Molecular Pathophysiology of Cloned $K_{ATP}$ Channels and Implications for Cardiovascular Disease

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Master of Philosophy

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Press release of this research by the University of Manchester:
“A tiny channel and a large vessel: a new clue for heart attack”............126

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# Abbreviations

<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>4-AP</td>
<td>4-Aminopyridine</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP binding cassette</td>
</tr>
<tr>
<td>ABCC8</td>
<td>ATP binding cassette transporter, subfamily 8 gene</td>
</tr>
<tr>
<td>ABCC9</td>
<td>ATP binding cassette subfamily C member 9</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AMI</td>
<td>Acute myocardial infarction</td>
</tr>
<tr>
<td>AMP-PNP</td>
<td>Adenylly-imidodiphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BK&lt;sub&gt;Ca&lt;/sub&gt;</td>
<td>Calcium-activated potassium channel</td>
</tr>
<tr>
<td>CaCC</td>
<td>Calcium-activated chloride channel</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CD-8</td>
<td>Cluster of differentiation 8</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CK</td>
<td>Creatine kinase</td>
</tr>
<tr>
<td>CK-MB</td>
<td>Creatine Kinase, Muscle and Brain (subunits)</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EAG</td>
<td>Ether-à-go-go</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiogram</td>
</tr>
<tr>
<td>ECs</td>
<td>Endothelial cells</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric-oxide synthase</td>
</tr>
<tr>
<td>ET-1</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>G-protein</td>
<td>Guanine nucleotide-binding protein</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine-5'-triphosphate</td>
</tr>
<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
</tr>
<tr>
<td>HEK-293T</td>
<td>Human embryonic kidney cells (transformed)</td>
</tr>
<tr>
<td>hsCRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>IC50</td>
<td>Half maximal inhibitory concentration</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular Adhesion Molecule 1</td>
</tr>
<tr>
<td>IP&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Inositol trisphosphate</td>
</tr>
</tbody>
</table>
k  Hill coefficient for positive site
K_{ATP}  ATP-Sensitive Potassium
KCl  Potassium Chloride
kHz  Kilohertz
K_{ir}  Inward rectifying potassium channel
Kir6.x  Inward rectifier subunit of the ATP-sensitive potassium channel
KOH  Potassium hydroxide
K_V  Voltage-gated potassium channel
K_V_{LQT}  Voltage-gated potassium channel – long QT interval
LDL  Low density lipoprotein
LVEF  Left ventricular ejection fraction
LVM  Left ventricular myocardium
MBS  Myosin-binding subunit
MD  Molecular dynamics
Mg{NDP}  Magnesium nucleotide diphosphate
MRP  Multidrug resistance protein
NAMD  Nanoscale molecular dynamics
NBD  Nucleotide binding domain
OD  Optical density
Op-amp  Operational amplifier
P_A  Arterial pressure
P_{AO}  Aortic pressure
PBS  Phosphate buffered saline
PCR  Polymerase chain reaction
PDB  Protein databank
PIP_2  Phosphatidylinositol4,5 biphosphate
PKA  Protein kinase
PKG  cGMP-dependent protein kinase
PLC  Phospholipase C
PMCA  Plasma membrane Ca2+ ATPase
POPC  1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
PSF  Protein structure file
RASMCs  Rat pulmonary artery smooth muscle cells
REM  Rapid eye movement
RMSD  Root mean square deviation
ROCK  Rho-associated coiled-coil containing protein kinase
Ry  Ryanodine
Ry-R  Ryanodine receptor
SM  Smooth muscle
SMCs  Smooth muscle cells
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
</tr>
<tr>
<td>SSCP</td>
<td>Single strand conformation polymorphism</td>
</tr>
<tr>
<td>SUR</td>
<td>Sulphonylurea receptor</td>
</tr>
<tr>
<td>TMD</td>
<td>Transmembrane domain</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TRP</td>
<td>Transient receptor potential</td>
</tr>
<tr>
<td>TRPC6</td>
<td>Transient receptor potential cation channel subfamily C member 6</td>
</tr>
<tr>
<td>TRPM4</td>
<td>Transient receptor potential cation channel subfamily M member 4</td>
</tr>
<tr>
<td>TRPV2</td>
<td>Transient receptor potential cation channel subfamily V member 2</td>
</tr>
<tr>
<td>UDP</td>
<td>Uridine diphosphate</td>
</tr>
<tr>
<td>V734I</td>
<td>Substitution variant in SUR2 protein (Valine to Isoleucine at position 734)</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion protein 1</td>
</tr>
<tr>
<td>VCmd</td>
<td>Command potential</td>
</tr>
<tr>
<td>VDCC</td>
<td>Voltage-dependent Calcium channel</td>
</tr>
<tr>
<td>VMD</td>
<td>Visual Molecular Dynamics</td>
</tr>
<tr>
<td>VSM</td>
<td>Vascular smooth muscle</td>
</tr>
<tr>
<td>W_A</td>
<td>Walker A motif</td>
</tr>
<tr>
<td>W_B</td>
<td>Walker B motif</td>
</tr>
<tr>
<td>α2-AR</td>
<td>α2-adrenoceptor</td>
</tr>
</tbody>
</table>
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Abstract

ATP-sensitive potassium (K\textsubscript{ATP}) channels are a class of ion channels involved in a multitude of cellular roles. The hallmark feature of these ion channels is that they are inhibited by a rise in intracellular ATP; thus, K\textsubscript{ATP} channels serve as a link between cell metabolism and cell electrical activity. K\textsubscript{ATP} channels are octameric complexes of two subunits: four Kir6.x subunits coassemble to form the channel pore, which is surrounded by four auxiliary SURx subunits that modulate ATP-dependent gating of the channel. Different combinations of Kir6.x and SURx subunits give rise to K\textsubscript{ATP} channels in different tissues.

In the heart, K\textsubscript{ATP} channel activation under ischemic conditions results in shortening of the cardiac myocyte action potential and reduces contractility. In this way, K\textsubscript{ATP} channels reduce calcium influx and ATP consumption at times of metabolic stress. In the vascular smooth muscle, K\textsubscript{ATP} channels modulate the membrane resting potential and control arterial tone. Genetic deletion of vascular K\textsubscript{ATP} channels in mice gives rise to a phenotype resembling Prinzmetal angina in humans. Here, we hypothesize that a genetic mutation in the cardiovascular K\textsubscript{ATP} channel in man may result in disease such as angina or cardiomyopathy.

In collaboration with the University of Pavia, 1123 patients diagnosed with acute myocardial infarction (AMI) were examined and genetically screened for mutations in the SUR2 gene (ABCC9). It was found that 11 patients presented AMI of vasospastic origin and each patient in this subgroup presented the same single-point mutation in ABCC9. The mutation results in the substitution of valine 734 into isoleucine (V734I) in the SUR2 protein. The main aim of this study was to define the pathophysiological mechanism underscoring the effects of the mutation, and the cause of acute myocardial infarction. The main results and findings are:

1) Wild-type channels composed of Kir6.2/SUR2A (cardiac), Kir6.2/SUR2B (endothelial) and Kir6.1/SUR2B (vascular smooth muscle) were reconstituted in a heterologous expression system and the sensitivity of these channels to intracellular MgATP and physiological nucleotide diphosphates (MgNDP) (MgADP, MgGDP and MgUDP) examined with the patch-clamp technique.

2) Electrophysiological analysis revealed that the mutation reduced the sensitivity to MgATP inhibition of Kir6.2/SUR2B channels but not of Kir6.2/SUR2A and
Kir6.1/SUR2B channels. Furthermore, the stimulatory effects of MgNDP were unaltered in mutant Kir6.2/SUR2A and Kir6.1/SUR2B channels. In contrast, mutant channels composed of Kir6.2 and SUR2B subunits were less sensitive to MgNDP activation.

3) Generation of a structural molecular homology model indicated that V734I may be situated in a vicinity of the MgATP binding site at SUR2.

4) The antianginal drug nicorandil activated Kir6.2/SUR2B channels containing the mutation, thus substituting for the loss of MgNDP stimulation, suggesting that this drug could be efficacious in the treatment of AMI associated with V734I.

In conclusion, these findings indicate that coronary spasm and AMI in patients presenting V734I are precipitated by an altered K\textsubscript{ATP} channel response to intracellular nucleotides. These findings suggest that vascular K\textsubscript{ATP} channels are a possible pharmacological target for the treatment AMI of vasospastic origin. Furthermore, the results of this research indicate that the region where V734I is located is a domain of the channel involved in ATP-dependent gating.
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Publications and press articles from this research


A press release for this paper, entitled “A tiny channel and a large vessel: a new clue for heart attack” has been presented by the University of Manchester at [http://www.manchester.ac.uk/aboutus/news/display/?id=10624](http://www.manchester.ac.uk/aboutus/news/display/?id=10624) (accessed on 23/03/14), and reported on the BBSRC website [http://www.bbsrc.ac.uk/news/health/2013/130910-pr-new-clue-for-heart-attack.aspx](http://www.bbsrc.ac.uk/news/health/2013/130910-pr-new-clue-for-heart-attack.aspx) (accessed on 23/03/14).

Additional publications obtained during the course of my MPhil


Conference presentations

- Keith J Smith, Aiste Adomaviciene, Andrew Chadburn, Enzo Emanuele, Paolo Tammaro. A mutation in SUR2A associated with myocardial infarction does not alter the functional properties of cloned cardiac K\(_{\text{ATP}}\) channels. Gordon Research Conference (Ion Channels), Mount Holyoke College, MA, USA, 8th-13th Jul 2012.
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Keith James Smith
Chapter 1

Introduction

1.1 The vascular circulations

The vascular circulation system is comprised of the pulmonary and systemic circuits. The pulmonary circulation is the component of the vascular system by which the pulmonary arteries transport deoxygenated blood from the heart to the lungs, and oxygenated blood is returned to the heart through the pulmonary veins (Martini, 2001; Levick, 2009). The systemic circulation describes the network of vessels through which oxygenated blood from the heart is distributed to the organs and tissues throughout the body, and deoxygenated blood is circulated back to the heart. The systemic circulation also contains the coronary circulation that distributes oxygenated blood to the walls of the heart, coupled with the drainage of oxygen-depleted blood (Martini, 2001; Levick, 2009). The organization of these key vascular components is highlighted in Fig. 1.1.

![Diagram of the human vascular system](image)

**Fig. 1.1** Main components of the human vascular system. The descending aorta supplies oxygenated blood to the organs and tissues in the thorax and abdominal cavity. The subclavian arteries and common carotid arteries transport oxygenated blood to the shoulders, upper limbs and the head, which is then drained by the internal jugular vein back to the heart. The superior
and inferior vena cava are the largest veins, delivering deoxygenated blood back to the heart from the upper and lower areas of the body. Renal vessels circulate blood through the kidneys.

1.2 Vascular function and structure

Blood vessels are divided into arteries, arterioles, capillaries, venules and veins. This distinction is based on the function and diameter of the vessel. Arteries mainly conduct oxygenated blood from the heart to the target organs, while veins drain oxygen-depleted blood back to the heart. Exceptions include the pulmonary and umbilical veins that channel oxygenated blood to the heart, and portal veins that conduct blood directly from one capillary bed into another. For example, the hepatic portal vein transports blood containing essential digested compounds from the gastrointestinal tract directly to capillary beds of the liver (Martini, 2001; Aaronson & Ward, 2007).

The walls of arteries and veins are composed of three layers, termed tunics. Specifically, the tunica intima, the tunica media and the tunica adventitia (Fig. 1.2). The tunica intima of both arteries and veins is formed by an internal sheet of endothelial cells (ECs) which shares substantial similarities to the endocardial layer of the heart (Greger & Windhorst, 1996a; Levick, 2009; Majesky et al., 2011). ECs are positioned above a complex of collagen, fibronectin, laminin and amorphous ground substance, collectively termed the basement membrane which also forms part of the tunica intima (Greger & Windhorst, 1996a; Levick, 2009; Majesky et al., 2011). The tunica intima forms the primary blood barrier interface and is enclosed by the tunica media. An internal elastic lamina and multiple interdigitating layers of smooth muscle cells (SMCs) form the tunica media. These layers are disposed externally to the elastic lamina, the extracellular matrix portion between the basement membrane and the SMCs (Greger & Windhorst, 1996a; Majesky et al., 2011). The tunica adventitia is the outermost layer, composed of an external elastic lamina, gripped by a thick mesh of connective tissue fibres that anchor the vessel to the surrounding tissue. The adventitia is interspersed with perivascular nerves, fibroblasts, and in large arteries such as the aorta, a network of smaller vessels known as vasa vasorum. It is also evident that the adventitia harbours dendritic cells, T-cells and progenitor cells (Greger & Windhorst, 1996a; Boron & Boulpaep, 2004; Majesky et al., 2011).
1.3 Variations in vessel wall histology

The diameter and tissue composition of blood vessels depends on their physiological location relative to the heart and the target organ. Arteries can be defined as: major (distensible) arteries, muscular arteries and small resistance vessels and arterioles. The major arteries are the largest artery type having a typical lumen diameter of ~ 10 - 25 mm in humans with a vessel wall thickness of up to ~ 2 mm. Muscular arteries are vessels of medium caliber (typical lumen diameter ~ 4 mm with a vessel wall thickness ~ 1mm). Arterioles have a typical lumen diameter of ~ 30 µm and a wall thickness in the region of 15-30 µm (Greger & Windhorst, 1996a; Levick, 2009). It is noteworthy however, that no precise delineation exists between the smaller vessel types and the arterioles (Klabunde, 2011).

**Major (distensible) arteries**

The major arteries contain a higher elastin fiber content than collagen and smooth muscle (SM). During cardiac systole, the elastic fibres are easily stretched to store energy and alleviate large waves in blood pressure ejected from the heart, a phenomenon known as compliance. When the pressure drops during cardiac diastole, the vessel’s elastic recoil re-elevates pressure and propels the blood onwards, producing a smoother continuous flow (Greger & Windhorst, 1996a; Martini, 2001; Levick, 2009).
Examples of major arteries are the aorta, the largest artery in the body, and the subclavian arteries that moderate high pressures from the aortic arch and the brachiophalmonic trunk (Greger & Windhorst, 1996a; Martini, 2001).

The tunica intima of the large arteries contains a pavement monolayer of ECs supported by a thick subendothelial layer of largely developed connective tissue which lines the internal elastic lamina. The broad tunica media of the major arteries consists of a high elastic fibre content compared to SM tissue (Greger & Windhorst, 1996a; Martini, 2001; Boron & Boulpaep, 2004; Levick, 2009). This is established throughout arterial development as SMCs, arranged spirally in the media, synthesize and secrete molecules of elastin. In this way, additional elastic layers are formed interposed between the SMC layers. Importantly, these elastic layers are “fenestrated” (from Latin meaning “windows”), presenting apertures that allow the diffusion of nutrients to cells situated deeper within the vessel wall (Greger & Windhorst, 1996a; Martini, 2001; Wagenseil & Mecham, 2012). A number of physiological changes and disorders of the arterial wall may lead to the development of arteriosclerosis (Xu, 2004; Levick, 2009) such as Ca²⁺ deposits in the tunica media, due to degenerated muscle tissue. Severe calcification may arise as a complication of diabetes mellitus (Martini, 2001). Another major cause of arteriosclerosis is the gradual fragmentation of elastin with age, resulting in the stiffer collagen having a greater effect on the properties of the vessel wall (Martini, 2001). Continual strain results in elastin becoming damaged and reduced, weakening the arterial wall. Collagen fibres are therefore more prone to additional stress, stimulating further collagen production. Because collagen lacks the flexibility of elastin, the arterial wall becomes hardened and the lumen diameter is increased. The overall loss of arterial compliance results in an elevation in cardiac workload (Greger & Windhorst, 1996a; Levick, 2009). Atherosclerosis, is also a form of arteriosclerosis characterized by localized plaque (Martini, 2001; Murphy & Lloyd, 2006). This condition is discussed in more detail in Section 1.7.

The tunica adventitia of the large arteries is occupied by fibroblasts, macrophages and sympathetic nerve terminals (Aaronson & Ward, 2007; Levick, 2009). Because the wall of these arteries is thick (~ 2 mm), oxygen delivery to the wall of the artery is supplemented by the vasa vasorum. These minute blood vessels (< 100 µm in lumen diameter) (Moritz et al., 2012) extend from the lumen (vasa vasorum internae) and branches of the main artery (vasa vasorum externae) into the adventitia of the large
arteries. Elastic fibres in the adventitia are scattered among collagen fibres in order to prevent the vessel wall being overstretched during high systolic pressures when the heart contracts, thus contributing to vessel compliance (Greger & Windhorst, 1996a; Martini, 2001; Aaronson & Ward, 2007; Levick, 2009).

*Muscular arteries*

Muscular arteries distribute the blood from the major arteries to specific organs. These arteries have a much lower elastin fiber content compared to that of the major distensible arteries. A further contrasting feature to the larger arteries is the tunica intima, which in muscular arteries contains more SM tissue and less elastin within the internal elastic lamina, making this layer more prominent (Greger & Windhorst, 1996a; Martini, 2001; Levick, 2009). The tunica media is composed of many concentric rings of SMCs, forming the most substantial proportion of the vessel wall (Martini, 2001; Aaronson & Ward, 2007). Collagen fibres organized longitudinally constitute most of the adventitia. The external elastic membrane lines the inside layer of the adventitia and is composed of fibroelastic tissue (Greger & Windhorst, 1996a; Martini, 2001; Aaronson & Ward, 2007; Levick, 2009).

*Resistance arteries and arterioles*

Muscular arteries further narrow in size to form the terminal resistance vessels. The tunica media in the resistance arteries is mainly occupied by SM tissue. These small arteries are histologically highly comparable to muscular arteries (Ward & Aaronson, 1999). However, the size of the tunica media, relative to the lumen diameter is larger compared to that found in the muscular arteries and the external elastic membrane is virtually absent. Thus, small changes in SM tone result in changes to the lumen diameter and alter blood flow (Greger & Windhorst, 1996a). Only a few sheets of SMCs are found in the tunica media which decreases to one or two layers in the smallest resistance vessels or arterioles. These small arterioles also present thinner internal and external elastic membranes (Greger & Windhorst, 1996a; Martini, 2001; Levick, 2009).

Local blood flow and blood pressure are predominantly determined by the resistance arteries. These vessels are abundantly innervated by autonomic nerves which provide substantial control over the arterial tone (Greger & Windhorst, 1996a; Klabunde, 2011).
Furthermore, endogenous (i.e. non-neuronal) regulatory mechanisms control the tone of these arteries. Organs need to adjust the blood flowing into them to ensure adequate oxygen and nutrient delivery, as well as removal of waste products. To accomplish this, tissues surrounding the arterioles release vasoactive substances that can either constrict or dilate the resistance vessels (Greger & Windhorst, 1996a; Levick, 2009; Klabunde, 2011). Examples of such substances include adenosine, carbon dioxide and hydrogen ions. In addition, the endothelium within arterioles regulates the activity of SMCs by releasing factors such as nitric oxide (NO), prostaglandins and endothelin-1 (ET-1) (Greger & Windhorst, 1996a; Garland & Weston, 2011).

The structural organization of the blood vessels also serves as a significant factor in determining the total vessel resistance. The overall resistance of vessels in series is:

\[ R_{ser} = R_1 + R_2 + R_3 + ... R_n \]  

(1)

Where \( R_{ser} \) is the total series resistance and \( R_n \) is the resistance of the \( n^{th} \) vessel. The total parallel resistance (\( R_{par} \)) is given by:

\[ \frac{1}{R_{par}} = \frac{1}{R_1} + \frac{1}{R_2} + \frac{1}{R_3} + ... + \frac{1}{R_n} \]  

(2)

Thus, vessels in series provide greater resistance than vessels in parallel. As a result, changing the resistance of a small number of vessels in parallel will have little effect on the total resistance. The vascular beds within each organ are mainly arranged in parallel (Levick, 2009; Klabunde, 2011). As a consequence, the blood flow to a particular organ can be altered (via regulation of the diameter of arterioles of that organ) without a large change of blood pressure and blood flow in the rest of the system.

**Veins**

Pericytic venules are the smallest venous vessels with a lumen diameter of approximately 15 µm. They are composed of an endothelium surrounded by connective tissue cells. Smooth muscle is formed again in larger venules where the vessel diameter ranges up to ~ 50 µm. The larger venules merge to form veins (Greger & Windhorst, 1996a; Martini, 2001; Bergan, 2006). Veins are also composed of a tunica intima, media and adventitia, however, in contrast to arteries, venous walls are thinner and contain a
lower muscular and elastic tissue composition. The tunica media is narrow and more loosely formed compared to arteries. Macrophages, fibroblasts, nonmyelinated axons, and schwann cells occupy the adventitia (Greger & Windhorst, 1996a). Medium-sized veins have a diameter of approximately 2-9 mm and have a relatively narrow tunica media. In the larger veins, the tunica adventitia is thick in comparison to the media (Martini, 2001; Ross & Pawlina, 2010). The superior and inferior vena cava are the largest veins. In the human body, valves formed by folds in the tunica intima, selectively block the passage of blood back against the direction of flow (Greger & Windhorst, 1996a; Martini, 2001; Bergan, 2006).

The walls of veins are narrow and elastic which accounts for their high compliance (Greger & Windhorst, 1996a; Martini, 2001; Bergan, 2006). More than half of the blood occupies the systemic veins at rest. Systemic veins therefore serve as a blood reservoir in rest conditions, in addition to regulating the return of blood to the heart. As a result of their capacitive characteristics veins are also termed capacitance vessels (Martini, 2001; Boron & Boulpaep, 2004; Bergan, 2006).

1.4 Smooth muscle

In general, SM is located in the walls of arteries and veins (as outlined above) as well as the lymphatic vessels and visceral organs such as the gut and urinary bladder (Greger & Windhorst, 1996b; Martini, 2001). Smooth muscle is defined as such because it is devoid of the striations that are typical of skeletal and cardiac muscle (Greger & Windhorst, 1996b; Martini, 2001). This is because the sarcomereres are organized in a non-parallel fashion, in contrast to the organization they have in the other muscle types (Greger & Windhorst, 1996b; Martini, 2001). Another striking feature of SM is that it exhibits long, steady contractions because the process of arrangement and detachment of the myosin cross-bridges is slow. This allows vascular smooth muscle (VSM) to function in a permanent tonic state of moderate contraction (basal tone), while using minimal metabolic energy to maintain this contraction (Greger & Windhorst, 1996b; Martini, 2001; Levick, 2009; Klabunde, 2011). More details of this process are covered in section 1.8. The main function of VSM is to regulate the vessel diameter and therefore local perfusion in organs and tissues (Klabunde, 2011).

SMCs are also characterized by an abundance of invaginations in the plasma membrane of approximately 50-100 nm depth, termed caveolae. These invaginations effectively
enlarge the total surface area of the membrane. It appears that caveolae are regions of the plasma membrane where membrane transport and receptor proteins tend to cluster. These include G-protein-coupled receptors, adrenergic receptors and ion channels (Hardin & Vallejo, 2006; Davies et al., 2010). It has been suggested that caveolae could serve as an important cellular location from which a number of signalling events originate (Levick, 2009; Davies et al., 2010; Klabunde, 2011). Tubules of the smooth endoplasmic reticulum are in close proximity to the caveolae that have been suggested to participate in the handling of \([\text{Ca}^{2+}]_i\) within the cell (Greger & Windhorst, 1996a).

### 1.5 The endothelium

The vascular endothelium is the internal cellular lining of blood vessels, forming the boundary between the blood and the rest of the vessel wall (Martini, 2001). All blood vessels in the circulation system are lined with endothelial tissue. This layer was initially thought to provide a smooth surface that facilitates blood flow. However, it soon became evident that it serves a much more complex role. ECs release factors that control the tone of the SMCs in their vicinity. These factors include: NO, prostaglandins and ET-1 (Garland & Weston, 2011; Klabunde, 2011). Specifically, NO is a potent vasodilator produced in vascular ECs which provides a signal mechanism to adjacent SMCs, promoting relaxation and lowering blood pressure. Enzymes termed eNOSs (endothelial nitric oxide synthases) convert L-arginine and \(O_2\) into NO.

ECs are also vital in hemostasis, where they moderate thrombus formation, preventing blood loss from damaged blood vessels, and in the accumulation of thrombocytes and wound healing (Esmon, 1995; Darbousset et al., 2012). A factor which can influence endothelial functioning is shear stress. A tangential force resulting from the dynamics of blood flow, acting on vascular ECs. A decrease in shear stress can result in EC proliferation and programmed cell death (apoptosis) (Paszkowiak & Dardik, 2003; Levick, 2009).

### 1.6 Perivascular nerve fibres

The vascular network is extrinsically mediated by the autonomic nervous system (ANS). The ANS is divided into the sympathetic and parasympathetic nervous systems (SNS and PNS). Sympathetic nerves trigger an accelerated physiological action when the body operates in modes of stress, described as a “fight or flight” response. The PNS
is involved in “rest and digest” responses, generally exhibited when an instant action is unnecessary. The parasympathetic system therefore promotes homeostatic processes during periods of rest. Both the sympathetic and parasympathetic systems are constantly active and function complementary to each other (Greger & Windhorst, 1996b; Martini, 2001).

VSM is mainly innervated by sympathetic nerves. Nerves embedded in the adventitia have beaded structure endings termed varicosities. These fibrous termini release chemicals when transport vesicles fuse with the plasma membrane releasing specific neurotransmitters. These chemicals are able to diffuse out to the SMC via the extracellular matrix. Contraction or relaxation of entire regions of SM can be stimulated by autonomic innervation of a single cell (Greger & Windhorst, 1996b; Martini, 2001; Levick, 2009). This is permitted by gap junction connections that link individual SMCs (Figueroa & Duling, 2009). Gap junctions are low resistance channels for current flow, providing electrical coupling between cells and therefore, fast signal propagation throughout the vessel wall. In this way, autonomic nerves serve to extrinsically mediate the flow of blood (Greger & Windhorst, 1996b; Levick, 2009).

1.7 The coronary arteries: physiology, pathophysiology and acute myocardial infarction

As outlined below (Section 1.17 - Aims of the project) my project centres on K\textsubscript{ATP} channels and the coronary circulation. Therefore this section will provide further details on this particular circulation.

The coronary circulation is a specialized arrangement of vessels that ensure the blood supply to the walls of the heart (Fig. 1.3). The coronary arteries stem from the left and right aortic sinuses; pockets within the base of the ascending aorta. These arteries distribute oxygenated blood to the muscular wall of the myocardium, while the cardiac veins channel the removal of deoxygenated blood from the heart to the coronary sinus, into the right atrium. The coronary arteries are low resistance vessels that course along the surface of the heart (Katz, 2006; Murphy & Lloyd, 2006). Although these vessels have been referred to as elastic arteries (Reese \textit{et al}., 2002), it should be pointed out that they have contrasting features compared to other elastic arteries. In particular, the tunica media is composed of a greater mass of SM tissue and a lower elastic fibre content (Reese \textit{et al}., 2002). Branches of these vessels that enter the myocardial tissue are
muscular distributive arteries (Reese et al., 2002). These branches further divide into the coronary resistance vessels which contribute significantly to the coronary vascular resistance. The resistance vessels eventually narrow and form an extensive capillary network (Reese et al., 2002; Murphy & Lloyd, 2006; Levick, 2009).

The coronary anatomy has a degree of variation among individuals. Mainly, this is defined by the way in which blood is supplied to the posterior descending artery (PDA – illustrated in Fig. 1.3 as a faded line coursing around the posterior side of the myocardium). This arterial branch supplies the posterior third of the interventricular septum, i.e. the internal dividing tissue between the left and right ventricle. In approximately 90% of human hearts the PDA is supplied by the right coronary artery, which is termed “right dominant”. The term “left dominance” refers to the remaining 10% of cases in which the PDA is supplied by the circumflex artery (Katz, 2006).

The right coronary artery mainly carries blood to the right atrium and sections of the left and right ventricles. In approximately 60% of individuals, branches of the right coronary artery supply blood to the sinuatrial node, a mass of cells located on the right atrium contained in a collagenous stroma (Katz, 2006; Murphy & Lloyd, 2006). The sinuatrial node is designated as the main pacemaker of normal heart beats, known as “sinus rhythm”. The sinoatrial node receives dual innervation from both sympathetic and parasympathetic nerves (Katz, 2006; Murphy & Lloyd, 2006; Klabunde, 2011). The atrioventricular node is a subendocardial assemblage of cells with sparse cross-striations, situated between the atria and the ventricles. In approximately 90% of individuals the atrioventricular node is supplied by branches of the right coronary artery (Murphy & Lloyd, 2006). This specialized group of cells electrically connect the atria and the ventricles. Atrioventricular nodal cells have low electrical coupling and thus, a low electrical conduction velocity. This provides an adequate delay for the propagation of electrical impulses from the atria to the ventricles, ensuring that blood is ejected from the atria prior to ventricular contraction (Katz, 2006; Murphy & Lloyd, 2006). Deprivation of blood supplied to the atria or ventricles can affect these nodes. In particular, decreased Ca\textsuperscript{2+} dependent action potentials in the atrioventricular node can result in a restriction of the conduction of electrical signals from the atria to the ventricles, known as an atrioventricular block (Boron & Boulpaep, 2004; Katz, 2006).

The left coronary artery carries blood to the left atrium and branches into the anterior interventricular artery and circumflex artery to feed the left ventricle. The anterior
ventricular artery further extends to reach the lowest section of the heart, termed the apex (Murphy & Lloyd, 2006) (see Fig 1.3). This zone is involved in the regulation of ventricular contractions (Kelly, 2011). The left coronary artery also distributes blood to the interventricular septum (Reese et al., 2002; Murphy & Lloyd, 2006; Levick, 2009).

![Diagram of the human heart](image)

**Fig. 1.3** Diagram of the anterior epicardial surface of the human heart, displaying the structural arrangement of the left and right coronary arteries and their branches.

The myocardium relies largely upon oxygen delivery via the coronary blood supply to uphold oxidative phosphorylation for the production of high energy phosphates, vital for normal contractions (Katz, 2006; Levick, 2009). A report by Waller et al. (1996b) indicates that narrowing of coronary vessels by more than approximately 70% in lumen diameter significantly occludes blood flow to the myocardial tissue. Significant narrowing of the vessel triggers the stimulation of sensory fibres in the heart that are linked via the cardiac plexus to the brainstem, located above the spinal cord. The signalling sequence is interpreted as chest pain, known as angina or angina pectoris (Katz, 2006). If occlusion to blood flow persists, cardiac myocytes are subject to irreversible injury and ultrastructural changes that can elicit a disturbance to the heart’s conduction system; this damage to the heart is termed a myocardial infarction. Occlusion to blood flow in the coronary arteries can occur due to either (i) plaque or (ii) intrinsic muscular spasm (Katz, 2006; Stern & Bayes de Luna, 2009; Klabunde, 2011; Lanza et al., 2011).
Arteriosclerosis results in thickening and hardening of medium and large arteries, coupled with a loss of elasticity. Atherosclerosis is a particular classification of the disease characterized by the accumulation of hard localized plaques (atheroma), which occlude blood flow (Willeit & Kiechl, 2000; Martini, 2001; Murphy & Lloyd, 2006). Atherosclerosis of the coronary vessels can result in significant narrowing and occlusion to blood flow, known as coronary artery disease. It is currently understood that the onset of atherosclerosis can be initiated by a damaged and/or dysfunctional endothelium (Ross, 1999; Martini, 2001; Murphy & Lloyd, 2006). For example, if endothelial NO synthesis is diminished this results in higher levels of platelets and adhesion molecules and subsequent inflammation and remodelling proceeds (Martini, 2001; Murphy & Lloyd, 2006).

Atherosclerosis commonly occurs in conditions of high circulating cholesterol levels (Ross, 1999; Martini, 2001). High levels of lipoprotein (a), a low level density lipoprotein are linked to atherosclerosis. In this condition, cholesterol rich lipoproteins are present in the blood for longer durations. These lipoproteins are then extracted from the blood by monocytes. Lipid filled monocytes termed foam cells, connect to the vascular endothelial lining and secrete growth factors. This causes SMC proliferation (Hopkins, 2013). In addition, monocytes, SMCs and ECs start to phagocytize lipids, which contribute to forming a plaque. As the process advances, ECs expand, leaving cracks between adjacent cells, forming sites that platelets can bind within. This can give rise to the formation of a blood clot (thrombosis) (Cines et al., 1998; Hopkins, 2013). The presence of plaques can result in the targeted tissue being deprived of blood (tissue ischemia), and therefore, oxygen (tissue hypoxia) and glucose causing cells to die. Myocardial cells are subject to severe injury beyond approximately 20-40 minutes of cardiac ischemia (Acosta, 2001; Verma et al., 2002; Katz, 2006). In some individuals with progressive coronary artery disease, increased collateralization of parallel vessel arrangements have been observed, providing alternative channels of blood flow in the myocardium (Klabunde, 2011).

Myocardial infarction due to non-atherosclerotic coronaries occurs in approximately 5% of patients (Waller et al., 1996a; Reynolds, 2012). In the event of spasm, the coronary circulation is occluded by an abrupt narrowing of the arterial wall, preventing the flow of blood into the myocardial tissue. Notably, the rapid onset of constriction is usually localized to a segment of the epicardial coronary arteries (Kaski et al., 1989;
This is envisaged to result from (i) hyperactivity of a coronary segment to vasoconstrictors, (ii) capacity of the vasoconstrictor to elicit spasm in the localized region (Lanza et al., 2011). As a result, cardiac myocytes are prone to oxygen and glucose deprivation, angina, and depending on the severity, an acute myocardial infarction (AMI) (Katz, 2006; Lanza et al., 2011). The type of angina that is normally associated with coronary spasm is known as variant or Prinzmetal angina (Lanza et al., 2011). In contrast to patients with angina due to plaque, patients presenting Prinzmetal angina usually exhibit normal exercise tolerance and development of AMI commonly occurs at rest (Hamilton & Pepine, 2000; Lanza et al., 2011). Interestingly, Murao et al. (1972) also found specific cases of Prinzmetal angina that occurred in the early hours of the morning, particularly during the phase of sleep correlating with rapid eye movement (REM). Evidently, the neurotransmitter, acetylcholine provokes and maintains this sleep phase, whereas atropine inhibits activity (Murao et al., 1972).

Myocardial ischemia due to coronary vasospasm is commonly observed with a characteristic upward deflection of the ST-segment on the electrocardiogram (Hamilton & Pepine, 2000; Boron & Boulpaep, 2004; Lanza et al., 2011). This is indicated on the diagrammatic representation of Fig. 1.4. Coronary artery spasm has been identified as a possible cause of AMI (Yasue et al., 2008; Lanza et al., 2011).

Fig. 1.4 The electrocardiogram, representing the transmission of the depolarizing wavefront from the atria to the ventricles. This phenomenon arises from the net effect of the cardiac myocyte action potentials. The P wave reflects atrial depolarization (contraction). The QRS wave signifies the period when depolarization is transmitted to the ventricles (ventricular
contraction) whereas the T wave is observed as the ventricles are repolarized. The ST-segment is the time at which depolarization of the ventricles reaches a plateau (contraction of the myocardium).

Elevation of the ST-segment is a consequence of transmural MI, when the necrotic region spans the full width of the myocardial wall area affected, running from the endocardium via the myocardium, to the epicardium (Katz, 2006). This effect occurs due to the ischemic insult having a depolarizing effect on the myocytes situated close to the epicardium. Electrode potentials of the healthy and damaged cells will be offset due to the depolarized resting potential of the damaged cell. However, the peak of the action potential will be the same. Consequently, there is a resultant voltage offset everywhere except the ST-segment which is observed to be higher with respect to the baseline (Boron & Boulpaep, 2004). This contrasts to ST-segment depression that is generally produced when the necrotic section does not run all the way through the myocardial wall. Subendocardial ischemia gives rise to this type of ST-deviation (Katz, 2006).

Currently, there is a limited understanding into the molecular determinants of vasospasm (Tun & Khan, 2001; Yasue et al., 2008; Stern & Bayes de Luna, 2009; Lanza et al., 2011). However, certain findings have highlighted endothelial dysfunction as a potential component (Kawano & Ogawa, 2004). Other factors suggested to cause coronary spasm are low-grade inflammation, smooth muscle hypersensitivity and increased autonomic tone (Tun & Khan, 2001; Yasue et al., 2008; Stern & Bayes de Luna, 2009; Lanza et al., 2011).

1.7.1 Clinical symptoms and pathology of AMI

As previously described, cardiac myocytes are exquisitely responsive to deteriorations in oxygen delivery. If cardiac ischemia resulting from muscular spasm of the coronary arteries (or from reduced blood supply due to plaques) is prolonged, this can give rise to an AMI (Murphy & Lloyd, 2006). The term “acute” indicates the event is sudden and presents a serious risk; immediate, unrelenting angina is a common preliminary symptom (in contrast to silent myocardial infarctions) (Thygesen et al., 2007). The distinct feature of myocardial necrosis is plasma membrane damage, as a result, cardiac enzymes including creatine phosphokinase and components of troponin leak from the damaged cardiac tissue into the blood (Katz, 2006). Clinically, the event is defined as “acute” by a deviation in the ST-segment and/or the detection of cardiac enzymes in the
blood (Aaronson & Ward, 2007). Further, cellular infiltration of neutrophils and macrophages in response to necrosis of myocardial tissue distinguishes acute infarction from acute ischemia (Murphy & Lloyd, 2006).

Pathologically, AMI is phased into acute, healing or healed infarction. The presence of polymorphonuclear leukocytes due to acute inflammation indicates the acute stage of infarction (Thygesen et al., 2007; Senter & Francis, 2009). Healing infarction is indicated by the detection of fibroblasts and mononuclear cells and an absence of polymorphonuclear leukocytes. Healed infarction normally develops around 6 weeks later and can be identified by the formation of scar tissue without cellular infiltration (Thygesen et al., 2007; Senter & Francis, 2009). Classification of the actual infarct zone is based on the magnitude of the lesion. The smallest infarcts are observed as focal microscopic areas of necrosis. Above this, small infarcts span less than 10% of the left ventricular myocardium (LVM). The infarct is termed moderate if it extends approximately 10-30% of the LVM, and large when spanning over 30% (Thygesen et al., 2007; Senter & Francis, 2009).

AMI can lead to various complications. Injured heart tissue conducts electrical impulses in an altered, slow fashion. This can give rise to a feedback circulation of electrical impulses within the heart, resulting in fast or abnormal rhythms known as arrhythmias. Lethal ventricular arrhythmias occurring during the acute phase of infarction can result in sudden cardiac death (Bunch et al., 2007). In particular, ventricular fibrillation is a rapid, uncoordinated heart rhythm and presents a major risk factor of sudden cardiac death (Murphy & Lloyd, 2006). Another form of arrhythmia is ventricular tachycardia, a condition characterized by a fast heartbeat, altering blood flow to the tissues and organs. Further complications that may occur are ventricular aneurysms, whereby a localized section of the ventricular wall balloons into a blood-filled sack (Auer et al., 2003). This condition usually occurs some days or weeks after an AMI and occurs in the infarcted zone due to the calcification of scar tissue during the healing phase (Karaahmet et al., 2009). This can obstruct the vessels passing out of the heart, preventing sufficient perfusion of the blood to the rest of the body, potentially leading to cardiogenic shock.
1.8 Molecular regulation of the arterial tone and coronary arterial tone

The arterial tone is determined by mechanisms that alter the internal contractile apparatus of the SMC. Contraction and relaxation of SM is mainly regulated by the cytosolic Ca$^{2+}$ concentration because Ca$^{2+}$ is crucial for the stimulation of the contractile apparatus of the cell (see Fig. 1.5). An uptake in Ca$^{2+}$ leads to the interaction between Ca$^{2+}$ and calmodulin, which in turn stimulates the phosphorylation of myosin light chains (Greger & Windhorst, 1996a; Martini, 2001; Levick, 2009). Consequently, the heads of the myosin filament bind to exposed sites on the actin molecule, allowing the actin myosin filaments to slide across each other, generating sufficient tension to contract the cell (Greger & Windhorst, 1996a; Martini, 2001; Levick, 2009; Klabunde, 2011). The actin and myosin filaments therefore form the contractile motor components of the SMC. When the cytosolic Ca$^{2+}$ concentration falls beneath 10$^{-7}$ M there is insufficient binding between Ca$^{2+}$ and calmodulin, thus no contraction is permitted (Rhoades & Bell, 2012). At concentrations greater than 10$^{-4}$ M, full occupation of the binding sites on the calmodulin molecule is achieved, consequently light chain phosphorylation is maximally primed (Rhoades & Bell, 2012). The cytoplasmic concentration of Ca$^{2+}$ varies within these extremities giving rise to sustained graded contractions. As previously described, this is imperative for the control of blood flow in the vascular system (Rhoades & Bell, 2012). At rest [Ca$^{2+}$], is very low, approximately 50 nM, whereas the extracellular concentration is dramatically higher, around 1.6 mM (Greger & Windhorst, 1996a; Chipperfield & Harper, 2000).

![Fig. 1.5 Ca$^{2+}$ dependent contractile apparatus of the SMC. The actin and myosin molecules form the cell’s contractile motors. The process is initiated by Ca$^{2+}$. Membrane-associated dense bodies anchor the actin filaments, thus facilitating contraction of the cell.](image-url)
Sourcing of Ca\(^{2+}\) into the cytoplasm is accomplished by two main mechanisms. Ca\(^{2+}\) can be channelled through voltage-dependent Ca\(^{2+}\) channels (VDCCs) from the extracellular space, or alternatively Ca\(^{2+}\) can be released by Ca\(^{2+}\) release channels located on the sarcoplasmic / endoplasmic reticulum (SR/ER) (Greger & Windhorst, 1996a). It is further established that the degree of contraction is also mediated and sustained by the Ca\(^{2+}\) sensitivity of the myosin filaments, which in turn depend on RhoA/Rho-kinase activity (Uehata et al., 1997). It is understood that this mechanism involves the inhibition of myosin phosphatase. Activation of Rho is by produced by receptor agonists that are coupled to cell membrane G-proteins. This results in the translocation of Rho to the cell membrane, stimulating Rho-kinase. The subsequent phosphorylation of myosin light chain phosphatase induces VSM contraction (Jalil et al., 2005).

**Intracellular organelles**

Intracellular Ca\(^{2+}\) stores in VSMCs serve a major function in the control of cytoplasmic Ca\(^{2+}\) levels. Ca\(^{2+}\) release channels present in the SR, and particularly the ER enable Ca\(^{2+}\) efflux into the cytosol (Somlyo et al., 1985). The two Ca\(^{2+}\) release channel subfamilies – the inositol 1,4,5-triphosphate (IP\(_3\)) -sensitive channels (IP\(_3\)-receptors) and ryanodine-sensitive channels (Ry-receptor) have been extensively characterized. Furthermore, these channels may be present in cluster formations according to certain findings (Nixon et al., 1994; Lesh et al., 1998). These Ca\(^{2+}\) release channels have much higher conductances (~ 100 pS) than that of VDCCs and so are effective components in elevating local Ca\(^{2+}\) levels (Wray & Burdyga, 2010).

IP\(_3\)-Receptors are tetrameric non-selective cation channels, regulated by IP\(_3\). Hormone interaction with a G-protein linked receptor instigates the release of an α-subunit-GTP-complex which activates plasmalemmal receptors coupled to phospholipase C (PLC). PLC cleaves the membrane lipid phosphatidylinositol 4,5 bisphosphate PIP\(_2\) into IP\(_3\) and diacylglycerol (DAG). The IP\(_3\) second messenger stimulates the IP\(_3\) receptor, producing an enhanced sensitivity to Ca\(^{2+}\) binding at a Ca\(^{2+}\) activatory site situated on the cytoplasmic side. Binding of Ca\(^{2+}\) at this site promotes trafficking of Ca\(^{2+}\) through to the cytoplasm, hence creating a positive feedback system - the mechanism by which remains uncertain (Martini, 2001; Aaronson & Ward, 2007; Levick, 2009; Klabunde, 2011). The ryanodine receptor complex (Ry-R) is also a tetrameric channel which
exhibits a positive feedback mechanism of Ca\(^{2+}\) efflux. Although these receptors are suppressed in excessive doses of Ca\(^{2+}\), these doses are greater than the concentration observed in the physiological environment (Greger & Windhorst, 1996a).

The Ca\(^{2+}\) ATPase pumps Ca\(^{2+}\) back into the sarcoplasmic reticulum. This lowers the [Ca\(^{2+}\)] below the level necessary to form Ca\(^{2+}\)-calmodulin complexes. Phospholamban, cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG) mediate the action of the Ca\(^{2+}\) - ATPase (Colyer, 1998). Ca\(^{2+}\) is also expelled into the extracellular medium by the Ca\(^{2+}\) ATPase and the Na\(^{+}\)/Ca\(^{2+}\) exchanger in the plasma membrane. The main driving agent of this process is PMCA, which is also regulated by calmodulin, cAMP- and cGMP-dependent kinases (Zylinska et al., 1998). Local changes in [Ca\(^{2+}\)], could be attributed to the Na\(^{+}\)/Ca\(^{2+}\) exchanger due to an imbalanced arrangement of proteins on the plasmalemma (Ashida & Blaustein, 1987; Moore et al., 1993).

Numerous molecular mechanisms act in concert to control the balance of [Ca\(^{2+}\)], levels and thus, arterial tone. Pharmacomechanical coupling involves the release of Ca\(^{2+}\) from IP\(_3\) gated channels in the SR, in response to hormones and neurotransmitters. A further contributory factor is the process of voltage-dependent Ca\(^{2+}\) channel activation in the plasma membrane due to membrane depolarization. This is termed excitation-contraction coupling (Martini, 2001; Aaronson & Ward, 2007; Levick, 2009). Small deviations in the membrane potential (3 mV) are sufficient to open VDCCs, generating influx of Ca\(^{2+}\) (Nelson et al., 1990). In addition, store operated Ca\(^{2+}\) channels in the plasma membrane open in response to a diminished Ca\(^{2+}\) reserve from the SR, facilitating Ca\(^{2+}\) influx (Gibson et al., 1998). The mechanism underscoring this process is unclear. Although, it has been described by Trepakova et al. (2000) that Ca\(^{2+}\) depletion stimulates store operated Ca\(^{2+}\) channels via Ca\(^{2+}\) influx factor (CIF) in mouse aortic SMCs. An additional factor which contributes to VDCC activity is the modulation of the membrane potential by Ca\(^{2+}\) - activated Cl\(^-\) channels (CaCCs). The release of Ca\(^{2+}\) from intracellular Ca\(^{2+}\) stores promotes CaCC opening and hence membrane depolarization.

*Flow-mediated dilation*

Blood vessels elicit a myogenic reaction to stretch of the vessel wall which can be induced by an increase in intraluminal pressure. Stretch of the SMC membrane results
in opening of stretch-activated ion channels promoting \( \text{Ca}^{2+} \) uptake and vasoconstriction. The vessel’s myogenic mechanism to stretch is termed the Bayliss effect (Coats et al., 2001). Parallel to this, elevations in pressure drive shearing forces along the vessel wall, potentiating the release of nitric oxide from the vascular endothelium. This process occurs via the stimulation of shear stress mechanosensors and produces vasodilation, known as the Schretzenmayr effect (Lüscher & Corti, 2004). Fisher et al. (2001) reported that the vascular endothelial growth factor receptor (a receptor tyrosine kinase) situated at the luminal interface of ECs, may function as a mechanosensor to shear stress. Further mediation of the vessel wall is accomplished through ion channels, g-proteins and intercellular junction proteins (Fisher et al., 2001). Thus, the endothelial response to alterations in blood flow is mediated by vasoconstriction and vasodilation mechanisms acting in concert.

1.8.1 Coronary control

The coronary arteries depend upon precise regulation in order to continuously meet the myocardial oxygen demand. The following mechanisms coactively modulate the coronary tone and therefore, the coronary vascular resistance.

Myocardial metabolism

Blood supply to the heart is regulated by changes in the metabolic state of the myocardium. This delivery is crucial for the oxidative production of ATP, and therefore cardiac contraction. When cardiac workload and myocardial oxygen demand are increased, adenosine is produced via enzymatic breakdown of adenosine monophosphate (AMP), which is derived from ATP and ADP. Because the concentration ratio of ATP to adenosine is of the order of 1000:1 (Klabunde, 2011), only a small amount of ATP is required for the production of adenosine. Through its action on A2A receptors in VSMCs, adenosine induces coronary relaxation (Rossen et al., 1994; Duncker & Bache, 2008; Deussenn et al., 2012), elevating blood flow to the heart. Formation of adenosine in the myocardium during hypoxia or ischemia produces a negative feedback mechanism to maintain oxygen delivery within a normal physiological level (Daut et al., 1990; Essop, 2007).

If cardiac perfusion pressure is reduced, an initial drop in blood flow occurs which is compensated for within a period of minutes (Feliciano & Henning, 1999; Klabunde,
A metabolic response is triggered by the decline in perfusion pressure, in turn stimulating coronary vasodilation. This sequence of events is termed autoregulation (Feliciano & Henning, 1999; Klabunde, 2011).

**Neurogenic regulation**

The coronary arteries are innervated by both the sympathetic and parasympathetic nervous systems (Levick, 2009; Klabunde, 2011). Sympathetic innervation of the heart results in only transient constriction of the coronary arteries. This is followed by vasodilation as sympathetic innervation also elevates heart rate via β-adrenoceptors. Subsequently, vasodilator metabolites are produced, thus suppressing vasoconstriction. This process is known as functional sympatholysis (Levick, 2009; Klabunde, 2011). Parasympathetic innervation produces a moderate vasodilation effect via the direct actions of acetylcholine on the coronary vessels. Conversely, if parasympathetic innervation elicits a significant reduction in myocardial oxygen demand, local metabolic processes contribute to coronary vascular tone, leading to constriction (Levick, 2009; Klabunde, 2011).

Adrenaline and noradrenaline are secreted by the sympathetic nerve termini and bind to adrenoceptors on the VSMC (Ramanathan & Skinner, 2005; Klabunde, 2011). Both α₁ (a Gq coupled receptor) and α₂ (a Gi coupled receptor) receptors are expressed in the coronary circulation. The α₁-adrenoceptor agonist noradrenaline is a vasoconstrictor, able to couple to the Gq-protein linked α-receptor and induce a conformational change in the α-subunit of the G-protein, releasing an α-GTP complex. This complex migrates within the cell membrane and binds to and activates PLC, essentially leading to liberation of IP₃ and the release of Ca²⁺ from intracellular calcium stores in the ER or SR. The contraction mechanism is therefore initiated (Boron & Boulpaep, 2004; Klabunde, 2011). Binding of adrenaline or noradrenaline to the α₂ can also stimulate contraction. The α-receptors are the dominant receptor type in the epicardial vessels, producing a constrictive response when activated. On the other hand, β₂ receptors are mainly expressed in the subendocardial vessels and trigger a dilation response (Greger & Windhorst, 1996a; Martini, 2001; Ramanathan & Skinner, 2005; Klabunde, 2011).

Parasympathetic innervation of the heart initiates in the dorsal efferent nuclei of the medulla oblongata, where signals are transmitted along the cardiac branches of the vagus nerve to the myocardium (Katz, 2006). Acetylcholine is a major neurotransmitter...
released by the parasympathetic nervous system at rest (Boron & Boulpaep, 2004; Katz, 2006). This substance acts on muscarinic acetylcholine receptors (M3R) on ECs of the coronary vessels, provoking vasodilation. Although acetylcholine also acts on M3Rs in SM tissue causing constriction, the vasodilation mechanism overrides, producing a modest dilatory effect in the presence of an intact endothelium (Miller et al., 1998; Klabunde, 2011). Notably, a report by el-Tamimi et al. (1994) demonstrated that patients with atherosclerosis or risk factors for coronary artery disease displayed acetylcholine-induced constriction, in contrast to endothelial-mediated dilation. In these cases, the endothelium of patients showed marked heterogeneous responses to acetylcholine and varying degrees of endothelial dysfunction, as opposed to a diffuse dysfunctional state.

**Circulating hormones**

The antidiuretic peptide hormone has a mild effect on the coronary tone although has been shown to trigger a constrictive response in patients under stress conditions. The atrial natriuretic peptide, vasoactive intestinal peptide and calcitonin gene-related peptides have been associated with endothelial-modulated vasodilation (Henning & Sawmiller, 2001; Chan et al., 2010; Barbato et al., 2012). Angiotensin II induces vasoconstriction. It is also connected to elevations in [Ca2+]i in addition to a rise in endothelin (Schmermund et al., 1999).

**Endothelial regulation**

NO released from vascular ECs has been shown to produce significant coronary vasodilation (Lüscher et al., 1990; Kugiyama et al., 1996). NO synthesis in ECs is accelerated by shearing forces caused by increased blood flow in the vessel lumen (Quyyumi et al., 1995). Bradykinin is also a powerful vasodilator that primes B2-kinin receptors, triggering the release of NO, prostacyclin and hyperpolarizing factor (Groves et al., 1995; Kuga et al., 1997). Prostacyclin (PGI2) derived from arachidonic acid within ECs, causes coronary vasodilation. PGI2 augments a vasodilation response by stimulating smooth muscle adenylyl cyclase, increasing cAMP.

ET-1 is produced by endothelin-converting enzyme (ECE), located on the EC membrane. This substance has a powerful constrictive action when bound to Gq-protein coupled ETa receptors on the SMC (Kinlay et al., 2001; Wiley & Davenport, 2001;
Klabunde, 2011). Although ET-1 also has a vasodilating action when bound to endothelial ET\textsubscript{B} receptors, the vast amount of experimental data has demonstrated the constrictive action of ET-1 to be overall the most prominent. Numerous substances promote the formation of ET-1 including, angiotensin II, vasopressin (antidiuretic hormone, ADH), thrombin and cytokines. Prostacyclin prevents the release of ET-1 (Klabunde, 2011).

Studies demonstrate constrictive effects of thromboxane A\textsubscript{2} (TxA\textsubscript{2}) on human coronary arteries (Schrör, 1990; Feng et al., 2011). Produced by circulating platelets during endothelial injury, the TxA\textsubscript{2} receptor may enhance the response of the neurotransmitter 5-hydroxytryptamine (5-HT or serotonin) regulated by 5-HT like receptors. In studies conducted on patients suffering from variant and chronic stable angina, the 5-HT like receptor may participate in moderating the extent of coronary contraction (Chester et al., 1993).

1.9 Ion transport and the membrane potential

Transporters use energy to move various ions against their concentration gradient through the plasma membrane into either the cytoplasm or extracellular fluid. In addition, the presence of ion channels allows ions to flow down their electrochemical gradients. As a result of both energy requiring (i.e. via transporters) and passive (i.e. via channels) transport mechanisms, a charge separation and therefore, a potential difference is introduced across the plasma membrane. This is referred to as the membrane potential (\(V_m\)). The activity of Voltage-Dependent Ca\textsuperscript{2+} Channels (VDCCs) is primarily dependent on the \(V_m\).

1.10 Modulation of the membrane potential and arterial vasomotor tone

The membrane potential of VSMCs is a major determinant of arterial contractile tone. Ion channels moderate the blood vessel tone via modulation of the \(V_m\). Under normal physiological conditions arterial SMCs have a resting potential ranging from -40 to -60 mV (Chen & Wagoner, 1991; Nelson & Quayle, 1995; Crane et al., 2003). Due to the high membrane permeability for K\textsuperscript{+} the resting potential of SMCs lies closely to \(E_K\). At rest, the VSM cell membrane is usually far more permeable to K\textsuperscript{+} ions than to the other ions which are present in the intra- and extra-cellular environments, such as Na\textsuperscript{+}, Cl\textsuperscript{-} and Ca\textsuperscript{2+}. This is because some K\textsuperscript{+} channels are open under resting conditions. This causes
$V_m$ to lie relatively close to the physiological Nernst potential for $K^+$ (~80 mV). Thus, $K^+$ channels have a major involvement in the setting of $V_m$ (Nelson & Quayle, 1995; Jackson, 2000). There is also evidence to suggest that $Cl^-$ channels have a substantial depolarizing effect on the membrane potential of arterial SMCs (Hirst & Edwards, 1989). Efflux of $Cl^-$ would account for observed membrane potentials *in vivo* (~ -50mV) being more positive compared to the $K^+$ equilibrium potential ($E_K$) of ~-83mV (Neild & Keef, 1985; Hirst & Edwards, 1989; Nelson *et al.*, 1990; Liang *et al.*, 2009).

The open-state dynamics of VDCCs are tightly controlled by the $V_m$. These channels have a low open probability in rest conditions, however, they are highly sensitive to changes in $V_m$. Hyperpolarization of just 3 mV can significantly enhance the open channel probability and increase $Ca^{2+}$ influx two-fold (Yuan, 1995). In this way, the VDCC open probability couples the fine tuning of $V_m$ to the extent of tonic contraction. Specifically, a relatively small displacement in the resting potential of arterial SMCs is sufficient to induce a considerable alteration in arterial diameter and the vascular resistance (Nelson *et al.*, 1990; Brayden & Nelson, 1992; Yuan, 1995).

Activation of the $Na^+/Ca^{2+}$ exchanger is also coupled with changes in $V_m$ and moderates $[Ca^{2+}]_i$ levels (Nelson *et al.*, 1990). This protein transporter serves to extrude $Ca^{2+}$ from the cell using the energy provided by the influx of $Na^+$ down its electrochemical gradient. However, the exchanger can also function in reverse when the cell is in a depolarized state (Karaki *et al.*, 1997).

### 1.11 Ion channels in VSM and ECs

There are four main species of ion channels involved in the regulation of the arterial tone of VSM cells. These are $K^+$ channels, voltage-dependent $Ca^{2+}$ channels, $Cl^-$ channels and non-selective cation channels. Channels selective for $Na^+$ have been reported in a minority of VSM. $Na^+$ currents were initially undetectable in freshly isolated human coronary myocytes, however, upon culture, large $Na^+$ currents were observed (Quignard *et al.*, 1997). These channels conveyed an atypical tetrodotoxin sensitivity and have been suggested to be involved in cell proliferation. More recently, expression of voltage-gated $Na^+$ channels were detected in cultured and diseased human aortic SMCs, although were unidentifiable in normal aorta (Meguro *et al.*, 2009). As my MPhil project centers on a subclass of $K^+$ channels (the ATP-sensitive potassium ($K_{ATP}$) channel) this section will focus more specifically on these types of ion channels.
In VSM activation of K⁺ channels results in hyperpolarization of the membrane potential, closure of voltage gated Ca²⁺ channels, and vasodilation, whereas inhibition of K⁺ channels will have the opposite effects (Smirnov et al., 1994). Thus, regulation of K⁺ channel activity is a major mechanism of vasodilation and vasoconstriction, both in normal and in pathophysiological conditions (Quayle et al., 1997; Sobey, 2001). Various types of potassium channels have been reported in VSM, as briefly described below.

**Voltage-gated (Kᵥ) potassium channels**

Kᵥ channels are key to the modulation of the Vₘ in VSM by responding to stimuli that trigger Vₘ depolarization (Tammaro et al., 2004; Firth et al., 2011). Kᵥ channels are composed of four subunits each consisting of six transmembrane domains (TMD). The 4ᵗʰ TMD acts as a voltage sensor, the structural segment of the channel that couples changes in Vₘ to channel gating (Choi & Abbott, 2010).

**Calcium-activated potassium channels (BKₐᵥ)***

The main feature of BKₐᵥ channels is their dual sensitivity to both [Ca²⁺]ᵢ and membrane depolarization. BKₐᵥ channels mediate Vₘ by reacting to alterations in [Ca²⁺]ᵢ and are suggested to participate in the setting of the myogenic tone in microvessels (Smirnov & Aaronson, 1992; Feng et al., 2009; Wan et al., 2013). The overall structure of BKₐᵥ channels is a tetrameric complex consisting of α and β subunits in a four by four arrangement. Positively charged residues are contained in the 4ᵗʰ transmembrane domain of each α-subunit, forming voltage sensors.

**Two-pore domain potassium channel**

The role of two-pore domain potassium channels is mainly to contribute to the setting of the resting Vₘ (Gurney et al., 2003; Enyedi & Czirják, 2010). Two-pore domain potassium channels consist of two pore-forming α-subunits, each consisting of four transmembrane domains. The absence of a voltage sensor explains the voltage independent nature of this subclass of channels. Although their activity is independent of voltage, they are regulated by other mechanisms including G-proteins, cyclic nucleotides, mechanical stretch and intracellular pH (Gurney et al., 2003; Sanders & Koh, 2006).
**Inward rectifying potassium channel (Kir)**

Kir channels are deemed to have two major roles in VSM. Primarily, these channels are thought to control $V_m$ and the basal tone in the resistance vessels. In addition, a rise in extracellular $K^+$, resulting in opening of Kir channels may produce a vasodilation response (Nelson & Quayle, 1995; Standen & Quayle, 1998). The main property of Kir channels is that their current versus voltage relationship deviates from a straight line. Thus, current measured at positive potentials is smaller than the current elicited by a voltage step of the same amplitude but of opposite sign. This phenomenon is called inward rectification of the current. It is mainly attributed to a voltage dependent block of the channel by cations such as $Mg^{2+}$ and polyamines (Reimann & Ashcroft, 1999). Kir channels are composed of only two transmembrane domains linked by a pore loop. There is no voltage sensor accounting for their weak voltage dependence. Both the N and C termini are located in the cytoplasm.

**Endothelial potassium channels**

At least 5 subtypes of $K^+$ channels have been reported to be expressed in ECs of the microvasculature (Jackson, 2005). Small conductance $Ca^{2+}$-activated $K^+$ channels (SK$_{Ca}$), stimulated by endothelium dependent vasodilators, and intermediate conductance $Ca^{2+}$-activated $K^+$ channels (IK$_{Ca}$) have been proposed. Kir channels have also been detected and it has been indicated that they could potentiate the hyperpolarization induced via SK$_{Ca}$ and IK$_{Ca}$ channels and facilitate propagation of the hyperpolarizing signal across ECs via gap junctions. K$_{ATP}$ channels serve to mediate the resting $V_m$ and $[Ca^{2+}]_i$, and there is also evidence that they have a protective role for endothelial function (Wang et al., 2007).

In addition, $K_V$ channels may provide a negative feedback process to restrict hyperpolarization in some ECs (Jackson, 2005). It is of note that although some voltage-gated ion channels have been identified in ECs, their functional contribution appears to be relatively minor (Nilius & Riemann, 1990). ECs are therefore described as “non-excitable” as they do not propagate action potentials.

**1.12 ATP-Sensitive potassium (K$_{ATP}$) channels**

K$_{ATP}$ channels are expressed in numerous cell types including, vascular and visceral SM, ECs, neurons, cardiac tissue, pancreatic cells and skeletal muscle (Inagaki et al.,
The hallmark feature of the $K_{ATP}$ channel is its regulation by the intracellular concentration of ATP. Thus, $K_{ATP}$ channels serve as molecular sensors that convert the cell’s metabolic energy status into electrical activity of the plasma membrane (Flagg et al., 2010).

### 1.12.1 Channel assemblies, architecture and gating

$K_{ATP}$ channels are octameric structures of two different subunits. Four inwardly rectifying $K^+$ channel (Kir6.x) subunits form the tetrameric pore (Inagaki et al., 1995; Sakura et al., 1995; Nichols, 2006), each associating with an ancillary sulphonylurea receptor (SUR) subunit to form the functional channel (Shyng & Nichols, 1997; Song & Ashcroft, 2001). The putative topology of the $K_{ATP}$ channel is displayed in Fig. 1.6. In the absence of the SUR subunit, a motif contained in the C-terminus of Kir6.x which serves as an ER retention signal halts the trafficking of functional $K_{ATP}$ channels to surface expression on the plasma membrane (Zerangue et al., 1999). A truncated variation of the Kir6.2 C-terminal (Kir6.2D C26 or Kir6.2D C36) was found to be an exception to this, by facilitating trafficking of functional channels to the plasma membrane, in the absence of SUR (Zerangue et al., 1999). The variation of Kir6.x and SURx channel compositions depends on the tissue type in the body. Kir6.2/SUR1 channels are expressed in pancreatic beta cells (Tarasov et al., 2004; Shi et al., 2005), whereas Kir6.2/SUR2A channels are mainly expressed in ventricular myocytes (Inagaki et al., 1996; Tammaro & Ashcroft, 2006). Assemblies of Kir6.1 and SUR2B are the predominant type in SM (Isomoto et al., 1996; Yamada et al., 1997; Miki et al., 2002) and Kir6.1, Kir6.2 and SUR2B are channel constructs of vascular endothelial tissue (Yoshida et al., 2004; Morrissey et al., 2005). Combinations of Kir6.2 and Kir6.1 subunits with SUR1 and SUR2B have also been detected in neurons (Dunn-Meynell et al., 1998; Karschin et al., 1998; Liss & Roeper, 2001).

The Kir6.1 and Kir6.2 subunits are encoded by the pore-forming inward rectifier genes $KCNJ8$ and $KCNJ11$, respectively (Nichols et al., 2013). Binding of ATP or ADP (in either the presence or absence of $Mg^{2+}$) to Kir6.x suppresses channel activity by reducing the mean channel open time and by lowering the mean open burst periods and the frequency to which they occur (Tucker et al., 1997; Drain et al., 1998; Craig et al., 2008). Binding of ATP to Kir6.2 occurs via an interaction between the $\beta$- and $\gamma$-
phosphate groups of the ATP molecule with residues in the N- and C-termini (including arginine at position 50 of the N-termini and lysine at position 185 of the C-termini) of the channel pore, respectively (Trapp et al., 2003; Tsuboi et al., 2004). Non-hydrolysable ATP analogues as well as ATP inhibit \( K_{\text{ATP}} \) channels. This indicates that channel inhibition is caused by direct nucleotide binding, without the requirement for hydrolysis (Findlay, 1988; Lederer & Nichols, 1989).

The SUR subunit is a member of the ATP binding cassette (ABC) transporter superfamily (Park & Terzic, 2010), encoded by the \( ABCC9 \) (ATP binding cassette transporter channel gene, subfamily C member 9) gene. SUR2A and SUR2B are transcript variants of alternative splicing, distinguished only by the sequence of their last 42 amino acids, arising from differential usage of two terminal exons (exon 38A - SUR2A and exon 38B - SUR2B) (Inagaki et al., 1996; Isomoto et al., 1996; Morrissey et al., 2005; Tammaro et al., 2006). \( K_{\text{ATP}} \) channel activation can be induced in two main ways; 1) via binding and/or hydrolysis of MgATP at the SUR binding domains, 2) through direct binding of Mg-nucleotide diphosphates (MgNDP) with SUR. Thus, the SUR subunit harnesses the energy of Mg-nucleotide binding and/or hydrolysis to provoke a conformational change in the channel, inherently extending the mean channel open time and open burst durations, and their occurrence (Babenko et al., 1998; Seino, 1999; Nichols, 2006). Direct structural information of the entire \( K_{\text{ATP}} \) channel at atomic resolution is not yet available. However, hydropathy analysis, a computational technique used to define a membrane topology, suggests SUR subunits contain 17 transmembrane helices (illustrated in Fig. 1.6) (Ashcroft & Gribble, 1998; Campbell et al., 2003). Furthermore, coimmunoprecipitation assays involving the use of antibodies to identify specific segments of the protein, have indicated that TMD0 of SUR has a close association with Kir6.x (Chan et al., 2003). The two cytoplasmic loops that contain the nucleotide binding domains (NBD1 and NBD2) are displayed in Fig. 1.6.

In the absence of nucleotides \( K_{\text{ATP}} \) channels exhibit intrinsic spontaneous activity. However, Kir6.1-forming channels are an exception to this in that MgATP or MgNDP are mandatory for channel activation (Satoh et al., 1998).
Fig. 1.6 Putative topology of the K\textsubscript{ATP} channel octameric structure. The channel pore is a formation of four Kir6.x subunits. Four SUR\textsubscript{x} modulatory subunits encase the pore. The tubular structures represent alpha helices. The P-loop forms the channel’s selectivity filter and the L\textsub{O} linker has been proposed to participate in channel gating.

1.12.2 Protective role of cardiac sarcolemmal K\textsubscript{ATP} channels during ischemia

Under normal physiological conditions, K\textsubscript{ATP} channels in the heart are closed. In conditions of low metabolism, a fall in cellular ATP levels results in channel activation. During ischemia, activation of cardiac K\textsubscript{ATP} channels has been shown to defend against myocardial stunning elicited by high Ca\textsuperscript{2+} influxes (Lascano \textit{et al.}, 2002; Flagg \textit{et al.}, 2010). Channel stimulation results in hyperpolarization of the membrane, reducing the action potential duration and therefore the time for Ca\textsuperscript{2+} influx via L-type Ca\textsuperscript{2+} channels. Decreased myocardial contraction preserves the ATP energy stores in the heart. Thus, in the absence of channel stimulation the cell would be subjected to an excessively high elevation of [Ca\textsuperscript{2+}]\textsub{i} and an overload in mitochondrial Ca\textsuperscript{2+} levels (Flagg \textit{et al.}, 2010). The subsequent increase in reactive oxygen species (ROS) can cause injury to muscle fibres and cell death. Hence, if K\textsubscript{ATP} channel function is compromised, arrhythmia and contractile dysfunction may ensue (Hodgson \textit{et al.}, 2003; Flagg \textit{et al.}, 2010).

A process termed reperfusion injury can also occur when the blood supply is returned to the tissue following the period of ischemia (Katz, 2006; Levick, 2009). During the
ischemic phase, the tissue is deprived of oxygen and nutrients. When the blood is returned to the section of tissue affected, inflammation results due to a release of chemicals including interleukins through oxidative stress. $K_{ATP}$ channels in the plasma membrane of cardiac myocytes have been identified to mitigate reperfusion injury in mice (Suzuki et al., 2002). Ischemic preconditioning is the process whereby short durations of ischemia protect the heart from injury due to prolonged ischemia and reduce infarct size (Katz, 2006; Levick, 2009; Klabunde, 2011). A reduction in $K_{ATP}$ channel activity and increase in Ca$^{2+}$ overload into the cell may inhibit the preconditioning process (Suzuki et al., 2002; Flagg et al., 2010).

1.12.3 Role of vascular $K_{ATP}$ channels

$K_{ATP}$ channels are widely expressed in the human vascular system (Quayle & Standen, 1994). Suppression of $K_{ATP}$ channel activity causes a decrease in K$^+$ efflux and membrane potential depolarization. This in turn increases cellular Ca$^{2+}$ influx and thus, vasoconstriction proceeds. The sulphonylureas glibenclamide and tolbutamide block vascular $K_{ATP}$ channels, resulting in constriction of the blood vessels (Yokoshiki et al., 1998; Ko et al., 2008). Conversely, vasodilation is induced via stimulation of $K_{ATP}$ channels, membrane hyperpolarization and decreased Ca$^{2+}$ influx. Synthetic $K_{ATP}$ channel openers include diazoxide and the antianginal drug nicorandil (Yokoshiki et al., 1998; Chowdhury et al., 2013). Emerging evidence continues to shed light into the numerous signalling events these channels are involved in and their major influence in the setting of the coronary arterial tone. Farouque et al. (2002) investigated $K_{ATP}$ channels in human conduit and resistance coronary vessels using glibenclamide, a potent $K_{ATP}$ channel blocker. Intracoronary infusion of glibenclamide reduced the conduit diameter and blood flow, and increased coronary vascular resistance, while having a partial effect on the coronary microvessels. In this case, $K_{ATP}$ channels are suggested to be of key importance in determining the basal coronary tone. In isolated human and porcine coronary artery SMCs, isometric tension recordings and patch-clamp electrophysiology revealed that application of pinacidil produced relaxing effects by augmenting $K_{ATP}$ channel activity (Gollasch et al., 1995). Application of glibenclamide counteracted the pinacidil-induced relaxation. In addition to the intrinsic regulation of the coronary artery SM tone, external regulation occurs via $K_{ATP}$ channel mechanisms derived in ECs and autonomic nerve terminals (Fig. 1.7).
As illustrated in Fig. 1.7, K_{ATP} channels in the autonomic nerve terminal also participate in the setting of the coronary arterial tone. Inhibition of K_{ATP} channels results in Ca^{2+} entry through VDCCs, elevating intracellular levels of noradrenaline (NA), which in turn binds to the α-1 adrenoceptor (α_1R) located on the SMC. PL-C is then stimulated which acts to cleave PIP_2 into IP_3, subsequently triggering the release of Ca^{2+} from the SR (Levick, 2009; Klabunde, 2011).

In ECs, activation of K_{ATP} channels permits the outflow of K^+, increasing the driving force for Ca^{2+} into the cell, stimulating the production of NO. The resulting free radical diffuses out of the EC and into the SM, prompting guanylyl cyclase receptor activity and accumulation of cGMP. Activation of protein kinase G follows. This exerts a hyperpolarizing effect on the vessel via phosphorylation of K^+ channels, leading to vasodilation. In addition, Ca^{2+} pumps are activated that drive Ca^{2+} out of the SMC. A further relaxing effect of NO occurs via its strong suppressive action against the release of endothelin-1 (ET-1), a powerful vasoconstrictor (Thorin & Clozel, 2010; Bourque et al., 2011).

The SM tone is also affected by deviations in the membrane potential of ECs that are relayed to SMCs directly through gap junctions, mediating VDCCs in the SM membrane. Innervation of a single SMC can be transmitted to adjacent cells, producing a unified contraction (Greger & Windhorst, 1996b; Boron & Boulpaep, 2004; Aaronson & Ward, 2007; Thomas, 2011).
Fig. 1.7 Regulation of VSM contraction by $K_{ATP}$ channel signalling events in the vascular wall. Closure of neuronal $K_{ATP}$ channels results in membrane depolarization and influx of $[Ca^{2+}]_i$. This in turn releases NA which acts on the $\alpha$-1 adrenoceptor ($\alpha_1$R), stimulating SM contraction via cleavage of PIP$_2$ into IP$_3$ and secretion of $[Ca^{2+}]_i$, from the ER. Inhibition of $K_{ATP}$ channels in the SM membrane causes membrane depolarization followed by an influx of $Ca^{2+}$ through VDCCs, inducing SM contraction. Opening of $K_{ATP}$ channels in ECs results in hyperpolarization and inflow of $Ca^{2+}$. This promotes the biosynthesis of NO which is dispersed to the SMC, causing relaxation.

1.1.2.4 Genetic deletions of $K_{ATP}$ channels in mice

Genetic deletion (knockout) of the $K_{ATP}$ channel gene in mice hearts resulted in $[Ca^{2+}]_i$ overload and injury to cardiac myocytes. These mice also presented arrhythmias and sudden death (Zingman et al., 2002). Miki et al. (2002) reported that knockout of the Kir6.1 subunit in mice (global knockouts) precipitates a phenotype mirroring Prinzmetal angina in humans. These mice displayed spontaneous coronary vasospasm, coupled with ST-segment elevations during the ECG procedure and atrioventricular block. The authors conclude that these symptoms were due to myocardial ischemia. Chutkow et al. (2002) further demonstrated the occurrence of episodic coronary vasospasm in mice.
genetically engineered for the global knock-out of the $K_{\text{ATP}}$ channel SUR2 subunit; the mice subsequently conveyed increased resting blood pressures and sudden death. Local coronary vasospasm was also present accompanied by ST segment elevation which was relieved by application of $\text{Ca}^{2+}$ channel inhibitors. The report by Chutkow et al. (2002) suggests that $K_{\text{ATP}}$ channels containing the SUR2 subunit are imperative in the control of episodic and tonic VSM tone. More recently, research conducted by Kakkar et al. (2006) was carried out to re-express the SUR2B subunit into the VSM of SUR2-null mice using a SM-specific promoter. However, re-insertion of the SUR2B subunit did not alleviate vasospasm observed in the mice. The authors propose a mechanism extrinsic to the SMC to underpin the vasospastic phenotype in these mice. They suggest a peripheral SM mechanism to be the pathogenic trigger of spasm. Experiments performed by Malester et al. (2007) lends credence to this hypothesis, highlighting a role for endothelial $K_{\text{ATP}}$ channels in the regulation of coronary blood flow by controlling the release of the vasoconstrictor, endothelin-1. These reports establish a role for $K_{\text{ATP}}$ channels in mediating the fine balance between vasodilation and vasoconstriction processes in the setting of the coronary vessel tone.

1.12.5 Pathology of $K_{\text{ATP}}$ channels in the cardiovascular system

$K_{\text{ATP}}$ channels have been associated with the pathogenesis of a number of cardiovascular diseases. The Cantu syndrome is linked to $K_{\text{ATP}}$ channel dysfunction (Harakalova et al., 2012). This is a rare condition that causes enlargement of the heart (cardiomegaly) and also excessive hair growth (hypertrichosis). The pathological origin has been connected to mutations in the SUR2 subunit (Harakalova et al., 2012). Impaired function of $K_{\text{ATP}}$ channels is also associated with cardiomyopathies (inflammation of the heart) in hypertensive rats (Zlatkovic et al., 2009).

Missense and frameshift mutations in cardiac $K_{\text{ATP}}$ channels have been observed to affect channel gating, consequently altering heart rhythms and increasing susceptibility to dilated cardiomyopathy (Bienengraeber et al., 2004). Specifically, mutations in the cardiac channel SUR2A subunit that map to sites proposed to be vital in the hydrolysis of ATP are suggested to be a cause of dilated cardiomyopathy by disruption of catalytic activity. An increasing amount of evidence suggests that abnormal vascular $K_{\text{ATP}}$ channels may be involved in the development of coronary vasomotor dysfunction and ischemic heart disease (Zingman et al., 2007; Flagg et al., 2010; Baczkó et al., 2011).
1.13 Aims of the project:

$K_{ATP}$ channels serve critical regulatory functions in the cardiovascular system. Furthermore, cardiac diseases have been ascribed to mutations that alter $K_{ATP}$ channel function. Recently, a heterozygous missense mutation was reported in a patient who had suffered an AMI of vasospastic origin (Minoretti et al., 2006). This mutation results in an amino acid substitution of valine at position 734 of SUR2 into isoleucine. At the gene level, this is underscored by a point mutation, which occurs as a single nucleotide variation (c.2200G>A) in exon 17 of the ABCC9 transporter gene, located on chromosome 12. This region encodes for a segment of the NBD1 on the SUR2 subunit. It is also noteworthy that exon 17 is present in both of the splice variants of SUR2: SUR2A and SUR2B. In collaboration with colleagues at the Division of Cardiology, Azienda Ospedaliera University and the Department of Health Sciences, University of Pavia, Italy, a large scale genetic screening was performed and an additional number (10) of AMI patients carrying the mutation were identified. As shown in Fig. 1.8, valine 734 is an evolutionarily conserved residue across numerous species (Minoretti et al., 2006).

**Fig. 1.8** Conservation of the valine 734 residue, highlighted within the amino acid primary structure segment taken from SUR2 NBD1 of various species.

The pathophysiological mechanism of this genetic mutation is unknown. We hypothesized that the V734I variant could alter the function of cardiac (Kir6.2/SUR2A) and/or vascular (Kir6.1, Kir6.2/SUR2B) $K_{ATP}$ channels, and that such an alteration could give rise to AMI. We also hypothesized that this mutation may reduce the $K_{ATP}$ channel current amplitude because SUR2 gene knockout in mice results in a condition resembling Prinzmetal angina (Chutkow et al., 2002) and mutations in SUR2 cause dilated cardiomyopathy (Bienengraeber et al., 2004). Coronary spasm in humans may
therefore result from a loss of VSM (Kir6.1/SUR2B), endothelial (Kir6.2/SUR2B) and/or cardiac (Kir6.2/SUR2A) channel activity. Therefore, the main aim of my project was to assess the impact of the V734I mutation on the functional characteristics of $K_{ATP}$ channels in the cardiovascular system.

Specific aims were:

(I) To characterize and compare the response of wild-type and mutant channels composed of Kir6.2/SUR2B (endothelial), Kir6.1/SUR2B (VSM) and Kir6.2/SUR2A (cardiac) subunits to a range of physiological nucleotides using variations of patch-clamp electrophysiology.

(II) To use computational homology modelling to predict the structure of the mutated NBD complex and to employ molecular dynamics simulations to deduce a ligand/receptor docked conformation and gain insights into the biophysical mechanism of the mutation.

(III) To examine the pharmacological response of $K_{ATP}$ channels harbouring the V734I mutation to the antianginal drug nicorandil using patch-clamp electrophysiology.
Chapter 2

Materials and methods

This section provides a detailed description of the experimental techniques used in my thesis. Although the experiments used to assess patient characteristics and genetics were conducted by our clinical collaborators Dr Piercarlo Minoretti, Dr Luigi Vignali and Dr Enzo Emanuele (Division of Cardiology, Azienda Ospedaliera University and the Department of Health Sciences, University of Pavia, Italy), and not myself directly, I have outlined these methods in Sections 2.2 – 2.5. This is to provide the reader with a description of how initial clinical and genetic data were collected. These data form the basis for my project. A more detailed explanation of the procedures used by our colleagues is reported in Minoretti et al. (2006) and Smith et al. (2013).

2.1 Materials and reagents

The molecular biology Mini/Midi prep kits and transfection reagents were purchased from Qiagen (UK), all other biochemical agents including $K_{ATP}$ channel activators and blockers were purchased from Sigma-Aldrich.

2.2 Study participants

All patients participating in the study provided written consent prior to enrollment. From January 2003 to May 2011 a total of 1123 patients with precocious AMI were assessed. Eleven carriers of the V734I variant were detected through genetic screening as described below. Thirty three AMI patients without the mutation were used as a control group by frequency matching for age and gender with subjects carrying the mutation.

2.3 Screening for genetic variants

Blood samples of all 1123 participants were collected and DNA was extracted from each sample for genetic and biochemical analysis. The DNA was probed for mutations in the $ABCC9$ gene using PCR technology and single strand conformation polymorphism (SSCP) analysis as described in Minoretti et al. (2006).
2.4 Clinical diagnosis of AMI

As mentioned above, this study included 1123 patients presenting precocious AMI. Subjects with valvular heart disease or previous coronary revascularisation procedures were not included in this study. AMI was diagnosed by the presence of the following three criteria: 1) anginal pain lasting >30 minutes refractory to sublingual nitroglycerin; 2) typical electrocardiographic changes including any of the following: i) ST segment elevation of ≥1 mm in at least 2 standard leads; ii) ≥2 mm in at least 2 contiguous precordial leads; iii) the presence of a left bundle branch block; iv) ST segment depression of >1 mm in 2 contiguous leads; v) inversion of the T waves of >1 mm in at least 3 contiguous leads (non–ST-elevation myocardial infarction); or 3) cardiac enzyme increases (creatine kinase (CK) >3 times the normal upper limit with significant CK-MB fraction or elevation of troponin T or troponin I).

2.5 Examination of coronary blood flow and measurement of cardiovascular biomarkers

Coronary angiography was used to determine obstruction to coronary blood flow by identifying global or focal irregularities of the vessel lumen. In general, this procedure involves the administration of a contrast medium into the patient’s coronary arteries, followed by a series of scans using mainly X-ray computed tomography (CT) or magnetic resonance imaging (MRI) to observe the coronary blood stream (Murphy & Lloyd, 2006).

The Left ventricular ejection fraction (LVEF) was monitored, in addition to patient medical histories including diabetes, hypertension and a history of ischemic heart disease. Age, gender and body mass index were also recorded. A range of biomarkers were measured including, total cholesterol, high density lipoprotein (HDL) and triglycerides. Rho-associated coiled coil containing protein kinase (ROCK) activity was assayed in peripheral blood leukocytes as the level of phospho-Thr853 in the phospho myosin-binding subunit (MBS) of myosin light chain phosphatase (Nohria et al., 2006). The Enzyme-linked immunosorbent assay (ELISA) was used to obtain plasma concentrations of ET-1. Commercial ELISA kits (R&D Systems, Minneapolis, MN, USA) were also used to identify a rise in additional markers of cardiovascular injury. These included; Interleukin-6 which is associated with tissue damage and inflammation, tumour necrosis factor (TNF-α, a cytokine involved in systemic inflammation), and C-
reactive protein (hsCRP) and adiponectin, where elevations in these markers provide an indication to a histopathological inflammatory response (Pearson et al., 2003).

2.6 Molecular Biology

(Wild-type Kir6.1 (accession number: NM_008428, species: mouse), Kir6.2 (NM_000525, species: human), SUR2A (NM_013040, species: rat) and SUR2B (AF087838, species: rat) cDNA were sub-cloned into the pcDNA3.1 vector prior to the beginning of my project). Although we did not have access to clones obtained from the same species, other previous studies have used combinations of clones obtained from different species to investigate genetic mutations in the K<sub>ATP</sub> channel that occur in human (e.g. Tarasov et al., 2007; Proks et al., 2013).

2.6.1 Site-directed mutagenesis

The V734I point mutation was performed using the QuickChange site-directed mutagenesis protocol (Stratagene, La Jolla, CA, USA), according to the manufacturer’s instructions. This procedure is based on the use of two oligonucleotide primers containing an internal mismatch with the template DNA (pcDNA 3.1 vector including SUR2A or SUR2B cDNAs) to create the desired mutation. The mismatches were designated towards the 5’ ends of the primers to ensure greater stability between the target and the primer, and efficient extension during the subsequent polymerase step. Temperature cycling was used to anneal the primers to the DNA vector. PfuTurbo DNA polymerase (Invitrogen, Carlsbad, CA, USA) was used to extend the primer strands. Methylated, non-mutated parental DNA was digested from the DNA sample by exposure to the restriction enzyme Dpn I, which selectively cuts up the methylated DNA. In this way, the parental methylated DNA (wild-type) was eliminated from the sample. The mutation in each of the plasmids was verified by DNA sequencing performed offsite (GATC Biotech, Germany).

2.6.2 Amplification of DNA plasmids in bacteria

In order to obtain a sufficient amount of DNA plasmid for heterologous expression and functional characterization the following steps were taken.
**Bacterial transformation**

XL1 Blue competent bacterial cells derived from Escherichia coli were purchased from Invitrogen (Carlsbad, CA, USA). These bacteria are non-infectious and extremely sensitive to U.V. rays to prevent risks to the operator. Uptake of the DNA vector into bacterial cells was carried out by heat shocking the DNA/bacterial solution as described in Green & Sambrook (2012). The pcDNA3.1 vector contains the “amp” gene, as a result only successfully transformed bacterial cells were able to survive and grow in the presence of ampicillin. In brief, a 1.5 ml plastic tube containing a suspension of bacteria (30 µl) and plasmid DNA (100 ng) was maintained on ice for 30 minutes and then transferred immediately into a water bath at 42 °C for 45 sec, followed by 5 minutes on ice. 1 ml of Luria broth (LB) supplemented with ampicillin was added to the bacterial suspension and incubated at 37 ºC on a shaking device set at 220 rpm. After one hour, SOC (Super optimal broth with catabolic repression) medium was added to provide further bacterial growth supplementation during the recovery phase, and enhance the overall transformation efficiency. Transformed bacteria were placed on an agar plate containing ampicillin (50 µg/µl) and left to grow overnight at 37 ºC. One colony per plate was collected and expanded in 50 ml of liquid medium (in the presence of 50 µg/ml ampicillin). The medium was incubated overnight at 37 ºC.

**Extraction and purification of plasmid DNA**

The Qiagen Plasmid Midikit (Qiagen) was used for the extraction and purification of plasmid DNA according to the manufacturer’s instructions. This kit is based on an initial lysis of the bacteria with a solution containing detergents such as sodium dodecyl sulphate (SDS). The plasmid DNA was then purified using an ionic exchange resin column. The sample was washed with 70% ethanol to remove salts along with small debris. Finally, an “elution buffer” was added to the column to elute the DNA into a 1.5 ml collection tube. The elution buffer contains TRIS which ensures the DNA is deprotonated and solubilized, and EDTA which inactivates nucleases, preventing degradation of the sample. This procedure yields approximately 80 µg of DNA extracted from 50 ml of bacterial suspension.
**Agarose gel electrophoresis**

An agarose gel was run in order to ensure that the purified DNA solution contained only the plasmid of interest. This approach involves the application of an electric field to separate the negatively charged DNA fragments in an agarose matrix. Smaller double stranded DNA molecules travel through the agarose medium at a greater velocity. A 0.5% agarose gel was prepared in 0.5 x Tris-borate buffer (TBE). The gel was then poured into the electrophoresis compartment and left to cool to room temperature. A DNA volume of 4 µl was added to 1 µl of loading buffer type II and then pipetted into the gel. Ethyldium bromide (EtBr) was also added to the gel (10 µl for 100 ml of gel). Under U.V. light EtBr acts as a fluorescent stain as it slots in between the base pairs of the DNA molecule. When bound, EtBr fluorescence is greatly enhanced and the DNA is illuminated, providing a tool to identify individual fragments. The separation process was set for 15 minutes at 100 V. Overall, the replication / purification process introduces a degree of strain on the DNA, therefore the plasmid DNA ring is subject to winding into coiled structures. Three conformations of the DNA plasmid were identified as linear, coil and supercoil.

**Spectrophotometry analysis**

Spectrophotometry was used to examine the concentration and purity of DNA. As DNA molecules highly absorb light at a wavelength 260 nm, the concentration can be determined by measuring the optical density (OD) of the sample at this wavelength. An OD of 1 is equivalent to 50 µg/ml of double stranded DNA (Green & Sambrook, 2012). Proteins are a potential source of contamination in DNA preparations and strongly absorb light at a wavelength of 280 nm. The ratio of absorption at 260 nm to 280 nm was therefore measured to establish the purity of the DNA in the sample. A ratio of cDNA purity ~ 1.9 indicated minimal contamination (Green & Sambrook, 2012). Samples were excluded when this ratio fell beneath this value.

**2.6.3 Cell culture**

Human embryonic kidney cells (HEK-293T) were grown in DMEM12 culture medium (Sigma) supplemented with 0.1% Gentamicin (50 µg/ml), 1% L-glutamine and 10% Fetal bovine serum, and were incubated at 37 °C in a humidified atmosphere containing 5% CO₂. Cells were passaged upon a confluence level of approximately 80%. The
passage procedure consisted of rinsing cells with phosphate buffered saline (PBS) followed by trypsinization and centrifugation at 1200 rpm for 3 minutes. A hemocytometer was used to count cells as shown in Fig 2.1. The total number of cells in the central square (indicated by the central blue ring) was counted under a microscope. This square has a volume of 100 nl, therefore the total number of cells per ml was obtained by multiplying the count by a factor of $10^4$. A value of $10^5$ cells were plated for each 3.5 mm petri dish.

**Fig 2.1** Diagrammatic representation of the hemocytometer device used to count cells for plates prior to experiments. A cell suspension is injected into the counting space groove beneath the glass cover slip and then viewed under an optical microscope. The scattered pellets in the grid view represent HEK-293T cells. Cells were counted within the area outlined by the blue ring in the central section of the grid.

### 2.6.4 Transfection of DNA into HEK-293T cells

Wild-type and mutant $K_{ATP}$ channels were expressed in HEK-293T cells for electrophysiological study. The HEK-293T cell type was used because it expresses low endogenous $K^+$ currents and has been used extensively in $K_{ATP}$ channel investigations and other ion channel studies (Kondo et al., 1998; Moran et al., 2003; Adomaviciene et al., 2013). The $K_{ATP}$ channel is formed by Kir6.x and SUR2.x subunits in a 1:1 ratio. As the primary sequence of SUR2.x subunit is approximately four times longer than that of the Kir6.x subunit, a four-fold greater amount of SUR2.x DNA was transfected to ensure that cells were exposed to an equal number of plasmids containing Kir6.x and SUR2.x (Tammaro et al., 2008). Specifically, cells were transfected with 0.5 µg Kir6.x, 2 µg SUR2 and 0.8 µg of the CD8 membrane antigen cDNA using Attractene (Qiagen,
Venlo, Netherlands) according to the manufacturer’s instructions. Attractene is a lipid-based transfection reagent. In this procedure, plasmid DNA is engulfed by liposomes which fuse to the cell’s plasma membrane to deliver DNA into the cell’s interior. The CD8 membrane antigen was used as it enables recognition of transfected cells (Jurman et al., 1994). Prior to experiments 2 µl of CD8 Dynabeads (Invitrogen, Carlsbad, CA, USA) were mixed into the cell bath and incubated for 2 minutes at room temperature. These beads are coated in antibodies specifically designed to adhere to the CD8 antigen. As a result of antibody-antigen binding, successfully transfected cells were identified by the presence of Dynabeads on the cell surface (Jurman et al., 1994). Patch-clamp experiments were performed 1-2 days after transfection. By counting proportions of cells that were positive to CD8 beads the overall yield of successfully transfected cells was approximately 40-60%.

2.7 Electrophysiology

The patch-clamp technique is a microelectrode recording method used to amplify bioelectrical signals of various cells (Hamill et al., 1981; Bretschneider & De Weille, 2006; Sakmann & Neher, 2009). This procedure permits recording of ionic currents from a single ion channel or multiple ion channels simultaneously.

2.7.1 Principles of the patch-clamp system

The patch-clamp procedure consists of an operational amplifier linked to both a recording electrode and a ground electrode, to measure electrical signals across a cell membrane. The cell recording chamber is observed under an inverting optical microscope. The system requires isolation from sources of electronic noise, including thermal current noise which exists within all components of the setup (Neher & Sakmann 1995; Ogden, 1994; Moran, 1996). A Faraday cage is built around the system to redistribute electronic noise away from the recording area and devices. Each component of the system is connected to a single ground point and mounted onto a pneumatically controlled anti-vibration table to prevent mechanical vibration effects from external sources.

Figure 2.2 displays the classical patch-clamp pre-amplification system in the whole-cell voltage clamp mode. In this mode, the amplifier sets the membrane potential to a specific value, allowing fluctuations in the current passing through the membrane to be
observed. When current passes through the membrane to the bath, the operational amplifier (op-amp) A1 measures a voltage drop across the resistor, detected as a difference in potential between the two inputs. An adjustment is then made to the A1 output and thus, the voltage across the feedback resistor. Consequently, current is driven back through the resistor bringing the inverting input back to the same potential as the non-inverting input (command potential or $V_{\text{Cmd}}$). In this way, current is injected into the cell holding the transmembrane voltage at $V_{\text{Cmd}}$. Op-amp A1 operates as a voltage clamp to the cell membrane (Bretschneider & De Weille, 2006; Walz, 2007; Sakmann & Neher, 2009). The inverting input of A1 serves only to sense the voltage, therefore high impedance inputs are employed to draw a minimal fraction of the current. As the output of A1 reflects the change in the current value op-amp A2 is used to subtract the output of A1 from $V_{\text{Cmd}}$. This is proportional to the voltage across the feedback resistor, and is an analogue of the membrane current (Bretschneider & De Weille, 2006; Sakmann & Neher, 2009). The combination of amplifiers A1 and A2 serve as a current-to-voltage converter. A ground reference electrode is inserted into the bath solution to complete the circuit, maintaining the bath solution at the ground potential by acting as an infinite source or sink of charge.
Fig. 2.2 Schematic diagram of the classical patch-clamp pre-amplification system (voltage-clamp mode) connected to a series resistance compensatory component, shown here in the whole cell configuration. A portion of the A2 output is added to the command by the summing amplifier A3 to compensate for the series resistance. Substantial amplification is applied via a Gain component built-in to the main unit (not shown).

When recording from whole cells, the series resistance existing between the headstage input and the ground electrode, excluding the membrane resistance, can present a significant recording offset. This is mainly the sum of the pipette resistance (due to the small size of the pipette tip), any access resistance such as that encountered due to connective tissue, and possible resistance arising from the use of agar bridges. When the value is substantial, a significant offset in voltage is introduced. Therefore, a series resistance compensation circuit is used to compensate for this (Bretschneider & De Weille, 2006; Walz, 2007; Sakmann & Neher, 2009). The signal is filtered using a low-pass anti-aliasing filter to “clean” it from extraneous noise and digitized using an analogue-to-digital converter for analysis.

2.7.2 Electrophysiological instrumentation specifically used in this study

An Axon Multiclamp 700B amplifier (Axon Instruments, California, USA) was connected to a recording chamber positioned in the line of sight of an Olympus TH4-200 inverting optical microscope (Shinjuku, Tokyo, Japan). Fine manoeuvrability of the micropipette was achieved using a Burleigh PCS-PS60 patch-clamp micromanipulator (Burleigh, Newton, New Jersey, USA). A low-pass 4-pole Bessel filter integrated within
the internal circuitry of the amplifier was used to filter the current signal. A key property of this type of filter is that it produces a constant delay in all frequency components of the signal. In this way, the shape of the signal is maintained which is of particular use when analyzing current signals in the time domain (Walz, 2007; Sakmann & Neher, 2009). An additional characteristic of this filter is that it produces low signal ‘ring’ in response to a step input, making it a practical choice when examining single channel gating events (Walz, 2007; Sakmann & Neher, 2009). The Multiclamp 700B Software Command interface (Axon Instruments, Foster City, CA) was used to set the filter and the gain, and control the capacitance compensation circuit built into the amplifier. The Clampfit9.2 software panel, accessed via a personal computer, commanded the Axon Digidata 1322A 16 bit digitizer (Axon Instruments, Foster City, CA).

A silver wire of 0.01 mm thickness encased in a layer of silver chloride was selected for the pipette electrode and the ground reference electrode. The silver chloride coating permits the reversible reaction; \( \text{Ag} + \text{Cl}^- \leftrightarrow \text{AgCl} + e^- \). This allows electrical current in the wire to be transferred to the ionic solution. It is important to consider that ions travel at different rates in different solutions. With respect to the intracellular and extracellular liquid mediums, this difference in rate results in a partial accumulation of charge across the liquid junction boundary, known as the liquid junction potential (Walz, 2007; Sakmann & Neher, 2009). This potential was therefore compensated before commencing experiments. Micropipettes were prepared using borosilicate glass which has a low dielectric constant and therefore, reduces capacitive transients during electrophysiological recordings (Hille, 2001). Micropipettes were pulled to a resistance of 1-2 MΩ using a Narishige PC-10 pipette puller. Pipette tips used in whole-cell noise experiments were also coated in a film of beeswax. This inert substance was employed to prevent the bath solution sliding up the external side of the internal pipette, providing a more effective dielectric separation between the internal and external solution, reducing the capacitive transients.

### 2.7.3 Preparation of electrophysiological solutions

Solutions were designed to give a \( K^+ \) reversal potential of 0 mV, and therefore no net driving force for \( K^+ \) current flow at this potential (see Table. 2.1). Where MgATP and/or MgNDP have been applied, this is indicated in the figure legends of Chapters 4
and 5. Note that the intracellular solution used to assess the ATP response in the absence of Mg$^{2+}$ was identical to the intracellular solution in Table 2.1, except that the MgCl$_2$ component was omitted and a Mg$^{2+}$-free salt of ATP was used in place. The whole cell current density experiments (Section 4.1) were conducted using standard intracellular solution in the absence of nucleotides. Nucleotide stocks were stored at -20°C and working solutions were prepared on the day of the experiments.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Concentration/mM</th>
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<tbody>
<tr>
<td>Extracellular solution</td>
<td>KCl</td>
</tr>
<tr>
<td></td>
<td>HEPES</td>
</tr>
<tr>
<td></td>
<td>MgCl$_2$</td>
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<td></td>
<td>CaCl$_2$</td>
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<tr>
<td>Intracellular solution</td>
<td>KCl</td>
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<tr>
<td></td>
<td>CaCl$_2$</td>
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<tr>
<td></td>
<td>EGTA</td>
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<td>HEPES</td>
</tr>
<tr>
<td></td>
<td>MgCl$_2$</td>
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</tbody>
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**Table 2.1** Composition of both the internal and external electrophysiological solutions.

### 2.7.4 Inside-out macroscopic recordings from HEK-293T membrane patches

A perfusion system was constructed from 8 glass tubes of 800 µm diameter. For Kir6.2/SUR2x, concentration responses were constructed by measuring the current at -80 mV in the presence of various concentrations of nucleotides relative to the current measured in control (nucleotide-free) solutions. These concentration responses were fitted using the Hill-Langmuir equation (Rang & Dale, 2011) of the form:

$$\frac{I}{I_0} = \frac{1}{1 + ([ATP]/IC_{50})^h}$$

(3)
where $I$ is the current in the presence of ATP, $I_0$ the current in control solution (ATP-free), $[ATP]$ is the ATP concentration, $IC_{50}$ the half maximal inhibitory concentration and $h$ is the Hill coefficient for inhibition. Concentration responses were recorded at a holding potential of -80 mV. Currents were filtered at 1.8 kHz and sampled at 4 kHz.

Kir6.1/SUR2B channels are closed in the absence of nucleotides (Yamada et al., 1997; Takano et al., 1998), therefore currents were recorded in the presence of 300 µM pinacidil (a K$_{ATP}$ channel opener) to stimulate channel activity. The concentration responses to ATP for this type of K$_{ATP}$ channel has a bimodal component and was fitted with the following equation:

$$\frac{I}{I_0} = \left( \frac{1}{1 + ([ATP]/IC_{50})^h} \right) \cdot \left( 1 - \left( \frac{1}{1 + ([ATP]/EC_{50})^k} \right) \right)$$

(4)

Where $EC_{50}$ is the half maximal effective concentration and $k$ is the Hill coefficient for activation.

### 2.7.5 Single channel recordings

To assess the intrinsic K$_{ATP}$ channel open probability ($P_o$) Kir6.2/SUR2B and Kir6.2/SUR2B-V734I single channel currents were recorded from inside-out membrane patches at a holding potential of -80 mV. Currents were filtered at 2 kHz and sampled at 10 kHz. The Gaussian fits to all-points amplitude histograms (Tammaro & Ashcroft, 2007b) was used to calculate the single channel current ($i$) and the intrinsic channel open probability ($P_o$) in the absence of nucleotides.
2.7.6 Perforated patch configuration

The perforated-patch configuration is a specialized patch-clamp procedure which is achieved by filling the internal pipette with an antifungal (such as amphotericin B or nystatin). Upon seal formation these agents form channel pores that enable the passage of small molecules and ions through the membrane while preventing the transmission of larger molecules (e.g. ATP or other nucleotides or organelles). This approach allows recordings to be made across the whole cell without dialyzing the cytoplasm (Bretschneider & De Weille, 2006; Walz, 2007).

The internal solution was prepared on the day of experiments. Amphotericin B (5 mg) was dissolved in DMSO (50 µl) to obtain a final concentration of 1 mM. A 5 µl aliquot of this stock was added to 1 ml of standard K_ATP intracellular solution in the absence of MgATP, to give a final concentration of 0.5 mg/ml. The solution was vortexed for 3 minutes and kept on ice. Before filling the micropipette with the amphotericin-internal solution mixture, the tip of the micropipette was initially dipped into amphotericin-free solution. In this way, some solution penetrates the pipette tip by capillary action creating an initial barrier between the solution containing amphotericin B and the cell membrane. This was carried out to avoid direct interaction of amphotericin B with the tip of the pipette, an effect that prevents gigaseal formation (Walz, 2007; Sakmann & Neher, 2009).

Effective electrical access with the cell interior was monitored by observing the time course of the capacitive transients in response to 10 mV voltage steps of 10 ms durations.

2.7.7 Measurements of whole-cell current density

The whole-cell recording protocol consisted of a sequence of voltage sweeps from -90 mV to +90 mV in 20 mV intervals. Each pulse was applied for 200 ms. The fastest whole-cell signals measured are in the range of ~ 2-5 kHz (Walz, 2007). Filtering was set at 6 kHz and the signal sampled at 20 kHz. The membrane capacitance was determined using the Multiclamp 700B Command software. The software calculates the current time integral from the area under the capacitive spike, divided by the voltage step value (Walz, 2007). The compensation circuit integrated within the Axon 700B amplifier was utilized to compensate the series resistance when the value exceeded 10 MΩ. Compensation was performed by optimizing the correction control, whilst
observing the stability of the current signal to prevent significant noise and oscillation occurring.

2.7.8 Stationary noise analysis

Stationary noise analysis was used to determine the $K_{ATP}$ channel $P_0$. This method centres upon measuring the mean and variance of a steady-state macroscopic current signal to deduce the $P_0$ (Hille, 2001). Currents were stimulated with hyperpolarizing voltage sweeps at -90 mV of 2 s durations and the mean current and variance were recorded. Currents were filtered at 6 kHz to capture brief gating events and sampled at 20 kHz. The mean macroscopic current can be described by:

$$ < I > = N P_o i $$  \hspace{1cm} (5)

where $N$ is the number of channels, $P_o$ is the open probability and $i$ is the single channel current. The variance is:

$$ \sigma^2(I) = N i^2 P_o (1 - P_o) $$ \hspace{1cm} (6)

where $(1 - P_o)$ is the probability of a channel being closed. Therefore, substituting equation 5 into equation 6 yields,

$$ \sigma^2(I) = i < I > - \left( \frac{< I >^2}{N} \right) $$ \hspace{1cm} (7)

Equation 7 can be used to deduce the value of $N$ which can then be substituted into equation 5 to find $P_o$, provided that $i$ is known.

2.7.9 Electrophysiology software and statistical analysis

Mean currents were analysed using the ANA electrophysiology program (Dr M. Pusch, CNR, Italy). Concentration responses were then constructed and further evaluated using programs written by Dr Tammaro in the IGOR Pro technical graphing environment (Wavemetrics, Lake Oswego, OR, USA). Single channel currents and single channel
open probabilities were computed using the Gaussian fits to all-points amplitude histograms tool in ANA. Mean currents and variance from noise recordings were also obtained using the ANA program and subsequently analysed in IGOR with a program written by Dr Tammaro. The Student’s two-tailed *t* test or ANOVA with post-hoc Bonferroni’s test were used for statistical comparison of data and *p* < 0.05 was considered to be significant. Data are given as mean±SEM and number of experiments (N).

### 2.8 Molecular modelling of the human SUR2B-V734I NBD1/NBD2 dimer

To construct a structural homology model of the NBD complex of human SUR2B-V734I, templates of known crystal structures were selected based on the following criteria: i) A resolution requirement of ~ ≤ 2 Å, ii) A maximum homology match score with the target sequence. Selected templates were: the human multidrug resistance protein 1 NBD1 (2CBZ); the cystic fibrosis transmembrane conductance regulator (CFTR) NBDs (2PZE); the alpha-hemolysin translocation ATP binding protein HlyB (2PMK) and a fusion complex of CFTR maltose/maltodextrin import ATP-binding protein (3GD7).

#### I) Co-ordinate file (pdb) and structure file (psf) preparation

Modeller version 9.8 (Sali & Blundell, 1993) was used to produce a 3D cartesian coordinate model by aligning the target with the template sequences by satisfaction of spatial restraints. This method involves scanning for known structural correlations identified in the template, expressed as conditional probability density functions. The program computed 50 models with a root mean square deviation (RMSD) ≤ 4.0 Å. The Ramachandran plot was employed to exclude models that had over 10% of residues outside of the designated plot region (Berg *et al.*, 2006). The final model selected held the highest Modeller scoring function and lowest RMSD of α-carbons of the target to the templates.

NBD1 and NBD2 were fitted into the dimer complex using ProFit 3.1 least squares fitting (McLachlan, 1982). The cytoplasmic domains of the multidrug ABC Transporter SAV1866 (2HYD) were chosen as the reference co-ordinates.

The Visual Molecular Dynamics (VMD) program version 1.9 (Humphrey *et al.*, 1996) was used to generate the protein structure file (psf), containing atom bond, angle, and
dihedral connectivity specifications and partial charges using the CHARMM topology file. The VMD application was used throughout the modelling procedure to display the molecule and analyse its structure.

II) Molecular dynamics

NAMD (Nanoscale molecular dynamics) 2.8 (Phillips et al., 2005) in conjunction with the Charmm27 force field parameter file were used to determine the new molecular model structure in its minimum global energy state. This procedure consisted of three main stages: minimization, heating and equilibration. The preliminary step involved performing an energy minimization to relax strained local arrangements by eliminating steric clashes. These clashes are introduced by co-ordinate estimation in the production of the psf file. An explicit water box with periodic boundary conditions was set around the dimer molecule, and Na\(^+\) and Cl\(^-\) ions were added to neutralize the charge of the system using the VMD extensions tool. Extensive minimization was conducted by restraining protein heavy atoms whilst allowing water molecules to equilibrate into natural interactive positions. The system was heated linearly from 0 K to 300 K at a constant pressure of 1 atmosphere. The system was then equilibrated at a constant temperature of 300 K for 10 ns. This stage is required to ensure that thermodynamic variables, such as potential energy, display a relatively constant fluctuation around a mean value with time.

III) Computing the mutation

The V734I point mutation in NBD1 was executed using the Mutator plug-in component of the VMD software. The mutated model was then minimized as described above.

IV) Docking of the ligand to the receptor

The receptor and ligand molecules were prepared using AutoDockTools 1.5.4. Gasteiger charges and Kollman atom charges were assigned to the ligand and receptor, respectively, and hydrogens were added. The AutoDockTools program was further used to map the region of the receptor for docking and determine grid parameters. The macromolecule was held as a rigid structure, whereas torsion flexibility was enabled for the MgATP ligand molecule. Interactive docking regions were set around the Walker A motifs of the binding domains. The program AutoDock 4.2 (Morris et al., 2009) and
the Lamarkian Genetic Algorithm protocol were used to compute the docking simulation of MgATP. Conformations were then analysed using AutoDock Tools 1.5.4.

V) VMD analysis and rendering

VMD was employed to compute distances between the Walker A, Walker B and Signature Sequence motifs, measured in Angstroms. The built-in Tachyon image processor of the VMD program was selected to render the final molecular graphics file based on texture quality, compatibility and fast rendering speed.

2.9 Design of illustrations in this thesis

To create the scientific illustrations in this thesis, I utilized drawing, painting, and text tools from a combination of graphic design programs, specifically, Microsoft PowerPoint 2013, Adobe Photoshop CS6, Microsoft Paint - Windows 8, CorelDraw and IGOR Pro.
Chapter 3

Patient medical characteristics

3.1 Patients considered in this study
A total of 1123 patients consecutively enrolled at the hospitals of our collaborators (Division of Cardiology, Azienda Ospedaliera University and the Department of Health Sciences, University of Pavia, Italy) were considered in this study. All of these patients presented with precocious (before the age of 60) AMI. For each patient, all exons of the ABCC9 gene were sequenced to probe for genetic mutations. Genetic screening was performed as previously described by Minoretti et al. (2006). This analysis lead to the identification of the V734I mutation in 11 individuals.

3.2 Assessment of coronary angiography and cardiovascular biomarkers
The 11 subjects possessing the V734I variant were frequency matched with a control group of 33 randomly selected AMI patients accounting for statistical similarity of age and gender (see Table. 3.1). A 1:3 ratio of study to control was intended (Armitage & Colton, 1999; Suresh et al., 2012). Patients within the subject group were aged between 40 and 59 years, while the control group ranged from 41 to 59 years. Angiographic analysis revealed that subjects carrying this gene variant exhibited focal narrowing of the coronary arteries caused by vasospasm (Fig. 3.1, A), in contrast to the controls who did not present these symptoms. Vasospasm was effectively alleviated by the administration of sublingual nitroglycerin (Fig. 3.1, B).
Fig. 3.1 A. Coronary angiogram of a male AMI patient carrying the V734I mutation, displaying a narrowing in the right coronary artery (indicated by the red arrow). This was symptomatic of all patients carrying the V734I mutation. B. The angiogram shows the arterial narrowing is abolished after the patient was treated with nitroglycerin, indicating that the narrowing occurred due to spasm. Inserts in the lower left corners of the angiograms display a magnified view of the area of the tract where spasm occurred.

Various clinical characteristics were recorded and compared for both sets of patients including: age, gender, diabetes, smoking and total cholesterol, LDL and triglyceride levels. Statistical analysis detected no change in any of these data parameters between subjects and controls, in addition to patient body mass index, and the two groups did not contrast significantly in terms of hypertension or a family history of ischemic heart disease. The patients’ left ventricular ejection fractions were recorded, in addition to the presence of circulating inflammatory markers. However, these variables displayed no significant differences. Arterial levels of stenosis were analysed according to 1, 2 or 3 vessel plaque-associated occlusions. This corresponds respectively to stenosis of the right coronary artery, left anterior descending artery and the circumflex artery, indicating the proportions of plaque-affected arteries. Occlusion to the left coronary artery presents a particularly serious risk because this artery feeds the two main branches that distribute the blood to the left ventricle. Table. 3.1 indicates that patients carrying the mutation displayed a lower occurrence of plaque-affected arteries. The data in Table. 3.1 also demonstrates that carriers of the variant displayed significantly elevated levels of leukocyte ROCK and plasma concentrations of ET-1.
<table>
<thead>
<tr>
<th></th>
<th>AMI patients without 734Ile (N = 33)</th>
<th>AMI patients with 734Ile (N = 11)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>51 ± 8</td>
<td>51 ± 7</td>
<td>0.786</td>
</tr>
<tr>
<td>Males, n</td>
<td>22</td>
<td>9</td>
<td>0.567</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>25 ± 3</td>
<td>26 ± 3</td>
<td>0.429</td>
</tr>
<tr>
<td>Smoking, n</td>
<td>15</td>
<td>6</td>
<td>0.861</td>
</tr>
<tr>
<td>Diabetes mellitus, n</td>
<td>7</td>
<td>4</td>
<td>0.546</td>
</tr>
<tr>
<td>Hypertension, n</td>
<td>20</td>
<td>8</td>
<td>0.717</td>
</tr>
<tr>
<td>Family history of ischemic heart disease, n</td>
<td>10</td>
<td>4</td>
<td>0.722</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>211 ± 40</td>
<td>198 ± 33</td>
<td>0.185</td>
</tr>
<tr>
<td>Low-density cholesterol (mg/dL)</td>
<td>139 ± 40</td>
<td>133 ± 52</td>
<td>0.236</td>
</tr>
<tr>
<td>High-density cholesterol (mg/dL)</td>
<td>35 ± 12</td>
<td>37 ± 14</td>
<td>0.421</td>
</tr>
<tr>
<td>Left ventricular ejection fraction (%)</td>
<td>70 ± 8</td>
<td>67 ± 9</td>
<td>0.116</td>
</tr>
<tr>
<td><strong>Number of stenosed arteries</strong></td>
<td></td>
<td></td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>8</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><strong>Inflammatory markers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsCRP (mg/L)</td>
<td>2.11 (0.78, 4.40)</td>
<td>2.00 (0.97, 5.19)</td>
<td>0.715</td>
</tr>
<tr>
<td>Interleukin-6 (pg/mL)</td>
<td>2.66 (1.32, 2.96)</td>
<td>1.41 (1.12, 2.17)</td>
<td>0.418</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>1.34 (1.06, 1.93)</td>
<td>59.1 ± 31.5</td>
<td>0.625</td>
</tr>
<tr>
<td>Adiponectin (µg/mL)</td>
<td>55.7 ± 39.1</td>
<td>0.92 (0.35, 2.01)</td>
<td><strong>0.016</strong></td>
</tr>
<tr>
<td>Leucocyte ROCK (%)</td>
<td>0.55 (0.22, 1.11)</td>
<td>4.55 (2.93-10.11)</td>
<td><strong>0.009</strong></td>
</tr>
<tr>
<td>Endothelin, pg/mL</td>
<td>3.05 (1.81-6.61)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table. 3.1** Characteristics of AMI patients with (n = 11) and without (n = 33) the 734Ile allele in *ABCC9*. Categorical data are expressed as numbers of patients. Continuous data with and without skewness are presented as: medians (25th, 75th percentiles) and means ± SDs, respectively. ROCK, Rho-associated coiled-coil containing protein kinase; TNF-α, tissue necrosis factor-α.
Chapter 4

Electrophysiological characterization of wild-type and mutant channel sensitivities to intracellular nucleotides

As previously described, the mutation V734I in ABCC9 was found in 11 patients affected by AMI of vasospastic origin. However, there was no knowledge of whether the mutation altered \( K_{\text{ATP}} \) channel function. This chapter includes a comprehensive comparison of the electrophysiological characteristics of \( K_{\text{ATP}} \) channels composed of Kir6.1/SUR2B, Kir6.2/SUR2B and Kir6.2/SUR2A in the absence and presence of the V734I mutation. These \( K_{\text{ATP}} \) channel combinations represent those found in VSM, ECs and cardiac myocytes, respectively.

4.1 Whole cell current density recordings

Several mutations in ion channels are associated with changes in the magnitude of the whole cell current (Ashcroft, 1999). Thus, the study began by comparing the magnitude of whole cell currents (wild-type and mutant). In order to compare the amount of current between cells expressing wild-type and mutant channels, the whole cell capacitance was first measured as an indirect measurement of the cell surface area (Sakmann & Neher, 2009). Therefore, normalization of the current to the cell capacitance yields the whole cell current density. The mean whole cell capacitance values recorded from HEK-293T cells expressing wild-type and mutant channels were 20.8±2.6 pF and 20.7±3.4 pF (Kir6.2/SUR2A), 18.3±4.0 pF and 15.4±1.6 pF (Kir6.2/SUR2B), 18.1±3.8 pF and 13.8±0.68 pF (Kir6.1/SUR2B), respectively.

As it took time for the pipette solution (free of nucleotides) to fully perfuse into the cell after the whole cell “break-in”, a series of voltage ramps running from -100 mV to +100 mV were recorded in 5 s intervals. This provided a clear display of the time required for the current to reach a steady value (see Fig 4.1). The current amplitude increased presumably because ATP was washed out of the cell which results in activation of \( K_{\text{ATP}} \) channels. The trace also shows a characteristic inwardly rectifying response. The time (in minutes) to reach a stable current signal were 6.73±0.34 (Kir6.2/SUR2A, \( N=8 \)) and 8.10±0.9 (Kir6.2/SUR2A-V734I, \( N=11 \)); 2.25±0.60
consistent with previous studies, channels composed of Kir6.1/SUR2B subunits mediated currents characterized by a lower level of inward rectification compared to channels composed of Kir6.2 and SUR2A or Kir6.2 and SUR2B subunits. The degree of inward rectification was quantified by measuring the ratio of the mean current measured at -90 mV to that measured at the +90 mV level (I_{-90}/I_{+90}, rectification index). Current sweeps corresponding to these voltages can be seen as the lowest current sweep and top sweep in each of the traces of Fig. 4.2 (A). The rectification indices were 1.53±0.093, N=10 (Kir6.2/SUR2A) and 1.68±0.077, N=8 (Kir6.2/SUR2A-V734I);
Mean whole cell current densities (Fig. 4.2, B) were $-304\pm87$ pA/pF (Kir6.2/SUR2A) and $-213\pm40$ pA/pF (Kir6.2/SUR2A-V734I), $-750\pm256$ pA/pF (Kir6.2/SUR2B) and $-578\pm193$ pA/pF (Kir6.2/SUR2B-V734I), and $-85\pm16$ pA/pF (Kir6.1/SUR2B) and $-201\pm80$ pA/pF (Kir6.1/SUR2B-V734I). Overall, whole cell analysis detected no change in the current density between wild-type and mutant channels.

**Fig. 4.2** (A) Whole cell current sweeps of 200 ms durations induced by stepwise voltage pulses from +90 to -90 mV in 20 mV increments, every 5 s. The holding potential was clamped at 0 mV between sweeps. Recordings were made from HEK-293T cells expressing cardiac and vascular, wild type and mutant channels as indicated. No ATP/NDPs were present in the intracellular solution. (B) Bar chart illustrating the subsequent mean current density at -90 mV for cardiac and vascular, wild type and mutant channels. In each case, wild type and mutant data were not significantly different. The number of experiments ranged from 7 to 13.

### 4.2 MgATP recordings from inside-out membrane patches

The NBD1 of the SUR2 subunit that houses the mutant residue is a site involved in the binding and hydrolysis of MgATP. To investigate whether the V734I mutation results in an altered response to MgATP, the response of channels composed of Kir6.2 and SUR2A, Kir6.2 and SUR2B, and Kir6.1 and SUR2B subunits to various concentrations
of intracellular MgATP were examined using the inside-out configuration of the patch-clamp technique (Fig. 4.3).

Kir6.2/SUR2B-V734I channels presented a reduced response to ATP inhibition in the presence of Mg\(^{2+}\) compared to Kir6.2/SUR2B channels. The IC\(_{50}\) values were 96 ± 21 µM for Kir6.2/SUR2B and 214 ± 47 µM for Kir6.2/SUR2B-V734I (P < 0.05, Table 4.1, B). However, at concentrations ≥ 1mM MgATP there was no difference in the current. On the other hand, the cardiac K\(_{ATP}\) channel type (Kir6.2/SUR2A and Kir6.2/SUR2A-V734I) presented equivalent responses to MgATP. The IC\(_{50}\) was 53±5 µM and 56±6 µM for Kir6.2/SUR2A and Kir6.2/SUR2A-V734I channels, respectively.

As previously described, VSM K\(_{ATP}\) channels (Kir6.1/SUR2B) are closed in the absence of intracellular MgADP (Yamada et al., 1997; Takano et al., 1998). Therefore 300 µM pinacidil, an activator of VSM K\(_{ATP}\) channels was used to stimulate channel activity in order to examine the response to MgATP. As displayed in Fig. 4.3, C, the response of Kir6.1/SUR2B channels to MgATP was biphasic. MgATP enhanced the magnitude of the current up to approximately a 30 µM concentration, above this level an inhibitory effect was observed. Importantly, Kir6.1/SUR2B and Kir6.1/SUR2B-V734I channels presented an identical response to MgATP (Table. 4.1, C).
**Fig. 4.3** A and B. i, Currents recorded from inside-out patches excised from HEK-293T cells expressing Kir6.2 and either SUR2x or SUR2x-V734I as indicated. The holding potential was clamped at −80 mV for the whole duration of the experiments. The solid bars indicate when MgATP (100 μM) was applied. The dashed lines represent the zero current level. ii, Mean relationship between [MgATP] and K$_{ATP}$ current (I), normalized to the current in the absence of nucleotide (I$_0$) for K$_{ATP}$ channels composed of Kir6.2 and either SUR2x or SUR2x–V734I subunits as shown. The smooth curve represents the best fit of Eq. (3) to the data. The number of experiments was 8–11 (A) and 18 (B) in each case. C. i Currents recorded from inside-out patches excised from HEK-293T cells expressing Kir6.1 and either SUR2B or SUR2B–V734I as described. Solid bars signify when various concentrations of MgATP were applied (a through f indicate 10, 30, 300, 1000, 3000 and 10,000 μM MgATP, respectively). 300 μM pinacidil was applied to the cell together with MgATP. The dashed lines indicate the zero current level. ii, Mean relationship between [MgATP] and K$_{ATP}$ current (I), normalized to the current in the absence of nucleotide (I$_0$) for K$_{ATP}$ channels composed of Kir6.1 and either SUR2B or SUR2B–V734I subunits, as shown. The curves were fitted using Eq. (4). The number of experiments was 7–10 in each case. The shaded bars in A, B and C ii indicate the predicted range of physiological ATP concentrations inside a generic cell.
4.3 Sensitivity of K\textsubscript{ATP} channels to MgATP in the presence of MgNDP

In the intact cell MgNDPs are also present, therefore these nucleotides were studied in combination with MgATP. A concentration of 500 µM MgNDP has been shown to effectively activate K\textsubscript{ATP} channels (Lederer & Nichols, 1989; Michailova et al., 2005; Tammaro et al., 2006). Therefore, a constant concentration of 500 µM MgNDP was selected to compare the activation potencies of these nucleotides when administered with MgATP. Specifically, 500 µM MgADP, MgUDP and MgGDP were separately applied in combination with various concentrations of MgATP (see Fig. 4.4).

Fig. 4.4 A and B. Mean relationship between [MgATP] and K\textsubscript{ATP} current (I), expressed relative to the current in the absence of nucleotide (I\textsubscript{0}) for experiments conducted in the presence of 500 µM of individual MgNDP, as indicated, for Kir6.2/SUR2x (i) and Kir6.2/SUR2x–V734I channels (ii). The continuous lines are the best fit of Eq. (3) to the data. The dashed curves correspond to the best fit of Eq. (3) to data obtained in the absence of MgNDPs (re-plotted from Fig. 4.3, A and B). The number of experiments was 4–10 (A) and 6–18 (B) in each case. C. Mean relationship between [MgATP] and K\textsubscript{ATP} current (I), expressed relative to the current in the absence of nucleotide (I\textsubscript{0}) for experiments conducted in the presence of 500 µM MgGDP, as
indicated, for Kir6.1/SUR2B (i) and Kir6.1/SUR2B–V734I channels (ii). Pinacidil (300 μM) was applied in combination with MgGDP. The continuous lines are the best fit of Eq. (4) to the data. The dashed curves correspond to the best fit of Eq. (4) to data obtained in the absence of MgGDP and re-plotted from Fig. 3.4, C. The number of experiments was 3 in each case.

The sensitivities of Kir6.2/SUR2A and Kir6.2/SUR2A-V734I channels to MgATP in the presence of MgNDP displayed an equivalent response. The IC₅₀ values for Kir6.2/SUR2A and Kir6.2/SUR2A-V734I channels were ~140 μM (+MgADP), ~170 μM (+MgUDP) and ~250 μM (+MgGDP) in each case (Table. 4.1, A). However, the capacity of MgNDP to further activate Kir6.2/SUR2B-V734I channels was reduced compared to Kir6.2/SUR2B channels. The IC₅₀ values measured for Kir6.2/SUR2B channels were 226±43, 455±82 and 617±76 μM when in the presence of 500 μM MgADP, MgUDP and MgGDP, respectively (Table. 4.1, B). Whereas Kir6.2/SUR2B-V734I channels were found to be 200 - 400 μM in all cases (Table. 4.1, B). For Kir6.1/SUR2B and Kir6.1/SUR2B-V734I channels, the sensitivity to MgATP was assessed in the presence of 500 μM MgGDP and 300 μM pinacidil. Table. 4.1, C shows that the response of these channels to these agents are equivalent. The percentage of current that remains unblocked in the presence of a physiological concentration of MgATP is also reported in Table. 4.1.
<table>
<thead>
<tr>
<th>Channel</th>
<th>MgATP</th>
<th>+ MgADP</th>
<th>+MgUDP</th>
<th>+MgGDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kir6.2/SUR2 A</td>
<td>53 ± 5 (8)</td>
<td>137 ± 53 (4) *</td>
<td>187 ± 36 (11) *</td>
<td>254 ± 39 (10) *</td>
</tr>
<tr>
<td>h (n)</td>
<td>1.0 ± 0.1 (8)</td>
<td>1.3 ± 0.1 (4)</td>
<td>1.3 ± 1.2 (11)</td>
<td>1.4 ± 0.1 (10)</td>
</tr>
<tr>
<td>% unblocked</td>
<td>4.9 ± 1.2 (8)</td>
<td>7.5 ± 4.5 (4)</td>
<td>11.4 ± 2.3 (11)*</td>
<td>13.7 ± 2.0 (10)*</td>
</tr>
<tr>
<td>Kir6.2/SUR2 A-V734I</td>
<td>56 ± 6 (11)</td>
<td>140 ± 28 (6) *</td>
<td>163 ± 25 (10) *</td>
<td>254 ± 31 (10) *</td>
</tr>
<tr>
<td>h (n)</td>
<td>1.0 ± 0.1 (11)</td>
<td>1.4 ± 0.1 (6)</td>
<td>1.5 ± 0.2 (10)</td>
<td>1.5 ± 0.1 (10)</td>
</tr>
<tr>
<td>% unblocked</td>
<td>5.7 ± 1.0 (11)</td>
<td>6.0 ± 1.7 (6)</td>
<td>10.4 ± 1.6 (10) *</td>
<td>12.1 ± 2.0 (10) *</td>
</tr>
<tr>
<td>Kir6.2/SUR2 B</td>
<td>96 ± 21 (18)</td>
<td>226 ± 43 (6) *</td>
<td>455 ± 82 (12) *</td>
<td>617 ± 76 (18) *</td>
</tr>
<tr>
<td>h (n)</td>
<td>0.9 ± 0.1 (18)</td>
<td>1.0 ± 0.1 (6)</td>
<td>1.1 ± 0.1 (12)</td>
<td>1.3 ± 0.1 (18)</td>
</tr>
<tr>
<td>% unblocked</td>
<td>10 ± 1 (18)</td>
<td>20 ± 4 (6) *</td>
<td>27 ± 5 (12) *</td>
<td>34 ± 4 (18) *</td>
</tr>
<tr>
<td>Kir6.2/SUR2 B-V734I</td>
<td>214 ± 47 (18)</td>
<td>274 ± 57 (7)</td>
<td>378 ± 72 (12)</td>
<td>374 ± 49 (16) *</td>
</tr>
<tr>
<td>h (n)</td>
<td>1.1 ± 0.1 (18)</td>
<td>1.0 ± 0.1 (7)</td>
<td>1.1 ± 0.1 (12)</td>
<td>1.2 ± 0.1 (16)</td>
</tr>
<tr>
<td>% unblocked</td>
<td>15 ± 3 (18)</td>
<td>21 ± 4 (7)</td>
<td>23 ± 3 (12)</td>
<td>23 ± 3 (16) *</td>
</tr>
</tbody>
</table>

Table 4.1, A: Mean data for Kir6.2/SUR2A and Kir6.2/SUR2A-V734I channels

<table>
<thead>
<tr>
<th>Channel</th>
<th>MgATP</th>
<th>+MgGDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kir6.1/SUR2B</td>
<td>2058 ± 606 (7)</td>
<td>317 ± 89 (3) *</td>
</tr>
<tr>
<td>h (n)</td>
<td>0.7 ± 0.2 (7)</td>
<td>0.7 ± 0.1 (3)</td>
</tr>
<tr>
<td>EC50 (n)</td>
<td>1.9 ± 0.7 (7)</td>
<td>0.6 ± 0.3 (3)</td>
</tr>
<tr>
<td>k (n)</td>
<td>0.9 ± 0.4 (7)</td>
<td>0.9 ± 0.5 (3)</td>
</tr>
<tr>
<td>% unblocked</td>
<td>66.4 ± 6.6 (7)</td>
<td>26.7 ± 2.3 (3) *</td>
</tr>
<tr>
<td>Kir6.1/SUR2B-V734I</td>
<td>2023 ± 811 (10)</td>
<td>290 ± 104 (3) *</td>
</tr>
<tr>
<td>h (n)</td>
<td>0.6 ± 0.2 (10)</td>
<td>0.9 ± 0.2 (3)</td>
</tr>
<tr>
<td>EC50 (n)</td>
<td>2.2 ± 0.8 (10)</td>
<td>0.9 ± 0.4 (3)</td>
</tr>
<tr>
<td>k (n)</td>
<td>0.9 ± 0.3 (10)</td>
<td>1.0 ± 0.4 (3)</td>
</tr>
<tr>
<td>% unblocked</td>
<td>60.5 ± 5.1 (10)</td>
<td>23.8 ± 1.8 (3) *</td>
</tr>
</tbody>
</table>

Table 4.1, B: Mean data for Kir6.2/SUR2B and Kir6.2/SUR2B-V734I channels

Table 4.1, C: Mean data for Kir6.1/SUR2B and Kir6.1/SUR2B-V734I channels

Table 4.1 A, B and C. Mean values of MgATP concentration producing half-maximal inhibition of the channel (IC50) and Hill coefficient (h) recorded in the absence of MgNDP
(column labelled “MgATP”) or in the presence of various MgNDP (500 µM), as indicated. Note that all experiments related to Kir6.1/SUR2B have been conducted in the presence of 300 µM pinacidil in the intracellular solution. * indicates statistical significance against data recorded in the presence of MgATP only; ° indicates statistical significance against wild-type.

4.4 Whole cell perforated patch recordings

Experiments in previous chapters and sections were recorded from inside-out patches in which nucleotides were fixed to physiological values. The next set of experiments were aimed to study the regulation of Kir6.2/SUR2B and Kir6.2/SUR2B-V734I channels in intact cells under different metabolic conditions. To achieve this, perforated patch clamp recordings were used. Fig 4.5 displays the capacitance current signal in response to 1000 ms steps to +10 mV from a holding potential of 0 mV. Currents were recorded every minute over a period of approximately 10 minutes. This was performed in order to visually monitor the extent of electrical access established during the perforation process. This was quantified by measuring the time course of the exponential decay of the capacitive transient over the time (Sakmann & Neher, 2009). Once the capacitive transients reached stability, whole cell K\textsubscript{ATP} currents were recorded in response to -80 mV sweeps (1 s durations) applied every 10 s from a holding potential of 0 mV. To study the effect of changes in the metabolic state of the cell on K\textsubscript{ATP} channel activity, currents were recorded in the absence and presence of sodium azide (NaAzide) (Fig. 4.6), an inhibitor of mitochondrial metabolism (Tammaro et al., 2006).
Fig 4.5 Capacitive current signals recorded in the perforated patch configuration in response to a 10 mV step pulse. Recordings were made from HEK-293T cells expressing Kir6.2 and either SUR2B (a) or SUR2B-V734I (b) channels. The capacitive transients were recorded in stages to visualize the progressive perforation of the membrane. (i) The first minute where the seal was rapidly forming and the membrane was beginning to perforate, (ii) a time 3 minutes later where the capacitative transients had gradually increased, (iii) and 10 minutes later when the transients had reached a stable time course.

Fig 4.6, A illustrates Kir6.2/SUR2B and Kir6.2/SUR2B-V734I currents recorded at -80 mV in the absence of NaAzide (control) or at various times of continuous exposure to NaAzide (100 µM). Fig. 4.6, B shows the mean current recorded in the absence and presence of NaAzide at various time points. Upon stability, the cell was perfused with K<sub>ATP</sub> extracellular solution containing a 50 µM concentration of glibenclamide. Only recordings where K<sub>ATP</sub> channel currents displayed a complete block by glibenclamide were used for analysis.
Fig 4.6 Perforated patch current response to 100 µM NaAzide. (A) Perforated patch traces taken at four different times throughout the experiment as labelled. Measurements were made from HEK-293T cells expressing Kir6.2 and either SUR2B or SUR2B-V734I channels. (B) Mean $I_{K_{ATP}}$ current measured at various time points after application of 100 µM NaAzide. The current was measured in response to 1 s steps to -80 mV, applied every 10 s. Mean current levels were estimated once the current attained a stable value, typically within the latter 500 ms of the sweep. Current amplitude is expressed relative to the amplitude of the current after full activation by NaAzide ($I_{Max}$). The cell was initially perfused for 100 s with whole cell external control solution, at this point (as indicated by the black arrow) 100 µM NaAzide was added to the extracellular solution for approximately 15 minutes, until currents fully stabilized. Note that the $I/I_{Max}$ ratio does not reach the value 1, this is due to variability in the time course of the response to NaAzide in the various cells. The grey block arrow illustrates the expected increase in the MgADP/MgATP ratio upon application of NaAzide.

4.5 Pharmacological response of Kir6.2/SUR2B and Kir6.2/SUR2B-V734I channels to nicorandil

Nicorandil is a potent vasodilator drug which acts on $K_{ATP}$ channels in the plasma membrane. It is therefore used to treat angina for its relaxing effects on the coronary arterial wall (Suryapranata, 1993; Reimann et al., 2001). As the mutation suppresses the
stimulatory action of MgNDP on Kir6.2/SUR2B channels we explored the possibility that nicorandil could remediate the loss of function effect of the channel variant. The effect of nicorandil was examined at two different concentrations (30 or 300 µM). MgGDP (500 µM) was also included in the intracellular solution which contained various concentrations of MgATP. MgGDP was used in these experiments because it is the nucleotide in the presence of which, the electrophysiological properties of Kir6.2/SURB and Kir6.2/SUR2B-V734I channels differ more substantially. It is therefore easier to examine the capacity of nicorandil to correct this effect. The experiment shown in Fig. 4.7 indicates that low doses of nicorandil gave rise to an even greater rightward shift.

Fig. 4.7 demonstrates that Kir6.2/SUR2B-V734I channels displayed a rightward shift in their MgATP response when 30 µM and 300 µM nicorandil was added. The IC_{50} values were 1364±350 (n=6) and 1050±202 (n=5) at the 30 µM and 300 µM concentrations, respectively.

![Fig. 4.7 A, Kir6.2/SUR2B-V734I current traces taken at the 1000 µM concentration of MgATP for (i) 30 µM and (ii) 300 µM nicorandil. B, MgATP response of Kir6.2/SUR2B-V734I channels conducted in the presence of 500 µM MgGDP, and 30 µM (triangles) or 300 µM (diamonds) nicorandil for Kir6.2/SUR2B-V734I channels. The dashed curves represent the MgATP response in the presence of 500 µM MgGDP for Kir6.2 and SUR2B or SUR2B-V734I recordings as indicated, taken from Fig. 4.4 B, i and ii, shown previously. In each case, nicorandil produced a strong rightward shift with IC_{50} values equal to 1364±350 µM and]
1049±202 at the 30 μM and 300 μM concentration, respectively, demonstrating the capacity of the drug to induce channel stimulation.
Chapter 5

Biophysical mechanism of the V734I mutation

5.1 Molecular structure analysis and biophysics of Kir6.2/SUR2B and Kir6.2/SUR2B-V734I channels

In order to gain insights into the biophysical mechanisms underlying the functional effects of the V734I mutation, the primary sequence of SUR2 was analysed. The V734 residue is situated 22, 78 and 98 amino acids from the Walker A motif W_A, signature sequence (SS) and Walker B (W_B) motif, respectively (Fig 5.1, B). However, because the tertiary structure of the protein cannot be immediately predicted from analysis of the primary structure, a molecular model was constructed (Fig 5.1, C). To gain a more complete understanding into the biophysical properties of the variant, the structural model was mutated during the computational process to contain the isoleucine residue.

From analysis of the molecular model (Fig. 5.1, C), NBD1 and NBD2 of the human SUR2B-V734I sequence formed a heterodimer, adhered by two molecules of MgATP. The dimer associated in a head-to-tail fashion with the MgATP ligands coordinating into the Walker A pockets. The mutated isoleucine residue in NBD1 is at ~20 Å, ~15 Å and ~27 Å from its adjacent W_A, W_B and linker region, respectively.
Fig. 5.1 A. Membrane topology of the SUR2B-V734I subunit, highlighting the cytoplasmic NBDs. The site of the mutation is displayed as a magenta star. B. Primary sequence of the human SUR2B-V734I NBD1. C. Molecular model of the human SUR2B-V734I NBD complex. NBD1 and NBD2 are represented by green and black ribbon structures, respectively. The isoleucine (mutant) residue is displayed in magenta by its Van der Waals spheres of interaction. In both NBD1 and NBD2, Walker A motifs are displayed in gold, Walker B motifs are in red and the Signature Sequences are shown in blue. The two molecules of ATP flanked between the Walker A motifs and the signature sequences are displayed as stick bonds, and Mg$^{2+}$ ions are shown as spheres. The coiled sections of the structure represent α-helices, while arrows symbolize β-sheets.
5.2 Molecular pathophysiological mechanism of the V734I mutation

Alterations in MgATP sensitivity of the mutant channel may be due to a number of factors. These include i) an impaired channel inhibitory response at the Kir6.x pore-forming subunit where ATP binds into, ii) an altered intrinsic (ligand-independent) open probability of the channel, iii) an impaired response of SUR to MgATP (Enkvetchakul & Nichols, 2003; Masia et al., 2005; Tammaro et al., 2005). These possibilities were therefore examined individually.

5.3 ATP sensitivity examined in the absence of Mg$^{2+}$ from inside-out membrane patches

To examine if V734I alters the sensitivity of the K$_{ATP}$ channel complex to ATP inhibition, the sensitivity of Kir6.2/SUR2B and Kir6.2/SUR2B-V734I channels was tested in the absence of Mg$^{2+}$. Under these conditions, ATP does not interact with SUR2 to stimulate channel activity; this enables the effects of the V734I mutation on ATP inhibition at Kir6.2 to be analysed in isolation from MgATP activation at SUR2. Consistent with previous studies (Tammaro & Ashcroft, 2007a), we found that in the absence of Mg$^{2+}$, wild-type Kir6.2/SUR2B channels were significantly more sensitive to ATP than in the presence of Mg$^{2+}$. Figure 5.2 shows that V734I does not affect the sensitivity of Kir6.2/SUR2B channels to ATP inhibition; the IC$_{50}$ and h being 13±3 µM and 1.1±0.1 (N=13) (Kir6.2/SUR2B) and 10±2 µM and 1.3±0.1 (N=9) (Kir6.2/SUR2B-V734I).
Fig. 5.2 Response of Kir6.2/SUR2B and Kir6.2/SUR2B-V734I channels to ATP in the absence of Mg\textsuperscript{2+}. (A) Currents recorded in inside-out patches excised from HEK-293T cells expressing Kir6.2 and either SUR2B or SUR2B-V734I as indicated. The membrane potential was held at -80 mV. ATP (100 µM) was applied as indicated by the horizontal bars. The dashed lines indicate the zero current level. (B) Mean relationship between [ATP] and K\textsubscript{ATP} current (I), expressed relative to the current in the absence of nucleotide (I\textsubscript{0}) for K\textsubscript{ATP} channels composed of Kir6.2 and either SUR2B or SUR2B-V734I subunits as indicated. The continuous lines are the best fit of Eqn. 3 to the data. The number of experiments was 8-11 (A) and 18 (B) in each case.

5.4 Single channel recordings from inside-out membrane patches

It is a well-established fact that the sensitivity of K\textsubscript{ATP} channels to ATP is affected by the intrinsic P\textsubscript{o} of the channel. Specifically, ATP binds with greater affinity to Kir6.2 during the channel closed phase (Fan & Makielski, 1999; Li \textit{et al.}, 2002; Craig \textit{et al.}, 2008). Thus, mutations that increase the P\textsubscript{o} result in a decrease in sensitivity of the channel to ATP inhibition, while mutations that reduce P\textsubscript{o} have an opposite effect. Therefore single channel analysis was used to examine the possibility that the V734I mutation caused an altered response of Kir6.2/SUR2B channels via an effect on the intrinsic channel P\textsubscript{o}. Single channel analysis revealed that the V734I mutation did not affect the unitary current \(i\) or the channel P\textsubscript{o}. These values were 4.9±0.1 pA and 0.34±0.04 (11), and 4.9±0.2 pA and 0.42±0.05 (11) for Kir6.2/SUR2B and
Kir6.2/SUR2B-V734I, respectively. Fig. 5.3 displays sample traces of single Kir6.2/SUR2B and Kir6.2/SUR2B-V734I channel recordings. Therefore, it can be excluded that the effects of the V734I mutation on channel sensitivity to nucleotides is secondary to a change in the intrinsic channel $P_o$.

**Fig. 5.3** Single channel currents recorded at -80 mV from inside-out patches excised from HEK-293T cells, expressing Kir6.2 and either SUR2B or SUR2B-V734I $K_{ATP}$ channels. The dashed line indicates the zero current level. The rapid electronic flicker seen during open bursts could be due to non-selected channel ions being temporarily drawn in to clog the mouth of the pore.

### 5.5 Whole cell noise analysis

Stationary noise analysis was used to deduce the $P_o$ of Kir6.2/SUR2A, Kir6.2/SUR2A-V734I, Kir6.1/SUR2B and Kir6.1/SUR2B-V734I channels. This procedure requires knowledge of the single channel current amplitude (see methods for stationary noise). In the case of Kir6.1/SUR2B and Kir6.1/SUR2B-V734I the single channel current amplitudes were first measured from single channel recordings. These values were 3.2±0.4 pA (Kir6.1/SUR2B) and 3.6±0.6 pA (Kir6.1/SUR2B-V734I) were used to compute the channel $P_o$. For Kir6.2/SUR2A channels, a unitary current of 4.0 pA was taken from Tammaro et al. (2006). Fig. 5.4, B shows that there was no change in $P_o$ between wild-type and mutant channels in both cases.
5.6 MgNDP sensitivity examined in the absence of MgATP from inside-out membrane patches

To investigate whether V734I altered the Kir6.2/SUR2B response to MgNDPs, the effect of MgADP, MgGDP and MgUDP were studied, independently, in the absence of MgATP. At a concentration of 500 µM, these nucleotides have little inhibitory effect on Kir6.2 but stimulate channel activity via SUR2 (Lederer & Nichols, 1989; Michailova et al., 2005; Tammaro et al., 2006). Fig. 5.5 shows that Kir6.2/SUR2B and Kir6.2/SUR2B-V734I channels were activated to the same extent by these nucleotides. This indicates that in the absence of MgATP, the V734I mutation does not alter the overall current amplitude of the channel after exposure to MgNDP. However, as described above, the response of Kir6.2/SUR2B-V734I channels was impaired when in the simultaneous presence of MgATP.
The times taken to reach half the maximal current amplitude were analysed to compare the rate of activation of Kir6.2/SUR2B and Kir6.2/SUR2B-V734I channels in response to individual MgNDPs (Fig. 5.5, C). The half-times were $\tau_{0.5} = 33218\pm6333$ s and $28959\pm7521$ s (500 µM MgADP) and $\tau_{0.5} = 2067\pm495$ s and $2227\pm276$ s (500 µM MgUDP) for Kir6.2/SUR2B and Kir6.2/SUR2B-V734I channels, respectively. Thus, the responsiveness of Kir6.2/SUR2B and Kir6.2/SUR2B-V734I channels to MgADP and MgUDP were not significantly altered. However, Kir6.2/SUR2B-V734I channels conveyed a significantly slower activation rate to 500 µM MgGDP compared to the Kir6.2/SUR2B channel ($\tau_{0.5} = 2160\pm252$ for Kir6.2/SUR2B and $\tau_{0.5} = 3150\pm401$ for Kir6.2/SUR2B-V734I).

Fig. 5.5 (A) Currents recorded in inside-out patches excised from HEK-293T cells expressing Kir6.2 and either SUR2B (i) or SUR2B-V734I (ii) channels. The membrane potential was held at -80 mV. ATP (10 mM) was applied to the patch at the beginning of the experiments to ensure that negligible leakage currents were present in each patch, while various MgNDP (500 µM) were subsequently applied as indicated. (B) Mean steady state Kir6.2/SUR2B and Kir6.2/SUR2B-V734I current measured in the presence of individual MgNDPs relative to the current in the absence of nucleotide ($I_0$). The number of experiments was 4-16 in each case. (C)
Mean $\tau_{0.5}$ of Kir6.2/SUR2B and Kir6.2/SUR2B-V734I current activation in the presence of individual MgNDPs. The number of experiments was 4-16 in each case.

5.7 Response of Kir6.2/SUR2B and Kir6.2/SUR2B-V734I channels to the non-hydrolysable MgATP analogue: MgAMP-PNP

The altered metabolic regulation of Kir6.2/SUR2B-V734I channels by MgATP might be a result of a structural change in the NBD1 that allows MgATP to act directly as a channel activator without being first hydrolysed to MgADP. To explore this further, concentration responses of Kir6.2/SUR2B and Kir6.2/SUR2B-V734I channels were conducted using the non-hydrolysable MgATP analogue, MgAMP-PNP (Fig. 5.6). The response to MgAMP-PNP of wild-type and mutant channels did not significantly differ. The IC$_{50}$ values were 56.3±14.6 $\mu$M (N=6) and 42.2±2.3 (N=7) and h values were 1.29±0.09 (N=6) and 1.84±0.17 (N=7), computed from Kir6.2/SUR2B and Kir6.2/SUR2B–V734I traces, respectively. These findings suggest that the altered response of mutant channels to MgATP is related to a change in the hydrolysis of MgATP within the NBD complex.
Fig. 5.6 Inside-out concentration response of Kir6.2/SUR2B and Kir6.2/SUR2B-V734I channels to MgAMP-PNP. (A) Current responses to 1000 µM AMP-PNP recorded under a constant holding potential of -80 mV. Traces are shown for both Kir6.2/SUR2B (i) and Kir6.2/SUR2B-V734I channels (ii). The dashed line reflects the zero current mark. The solid lines indicate the brief time when the concentration was applied. (B) MgAMP-PNP concentration response curve for Kir6.2/SUR2B and Kir6.2/SUR2B-V734I channels as shown. Data points for each concentration were not significantly different.
Chapter 6

Discussion

6.1 Main findings of this research

The major finding from this research is that the human missense mutation in \textit{ABCC9} (V734I), which is associated with precocious AMI (Minoretti \textit{et al.}, 2006), impairs the response of vascular $\text{K}_{\text{ATP}}$ channels composed of Kir6.2 and SUR2B-V734I subunits to intracellular nucleotides. In general, $\text{K}_{\text{ATP}}$ channel compositions of Kir6.2 and SUR2B have been detected in vascular ECs, in addition to sympathetic neurons (Dunne \\& Petersen, 1986; Karschin \textit{et al.}, 1998; Yoshida \textit{et al.}, 2004). The V734I mutation altered the response of Kir6.2/SUR2B channels to both MgATP and MgNDP. These two effects combined produced a loss of Kir6.2/SUR2B-V734I current. The anti-anginal drug nicorandil activated Kir6.2/SUR2B-V734I channels, substituting for the loss of MgNDP stimulation, indicating that this drug could prove effective in the treatment of spasm in patients presenting V734I.

All eleven patients carrying the V734I mutation displayed severe focal coronary spasm. In general, obstruction to the coronary arteries can result in acute coronary syndrome. This condition occurs predominantly at rest and is typically linked to ST-segment elevation on the ECG which can result in AMI or lead to arrhythmias in the young adult (Tun \\& Khan, 2001; Perron \\& Sweeney, 2005; Kolansky, 2009; Lanza \textit{et al.}, 2011; Reynolds, 2012). This condition can be life threatening. Furthermore, symptoms of angina persist in a large number of patients irrespective of current medical treatments (Tun \\& Khan, 2001; Perron \\& Sweeney, 2005; Kolansky, 2009; Lanza \textit{et al.}, 2011; Reynolds, 2012). The main genetic risks associated with coronary spasm to date are polymorphisms of NO synthase (Yasue \textit{et al.}, 2008). This research indicates that the V734I mutation in the SUR2 subunit of the $\text{K}_{\text{ATP}}$ channel may also give rise to coronary spasm.

6.2 Assessment of patient characteristics

A range of parameters were assessed to compare the medical characteristics of AMI patients carrying the mutation with an AMI control group. The gender of the patients
were recorded as cardiovascular disease is more common in males, potentially due to the benefit of oestrogen in stimulating nitric oxide synthase activity (Aaronson & Ward, 2007). From the cohort of patients seen in Table 3.1, mutation occurred in 9 males compared to 2 females. However, due to the small sample size available, it is not possible to establish if the severity of the effects of this mutation correlates with either of the two genders. The age of the patients were also recorded as arteriosclerosis is found to develop with age and the presence of diabetes mellitus was assessed as patients with this condition are subject to a higher risk of atherosclerosis (Aaronson & Ward, 2007). Whether the patients were smokers or non-smokers was also documented because the tobacco compound contains substances that coagulate the blood and can elicit injury to the vascular structure (Aaronson & Ward, 2007). Total cholesterol levels, LDL and triglycerides are linked to atherosclerosis of the coronary vessels. Heterozygous carriers of the V734I mutation were not significantly different from the control group in any of the parameters outlined above.

Measurements of the inflammatory biomarkers hsCRP, Interleukin-6, TNF-α and Adiponectin were measured as inflammation has been associated with coronary disease, whereby rupture of the plaque’s fibrous cap in atherosclerotic vessels can elicit an inflammatory response (Napoleão et al., 2007). However, these markers displayed no significant variation between subject and control, suggesting that coronary spasm in patients carrying the V734I mutation was not directly brought on by inflammation. High cholesterol levels can be an indication of the presence of arterial plaques. Importantly, subjects harbouring V734I did not display significantly heightened cholesterol levels compared to controls. Further to this, patients carrying the V734I mutation displayed a significantly lower number of plaque-infected arteries compared with the control group, with 7 individuals showing no evidence of vessel stenosis and 4 of the remaining 11 presenting only 1 plaque-affected artery. This data supports the hypothesis that AMI in patients carrying the V734I mutation originates from a non-atherosclerotic mechanism. A further key finding was that V734I-patients presented elevated levels of ET-1 and Leukocyte ROCK. The impact of these markers associated with cellular function is discussed in Section 6.7 (Functional implication of V734I).

Coronary spasm in patients with V734I was treated with nitroglycerin by sublingual administration. By way of conversion into nitric oxide, nitroglycerin is a standard vasodilator drug administered to patients suffering from vasospasm (Moncada et al.,
1991; Kugiyama et al., 1996). In all patients spasm was alleviated as a result of this treatment.

### 6.3 Molecular composition of SUR2-containing K\textsubscript{ATP} channels in the cardiovascular system

A considerable number of findings suggest that co-expression of Kir6.1 and SUR2B subunits reconstitute the VSM channel type (Isomoto et al., 1996; Yamada et al., 1997). Assemblies of Kir6.1, Kir6.2 and SUR2B have been detected in ECs (Yoshida et al., 2004; Morrissey et al., 2005) whereas the channel composition within ventricular myocytes mainly consists of the Kir6.2 and SUR2A subunits (Inagaki et al., 1996). K\textsubscript{ATP} channels (including those containing Kir6.1) are also expressed in sympathetic neurons (Dunn-Meynell et al., 1998; Karschin et al., 1998). Hence, the tissue distribution of Kir6.x and SUR2 in the cardiovascular system is diverse. In view of this, the effects of SUR2-V734I in combination with both Kir6.1 and Kir6.2 isoforms were investigated.

### 6.4 Role of K\textsubscript{ATP} channels in the control of coronary artery function

Coronary SM K\textsubscript{ATP} channels participate in the regulation of coronary tone including vasodilator responses to exercise and hypoxia (Hibino & Kurachi, 2006). Knockout mice for either Kir6.1 or SUR2 presented an identical phenotype of coronary vasospasm closely resembling Prinzmetal angina in humans (Chutkow et al., 2002; Miki et al., 2002). The coronary SM tone ultimately depends on the contractile state of VSMCs. VSM activity is regulated by both intrinsic factors as well as by SM-extrinsic mechanisms. Autonomic nerves and ECs are key to this control (Yasue et al., 1974; Garland & Weston, 2011). K\textsubscript{ATP} channels are present in both ECs and nerve terminals and may indirectly participate in the control of coronary blood flow.

K\textsubscript{ATP} channels in sympathetic neurons mediate the membrane potential and consequently, [Ca\textsuperscript{2+}]. This ultimately controls the secretion of NA. An association has been presented between hyperactivity of autonomic nerves and angina of vasospastic origin in man (Yasue et al., 1974). Injection of the K\textsubscript{ATP} channel blocker glibenclamide in dog atria augmented coronary vascular resistance during cardiac sympathetic nerve stimulation (Mori et al., 1995). More recently, Burgdorf et al. (2004) demonstrated that opening of pre-synaptic K\textsubscript{ATP} channels mediated suppression of NA overflow from rat...
heart during coronary low flow ischemia. In ECs, activation of K<sub>ATP</sub> channels by α<sub>2</sub>-AR stimulation contributes to the generation of NO (Hibino & Kurachi, 2006).

6.5 Examination of the electrophysiological characteristics of wild-type Kir6.2/SUR2B, Kir6.2/SUR2A and Kir6.1/SUR2B channels

Consistent with previous studies (Reimann et al., 2000; Tammaro & Ashcroft, 2007a) channels composed of Kir6.2/SUR2B subunits displayed a slightly reduced inhibitory response to ATP compared to Kir6.2/SUR2A channels when 2 mM Mg<sup>2+</sup> was included in the internal solution. This differential response to MgATP is understood to arise from the variation in the last 42 amino acids between the SUR2A and SUR2B isoforms. It has been proposed that this variation may result from different rates of ATP hydrolysis or MgATP being more tightly bound with SUR2B than SUR2A (de Wet et al., 2010). The observation that channels composed of Kir6.1/SUR2B subunits were closed in the absence of nucleotides and conveyed a biphasic response to MgATP is also consistent with previous findings (Yamada et al., 1997; Takano et al., 1998).

6.6 Nucleotide sensitivities of Kir6.2/SUR2B and Kir6.2/SUR2B-V734I channels

The IC<sub>50</sub> for MgATP inhibition of Kir6.2/SUR2B-V734I was decreased relative to wild-type channels. However, in the physiological range of MgATP concentrations (i.e. ~0.5 - 3 mM) there was no significant difference between Kir6.2/SUR2B and Kir6.2/SUR2B-V734I channel currents. In the simultaneous presence of MgATP and MgNDP, which more closely resembles the intracellular milieu, the amplitude of the Kir6.2/SUR2B-V734I current was lower than that measured for wild-type channels. In this respect, V734I was shown to result in an overall loss of K<sub>ATP</sub> channel current.

6.7 Functional implication of mutation V734I

Kir6.2/SUR2B assemblies are found in ECs and synaptic terminals. Therefore, the potential effects associated with a loss of K<sub>ATP</sub> channel activity in these cell types are discussed below.
(i) Kir6.2/SUR2B-V734I channels in vascular ECs

In conditions when the myocardial oxygen demand is increased, the diminished stimulatory effect of MgNDPs upon Kir6.2/SUR2B-V734I channels would have a depolarizing effect on the EC. Consequently, there would be a weaker driving force for Ca\(^{2+}\) influx through voltage-independent Ca\(^{2+}\) channels (Nilius & Droogmans, 2001). The drop in [Ca\(^{2+}\)]\(_i\) in the EC is expected to decrease the synthesis of NO. Previous studies have linked coronary vasospasm to a reduced endothelial NO reserve (Kugiyama et al., 1996). NO is synthesized from the amino acid L-arginine via the constitutive calcium-calmodulin-dependent enzyme NOS. One possibility could be that the loss of function effect of the mutation, which occurs as a reduction in K\(_{\text{ATP}}\) channel activity acts to reduce the driving force for Ca\(^{2+}\) uptake into vascular ECs, restricting NO synthesis. Production of cytochrome P450 metabolites may also decline. Both of these processes would otherwise produce greater vasodilation effects. In addition, the depolarization induced by a closure of endothelial K\(_{\text{ATP}}\) channels can be transmitted to the SMCs via gap junctions, triggering SM contraction.

ET-1 is a powerful and long-lasting vasoconstrictor peptide produced by vascular ECs (Inoue et al., 1989) and at elevated concentrations has been proposed to trigger coronary spasm (Toyo-oka et al., 1991). Patients carrying the V734I mutation presented significantly elevated circulating levels of ET-1 compared to the control group. This is consistent with research conducted by Malester et al. (2007), who recently highlighted a role for endothelial K\(_{\text{ATP}}\) channels as mediators of coronary vessel tone by controlling the secretion of ET-1. They demonstrated that blockade of K\(_{\text{ATP}}\) channels increased ET-1 release from isolated human coronary ECs. It is also noteworthy that an inverse relationship appears to exist between endothelial NO availability and ET-1 release/activity (Bourque et al., 2011). One possibility is that a reduction in the availability of NO in vascular ECs is potentiated by a loss of endothelial K\(_{\text{ATP}}\) channel current due to the V734I mutation. This could contribute to the elevation in ET-1 seen in patients carrying the mutation.

In consideration to a possible loss of K\(_{\text{ATP}}\) channel current and factors associated with endothelial dysfunction, it is important to consider the coupling effect between acetylcholine released from autonomic nerve terminals and the NO availability in vascular ECs. Acetylcholine is released by parasympathetic nerves at rest and its action in the presence of an impaired endothelial response, specifically, a decline in endothelial
NO availability has been implicated as a possible trigger of coronary artery spasm (Furchgott & Zawadzki, 1980; Kawano & Ogawa, 2004; Kaneda et al., 2006). Under normal conditions, the effect of acetylcholine on the vessel is mainly relaxation by priming M₃ receptors on vascular ECs (Kawano & Ogawa, 2004). However, acetylcholine also binds to M₃ receptors on VSMCs to promote contraction. In conditions when endothelial NO availability is reduced, the acetylcholine-induced contraction of VSM becomes more substantial relative to the dilatory mechanism. Thus, in conditions of endothelial dysfunction and reduced levels of NO in ECs, acetylcholine would have a vasoconstrictive effect which could be a contributing factor to vasospasm.

A further observation was that significantly high Leukocyte ROCK activity was detected in subjects carrying the V734I variant, compared to the control group. Leukocyte ROCK is a recognized marker of coronary vasospasm (Hung et al., 2012). It is also notable that endothelial NO has been reported to have a vasodilation effect on rat aorta via inhibition of Rho-kinase-mediated contraction (Chitaley & Webb, 2002). It is possible that in the presence of a reduced endothelial NO reserve, ROCK activity is elevated, promoting vascular constriction. A closure of K_{ATP} channels in the vascular endothelium of human coronary arteries would induce membrane depolarization, an influx of Ca^{2+} and consequently inhibit NO synthesis. Of further interest, is that fasudil, a Rho-kinase inhibitor has been observed to avert acetylcholine-induced coronary spasm in patients with Prinzmetal angina (Masumoto et al., 2002).

(ii) Kir6.2/SUR2B-V734I channels in the nerve terminal

The pathophysiological effects of Kir6.2/SUR2B-V734I channels could also arise from an alteration in neuronal K_{ATP} channel activity. A loss of K_{ATP} channel current would induce greater Ca^{2+} influx into the nerve terminal, promoting the secretion of NA. This neurotransmitter would then act on the SM α-adrenoceptor, priming the contraction mechanism via release of Ca^{2+} from intracellular stores. A rise in activity of autonomic nerves has been proposed to trigger attacks of Prinzmetal angina in humans through activation of α-receptors in the large coronary arteries (Yasue et al., 1974).

The possibility that AMI and coronary spasm originate through an impaired K_{ATP} channel response in ECs and/or autonomic nerves is consistent with the fact that SUR2B knock-out mice suffer from a form of angina resembling Prinzmetal angina in humans, despite re-expression of the channel into the VSM (Kakkar et al., 2006).
6.8 Metabolic regulation of channels containing V734I during perforated patch recordings

The metabolic regulation of Kir6.2/SUR2B and Kir6.2/SUR2B-V734I channels was assessed using the perforated patch configuration. In the absence of NaAzide there was only a small difference between the residual Kir6.2/SUR2B and Kir6.2/SUR2B-V734I channel currents. However, upon application of NaAzide, V734I-channels displayed an initial reduction in $K_{ATP}$ channel current and required greater durations to reach a maximum stable current amplitude, relative to Kir6.2/SUR2B channels. These recordings indicate the more substantial effect of V734I to be a loss of $K_{ATP}$ channel current in conditions when the cellular ATP/ADP ratio falls, for instance, during times of metabolic stress. In contrast, during conditions of high metabolism when the ATP/ADP ratio is high, the slight decrease in ATP inhibition in the presence of Mg$^{2+}$ might have a protective effect. This is consistent with the fact that the patients in this study carrying the V734I mutation suffered an AMI in adulthood.

6.9 Molecular mechanism

The V734I mutation caused two effects on Kir6.2/SUR2B channels: i) a decrease in sensitivity to MgATP block and ii) a decrease in MgNDP activation in the presence but not in the absence of MgATP. The first effect appeared to be Mg$^{2+}$-dependent as Kir6.2/SUR2B and Kir6.2/SUR2B-V734I channels presented identical sensitivities to ATP in the absence of Mg$^{2+}$. Note that in the absence of intracellular Mg$^{2+}$, ATP only interacts with the inhibitory site on Kir6.x and not with the NBDs of SUR (Nichols, 2006). Therefore, it appears that the binding affinity/efficacy of ATP at Kir6.2 was unaltered. Single channel analysis also indicates that the intrinsic $P_o$ of the channel was unaffected by the mutation.

Current data suggests that hydrolysis of MgATP at the nucleotide binding domains of SUR2 is required to induce channel activation (Matsuo et al., 2002; de Wet et al., 2010). To examine whether the mutation results in a conformational change that enables MgATP to act directly as a channel activator (without first hydrolyzing to MgADP), experiments were performed using the non-hydrolysable MgATP analogue, AMP-PNP. However, Kir6.2/SUR2B and Kir6.2/SUR2B-V734I channels were equally responsive to this analogue, suggesting MgATP hydrolysis to be necessary for channel stimulation.
The mutation may alter binding and/or hydrolysis of MgATP at SUR2. In the case of an alteration in binding affinity, it is possible that the mutation enhances the binding of MgATP at NBD1, promoting channel activation. It is possible that a greater occupancy of MgATP at SUR could antagonize binding of the more potent activators such as MgGDP in a competition dependent manner. This could explain the reluctance of Kir6.2/SUR2B-V734I channels to further activation (Fig. 4.4, B). A further possibility is that the mutation alters the rate of MgATP hydrolysis at SUR2. Remarkably, recent evidence by de Wet et al. (2010) suggest that a slowdown, rather than an increase, in the turnover rate of hydrolysis produces greater channel activation by SUR. In light of these recent findings, it could be envisioned that the mutation results in a reduced rate of MgATP hydrolysis, resulting in longer durations of MgADP bound states at SUR2B, increasing Po. Theoretically, this could account for the fact that Kir6.2/SUR2B-V734I channels were less sensitive to ATP inhibition in the presence of Mg2+. This could also have an antagonistic side effect on the binding and occupancy of the more powerful activating nucleotides, such as MgGDP. This could explain the reduced responsiveness observed for Kir6.2/SUR2B-V734I to further activation compared to the wild-type (Kir6.2/SUR2B) channel response, when 0.5 mM MgGDP was applied in combination with MgATP.

A final observation was that Kir6.2/SUR2B-V734I channels conveyed a 1.6 fold slower activation rate compared to Kir6.2/SUR2B channels when perfused with 0.5 mM MgGDP in the absence of MgATP. The peak current amplitudes in both cases were equivalent after 2 minutes of perfusion. The slowed response rate of Kir6.2/SURB-V734I channels to this nucleotide could be due to a delayed response of the signal transduction of MgGDP binding into channel opening.

6.10 Computational modelling and biophysical analysis of the SUR2B-V734I NBD dimer

A combination of homology modelling and molecular dynamics tools were employed to construct a ligand-docked structural model of the mutated SUR2B NBD complex. The V734I residue mapped to a region between the Wa and Wb motifs, at a distance of 20 Å, 15 Å and 27 Å from the Wa, Wb and SS, respectively. In agreement with previous research into ABC transporters, the MgATP coordinates via intermolecular electrostatic interactions into the Wa pocket of one NBD, where it is penned by the SS of the
adjacent NBD (Fig. 3.9, C). Research by Ramakrishnan et al. (2002) indicates that the lysine residue in the W\textsubscript{A} motif (GXXXXGK(T/S)) may provide stability to the bound MgATP molecule by binding to the phosphate oxygen atoms of the nucleotide. Glycine residues in the SS may serve an important role in the interaction of the receptor with MgATP. The side chain of glycine consists of only a single hydrogen atom, facilitating a close contact between the phosphates of MgATP and the framework of the SS (Karp, 2009). Glycine also has relatively high flexibility compared to other amino acids, enabling sections of the backbone to possess hinge-like properties (Karp, 2009).

The W\textsubscript{B} motif is suggested to serve a role in coordinating the ligand into the W\textsubscript{A} pocket (Campbell et al., 2003). The aspartate residue has been proposed to steer the MgATP molecule into the W\textsubscript{A} binding groove by a coordinated interaction with the Mg\textsuperscript{2+} ion (Gribble et al., 1997; Campbell et al., 2003). Furthermore, mutations in the W\textsubscript{B} aspartate residue have been shown to abrogate the stimulatory effects of MgADP (Gribble et al., 1997). Additional ABC transporter research (Karpowich et al., 2001; Verdon et al., 2003) implicate a role for the W\textsubscript{B} motif in the hydrolysis of MgATP, where mutations in W\textsubscript{B} decrease ATPase activity. The negativity of the glutamate may assist in guiding the attacking water molecule into a position where it can interact to hydrolyze MgATP (Karpowich et al., 2001; Verdon et al., 2003).

A case in point was a recent study by Park & Terzic (2010) which indicates that the mutated (V734I) residue might be situated in a vicinity where the NBD1 of neighbouring subunits interact. Recent findings have also demonstrated that various disease associated mutations in the K\textsubscript{ATP} channel are located at the interface between subunits (Ashcroft, 2005; Nichols, 2006). It is possible that V734I is situated in a region of interaction with the C42 amino acid chain of adjacent subunits, thereby altering binding and/or hydrolysis of nucleotides in SUR2B.

**6.11 Could nicorandil be used to treat patients with V734I?**

Nicorandil is an anti-anginal drug used in Europe and Japan (Reimann et al., 2001; Horinaka et al., 2010). This drug is a K\textsubscript{ATP} channel opener and an effective vasodilator of the coronary vessels (Suryapranata, 1993; Reimann et al., 2001). Furthermore, nicorandil has a limited effect on heart rate, blood pressure and cardiac contraction, and does not elicit a depressant effect on atrioventricular conduction compared to some Ca\textsuperscript{2+} channel blockers (Kinoshita & Sakai, 1990). A recent study (Reimann et al., 2001)
indicated that the binding site for nicorandil is located in the 17th transmembrane helix of SUR2 and that transmembrane helices 13-16 also participate in the transduction of drug binding into channel opening (Reimann et al., 2001). The observation that nicorandil only significantly augments channel activation in the presence of nucleotides could suggest that this drug acts through allosteric interaction, enhancing the signal transduction of SUR2-nucleotide binding into channel opening. Our experiments show that nicorandil potentiated activation of Kir6.2/SUR2B-V734I channels, therefore substituting for the loss of current observed in response to MgATP + 0.5 mM MgGDP. Application of both 30 and 300 µM nicorandil produced a substantial rightward shift to the concentration response. It should be considered that the standard administered doses of this drug into the blood stream are estimated to fall within the nanomolar range (Kinoshita & Sakai, 1990; Frydman, 1992; Suryapranata, 1993). Therefore, it could be extrapolated from these concentration responses that lower doses of nicorandil may restore activity of the mutant channel to normal levels at times when the cellular ATP/NDP ratio falls. In this way, the mechanism of action of nicorandil could be used to target the vascular K\textsubscript{ATP} channel variant (Kir6.2/SUR2B-V734I) for the treatment of spasm in patients carrying V734I.

### 6.12 Possible future investigations

To gain a more precise understanding into the functional impact of the V734I variant, human coronary samples containing the mutation would be required. Viral mediated gene transfer (VMGT) is a method which could be utilized to express the mutation in isolated human or rat arteries (Cable et al., 1999; Morishige et al., 2000; Fischer et al., 2002). However, in order to perform this technique, vessels need to be maintained in culture for several days to enable viral infection. Culturing of isolated vessels can exert multiple changes on the vessel, such as remodelling of the arterial wall and alterations in vessel function and gene expression (Thorne & Paul, 2003; Manoury et al., 2009). Alternatively, a transgenic mouse model could be generated to further examine the pathophysiological consequences of the mutation. Specifically, mice could be engineered to harbour V734I. This would enable electrocardiographic studies and analysis of vasospasm, as well as measurements of cardiovascular biomarkers, including ET-1. From a pharmacological point of view, the molecular homology model that has been generated could be used for computational virtual screening of a range of
small molecules. This search could potentially lead to the identification of molecules of novel chemical structure which interact with wild-type or mutant channels. To conclude, the study presented in this thesis should provide a solid base for achieving these important goals.
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A tiny channel and a large vessel: a new clue for heart attack

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Scientists at The University of Manchester and medical institutes in Italy have identified a gene variant that predisposes people to a special type of heart attack.

Their research, published in the International Journal of Cardiology could lead to the development of new drugs to treat the problem.

Dr Paolo Tammaro, who led the team, said: “Heart attacks happen when the blood supply to the heart is reduced by the narrowing or blocking of the coronary artery – the vessel that supplies the heart with oxygen and nutrients. Often this is due to fatty deposits which narrow the vessel.

However, in some people with perfectly clean arteries, the vessel suddenly constricts shutting off the blood supply. We have discovered that this process, known as vasospasm, can be associated with a rare variant of a particular gene.”

Dr Enzo Emanuele, from the University of Pavia, who screened the patients, said: “We knew that this type of heart attack occurs in about 6% of patients and that many of them have a genetic predisposition, but we did not know the gene responsible. Now that it is identified it will be possible to predict who is at risk and to treat them accordingly.”

The gene identified by the team encodes a protein termed KATP channel. This protein forms microscopic gated pores that allow potassium ions to move into and out of the cells, in this way giving rise to electrical impulses.

Dr Tammaro and research scientist Keith Smith, both based at the Faculty of Life Sciences at The University of Manchester, added: “These channels are abundant in the cells forming the wall of coronary arteries, and the electrical impulses they generate govern this artery’s diameter. Due to the mutation we have identified, the KATP channel in the coronary artery can no longer fulfill this delicate process.”

The team, whose work was supported by the BBSRC (Biotechnology and Biological Sciences Research Council), now plans to approach pharmaceutical companies with their findings, aiming to design novel drugs that could interact with this new target.