasorelaxation. Conduit artery segments were pre-constricted with 1 μM phenylephrine in the absence or presence of XE991 (1 μM), HMR1556 (1 μM), or linopirdine (1-5 μM) then, challenged with forskolin at a concentration range of 1 nM to 3 μM, added cumulatively. Forskolin reduced the constriction activated by phenylephrine in a concentration-dependent manner in the absence or presence of Kv7 channel blockers, as shown in figure 3.19A. The effects of blockers on forskolin concentration-relaxation are shown in figure 3.19B. None of the Kv7 channel blockers affected the maximum relaxation response to forskolin, which relaxed vessels with pEC50 =7.25 ± 0.04 (EC50= 57 nM, n=6), reaching a maximum response of 104 ± 2 % at 1 μM. In the presence of 1 μM HMR1556, the forskolin concentration response unchanged with pEC50 =7.29 ± 0.05 (EC50 52 nM, n=6). In contrast, both linopirdine and XE991 shifted the forskolin concentration-response curve to a higher concentration. When 1 μM of linopirdine was present, forskolin pEC50 value was 6.98 ± 0.07 (EC50 104 nM, n=7, P=0.0045 via ANOVA along with Dunnett’s post-tests); in the presence of 5 μM of linopirdine, it was 6.8 ± 0.04 (EC50 = 159 nM, n=4, P<0.0001) and in the presence of 1 μM XE991, it was 6.72 ± 0.07 (EC50 = 190 nM, n=4, P<0.0001).
PHARMACOLOGICAL DISSECTION OF Kv7 CHANNEL

A

PE

HMR1556

Linopirdine

XE991

[forskolin], (M)
Figure 3.19 Effect of Kv7 channel blockers on forskolin-induced vasorelaxation.
A. Representative traces showing concentration-dependent relaxation evoked by forskolin in arteries constricted with phenylephrine in the absence or presence of 1 µM HMR1556, XE991 or linopirdine. Calibration bars are 1 mN vertical and 10 min horizontal B. Log concentration-response curves were for forskolin-mediated vasorelaxation of conduit arteries in the absence or presence of linopirdine (1 or 5 µM), XE991 (1 µM) or HMR1556 (1 µM). Relaxation was measured as percentage of the phenylephrine-induced constriction just prior to the addition of forskolin. Data plotted as mean ± s.e.m of vessels from n number of animals.
3.2.4.3 *Kv7 potassium channels in treprostinil-induced dilation of pulmonary artery*

Prostacyclin causes vasodilation by stimulating IP receptors, which also couple to the Gαs protein and stimulate AC, cAMP, PKA pathway (Narumiya *et al.*, 1999; Majed and Khalil, 2012; Clapp and Gurung, 2015). Vasorelaxation responses to treprostinil were compared in conduit and small IPA. Arteries were constricted with 30 nM U46619 and then challenged with treprostinil at concentrations from 0.1 nM to 1 μM. Treprostinil induced relaxation in a concentration-dependent manner, but the peak relaxation appeared to be higher in conduit vessels (figure 3.20A). Figure 3.20B compares the concentration-response relationships for treprostinil in conduit and small arteries. The maximum relaxation, reached at around 1 μM treprostinil was 86 ± 11 % (n=3) in conduit arteries and 49 ± 9 % (n=3, P=0.0603 unpaired two-tailed t-test) in small vessels. The mean treprostinil pEC\(_{50}\) was similar for conduit and small vessels, being 7.18 ± 0.18 (EC\(_{50}\) =66 nM) and 7.85 ± 0.37 (EC\(_{50}\) = 14 nM), respectively.

Similarly, the relaxing effect of treprostinil was also tested in conduit vessels that were pre-constricted with 1 μM phenylephrine. Treprostinil induced concentration-dependent relaxation (figure 3.21A). The concentration-response curves for treprostinil in conduit arteries is shown in figure 3.21B. The maximum relaxation response induced by treprostinil was 76 ± 4 % (n=6) and the pEC\(_{50}\) was 7.92 ± 0.16 (EC\(_{50}\) =12 nM).

The sensitivity of the conduit arteries to treprostinil was significantly higher when they were pre-constricted by 1 μM phenylephrine compared with 30 nM U46619 (P value was 0.026 unpaired two-tailed t-test), but, the maximum response, reached by treprostinil was comparable.

To test whether Kv7 channels are involved in mediating the relaxing effect of treprostinil in pulmonary artery, the ability of Kv7 channel blockers to interfere with its effect was investigated. Conduit pulmonary arteries were constricted with 1 μM phenylephrine in the presence of 1 μM linopirdine, XE991 or HMR1556, then challenged with treprostinil at same concentrations range as control condition. Treprostinil reduced the constriction evoked by phenylephrine in a concentration-dependent manner, as presented in figure 3.21A. The concentration-response relationship for treprostinil in the presence of Kv7 blockers compared with control condition, as shown in figure 3.21B. HMR1556 did not inhibit the relaxation induced by treprostinil, but, linopirdine and XE991 both caused marked inhibition at all effective treprostinil concentrations. The maximum relaxation response was reduced to 42 ± 4 % (n=6, P<0.0001, by One-way ANOVA followed with Dunnett’s post-tests) in the presence of 1 μM linopirdine and 32 ± 3 % (n=6, P<0.0001) in the presence of 1 μM XE991. In contrast, none of the Kv7 channel blockers affected the pEC\(_{50}\) of treprostinil.
which was $7.78 \pm 0.29$ (EC$_{50}$ = 17 nM) in the presence of linopirdine and $7.87 \pm 0.27$ (EC$_{50}$ = 14 nM) in the presence of XE991.

**Figure 3.20 Effect of artery size on treprostinil-induced vasorelaxation.**

A, Representative traces showing concentration-dependent relaxation induced by treprostinil in conduit and small arteries preconstricted with 30 nM U46619. Calibration bars are 1 mN vertical and 10 min horizontal. B, log concentration-relaxation curves induced by treprostinil (0.1 nM - 1 µM) added cumulatively to conduit or small arteries pre-constricted with 30 nM U46619. Relaxation was measured as percentage of the U46619-evoked constriction just before the addition of treprostinil. Data plotted as mean ± s.e.m of vessels from 3 animals.
PHARMACOLOGICAL DISSECTION OF Kv7 CHANNEL

A

PE

HMR1556

Linopirdine

XE991

[treprostinil], (M)
Figure 3.21 Treprostinil-induced vasodilation was inhibited by pan Kv7 channel blockers.

A. Representative traces showing concentration-dependent relaxation induced by treprostinil in conduit arteries preconstricted with 1 µM phenylephrine in the absence or presence of 1 µM linopirdine, XE991 or HMR1556. Calibration bars are 1 mN vertical and 10 min horizontal. B, Log concentration-response curve for treprostinil-evoked relaxation of conduit arteries in the absence or presence of HMR1556, linopirdine or XE991. Relaxation was measured as percentage of the phenylephrine-induced constriction just prior to the addition of treprostinil. Data plotted as mean ± s.e.m of arteries from 6 animals.
3.2.5  Kv7 potassium channels in cGMP-mediated vasodilation

3.2.5.1  Effects of Kv7 blockers on relaxation to NO donors

To investigate whether Kv7 K+ channels are involved in mediating cGMP-dependent vasorelaxation in pulmonary artery, we tested if Kv7 channel blockers could interfere with the relaxing effects of nitric oxide donors, SNP and GTN. Phenylephrine was used to constrict vessels in the absence or presence of 1 μM XE991, linopirdine or HMR1556, (figure 3.22A), and then challenged with SNP at concentrations from 0.1 nM to 3 μM, which were added cumulatively. SNP concentration-response relationships in the absence or presence of Kv7 blockers were compared in figure 3.22B. HMR1556 had no effect on the relaxation induced by SNP at any concentration. In contrast, linopiridine and XE991 significantly reduced the maximum relaxation response to SNP from 86 ± 3 % (n=9) in control conditions to 54 ± 2 % (n= 7, P<0.0001 by ANOVA followed by Dunnett’s post-test) after linopiridine addition and 39 ± 4 % (n=5, P<0.0001) upon exposure to XE991. The pEC50 of SNP was similar in control conditions (7.72 ± 0.07), with presence of linopiridine (7.67 ± 0.07) or XE991 (7.4 ± 0.22).

The effect of GTN on vessels that were constricted with phenylephrine in the absence or presence of Kv7 K+ channel blockers applied at 1 μM, is illustrated in figure 3.23A. In figure 3.23B compares the GTN concentration-relaxation curves obtained under various conditions. HMR1556 had no effect on relaxation induced by GTN at any concentration, but the maximum relaxation response was significantly reduced to GTN with linopiridine and XE991, which was 61 ± 2 % (n=6) in control condition, but dropped to 28 ± 2 % (n= 6, P<0.0001) in the presence of linopiridine and 22 ± 2 % (n=6, P<0.0001) in the presence of XE991. None of the drugs altered the pEC50 of GTN, which in control condition was 7.44 ± 0.07; when linopiridine was present, it was 7.34 ± 0.14 and 7.36 ± 0.15 when XE991 was present.
Figure 3.22 SNP mediated vasorelaxation was impaired by Kv7 channel blockers. 
A. Representative traces showing concentration-dependent relaxation were induced by SNP in conduit arteries pre-constricted with 1 µM phenylephrine in the absence (upper panel) or presence (lower panel) of 1 µM XE991. Calibration bars are 1 mN vertical and 10 min horizontal. B. Log concentration-response curves were for SNP-induced relaxation of conduit arteries in the absence or presence of 1 µM HMR1556, linopirdine or XE991. Relaxation was measured as percentage of the phenylephrine-induced constriction just prior to the addition of SNP. Data plotted as mean ± s.e.m of vessels from n animals.
Figure 3.23 GTN mediated vasorelaxation was impaired by Kv7 channel blockers. 

A, Representative traces showing concentration-dependent relaxation induced by GTN in conduit arteries preconstricted with 1 µM phenylephrine in the absence or presence of 1 µM linopirdine. Calibration bars are 1 mN vertical and 10 min horizontal. 

B, Log concentration-response curves for GTN-induced relaxation of conduit arteries in the absence or presence of 1 µM HMR1556, linopirdine or XE991. Relaxation was measured as percentage of the phenylephrine-induced constriction just before the addition of GTN. Data plotted as mean ± s.e.m of vessels from 6 rats.
3.2.5.2 Effects of Kv7 blockade on relaxation responses to guanylate cyclase activator

To further investigate the role of Kv7 channels as a downstream target of the cGMP signalling pathway, we tested the effects of Kv7 channel blockers on vasodilation due to NO-independent activation of sGC. BAY41-2272 is a NO-independent, sGC enzyme stimulator that causes an increase in the sGC activity, resulting in the cGMP up-regulation (Stasch et al., 2002; Schmidt et al., 2003; Evgenov et al., 2006). It was applied at 1 nM to 3 μM to artery segments constricted with 1 μM phenylephrine in the absence or presence of 1 μM linopirdine, XE991 or HMR1556. BAY41-2272 reduced the phenylephrine mediated constriction in a concentration-dependent manner with or without the presence of Kv7 K⁺ channel blockers (Figure 3.24A). Comparison of the BAY41-2272 concentration-response relationship before and after applying a Kv7 blocker (Figure 3.24B) shows that neither HMR1556, linopirdine nor XE991 affected the maximum relaxation, while HMR1556 did not affect the log concentration-response relationship; however, linopirdine and XE991 both caused a shift to the right. BAY41-2272 relaxed vessels with a maximum response of 115 ± 9 % (n=11) and pEC₅₀ of 7.47 ± 0.20 (EC₅₀ =34 nM). In the presence of linopirdine, the pEC₅₀ was significantly decreased to 6.97 ± 0.04 (EC₅₀ =107 nM, P=0.032, One-way ANOVA with Dunnett’s post-test). In the presence of XE991 the pEC₅₀ was decreased to 6.91 ± 0.06 (EC₅₀ =124 nM, P<0.013).
Figure 3.24 Effect of Kv7 channel blockers on BAY41-2272-induced vasorelaxation.

A. Representative traces showing concentration-dependent relaxation induced by BAY41-2272 in conduit arteries preconstricted with 1 µM phenylephrine in the absence or presence of 1 µM XE991. Calibration bars are 1 mN vertical and 20 min horizontal. B, Log concentration-response curve for BAY41-2272-induced relaxation of conduit arteries in the absence or presence of 1 µM XE991, linopirdine or HMR1556. Relaxation was measured as percentage of the phenylephrine-induced constriction just prior to the addition of BAY41-2272. Data plotted as mean ± s.e.m of arteries from 11 animals.
3.2.5.3  

*Kv7 channels in endothelium-dependent vasodilation*

Endothelial cells release NO under basal conditions and in response to different stimuli, which suggest endothelium-dependent vasorelaxation due to NO activating the sGC-cGMP signalling pathway (Loscher, 1991). Muscarinic agonist promotes endothelial cells to produce NO (Loscher, 1991). The relaxing impact by carbachol, a muscarinic receptor agonist, was evaluated in the presence or absence of Kv7 channels blocker to test whether Kv7 K⁺ channels have a role in mediating endothelium-dependent vasodilation in pulmonary artery. Conduit vessels that were pre-constricted with 1 μM phenylephrine, with or without 1 μM HMR1556, linopirdine or XE991, were then challenged with carbachol at a concentration range of 1 nM to 30 μM. The phenylephrine-evoked constriction was reduced by carbachol in a concentration-dependent manner, irrespective of vessels being exposed to Kv7 channel blocker (Figure 3.25A). The concentration-response relationships for carbachol-induced relaxation (Figure 3.25B) show that HMR1556 had no effect. The carbachol pEC₅₀ was 6.45 ± 0.09 (n=6) and maximum relaxation was 93 ± 3 %. In the presence of either of the pan Kv7 blockers, the maximum relaxation and pEC₅₀ of carbachol were significantly reduced. The maximum relaxation fell to 69 ± 3 % (P<0.0001 one-way ANOVA followed by Dunnett’s multiple comparisons test, n= 6) in the presence of linopirdine and 64 ± 2 % (n=5, P<0.0001) in the presence of XE991. Linopirdine reduced the carbachol pEC₅₀ to 6.11 ± 0.09 (P=0.013) and XE991 reduced it to 6.12 ± 0.07 (P=0.018).

To further investigate Kv7 K⁺ channels as downstream targets in endothelium-dependent vasorelaxation, endothelium-dependent dilation to the Ca²⁺ ionophore, A23187, was tested in control condition and after exposure to a Kv7 channel blocker. Phenylephrine-constricted vessels were challenged with A23187 at concentrations ranging from 0.3 nM to 1 μM. A23187 reduced the constriction in a concentration-dependent manner in the absence or presence of HMR1556 (Figure 3.26A). The concentration-response relationship for A23187-induced relaxation is compared in the presence and absence of Kv7 blockers in figure 3.26B. A23187 relaxed vessels with a maximum response of 61 ± 4 % (n=8) and pEC₅₀= 7.85 ± 0.2 (EC₅₀ =14 nM). HMR1556 had no significant effect on the maximum response and the pEC₅₀ of A23187. The A23187-induced relaxation was almost abolished in the presence of XE991 or linopirdine.
Figure 3.25 Carbachol-mediated relaxation was impaired by Kv7 channel blockers.

A, Representative traces showing concentration-dependent relaxation were induced by carbachol in conduit arteries pre-constricted with 1 μM phenylephrine in the absence (upper panel) or presence of 1 μM XE991 (lower panel). Calibration bars are 1 mN vertical and 5 min horizontal. B, Log concentration-response curves were for carbachol-mediated relaxation of conduit arteries in the absence or presence of 1 μM XE991, linopirdine or HMR1556. Relaxation was measured as percentage of the phenylephrine-induced tone just prior to the addition of carbachol. Data plotted as mean ± s.e.m of arteries from 6 animals.
**Figure 3.26 Effects of Kv7 channel blockers on A23187-mediated vasodilation.**

A. Representative traces showing concentration-dependent relaxation induced by A23187 in conduit arteries preconstricted with 1 μM phenylephrine in the absence or presence of 1 μM XE991. Calibration bars are 1 mN vertical and 20 min horizontal. B, Log concentration-response curve for A23187-induced relaxation of conduit arteries in the absence or presence of 1 μM HMR1556, linopirdine or XE991. Relaxation was measured as percentage of the phenylephrine-induced constriction just prior to the addition of A23187. Data plotted as mean ± s.e.m of vessels from n animals.
3.2.5.4 **Kv7 channels contribute to sildenafil-induced relaxation of pulmonary artery**

The phosphodiesterase-5 inhibitor, sildenafil, raises smooth muscle cGMP level by preventing its breakdown (Pauvert et al., 2003; Freitas et al., 2012; Hussain et al., 2017). Vasorelaxation responses to the PDE5 inhibitor, sildenafil, were studied in conduit and small IPA pre-constricted with 30 nM U46619. They were challenged with sildenafil at a concentration range of 0.1 nM to 10 μM. Sildenafil induced concentration-dependent relaxation in both conduit and small IPA (Figure 3.27A). Concentration-response relationships are compared in figure 3.27B. Sildenafil relaxed vessels above 1 nM, producing a maximum 80 ± 3 % (n=4) relaxation in conduit arteries and 51 ± 5 % (n=3) in small arteries. The maximum response was significantly smaller (P=0.0053 unpaired t-test) in small arteries. Both small (8.74 ± 0.44; EC$_{50}$ = 2 nM) and conduit (8.53 ± 0.2; EC$_{50}$ = 3 nM) arteries had similar mean pEC$_{50}$.

In the same manner, the relaxing effect of sildenafil was tested in conduit arteries pre-constricted with 1 μM phenylephrine. Sildenafil (0.1 nM to 10 μM) evoked concentration-dependent relaxation (Figure 3.28A).

The concentration-response curve for sildenafil-mediated relaxation is shown in figure 3.28B. The relationship was not clearly sigmoidal and the maximum response may not have been reached. Nevertheless, nearly 100% relaxation was achieved at 10 μM sildenafil and 50 % relaxation between 10 and 100 nM. As a clear maximum was not reached, the EC$_{50a}$ was determined as the sildenafil concentration evoking a 50 % reduction of the phenylephrine-activated tension. The mean relaxation achieved by sildenafil at 10 μM was 100 ± 2 % (n=8), and the pEC$_{50a}$ was 7.05 ± 0.4 (EC$_{50a}$ 89 nM).

The sensitivity of the conduit arteries to sildenafil was significantly higher when they were pre-constricted by 30 nM U46619 compared with 1 μM phenylephrine (P=0.0076 unpaired two-tailed t-test), but the maximum response, reached by sildenafil was significantly larger in vessels pre-constricted by 1 μM phenylephrine compared with 30 nM U46619 (P=0.0025).

We evaluated whether Kv7 blockers had a role in interfering the relaxing effect induced by sildenafil in conduit arteries, and examine whether Kv7 channels play any role as a downstream target, in mediating the relaxing effect of sildenafil. Vessel segments constricted with 1 μM phenylephrine and then challenged by sildenafil (0.1 nM to 10 μM). Sildenafil evoked concentration-dependent relaxation in the presence of 1 μM linopirdine or HMR1556 (figure 3.28A).

The concentration-response relationship for sildenafil-induced relaxation measured after adding a Kv7 channel blocker (Figure 3.28B). Linopirdine shifted the sildenafil concentration-response curve to the right, reducing the pEC$_{50a}$ to 6.07 ± 0.04
(EC$_{50a}$ =0.84 μM, P=0.018) in its presence. HMR1556 did not affect the pEC$_{50a}$. The maximum relaxation reached by sildenafil at 10 μM was not affected by the presence of Kv7 blockers.

Figure 3.27 Effect of artery size on sildenafil-induced vasodilation.
A, Representative traces showing concentration-dependent relaxation induced by sildenafil in small and conduit arteries constricted with 30 nM U46619. Calibration bars are 1 mN vertical and 10 min horizontal. B, log concentration-relaxation curves induced by sildenafil (0.1 nM - 10 μM) added cumulatively to conduit and small pulmonary arteries pre-constricted with 30 nM U46619. Relaxation was measured as percentage of the U46619-evoked constriction just before the addition of sildenafil. Data plotted as mean ± s.e.m of n number of animals.
**Figure 3.28 Effect of Kv7 channel blockers on sildenafil-induced vasorelaxation.**

A. Representative traces showing concentration-dependent relaxation were induced by sildenafil in conduit arteries pre-constricted with 1 µM phenylephrine in the absence (upper panel) or presence (lower panel) of 1 µM linopirdine. Calibration bars are 1 mN vertical and 10 min horizontal. B, Log concentration-response curves were for sildenafil-mediated relaxation of conduit arteries in the absence or presence of 1 µM HMR1556 or linopirdine. Relaxation was measured as percentage of the phenylephrine-induced constriction just prior to the addition of sildenafil. Data plotted as mean ± s.e.m of n animals.
3.2.5.5  Mechanism of β-adrenergic activation of Kv7 channels

Activation of Gαs coupled receptors leads to the dissociation of the Gα subunit from the Gβγ complex, both of which can mediate signalling (Khan et al., 2013). Gβγ complex was recently proposed to have a positive regulator action on the Kv7.4 channel in renal artery smooth muscle (Stott et al., 2015a) and this could underlie the apparent role of Kv7 channels in isoprenaline and treprostinil dilation of pulmonary artery. To test the potential role of Gβγ in the isoprenaline-induced relaxation of pulmonary artery, the ability of gallein, a Gβγ subunits inhibitor to interfere with the relaxation was tested. The role of the Gα subunit, which results in the activation of PKA, was investigated in a similar way, using the PKA inhibitor 8- Bromoadenosine- 3', 5'- cyclic monophosphorothioate, Rp- isomer (Rp-8-Br-cAMPS), sodium salt. With 1 μM phenylephrine, arteries were constricted with or without 100 μM gallein or Rp-8-Br-cAMPS, and then challenged with isoprenaline 0.3 nM to 1 μM. The concentration-response relationship for isoprenaline-induced relaxation is compared with or without gallein or Rp-8-Br-cAMPS in figure 3.29. Neither gallein nor Rp-8-Br-cAMPS had an impact on the relaxation to isoprenaline at any concentration.

![Figure 3.29](image_url)

**Figure 3.29** The effect of protein kinase A and G-protein βγ subunits inhibitor on isoprenaline-mediated vasodilation.

Log concentration-response curve for isoprenaline-induced relaxation of arteries in the absence or presence of 100 μM Rp-8-Br-cAMPS or gallein. Relaxation was measured as percentage of the phenylephrine-induced constriction just prior to the addition of isoprenaline. Data plotted as mean ± s.e.m of n number of animals.
3.3 Summary

The results of the experiments in this chapter can be summarised as follows:

1. Vessel size affected the response of PAs to Kv7 blockers, which produced larger constriction in conduit vessels. In contrast, Kv7 activators induced similar relaxation in conduit and small vessels. Drugs used therapeutically in PAH (sildenafil and treprostinil), induced larger relaxation of conduit vessels compared to small vessels.

2. Kv7.1 channel blockers contracted pulmonary artery, but only if the tension was first elevated by a different vasoconstrictor. Thus Kv7.1 channels may be functionally active in PAs, but have no role in mediating resting tone.

3. The Kv7.1 channel activators, RL-3 and ML277, are potent dilators of pulmonary artery and their effects are selectively prevented by Kv7.1 channel blocker.

4. The concentration-response curve to cAMP-mediated vasodilation, but not to treprostinil, shifted rightwards by the presence of pan Kv7 blocker without affecting the maximum response. The maximum response to treprostinil significantly decreased in the presence of Kv7 blockers.

5. The maximum response to cGMP-mediated vasodilation significantly decreased by Kv7 channel blockers.

6. Kv7 channels are involved in mediating the vasodilating effects of sildenafil and treprostinil.
CHAPTER 4
ORGANOID CULTURING AND Kv7 CHANNEL KNOCKDOWN
4.1 Background
Organoid culture for different tissues has gained interest for conducting physiological and pharmacological experiments, particularly for use with techniques to knockdown gene expressions such as small interference RNA (siRNA) transfection techniques (Guibert et al., 2005; Manoury et al., 2009). The process, from the start of siRNA transfection of ex-vivo tissues until knockdown of the target protein, requires culturing of isolated vessels for 2 – 4 days (Lin et al., 2004; Yu et al., 2004). Many studies showed that the three-dimensional complex structure and the contraction ability of arteries were maintained during culturing, despite some changes in the mRNA expression, including downregulation of the angiotensin II type-1 receptor and upregulation of mRNA for the endothelin ET-B receptor and serotonin-2A receptor (Möller et al., 2002; Luo et al., 2004; Johnsson et al., 2008). Furthermore, there was upregulation of intracellular Ca\(^{2+}\) stores and store-operated Ca\(^{2+}\) entry (Bergdahl et al., 2005), as well as downregulation of L-type Ca\(^{2+}\) channel expression (Dreja et al., 2001). Guibert et al. (2005) reported that culturing pulmonary arteries for 4 days did not change the histological features or the contractile phenotype of the vessels, but it did cause hypersensitivity to K\(^{+}\) challenges, a stronger contraction response to serotonin and impaired relaxation to carbachol. Additionally, Manoury et al. (2009) showed that culturing IPA changes the pharmaco-mechanical properties of the vessel, with culturing of vessels for around four days inducing changes that were similar to the effects of chronic hypoxia. These changes included depolarisation of PASMC, enhanced resting Ca\(^{2+}\) influx, an increase in intrinsic tone and induction of spontaneous vascular constriction. Such changes may limit the use of cultured IPA for pharmacogenomic studies. During the incubation, many factors affect the pharmaco-mechanical properties of the vessel: Bakker et al. (2000) showed the presence of serum in the culture medium can affect properties of the incubated vessels by showing a progressive constriction and undergoing eutrophic remodelling, and Manoury et al. (2009) reported that removing the endothelium and oxygenating the medium were helpful to maintain normal function of the vessel. We, therefore, optimised the conditions for culturing IPA to keep the vessels for three days without significant changes in the contractile properties or the relaxation response, particularly to Kv7 modulators. This allowed us to conduct pharmaco-genomic experiments using siRNA knockdown.

The results presented in chapter 3 are consistent with a role of Kv7 K\(^{+}\) channels in mediating the vasodilator effect of Kv7 modulators and the NO-sGC-cGMP pathway. The
physiological function of each member of the Kv7 channel family; however, was not addressed precisely, because of the lack of specific modulators for each subtype. Through the transfection of exogenous siRNA, the process of RNA interference (RNAi) is a powerful approach to knockdown targeted ion channels and enable the function of the channel to be studied (Elbasher et al., 2001; Gurney and Hunter, 2005). Therefore, it can be considered an elegant alternative to a specific Kv7 channel blocker. To our knowledge, KCNQ siRNA has not previously been used to knockdown Kv7 channel in pulmonary arteries. We, therefore, attempted to explore the exact role of each Kv7 subtype expressed in PASMCs by transfecting intact arteries separately with KCNQ1,4 and 5 siRNA, to knockdown the respective proteins.

Delivering siRNA to the target cells, particularly in intact tissue, is the main constraint to achieving an efficient knockdown of the targeted protein, and can be challenging (Gurney and Hunter, 2005). RNAi methods were reported to knockdown TASK-1 and TASK-2 channels in rat PASMCs (Gurney and Hunter, 2005; Gönczi et al., 2006). Nevertheless, our laboratory has not been able to reliably knockdown the expression of ion channel proteins in rat isolated arteries, using published methods. Therefore, we investigated the use of transfecting agents that are reported to show high efficiency in delivering siRNA, such as lipofectamine RNAiMAX and N-TER. Lipofectamine RNAiMAX is a cationic lipid-based transfecting reagent. It showed high efficacy in delivering siRNA into neural stem cells, human mesenchymal stem cells and human embryonic stem cells (Tian et al., 2007; Zhao et al., 2008; Kamaci et al., 2011). It requires a low concentration of siRNA to give maximal viability in the transfected cells, and it has a lower cytotoxic effect when compared with other transfecting reagents (Kamaci et al., 2011). It was also used successfully with hard to transfect cells such as mesenchymal stem cells (De Becker et al., 2007). Other studies; however, showed that using the peptide N-TER is superior to lipid-based siRNA transfection reagents for hard to transfect cells and provides quicker delivery of siRNA, but with less toxicity and higher stability. N-TER forms nanoparticles with siRNA which enables it to be transported across the plasma membrane into the cytoplasm, where it is rapidly released (Morris et al., 1997; Simeoni et al., 2003). Although the system was developed for use with cell lines, it has been used to deliver microRNA activators and inhibitors into mouse embryonic explanted organ (Rebustini, 2017). We, therefore, tested the ability of lipofectamine RNAiMAX and N-
TER to deliver siRNA and dsRNA into PASMCs of intact arteries and attempted to optimise conditions for selectively knocking down Kv7 protein subtypes.

Having optimised conditions for organ culture and Kv7 subunit knockdown, these techniques were used to investigate the roles of Kv7 subtypes in the modulatory effects of Kv7-selective drugs on pulmonary artery. Joshi et al. (2009) reported that the mRNA coding for KCNQ1, 4 and -5 are expressed in PASMCs of rat and that the KCNQ4 transcript is the most abundant, but the expression of Kv7 proteins has not been reported. Therefore, we investigated whether the expressed KCNQ genes in IPA smooth muscle cells are translated into the respective proteins and the extent of each Kv7 protein subtype expression.

In summary, the aims of the experiments reported in this chapter were to:

1. Optimise the culture conditions to maintain the contractile properties of IPA for at least three days.
2. Optimise a transfection protocol for delivering KCNQ siRNA into PASMCs of intact arteries.
3. Explore the protein expression of Kv7 channel subtypes in PASMCs.
4. Investigate the effects of knocking down each Kv7 subtype on the abilities of Kv7 modulators to alter pulmonary artery tone.
4.2 Results

4.2.1 Pharmaco-contractile properties of cultured intrapulmonary artery

4.2.1.1 Effects of organ culture on the contractile responses of IPAs stimulation with KCl or phenylephrine

The influence of different organ culture conditions on the contractile properties of conduit vessels were studied on the third day of their culturing. Vessels were incubated in PSS, buffered either with HEPES or bicarbonate, or medium buffered with bicarbonate, in the absence or presence of 0.5 or 1 % antibiotic mixture (10000 units penicillin, 10 mg streptomycin and 25 µg amphotericin B per ml). The vessels were cultured in 4 well culture plates and the plates were either sealed or unsealed when the vessels were cultured in HEPES-buffered PSS. However, the plates were unsealed in other conditions. Parafilm was used to seal the lids to avoid PSS exposure to incubator air and the environment. The plates were kept in the incubator at 37°C with 5 % CO₂ in air, and the solution was changed every 24 hours. After incubation, vessels were exposed to 50 mM KCl to test whether they were contractile. The cultured vessels maintained their constriction ability by responding to 50 mM KCl; however, the average constriction responses in some conditions were significantly decreased when compared with the contractile response of freshly isolated IPA, as shown in figure 4.1A. Further, the average constriction did not decrease in vessels cultured in bicarbonate-buffered medium in the presence of antibiotics. Many studies showed that cultured IPA will become hypersensitive to potassium after three days of culturing (Guibert et al., 2005; Manoury et al., 2009). To test if the same would happen with our cultured vessels, we added 10 mM K⁺ to the bath to increase the K⁺ concentration to 15mM, which is a sub-threshold concentration for inducing constriction in freshly isolated vessels (Casteels et al., 1977a; Guibert et al., 2005). Figure 4.1A shows that the control vessels did not respond to the application of 10 mM KCl; however, the vessels in most of the culture conditions did constrict to 10 mM KCl and the constriction of vessels cultured in bicarbonate-buffer medium was not significantly different from the control vessels. Therefore, the results from the above sets of experiments, as shown in figure 4.1A, suggest that the best condition for keeping vessels responsive to KCl is by culturing pulmonary artery segments in bicarbonate-buffered medium in the presence of 1 % antibiotics, as it did not cause a significant decrease in the contractile response to 50 mM KCl or a considerable increase in hypersensitivity to 10 mM KCl.
Figure 4.1 Effects of different culturing conditions on the IPA constriction to KCl and phenylephrine

Summary of contractile responses to 50 mM or 10 mM KCl (A) and 1 µM PE (B) of conduit intrapulmonary arteries. Control represents freshly isolated IPA. Responses to KCl and PE were measured 5 min and 30 min after exposure, respectively. Data plotted as mean ± s.e.m of vessels from n animals. * P<0.05, **P<0.01 versus control vessels constricted with 50 mM KCl. # P<0.05, ## P<0.01 versus control vessels constricted with 10 mM KCl. M: bicarbonate-buffered medium.

The effect of incubation conditions on the response of vessels to 1 µM phenylephrine was also tested. The average constriction of cultured vessels, measured relative to the response of 50 mM KCl, was compared with responses seen in the control vessels (Figure 4.1B). There was no significant change in the phenylephrine-evoked contraction on day 3 of culturing compared to the freshly isolated arteries. However, three vessels incubated in HEPES based PSS in a sealed container did not respond or responded poorly (<0.2 mN) to 1 µM phenylephrine. Hence, these were not included.
4.2.1.2 Influences of organ culture on responses of intrapulmonary arteries to Kv7 channel blockers

To investigate the influence of different culture conditions on the constrictor responses of IPA to Kv7 channel blockers, blood vessels were challenged with linopirdine or XE991 at concentrations ranging from 10 nM to 50 µM, added cumulatively. Figure 4.2A compares the relationship between linopirdine concentration and contraction when arteries were cultured under different conditions or freshly isolated. Responses were measured as a percentage of the contraction induced by adding 50 mM KCl to the vessel. Constriction to linopirdine was maintained in all culture conditions and the maximum constriction was produced at around 10 µM linopirdine. Table 4.1 lists the maximum response and pEC\textsubscript{50} of linopirdine in control vessels and vessels incubated under different conditions. The maximum response was significantly increased (P<0.01 versus control vessels) in vessels incubated in HEPES-buffered PSS containing 1% antibiotic mixture in a sealed container. In all other conditions, differences between cultured and control vessels did not reach statistical significance. Importantly, cultured vessels did not lose their response to linopirdine. The sensitivity of the vessels to linopirdine, indicated by the pEC\textsubscript{50}, was not significantly altered in any condition.

Figure 4.2B shows that contraction responses to XE991 were also maintained after three days of culturing IPAs. The maximum constriction was reached at 10 µM XE991. In some culture conditions, the maximum response and/or pEC\textsubscript{50} for XE991 were significantly elevated on day three of culturing when compared to freshly isolated arteries, as shown in table 4.1.

The condition that retained contractile responsiveness of vessels, which was closest to that found in freshly isolated arteries for both linopirdine and XE991, was bicarbonate-buffered medium, with or without 1% antibiotics, maintained in an unsealed four-well culture plate in a standard 37°C incubator.
Figure 4.2 Response of fresh and cultured IPA to linopirdine and XE991
Concentration-response relationships for contraction are induced by linopirdine (A) or XE991 (B) in freshly isolated and cultured pulmonary arteries. Vessels were cultured for 3 days in the conditions indicated by the symbols in the legend. Constriction is plotted as a percentage of the contraction induced by 50 mM KCl. Each point was plotted as the mean ± s.e.m of experiments on vessels from n number of animals.
Table 4.1 Effects of different incubation conditions on the maximum response (Max.) and pEC$_{50}$ of linopirdine and XE991.
(n) represents the number of animals used for each set of experiments. * P<0.05, **P<0.01 versus freshly isolated IPA (control).

<table>
<thead>
<tr>
<th>Condition</th>
<th>Max. (%)</th>
<th>pEC$_{50}$</th>
<th>n</th>
<th>Max. (%)</th>
<th>pEC$_{50}$</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>55 ± 5</td>
<td>5.91 ± 0.16</td>
<td>9</td>
<td>66 ± 7</td>
<td>5.75 ± 0.30</td>
<td>11</td>
</tr>
<tr>
<td>PSS HCO$_3^-$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unsealed</td>
<td>31 ± 7</td>
<td>6.75 ± 0.43</td>
<td>3</td>
<td>50 ± 10</td>
<td>6.88 ± 0.44</td>
<td>4</td>
</tr>
<tr>
<td>PSS HEPES</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unsealed</td>
<td>89 ± 12</td>
<td>6.87 ± 0.91</td>
<td>3</td>
<td>126 ± 17 *</td>
<td>6.85 ± 0.28</td>
<td>3</td>
</tr>
<tr>
<td>PSS HEPES sealed</td>
<td>62 ± 6</td>
<td>6.25 ± 0.07</td>
<td>9</td>
<td>104 ± 13 *</td>
<td>6.96 ± 0.31 *</td>
<td>9</td>
</tr>
<tr>
<td>PSS HEPES sealed 0.5% Abs</td>
<td>91 ± 10</td>
<td>6.14 ± 0.14</td>
<td>3</td>
<td>121 ± 9 *</td>
<td>5.94 ± 0.13</td>
<td>3</td>
</tr>
<tr>
<td>PSS HEPES sealed 1% Abs</td>
<td>105 ± 10</td>
<td>6.23 ± 0.12**</td>
<td>3</td>
<td>134 ± 12 **</td>
<td>6.42 ± 0.17</td>
<td>3</td>
</tr>
<tr>
<td>Medium HCO$_3^-$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unsealed</td>
<td>61 ± 4</td>
<td>5.51 ± 0.56</td>
<td>3</td>
<td>86 ± 13</td>
<td>6.1 ± 0.22</td>
<td>3</td>
</tr>
<tr>
<td>Medium HCO$_3^-$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unsealed 1% Abs</td>
<td>66 ± 12</td>
<td>5.86 ± 0.23</td>
<td>7</td>
<td>80 ± 14</td>
<td>6.33 ± 0.33</td>
<td>7</td>
</tr>
</tbody>
</table>
4.2.1.3 Influence of organ culture on responses of intrapulmonary arteries to flupirtine
We next tested whether the vasodilatory response of flupirtine is maintained in conduit IPA segments after three days of organ culture. Freshly isolated vessels or vessels cultured in various conditions were pre-constricted with 1 μM phenylephrine, and then challenged with flupirtine at 0.01 μM to 200 μM, added cumulatively. The same volume of vehicle was added to time-controlled vessels contracted in the same way with phenylephrine. Flupirtine reduced constriction in a concentration-dependent manner in both the control arteries and in cultured IPA. The concentration-response relationships in freshly isolated IPA and cultured arteries are compared in figure 4.3A. The relaxation induced at the highest concentration of flupirtine tested appeared to approach a maximum, at 89 ± 4 % (n=11) in control and 87 ± 5 % (n=3) in vessels cultured in bicarbonate-buffered medium with 1 % antibiotics. As a clear maximum level was not reached, an apparent EC\textsubscript{50} (EC\textsubscript{50a}) was measured as the concentration of flupirtine that impaired phenylephrine-evoked vasoconstrictor tone by 50%. The mean pEC\textsubscript{50a} was similar in freshly isolated vessels and vessels cultured in medium. The pEC\textsubscript{50a} of flupirtine in control vessels and vessels cultured in different conditions are listed in table 4.2. Some incubation conditions appeared to cause a small loss of sensitivity to flupirtine (fig 4.3A), but in all conditions the pEC\textsubscript{50a} values did not differ significantly from that measured from control arteries.

To ensure that the data obtained for flupirtine was due to its relaxant effect and not due to the loss of phenylephrine-induced tone during the experiment and/or the effect of DMSO, time control experiments were run in parallel for each condition. Figure 4.3B shows that the phenylephrine-induced constriction was well maintained during experiments on cultured vessels. In fact, a 25% inverse tone was generated during the experiments, and the mean tone was nearly doubled by the end of the experiments on vessels incubated in HEPES-buffered PSS in the absence of antibiotics.

The above results show that the relaxant effect of flupirtine is little affected by the conditions used to culture IPAs. In particular, vessels cultured in HEPES-buffered PSS without antibiotics, or in bicarbonate-buffered medium with antibiotics, responded to flupirtine in essentially the same way as freshly isolated IPA.
**Figure 4.3 Response of fresh and cultured IPA to flupirtine and vehicle**

Log concentration-response curves were for flupirtine A, with its time control B, in freshly isolated IPA and vessels cultured for three days in the conditions indicated by the symbols in the legend. The vessels were pre-constricted with 1 µM phenylephrine. Time control vessels were treated by adding the same amount of vehicle parallel to the addition of flupirtine. Relaxation was measured as percentage of the phenylephrine-induced constriction just before the addition of flupirtine (A) or vehicle (B). Data plotted as mean ± s.e.m of n number of vessels from different rats.

**Table 4.2 Effects of different incubation conditions on the maximum response and pEC$_{50a}$ of flupirtine.**

(n) represents the number of animals used for each set of experiments.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Maximum (% )</th>
<th>pEC$_{50a}$</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>89 ± 4</td>
<td>4.58 ± 0.04</td>
<td>11</td>
</tr>
<tr>
<td>PSS HEPES sealed</td>
<td>94 ± 2</td>
<td>4.49 ± 0.03</td>
<td>4</td>
</tr>
<tr>
<td>PSS HEPES sealed 0.5% Abs</td>
<td>81 ± 13</td>
<td>4.26 ± 0.31</td>
<td>3</td>
</tr>
<tr>
<td>PSS HEPES sealed 1% Abs</td>
<td>80 ± 8</td>
<td>4.44 ± 0.01</td>
<td>3</td>
</tr>
<tr>
<td>Medium HCO$_3^-$ Unsealed 1% Abs</td>
<td>87 ± 5</td>
<td>4.58 ± 0.15</td>
<td>3</td>
</tr>
</tbody>
</table>
**4.2.2 Optimisation of siRNA transfection**

Bicarbonate-buffered medium containing 1% antibiotic mixture was selected to culture isolated IPAs for three days when attempting siRNA knock-down. Experiments were performed to identify the most effective transfecting reagent and optimise conditions for inducing siRNA transfection of *ex vivo* arteries and knockdown of Kv7 proteins. Two different transfecting reagents, RNAiMAX or N-TER, were investigated by adjusting the volume added to the transfecting medium and varying the final concentration of a fluorescence labelled siRNA (*Silencer®* FAM Labelled Negative Control) or dsRNA (*BLOCK-iT™* Alexa Fluor®, which is red fluorescent control), which were used as markers of transfection. The FAM-labelled siRNA has green emission under a fluorescence microscope and was monitored using an emission filter with a peak at 525 nm. The labelled dsRNA emitted a red signal and was monitored at 593 nm.

The reverse permeabilisation protocol was used by others to deliver KCNQ4 siRNA into smooth muscle cells in renal artery segments (Chadha *et al.*, 2012b). However, FAM labelled siRNA or *BLOCK-iT™* Fluorescent Oligo were not detectable in IPA when used at concentrations of up to 100 nM (Figure 4.4). As an alternative to improve the transfection, we repeated the reverse permeabilisation protocol but added transfecting reagent as well. Either lipofectamine (12 µl) or N-TER (12 µl), was added with 100nM labelled siRNA or dsRNA. This approach was also unsuccessful (Figure 4.4).

Other protocols using transfecting reagents without reverse permeabilisation were then explored. We used only the red *BLOCK-iT* Fluorescent Oligo as the indicator of transfection, as there was green autofluorescence in pulmonary artery sections. Autofluorescence was very small in the red region of the spectrum, as shown in figure 4.5.
Figure 4.4 Imaging of PA sections treated with fluorescent labelled siRNA or dsRNA, using the reverse permeabilisation protocol.
Representative fluorescence images of IPA sections (10 µm) treated with 100 nM FAM-labelled siRNA (A) or BLOCK-iT™ Fluorescent Oligo (B) in the absence or presence of lipofectamine or N-TER as indicated. Sections were imaged using an epifluorescence microscope to detect green fluorescence (A) or red fluorescence (B). Controls represent vessels subject to permeabilisation in the presence of transfecting agent only. Scale bar is 1000 µm.
Figure 4.5 Autofluorescence in PA sections
Representative fluorescence images of freshly isolated IPA sections (10 µm) captured by an epifluorescence microscope to detect green fluorescence (A) or red fluorescence (B). Scale bar is 200 µm.

The volume of transfection reagent and the concentration of the fluorescent dsRNA were varied. In preliminary experiments, the efficacies of four concentrations of each transfection reagent were investigated by monitoring the uptake of fluorescent dsRNA by cells in the artery. The concentration of the labelled dsRNA was increased with an increasing volume of transfecting reagent, as indicated in table 4.3.

Table 4.3 Concentration of BLOCK-iT™ Fluorescent dsRNA and volume of transfecting reagent added to 1 ml well with ex-vivo arteries.

<table>
<thead>
<tr>
<th>Final concentration (nM)</th>
<th>Lipofectamine volume (µl)</th>
<th>OR</th>
<th>N-TER volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>3</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>50</td>
<td>6</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>100</td>
<td>12</td>
<td></td>
<td>12</td>
</tr>
<tr>
<td>200</td>
<td>20</td>
<td></td>
<td>16</td>
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</table>
The transfection efficiency varied considerably. The fluorescence emission from vessel sections increased with the increasing volume of the reagent and concentration of the fluorescent-dsRNA. Emission was negligible at the lowest concentration tested, but there was clear uptake of the fluorescent dsRNA when it was present at 100 nM with 12 µl of lipofectamine. The signal was strongest when the lipofectamine volume was 20 µl and concentration of dsRNA was 200 nM. Importantly, fluorescence was apparent in the medial layer of the artery, containing the smooth muscle cells. Figure 4.6 illustrates the results of transfection using lipofectamine and fluorescent dsRNA at these concentrations. Figure 4.7 illustrates the effect of N-TER and dsRNA concentration on the uptake of the labelled dsRNA by IPA. The images of transfected vessel sections show that fluorescence increased with the increase of N-TER volume and concentration of labelled dsRNA. The fluorescence emission was highest at 200 nM dsRNA and 16 µl N-TER (Figure 4.7). There was stronger fluorescence in arteries treated with 100 nM or 200 nM dsRNA with N-TER as the reagent, compared to arteries treated with lipofectamine RNAiMAX and similar dsRNA concentrations. N-TER, therefore, appeared to be superior to lipofectamine RNAiMAX as a transfecting reagent to deliver labelled dsRNA into PASMCs of intact ex-vivo arteries.
Figure 4.6 Images of sections of PA transfected with fluorescent labelled-dsRNA using lipofectamine RNAiMAX
Representative fluorescence images of IPA sections (10 µm) transfected with 3-20 µl lipofectamine and 25-200 nM BLOCK-iT™ Alexa Fluor (Red fluorescent). Sections were imaged with an epi-fluorescence microscope and red filter. IPA segments were incubated with the transfection mixture for 24 hours. The control represents vessels treated with 20 µl lipofectamine. Scale bar is 200 µm.
Figure 4.7 Images of sections of PA transfected with fluorescent dsRNA using N-TER
Representative fluorescence images of IPA sections (10 µm) transfected with 25-200 nM fluorescent labelled dsRNA (BLOCK-iT™ Alexa Fluor Red) and 3-16 µl N-TER. Red filter of the epifluorescence microscope was used. IPA segments were incubated with the mixture for 24 hours. The control represents vessels treated with 16 µl N-TER or 200 nm labelled dsRNA. Scale bar is 200 µm.
To further evaluate the delivery efficiency of the reagents, we conducted preliminary functional experiments on vessels using the same transfecting reagents and protocol, but substituting KCNQ siRNA for the fluorescence labelled dsRNA. The concentration of siRNA in the final mixture was 100 nM or higher, because such concentrations were needed to deliver measurable fluorescent dsRNA to the vessels. 100 nM KCNQ4 siRNA was reported to achieve 60% knockdown of Kv7.4 protein in ex vivo renal arteries when using the reverse permeabilisation technique (Chadha et al., 2012b). The effect of knockdown on vessel function was evaluated by comparing responses to Kv7 modulators in vessels targeted either by KCNQ siRNA or scrambled siRNA (control). The contractile responses of treated pulmonary arteries to KCl was also tested to assess if the transfection protocol altered vasoreactivity.

4.2.2.1 Effect of lipofectamine RNAiMAX transfection system on pulmonary artery function

To test if the contractile response of cultured IPAs was maintained in the presence of the lipofectamine RNAiMAX reagent, we cultured the isolated arteries for three days with either 15, 20 or 25 µl lipofectamine RNAiMAX present during the first two days. The medium containing 1% antibiotics was changed every 24 hours. Preliminary results from isometric tension experiments show that the artery segments cultured for three days maintained their contractile response to 50 mM KCl (Figure 4.8A). The limited number of experiments cannot be statistically analysed; however, the results suggest that contractile responses may be better maintained in the presence of 15 or 20 µl of the transfecting reagent. The vessels were also challenged with 10 mM KCl and the preliminary results show a substantial response of the vessels (Figure 4.8A). Constriction reached nearly 50% of the 50 mM KCl constriction in vessels cultured with 15 µl of the reagent but did not increase with increasing concentration of the transfecting reagent. Therefore, no correlation between the transfecting reagent and hypersensitivity to potassium was noted.

Preliminary experiments were also performed with two different concentration of KCNQ4 or KCNQ5 siRNA and 15 or 20 µl of transfecting reagent. The purpose at this point was to assess the best condition for knocking down gene expression. Control vessels were treated similarly, but using scrambled siRNA. Vessels were incubated in a siRNA-lipofectamine mixture, changed with freshly prepared transfection mixture after 24 hours and with fresh medium after 48 hours.
On day 3, arteries were challenged with a cumulative addition of linopirdine at 0.03 to 30 µM. The concentration-response curves for the preliminary experiments showed no obvious differences between vessels exposed to KCNQ or scrambled siRNA when the volume of lipofectamine RNAiMAX was 15 µl and siRNA concentration was 150 nM (Figure 4.8B). Although only one pilot experiment was performed, the concentration-response curves for linopirdine seemed to be shifted to the right with a reduced maximum response when 200 nM KCNQ4 siRNA and 20 µl transfecting reagent were used, as shown in figure 4.8B. The response to linopirdine did not show any impairment with the KCNQ5 siRNA.

Figure 4.8 Effect of lipofectamine RNAiMAX and targeting KCNQ4 or KCNQ5 genes on the constrictive response of IPA. 
A, Average response of IPAs segments to 50 mM KCl on day 3 of culturing when 15, 20 or 25 µl lipofectamine RNAiMAX was present in the first 2 days of culturing. Response to KCl was measured 5 min after exposure. Data plotted as mean ± s.e.m of experiments on vessels from n animals. B, log concentration-response relationship for contraction induced by linopirdine in vessels targeted by 150nM (left panel) or 200 nM (right panel) of KCNQ4 or -5, or scrambled siRNA. Constriction is plotted as percentage of the tension induced by 50 mM KCl. Each point was plotted by the data obtained from 1 experiment.
Pilot experiments also investigated the response to retigabine in IPAs treated with lipofectamine and by siRNA. Artery segments were pre-constricted with 1 μM phenylephrine and then challenged with retigabine at a concentration range of 0.01 μM to 200 μM, added cumulatively. Retigabine evoked in a concentration-dependent relaxation of vessels treated with 15 or 20 μl lipofectamine and 150 or 200 nM KCNQ or scrambled siRNA. Concentration-response curves show that the relaxation response to retigabine was not impaired in any condition (Figure 4.9A).

Similar experiments were carried out to test RL-3 on vessels incubated with KCNQ1 or scrambled siRNA. Figure 4.9B shows that the concentration-response curves for RL-3 were similar in vessels treated with lipofectamine at 15 or 20 μl and siRNA at 150 or 200 nM. There was also no obvious difference between KCNQ1 and scrambled siRNA. The curves show that the relaxant response of the control and KCNQ1 targeted vessel was maintained on the third day of culture, and no reduction in relaxation could be noted in KCNQ1 targeted *ex-vivo* arteries.

Overall, the preliminary functional experiments found that arteries treated with transfection reagents maintained the responsiveness to Kv7 modulators. The results did not show any marked changes in the responses of IPAs to Kv7 modulators when lipofectamine RNAiMAX was used as a transfecting reagent.
Figure 4.9 Effect of targeting KCNQ1,4 or -5 genes on the response of IPA to Kv7 activators
A. Log concentration-response curves were for retigabine-induced relaxation in vessel transfected by scrambled siRNA (control) and 150nM (left panel) or 200 nM (right panel) of KCNQ4 and KCNQ5 siRNA. B. Log concentration-response relationship for RL-3 evoked relaxation in vessels treated with scrambled siRNA (control) and with 150nM (left panel) or 200 nM (right panel) KCNQ1 siRNA. Relaxation was measured as percentage of the phenylephrine-induced constriction just before the addition of Kv7 channel activator. Each curve represents the data from one experiment.
4.2.2.2 Effect of N-TER Nanoparticle transfection system on pulmonary artery function

Before the transfecting of ex-vivo arteries with a KCNQ siRNA by using N-TER reagent, we tested the effect of N-TER on the constricive response of cultured IPAs to 50 and 10 mM KCl. The vessels were cultured in the medium containing 1 % antibiotics for three days in the presence of either 12, 16 or 20 µl of N-TER during the first two days. The medium was changed every 24 hours. All vessels maintained their ability to contract on day three of culture, as they constricted to 50 mM KCl (Figure 4.10A). There was no marked difference when comparing vessels cultured with 12 or 16 µl of N-TER; however, the response to 50 mM KCl clearly reduced when N-TER volume was increased to 20 µl.

The sensitivity of the vessels to KCl was increased when cultured with N-TER reagent as they markedly contracted to 10 mM KCl (Figure 4.10A). Contraction of vessels reached 36% and 17.5% of the 50 mM KCl in vessels cultured with 12 µl and 16 µl of N-TER respectively. The increase in hypersensitivity might not be related to the transfection reagent because sensitivity to KCl did not increase with increasing N-TER volume.

Preliminary experiments were also done to evaluate the constrictive response of IPA to linopirdine when N-TER reagent was used to deliver KCNQ siRNA. To assess the best condition for knocking down of KCNQ gene expression, two different concentrations of KCNQ4 and KCNQ5 siRNA and 12 or 16 µl of the reagent were tested. Control vessels were treated similarly, but scrambled siRNA was used. Vessels were cultured in a siRNA N-TER mixture, which was changed with freshly prepared mixture after 24 hours and with fresh medium after 48 hours. On day 3, transfected arteries were challenged with cumulative addition of linopirdine ranging from 0.03 to 30 µM. Figure 4.10B shows the concentration-response relationships for linopirdine-induced constriction in vessels treated with 12 or 16 µl N-TER and 100 or 200 nM KCNQ or scrambled siRNA. The maximum response to linopirdine reduced from 99% in control vessels to 67% and 65% in vessels transfected with 100 nM KNCQ4 and KCNQ5 siRNA respectively. The maximum response was also decreased when vessels transfected with 200 nM siRNA, which was 103% and 65% in vessels transfected with 200 nM scrambled and KNCQ4 siRNA respectively. However, the concentration-response curves of vessels treated with 200 nM KCNQ5 shifted markedly to the left, and the vessels constricted significantly at 30 nM of linopirdine.
Preliminary results from the above experiments show that the response to linopirdine was impaired in KCNQ4 and KCNQ5 targeted vessels when N-TER nanoparticle transfection siRNA system was used. The impairment was clear when 100 nM KCNQ siRNA and 12 µl N-TER were used.

Figure 4.10 Effect of N-TER reagent on the constrictive response of IPA transfected with KCNQ siRNA.

A. Average response to 50 mM KCl on day 3 of culture when vessels incubated in the medium containing 12, 16 or 20 µl N-TER reagent during the first 2 days of culture. Response to KCl was measured 5 min after exposure to KCl. Data plotted as mean ± s.e.m of experiments on vessels from n animals. B. log concentration-response curves were for contraction induced by linopirdine in vessels transfected by 100 nM (left panel) or 200 nM (right panel) KCNQ4 or KCNQ5, or scrambled siRNA (control). The constriction was plotted as percentage of the tension induced by 50 mM KCl. Each curve was plotted by the data obtained from one experiment.
Preliminary experiments were also conducted to investigate the response to Kv7 activators in IPA segments treated with N-TER and siRNA. Artery segments were pre-constricted with 1 μM phenylephrine and then challenged with a concentration range of retigabine (0.01 μM to 200 μM), added cumulatively. Retigabine reduced phenylephrine-evoked constriction in a concentration-dependent manner of vessels treated 12 or 16 μl N-TER and 100 or 200 nM KCNQ or scrambled siRNA. Figure 4.11A shows the concentration-response curves to retigabine was not impaired in vessels transfected with KCNQ5 siRNA when compared to control vessels. The relaxation response curves to retigabine markedly shifted leftward when vessel was treated with 100 nM KCNQ4 and 12 μl N-TER reagent. However, the response to retigabine was impaired when vessels were treated with 200 nM KCNQ4 siRNA compared to the control vessel, and the maximum response was reduced from 98% (control vessels) to 67% (KCNQ4 targeted vessels).

Similar experiments were carried out to test the relaxant effect of RL-3 on artery segments incubated with scrambled or KCNQ1 siRNA. Figure 4.11B shows that the relaxant response of the control and KCNQ1 targeted vessels was maintained on the third day of culture, and concentration-response relationships for RL-3 were similar in vessels treated with 12 μl N-TER and 100 nM KCNQ1 or scrambled siRNA. However, the curves of RL-3 induced relaxation shifted to the right and the maximum response induced by RL-3 decreased from 98% (control vessels) to 74% in vessels transfected with 200 nM KCNQ1 siRNA.

As only one experiment was conducted for each condition, the data cannot be statistically analysed to draw a scientific conclusion. Therefore, more experiments are necessary to understand the effect of the KCNQ gene knockdown on the response of IPAs to Kv7 activators.

Following the fluorescence images and responses of the vessels transfected with KCNQ1, 4 and -5 siRNA, to Kv7 modulators when either lipofectamine or N-TER reagent was used, 16 μl N-TER transfection reagent and 200 nM KCNQ siRNA was selected as the best condition to perform siRNA knockdown of KCNQ genes, and then assess the effect on artery function. Therefore, we decided to conduct further experiments under the same condition to knockdown Kv7 protein subtypes and investigate the role of each subunit in rat IPA.
Figure 4.11 Effect of targeting KCNQ genes on the response of IPA to Kv7 activators. 
A, Log concentration-response relationship for retigabine-induced relaxation in control vessel and vessels transfected with 100nM (left panel) and 200 nM (right panel) of KCNQ4 or -5, or scrambled siRNA. B, Log concentration-response curves were for RL-3 induced relaxation of the control vessel or vessels treated with 100 nM (left panel) or 200 nM (right panel) of KCNQ1 or scrambled siRNA. Relaxation was measured as percentage of the phenylephrine-evoked contraction just prior to the addition of Kv7 channel activator. Each curve represents the data obtained from one experiment.
4.2.3 Expression profile of Kv7 channel subunits in intrapulmonary artery

The expression of Kv7 subunit proteins in IPA was investigated using Western blots. Optimal conditions for the detection of Kv7.4 protein in lysates from IPA were determined by varying the amount of protein loaded into each well, the presence of milk in the primary antibody diluting buffer, and heating of the sample in the presence of reducing agent, before loading on the gel. Blots of IPA lysates were probed with antibodies against the Kv7.4 protein and bands with a molecular weight ~75 kDa were detected as illustrated in figure 4.12. This is consistent with the predicted molecular weight of Kv7.4 protein (~77 kDa) (Jepps et al., 2011; Chadha et al., 2012b; O'Donnell et al., 2017; Lindman et al., 2018). The image of blot shows that clearer bands of Kv7.4 protein could be detected when loading 25 µg of protein on to the wells than loading 12.5 µg of the prepared sample. The presence of 1% milk in the primary antibody diluting buffer masked many non-specific bands, but multiple bands were still apparent. Heating the sample in laemmli buffer at 95°C for 5 minutes resulted in a single band of protein at the target size, instead of double bands close to each other. The results suggest that the best condition to detect Kv7.4 protein by western blot method is to load 25 µg of sample lysate in each well, heat the samples at 95°C for five minutes before loading and using a primary antibody at a dilution of 1:500 with 1 % non-fat dry milk. A secondary antibody dilution of 1:3000 produced a strong band.

Taking the same approach, we tried to detect Kv7.1, 4 and -5 proteins, using α-tubulin as a loading control. We began with lysates extracted from whole lung tissue and brain. Two types of membrane, polyvinylidene difluoride (PVDF) and nitrocellulose were compared for optimising the immunoblotting conditions and detecting Kv7 protein subunits. The blots were treated with antibodies directed against α-tubulin and Kv7.1, Kv7.4 or Kv7.5 proteins. The results are illustrated in figure 4.13. As found with IPA, a clear band with a molecular weight slightly above 75 kDa was detected from lysates of both lung and brain samples when the blot was probed with the Kv7.4 primary antibody. There was no clear difference in the band intensity between either type of membrane. The blots probed with antibody against the Kv7.1 subunit showed a clear band at a molecular weight just below 75 kDa in the lung lysate sample, but the band from sample of brain lysate was faint. The band’s molecular weight was consistent with the molecular weight of...
Kv7.1 protein, which is ~75 kDa (Goldman et al., 2009; Ma et al., 2015). The bands were clearer on the PVDF membrane than the nitrocellulose membrane.

![Immunoblotting of Kv7.4 protein in IPA lysate under different conditions.](image)

<table>
<thead>
<tr>
<th>Protein loaded/well (µg)</th>
<th>12.5</th>
<th>25</th>
<th>12.5</th>
<th>25</th>
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<tr>
<td>Milk in primary Ab. Sol.</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>Primary Ab. conc. 1:500</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Secondary Ab. conc.</td>
<td>1:3000</td>
<td>1:3000</td>
<td>1:3000</td>
<td>1:10000</td>
<td>1:3000</td>
<td>1:3000</td>
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<td>Heated at 95°C</td>
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**Figure 4.12 Immunoblotting of Kv7.4 protein in IPA lysate under different conditions.**

Representative blot of Kv7.4 channel in total protein of IPA lysate under different conditions. Nitrocellulose membrane was used for all blots. Ab: antibiotics; Sol: solution; conc.: concentration.

Bands with high intensity of around 100 kDa could be detected on blots of brain lysate when Kv7.5 antibody was applied to either PVDF or nitrocellulose membranes, as shown in figure 4.13. The result is consistent with the detection of Kv7.5 protein, as its molecular weight is predicted to be ~102 kDa (Lerche et al., 2000; O'Donnell et al., 2017). No band with a molecular weight ~ 100 kDa was detected from blots of lung lysate.

Bands with a molecular weight slightly above 50 kDa were detected in all lysate samples when an antibody against α-tubulin was used (Vu et al., 2017). The bands were of higher intensity in brain lysate compared to lung lysate in all blots. No clear difference in band intensity was detected between PVDF and nitrocellulose membranes.
Figure 4.13 Immunoblots of Kv7 protein subunits and α-tubulin in lung and brain lysate.
Representative blots of Kv7 channels and α-tubulin expression in total protein of lung and brain lysate. 25 µg of lysate loaded on to each well. Immunoblot was performed with Kv7.1, 4 and -5 and α-tubulin antibodies on PVDF and nitrocellulose membrane.

The relative expression of Kv7.1 and Kv7.4 in lung versus brain lysate was estimated by normalising the density of the Kv7 band to the associated α-tubulin band (Figure. 4.14). The expression of Kv7.1 protein was 26-fold higher in lung than brain. Kv7.4 protein expression was around 7-fold higher in lung than in brain.
Figure 4.14 Expression of Kv7.1 and Kv7.4 protein in lung lysate relative to brain lysate.

Histogram represents Kv7.1 and Kv7.4 expression folds in lung lysate relative to brain lysate. Kv7.4 and Kv7.1 band were normalised to their respective α-tubulin band before calculating the ratio of the protein in lung to brain. The detected bands on PVDF membrane were analysed to plot the histograms. Data corresponds to sample obtained from one animal.

Similarly, the detection of Kv7.1, 4 and -5 proteins were investigated in lysates from control IPA and aorta (for Kv7.4 only). The maintenance of Kv7 protein expression on day three of ex-vivo culture was also tested. In these experiments, PVDF membrane was used for blotting. As shown in figure 4.15a, Kv7.4 protein was expressed in IPA and aorta, and expression was still apparent in IPA after 3 days of culture. The expression of Kv7.4 appeared to reduce on incubating the vessels for three days (Figure 4.15b), but only one experiment was performed. Preliminary results also suggest that the expression of Kv7.4 protein in IPA lysate is around 6-fold higher than in aorta lysate (Figure 4.15b).

Kv7.1 protein bands were also detected in lysates from control and cultured IPAs (Figure 4.16a). The expression of Kv7.1 protein did not appear to be affected by culturing the vessel for 3 days (Figure 4.16c), but this experiment was only repeated twice. No band with a molecular weight of around 100 kDa was detected in IPA lysates when the Kv7.5 antibody was used (Figure 4.16b), although a strong band of α-tubulin was detected from the same sample.

As there were only one or two experiments conducted for each condition, the data cannot be statistically analysed to draw a scientific conclusion. Therefore, more experiments are necessary to understand the expression of Kv7 subunit proteins in IPA and
the effect of culturing on the Kv7 protein expression before being able to evaluate the success of Kv7 protein knockdown.

**Figure 4.15 Immunoblotting of Kv7.4 protein and its expression in conduit IPA and aorta lysate.**

A. Representative blots of Kv7.4 channel expression and α-tubulin in conduit IPA and aorta lysate of control vessel and IPA segments incubated for three days. 25 µg of lysate loaded in each well. Immunoblot was performed with Kv7.4 and α-tubulin antibodies on PVDF membrane. B. Relative expression of Kv7.4 protein from control IPA segments compared with aorta on day zero and IPA segments incubated for three days. Kv7.4 bands were normalised to their respective α-tubulin band. Data corresponds to sample obtained from vessels of n number of animals.
Figure 4.16 Immunoblots of Kv7.1 and Kv7.5 protein expression in IPA.
A and B, Representative blots of Kv7.1 and Kv7.5 channel expression, respectively, and their respective α-tubulin bands in IPA lysate of control vessels and vessels incubated for three days (only for Kv7.1 protein). 25 µg of lysate loaded in each well. Immunoblot was performed with Kv7.1, Kv7.5 and α-tubulin antibodies on PVDF membrane. C, Relative expression of Kv7.1 protein from control IPA segments compared with vessels incubated for three days. Kv7.1 bands were normalised to their respective α-tubulin bands. Data corresponds to sample obtained from vessels of two animals.
4.2.4 PASMCs transfection with KCNQ siRNA in ex vivo arteries

The N-TER nanoparticle transfection system was used to achieve the transfection of PASMCs with siRNA directed against the KCNQ1, KCNQ4 and KCNQ5 genes. The optimal protocol developed in the previous section was used.

The effect of vessel transfection with KCNQ siRNA was first investigated on responses to KCl and phenylephrine. As shown in figure 4.17A, all vessels contracted to 50 mM KCl and contraction of transfected conduit artery segments was well maintained at approximately 3 mN, although smaller than the contraction developed by freshly isolated arteries. There was no significant difference in average contraction between vessels transfected with scrambled siRNA (control) and KCNQ1, 4 or -5 siRNA.

All vessels showed a small contraction response to 10 mM KCl (Figure 4.17A) ranging from 12 to 18 % of the 50 mM KCl-induced constriction. However, there was no significant difference between the responses of control vessels compared with KCNQ siRNA treated vessels.

The responses of transfected arteries to phenylephrine were also tested. The constriction response was well maintained in all vessels (Figure 4.17B) and there was no significant difference in the phenylephrine-evoked constriction between control and KCNQ-targeted vessels. The contraction of control vessel to 1 µM phenylephrine was around 50 % of the response to 50 mM KCl, which was smaller than the contraction developed by freshly isolated arteries.
Figure 4.17 IPA constriction to KCl and phenylephrine maintained after transfection with KCNQ siRNA.

Summary of contractile responses to 50 mM or 10 mM KCl (A) and 1 µM PE (B) of intrapulmonary arteries transfected with 200 nM KCNQ1,4 and -5 siRNA. The control represents arteries transfected with 200 nM scrambled siRNA. Responses to KCl and PE were measured 5 min and 30 min after exposure, respectively. Data plotted as mean ± s.e.m of vessels from 11 (KCl) and 10 (PE) animals.
4.2.4.1 Effect of KCNQ siRNA transfection on the IPA response to linopirdine

To compare vasoconstrictor responses of arteries transfected with scrambled or KCNQ siRNA, blood vessels were challenged with linopirdine over a concentration range of 30 nM to 30 μM, added cumulatively. Figure 4.18 shows the concentration-response relationship for contraction evoked by linopirdine in control and KCNQ1,4 or -5 siRNA transfected vessels. The maximum response was reached at 10 μM linopirdine and as seen in freshly isolated arteries, there was a sharp loss of linopirdine-evoked contraction at higher concentrations. The contraction-response curves were almost identical in vessels transfected with KCNQ4 or scrambled siRNA. In contrast, vessels transfected with KCNQ1 or KCNQ5 siRNA showed a reduced maximum response to linopirdine. The mean maximum constriction, measured as a percentage of the response to 50 mM KCl, was 125 ± 7 % (n=11) in control arteries, 92 ± 5 % (n=11, P=0.0021 One-way ANOVA followed by Dunnett’s multiple comparisons test was applied) in vessels transfected with KCNQ1 siRNA and 92 ± 5 % (n=11, P=0.0021) in vessels transfected with KCNQ5 siRNA. The mean pEC<sub>50</sub> was not affected by siRNA transfection in any condition.

![Figure 4.18](image-url)

Figure 4.18 Effect of targeting KCNQ1, 4 and -5 genes on the constrictive response of IPA to linopirdine.

Log concentration-response curves were for contraction induced by linopirdine in vessels transfected with 200 nM KCNQ1,4 or -5 or scrambled siRNA. The constriction was plotted as percentage of the tension induced by 50 mM KCl. Each point was plotted as the mean ± s.e.m of experiments on vessels from 11 animals.
4.2.4.2 Effect of knocking down KCNQ genes on the IPA response to Kv7 activators

The effect of transfecting arteries with KCNQ1, 4 or -5 siRNA was also tested on responses to Kv7 activators flupirtine and RL-3. Control and KCNQ-targeted artery segments were pre-constricted with 1 μM phenylephrine and then challenged with flupirtine at concentrations ranging from 0.1 μM to 200 μM, added cumulatively. The flupirtine concentration-response curve is compared in control and KCNQ1,4 and -5 targeted arteries, as shown in figure 4.19A. Responses to flupirtine were similar and maintained among all groups, with perhaps a slight shift leftward in KCNQ1 targeted vessels. However, there was no significant change in the pEC_{50} or maximum relaxation recorded from any KCNQ-targeted vessel when compared to the control vessels. The mean pEC_{50} was 4.65 ± 0.1 (EC_{50} = 22 μM; n=8) in control, 4.76 ± 0.16 (EC_{50} = 17 μM; n=6) in KCNQ1 targeted-vessels and 4.46 ± 0.23 (EC_{50} = 35 μM; n=8) in KCNQ5 targeted-vessels. The maximum relaxation reached at 200 μM flupirtine was also similar, being 78 ± 7 % in control and 91 ± 11 % and 103 ± 22 % (P=0.39) in KCNQ1 and KCNQ5 targeted-vessels respectively.

At the end of each experiment, 1 μM nifedipine was added to fully block contraction mediated by voltage-gated Ca^{2+} entry. Figure 4.19A manifests that nifedipine showed no further relaxation of the vessels, confirming that the maximum relaxation response to Kv7 channel activator was achieved at 200 μM flupirtine, the highest concentration tested.

The relaxant response to RL-3 was also compared in control and KCNQ siRNA transfected arteries. The concentration-response relationship for RL-3-induced relaxation is illustrated in figure 4.19B for vessels transfected with KCNQ1,4 and -5 siRNA or scrambled siRNA. The concentration-response curves following Kv7.1 knockdown shifted to the right when compared to the vessels transfected with scrambled siRNA. An F test was used to examine if one curve fits for both control and KCNQ1-targeted data sets. The result showed the lack of fit of one curve for both data sets with P value equals to 0.0072. However, the difference between pEC_{50a} values did not reach statistical significance. The mean pEC_{50a} for RL-3 was 6.28 ± 0.11 (EC_{50a} = 0.62 μM; n=7) in control vessels and 6.07 ± 0.1 (EC_{50a} = 0.97 μM; n=7) in KCNQ1-targeted vessels. The relaxation induced at the highest concentration of RL-3 tested was also not statistically different between control vessels (91 ± 5 %) and vessels transfected with KCNQ1 siRNA (77 ± 8 %).
Figure 4.19 Effect of targeting KCNQ1,4 and -5 genes on the IPA response to Kv7 activators.
Log concentration-relaxation relationship for flupirtine (A) and RL-3 (B) induced relaxation in vessels transfected with 200 nM scrambled or KCNQ1,4 and -5 siRNA. Relaxation was measured as percentage of the phenylephrine-induced contraction just before the addition of Kv7 channel activator. Each point was plotted as the mean ± s.e.m of experiment on vessels from n number of animals.
4.2.4.3 Effect of KCNQ siRNA transfection on the IPA response to sildenafil and treprostinil

The results in the previous chapter showed that Kv7 K\(^+\) channels are involved in mediating the relaxing effect of sildenafil and treprostinil. Therefore, we decided to investigate the effect of transfecting PASMCs with KCNQ4 siRNA in intact *ex-vivo* arteries on responses of treprostinil and sildenafil. KCNQ4 gene was targeted to investigate if the Kv7.4 channel involved in mediating the effect of treprostinil and sildenafil, as Joshi *et al.* (2009) showed that KCNQ4 was the most expressed subtype of KCNQ family in PASMCs.

Vasorelaxation responses to treprostinil were compared in control and KCNQ4-targeted vessels. Arteries were constricted with 1 μM phenylephrine and then challenged with treprostinil at concentrations from 0.1 nM to 1 μM. Figure 4.20A shows the concentration-response relationship for treprostinil in KCNQ4 and scrambled siRNA transfected vessels. The relaxation to treprostinil was maintained in both groups, and responses to treprostinil were similar among both groups. The maximum relaxation, reached at around 1 μM treprostinil was 109 ± 19 % (n=5) in control vessels and 101 ± 4 % (n=8) in KCNQ4-targeted vessels. The mean pEC\(_{50}\) for treprostinil was similar in vessels transfected with scrambled and KCNQ4 siRNA, being 7.53 ± 0.3 (EC\(_{50}\) =30 nM) and 7.7 ± 0.07 (EC\(_{50}\) =20 nM), respectively.

Similarly, the relaxation response to sildenafil was tested in control and KCNQ4 targeted-vessels. The pre-constricted vessels with 1 μM phenylephrine were challenged with sildenafil at a concentration range of 0.1 nM to 10 μM, added cumulatively. The concentration-response curve for sildenafil-mediated relaxation is shown in figure 4.20B. Knocking down Kv7 protein did not significantly affect sildenafil-mediated relaxation, but there was a very slight shift to the right in KCNQ4-targeted arteries. The sildenafil concentration-response relationship was not sigmoidal and the maximum response was not clearly reached. Nevertheless, nearly 100% of relaxation was achieved at 10 μM sildenafil. The mean pEC\(_{50a}\) for sildenafil in control vessels and KCNQ4-targeted vessels was 6.18 ± 0.17 (n=9) and 6 ± 0.16 (n=8) respectively. The mean relaxation achieved by sildenafil at 10 μM was 100 ± 4 % in control vessels and 92 ± 3 % in KCNQ4 vessels.
Figure 4.20 Effect of transfecting intact IPA with KCNQ4 siRNA on relaxant response of treprostinil and sildenafil.
Log concentration-relaxation relationship for treprostinil (A) and sildenafil (B) induced relaxation in vessel segments transfected with 200 nM scrambled (control) and KCNQ4 siRNA. Relaxation was measured as percentage of the phenylephrine-induced contraction just before the addition of treprostinil or sildenafil. Each point was plotted as the mean ± s.e.m of vessels from n number of animals.
4.2.5 **Summary**

The results of the experiments in this chapter can be summarised as follows:

1. IPA segments maintained in culture for up to 3 days retained their contractile response to KCl, phenylephrine and Kv7 modulators.
2. The optimum condition to keep vessels for three days *ex vivo*, while retaining normal responsiveness to KCl (10 and 50 mM) and Kv7 modulators, is to culture segments in the bicarbonate-buffered medium in the presence of 1% antibiotics.
3. The reverse permeabilisation technique was unsuccessful in transfecting IPA segments with siRNA or dsRNA.
4. N-TER transfecting reagent was more efficient than lipofectamine RNAiMAX in delivering fluorescein-labelled dsRNA into PASMCs of isolated IPA.
5. The optimal concentration of dsRNA to transfect PASMCs in intact IPA was 200 nM when using 16 µl N-TER in 1 ml medium.
6. Transfecting IPA segments with KCNQ1, 4 or -5 siRNA using N-TER transfection reagent did not affect the contractile responses of the vessels to KCl or phenylephrine.
7. Kv7.1 and Kv7.4 proteins are expressed in IPA.
8. Kv7.1 and Kv7.5 protein knockdown impaired the maximum response to linopirdine.
CHAPTER 5
GENERAL DISCUSSION
5.1 Main findings of the project

- Vessel size affects the response of IPAs to Kv7 channel blockers, sildenafil, treprostinil and phenylephrine. Each of these drugs produced larger maximum responses in conduit vessels. In contrast, Kv7 channel activators and U46619 induced a similar response in conduit and small vessels.
- Kv7.1 and Kv7.4 proteins are expressed in IPAs.
- Kv7.1 channel activators were potent vasorelaxants of IPAs (EC$_{50}$ ~ 0.3 µM) and their effects were selectively prevented by a Kv7.1 channel blocker.
- The maximum response to cGMP-mediated vasodilation, sildenafil and treprostinil were significantly reduced by pan-Kv7 channel blockers, but the EC$_{50}$ values were unaltered.
- The concentration-response curves to cAMP-generating vasodilators were shifted to the right in the presence of Kv7 channel blockers without affecting the maximum response.
- IPA segments maintained in organ culture for up to 3 days retained their contractile responses to KCl, phenylephrine and Kv7 modulators.
- The optimum condition for keeping IPA segments for three days ex-vivo, while maintaining a normal response to KCl and Kv7 modulators, was to culture the segments in a bicarbonate-buffered medium in the presence of 1% antibiotics.
- The N-TER transfecting reagent was more efficient than lipofectamine RNAiMAX in delivering fluorescein-labelled dsRNA into PASMCs of isolated IPAs.
- The optimal concentration of dsRNA to transfect PASMCs in intact IPAs was 200 nM when using 16 µl N-TER in 1 ml of the medium.
- Transfecting the artery segments with KCNQ1 and KCNQ5 siRNA impaired the maximum response to linopirdine, but transfecting vessels with KCNQ4 siRNA had no effect.
5.2 Response of conduit and small IPAs to vasoactive agents.

High concentrations of KCl lead to the constriction of pulmonary artery by depolarizing the PASMCs (Casteels et al., 1977a), which consequently causes an influx of Ca\(^{2+}\) through the VDCC. KCl (50 mM), was used to evaluate the viability of isolated vessels, and to evoke a maximum constriction (Gurney and Howarth, 2009) against which responses induced by other vasoconstrictor agents could be compared. The conduit and small IPAs showed distinct responses to 50 mM KCl, which caused the small vessels to constrict with a larger amplitude than the conduit vessels. This is consistent with the findings of a study by Leach et al. (1989), which showed that 75 mM KCl caused significantly larger contractile responses in small IPAs compared to the conduit vessels. It is also consistent with a study by Gurney and Howarth, (2009), who also showed that the maximum constriction to K\(^{+}\) was higher in the resistance vessels. However, they showed that KCl produced contraction with similar EC\(_{50}\) of around 40 mM in the conduit and resistance vessels. Thus, KCl had a similar potency in each vessel, but produced a larger maximum contraction in resistance arteries.

The responses of conduit and small IPAs to two of the most commonly used vasoconstrictors, phenylephrine and U46619, were different. The conduit arteries displayed a larger constrictor response to phenylephrine than the small IPAs, which is consistent with previous studies, where the response to phenylephrine was considerably larger in the conduit vessels (Leach et al., 1992; Gurney and Howarth, 2009). It has been shown that the size of the pulmonary artery also has a significant effect on the vascular response to serotonin and noradrenaline, which induced a negligible constriction in small IPAs (Priest et al., 1997; Gurney and Howarth, 2009). The weak response to phenylephrine in the small IPAs could be related to a lower expression of \(\alpha_1\)-receptors, which consequently, may be less involved in regulating vascular resistance. Contrast with this idea, the conduit and small IPAs showed a similar constriction response to 30 nM U46619. Conduit and small (or resistance) vessels also responded similarly to other vasoconstrictor agents, such as PGF\(_{2\alpha}\) (Leach et al., 1989; Priest et al., 1997; Gurney and Howarth, 2009). Thus, the pathways downstream of GPCRs appear to be comparable in both types of vessels. In this study, the small vessels produced a more robust response to U46619 than to phenylephrine, while the amplitudes of contraction of conduit arteries in response to phenylephrine and U46619
were not significantly different. Therefore, U46619 was selected as the vasoconstrictor to evoke tension in small or large vessels when testing responses to vasorelaxant drugs.

The Kv7 channel blockers, linopirdine and XE991, act as powerful constrictors of small IPAs (Joshi et al., 2006). The blockade of Kv7 channels resulted in the depolarisation of PASMCs, vasoconstriction and consequently, elevation of pulmonary artery pressure (Joshi et al., 2009). The data obtained in the current project is consistent with previous findings, as XE991 and linopirdine acted as potent vasoconstrictors of the conduit and small (only linopirdine-tested) vessels. This supports the idea that Kv7 channels are involved in regulating the basal tone and RMP of PASMCs (Joshi et al., 2006, 2009). Linopirdine constricted conduit and small IPAs with EC50 of 3.7 and 2.0 μM, respectively. The maximum constriction was reached at around 10 μM linopirdine. These values are of the same order as the EC50 for linopirdine of around 1 μM reported for rats (small arteries) and mice (resistance arteries) and a maximal effect reached at 10 μM linopirdine (Joshi et al., 2006). The EC50 for linopirdine in the conduit and small vessels is also similar to the IC50 value (2-4 μM) for inhibiting the M-current in sympathetic ganglia and hippocampal neurons (Lamas et al., 1997; Schnee and Brown, 1998; Wang et al., 1998). In this project, the mean maximum response to linopirdine had a significantly larger amplitude in the conduit (~ 56% of 50 mM induced constriction) than in the small (~20 % of 50 mM KCl induced constriction) IPAs. It was lower than the ~ 85% of what reported by Joshi et al. (2006), for small vessels. This difference in maximum response compared to the previous study could be due to different experiment conditions. In Joshi et al. (2006), the applied basal tension was 5 mN and the CaCl2 concentration in PSS was 1 mM. In this project, the applied basal tension was 4 mN, and a CaCl2 concentration of 1.8 mM was used. The Greenwood lab suggested that the response to Kv7 blockers is greater when applied tension is increased (Chadha et al., 2012a). The different responsiveness of conduit and small vessels to linopirdine suggests that it would be injudicious to extrapolate or generalise the results obtained in conduit IPAs to small IPAs or vice versa.

XE991 is 10 times more potent than linopirdine in inhibiting Kv7 channels (Joshi et al., 2006). Consistent with that, the XE991 EC50 for contracting IPA was around 0.4 μM in small IPAs (Joshi et al., 2006). In this study, XE991 constricted conduit IPAs with an EC50 of 1.78 μM in conduit vessels, which was four times that reported for small IPA. This difference could be due to variation in the size of the
vessels studied, as it was noted that the EC$_{50}$ for linopirdine appeared higher in conduit vessels compared to small vessels, although the difference did not reach statistical significance. The potency of XE991 as a vasoconstrictor is, however, close to the IC$_{50}$ value for inhibition heteromeric KCNQ2/3 channels (IC$_{50}$ ~1 μM) (Wang et al., 1998), homomeric KCNQ4 channels (IC$_{50}$ ~5.5μM) (Søgaard et al., 2001; Yeung et al., 2007) and homomeric Kv7.1 (IC$_{50}$ ~1 μM) (Wang et al., 2000), but far from the IC$_{50}$ value (~65 μM) to inhibit KCNQ5-encoded channels (Schroeder et al., 2000) and Kv7.1/KCNE channels (11.1 μM) (Wang et al., 2000). These data suggest that Kv7.4 K$^+$ channels are the most likely to mediate linopirdine- and XE991-induced pulmonary vasoconstriction, because heteromeric KCNQ2/3 channels and KCNQ2 mRNA are not expressed in pulmonary arteries. Moreover, the KCNQ5 mRNA level was low (Joshi et al., 2009) and in the current study the protein could not be detected. Additionally, the Kv7.1 channel blocker (HMR1556) failed to affect basal tone, consistent with the findings of other studies on basal vascular tone (Towart et al., 2009; Chadha et al., 2012a; Tsvetkov et al., 2017). This also suggests that the Kv7.1 isoform is not involved in the regulation of RMP, or it makes an insignificant contribution, so that its inhibition alone was insufficient to depolarise the membrane sufficiently to activate VDCCs. A possible reason for the inactivity of the Kv7.1 channel at the RMP of PASMCs could be its association with the KCNE4 protein in the vasculature (Yeung et al., 2007; Zhong et al., 2010). KCNE4 was reported to efficaciously silence the activity of the Kv7.1 channel (Grunnet et al., 2005).

The maximum constriction response to XE991 was reached at 10 μM, which is consistent with the results of Joshi et al. (2006). The maximum response was lower by around 30% compared to their finding, presumably for the same reasons as discussed for linopirdine.

The constrictor effects of the Kv7 blockers are likely to be mediated by an action on Kv7 channels, as they act selectively on Kv7 channels up to a concentration of 10 μM. At higher concentrations (50 μM), linopirdine and XE991 induced vasorelaxation. This is also consistent with the findings of other studies (Zaczk et al., 1998; Joshi et al., 2006). As it is a non-specific effect, it was not investigated further.

The effects of linopirdine and XE991 in many vascular beds have been reported, including the portal vein, aorta, renal and mesenteric arteries (Yeung et al., 2007; Mackie et al., 2008; Joshi et al., 2009; Chadha et al., 2012a, b). Joshi et al.
reported that linopirdine and XE991 contracted IPAs most strongly among a range of vascular beds, with negligible contraction of tail, cerebral, femoral and coronary arteries, but small constriction of the renal and mesenteric arteries of the rat. Furthermore, they showed that linopirdine raised the mean pulmonary artery pressure without affecting systemic pressure. They, therefore, suggested a larger role for Kv7 channels in IPAs compared to other vascular beds. Jepps et al. (2011) reported, however, that linopirdine and XE991 produced robust vasoconstriction of the thoracic aorta and it was significantly impaired in SHR. Additionally, they showed that applying 1 µM S-1 or 10 µM XE991, respectively, increased or decreased the outward K+ currents recorded from isolated mesenteric artery myocytes. These effects on K+ currents were also impaired in myocytes from SHR. These data also suggested the importance of Kv7 channels in regulating vascular tone and the RMP in these cells. While Chadha et al. (2012a) reported that linopirdine constricted the mesenteric artery, consistent with the findings of Joshi et al. (2006, 2009), the contraction was only ~ 5 % of the response to 50 mM KCl. The response was, however, amplified if the drug was applied to already contracted arteries.

Knockdown experiments using siRNA were conducted to gauge the contribution of each Kv7 channel α-subunit to the vasoconstrictor effect of Kv7 blockers. The maximal constriction response to linopirdine was significantly impaired to a similar degree in IPA segments treated with siRNA targeted against KCNQ1 or KCNQ5, but was not affected in vessels incubated with siRNA against KCNQ4. This result is consistent with the involvement of K7.1 and Kv7.5 channels, either as monomers or perhaps more likely as heteromeric Kv7.1/Kv7.5 channels. The lack of effect of KCNQ4 knockdown suggests that the monomeric Kv7.4 or heteromeric Kv7.4/Kv7.5 channels play no role. This finding is consistent with a study conducted by Oliveras et al. (2014), where they showed Kv7.1 and Kv7.5 subunit associate to form Kv7.1/Kv7.5 heteromeric channels with unique pharmacological and electrophysiological properties. Although they showed that the channels are mainly retained at the ER, they also showed retigabine could increase currents mediated by Kv7.5 and Kv7.1/Kv7.5 channels by more than 2 fold. However, this idea is inconsistent with the Western blot results, which could not detect Kv7.5 protein as well as with most other studies on vascular Kv7 channels (Mani et al., 2009; Jepps et al., 2011; Chadha et al., 2012b; Brueggemann et al., 2014b). It is possible that the Kv7.5 protein is present in IPA, but expressed at a low
level, either as a monomeric or heteromeric channel with Kv7.1. It seems unlikely that the Kv7.4 channels play no role in regulating IPA tone, since it was clearly detected both at mRNA level (Joshi et al., 2009) and from Western blotting. Moreover, the down-regulation or knockdown of Kv7.4 protein was shown to weaken the response of systemic vessels to Kv7 modulators implicating them a mediator in these vessels. A problem with my study is that there was insufficient time to test how efficient the Kv7 α-subunit protein were knocked down. To understand the exact role of each sub-unit in the Kv7 channels of IPA, it will be necessary to evaluate knockdown success by Western blotting, so that the degree of protein reduction can be correlated with the change of vascular response to Kv7 modulators.

In agreement with the previous report (Joshi et al., 2009), the two commonly-used Kv7 activators, flupirtine and retigabine, inhibited pulmonary artery vasoconstriction. They were effective against tension induced by phenylephrine or U46619, in conduit and small IPAs (only flupirtine). The results extend the previous findings by showing that the drugs were equally effective in small and conduit arteries and their effects were independent of the vasoconstrictor present. Flupirtine reversed the constriction of conduit and small IPAs with similar EC\textsubscript{50} values of 21 and 37 µM, respectively. The data are consistent with the reported EC\textsubscript{50} for flupirtine in small IPA of 62 µM (Joshi et al., 2009). The potency of flupirtine in this study is slightly lower than its effect in activating the native M-current of visceral sensory neurons (EC\textsubscript{50}= 10-20 µM) (Wladyka and Kunze, 2006) and Kv7 channels in hippocampal neurons (EC\textsubscript{50}= 6.1 µM) and dorsal horn neurons (EC\textsubscript{50}= 5.4 µM) (Klinger et al., 2012). Retigabine was 4 times more potent than flupirtine at reversing the constriction of IPAs, with EC\textsubscript{50} of 5 µM in conduit arteries. A similar 5 fold difference in EC\textsubscript{50} value was reported previously (Joshi et al., 2009). Similar potency has been reported for retigabine activation of Kv7 channels. Retigabine activated KCNQ2/3 channels with EC\textsubscript{50}= 5.2 µM (Main et al., 2000), the M-currents in sympathetic neurons with EC\textsubscript{50} ~1 µM, the Kv7.4 channel expressed in Chinese hamster ovary cells with EC\textsubscript{50}= 5.2 µM (Tatulian et al., 2001) and KCNQ3/5 channels with EC\textsubscript{50}= 1.4 µM (Wickenden et al., 2001), of these channels only the Kv7.4 subtype is expressed in IPA (Western blot results), while the KCNQ2 and KCNQ3 mRNA are not expressed in the IPA of rat (Joshi et al., 2009). The potencies of retigabine and flupirtine are compatible with the idea of Joshi et al. (2009) that Kv7 activators induce pulmonary vasodilation by activating Kv7 channels.
Joshi et al. (2009) showed that PASMCs express KCNQ1, 4 and 5 mRNA, with the KCNQ4 subunit being the most expressed and KCNQ5 the least expressed. Western blots confirm these findings by showing that the Kv7.4 and Kv7.1 proteins are highly expressed in IPAs, but Kv7.5 protein could not be detected, although it was detected in brain tissue. As flupirtine and retigabine do not act on the Kv7.1 channel (Schenzer et al., 2005; Wuttke et al., 2005; Joshi et al., 2009), and no Kv7.2 or Kv7.3 mRNA were expressed in IPAs (Joshi et al., 2009), the effects of flupirtine and retigabine must be mediated by modulating homomeric or heteromeric KCNQ4 and/or KCNQ5 channels. The high abundance of Kv7.4 channels makes them the most likely target, but Kv7.5 could be expressed at a level too low to be detected, but sufficient to combine to a functional channel.

The siRNA knockdown studies found that responses to flupirtine of vessels incubated with siRNA against KCNQ1, 4 or 5 were not impaired. This contrasts with the findings that had shown the role of Kv7 channels in the IPA (Joshi et al., 2006, 2009; Morecroft et al., 2009) and in other vascular beds (Mani et al., 2009; Jepps et al., 2011; Chadha et al., 2012b; Zavaritskaya et al., 2013; Brueggemann et al., 2007, 2011a, 2014). This could have been due to insufficient or unsuccessful knockdown. Although we demonstrated successful uptake of fluorescent dsRNA by PASMC, this does not guarantee gene knockdown. Western blot analysis of Kv7.4 channel expression is required to determine successful knockdown of the Kv7 channel subunit in IPA was achieved. Unfortunately, this cannot be checked for Kv7.5, due to its low abundance. As KCNE subunits also influence the properties including the pharmacology (Schwake et al., 2006; Abbott, 2014) of Kv7 channels, their expression should also be assessed to get a full picture of the Kv7 channels that mediate responses of Kv7 activators.

Potassium channel openers cause the K⁺ flux to increase across the cell membrane, hyperpolarising the membrane potential below the threshold for opening VDCC, thereby resulting in vasorelaxation (Clapp et al., 1993). Increasing the extracellular [K⁺] depletes the transmembrane [K⁺] gradient, reducing the resting K⁺ efflux and resulting in pulmonary artery constriction due to depolarisation of the PASMC membrane. Raising the extracellular [K⁺] to 55 mM depolarises the membrane potential of rabbit PASMC to around -26 mV, and raising it to 80 mM causes depolarisation to around -18 mV (Casteels et al., 1977a). By depleting the transmembrane K⁺ gradient, raising the extracellular K⁺ concentration also prevents
change in $K^+$ channel activity from altering the membrane potential. It, therefore, prevents $K^+$ channel activators from hyperpolarising the membrane of PASMCs and causing vasodilation (Hamilton et al., 1986). In this study, flupirtine was less effective in relaxing conduit and small IPAs when pre-constricted with 80 mM [K+] compared to vessels constricted with phenylephrine or U46619. At 10 µM, flupirtine had especially no effect on $K^+$ constricted IPA, but at higher concentrations relaxation efficiency of flupirtine was only partially inhibited in vessels pre-constricted with $K^+$. Thus, this finding argues against Kv7 channel activator as the only mechanism by which flupirtine evokes relaxation. Since inhibition of $K^+$-induced contraction is a hallmark of Ca$^{2+}$ antagonists, it is likely that flupirtine can act as a Ca$^{2+}$ antagonist, similar to the Ca$^{2+}$ channel inhibition reported for retigabine (Mani et al., 2013).

Although I was unable to confirm a major role for Kv7.4 or Kv7.5 channel in mediating IPA dilation by Kv7 activators, both have been implicated, although there is disagreement over which is the key subunit. Jepps et al. (2011) showed that Kv7.4 channel downregulation in hypertension model inhibited Kv7 currents and responses of mesenteric arteries and aorta to Kv7 activators, while Chadha et al. (2012b) concluded that the Kv7.4 channel is a major mediator of relaxation in renal arteries of the rat. In contrast, other studies, also using siRNA knockdown strategies, (Mani et al., 2009; Brueggemann et al., 2007, 2011a, 2014) provided evidence that currents recorded from mesenteric and aortic arterial myocytes or A7r5 smooth muscle cells were generated by Kv7.5 or Kv7.4/7.5 channels. It is possible that different Kv7 channels are involved in regulating vascular tone and RMP in different vascular beds.

Although we have been unable to confirm the Kv7.4 or Kv7.5 involvement in IPA responses to flupirtine and retigabine, the effects of ICA069673 are consistent with a role of Kv7.4 channel in IPA of rat. ICA069673 relaxed IPA by up to 100% with $EC_{50} = 6 \mu M$. This drug has been shown to robustly activate homomeric Kv7.4 channels at $\sim 10 \mu M$, to have little effect on Kv7.1 or Kv7.5 channels and an intermediate effect on heteromeric Kv7.4/Kv7.5 channels (Amato et al., 2011; Brueggemann et al., 2014a; Provence et al., 2015).

The relaxation to ICA069673 was unaffected by blocking Kv7.1 channels with HMR1556. Importantly, while ICA069673 relaxed arteries constricted with PE, it had especially no effect on arteries constricted with 80 mM K. Thus, unlike retigabine and flupirtine, ICA069673 did not appear to act as a Ca$^{2+}$ channel antagonist. The most
likely explanation for the vasorelaxation response to ICA069673 is therefore that it activated Kv7.4 channels, either in a monomeric or a heteromeric with Kv7.5 channels.

5.3 Effect of activating Kv7.1 channels on intrapulmonary arteries

It has been shown that the Kv7.1 channel is expressed in VSMCs of rodents and humans, both in terms of the mRNA and protein levels (Ohya et al., 2003; Yeung et al., 2007; Mackie et al., 2008; Joshi et al., 2009; Zhong et al., 2010; Jepps et al., 2011; Ng et al., 2011; Chadha et al., 2012a). Kv7.1 subunit in IPA has been reported at the mRNA level (Joshi et al., 2006). I have now shown that Kv7.1 protein is expressed in IPA at a level comparable with an expression of the Kv7.4 protein. Pharmacological studies showed that the Kv7.1 channel activator, RL-3, induced vasorelaxation in the pre-contracted thoracic aorta (EC50= 4 µM), intrapulmonary (EC50= 3 µM) and mesenteric arteries (EC50= 0.5 µM) of rats (Chadha et al., 2012a). Furthermore, RL-3 induced enhancement of KCNQ1-generated currents in Xenopus oocytes overexpressing the Kv7.1 channel. RL-3 was selective to the Kv7.1 channel, because it did not affect KCNQ4 or KCNQ5-generated currents in the same system (Chadha et al., 2012a).

HMR1556 is a Kv7.1 blocker which inhibited the currents conducted by KCNQ1 without having any effect on the currents generated by overexpression of KCNQ4 and KCNQ5 (Chadha et al., 2012a). On the other hand, Tsvetkov et al. (2017), reported that RL-3 dilation was unchanged in Kv7.1 knockout mice, compared with wild mice. They also found that the relaxation was unaffected by Kv7.1 blockers, chromanol 293B or HMR1556, and concluded that the Kv7.1 channel is not involved in the RL-3-mediated relaxation of arteries in mice. The same author found that ML277, a potent Kv7.1 activator with EC50 of 260 nM (Mattmann et al., 2012), also induced relaxation of mesenteric arteries, but with an EC50 ~ 100 µM (Tsvetkov et al., 2017). The picture is further complicated by a report that RL-3 had no vasorelaxant effect on pre-constricted coronary arteries of rats (Khanamiri et al., 2013), and other reports showing that ML277 was ineffective in the coronary artery of pigs (Chen et al., 2016), human chorionic plate arteries (Wei et al., 2018), and the mesenteric artery of mice (Tsvetkov et al., 2017). In rat IPA RL-3 reversed the vasoconstriction induced by phenylephrine or U46619 in both conduit and small IPAs, with EC50 of 0.45 and 0.56 µM, respectively. It proved to be around 6 fold more potent than previously reported for rat PA, but of similar potency to the dilation of rat mesenteric arteries (Chadha et al., 2012a). Thus, it appears that the
activation of Kv7.1 channels is effective in inducing pulmonary vasorelaxation in the rat. Further support was provided by the efficacy of ML277, which also evoked relaxation of rat pulmonary artery with EC$_{50}$ values of 180 nM in the conduit IPAs. The ML277 EC$_{50}$ value was almost the same as that observed for its action on heterologously expressed Kv7.1 channels (Mattmann et al., 2012). The maximum relaxations induced by RL-3 and ML277 were ~93 and ~83%, respectively. Thus, the activation of Kv7.1 channels evoked profound relaxation of the IPAs of rats. Interestingly, the efficacy and potency of the Kv7.1 activators were at least as high as found for the vasorelaxant effects of the Kv7.2-Kv7.5 channel activators, flupirtine and retigabine. The maximum effect of RL-3 was similar to the maximum relaxation response produced in mesenteric artery, but double that observed by Chadha et al. (2012a) in the pulmonary artery. A possible explanation is that Chadha et al. (2012a) did not wait long enough for the drug to take full effect before applying increments in drug concentration because RL-3 was slow to produce a response, but it took around 30 minutes for the response to reach a steady state.

The inconsistent findings with RL-3 and ML277 could be due to variations in vascular beds and also among species. A possible explanation for these variations is the association of different KCNE subunits with the Kv7.1 protein, which significantly affects the pharmacology and biophysical properties of the channel (Barhanin et al., 1996; Yeung et al., 2007; Melman et al., 2002, 2004; Grunnet et al., 2005; Jespersen et al., 2005; Zhong et al., 2010).

Data from the siRNA knockdown experiments showed the EC$_{50}$ and the maximum response for RL-3 was unaffected in vessels incubated with KCNQ1, 4 or 5 siRNA compared to control vessels. The lack of effect of KCNQ1 knockdown suggests that the monomeric channels play no role in mediating the vasorelaxant effect of RL-3. It seems unlikely that the Kv7.1 channel play no role in mediating the vasorelaxant effect of Kv7.1 activator, since it was clearly detected both at mRNA level (Joshi et al., 2009) and from western blotting, and the pharmacological studies in my project. Furthermore, Chadha et al. (2012a), suggests that the RL-3 relaxation is at least partly mediated via activating Kv7.1 channel. A problem with my study is that there was insufficient time to test how efficient the Kv7.1 protein was knocked down. To understand the role of Kv7.1 channels of IPA. It will be necessary to evaluate
knockdown success by western blotting, so that the degree of protein reduction can be correlated with the change of vascular response to Kv7.1 modulators.

Consistent with action on K+ channels, ML277 and RL-3 were both more effective at inhibiting agonist-induced vasoconstriction, compared with 80 mM K+ induced vasoconstriction. The inhibition by K+ was, however, incomplete. This suggests that, although K+ channel activation could account for most of the relaxation induced by these drugs, at least part of the relaxation must result from a mechanism that is independent of Kv7.1 channel activation. A possible explanation for the non-selective effects of RL-3 and ML277 is that, in common with retigabine and flupirtine, they inhibit L-type Ca2+ channels (Mani et al., 2013). This was not tested, but it could explain the inhibition of depolarisation-induced contraction at higher concentrations. Drugs that activate KATP channels cause vasodilation by hyperpolarising SMCs, and inhibiting the influx of Ca2+ (Clapp et al., 1993; Brayden, 2002). KATP channels were not involved in the RL-3-mediated relaxation of pulmonary artery, because it was not affected in the presence of glibenclamide, a selective KATP channel blocker (Robertson and Steinberg, 1990).

HMR1556 and chromanol 293B selectively inhibit the monomeric Kv7.1 channel, although HMR1556 is more potent (Gogelein et al., 2000; Wang et al., 2000; Chadha et al., 2012a). Neither drug has been found to contract systemic blood vessels under basal tone (Chadha et al., 2012a), and this consistent with my findings. However, in the presence of minimal pre-tone, HMR1556 caused contraction of around 10% of the response evoked by 50 mM KCl in rat IPAs. This agrees with a report by Chadha et al. (2012a), who found that it did not contract mesenteric arteries under the same conditions, but they showed that HMR1556 caused a contraction of around 10% of the contraction induced by 60 mM KCl in IPA of rat.

Chadha et al. (2012a) showed that HMR1556 attenuated KCNQ1-mediated currents in Xenopus oocytes cells without affecting KCNQ4 and KCNQ5 generated currents. Thus, Kv7.1 channels may become activated in the presence of receptor agonist and counteract or limit the agonist-induced tone. Another possible explanation is that Kv7.1 channels are active at the resting potential but its blockade alone cannot depolarise the membrane sufficiently to reach the threshold to open VDCC. HMR1556 was reported to reverse the RL-3 induced relaxation of rat mesenteric and pulmonary artery (Chadha et al., 2012a), but its effects were tested only at 10 µM and
against one concertation of RL-3. I studied the effect of HMR1556 on IPA over a range of concentrations and found that it antagonised RL-3 mediated relaxation at concentrations as low as 0.1 µM. The antagonism was concentration dependent and shifted RL-3 concentration-response curve to the right, although Schild analysis indicated that the mechanism of blocking was not competitive, because the slope of the Schild plot was far lower than 1 (Arunlakshana and Schild, 1959). The slope value does not obey the competitive binding theory where the concentration ratio of agonist increases linearly with the increment of antagonist concentration over a wide range of antagonist concentrations with a slope of unity (Arunlakshana and Schild, 1959; Neubig et al., 2003; Dougall and Unitt, 2015) as the competitive antagonist causes rightward displacement of the concentration-response curve without any change in the maximal response (Arunlakshana and Schild, 1959; Neubig et al., 2003). Therefore, our result suggests that RL-3 and HMR1556 have distinct binding sites or interaction of RL-3 with more than one target protein (receptor) (Neubig et al., 2003; Dougall and Unitt, 2015). As yet, however, there are no reports of how either drug interacts with the Kv7.1 channels. 1 µM HMR1556 also shifted the ML277 concentration-response curve to the right with a significant increase in the EC\textsubscript{50} value.

Importantly, HMR1556 did not interfere with the relaxation of IPA activated by retigabine or levcromakalim. This data supports the hypothesis that HMR1556 interfered with RL-3 or ML277 relaxation by interacting selectively with the Kv7.1 channel.

Chromanol 293B inhibits KCNQ1/KCNE1 subunits with an IC\textsubscript{50} of ~7 µM, while it inhibits homomeric Kv.1 channel with an IC\textsubscript{50} ~27 µM and heteromeric (KCNQ2/KCNQ3) channels with IC\textsubscript{50} of ~100 µM (Lerche et al., 2007). As previously reported (Yeung et al., 2007; Ng et al., 2011; Chadha et al., 2012a), it lacked the ability to induce vascular tone. It also failed to inhibit the relaxation produced by Kv7.1 activators when used at concentrations up to 10 µM. It was ineffective both as the racemate or as the active negative enantiomer, which showed 7 fold higher potency (Seebohm et al., 2001). Chromanol 293B also failed to reverse the RL-3-produced relaxation in mesenteric arteries of mice, although it partially reversed (by ~ 10%) RL-3-induced relaxation of rat mesenteric arteries (Tsvetkov et al., 2017). Chromanol 293B (+293B and -293B) also did not interfere with the relaxation of rat IPA induced by retigabine. As the potency of chromanol 293B is affected by the subunits that co-
assemble with Kv7.1, it is possible that higher concentrations of the drug may be needed to counteract Kv7.1 activation and IPA relaxation. Thus, the lack of effect of chromanol does not rule out Kv7.1 channel involvement in responses to RL-3.

Overall, the data in this study suggest that the Kv7.1 channel is likely a functional and druggable target in the IPAs of rats, and that activation of Kv7.1 channels evokes profound vasodilation. On the other hand, the data also show that Kv7.1 channels play no appreciable role in regulating the basal resting tone of IPA. As the Kv7.1 protein is expressed in IPA and activating the Kv7.1 channel, could provide a strategy for treating PAH. Human IPAs should, therefore, be investigated to determine if they show a similar sensitivity to Kv7.1 activators.

5.4 Role of Kv7 potassium channels in cGMP-mediated vasodilation

The pan-Kv7 channel blockers linopirdine and XE991 impair the maximal response to cGMP-mediated vasodilators. This was seen with the cGMP-dependent vasodilators SNP, GTN and BAY41-2272, although the effects on relaxation to the later compound was much less marked. To the best of my knowledge, this is the first study to demonstrate a functional correlation between cGMP and Kv7 channels in pulmonary arteries.

Stott et al. (2015b) reported that in rat aorta, 10 µM linopirdine shifted the concentration-response curve to the right for SNP, atrial natriuretic peptide (ANP) and C-type natriuretic peptide (CNP), while in the renal artery interfered only with the effect of ANP. They also showed that cGMP increased the currents conducted by Kv7.4 channels in human embryonic kidney (HEK)293 cells. In pulmonary artery, linopirdine was effective at 1 µM. Given that HMR1556 had no effect on relaxation response to the same cGMP-dependent vasodilators, Kv7.1 channels were not the mediators of the response (Stott et al., 2015b). HMR1556 was ineffective at a concentration causing a substantial block of relaxation to RL-3. Thus, Kv7.4, Kv7.5 or heteromeric Kv7.4/Kv7.5 channels are the likely candidates for the linopirdine and XE991-sensitive component of the relaxation. This is consistent with the findings of Stott et al. (2015b), who proposed Kv7.4 and/or Kv7.5 channels are a target for cGMP.

cGMP dependent vasodilation has been studied for several decades and numerous mediators have been proposed, such cGMP-dependent protein kinases, cGMP inhibited- and cGMP stimulated-PDE, and cGMP-gated cation channels (Lincoln and

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cGMP can interact with protein targets (Smolenski et al., 1998; Schmidt et al., 2003), although relaxation is thought to result mainly from the activation of cGMP dependent PKG (Dhanakoti et al., 2000). The kinase has many downstream targets, including IP₃R, Phospholamban, SOC and vasodilator-stimulated phosphoprotein (Smolenski et al., 1998). Thus, although our evidence suggests the involvement of Kv7 channels in the NO/cGMP signalling pathway, it is unlikely to be the sole mediator of relaxation. As linopirdine and XE991 reduced the maximum response to cGMP-dependent vasodilators by 30-40%, a substantial proportion of the response may depend on Kv7 channels.

Sildenafil activates cGMP-dependent dilation by preventing cGMP metabolism. Dilation to sildenafil was also inhibited by linopirdine, but it is less clear how. The log concentration-response curve to sildenafil was often biphasic. The maximum response for sildenafil in conduit IPA is significantly higher than those with smaller IPA, but there was no difference between EC₅₀ value suggesting that sildenafil may have better efficacy in conduit vessels compared to small IPA. Linopirdine appeared to preferentially inhibit the first component, below 1 µM sildenafil. As sildenafil inhibits PDE5 with IC₅₀ ~ 10 nM, the second phase of dilation at sildenafil > 1 µM may reflect an off-target action of sildenafil. When cGMP synthesis was stimulated with the GC activator BAY41-2272, the resulting relaxation showed much lower sensitivity to linopirdine and XE991 than seen with other cGMP dependent dilation. This argues against the involvement of Kv7 channels in cGMP-dependent dilation. On the other hand, cGMP-signalling is compartmentalised in cells (McCormick and Baillie, 2014) and it is possible that the cGMP generated by BAY41-2272 is the wrong place to activate Kv7 channels. Since the effects of linopirdine and XE991 were more pronounced on SNP and GTN-induced dilation than on the dilation to sildenafil and BAY41-2272, it is possible that Kv7 channel activation involves a cGMP-independent component. SNP and GTN both generate NO and NO is known to produce some of its effects independently of cGMP (Adachi et al., 2004; Adachi, 2010).

Further evidence for Kv7 channels as a target in cGMP-mediated vasodilation comes from the ability of linopirdine and XE991 to inhibit endothelium-dependent dilation. Muscarinic agonist, carbachol, promotes endothelial cell production of NO, which diffuses to the smooth muscle cells and activates the sGC-cGMP signalling pathway (Loscher, 1991). The vasorelaxant effect of carbachol was compromised by
linopirdine and XE991, but not by HMR1556. Endothelium-dependent relaxation can also be stimulated by the Ca\(^{2+}\) ionophore, A23187, which cause Ca\(^{2+}\) entry into endothelial cells and activation of the Ca\(^{2+}\)-dependent synthase. The concentration-response curve to A23187 was also impaired by linopirdine and XE991, but not significantly by HMR1556. As seen with relaxation induced by SNP and GTN, the Kv7 channel blockers reduced the maximum response to both carbachol and A23187, consistent with a component of relaxation being mediated by Kv7 channels. As the vasodilation were all affected in the same way by Kv7 channel blockers, the inhibition of endothelial-dependent dilation reflects the action of the blockers on the smooth muscle cells.

This result further supports the involvement of Kv7.4 or Kv7.4/Kv7.5 channel and not Kv7.1 sub-unit in mediating vasodilation induced by the release of NO from endothelial cells. Hence, these data and the study conducted by Joshi et al. (2009), provide a better picture on the importance and the role of Kv7 channels in regulating pulmonary vascular tone and shows Kv7 channels are more likely participate to the mechanism of NO-induced vasodilation. It would be of interest to investigate if the downregulation of Kv7 channels, particularly the Kv7.4 sub-unit, plays any role in mediating endothelial dysfunction that occurs in the arteries of pulmonary hypertensive animals (Humbert et al., 2004).

### 5.5 Role of Kv7 potassium channels in cAMP-mediated vasodilation

Several studies have shown a contribution of Kv7 channels to cAMP-dependent relaxation of different blood vessels and in response to various endogenous and exogenous agents. These studies were based on pharmacological and/or molecular approaches, mainly using pan-Kv7 channel blockers, which attenuated cAMP-dependent vasorelaxation. Chadha et al. (2012b) proposed the involvement of Kv7 channels in β-adrenoceptor mediated relaxation of rat renal arteries, since the maximal isoproterenol-mediated vasodilation was significantly impaired by 10 µM linopirdine or by knocking down Kv7.4 channel expression \textit{ex-vivo}. They showed that isoproterenol increased Kv7 channel generated currents, whereas linopirdine had the opposite effect (Chadha et al., 2012b). Stott et al. (2015a) also showed the maximum isoproterenol relaxation to be impaired by the addition of 10 µM linopirdine. Our result, show a rather subtle effect of Kv7 channel blocker on cAMP-mediated relaxation of IPA.
Linopirdine and XE991 noticeably shifted the isoprenaline-concentration relaxation curve to the right, without affecting the maximum response. This difference could be a feature of different vascular beds, with Kv7 channels displaying artery-specific contributions to cAMP-dependent vasodilation. Many studies have shown that the vasodilatory effect of cAMP-producing molecules can require PKG “cross-activation” by cAMP (Toyoshima et al., 1998; Hill et al., 2016). Thus, there could be indirect involvement of Kv7 channels in the cAMP-mediated relaxation, via the cGMP pathway.

Another factor supporting the involvement of Kv7 channels in producing vasodilation in response to activating β-adrenoceptors is its inhibition by 1 µM XE991, which was more pronounced than inhibition by 1 or 5 µM linopirdine. This result is consistent with the differential potencies of XE991 and linopirdine as Kv7 channel blockers. Since HMR1556 did not affect the isoprenaline-induced relaxation, Kv7.1 channels were not involved. Therefore, it is likely that Kv7.4 or Kv7.5 monomers or Kv7.4/Kv7.5 heteromer were involved in mediating the effect in the rat IPA.

In contrast to the reports of Chadha et al. (2012b) and Stott et al. (2015a), Mani et al. (2016b) found that isoproterenol activated Kv7.5 currents in A7r5 cells via β-AR and the PKA-dependent signalling pathway. Our results do not differentiate between the Kv7 channel types involved. Further molecular and electrophysiological studies are necessary to identify the composition of the Kv7 channels contribute to isoprenaline-induced vasodilation of rat IPA.

Since Barman et al. (2003) identified BK_{Ca} channels as a target of PKA-independent and PKG-dependent signalling in PASMC and the same findings were reported in coronary artery SMCs (Minami et al., 1993; White et al., 2000), we considered the possibility that the Kv7 channel blockers might interfere with isoprenaline-induced relaxation by blocking BK_{Ca} channels. In IPA, however, the BK_{Ca} inhibitor iberiotoxin did not interfere with the relaxation response to isoprenaline, resulting BK_{Ca} channels out as target of cAMP in IPA.

Linopirdine (1 and 5 µM) and XE991 (1 µM) also attenuated the vasodilating effect of forskolin, an AC activator, in IPA. This agrees with the earlier studies on rat renal artery (Chadha et al., 2012b), and mouse basilar artery (Lee et al., 2015), although these studies used a 10-fold higher concentration of linopirdine. As seen with isoprenaline, Kv7 channel blockers shifted the forskolin concentration-response curve to the right, giving an increasingly higher EC_{50} with increasing concentration of
linopirdine. The maximum relaxation response demonstrated no change as a result of adding a Kv7 channel blocker. Also, as seen when isoprenaline was used to activate relaxation, HMR1556 had no effect on the forskolin-induced relaxation. The similar effects of Kv7 channel blockers on isoprenaline and forskolin-mediated relaxation support the hypothesis that cAMP was responsible for recruiting Kv7 channels, either directly or indirectly.

A recent study proposed that Gβγ subunits are crucial for Kv7.4 channel activation in blood vessels (Stott et al., 2015a). Gβγ was found to increase the activation rate and amplitude of Kv7 currents in HEK cells (Stott et al., 2015a). In addition, the Gβγ inhibitor, gallein, weakened the isoproterenol-induced vasorelaxation in a similar manner to linopirdine and had the opposite effect to Gβγ on Kv7 currents (Stott et al., 2015a). In IPA, gallein was found to have no effect on the isoprenaline-induced relaxation. Our results do not, therefore, support a role for Gβγ subunit in mediating the relaxation effect of β-adrenergic stimulation in IPA.

It is understood that Gas/cAMP/PKA pathway was the general vasodilation pathway for isoprenaline action (Mani et al., 2016b). However, our results had demonstrated inconsistencies with the previous study as well as with many other similar studies since the PKA inhibitor had not impeded the isoprenaline-induced vasorelaxation. The abnormality in the abovementioned result could be due to the insufficient permeation of the PKA inhibitor into the cell or the chemical that had become defective as a result of storage condition or preparation method.

Treprostinil activates AC and the cAMP signalling pathway by stimulating IP receptors (Olschewski et al., 2006; Cunningham et al., 2016). It was therefore surprising that Kv7 channel blockers reduced the maximal relaxation of IPA to treprostinil, which was not seen with isoprenaline and forskolin-induced relaxation. The effects of the Kv7 channel blockers on treprostinil-induced relaxation resembled their effects on cGMP-dependent dilation, rather than cAMP-mediated dilation. It is therefore of interest that the relaxation response to treprostinil of rat, porcine and human pulmonary arteries was recently shown to be impaired in the presence of the NO synthase inhibitor, L-NAME, and in endothelium-denuded arteries (Fuchikami et al., 2017). These findings indicate that part of the relaxation was due to NO release from the endothelium and acted via the NO/cGMP signalling pathway. Similar to cAMP and
cGMP-dependent relaxation, the response to treprostinil was not affected by HMR1556, ruling out Kv7.1 channels in the response.

Consistent with the involvement of the endothelium in responses to treprostinil, relaxant responses were larger in conduit IPA compared with smaller IPA. This result is also consistent with the findings of Fuchikami et al. (2017) noted above. Our results also showed that the sensitivity of conduit arteries to treprostinil was higher when they were pre-constricted by 1 μM phenylephrine, as compared to 30 nM U46619. This contrasts with responses to sildenafil, which acts directly on PASMCs and is not more effective in PE stimulated arteries.

The results of siRNA knockdown studies to investigate the Kv7 channels involved in cyclic nucleotide-dependent dilation were inconclusive. Concentration-response curves to sildenafil and treprostinil were unchanged in vessels incubated with KCNQ4 siRNA, as compared to vessels incubated with scrambled siRNA. Without measuring the level of Kv7 knockdown, we cannot be sure that protein expression was successfully reduced.

5.6 Optimising IPA vessel culture ex-vivo.
Cultured IPA segments maintained their ability to contract for up to three days after isolation, in terms of their responses to KCl, phenylephrine and Kv7 channel blockers. Contractile responses were noticeably altered in several culture conditions, compared to the control condition. The main changes were hypersensitivity to K⁺, a decrease in vascular response to 50 mM KCl and changes in the maximum response and/or potency of Kv7 channel blockers. Among the conditions tested the optimum for keeping the IPAs for three days ex-vivo was to use bicarbonate-buffered medium in the presence of 1% antibiotics and 10% BSA. In these conditions, there was no significant change in the parameters measured, compared to the control vessels.

Retaining the contractile ability after 3 days of culturing indicates that the PASMCs maintained their contractile phenotype and did not convert to a proliferative phenotype, which was detected after culturing isolated SMCs (e.g. Owens, 1995; Thyberg, 1996). Our data are consistent with other studies in which vascular SMCs retained their contractile phenotype following culture of vessel segments ex-vivo (Lindqvist et al., 1999; Guibert et al., 2005; Manoury et al., 2009). Manoury et al. (2009) reported a decrease in the contractile response to 50 mM KCl on day 3 of
culturing in HEPES-based DMEM containing 1% antibiotic and L-glutamine or glutamax. They also showed that, although freshly dissected IPA had a negligible response to 10 mM KCl (Total [K+] was 15 mM), after culture for 3 days vessels contracted significantly. Guibert et al. (2005) found that culturing pulmonary arteries for 4 days in HEPES-based DMEM, in the absence or presence of serum, caused a marked hypersensitivity to KCl (10 to 40 mM) in the main and first-order IPA. Resende et al. (2004) and Lartaud et al. (2007) reported that the contractile response of aorta to a high concentration of KCl was not reduced after 3 days of culturing in modified DMEM, supplemented with an antibiotic mixture and L-glutamine. Our data show that the average constriction of IPA to 10 and/or 50 mM KCl changed significantly in all conditions, except when the vessels were cultured in bicarbonate-buffered medium in the presence of the antibiotic, or in vessels incubated in HEPES-based PSS with no antibiotics. The variation in findings of the different groups might be due to variation in the medium type, culture environment or duration of culture i.e., 3 versus 4 days (Guibert et al., 2005). The buffering system is likely to be important, as HEPES was reported to have a cytotoxic effect, especially in medium exposed to light (Zigler et al., 1985), and to reduce the vascular contraction in response to prostaglandins (Altura et al., 1980). When HEPES-based PSS was used, contraction to KCl was only maintained in one condition. In this respect, the decrease in response of cultured IPA to 50 mM KCl is consistent with Guibert et al. (2005) and Manoury et al. (2009), who cultured in a HEPES-based medium rather than HEPES-buffered PSS. The reduction in response might, therefore, be due to the effect of HEPES. On the other hand, vessels cultured in bicarbonate-buffered PSS showed a deterioration in response to KCl, both in the magnitude of contraction and an increase in sensitivity. PSS lacks the amino acids, proteins and nutrients that are essential for the normal growth of PASMCs, Guibert et al. (2005) and Manoury et al. (2009) used medium in their studies, rather than PSS. The presence of the additional factors in medium may, therefore, help to preserve function. Our results further show that adding antibiotics to PSS did not improve the maintenance of contractility or prevent the increment in vascular response to 10 mM KCl.

Modifying the content of DMEM can affect the contractile properties of cultured vessels (Lindqvist et al., 1999; Bakker et al., 2000); the presence of serum causing a decrease in the contraction force of cultured rat tail arteries. In contrast, Guibert et al. (2005) found that the presence or absence of serum did not affect the contractile
phenotype of IPA or other effects of culturing the vessels. Replacing foetal calf serum with insulin-transferrin-selenium, reduced the development of spontaneous rhythmic contractions and hypersensitivity to K⁺ only in second order IPA. The reduction in vascular response to high concentrations of K⁺ after organ culture could be due to the development of intrinsic tone, associated with membrane depolarisation (Platoshyn et al., 2004; Manoury et al., 2009). However, we did not test the response of vessels to nifedipine or Ca²⁺ removal, under basal tension, to investigate the presence of the intrinsic tone. Loss of response to high [K⁺] could alternatively reflect a reduction of protein synthesis, which consequently reduces contractile protein and contraction force (Lindqvist et al., 1999; Thorne et al., 2002; Guibert et al., 2005).

Increased contraction of cultured arteries to 10 mM KCl suggest that the PASMCs are more depolarised, so smaller increase in [K⁺] is needed to reach the membrane potential for opening the Ca²⁺ channel to trigger contraction. The hypersensitivity to KCl seen here is consistent with findings in cultured porcine coronary arteries and rat IPAs (Thorne et al., 2002; Guibert et al., 2005; Manoury et al., 2009). Hypersensitivity was observed whether arteries were cultured in medium or PSS. Manoury et al. (2009) reported that oxygenating the IPA during organ culture reduced the hypersensitivity to 10 mM KCl.

The depolarisation of PASMCs in cultured arteries was shown to result from the loss of K⁺ channels that contribute to RMP (Manoury et al., 2009). This results in relatively increased constriction to 1mM 4-AP. In this study, the constriction response to TEA was increased from the third day of culture and was markedly elevated on the fourth day. In contrast, our results showed that the constriction response of cultured vessels to linopirdine and XE991 was maintained in all conditions for up to 3 days. This suggests that Kv7 channel expression is unchanged in culture. In fact, responses to Kv7 channel blockers were often enhanced after culture. This may be due to the presence of PASMC depolarization, which would increase the open probability of Kv7 channel at rest. The maximum contraction and/or the pEC₅₀ were significantly increased for XE991 and linopirdine in all conditions employing PSS, except when buffered with bicarbonate.

Preliminary results confirmed that a considerable amount of Kv7.1 and Kv7.4 protein was present in IPA after 3 days of culture in DMEM with 10% BSA and 1% antibiotic. Kv7.4 protein proved to be slightly decreased, but Kv7.1 protein was almost the same.
However, due to the low number of experiments, statistical analysis could not be performed to evaluate if the change in Kv7.4 protein was significant.

The maximum relaxation and pEC_{50a} of flupirtine were generally not affected by the culture conditions used, although in HEPES-buffered PSS with antibiotics there may have been a small loss in sensitivity. Thus Kv7 channel expression and function were well maintained regardless of the culturing conditions. This finding adds support to the idea that the increased effectiveness of Kv7 channel blockers was due to PASMC depolarization. As a consequence, Kv7 channels are well suited to siRNA knockdown experiments, which require culturing vessels for at least 3 days. Our result is in contrast to the impairment of relaxation responses of IPA to pinacidil following 24 hrs of culturing (Thorne et al., 2002). It is possible that, like several other K^+ channels, K_{ATP} channel (the target of pinacidil) expression is reduced following organ culturing.

The average contraction to phenylephrine, measured relative to the constriction produced by 50 mM KCl, was not changed significantly on day 3 of culturing, compared to the response of fresh vessels. Phenylephrine was, therefore, a suitable agent to constrict cultured vessels for studying vasodilation drugs. The optimal culture conditions determined here, therefore, provided an improvement over the conditions used by Manoury et al. (2009), in which the contraction response of IPA to phenylephrine increased after 3 days of culture, although it was returned to normal by day 4. Despite differences in culture condition, our result agrees with that reported by Binko et al. (1999), where phenylephrine-induced contraction was not markedly changed in endothelium-denuded aortic rings following 24 hrs in organ culture. The failure of some cultured vessels to constrict to phenylephrine could be due to the induction of NO synthase, which leads to NO and peroxynitrite generation (Binko et al., 1999). Damage to the endothelium before culturing rat aorta was found to induce the production of NO synthase (Binko et al., 1999). However, in a separate study removal of endothelium before organ culture was found to help maintain the vascular response to phenylephrine following several days of culturing. For these reasons, a small number of vessels that failed to respond to PE after organ culture were excluded from the analysis. It is assumed that these vessels were damaged and lost viability.

The presence of serum in culture medium caused a reduction in rat tail artery contractility, while enhancing of cell proliferation, compared to medium without serum (Lindqvist et al., 1999). Serum also caused eutrophic remodelling of rat cremaster...
arteries, which progressively constricted during culture, although it was beneficial for the maintenance of endothelial function (Bakker et al., 2000). However, in the presence of albumin only, vessels maintained myogenic tone and vascular responses to agonists, without remodelling of the artery (Bakker et al., 2000). For this reason, we preferred to use a serum-free medium for culturing IPA.

Clearly many factors can influence the effects of organ culture on artery contractility and viability. As there was insufficient time to test the effects of each factor in different culturing conditions, we selected the best conditions from those studied to culture vessels for siRNA knockdown experiments. Further modifications could be investigated to provide conditions that maintain artery function even closer to control over prolonged periods of culture.

5.7 Optimisation of siRNA transfection

The reverse permeabilisation technique which has been widely used to load arteries with luminescent proteins (Morgan et al., 1984) and oligonucleotides, including siRNA (Chadha et al., 2012b; Martinsen et al., 2014) was not successful in transfecting IPA segments with siRNA or dsRNA. It is unclear why the technique failed, but several other researchers in the laboratory also failed to reproducibly transfect arteries using reverse permeabilization. It may be that the adventitial layer of the arteries provides a greater barrier to siRNA uptake (Nabzdyk et al., 2017) in IPA compared with other vessels. Both N-TER and lipofectamine RNAiMAX were successful in delivering fluorescein-labelled dsRNA into PASMCs of intact ex-vivo IPA. However, the N-TER transfecting reagent was more efficient than lipofectamine RNAiMAX. The optimal concentration of dsRNA to transfect PASMCs in intact IPA was 200 nM when using 16 µl N-TER in 1 ml medium.

Interestingly, however, lipofectamine RNAiMAX and N-TER reagent failed to transfect ex-vivo IPA by fluorescent labelled siRNA when they were added to the reverse permeabilisation protocol. This could be due to insufficient incubation time of the vessels with the transfection solutions and the transfection condition.

Fluorescence images of transfected arteries showed that fluorescence emission from vessel sections increased with the increasing volume of the reagent and concentration of fluorescent-dsRNA. The strongest fluorescence signal was detected when the concentration of labelled dsRNA was 200 nM, in 20 µl lipofectamine
RNAiMAX or 16 µl N-TER per 1 ml of medium. The fluorescence signal was stronger when using the N-TER reagent, suggesting that the loading efficiency of N-TER was better than lipofectamine RNAiMAX. This result is consistent with other studies showing that N-TER was superior to lipid-based siRNA transfection reagents for cells difficult to transfect (Morris et al., 1997; Simeoni et al., 2003), and with a study showing that N-TER was effective in transfecting SMCs (Rebustini, 2017). Preliminary results also suggest that contractile responses to 50 mM KCl were better maintained, with less hypersensitivity to KCl, in vessels cultured with the N-TER reagent for 2 days, compared to vessels incubated with lipofectamine. This may be due to the less cytotoxic effect of N-TER compared to lipofectamine (Morris et al., 1997; Simeoni et al., 2003). Contractile response to phenylephrine were also well maintained in vessels incubated with N-TER transfection reagent and KCNQ or scrambled siRNA.

Preliminary functional studies on arteries transfected with KCNQ1, 4 or 5 siRNA suggested that the knockdown of Kv7 proteins was possibly most successful when the KCNQ siRNA concentration was 200 nM with 16 µl N-TER in 1 ml of medium, but was unsuccessful when lipofectamine RNAiMAX was used. The preliminary functional data and the fluorescence images were concerned to select optimal transfection condition to knockdown Kv7.1, 7.4 and Kv7.5 proteins. The arteries targeted with 200 nM KCNQ4 siRNA initially showed a reduced maximum response to linopirdine and retigabine compared with arteries targeted with scrambled siRNA. However, when the functional experiments were repeated, statistical comparisons did not detect a significant effect of KCNQ4 knockdown. Therefore, western blot measurement of protein expression will be required to assess the true success of the transfection protocol.
5.8 Future work

Future research should conduct electrophysiological studies on PASMCs as this allows the ionic currents mediated by Kv7 channels to be studied directly. Knocking down Kv7 proteins in isolated PASMCs may be easier than in the vessels and could directly determine how the subunits affect channel behaviour. This would be important to establish the involvement of Kv7.1 channels in mediating the vasorelaxant effects of Kv7.1 activators.

To understand the exact role of each sub-unit in the Kv7 channels of IPA, it will be necessary to verify that Kv7 subunits were knocked down by siRNA by western blotting, so that the degree of protein reduction be correlated with the change of the artery response to Kv7 modulators.

Heteromeric Kv7 channels are thought to be present in many blood vessels and may play a significant role in regulating RMP, and vascular tone (Brueggemann et al., 2011a; 2014; Chadha et al., 2014). Immunohistochemistry could be used to detect Kv7 channel subunits in isolated cells and tissue sections to verify their expression in PASMCs. Such studies should extend to KCNE auxiliary proteins, which modulate the electrophysiological properties and pharmacology of Kv7 channels (Schwake et al., 2006; Abbott, 2014). Expression of the KCNE subunits could also be assessed using western blots.

Our results and Fuchikami et al. (2017) study suggest that the endothelium is involved in relaxant responses to treprostinil. Therefore, it would be interesting to test vascular response to treprostinil in the endothelium-denuded vessel to see if the responses to treprostinil behave more like forskolin and isoprenaline.

The relaxation response induced by BAY41-2272 showed much lower sensitivity to pan-Kv7 blockers than seen with other cGMP dependent dilation. For this reason, it will be important to test if other sGC activators behave like BAY41-2272. If so, it will be necessary to further investigate for understanding the pathway by which Kv7 channels mediate cGMP dependent vasodilation.

Chadha et al. (2012b) proposed that the involvement of Kv7 channels in β-adrenoceptor mediated relaxation, and our data showed that pan-Kv7 channel blockers impaired isoprenaline and forskolin-mediated relaxation. As there are studies showing cross-activation between PKA and PKG-dependent pathways, it necessary to test if isoprenaline and forskolin effect on Kv7 channels is mediated by PKG.


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