STUDIES OF THE MECHANISMS OF ACTION OF
NICORANDIL ON RAT AND PIG SMALL ARTERIES

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by

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Abstract

Studies of the mechanisms of action of nicorandil on rat and pig small arteries

Nicorandil is used clinically as an anti-anginal drug. It has been found to have both $K^+$ channel opening and nitrovasodilator actions. Here, wire myography, using a small vessel myograph, was used to study the mechanism of action of nicorandil on rat mesenteric and pig coronary small arteries. Further, rat mesenteric arteries were acutely dissociated to yield single smooth muscle cells for whole cell patch clamp recordings.

Concentration-dependent nicorandil relaxations of rat mesenteric arterial rings, measured in the presence of L-NAME to block NO synthesis, were inhibited either by the ATP-sensitive $K^+$ ($K_{ATP}$) channel inhibitor glibenclamide, or by the guanylyl cyclase inhibitor LY 83583. When applied together, glibenclamide and LY 83583 were more effective than either agents alone. Nicorandil was also less potent at inhibiting 60 mM $K^+$ contractions. These results are consistent with nicorandil causing relaxation both by activation of $K_{ATP}$ channels and of guanylyl cyclase in this preparation. In whole cell patch clamp experiments, nicorandil activated a $K^+$ current that was inhibited by glibenclamide, but unaffected by the inhibitor of large conductance calcium-activated $K^+$ (BK$_{Ca}$) channels iberiotoxin. Single channels activated by nicorandil, resolved in whole-cell recordings, had a conductance of 31 pS in symmetrical high $K^+$ solutions.

Although BK$_{Ca}$ channel activation has been reported to be involved in the vasorelaxant action of nitrovasodilators in some studies, iberiotoxin (IbTX) did not affect nicorandil-induced relaxations of the rat mesenteric artery, nor did it inhibit nicorandil-induced $K^+$ currents. Similarly, IbTX and charybdotoxin (ChTX) failed to inhibit relaxations produced by the pure nitrovasodilators glyceryl trinitrate and SNAP. Interestingly, however, in the absence of L-NAME, ChTX significantly inhibited SNAP dose response curves, implying the activation of BK$_{Ca}$ channels under these conditions. Thus, in the rat mesenteric arterial preparation used here, the activation of $K^+$ channels by SNAP appears to be dependent on endogenous NO production. In the absence of endothelial NO, the drug produced full relaxation via $K^+$ channel-independent mechanisms.

The $K_{ATP}$ channel opening ability of nicorandil was postulated to be potentiated by cellular stress because such channels are sensitive to the metabolic state of the cell. In rat mesenteric arteries, the potency of nicorandil was enhanced by metabolic inhibition with carbonyl cyanide m-chlorophenylhydrazone (CCCP) and 2-deoxyglucose. This effect was then studied more fully in pig coronary arteries where metabolic inhibition with CCCP or zero glucose solution, external acidosis, and adenosine receptor activation all increased the potency of nicorandil to cause vasorelaxation. These effects were abolished by glibenclamide or by high $[K^+]_o$. These findings are consistent with the procedures used enhancing the activity of $K_{ATP}$ channels to increase the vasorelaxant potency of nicorandil, and suggest that nicorandil may be more effective in ischaemic tissues.
Appendix 1
Abbreviations

AA arachidonic acid
ABC superfamily ATP-binding cassette superfamily
AC adenylyl cyclase
ACE angiotensin converting enzyme
ACh acetylcholine
A-D interface analogue to digital interface
Ang II angiotensin II
ANOVA analysis of variance
ANP atrial natriuretic peptide
4-AP 4-aminopyridine
BK bradykinin
BK_Ca channel large conductance calcium-activated K^+ channel
BQ 123 D-Asp-L-Pro-D-Val-L-leu-D-Trp
2-CA 2-chloroadenosine
cAMP cyclic 3', 5' adenosine monophosphate
CCCP carbonyl cyanide m-chlorophenylhydrazone
CFTR protein cystic fibrosis transmembrane regulator protein
cGMP cyclic 3', 5' guanosine monophosphate
CGRP calcitonin gene related peptide
CGS-21680 HCl 2-p-(2-carboxyethyl)pentylamino-5'-N-ethylcarboxamidoadenosine hydrochloride
ChTX charybotoxin
DAT digital audio tape
DAG diacylglycerol
DPCPX 8-cyclopentyl-1, 3,-dipropylxanthine
2-DG 2-deoxyglucose
EC_{50} effective concentration for 50 % relaxation
E_Cl Cl^- equilibrium potential
EDHF endothelium derived hyperpolarising factor
EDRF endothelium derived relaxant factor
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tbody>
<tr>
<td>EET</td>
<td>epoxyeicosotetraenoic acid</td>
</tr>
<tr>
<td>E&lt;sub&gt;Ca&lt;/sub&gt;</td>
<td>equilibrium potential for Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
</tr>
<tr>
<td>E&lt;sub&gt;K&lt;/sub&gt;</td>
<td>equilibrium potential for K&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>E&lt;sub&gt;Na&lt;/sub&gt;</td>
<td>equilibrium potential for Na&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>E&lt;sub&gt;Rev&lt;/sub&gt;</td>
<td>reversal potential</td>
</tr>
<tr>
<td>ET</td>
<td>endothelin</td>
</tr>
<tr>
<td>GTN (NTG)</td>
<td>glyceryl trinitrate</td>
</tr>
<tr>
<td>HDL</td>
<td>high density lipoprotein</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine (serotonin)</td>
</tr>
<tr>
<td>IbTX</td>
<td>iberiotoxin</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>effective concentration for 50 % inhibition</td>
</tr>
<tr>
<td>IP&lt;sub&gt;3&lt;/sub&gt;</td>
<td>inositol 1, 4, 5-trisphosphate</td>
</tr>
<tr>
<td>I-V relationship</td>
<td>current-voltage relationship</td>
</tr>
<tr>
<td>K&lt;sub&gt;ATP&lt;/sub&gt; channel</td>
<td>ATP-sensitive K&lt;sup&gt;+&lt;/sup&gt; channel</td>
</tr>
<tr>
<td>[K+]&lt;sub&gt;i&lt;/sub&gt;</td>
<td>intracellular K&lt;sup&gt;+&lt;/sup&gt; concentration</td>
</tr>
<tr>
<td>[K+]&lt;sub&gt;o&lt;/sub&gt;</td>
<td>extracellular K&lt;sup&gt;+&lt;/sup&gt; concentration</td>
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<td>Kir channel</td>
<td>inward rectifier K&lt;sup&gt;+&lt;/sup&gt; channel</td>
</tr>
<tr>
<td>Kv channel</td>
<td>delayed rectifier potassium channel</td>
</tr>
<tr>
<td>L-type Ca&lt;sup&gt;2+&lt;/sup&gt; channel</td>
<td>long lasting type Ca&lt;sup&gt;2+&lt;/sup&gt; channel</td>
</tr>
<tr>
<td>LAD coronary artery</td>
<td>left anterior descending coronary artery</td>
</tr>
<tr>
<td>LDL</td>
<td>low density lipoprotein</td>
</tr>
<tr>
<td>L-NAME</td>
<td>Nω-nitro-L-arginine methyl ester</td>
</tr>
<tr>
<td>LY 83583</td>
<td>6-anilino-5, 8-quinolinedione</td>
</tr>
<tr>
<td>MLCK</td>
<td>myosin light chain kinase</td>
</tr>
<tr>
<td>NA</td>
<td>noradrenaline</td>
</tr>
<tr>
<td>NANC transmission</td>
<td>non-adrenergic non-cholinergic transmission</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>NPY</td>
<td>neuropeptide Y</td>
</tr>
<tr>
<td>ODQ</td>
<td>1H-[1, 2, 4] oxadiazolo [4, 3-a] quinoxalin-1-one</td>
</tr>
<tr>
<td>pD&lt;sub&gt;2&lt;/sub&gt;</td>
<td>negative log&lt;sub&gt;10&lt;/sub&gt; of the EC&lt;sub&gt;50&lt;/sub&gt;</td>
</tr>
<tr>
<td>PAF</td>
<td>platelet activating factor</td>
</tr>
<tr>
<td>pGC</td>
<td>particulate guanylyl cyclase</td>
</tr>
</tbody>
</table>
PGI$_2$ prostaglandin I$_2$ (prostacyclin)

PIP$_2$ phosphotidyl-inositol-4, 5,-bisphosphate

PKA cAMP-dependent protein kinase

PKC protein kinase C

PKG cGMP-dependent protein kinase

PLA$_2$ phospholipase A$_2$

PLC phospholipase C

P$_o$ open probability

PSS physiological salt solution

S.E.M. standard error of the mean

sGC soluble guanylyl cyclase

SHR rats spontaneously hypertensive stroke prone rats

SIN-1 3-morpholinosydnnonimine

SK$_{Ca}$ channel small conductance calcium-activated K$^+$ channel

SNAP (±) S-nitroso-N-acetylpenicillamine

SNP sodium nitroprusside

SR sarcoplasmic reticulum

SubP substance P

SUR sulphonylurea subunit

T-type Ca$^{2+}$ channel transient type Ca$^{2+}$ channel

TEA$^+$ tetraethylammonium ions

TXA$_2$ thromboxane A$_2$

VIP vasoactive intestinal peptide

VLDL very low density lipoprotein

V$_M$ membrane potential

CHAPTER 1

Introduction
1.1 General Introduction

The research presented in this thesis involves the study of potassium channels, in particular ATP-sensitive K⁺ or K<sub>ATP</sub> channels in small arteries under normoxic and ischaemic-like conditions, and the responses of vasodilator drugs under these different conditions. The contractile state of smooth muscle in small arteries play a role in maintaining the calibre of the vessel and determining mean arterial blood pressure. The cellular mechanisms involved in this was better understood after the discovery of ion channels. The basis of excitability in nerves and non-nerve cells, such as cardiac and smooth muscle, was determined through the study of ion channels. It is now clear that channels selective for K⁺ ions are involved in the maintenance of blood pressure and flow through small vessels by controlling the contractility of vascular smooth muscle cells. One such channel is the K<sub>ATP</sub> channel, which is a K⁺ selective channel and is characterised by its sensitivity to intracellular ATP. K<sub>ATP</sub> channels were discovered less than twenty years ago, and were first described in vascular smooth muscle by Standen <i>et al</i>, in 1989. Since then extensive research has been carried out to define their physiological roles, and more recently their primary amino acid sequence.

I therefore aim to provide an introduction to the importance of the cardiovascular system, in particular the role of and control by endogenous agents of small arteries. I will also give an up to date review of the research carried out on vascular K⁺ channels, concentrating on K<sub>ATP</sub> channels. Due to the importance of these channels in small arteries, drugs that open them provide therapeutic potential for the treatment of vascular disorders.

1.2 The Vasculature

The purpose of the cardiovascular system is to supply oxygen and nutrients to, and remove waste products from all the regions of the body. This is achieved by the heart and a network of blood vessels. The cardiovascular system has been known about for hundreds of years. The existence of the heart was known to the Greeks, the word ‘cardiac’ was developed from the Greek word for the heart, ‘kardia’. The cardiovascular system is essentially made up of a pump, which is the heart, and a series of distributing and
collecting tubes, which make up the extensive vascular system of the body (Vander et al, 1990).

The concept that the cardiovascular system was a circular system, with two different circuits, was first demonstrated by the British physiologist William Harvey, in 1628. The heart consists of four chambers; two atria and two ventricles. The atria receive blood from the venous circulations. Blood is expelled from the ventricles into the arterial circulations. One vascular circuit consists of the right side of the heart and pulmonary circulation. This circuit brings oxygenated blood to the heart. The right atrium receives blood from around the body, via the vena cava. Blood enters the right ventricle from the right atrium, and is then pumped through the pulmonary circulation. The blood passes through the lungs where carbon dioxide is released in exchange for the uptake of oxygen by haemoglobin. The oxygenated blood re-enters the heart, via the pulmonary veins, into the left atrium. The second vascular circuit is the flow of blood round the body, originating from the left-hand side of the heart. The blood from the left atrium flows through into the ventricle where it is pumped out into the aorta. The aorta and arterial vascular system supplies blood to the rest of the body. Valves present in the heart ensure that a unidirectional flow occurs (Berne & Levy, 1990).

The blood in the aorta flows into ever decreasing sized vessels, from arteries to arterioles and then capillaries. The functional and structural characteristics of the blood vessels change with successive branching. Blood vessel walls are essentially made up of three main components; the intima, the media and the adventitia. The capillaries have only an intima which consists of a layer of endothelial cells. Other vessels have layers of elastic tissue and smooth muscle cells which make up the media. The adventitia is mainly composed of connective tissue. Figure 1.1 shows schematically the components that make up the arterial circulation (Vander et al, 1990).

The aorta has a large lumen and a predominately elastic structure. This allows the vessel to expand as blood is expelled from the ventricle during systole. In comparison, the arteries are more muscular and in the arterioles the smooth muscle layer predominates. The arteries carry oxygenated blood to all the organs of the body. Near to the tissues they supply, they branch out to form arterioles. These small vessels determine the mean arterial blood pressure and control the flow of blood to individual organs. Flow through the arterioles smoothes the pulsate flow that occurs in the arteries, leading to a constant flow of
blood through the capillaries (Vander et al., 1990). Since these arteries are important in controlling both total peripheral resistance and the regional distribution of blood flow they are a primary site of action for vasodilators and vasoconstrictors. The contractile state of the smooth muscle cells in the arteriolar wall determines the blood flow to a particular region of tissue. The smooth muscle cells can relax, causing vasodilatation of the vessel, or contract to cause vasoconstriction. The contractile state of these cells, and therefore the vessel diameter, is controlled by local and extrinsic factors.

**Figure 1.1** The schematic dimensions of the arterial system.

The arterial vessels are composed of layers of adventitia, media and intima.

Expanding out from the arterioles is a network of capillaries which permeate almost every tissue of the body. Typically, capillaries consist of a single endothelial layer which ensures their close proximity to the cells of the tissue. The capillary wall is highly permeable to water and almost all plasma solutes. There are four basic mechanisms by which substances move across the capillary wall; diffusion, vesicular transport, bulk flow and carrier-mediated transport which occurs only in the brain. In most capillaries diffusion is the main mechanism by which movement of nutrients, oxygen and metabolic-end
products occurs across the capillary wall. Exchange of substances by diffusion is also aided by filtration and reabsorption. In contrast, ions and polar molecules which are poorly soluble in lipid pass across by vesicular transport. A hydrostatic pressure exists between the plasma, in the capillary, and the interstitial fluid, surrounding the tissue. This pressure, usually about 35 mmHg at the arterial end, leads to the bulk flow of plasma and soluble substances across the capillary wall into the interstitial fluid. Since the capillary offers no resistance to flow this hydrostatic pressure decreases to approximately 15 mmHg at the venous end of the capillary. The filtration of plasma out of the capillary causes a build up of insoluble proteins in its lumen. This generates an osmotic pressure of about 28 mmHg along the capillary. At the arterial end the higher hydrostatic pressure (~35 mmHg) encourages the filtration of plasma out of the capillary, however at the venous end the osmotic pressure is greater (~28 mmHg) which favours the reabsorption of fluid into the capillary. Control of filtration and reabsorption in capillaries is important for maintaining blood volume levels. Physiological regulation is mainly due to changes in diameter of the small arteries and arterioles. Dilatation of these vessels would increase hydrostatic pressure therefore promoting filtration. Vasoconstriction would decrease capillary pressure, hence there would be a net movement of fluid into the capillaries (Vander et al., 1990). From the capillaries, the blood flows into venules and then veins, to be transported back to the heart.

Veins and venules have large lumens surrounded by some elastic tissue and smooth muscle cells making them much thinner walled, compared to arteries. The composition of the vessels in the venous circulation is shown diagramatically in figure 1.2. The peripheral veins contain valves to ensure that flow of blood is towards the heart.

**Figure 1.2** Diagrammatic compositions of the vessels of the venous system.

EC, endothelial cell; SMCs, smooth muscle cells

![Diagram showing the composition of vessels in the venous system.](image-url)
1.2.1. *Coronary circulation.*

The heart is characterised by its high metabolic demands and oxygen consumption, and so the coronary circulation offers an highly efficient blood supply to this organ. Even though it only makes up about 4% of the total blood distributed to the major organs, coronary flow guarantees that the cardiac muscle is supplied with sufficient nutrients and oxygen. The coronary vascular bed consists of large arterial vessels, small resistance vessels, capillaries and veins. Like other circulations, the coronary vascular resistance is mainly controlled by the arterioles (Daut *et al*, 1994a). The vascular resistance can be altered by a variety of physiological and pharmacological stimuli. The different vessels respond in a heterogeneous manner to different stimuli. Under normal conditions, up to 50% of the vascular resistance is provided by the arterioles. Autoregulatory control of coronary vessels is mainly achieved in the smaller resistance vessels, those with a diameter of less than 100 μM. The larger resistance vessels, those with diameters of 100 μM or more, are under the regulation of humoral or neuronal factors (Marcus *et al*, 1990). This difference in responses allows the coronary circulation to finely control vascular resistance and precisely regulate perfusion of oxygen and nutrient delivery to the myocardium.

1.3 Resistance Vessels

Resistance vessels are composed of small arteries and arterioles, and are the major site for the maintenance of peripheral resistance. The tone of the smooth muscle cells in these vessels determines the arterial diameter and is controlled by neuronal and humoral (extrinsic) factors. They exist in a partially contracted state from which they can constrict further or dilate depending on the demand of blood of a particular organ. This partially constricted state of an artery has been termed myogenic tone by Bayliss in 1902. The myogenic tone is under the control of local autoregulation, in response to factors such as the pressure and the flow of blood.

The resistance vessels play a role in the regulation of tissue perfusion. In the coronary circulation there is a close link between the metabolic requirements of the heart and the magnitude of coronary blood flow (Daut *et al*, 1994b). The blood vessels are mainly controlled by local factors such as those released from the endothelium. The role of both extrinsic and local controls will be discussed below.
1.3.1. *Extrinsic controls.*

The control of resistance vessels by extrinsic factors is by innervation and circulating hormones. Neuronal control occurs via adrenergic, cholinergic and non-cholinergic non-adrenergic (NANC) such as calcitonin gene related peptide (CGRP), purinergic and substance P (Sub P)-mediated transmissions. Humoral control includes 5-hydroxytryptamine (5-HT, serotonin), adenosine, bradykinin (BK), vasopressin and angiotensin II (Ang II).

Resistance vessels receive a rich supply of sympathetic post ganglionic nerve fibres. These neurones release noradrenaline (NA) which produces vasoconstriction via $\alpha$-adrenoreceptor stimulation. Smooth muscle cells of blood vessels possess many $\alpha_1$-adrenoreceptors. However some vasoconstriction is mediated through stimulation of $\alpha_2$-adrenoreceptors which are also present on the cell membrane (Hirst & Neild, 1980). Briefly, $\alpha$-adrenoreceptors are coupled to phospholipase C (PLC) which causes an increase in inositol 1, 4, 5-trisphosphate ($IP_3$) production and release of $Ca^{2+}$ from intracellular stores (reviewed in Summers & McMartin, 1993). Hashimoto *et al* (1986) demonstrated the activation of PLC by NA in the rabbit mesenteric artery. This would therefore initiate contraction and ultimately constriction of the vessels. Vascular smooth muscle also possess $\beta$-adrenoreceptors. In contrast to $\alpha$-adrenoreceptors their activation by NA leads to vasodilatation. Stimulation of $\beta$-adrenoreceptors causes vasodilatation in a number of vascular preparations, including dog coronary (Narashigie *et al*, 1994) and rat basilar arteries (Kitazono *et al*, 1993).

Activation of cholinergic receptors *in vivo* by either vagal stimulation or acetylcholine (ACh) infusion produces vasodilatation. This ACh-mediated vasorelaxation is endothelium dependent (Vanhouette, 1989). ACh acts via muscarinic receptors present on endothelial cells to result in the production of nitric oxide (NO) which then freely diffuses across the cell membrane into the smooth muscle cells (Furchgott & Zawadzki, 1980). NO then causes relaxation via multiple mechanisms in the cell. $M_3$-cholinergic receptors are also present on smooth muscle cells, and their stimulation by ACh results in vasoconstriction (reviewed in Coulquhoun *et al*, 1987). This effect has been shown to be coupled through PLC leading to the production of $IP_3$ and diacylglycerol (DAG) (Bonev & Nelson, 1993a).
ATP is present as a primary mediator (Evans et al, 1992) or as a co-transmitter (Burnstock, 1990; von Kugelgen & Starke, 1991) in the peripheral nervous system. The vasoconstrictor effect of ATP is due to the stimulation of P2X-purinergic receptors (Ralevic & Burnstock, 1988). Recent evidence suggests that the P2X-purinergic receptor is a non-selective cation channel, and so vasoconstriction is caused by increased Ca^{2+} entry (Bean, 1992). ATP can also act through P2Y-purinergic receptors which are coupled to cAMP generation (reviewed in Burnstock, 1989).

Substance P (Sub P) containing terminals are abundant in the walls of many blood vessels. The vasculature shows a mixture of vasodilatation and vasoconstriction in response to Sub P. The actions of Sub P are mediated by neurokinin receptors. Vasodilatation is endothelial-dependent, whereas neurokinin receptors on smooth muscle cells mediate constriction (reviewed in Maggio, 1988).

Calcitonin gene related peptide (CGRP) is also involved in NANC transmission. CGRP is a 37 amino acid peptide transmitter contained in NANC sensory nerve endings in the vasculature (Holzer & Lippe, 1992). The peptide is thought to be involved in autoregulation, being released in response to mechanical stress on the vessel wall (Hong et al, 1994). CGRP causes vasodilatation in a number of vascular preparations including; rabbit mesenteric artery (Nelson et al, 1990a), rabbit ophthalmic artery (Zschauer et al, 1992), human uterine arteries (Nelson et al, 1993), rat cerebral vessels in vivo (Hong et al, 1994) and pig coronary arteries (Miyoshi & Nakaya, 1995).

Other extrinsic controls include circulating substances which act on blood vessels, either directly on the vascular smooth muscle cells or via the production of endothelium-derived factors. The main humoral factors discussed here are 5-HT, BK, vasopressin and Ang II.

5-HT has an important role as a neurotransmitter as well as functioning as a hormone in the vascular system. The mediator has several effects on the vasculature, depending on the size of the vessels. In large vessels, 5-HT predominately causes vasoconstriction, mediated through 5-HT_{2a} receptors present on vascular smooth muscle (Bonev & Nelson, 1996). The vasodilator effect of 5-HT on resistance vessels is mediated through 5-HT_{1} receptors which is seen after inhibition of the vasoconstrictor effect. Several different mechanisms are thought to be employed, such as endothelial release of NO to cause vasodilatation and inhibition of NA release from sympathetic nerve terminals.
(van Neuten et al, 1981; Houston & Vanhoutte, 1988). As well as causing vasodilatation of these arteries, 5-HT brings about constriction of venules therefore increasing filtration of fluids in the capillaries (reviewed in Houston & Vanhoutte, 1986).

Adenosine acts as a vasodilator in many vascular beds. The vascular effects of adenosine have mainly been attributed to stimulation of A₂-adenosine receptors (reviewed in Olsson & Pearson, 1990; Kleppisch & Nelson, 1995). However, some research suggests that actions may also be mediated via the A₁-adenosine receptor subtype (Merkel et al, 1992). The type of receptor that adenosine activates appears to be both tissue- and species-dependent. A₂-receptor mediated actions of adenosine occur via the stimulation of adenylyl cyclase (AC) which leads to an increase in the second messenger, cAMP (reviewed in Fredholm et al, 1994).

BK is a plasma derived vasoactive peptide formed by the action of enzymes on protein substrates called kininogens. It causes vasodilatation in numerous vascular beds via an endothelium-dependent process. Its vasodilator effect is due to its action on receptors on endothelial cells, leading to the stimulation of phospholipase A₂ (PLA₂) and the subsequent generation of prostaglandin I₂ (prostacyclin, PGI₂) (reviewed in Rang et al, 1992).

Vasopressin is a posterior pituitary peptide hormone whose primary action is in the kidney. However, the peptide is powerful vasoconstrictor (Rang et al, 1992). Vasopressin, acting via V₁ receptors on smooth muscle cells, ultimately causes constriction through increasing intracellular Ca²⁺ and possibly inhibition of K⁺ channels (Wakatsuki et al, 1992).

Renin is released into the blood from specialised cells in the kidney. It acts on a plasma globulin, termed angiotensinogen, which splits a decapeptide from its N-terminus forming angiotensin I. Angiotensin I is acted on by second proteolytic enzyme, angiotensin converting enzyme (ACE). This enzyme removes two further amino acids resulting in the formation of the active metabolite Ang II (Rang et al, 1992). Ang II has been shown to act as a powerful vasoconstrictor. ACE is present at many sites in the vascular system including the heart, brain, skeletal muscle and kidney, therefore the local formation of Ang II provides localised control of blood flow (reviewed in Valloton, 1987).
1.3.2. *Local controls.*

Myogenic tone, achieved by self-regulation or autoregulation of the vessels is an intrinsic property of arterial smooth muscle. The myogenic tone is related to the circumferential tension of the arterial wall rather than muscle length (Johnson, 1986). An increase in pressure usually induces a decrease in the diameter of arterioles, which is dependent on external Ca\(^{2+}\) (reviewed in Daut *et al.*, 1994b). If this positive feedback loop between pressure and vasoconstriction continued unchecked, an unstable situation would arise. However, a change in membrane potential is found to precede the change in vascular tone (Harder, 1984). The increased wall tension leads to membrane depolarisation and the opening of voltage operated Ca\(^{2+}\) channels resulting in vasoconstriction. The increase in cellular Ca\(^{2+}\) and change in membrane potential opens large conductance Ca\(^{2+}\)-activated K\(^{+}\) (BK\(_{Ca}\)) channels which initiates hyperpolarisation and acts as a negative feedback loop to limit depolarisation. Figure 1.3. is a flow diagram showing how pressure induced vasoconstriction leads to a stable myogenic tone (Daut *et al.*, 1994b).
1.3.3. Importance of the endothelium.

Endothelial cells are more than just a physical barrier between the interstitial and plasma fluids. The layer of endothelial cells have a large number of functions, summarised in table 1.1. Only about 20 years ago was the layer of endothelial cells, present in the whole of the vascular system, shown to play an active role in the control of the calibre of the vessel. The first vasoactive substance shown to be released by endothelial cells was PGI₂. Since then the endothelium has been shown to produce other factors, importantly endothelium derived relaxant factor (EDRF, NO), endothelin and endothelium derived hyperpolarising factor (EDHF) (Rang et al, 1992).
Table 1.1. Important roles of endothelial cells.

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<table>
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<tr>
<td>1</td>
<td>Serve as a physical lining in the heart and blood vessels</td>
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<tr>
<td>2</td>
<td>Secrete endothelium derived factors which mediate responses in vascular smooth muscle cells</td>
</tr>
<tr>
<td>3</td>
<td>Secrete substances that promote angiogenesis</td>
</tr>
<tr>
<td>4</td>
<td>Regulate the transport of macromolecules and other substances between the plasma and the interstitial fluid</td>
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<tr>
<td>5</td>
<td>Regulate platelet activity</td>
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<tr>
<td>6</td>
<td>Synthesise active hormones from inactive precursors</td>
</tr>
<tr>
<td>7</td>
<td>Extract or degrade hormones and mediators</td>
</tr>
<tr>
<td>8</td>
<td>Undergo contractile activity to regulate capillary permeability</td>
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Prostaglandins are a member of the eicosanoid family which are formed de novo from phospholipids. The main source of prostaglandins is arachidonic acid (AA). Many different stimuli, such as cell damage, cause the liberation of AA from its esterified form in the membrane phospholipids. The free AA is then metabolised by the enzyme cyclooxygenase. The constitutive form of the enzyme is bound to the endoplasmic reticulum of endothelial cells. Cyclooxygenase acts on AA leading to the formation of prostacyclin (PGI₂), prostaglandins G₂, H₂ and E₂ (reviewed in Salmon & Higgs, 1994; Coleman, 1994). Prostacyclin is the main prostaglandin synthesised in the endothelium. It acts via IP-receptors on vascular smooth muscle cells to cause vasorelaxation. Vasodilatation is brought about via stimulation of AC and increases in cAMP (Holzmann et al, 1980).

Another vasodilator produced by the endothelial cells is nitric oxide (NO) which has a very short half-life. Since its discovery in 1980, it has proved to be an important mediator of vascular tone. The substrates for NO are L-arginine and oxygen. NO is derived from these substrates by the enzyme nitric oxide synthase (reviewed in Moncada et al, 1991). The constitutive form of the enzyme is present under physiological conditions and is controlled by cellular Ca²⁺ (Knowles et al, 1989). Local hormones such as BK and
Sub P act on endothelial cells to increase the production of NO (Palmer et al, 1987). NO diffuses across the membranes into the muscle layer where it activates soluble guanylyl cyclase (sGC), leading to the formation of cyclic 3', 5' guanosine monophosphate (cGMP). This second messenger acts via multiple mechanisms to cause vasorelaxation (reviewed in Lincoln, 1989). Activation of GC is probably the primary mechanism whereby NO induces relaxation of vascular smooth muscle, however other proteins could also be involved. In the rabbit aorta, NO-induced relaxations were resistant to inhibition by methylene blue, a blocker of GC (Bolotina et al, 1994). Proposed target proteins for NO include K+ channels (Bolotina et al, 1994; Murphy & Brayden, 1995a) and the sarcoplasmic reticulum Ca2+ ATPase pump (Popescu et al, 1985).

In contrast to the vasodilators prostacyclin and NO, endothelial cells also produce endothelin (ET) (reviewed in Rubanyi & Polokoff, 1994). Endothelin is a potent, long-lasting vasoconstrictor peptide. The 21 residue peptide was discovered by Yanagisawa and colleagues in 1988. The active peptide is cleaved from a precursor protein, termed big ET, by an endothelin converting enzyme. Once synthesised, ET acts in a paracrine manner on the smooth muscle cells (Rang et al, 1992). Miyoshi et al (1992) showed that ET decreased the excitable nature of arterial smooth muscle cells by causing depolarisation. Two types of ET receptors have thus far been cloned and sequenced, designated ET_A and ET_B. Vasoconstriction is coronary arteries is thought to be mostly mediated by ET_A receptors because endothelin-induced contractions were reversed by D-Asp-L-Pro-D-Val-L-leu-D-Trp (BQ 123), a selective ET_A receptor antagonist (Pernow & Modin, 1993).

EDHF is, as yet, an unknown diffusible agent released by the endothelium in response to vasodilator drugs or shear stress-induced increases in cellular Ca2+. The exact nature of the substance is unknown but it has been shown to differ from either NO or prostacyclin (reviewed in Mombouli & Vanhoutte, 1997). The most likely candidates, thus far, for EDHF are epoxyeicosotetraenoic acids (EETs) and endocannabinoids. Bioassay for the identification of EDHF has proven to be difficult due to its short half life and the hydrophobicity of its derivatives. EETs are metabolites of the arachidonic acid pathway, and are produced by the cytochrome P_450 monooxogenase pathway. Inhibitors of the P_450 pathway blocked EET-induced relaxations in pig and bovine coronary arteries (Graier et al, 1996). However these blockers are non-specific and have been shown to inhibit K+ channels directly (Yuan et al, 1995). Another possible candidate for EDHF is anandamide,
the endogenous cannabinoid receptor agonist. In rats, application of anandamide resulted in hypotension (Varga et al, 1996). In the isolated rat mesenteric artery, cannabinoid receptor antagonists inhibited endothelial dependent relaxations to carbachol even in the presence of NOS and P450 inhibitors. Subsequently this effect was blocked by increasing external K+ concentrations and therefore preventing hyperpolarisation (Randall et al, 1996).

1.4 Control of Contraction in Vascular Smooth Muscle

The contraction of vascular smooth muscle is controlled by neurotransmitters, hormones and local factors released from the endothelial cells. As in other types of muscle, intracellular free Ca2+ is the central second messenger in smooth muscle contraction. Strict regulation is essential and is reflected by the submicromolar concentrations of Ca2+ present in the cytosol, compared to 1-2 mM Ca2+ externally, which have been measured using various Ca2+-sensitive indicators (Horowitz et al, 1996). At rest, Ca2+ levels in the cell has been estimated to be 50-150 nM depending on the preparation and indicator used. In response to many different neuronal or humoral stimuli, intracellular Ca2+ levels rise to approximately 600-800 nM. This increase does not seem very large but is sufficient to initiate contraction and reflects the efficiency of the numerous feedback mechanisms present to prevent excessive increases (Williams & Fay, 1986). The rise in intracellular Ca2+ to initiate contraction comes from two sources; intracellular stores of calcium in the sarcoplasmic reticulum (SR) and extracellular calcium. The release of intracellular Ca2+ is coupled to the binding of second messengers to specific SR membrane receptors. The increase in cellular Ca2+ levels in response to vasoactive agents has been shown to be biphasic. The initial increase in Ca2+ is thought to be due to the release of Ca2+ from intracellular stores. A smaller, more sustained phase is believed to be due Ca2+ entry across the cell membrane (McCarron et al, 1992). The plasma membrane has both voltage-operated and receptor-operated Ca2+ channels which allow an influx of external Ca2+ upon activation.

Relaxation is accompanied by a decrease in intracellular Ca2+ levels. Calcium can be removed from the cell by the plasma membrane Ca2+ ATPase or the Na+/Ca2+ exchanger. Calcium can also be taken up into the SR via the membrane-bound Ca2+
ATPase. Specific Ca$^{2+}$ binding molecules exist in the cytoplasm and SR to act as cell buffers to maintain cytosolic Ca$^{2+}$ levels low (reviewed in Kuriyama et al, 1995).

I will first outline the mechanics of contraction in vascular smooth muscle. Then the Ca$^{2+}$ transport processes which contribute to increasing and decreasing cytosolic Ca$^{2+}$ will be described in further detail.

1.4.1. Contractile machinery in vascular smooth muscle.

Smooth muscle differs from skeletal and cardiac muscle in that it contains no troponin. In cardiac and skeletal muscle, the troponin complex inhibits the interaction between actin and myosin filaments. The binding of Ca$^{2+}$ to troponin causes a conformational change which allows the myosin to bind to actin filaments, thus initiating contraction (Vander et al, 1990). In smooth muscle, Ca$^{2+}$ controls the cross-bridge activity by regulating the enzymatic phosphorylation of myosin. Cellular Ca$^{2+}$ interacts with calmodulin, a Ca$^{2+}$ binding protein. Four Ca$^{2+}$ ions bind to one calmodulin molecule. The Ca$^{2+}$-calmodulin complex binds to a protein kinase, termed myosin light chain kinase (MLCK) thereby activating it. MLCK then uses ATP to phosphorylate myosin. The phosphorylated myosin is then able to bind to actin and undergo cross-bridge cycling, see figure 1.4. (Horowitz et al, 1996). Therefore in smooth muscle cross-bridge activity is turned on by Ca$^{2+}$-mediated changes in myosin. In contrast, in striated muscle Ca$^{2+}$ mediates changes in the thin (actin) filament (Allen & Walsh, 1994). MLCK in smooth muscle provides an important site for the regulation of contraction. Other regulatory proteins, termed calponin and caldesmon, are associated with the actin filaments (Kuriyama et al, 1995). They binds to actin filaments, thus preventing their interaction with myosin. These regulatory proteins are involved in Ca$^{2+}$-independent contraction mechanisms (section 1.4.2). The summary diagram in figure 1.4 shows the mechanism of contraction in vascular smooth muscle.

Relaxation is brought about by dephosphorylation of the myosin filaments by a phosphatase called myosin light chain phosphatase. The phosphatase is found to be bound tightly with myosin, suggesting that it is targeted for its substrate (Horowitz et al, 1995). Second cellular messengers are also able to regulate contraction and relaxation, for example cAMP through activation of cAMP dependent protein kinase (PKA) is able to phosphorylate MLCK thus decreasing its affinity for the Ca$^{2+}$-calmodulin complex (Nishikawa et al, 1984). Relaxation can also be brought about by cGMP which acts via
cGMP dependent protein kinase (PKG) to decrease cytosolic Ca\(^{2+}\) levels. PKG phosphorylates target proteins such as the smooth muscle membrane Ca\(^{2+}\) ATPase pump (Yoshida et al., 1991) therefore increasing Ca\(^{2+}\) extrusion from the cell. Protein elements important in transporting Ca\(^{2+}\) in and out of the cytosol are discussed now.

**Figure 1.4. Schematic arrangement of the contraction of smooth muscle and the role played by Ca\(^{2+}\).**

The Ca\(^{2+}\)-calmodulin complex regulates the myosin through MLCK. Calponin is a regulatory protein on the actin filaments.

CaM, calmodulin; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; PKC, protein kinase C

1.4.2. **Ca\(^{2+}\) independent contraction.**

Changes in intracellular Ca\(^{2+}\) are generally accepted as the physiological regulator of smooth muscle contraction. However, there is growing evidence of Ca\(^{2+}\)-independent
activation of the contractile system. Contraction is seen in vascular smooth muscle in the absence of intracellular Ca\(^{2+}\) (Singer, 1996).

The main mediator of this alternative pathway is PKC. The kinase is activated by the endogenous lipid DAG. Insights into the role of PKC in force production have been inferred from responses to PKC activators, such as phorbol esters, and with the use of putative PKC inhibitors. A role for PKC in regulating smooth muscle contraction was proposed over ten years ago (Rasmussen et al., 1984). Since then contractile responses to PKC, using phorbol esters, have been implicated in a variety of vascular smooth muscle preparations including dog basilar (Baraban et al., 1985), pig carotid (Singer, 1990) and rabbit renal and femoral (Ratz, 1990) arteries. Direct evidence for this PKC regulatory pathway has been demonstrated by the ability of physiological stimuli, such as Ang II, ET and NA, to increase DAG and PKC levels in smooth muscle (Lee & Severson, 1994; Rokolya et al., 1994). In addition, immunoblotting techniques have been used to demonstrate the activation of PKC isozymes by contractile stimuli (Singer, 1996).

The mechanism of PKC-induced activation of arterial smooth muscle, independent of the Ca\(^{2+}\)-CaM myosin phosphorylation, is thought to be through the actin-associated regulatory proteins, calponin and caldesmon. PKC is able to phosphorylate calponin and release it from the thin filaments (Winder & Walsh, 1993), therefore allowing actin-myosin interaction. Inhibition of contraction can be seen with the application of recombinant calponin which is resistant to phosphorylation by PKC. Contraction can also be inhibited by the addition of a PKC blocker, the application then of exogenous PKC results in contraction (Naka et al., 1990). Similarly, caldesmon has been shown to be phosphorylated by phorbol esters in vitro. However, in vivo the protein is thought to be phosphorylated by a mitogen-activated protein (MAP) kinase. MAP-kinases are a family of serine/threonine protein kinases involved in a signalling cascade leading to a number of different physiological response including smooth muscle contraction. MAP kinase isoforms have been identified in vascular smooth muscle, and their activity has been shown to be increased by vasoconstrictor agents (Singer, 1996). Structural studies show that caldesmon has two phosphorylation sites where MAP kinase is thought to act. Phosphorylation of the regulatory protein results in its dissociation from actin and ultimately contraction. PKC is further able to control this MAP kinase signalling pathway by regulating the activity of the protein kinase (Khalil & Morgan, 1993).
1.4.3. **Ca\(^2+\) entry across the cell membrane.**

Ca\(^2+\) entry across the cell membrane occurs through voltage operated Ca\(^2+\) channels which are sensitive to changes in the membrane potential (see section 1.5). Another Ca\(^2+\)-influx pathway is activated by depletion of intracellular Ca\(^2+\) stores. Ca\(^2+\) influx can also occur through agonists acting on non-selective cation channels (reviewed in Horowitz *et al.*, 1996; Missiaen *et al.*, 1992). The characterisation of Ca\(^2+\) channels in vascular smooth muscle cells has proven to be difficult due to the need for isolation of viable cells for electrophysiological recordings and because of the heterogeneity of smooth muscle cells observed.

Of the currently recognised voltage operated Ca\(^2+\) selective channel subtypes, only transient (T)- and long lasting (L)-type Ca\(^2+\) channels are found to be present in vascular smooth muscle (reviewed in McDonald *et al.*, 1994). The L-type is thus far the more studied of the two channels. The major regulatory factor of these channels is voltage. Changes in voltage or membrane potential alter the cell's excitability. Excitability of a smooth muscle cell is increased by depolarisation, i.e. when the membrane potential is made more positive. Depolarisation of the cell leads to activation of voltage operated Ca\(^2+\) channels, whilst making the membrane potential negative promotes deactivation of channels. The channels start to activate or open significantly at about -40 mV and the majority of channels are open by 0 mV. The channel is very voltage dependent. The proportion of the time a channel is open, or its open probability (P\(_o\)), increases steeply with membrane depolarisation (Nelson *et al.*, 1990\(^a\)). Intracellular Ca\(^2+\) itself also regulates the channels. Calcium in the cytosol reversibly inhibits L-type mediated Ca\(^2+\) influx, and this has been demonstrated in the rabbit portal vein (Ohya *et al.*, 1988). In some smooth muscle, DAG through the activation of PKC also stimulates a Ca\(^2+\) current (Vivaudou *et al.*, 1988). Similarly, phorbol esters, activators of PKC, can also increase L-type channel-mediated Ca\(^2+\) flux (Litten *et al.*, 1987), suggesting that phosphorylation may also play a regulatory role.

Voltage independent, receptor operated cationic channels (see figure 1.5) have also been identified in smooth muscle. One such example is the P\(_{2X}\) purinergic receptor operated channel which is activated by ATP (Benham & Tsien, 1987). The channel is a non-selective cation channel, it is permeable to Ca\(^2+\), Na\(^+\) and K\(^+\) (Bean, 1992). Ca\(^2+\) is highly permeant through the P\(_{2X}\) channel, showing 3:1 selectivity of Ca\(^2+\) over Na\(^+\) at near
physiological concentrations (Benham & Tsien, 1987). The resulting depolarisation would cause the activation of voltage operated Ca\(^{2+}\) channels.

1.4.4. Ca\(^{2+}\) extrusion across the cell membrane.

There is a large difference in Ca\(^{2+}\) concentration across the cell membrane which leads to a continuous leak of Ca\(^{2+}\) into the cell. The plasma membrane Ca\(^{2+}\) ATPase pump and the Na\(^{+}\)/Ca\(^{2+}\) exchanger adjust their activity accordingly to maintain cytosolic Ca\(^{2+}\) levels low, see figure 1.5 (reviewed in Horowitz et al, 1996; Missiaen et al, 1992).

The cardiac Na\(^{+}\)/Ca\(^{2+}\) exchanger has been purified, sequenced and cloned (Nicoll & Philipson, 1991). In contrast, information about the Na\(^{+}\)/Ca\(^{2+}\) exchanger in smooth muscle is limited. The vascular smooth muscle exchanger is thought to be similar to the Na\(^{+}\)/Ca\(^{2+}\) exchanger present on cardiomyocytes. Investigators have predicted the exchanger to have a stoichiometry of 3Na\(^{+}\): 1Ca\(^{2+}\), and Ca\(^{2+}\) can be transported outward or inward (McCarron et al, 1994). Regulation of the Na\(^{+}\)/Ca\(^{2+}\) exchanger is believed to be through cGMP and activation of PKG. Recent evidence has suggested that the exchanger is also modulated by phosphotidyl-inositol-4, 5-bisphosphate (PIP\(_2\)), a cellular messenger. PIP\(_2\) activates the exchanger by binding to a domain on its intracellular surface. Molecular studies have predicted that the amino acid residues in this intracellular region of the exchanger play a role in its inactivation, and the binding of PIP\(_2\) inhibits this autoregulation (reviewed in O'Donnell & Owen, 1994).

The second mechanism of Ca\(^{2+}\) extrusion across the smooth muscle cell membrane is the Ca\(^{2+}\) ATPase pump (figure 1.5). Its activity varies considerably with changing cytosolic Ca\(^{2+}\). The Ca\(^{2+}\) pump is potentially electrogenic, exchanging Ca\(^{2+}\) for H\(^{+}\) (Furukawa et al, 1989). Agonists that cause Ca\(^{2+}\) mobilisation, such as carbachol, inhibit the pump. This inhibition is only seen in intact cells, suggesting that intracellular components are essential for the block (Missiaen et al, 1988). Activators of the Ca\(^{2+}\) ATPase pump include calmodulin which increases the affinity for Ca\(^{2+}\) and the maximal rate of pumping (Raeymaekers & Wuytack, 1993). The Ca\(^{2+}\) pump is also regulated by various protein kinases, such as PKG which activates it. However this activation may be indirect, through the phosphorylation of an intermediate protein (Vrolix et al, 1988; Yoshida et al, 1991).
1.4.5. Ca$^{2+}$ release from intracellular stores.

The sarcoplasmic reticulum (SR) acts as the major internal store for Ca$^{2+}$ in smooth muscle cells. Two Ca$^{2+}$ release channels have been classified in the SR of smooth muscle; the inositol 1, 4, 5-trisphosphate (IP$_3$) receptor and the ryanodine receptor, see figure 1.5 (reviewed in Missiaen et al, 1992; Pozzan et al, 1994).

Using caged IP$_3$-compounds, it has been shown that IP$_3$ releases Ca$^{2+}$ in smooth muscle cells (Walker et al, 1987). Berridge (1989) then showed that injection of IP$_3$ into Xenopus oocytes induced internal mobilisation of Ca$^{2+}$. Repetitive injections led to desensitisation, probably due to depletion of internal stores. IP$_3$ is a soluble product of PLC activity, typically generated upon G-protein coupled receptor stimulation (Berridge, 1993). The second messenger mobilises Ca$^{2+}$ from internal stores via a receptor-specific mediated mechanism (Berridge & Irvine, 1989). The smooth muscle IP$_3$ receptor is made up of four similar subunits with a Ca$^{2+}$ selective channel running down the middle. Cloning revealed that various isoforms of the receptor existed. IP$_3$ receptors are formed from at least four different genes which gives rise to structural diversity and to different functional properties (reviewed in Mikoshiba et al, 1993; Sienaert et al, 1996). As well as their functional differences, the IP$_3$ receptors are also influenced by phosphorylation by PKA (Nahorski et al, 1994) and by cytosolic Ca$^{2+}$. At low internal Ca$^{2+}$ concentrations, up to 300 nM, Ca$^{2+}$ acts in a positive manner to stimulate the receptor to increase release of Ca$^{2+}$. However, at higher Ca$^{2+}$ levels, a negative feedback loop operates to inhibit Ca$^{2+}$ release (lino & Tsukioka, 1994). This negative feedback mechanism limits excessive Ca$^{2+}$ accumulation in the cytoplasm.

Ryanodine was traditionally used to characterise Ca$^{2+}$ release from the SR in skeletal muscle. However, it has been shown to affect the contractile behaviour of a number of smooth muscle preparations, suggesting the presence of ryanodine receptors (Gerthoffer et al, 1988; Bourreau et al, 1991). Indeed, [$^3$H] ryanodine was used to label Ca$^{2+}$ release channels in smooth muscle (Zhang et al, 1993). The ryanodine receptor is a Ca$^{2+}$ release channel that becomes activated when cytosolic Ca$^{2+}$ levels increase, giving rise to a phenomenon known as “Ca$^{2+}$-induced Ca$^{2+}$-release” (Callewaett, 1992). It has been proposed that Ca$^{2+}$ influx across the plasma membrane opens SR ryanodine receptors (Ito et al, 1991). The ryanodine receptor is presumed to have a homotetrameric structure, similar to the IP$_3$ receptor (reviewed in Sorrentino & Volpe, 1993). Other then cytosolic
Ca$^{2+}$, cyclic adenosine diphosphoribose has been identified as a putative endogenous regulatory agonist for the ryanodine receptor (Galione, 1992).

1.4.6. Ca$^{2+}$ uptake into intracellular stores.

Ca$^{2+}$ ATPase pumps are also present on the SR of smooth muscle cells and are responsible for the uptake of Ca$^{2+}$ back into the stores (see figure 1.5). The Ca$^{2+}$ pump found on SR membranes is slightly smaller in size than those found on the plasma membrane (reviewed in Missiaen et al, 1992). Like the Ca$^{2+}$ ATPase pump found on the cell surface, the SR Ca$^{2+}$ ATPase is stimulated by Ca$^{2+}$ and is also dependent on Mg$^{2+}$ for activity (Wu & Lytton, 1993). The main regulator of the pump is phospholamban. This is a small 52 amino acid peptide which when unphosphorylated binds to the SR Ca$^{2+}$ ATPase pump, thus inhibiting it. Phosphorylation of the peptide releases it from its binding site and so the rate of Ca$^{2+}$ transport through the pump is increased (Tada, 1992). Investigators have shown that the phosphorylation of phospholamban in smooth muscle is associated with increases in cGMP levels (Karczewski et al, 1992). PKG may phosphorylate the peptide, resulting in increased Ca$^{2+}$ sequestration and ultimately relaxation of the smooth muscle.

**Figure 1.5. Summary diagram showing the protein elements involved in controlling cytosolic Ca$^{2+}$ in vascular smooth muscle cells.**

G, heterometic GTP-binding protein; PLC, phospholipase C; PIP$_2$, phosphotidyl-inositol-4, 5,-phosphate; IP$_3$, inositol 1,4,5-trisphosphate; ROCC, receptor operated Ca$^{2+}$ channel; VOCC, voltage operated Ca$^{2+}$ channel; SR, sarcoplasmic reticulum.
1.5 Membrane Potential in Vascular Smooth Muscle

As discussed above, many different strategies are employed by vascular smooth muscle to control cytosolic Ca\textsuperscript{2+}. A change in the membrane potential of a cell is another such mechanism. Like all animal cells, smooth muscle cells possess a membrane potential, i.e. the existence of a potential difference between its interior and exterior environments. A membrane potential is created because of the active transport of ions across a relatively non-conductive cell membrane leading to an uneven distribution of ions across it (Hille, 1992). Figure 1.6. shows the distributions of the major ions, Ca\textsuperscript{2+}, Na\textsuperscript{+}, K\textsuperscript{+} and Cl\textsuperscript{-}, across the membrane of a vascular smooth muscle cell.

**Figure 1.6.** Relative internal and external concentrations of K\textsuperscript{+}, Na\textsuperscript{+}, Ca\textsuperscript{2+}, Cl\textsuperscript{-} in a vascular smooth muscle cell.

The arrows indicate the ions permeant at rest. Values obtained from Hirst & Edwards, 1988 and Nelson & Quayle, 1995. The concentrations of the ions is given in mM.

Equilibrium potentials for K\textsuperscript{+} (E\textsubscript{K}), Na\textsuperscript{+} (E\textsubscript{Na}), Cl\textsuperscript{-} (E\textsubscript{Cl}), Ca\textsuperscript{2+} (E\textsubscript{Ca}); E\textsubscript{R}, resting membrane potential (at 37 °C).

Changes in membrane potential occur when the plasma membrane becomes more or less permeable to one or more species of ion. An ion will move either into or out of a cell in an attempt to reach equilibrium. Each species of ion will be subject to an electrochemical driving force across the membrane that depends on the membrane potential and its relative concentrations inside and out. Ions will diffuse down their concentration gradient causing a change in potential and a build up of electrical force. The electrical force opposes the movement of the ions down their concentration gradient. An equilibrium
potential is eventually reached when the electrical and diffusional forces balance each other. Ions move to reach their equilibrium potential ($E_i$), i.e. the voltage where zero net flow occurs, and this can be determined for an ion using the Nernst equation

$$E_i = \left( \frac{R \times T}{Z \times F} \right) \ln \left( \frac{[A]_o}{[A]_i} \right)$$

where $R$ is the gas constant (8.314 VCK$^{-1}$ mol$^{-1}$); $T$ is the absolute temperature ($T = 273 + ^\circ C$); $Z$ is the charge on the ion; $F$ is the Faraday constant (9.648 x 10$^4$ C. mol$^{-1}$); and $[A]_o$ and $[A]_i$ are the concentrations of the ion outside and inside the cell respectively, given in figure 1.6. (reviewed in Hille, 1992).

Smooth muscle cells at rest have a resting membrane potential ($E_R$) which is determined by the relative permeabilities to $K^+$ and $Cl^-$. Smooth muscle cells in vitro have been measured to have an $E_R$ in the range of -40 to -70 mV (Harder, 1984; Nelson et al, 1990$^b$). In vivo, the measured membrane potentials are between -40 and -55 mV (Neild & Keef, 1985; Nelson et al, 1990$^b$). Using the Nernst equation, at 37 $^\circ C$ and at physiological $K^+$ concentrations, the equilibrium potential for $K^+$ ($E_K$) is calculated to be about -84 mV (figure 1.6). If the membrane was permeable to only $K^+$ then $E_R$ would equal $E_K$ because the movement of $K^+$ ions would eventually equilibrate at -84 mV. However the measured $E_R$ is clearly more positive suggesting that the membrane is permeable to other ions at rest. At rest, the membrane is also permeable to $Cl^-$ (Hirst & Edwards, 1988), and therefore the $E_R$ is situated between the $Cl^-$ equilibrium potential ($E_{Cl}$) which is -31 mV and $E_K$. The outward diffusional movement of $K^+$ is balanced by the outward diffusional movement of $Cl^-$. Zero net current flow is reached when the diffusional and electrical forces of both ions balance, this potential is known as the reversal potential ($E_{Rev}$).

The movement of other ions is then needed for the initiation of contraction in vascular smooth muscle. Changes in membrane potential act in concert with the other mechanisms to alter blood vessel diameter. The trigger for contraction is the influx of $Ca^{2+}$ or $Na^+$ which makes the membrane potential more positive. This excitatory process is called depolarisation and is limited by the movement of $K^+$. The efflux of $K^+$ causes hyperpolarisation, that is their outward movement shifts the membrane potential in the negative direction, back towards $E_R$. 

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1.5.1 Membrane potential and control of $[Ca^{2+}]_i$.

There is a steep relationship between membrane potential and tone, such that even a few millivolts change can cause significant changes in vessel diameter (Nelson et al., 1990b). The membrane potential regulates smooth muscle contractility through voltage operated Ca$^{2+}$ channels which alters Ca$^{2+}$ influx. Voltage operated Ca$^{2+}$ channels have a very steep dependence on the membrane potential (Nelson & Quayle, 1995). The Na$^+$/Ca$^{2+}$ exchanger is also dependent on the membrane potential (Läuger & Apell, 1987). Depolarisation decreases the Ca$^{2+}$ extrusion leading to an increase in intracellular Ca$^{2+}$. Membrane potential also modulates the synthesis of IP$_3$ and therefore the mobilisation of Ca$^{2+}$ from intracellular stores (Ganitkevich & Isenberg, 1993). Membrane hyperpolarisation has been shown to inhibit IP$_3$ synthesis (Itoh et al., 1992). All these factors contribute to the steep dependence of intracellular Ca$^{2+}$ on membrane potential. It is now possible to appreciate the importance of the smooth muscle membrane potential in determining the arterial tone of vessels. Therefore any pharmacological or physiological agent that alters the membrane potential will have a significant effect on vessel diameter. Because the efflux of K$^+$ ions causes hyperpolarisation and so limits the degree of contraction, a number of endogenous mediators cause vasodilatation, in part, through increasing K$^+$ flux, for example CGRP (Holzer & Lippe, 1992), adenosine (Kleppisch & Nelson, 1995) and NO (Boilotina et al., 1994). In contrast, inhibition of K$^+$ flux would cause membrane potential depolarisation and vasoconstriction. Other endogenous vasoactive agents, such as endothelin, cause vasoconstriction, in part, by decreasing K$^+$ flux (Sumner et al., 1992).

1.5.2. Changes in membrane potential and current flow.

Ions are charged particles, and the non-conductive nature of the membrane sets up a separation of charges between its intracellular and extracellular environments. The net movement of charges or ions is the flow of electrical current. The size of current is determined by two factors; the potential difference and the electrical conductance between the two environments. Potential difference or voltage is defined as the work needed to move a unit charge in a frictionless manner between two points. And conductance is the ease of movement of charges from one point to another. Current (I) is defined by Ohm’s Law,
\[ I = GV \]  \hspace{1cm} (2)

where current is measured in amperes (A); \( V \) is the voltage measured in volts (V) and \( G \) is the conductance measured in siemens (S). The conductance is the reciprocal of resistance (R), therefore

\[ G = \frac{1}{R} \]  \hspace{1cm} (3)

Resistance is measured in ohms (\( \Omega \)) and is the obstruction to current flow. In cell membranes, ions move through distinct conductive regions, for example ion channels. The membrane has many such ion channels in parallel so a measure of whole cell conductance tells us how many ionic channels are open at a given voltage. \( V \) in equation (2) could be said to represent the membrane potential \( (V_m) \). However the driving force on an ion leading to an ionic current arises not only because of a potential difference, but also because a concentration gradient of an ion that exists between the inside and outside of the cell. Therefore equation (2) has to be rewritten,

\[ I = G(V_m - E_i) \]  \hspace{1cm} (4)

where \( E_i \) (in V) is the equilibrium potential for an ion and \( (V_m - E_i) \) is the driving force on a particular ion (Hille, 1992).

Measurement of membrane potential allows investigators to monitor functional changes in vessels. Membrane potential can be measured directly in vascular tissue using sharp microelectrodes (e.g. Brayden, 1990) or an indication of it can be inferred from changes in isometric force of ring segments (Mulvany, 1985) or from changes in diameter of pressurised arteries (Duling et al, 1981). The underlying changes in channel activity have to be measured on isolated single cells. Each channel type has its own identity and measurement of ionic current helps to profile its biophysical properties.

A major advance in studying ion channels came with the development of the patch-clamp technique. In 1976, Neher and Sakmann were the first to use this technique to resolve single channels in membranes of frog skeletal muscle. They recorded from a tiny area of cell membrane (a “patch”) by pressing a clean micropipette against the cell. In 1981 they reported the development of the “gigaohm seal”, called so because the fire polished micropipettes formed mechanically stable, very high resistance seals, in the order of gigahoms, with the cell membrane. This greatly decreased the noise of the recording.
and allowed different recording modes. One such configuration is the whole cell recording which is achieved by rupturing the cell membrane within the patch pipette. The whole cell configuration is extensively used to study macroscopic currents, that is the ionic flux through all the channels in the cell membrane (reviewed in Sakmann & Neher, 1984). The technique allowed researchers to study currents in small cells, such as smooth muscle cells which were inaccessible previously.

In conjunction with the patch clamp technique, voltage clamping is also used to study ionic currents. The voltage clamp offers a quantitative measure of currents flowing through ion channels. The experimental technique was developed by Marmont (1949), Cole (1949) and Hodgkin, Huxley and Katz (1949, 1952) for use on the giant squid axon. To “voltage clamp” is to electronically control the potential across the cell membrane. Under physiological conditions, a change in potential causes a change in current which causes further changes in potential. By voltage clamping a cell, a pre-determined voltage is applied and the current this produces is measured. The technique ensures that the current measured is directly due to ionic movements (Halliwell et al., 1994).

Therefore the whole cell voltage clamp technique is used to measure ionic currents in small cells. Plots of current against voltage give current-voltage (I-V) relationships which give information on the channels underlying the current. In my experiments, I have used the whole cell voltage clamp technique to measure currents through K+ channels (Chapters 3 & 4).

1.6 K+ Channels in Vascular Smooth Muscle

Ion channels are macromolecular proteins incorporated into cell membranes. Many channels open and close to produce the responses of vascular smooth muscle cells. The membrane potential is essentially controlled by K+ efflux through ion selective channels. Four distinct types of K+ channels have been identified in arterial smooth muscle: voltage-dependent K+ (Kv) channels, inward rectifier K+ (Kir) channels, large conductance calcium dependent K+ (BKCa) channels and ATP-sensitive K+ (KATP) channels (reviewed in Nelson & Quayle, 1995).
This section will give information about the properties and roles of these channels in vascular smooth muscle. The emphasis will be on $K_{\text{ATP}}$ channels which form the major topic of the experimental work presented in this thesis.

1.6.1. $K^+$ channels and the regulation of membrane potential.

As hinted in the previous section (1.5.1.), vasoactive substances acting through $K^+$ channels are able to alter vessel diameter. For example, activation of $K_{\text{ATP}}$ channels with the opener, cromakalim, hyperpolarised pressurised cerebral arteries. Concurrently there was a vasodilatation from $-160 \ \mu m$ to $360 \ \mu m$ (Nelson & Quayle, 1995). In contrast, inhibition of $B_{\text{KCa}}$ channels by charybdotoxin (ChTX), a channel blocker, depolarised similar arteries and constricted them from $-180 \ \mu m$ to $120 \ \mu m$ (Brayden & Nelson, 1992).

$K^+$ channels in vascular smooth muscle cells have very low levels of activity at physiological membrane potentials (-40 to -60 mV). Under physiological conditions of 6 mM $[K^+]_o$ and 140 mM $[K^+]_i$, the $E_K$ is approximately -84 mV and opening of $K^+$ channels leads to $K^+$ movement out of the cell. $K^+$ efflux (given as $J_K$, in mol.s$^{-1}$) is related to channel properties by,

$$J_K F = I_K = N_i P_o$$

where $F$ is Faraday's constant; $I_K$ is the whole cell $K^+$ current; $N$ is the number of channels in the cell; $i$ is the single channel current; and $P_o$ is the open probability of the channel. The patch clamp technique can be used to measure $I_K$ and $i$. As the following example illustrates very few $K^+$ channels are open at physiological potentials. Extrapolation of data based on arterial smooth muscle cells (Volk & Shibata, 1993) gave single channel current values of 0.07 pA and 0.17 pA, and $P_o$ of 0.003 and 0.015, for $K_v$ channels at -60 and -40 mV respectively. Now assuming that the number of channels in a cell is 1000 (Robertson et al, 1993), the whole cell $K_v$ current can be calculated from equation (4). Values for whole cell current would be 0.21 pA at -60 mV and 2.6 pA at -40 mV. Even though $K^+$ channels have little activity at these potentials, even small changes can have major effects on membrane potential and eventually vessel diameter (Nelson & Quayle, 1995).

Few $K^+$ channels need to open to cause changes in membrane potential because the resting input resistance in vascular smooth muscle is very high, in the order of 1-10 GΩ. The total membrane resistance or input resistance is so high because of the low density of
some K\(^+\) channels in the membrane. The following example illustrates the relationship between conductance and voltage. It can be assumed that the membrane potential actually results from two parallel conductances, a K\(^+\)-selective conductance representing all the K\(^+\) channels (\(G_k\)) and a leak conductance (\(G_L\)) which is due to the movement of Na\(^+\), Ca\(^{2+}\) and Cl\(^-\) ions. The leak current can be assumed to have a reversal potential (\(E_L\)) of 0, that is the voltage at which no net current flows, then

\[
V_m = \left[ \frac{G_k}{G_k + G_L} \right] E_k
\]

If the \(E_k\) is -85 mV and membrane potential (\(V_m\)) is -60 mV, an input resistance of 2 G\(\Omega\) corresponds to a total cell conductance of 500 pS (from equation (3)). Therefore from equation (5) \(G_k\) is 353 pS and \(G_L\) is 147 pS. If a typical K\(^+\) channel unitary conductance is taken as 20 pS (Nelson et al, 1990\(^b\)), the opening of one channel will increase the \(G_k\) to 373 pS, and the \(V_m\) will increase to approximately -61 mV. One channel opening changes the membrane potential by approximately 1 mV, and 20 or 50 channels opening will change the \(V_m\) to -71 mV or -77 mV respectively. This shows that even small changes in K\(^+\) channel opening will cause significant hyperpolarisation, and are sufficient to reduce force and increase vessel diameter. If for example, a smooth muscle cell has 1,000 \(K_{ATP}\) channels, then increasing \(P_o\) from 0 to 0.01 would change the membrane potential from -60 mV to -67 mV. And since voltage operated Ca\(^{2+}\) channels have been shown to be very sensitive to voltage (Nelson et al, 1990\(^b\)), the membrane potential is a powerful factor in controlling arterial smooth muscle tone.

1.6.2. Delayed rectifier K\(^+\) (Kv) channels.

Kv channels belong to the voltage-gated superfamily of ion channels, whose members include Na\(^+\) and Ca\(^{2+}\) channels. The main characteristics of all voltage operated channels are a voltage sensor and an ion selective pore. Kv channels show voltage-dependent activation, that is they open upon depolarisation (Volk & Shibata, 1993). Many Kv channels undergo inactivation with prolonged membrane depolarisation. In response to a long depolarisation, the current through Kv channels of vascular smooth muscle increases to a peak over time and then slowly decays due to voltage-dependent inactivation (Nelson & Quayle, 1995).
Based on analysis of their molecular structure, several families of Kv channels have been identified. The Shaker locus in the Drosophila provided the sequence for the first voltage-gated K⁺ channel (Tempel et al, 1987). Further studies from the Shaker channel gene led to the isolation of three more genes, termed Shab, Shal and Shaw (Wei et al, 1990). Alternative splicing then gives rise to further diversity in the four subfamilies. Several K⁺ channel genes have since been identified in the rat, mouse and human genome (reviewed in Jan & Jan, 1992, Grissmer, 1997). Each channel consists of α subunits and β regulatory subunits (Pongs, 1992). The subunits usually form homotetrameric channels however heteromers can also form, resulting in different channel kinetics (Wang et al, 1993). Only the α subunits have putative glycosylation sites, whereas both subunits have possible sites for phosphorylation. The complete channel is an octomer consisting of four α and four β subunits (MacKinnon, 1991a). The α subunits span the cell membrane and form the K⁺-selective pore (MacKinnon, 1991b), and their expression alone is sufficient to form a functional channel. The β subunits bind non-covalently to the α subunits on their intracellular surface and play a modulatory role. Co-expression of α and β subunits leads to an increased speed of inactivation. Each α subunit is made up of six transmembrane regions, termed S1 to S6, with hydrophilic amino acids linking each domain (see figure 1.7A). The N- and C-terminals are in the cytoplasm. The H5 region and parts of the S5 and S6 domains form the permeation pathway for K⁺ ions (Jan & Jan, 1992). Cloned Kv channels have been used extensively to study voltage sensing and ion selectivity properties. Mutagenesis of the wild type (native) channel was done to determine which amino acids lined the putative pore region, and those that were important for K⁺ selectivity. In this way it was found that the removal of a tyrosine-glycine pair of residues eliminated the ability of the Kv channel to select K⁺ over Na⁺ ions (Heginbotham et al, 1992). It was found that a small sequence of residues, including the tyrosine-glycine amino acids, were conserved among most members of the K⁺ channel family, including the inward rectifier K⁺ (Kir) gene families. The four H5 regions, from each subunit, come together to form the pore (figure 1.7B). Using chimeric Kv channels, Lopez et al (1994) showed that regions in the S6 segment formed part of the ion permeation pore as well. Kv channels also possess intrinsic voltage gating properties because their open probability increases with depolarisation, thus suggesting that they must have a voltage-sensor (reviewed in Papazian & Benzanilla, 1997). Molecular studies have shown that the S4 transmembrane segment of each α subunit acts as the voltage sensor. Every third residue in this domain is a positively
charged lysine or arginine amino acid. Site directed mutagenesis studies of these residues to neutral glutamine residues showed that the S4 region was indeed important for voltage-sensing (reviewed in Guy & Conti, 1990; Papazian et al, 1991). Recently, it has been proposed that residues in the S2 and S4 segments may also be involved in voltage sensing (Seoh et al, 1996).

Figure 1.7  Kv channel structure.

(A) Putative structure of one α subunit of the Kv channel. (B) Four α subunits come together to form a functional K⁺ channel. Redrawn from Jan & Jan, 1992.

Known inhibitors of Kv channels in vascular smooth muscle are 4-aminopyridine (4-AP) (Okabe et al, 1987), phencyclidine, quinidine (Beech & Bolton, 1989) and tedisamil (Pfunder & Kreye, 1992). Inhibition of Kv channels with a blocker would lead to
depolarisation and constriction of arteries. In vascular smooth muscle, 4-AP has been used to separate Kv currents from other membrane potential dependent currents (Beech & Bolton, 1989) such as those carried by the BK_{Ca} channels, but 4-AP is not completely selective (Gelband & Hume, 1992). Tetraethylammonium (TEA⁺) ions also inhibit Kv channels, but it is non-selective in that it also blocks other K⁺ channels such as BK_{Ca} channels (Beech & Bolton, 1989).

In vascular smooth muscle, voltage dependent K⁺ currents have been identified in several tissues including rabbit portal vein (Beech & Bolton, 1989); canine renal artery (Gelband & Hume, 1992) and rabbit coronary artery (Ishikawa et al, 1993a). There is some evidence to suggest that Kv channels may be modulated by endogenous agents. For example, a 4-AP sensitive K⁺ current was inhibited by a H₁-histamine receptor agonist (Ishikawa et al, 1993b). Their role in airway smooth muscle is to limit the degree of depolarisation (Fleischmann et al, 1993). Because of their voltage-dependence, Kv channels will open during depolarisation, thus initiating repolarisation of the cell (Knot & Nelson, 1995). Little is know about which channel subtypes are expressed in vascular smooth muscle, though recent studies have suggested the presence of Kv1.2 and Kv2.2 (Christie, 1995).

1.6.3. Inward rectifier K⁺ (Kir) channels.

Inward rectification was first described in skeletal muscle by Katz, in 1949. However, since then inward rectifiers have been described in many other cell types, including vascular smooth muscle cells. The Kir channels were so named because they exhibit a steep inward rectification which leads to them conducting an inward K⁺ current much more readily than an outward K⁺ current. Their rectification is caused by a strong voltage dependent block by intracellular polyamines and Mg²⁺ (Lopatin et al, 1994; Lopatin et al, 1995).

Molecular biology techniques over the last few years have revealed the genes encoding the inward rectifying K⁺ channels. Unlike other known K⁺ channels, the channels are encoded by a new gene family, termed Kir (reviewed in Doupnik et al, 1995). Thus far six Kir gene subfamilies have been recognised, each showing differences in the transmembrane and pore regions. The subfamilies have been designated Kir1.x-Kir6.x. The classical inward rectifiers, those that exhibit a strong rectification, are members of the
Kir2.x, Kir3.x and Kir4.x subfamilies. The first of these, Kir2.1 (formerly known as IRK), was cloned from a mouse macrophage cell line in 1993 (Kubo et al., 1993). Since then the Kir2.1 gene has been found on chromosome 17 of the human genome (Raab-Graham et al., 1994). These channels are characteristic of the native Kir channels found in many tissues. Outlined in table 1.2. are the currently recognised strong inward rectifier subfamilies (reviewed in Quayle et al., 1997). The cloned DNAs code for proteins which are about 400 amino acids in length. Hydrophobicity plots have predicted the protein to contain two transmembrane regions, called M1 and M2. Between them is a highly conserved P region which forms part of the $K^+$ selective pore (reviewed in Nichols & Lopatin, 1997). The amino- and carboxyl-terminals are thought to lie on the intracellular side of the membrane. This will give rise to the predicted structure shown in figure 1.7. (section 1.6.5.) Yang et al. (1995) determined the stoichiometry of the Kir channel to be similar to the Kv channel, that is four subunits come together to form a functional channel.

Table 1.2. Subfamilies and members of the strong inward rectifying $K^+$ channels.

Their tissue expression is also given, as is their likely functions in the tissues. Reproduced from Quayle et al., 1997.

<table>
<thead>
<tr>
<th>Subfamily and members</th>
<th>Example of clones</th>
<th>Expression</th>
<th>Possible function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kir2.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kir2.1</td>
<td>IRK$_1$</td>
<td>Brain, heart and skeletal muscle</td>
<td>Maintain resting membrane potential</td>
</tr>
<tr>
<td>Kir2.2</td>
<td>HRK$_1$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kir2.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kir3.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kir3.1</td>
<td>GIRK</td>
<td>Brain and heart</td>
<td>SA node and arterial channel activated by muscarinic-receptor stimulation</td>
</tr>
<tr>
<td>Kir3.2</td>
<td>GIRK$_2$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kir3.3</td>
<td>GIRK$_3$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kir3.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kir4.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kir4.1</td>
<td>BIR$_{10}$</td>
<td>Brain</td>
<td></td>
</tr>
</tbody>
</table>

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Channels from the Kir2.0 subfamily were used to elucidate the mechanism of rectification. Some inward rectification is caused by the intracellular block of outward current by Mg\textsuperscript{2+}. However, in strong inward rectifiers the removal of intracellular Mg\textsuperscript{2+} still leaves substantial rectification (Matsuda, 1991). The strong inward rectification can be accounted for by block by the polyamines, spermine and spermidine (Lopatin \textit{et al}, 1994; Ficker \textit{et al}, 1994; Fakler \textit{et al}, 1995). Mutagenesis studies have illustrated that amino acids deep within the pore are responsible for the channel’s rectification properties. Experiments showed that the residue at position 172, present on the M2 transmembranal domain of the channel, was important for determining rectification. In the Kir2.0 and 3.0 subfamilies, this residue is a negatively charged aspartic acid, however in weak inward rectifier channels, such as the Kir1.0 subfamily, the amino acid is a neutral asparagine. Point mutations in the Kir1.1 channel of the 172 residue from asparagine to aspartic acid conferred strong rectification properties onto the channel (Stanfield \textit{et al}, 1994; Wible \textit{et al}, 1994). However in the Kir2.1 channel, removing the charge of the 172 residue had little effect on its inward rectification (Wible \textit{et al}, 1994). The polyamines are large molecules, therefore it was likely that another amino acid residue is also needed for their binding. Subsequently, a glutamine residue in the intracellular C-terminal, position 242, was also shown to be important for polyamine binding (Yang \textit{et al}, 1995\textsuperscript{b}). Mutation of both residues, at positions 172 and 242, was needed to remove strong rectification of the Kir2.1 channels. These findings suggest that part of the C-terminal is involved in forming the ion pore region of Kir channels along with the H5-loop.

External Ba\textsuperscript{2+} has been shown to be an effective inhibitor of Kir currents in arterial smooth muscle. The block is voltage dependent and increases with hyperpolarisation, the $K_i$ for external Ba\textsuperscript{2+} is 2 \textmu M (Quayle \textit{et al}, 1993; Robertson \textit{et al}, 1996). External Ba\textsuperscript{2+} is therefore the most selective blocker available. At millimolar concentrations, externally applied Cs\textsuperscript{+} also blocks the channels in a voltage-dependent manner. At -60 mV, the $K_i$ for Cs\textsuperscript{+} is approximately 3 mM (Quayle \textit{et al}, 1993; Robertson \textit{et al}, 1996).

Studies, using voltage clamp with intracellular microelectrodes in intact arterial segments, detected the presence of Kir currents in resistance vessels of the mesenteric (Edwards & Hirst, 1988) and cerebral (Edwards \textit{et al}, 1988) vascular beds. Application of Ba\textsuperscript{2+} caused membrane depolarisation in these vessels (Edwards & Hirst, 1988) implying that Kir channels contributed towards the resting K\textsuperscript{+} conductance. Kir currents have also been
recorded in single smooth muscle cells from resistance-sized arteries (Quayle et al., 1993; Quayle et al., 1996). Kir channels seem to play a greater role in smaller resistance vessels than in the larger conduit vessels where little Kir currents have been detected (Quayle et al., 1996). It seems that the main function of the inward rectifier K⁺ channels in vascular smooth muscle is to maintain the resting membrane potential of resistance vessels. The Kir channels in vascular smooth muscle are likely to be products of the strong inward rectifier Kir2.0 subfamily. Kir2.0 mRNA has been detected in other types of muscle (Kubo et al., 1993).

1.6.4. *Large conductance Ca²⁺ dependent K⁺ (BKCa)* channel.

BKCa channels are named such because they are modulated by cytosolic Ca²⁺ and they pass K⁺ easily which gives rise to their large single channel conductance. The open probability of these channels increases with elevation in intracellular Ca²⁺ and membrane depolarisation (Blatz & Magleby, 1986). They were first identified in cultured skeletal muscle (Pallota et al., 1981) and in chromaffin cells (Marty, 1981). Since then investigations have shown that BKCa channels are present in virtually all smooth muscle cell types.

In 1991, Atkinson and colleagues reported the cloning of a component of the BKCa channel from *Drosophila slo* locus. Then in 1993, Butler et al. identified a mammalian homologue (mslo) in mice. The putative structure implied that the channels belonged to the superfamily of voltage gated K⁺ channels, with α subunits composed of six membrane-spanning regions. The channel has a similar stoichiometry to Kv channels. Associated with the α subunits are smaller β subunits which play a modulatory role (Knaus et al., 1994). Alternative gene splicing is thought to be responsible for the generation of isoforms of BKCa channels (Inoue et al., 1985).

Pharmacological agents have proved to be useful in dissecting the role of the BKCa channel. Peptides from scorpion venom have been found to be highly specific inhibitors of the channel. Two such potent inhibitors are charybdotoxin (Miller et al., 1985) and iberiotoxin (IbTX) (Giangiacomo et al., 1992). Charybdotoxin also blocks some subtypes of Kv channels but not those present in vascular smooth muscle (Galvez et al., 1990). Externally applied TEA⁺ also blocks BKCa channels with a Kᵢ of about 200 μM (Villarroel et al., 1988; Langton et al., 1991). Few BKCa channel openers have been described.
Recently, NS 1619 (Olesen et al., 1994; Holland et al., 1996) and related compounds have been developed as synthetic openers of BK$_{Ca}$ channels.

BK$_{Ca}$ currents have been reported in many vascular preparations including mesenteric arteries (Benham et al., 1986; Langton et al., 1991) and coronary smooth muscle cells (Toro et al., 1991). Recently the molecular structure of the vascular smooth muscle BK$_{Ca}$ channel has also been worked out. The channel, like other BK$_{Ca}$ channels, is a complex composed of at least two subunits; a pore forming $\alpha$ subunit and a modulatory $\beta$ subunit. The $\beta$ subunit is thought to be needed for modulating Ca$^{2+}$ sensitivity of the channel (Knaus et al., 1994; Tanaka et al., 1997). One proposed functional role for BK$_{Ca}$ channels in resistance vessels is that they may limit the degree of myogenic tone (reviewed in Nelson, 1993), thereby preventing over-constriction of the vessel. Blockers of the BK$_{Ca}$ channels cause depolarisation and constriction of pressurised coronary (Brayden & Nelson, 1992) and saphenous arteries (Berczi et al., 1992). Consistent with the idea that BK$_{Ca}$ channels may be involved in controlling vascular tone, many endogenous vasoactive substances are able to regulate them. Vasoconstrictors, such as Ang II (Toro et al., 1990) and endothelin (Sumner et al., 1992) depolarise vascular smooth muscle, in part, via the inhibition of BK$_{Ca}$ channels. In contrast BK$_{Ca}$ channel activation would cause hyperpolarisation and relaxation as shown in coronary arteries (Scornik et al., 1993). Activation of BK$_{Ca}$ channels by vasodilators is reported to be mediated by phosphorylation through PKA (Sadoshima et al., 1988; Taguchi et al., 1995). There is also some evidence showing direct G-protein coupling (Scornik et al., 1993) that is independent of phosphorylation by PKA. In other reports increases in cGMP leading to activation of PKG have also been shown to activate BK$_{Ca}$ channels (Williams et al., 1988; Robertson et al., 1993; Taniguchi et al., 1993). There is also evidence showing that NO, a potent endothelial-derived vasodilator, is able to act on BK$_{Ca}$ channels directly (Bolotina et al., 1994).

1.6.5. $ATP$-sensitive $K^+$ ($K_{ATP}$) channels.

Noma (1983) first described the presence of $K^+$ channels in the heart that were sensitive to cytosolic ATP levels. These channels were closed by high concentrations of ATP and activated by ADP. Subsequently, such channels were found in $\beta$-cells of the pancreatic islets (Cook & Hales, 1984), neurones (Ashford et al., 1988) and skeletal muscle (Spruce et
These channels are called ATP-sensitive or ATP-dependent K⁺ (K_{ATP}) channels. Even up to 1994, the physiological roles of K_{ATP} channels were poorly understood, with the exception of their function in pancreatic β-cells where they act as the primary determinants of insulin release (Ashcroft, 1988). It has been suggested that K_{ATP} channels play a cardioprotective role in the heart under conditions of cellular metabolic stress (Wilde & Janse, 1994). The outward movement of K⁺ ions results in a reduction in contractility and so protects the cardiomyocytes by decreasing oxygen demand (Escande & Cavero, 1992). In skeletal muscle, K_{ATP} channels may decrease the excitability of the cells. Sustained muscular activity decreases ATP concentrations thus increasing the activity of K_{ATP} channels (Davies, 1990; reviewed in Davies et al, 1991).

K_{ATP} channels are highly selective for K⁺ over other ions. In skeletal muscle K_{ATP} channels, the relative permeability to Na⁺ (p_{Na}/p_{K}) is around 0.01 (Spruce et al, 1987). The current flowing through K_{ATP} channels is essentially voltage independent but shows some rectification at positive potentials. This arises due to a voltage dependent block by internal cations such as Mg^{2+} (Findlay, 1987a). The single channel conductance when symmetrical K⁺ concentrations were used ranges from 80-90 pS in cardiac cells, 40-60 pS in skeletal muscle and 50-75 pS in pancreatic β-cells (reviewed in Ashcroft, 1988). Only ATP applied to the intracellular surface can produce channel inhibition. The K_i or half maximal block of the K_{ATP} channel by ATP ranges from 13 μM in cell lines to 500 μM in some studies in cardiomyocytes. Similarly the reported Hill coefficient ranges from one in skeletal muscle to six in cardiac muscle. It is therefore speculated that one ATP molecule is sufficient for closing the channel in skeletal muscle, however in cardiac cells there may be co-operativity between multiple binding sites (reviewed in Ashcroft, 1988). Evidence from skeletal and cardiac muscle shows that the channel openings occur in bursts with long closed periods in between (Zilberter et al, 1988). Intracellular ATP affects channel kinetics by increasing the time between bursts and reducing the length of bursts but it has no effect on single channel amplitudes (Spruce et al, 1987). The channel has at least two open states and several closed states. In addition to ATP being a channel inhibitor it is also required for the maintenance of channel activity. In the absence of ATP, isolated cell membrane patches of cardiomyocytes exhibited spontaneous openings of K_{ATP} channel (Trube et al, 1984). However the channel activity declined with time and this was termed "channel run down". Both Mg^{2+} and ATP were needed for the prevention or reversal of this phenomenon (Findlay, 1987b). Non-hydrolysable analogues of ATP were unable to
substitute for ATP, hence it seems likely that phosphorylation of the channel is a perquisite for opening (Ohno-Shosaku et al, 1987). Although $K_{ATP}$ channels have been identified and named due to their sensitivity to ATP, the levels of ATP in the cytosol are in the millimolar range and are usually well buffered (Gudbjarnason, 1970). Nucleotide diphosphates, such as UDP, GDP, CDP and IDP have been shown to stimulate $K_{ATP}$ channels in the absence of cellular ATP (Dunne & Peteson, 1986; Lederer & Nichols, 1989; reviewed in Terzic et al, 1994).

After years of intensive research, the structure of the $K_{ATP}$ channel was only determined recently. Initial biophysical studies showing inward rectification of the native $K_{ATP}$ channel suggested that it was likely to be a member of the Kir channel family. In 1993, Ho et al cloned and expressed an inward rectifying, ATP-regulated channel which they termed ROMK1 (now called Kir1.1a). The channel was regulated by ATP in that it was activated by MgATP, however it differed from native $K_{ATP}$ channels because very high concentrations of cellular ATP were needed for inhibition. Then in 1994, Ashford and colleagues isolated a cDNA coding sequence from the rat heart by the polymerase chain reaction. Their 417 amino acid sequence which they called rc$K_{ATP}$-1, a member of the Kir3.x subfamily, possessed all the essential features of the native cardiac $K_{ATP}$ channel when expressed. However shortly afterwards, Krapivinsky et al (1995) illustrated that the rc$K_{ATP}$-1 subunit formed a heterodimer with another cloned subunit of the Kir3.0 subfamily, termed GIRK1 (now called Kir3.1). The two subunits express a $K^+$ channel that is stimulated by ACh acting on muscarinic receptors. Meanwhile in 1995, Inagaki and co-workers isolated a cDNA encoding a novel member of the Kir family from the rat pancreatic islet. The channel was called u$K_{ATP}$-1 (now called Kir6.1) and formed the start of a new subfamily of weak inward rectifiers, termed Kir6.x. Single channel patch clamp studies of the expressed protein in HEK 293 cells revealed that the channel was inhibited by 1 mM intracellular ATP and had a unitary conductance of approximately 70 pS. The channel was also activated by the potassium channel opener diazoxide, however it was insensitive to glibenclamide, a sulphonylurea $K_{ATP}$ channel blocker. This provided evidence that the $K_{ATP}$ channel and the sulphonylurea receptor were separate entities. The mRNA for the channel was found to be expressed in many rat tissues, including skeletal muscle and heart but surprisingly not in insulin-secreting cells. The search for the $K_{ATP}$ in insulin-secreting cells led Bond et al (1995) to clone an inward-rectifying $K^+$ channel from insulinoma cells. The predicted amino acid sequence shared approximately 72 %
homology with the cloned cardiac $K_{ATP}$ channel (rc$K_{ATP}$-1). Although the newly cloned channel showed $K^+$ selectivity, inward rectification and block by external $Ba^{2+}$ ions, it was however insensitive to 1 mM internal ATP and not activated by $K^+$ channel openers. Therefore even though this was a new Kir channel found in $\beta$-cells, it was not the $K_{ATP}$ channel. The discovery of the $K_{ATP}$ channel in $\beta$-cells came when Inagaki et al (1995\textsuperscript{b}) cloned a second channel of the Kir6.x subfamily, termed Kir6.2, from a human genomic library. Shortly afterwards, Sakura et al (1995) cloned a similar channel from a rat insulinoma cell line. The Kir6.2 mRNA was found to be strongly expressed in $\beta$-cells, brain, skeletal and cardiac muscle. The cDNA coded for a 390 amino acid protein with two putative membrane-spanning regions and a highly conserved H5 pore domain. Both the Kir6.1 and Kir6.2 had possible sites for phosphorylation but lacked any obvious sequences for ATP binding (figure 1.8A). Unlike Kir6.1, expression of the Kir6.2 subunits alone was insufficient to produce active channels. However when they were co-expressed with the sulphonylurea receptor subunit the channels exhibited properties similar to native $\beta$-cell $K_{ATP}$ channels (Inagaki et al, 1995; Sakura et al, 1995).

The search for the sulphonylurea receptor started a few years earlier when photoaffinity labelling with $[^3H]$ glibenclamide led to the identification of a 150 kDa protein (Bernardi et al, 1988). Aguilar-Bryan et al (1990) were able to solubilise labelled proteins from $\beta$-cells using detergents. The solubilised membrane retained active sulphonylurea receptors. The breakthrough then came with the successful cloning of the sulphonylurea receptor (Aguilar-Bryan et al, 1995; Thomas et al, 1995). The sulphonylurea receptor, called SUR, was found to be a member of the ATP-binding cassette (ABC) superfamily (Philipson & Steiner, 1995). Other members include the P-glycoprotein proteins which cause resistance to chemotherapeutic drugs and the Cystic Fibrosis Transmembrane Regulator (CFTR) protein whose mutations cause cystic fibrosis. SUR is thought to be composed of multiple transmembrane domains and two nucleotide binding sequences (figure 1.8B). Sakura et al (1995) found that the activity of the Kir6.2 was only seen when the cDNA was co-transfected with that for the sulphonylurea receptor. Western blotting revealed that the SUR subunit has a molecular weight of 140 kDa and the Kir6.2 subunit has a molecular weight of approximately 38 kDa. The whole cell currents measured from the Kir6.2/SUR complex co-expression exhibited $K^+$ selectivity, weak inward rectification, and block by $Ba^{2+}$ ions and intracellular ATP. Inagaki et al (1995) went on to show that reconstitution of the SUR/Kir6.2 subunits gave rise to a channel with
a conductance of 76 pS, inhibited by sulphonylureas and activated by diazoxide. Gene mapping data also shows that the two genes occur consecutively on chromosome 11 of the human genome (Inagaki et al, 1995). Some members of the ABC transport superfamily can function as ion channels and their ionic selectivity is determined by the transmembranal domains. An example of an ABC protein that acts as ion channel is the CFTR protein which forms a Cl⁻-selective channel in epithelial cells (reviewed in Collins, 1992). However the SUR subunit is thought to act as a channel regulator because expression of SUR alone does not show any channel activity (Ämmälä et al, 1996). The mechanism by which the ABC protein controls the activity of the K⁺ channel is unknown. Hypotheses include direct protein-protein interactions leading to a conformational change, or the translocation of a mediator by the ABC protein which is then able to regulate the ion channel (Demolombe & Escande, 1996). Gribble et al (1997) demonstrated that the co-expression of the SUR and Kir 6.2 formed channels that mimicked many of the properties of the native β-cell Kₐ₅₆ channel. The channel displayed metabolic sensitivity, K⁺ selectivity and inhibition by glibenclamide and intracellular ATP. These experiments suggest that native Kₐ₅₆ channels of β-cells are composed of at least two subunits; Kir 6.2 and SUR.

The actual site that conferred ATP sensitivity was unknown until Tucker et al (1997) presented evidence to show that the site of ATP binding was on the Kir subunit. They found that neutralisation of a positively charged lysine residue at position 185 decreased the channel's sensitivity to ATP. The SUR subunit is required for modulation of channel activation and sensitivity to sulphonylureas and to channel openers. The SUR subunit is also involved in the MgATP-dependent regulation of the channel. Amino acid residues in the second nucleotide binding domain of the SUR subunit have been found to be involved in MgATP-induced activation of the channel (Nichols et al, 1996). Recently a second cardiac SUR receptor has been cloned, termed SUR2 (Chutow et al, 1996), hence the first SUR has been renamed SUR1. Northern blot analysis showed that SUR2 was expressed in heart and skeletal muscle, and co-expression of SUR2 and Kir6.2 gives channels with similar properties to native Kₐ₅₆ channels (Inagaki et al, 1996).
Figure 1.8  The proposed structure of the protein subunits that make up the $K_{ATP}$ channel.

(A) The putative structure of the Kir 6.2 channel subunit. The 390 amino acid protein is thought to have two transmembrane domains (M1 and M2) and a highly conserved H5 region which forms the $K^+$ selective pore. (B) The SUR1 subunit is a member of the ATP binding cassette superfamily. It is made up of 1582 amino acid residues and is predicted to have 13 membrane spanning segments and two intracellular nucleotide binding domains (NBDs).

The $K_{ATP}$ channels are now known to be composed of Kir and SUR subunits, the overall composition and stoichiometry of the channel is postulated to be similar to Kv channels. Recent evidence suggests that native $K_{ATP}$ channels are formed as an octameric complex composed of four Kir and four SUR subunits, but a stoichiometry of 1 SUR: 1 Kir6.2 is sufficient for channel formation (Shyng et al, 1997). A triple fusion of 1 SUR: 2 Kir6.2 does not form a $K_{ATP}$ channel (Clement IV et al, 1997a). A native $K_{ATP}$ channel is therefore made up of sixty transmembrane domains (Clement IV et al, 1997b), with a molecular weight of approximately 1000 kDa.
The $K_{ATP}$ channel represents a potential therapeutic target. This has lead to the discovery of many activators and inhibitors of the channel, some of which are used clinically. Examples include the use of sulphonylureas for non-insulin dependent diabetes mellitus, and as I will discuss later, the use of nicorandil, a $K_{ATP}$ channel opener, for angina (section 1.8.5).

Sulphonylureas, such as glibenclamide and tolbutamide, are the best known inhibitors of $K_{ATP}$ channels (reviewed in Ashcroft & Ashcroft, 1992). The ability of the sulphonylureas to cause hypoglycaemia via the stimulation of insulin secretion was elucidated by Loubatières in the early 1940s, however it took over twenty years to show that sulphonylureas worked by inducing electrical activity and decreasing $K^+$ permeability. Then in the 1980s, the patch clamp technique was used to demonstrate that these compounds were specific blockers of the $K_{ATP}$ channels (reviewed in Edwards & Weston, 1993). The sulphonylurea drugs were shown to inhibit whole cell $K_{ATP}$ currents in isolated pancreatic $\beta$-cells (Ashcroft et al, 1987). They were also selective inhibitors of $K_{ATP}$ channels in cardiac myocytes where glibenclamide has a $K_i$ of $\sim 7$ nM (Findlay, 1992). Similarly, glibenclamide inhibits $K_{ATP}$ currents in mammalian skeletal muscle with a $K_i$ of between 60 and 190 nM (Allard & Lazdunski, 1993; Barrett-Jolly & Davies, 1997). Glibenclamide has since been shown to inhibit whole cell $K^+$ currents in smooth muscle cells of coronary (Xu & Lee, 1994) and mesenteric arteries (Beech et al, 1993; Quayle et al, 1995). There is compelling evidence to show that glibenclamide and other sulphonylureas bind to the SUR subunit which is an integral part of native $K_{ATP}$ channels. Indeed, glibenclamide has been used to purify the sulphonylurea receptor subunit (Aguilar-Bryan et al, 1990). The SUR receptor has since been shown to be intimately linked to Kir subunits, thus forming functional $\beta$-cell $K_{ATP}$ channels (Inagaki et al, 1995; Gribble et al, 1997). The specificity of sulphonylureas for the $K_{ATP}$ channel subunits is mostly assured (Wellman et al, 1996), however, there is some evidence from cloned channel studies to suggest that the SUR subunit may couple with other Kir subunits (Ämmälä et al, 1996). Some sulphonylureas have also been shown to inhibit other voltage-activated $K^+$ channels (Beech et al, 1993) but at much higher concentrations than that needed to block $K_{ATP}$ channels. Other non-selective $K_{ATP}$ channel blockers are summarised in table 1.2 (reviewed in Quayle et al, 1997).
Table 1.2  Some inhibitors of $K_{ATP}$ channels.

$K_i$, half maximal inhibition; U-37883, 4-morpholinecarboximidene-N-1-adamantyl-N'-cyclohexylhydrochloride

<table>
<thead>
<tr>
<th>NAME</th>
<th>$K_i$</th>
<th>NOTES</th>
<th>EXAMPLE REFERENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glibenclamide</td>
<td>20nM-3μM</td>
<td>(see text)</td>
<td>Beech et al, 1993</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Quayle et al, 1995</td>
</tr>
<tr>
<td>Tolbutamide</td>
<td>380μM</td>
<td>A sulphonylurea drug</td>
<td>Belles et al, 1987</td>
</tr>
<tr>
<td>Extracellular $Ba^{2+}$</td>
<td>~100μM (at -60mV)</td>
<td>$K_i$ for non vascular smooth muscle. Inhibition increases with membrane hyperpolarisation.</td>
<td>Bonev &amp; Nelson, 1993*</td>
</tr>
<tr>
<td>TEA$^+$</td>
<td>~7mM</td>
<td>Inhibits other $K^+$ channels</td>
<td>Beech et al, 1993</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Davies et al, 1989</td>
</tr>
<tr>
<td>5-hydroxydecanoate</td>
<td>0.16μM</td>
<td>Not tested on smooth muscle.</td>
<td>Notsu et al, 1992</td>
</tr>
<tr>
<td>Phentolamine</td>
<td>0.7μM</td>
<td>Inhibition only if applied intracellularly</td>
<td>Plant &amp; Henquin, 1990</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Dunne, 1991</td>
</tr>
<tr>
<td>U-37883</td>
<td>~1μM-0.26μM (Xenopus oocytes)</td>
<td>Does not effect pancreatic β-cells. Effect on vascular tissue unknown.</td>
<td>Meisheri et al, 1993</td>
</tr>
</tbody>
</table>

Activators of vascular $K^+$ channels cause hyperpolarisation of cells by causing $K^+$ efflux and moving the membrane potential towards $E_K$ (Challinor-Roger & McPherson, 1994). This hyperpolarisation results in the closure of voltage-operated $Ca^{2+}$ channels and a decrease in cellular $Ca^{2+}$ and vasorelaxation (Nelson et al, 1990). Several different criteria have to be met before a drug is classified as a $K_{ATP}$ channel opener (Quast, 1993; Nelson & Quayle, 1995):

I. The compound should be more effective at relaxing vessels at low external $K^+$ concentrations than that higher concentrations. At high $[K^+]_o$, there is little difference in ion concentrations between the internal and external environment and so upon channel activation there is an insufficient driving force for $K^+$ efflux.

II. A potassium channel opener should cause increases in $^{86}$Rb or $^{42}$K efflux leading to membrane hyperpolarisation of the cell.

III. The action of $K_{ATP}$ channel openers should be blocked by inhibitors such as glibenclamide.
Finally the compound should activate $K_{ATP}$ current in single cells.

Many structurally different compounds have been developed which all activate $K_{ATP}$ channels (reviewed in Andersson, 1992; Quast et al, 1994). Clinically, most are used for the treatment of cardiovascular disorders such as hypertension and angina. Synthetic $K_{ATP}$ channel openers include cromakalim, pinacidil, nicorandil, minoxidil sulphate and diazoxide. Vasodilatation to these compounds is blocked by glibenclamide but is unaffected by IbTX or ChTX (Standen et al, 1989; Wiener et al, 1991). The role of these drugs as vasodilators is discussed in section 1.8.4.

Over the last few years, the structure of the $K_{ATP}$ channel has been successfully elucidated in $\beta$-cells, and to some degree in cardiac and skeletal muscle. However intriguing questions remain, especially about the structure of the channel in vascular smooth muscle. Thus far, Northern blots from stomach tissue do not show the expression of Kir6.2 subunits. Kir6.1 mRNA has been detected in stomach, small intestine and colon (Inagaki et al, 1995; Isomoto et al, 1996). Native $K_{ATP}$ channels have been described in many types of smooth muscle, including vascular smooth muscle cells (Standen et al, 1989; Silberberg & van Breeman, 1992; Dart & Standen, 1993; Clapp et al, 1993; Xu & Lee, 1994; Quayle et al, 1994; Quayle & Standen, 1994). Studies on excised patches from the rabbit portal vein gave a $K_0$ value for ATP of 29 $\mu$M (Kajioka et al, 1991) or 200 $\mu$M (Kamouchi & Kitamura, 1994). In a similar manner to other tissues, the effect of ATP on single channel kinetics is to decrease channel openings by reducing the duration of burst of openings (Kamouchi & Kitamura, 1994). In ATP-free pipette solution a background $K^+$ current was detected which was then inhibited with the release of caged ATP by flash photolysis (Clapp & Gurney, 1992). The $K_{ATP}$ current was also blocked with 1 mM ATP in the intracellular solution. In excised patches, rundown of current was observed. This was subsequently reversed with MgATP (Kamouchi & Kitamura, 1994). Excised and cell-attached patches have been extensively used to resolve the conductances of $K_{ATP}$ channels from single channel recordings. The reported conductances in vascular smooth muscle show a large variability, however they fall into two broad groups; small/medium conductance and large conductances (reviewed in Quayle et al, 1997). Small/medium conductances have been measured in smooth muscle cells of the portal vein (Kajioka et al, 1990; Kamouchi & Kitamura, 1994), coronary (Miyoshi & Nakaya, 1991; Dart & Standen, 1995) and mesenteric arteries (Zhang & Bolton, 1995). Under conditions of symmetrical
high K⁺ (140 mM [K⁺]o, 140 mM [K⁺]i) conductances in the range of 20 to 50 pS were measured. Large conductance channels between 130 and 160 pS in high [K⁺]o and [K⁺]i have been reported in smooth muscle cells of mesenteric, tail and renal arteries (Standen et al 1989; Lorenz et al, 1992; Furspan & Webb, 1993). Data from whole cell macroscopic currents and excised patches single channels have been used to estimate channel numbers in a cell. Estimates thus far suggest that the K_ATP channel occurs in low density in the cell membrane. Most reports have calculated the number of K_ATP channels to be in the order of a few hundred. Measurements on currents activated by levcromakalim gave values between 300 and 500 (Noack et al, 1992; Bonev & Nelson, 1993b; Clapp et al, 1994). These low numbers give rise to high input resistances. The variation in reported properties suggests that different isoforms of the K_ATP channel may exist.

In common with other muscle cells, cytosolic ATP levels in vascular smooth muscle cells are high, in the millimolar range. Therefore it is thought that the primary role for ATP is to keep the activity of the K_ATP channel low and enable other factors to regulate it. Nucleotide diphosphates are also involved in the modulation of the channel. In excised patches, nucleotide diphosphates were able to activate a glibenclamide-sensitive K⁺ current in the presence of channel openers (Kajioka et al, 1991; Kamouchi & Kitamura, 1994). Recent molecular cloning evidence suggests the presence of a K_ATP channel which differs from the classical K_ATP channels cloned thus far. Yamada et al (1997) analysed the K⁺ channels composed of SUR2 and Kir6.1 subunits, co-expressed in HEK cells. In cell attached patches, pinacidil and nicorandil activated a K⁺ current with a single channel conductance of about 33 pS, and which was inhibited with glibenclamide. Surprisingly, in the absence of intracellular ATP the channel did not open and nucleotide diphosphates were able to induce channel activity.

The physiological role of K_ATP channels has been assessed in vascular tissue. K_ATP channels contribute towards membrane hyperpolarisation, and are sensitive to the metabolic state of the cell. The activation of K_ATP channels during impairment of metabolism occurs due to cytosolic changes in the cell and also because of the release of vasoactive agents from the surrounding tissue. A consequence of cellular metabolic stress is a fall in cytosolic ATP levels and an increase in ADP levels. As discussed previously, this promotes the opening of K_ATP channels. Activation of K_ATP channels has been shown to occur in the presence of metabolic poisons and hypoxia. Patch clamp recordings have
illustrated the activation of a $K_{\text{ATP}}$ current when oxidative phosphorylation and glycolysis were inhibited (Silberberg & van Breeman, 1992; Conway et al, 1994; Teramoto & Brading, 1996). Similarly adenosine, which is released during hypoxia or ischaemia, has also been shown to induce glibenclamide-sensitive currents in vascular smooth muscle (Dart & Standen, 1993; Kleppisch & Nelson, 1995). The role of $K_{\text{ATP}}$ channels in ischaemia is discussed in greater detail in chapter 5. Many substances released in vivo regulate $K_{\text{ATP}}$ channels (reviewed in Quayle et al, 1997). Daut and colleagues (1994b) reviewed the contribution of $K_{\text{ATP}}$ channels to the resting $K^+$ conductance in the coronary circulation. Here, $K_{\text{ATP}}$ channels contribute towards the resting membrane potential and may play a role in maintaining basal coronary tone in vivo (Samaha et al, 1992).

Endogenous vasodilators and vasoconstrictors play an important role in regulating vascular $K_{\text{ATP}}$ channel activity. Channels are opened by vasodilators that activate PKA (and PKG), and are closed by constrictors that activate PKC. Many vasoactive substances can also act on other ion channels leading to general alteration in smooth muscle activity (reviewed in Quayle et al, 1997). Relaxations to a number of PKA-coupled vasodilators are inhibited by glibenclamide (e.g. Quayle et al, 1994). Vasodilators coupled to PKA include CGRP (Holzer & Lippe, 1992), NA (Kitazono et al, 1993), prostacyclin (Holzmann et al, 1980) and adenosine (Kleppisch & Nelson, 1995). These vasodilators generally act on a G-protein coupled receptor on the cell membrane, resulting in the activation of $G_\alpha$-protein, which in turn stimulates AC. The enzyme catalyses the formation of cAMP which then goes onto activate PKA. In vascular smooth muscle, CGRP has been shown to activate $K_{\text{ATP}}$ currents in cells of the mesenteric (Nelson et al, 1990b; Quayle et al, 1994); cerebral (Kleppisch & Nelson, 1995) and coronary arteries (Miyoshi & Nakaya, 1995). CGRP stimulates AC (Holzer & Lippe, 1992) which predictably increases cAMP levels and so activation of PKA occurs. Rp-cAMPS, a membrane permeant inhibitor of PKA was shown to inhibit CGRP-induced $K^+$ currents (Miyoshi & Nakaya, 1995). Similarly, H-89, another blocker of PKA was also shown to inhibit CGRP-activated $K_{\text{ATP}}$ currents in pig coronary cells (Wellman et al, 1996b). Other peptides, such as vasoactive intestinal peptide (VIP) have also been shown to produce glibenclamide-sensitive hyperpolarisation in rabbit cerebral arteries (Standen et al, 1989). Endothelial factors such as prostacyclin can also cause smooth muscle relaxation. Iloprost, a stable analogue of prostacyclin, elevates cAMP levels (Kukovetz et al, 1979; Holzmann et al, 1980), and functional studies suggests that its vasorelaxation action is, in part, via the activation of
**K\textsubscript{ATP}** channels (Jackson et al, 1993). PKG is stimulated by the second messenger cGMP which in turn is produced by GTP by the enzyme guanylyl cyclase (GC). Two isoforms of the enzyme exist; a soluble GC (sGC) present in the cytoplasm and a membrane-bound particulate GC (pGC). Certain peptide vasodilators, such as atrial natriuretic peptide (ANP) (Ruskoaho, 1992) and C-type natriuretic peptide, produce their actions through the generation of cGMP by activating pGC. ANP has been shown to activate single K\textsubscript{ATP} channels in cultured vascular smooth muscle cells (Kubo et al, 1994). Similar currents were activated by 8-Br-cGMP, the membrane permeant analogue of cGMP. Both currents were then blocked with methylene blue, a GC inhibitor. In another experiment, Miyoshi et al (1994) demonstrated the activation of K\textsubscript{ATP} channels by NO-derived cGMP in coronary artery explants. NO has been shown to stimulate sGC (Koesling et al, 1991) leading to cGMP production. At the same time as the increase in cGMP occurred, a glibenclamide-sensitive K\textsuperscript{+} current was measured (Miyoshi et al, 1994).

In a similar manner to vasodilators, vasoconstrictors act on membrane bound G-proteins which are linked to G\textsubscript{q}-proteins. G\textsubscript{q}-protein stimulates phospholipase C leading to the generation of IP\textsubscript{3} and diacylglycerol (DAG). While IP\textsubscript{3} causes Ca\textsuperscript{2+} release from intracellular stores, DAG activates PKC (Berridge, 1993). The protein kinase phosphorylates target proteins such as the K\textsubscript{ATP} channel (Bonev & Nelson, 1996). In arterial smooth muscle cells isolated from rabbit mesenteric arteries, currents activated by pinacidil were inhibited by neuropeptide Y (NPY), NA (via \(\alpha_2\) receptors), 5-HT (through 5-HT\textsubscript{2} receptors) and HA (by H\textsubscript{1} receptor stimulation) (Bonev & Nelson, 1996). Bonev and Nelson went on to show that the K\textsuperscript{+} currents were inhibited with activators of PKC and a DAG analogue. In contrast, D609 and GF 109203X, inhibitors of PLC and PKC respectively, reduced the inhibitory effects of the vasoconstrictors (Bonev & Nelson, 1996). There are few other studies illustrating the inhibition of K\textsubscript{ATP} channels by PKC. Other vasoconstrictors, for example Ang II (Miyoshi & Nakaya, 1991; Kubo et al, 1997), ET (Miyoshi et al, 1994) and vasopressin (Wakatsuki et al, 1992) inhibit K\textsubscript{ATP} channels however their cellular transduction mechanism has not been elucidated yet. Figure 1.9. summaries the role of the protein kinases in regulating vascular smooth muscle K\textsubscript{ATP} channels. The resulting level of K\textsubscript{ATP} channel activity will depend on the activity of the two protein kinases, PKA and PKC, as well as other cellular regulators.
1.7 Diseases of the Vascular System

1.7.1. Hypertension

Hypertension is a common progressive disorder which is defined as chronically increased systemic arterial pressure. If it is not effectively treated, it can lead to an increased risk of coronary thrombosis, strokes and renal failure. Although changes in diet and exercise can be beneficial treatment, many hypertensive patients are treated pharmacologically. Antihypertensive drugs have to be used for an indefinite period, so the avoidance of side effects is an important consideration (Mills, 1989). Hence there is a clinical need for the development of satisfactory therapeutic regimes. As better drugs have been developed for
the treatment of hypertension, the drug regime a patient undergoes has also changed progressively (reviewed in Gavras et al, 1997). During the 1960s, centrally acting sympathetic-blocking agents were the main choice of treatment, however they caused severe side effects. Since then drugs with fewer side effects have been developed and are used, namely β-adrenoreceptor antagonists (β-blockers) and diuretics. More recently, drugs acting directly on small resistance arteries have been introduced. Examples include the Ca²⁺ channel antagonists, angiotensin converting enzyme (ACE) inhibitors and nitrovasodilators. The mechanism of action of these drugs is discussed in the next section.

There are few recognisable and treatable causes of hypertension (Vander et al, 1990). Severe episodes of hypertension can be caused by tumours of the chromaffin tissue which lead to excess catecholamine secretion. Other recognisable causes of hypertension include tumours of the adrenal cortex resulting in the release of steroid hormones and stenosis of the renal artery. However for over 95 % of persons with hypertension no obvious cause is found. Hypertension resulting from an unknown cause is termed essential or primary hypertension.

Hypertension is essentially due to the narrowing of arterioles which results in a rise in peripheral vascular resistance and therefore blood pressure. The exact nature of the arteriolar vasoconstriction is unknown but many different hypotheses have been put forward. Excessive Na⁺ ingestion may be a contributory factor in genetically pre-disposed people, as is obesity (Vander et al, 1990). Changes in ion transport have been identified in animal models of hypertension. In the spontaneously hypertensive stroke prone (SHRSP) rat, dilatation of small cerebral arteries by high external K⁺ is attenuated (McCarron & Halpern, 1990).

1.7.2. Angina

The pain associated with angina occurs when the blood supply to the heart is insufficient for its metabolic needs. Anginal pain has a characteristic distribution in the chest, arm and neck. Two kinds of angina are clinically recognised. Angina of effort (angina pectoris) is always produced by an increased demand for blood supply to the heart which occurs during exercise and excitement, and is usually due to narrowing of the coronary vessels resulting in decreased blood flow. Variant angina (Prinzmetal’s angina) is believed to be caused by a coronary artery spasm, sometimes alone but more commonly superimposed on
atheromatous narrowing (Rang et al., 1992). It is far less common, hence most studies for drug development have concentrated on the treatment of angina pectoris. However drugs used clinically today are able to relieve both types of angina to some degree. Anti-anginal drugs have been developed to either improve myocardial perfusion or reduce the metabolic demand on the heart, or both. Two main groups of drugs are used for the treatment of angina; the organic nitrates and the calcium channel antagonists. They act as vasodilators on coronary vessels to increase blood flow to the heart muscle. β-adrenoreceptor antagonists are also used as anti-anginal drugs because they affect the metabolic demand of the heart. Certain potassium channel openers have recently emerged as new therapeutic agents for angina (Cook, 1988; Cook & Chapman, 1993).

Narrowing of the arteries or coronary atherosclerosis is a major determinant in causing angina. The formation of an atherosclerotic plaque is initiated by injury to the arterial vessel wall. Risk factors in cardiovascular disease such as hyperlipidemia and hypertension damage the endothelium and alter endothelial cell function (Haller, 1996). The lesion leads to the invasion of pro-inflammatory cells, mainly macrophages and T-lymphocytes, into the endothelial cell layer. An essential prerequisite for atherosclerotic plaque formation is the accumulation of cholesterol esters. The inflammatory cells and the endothelial cells also release chemotactic factors resulting in the adherence of platelets and the development of a thrombus. This occlusion of the vessel leads to an interruption of the blood flow to the myocardium which can result in ischaemia or death ("infarction") of the tissue beyond.

Accumulation of cholesterol in macrophages occurs only during the diseased state due to modification of the naturally occurring lipid carrying molecules. Lipids are hydrophobic and so they are transported in the plasma as lipoproteins. Lipoproteins are made up of a core of hydrophobic lipids, consisting of triacylglycerols and cholesteryl esters, and a surface monolayer of phospholipids, unesterified cholesterol and proteins (apoproteins). Different types of lipoproteins exist and are classified according to their density; chylomicrons, high density lipoprotein (HDL), low density lipoprotein (LDL), very low density lipoprotein (VLDL). Dietary cholesterol is transported as chylomicrons to the liver where they may be secreted in the bile or be transported in the plasma as VLDLs. These can then go into muscle or adipose tissue where the triacylglycerols are hydrolysed by a surface-bound lipoprotein lipase. The smaller lipoprotein is now termed a
LDL and acts as the major reservoir of cholesterol for the synthesis of steroids and plasma membranes. These are also the most atherogenic of all the lipoproteins. HDLs carry cholesterol molecules that have been degraded from plasma membranes upon cell death (Goldstein et al, 1983). High levels of modified LDL are found to be present at sites of atherosclerotic lesions. Modification of LDL does not normally occur in the plasma due to a high antioxidant content. LDLs migrate into the vessel wall where they are oxidatively modified. Damaged endothelial cells, smooth muscle cells and macrophages present at the site release free radicals which are involved in the oxidation process. Superoxide ions initiate the peroxidation of LDL which eventually results in the formation of highly reactive aldehydes and ketones. These combine covalently with the surface apoprotein, thereby modifying the LDL and allowing it to be recognised by scavenger receptors present on the macrophage surface. They are then rapidly taken up and laid in the cells leading to the formation of foam cells (Witzum & Steinberg, 1991). Vascular endothelial cells in culture and in vivo have also been shown to internalise oxidised LDL through another independent receptor-mediated pathway (Sawamura et al, 1997). Oxidised LDL has the ability to stimulate the production of chemotactic factors and cytokines from endothelial cells and proinflammatory cells. This atherosclerotic plaque provides the grounds for the formation of a thrombus.

1.7.3. Arterial thrombus

The arterial thrombus, associated with atherosclerosis, is largely composed of platelets and T-lymphocytes. In an undamaged tissue, the endothelium plays an active role in the prevention of the formation of a thrombus. It releases anti-coagulation agents such as prostacyclin, plasminogen and heparin. Endothelial NO also inhibits platelet adhesion to the internal lining of the blood vessel, thus basal NO synthesis protects against thrombus formation and preserves the normal structure of the vessel wall (Lüscher, 1990). Apoproteins on the surface of modified LDLs compete with plasminogen at its receptor site, therefore decreasing the anti-coagulation properties of the endothelium and promoting the generation of a thrombus (Hajjer et al, 1989). Platelets adhere to the thrombus and are activated leading to a change of shape (Ware et al, 1993). The shape change causes the release of various biologically active substances either from intracellular stores or synthesised de novo. Fibrinogen, ADP and 5-HT are released from storage granules
whereas platelet activating factor (PAF) and thromboxane A$_2$ (TXA$_2$) are synthesised upon activation (Rang et al, 1992). These agents are potent aggregating substances and lead to the adherence of more platelets (Furie & Furie, 1992). The arterial thrombus is also able to bind and activate blood coagulation factors. Fibrin, the final product of the blood clotting cascade, is insoluble and traps blood cells and therefore reinforces the platelet plug. The aggregated platelets also play their role in the facilitation of blood coagulation by the availability of negatively charged phospholipids on their surface. These act as a binding site for the interacting coagulation factors. Thrombin, the enzyme involved in the formation of fibrin from fibrinogen, is also chemotactic and like TXA$_2$ can increase the aggregation of further platelets (Rang et al, 1992). This eventually leads to a self-perpetuating response which is essentially irreversible.

1.7.4. Ischaemia

Even though ischaemia is not a disease of the vascular system, it arises due to vasoconstriction (or blockage) of arterioles supplying an organ. This is particularly significant in the heart where insufficient blood supply to the myocardium can lead to death. Coronary ischaemia is usually due to the presence of atherosclerosis and results in anginal pain. The thrombus can ultimately cause myocardial infarction. Insufficient blood supply to the myocardium decreases intracellular ATP. This leads to the inhibition of ATP-dependent processes, in particular the Na$^+$/Ca$^{2+}$ exchanger and the Ca$^{2+}$ ATPase pumps on the plasma membrane and the sacroplasmic reticulum membrane. The resulting increase in cellular calcium is disastrous to the cell resulting in their death. The decrease in cellular ATP also activates K$_{ATP}$ channels (Kanatsuka et al, 1992). This a protective mechanism to redistribute blood around the ischaemic region. The process is irreversible, therefore the prevention of ischaemic damage is an important therapeutic consideration (Rang et al, 1992). The changes in metabolite concentrations, that occurs in ischaemia, is discussed in greater detail in chapter 5.

1.8 Pharmacological Vasodilators

A wide variety of drugs are able to cause vasodilatation by producing relaxation of vascular smooth muscle. Many of these drugs are used clinically for the treatment of
vascular disorders such as hypertension and angina. The drugs employ a number of
different mechanisms to cause vasorelaxation, including closing of Ca\(^{2+}\) channels, opening
of K\(^+\) channels, mimicking the actions of the endogenous vasodilator, NO, and inhibition
of the endogenous vasoconstrictor, Ang II. The pharmacology of vasodilators, namely
Ca\(^{2+}\) channel antagonists, ACE inhibitors, nitrovasodilators, K\(^+\) channel openers and finally
nicorandil is discussed below.

1.8.1. *Angiotensin converting enzyme (ACE) inhibitors.*

Ang II is a highly active endogenous vasoconstrictor. It is a product of the renin-
angiotensin system (reviewed in Valloton, 1987). ACE forms an important enzyme in this
pathway, the active product Ang II is formed from angiotensin I, the substrate for the
enzyme. Different isoforms of the enzyme ACE are present in different vascular beds, thus
providing a localised production of Ang II (Cockcroft *et al*, 1995). The renin-angiotensin
pathway plays a role in the pathogenesis of such diseases as hypertension, and hence is an
important target for therapeutic intervention. The pathway can be influenced at different
points, however the development of ACE inhibitors has proved the most productive
(reviewed in Johnston & Burrell, 1995; Abdelrahman *et al*, 1993). Several specific ACE
inhibitors have been developed, for example captopril, enalapril and ramipril (Cockcroft *et
al*, 1993). The drugs are analogues of the terminal dipeptide of angiotensin I, the naturally
occurring substrate. ACE is a carboxypeptidase which cleaves the basic residues, leucine
and histamine, from angiotensin I to give the active product. The ACE inhibitors compete
for the active site on the enzyme. They consist of a proline, a basically charged amino acid
residue, and a non-peptide molecule (Rang *et al*, 1992).

These compounds act selectively on vascular tissue to cause a fall in arterial
pressure and because ACE is present in high levels in certain vascular beds, such as in the
coronary circulation, the drugs have preferential action there. A side effect associated with
the use of ACE inhibitors is hypotension. In some patients renal failure can also develop
because filtration through the glomerulus is usually maintained by Ang II-induced
constriction of renal arterioles.
1.8.2. \( \text{Ca}^{2+} \) channel antagonists.

A pharmacologically and chemically diverse group of compounds make up the family of \( \text{Ca}^{2+} \) channel antagonists. They include verapamil, dihydropyridines (such as nifedipine), diltiazem and prenylamine. Their main mechanism of action is the prevention of \( \text{Ca}^{2+} \) entry across the cell membrane by inhibition of voltage operated \( \text{Ca}^{2+} \) channels (Cauvin \textit{et al}, 1983). The heterogeneity of \( \text{Ca}^{2+} \) channels ensures that the drugs exhibit specificity for channels in the cardiovascular system. The main channel occurring in vascular smooth muscle is the L-type \( \text{Ca}^{2+} \) channel (reviewed in McDonald \textit{et al}, 1994). The \( \text{Ca}^{2+} \) channel antagonists preferentially inhibit this type of channel, so their side effects in the central nervous system is limited, where N-, P-, and Q-type channels are prevalent (Tsien \textit{et al}, 1988). The drugs do not block \( \text{Ca}^{2+} \) release from intracellular stores such as IP\(_3\)- or ryanodine sensitive-\( \text{Ca}^{2+} \) channels in the sarcoplasmic reticulum. The physiological selectivity of the channels is also increased because they show voltage dependent inhibition, that is their block of channels is more prominent at depolarised potentials (Rang \textit{et al}, 1992).

The exact nature of block by these compounds has been investigated, and it was found that they act with more complexity than by simply blocking the channel pore. Experiments using excised patches showed that \( \text{Ca}^{2+} \) channels existed in multiple states. Upon depolarisation a channel can exist in one of three states; mode 0 when the channel is closed, mode 1 when a channel has a low probability of opening or mode 2 where a channel has a high opening probability (Hess \textit{et al}, 1984). Dihydropyridine antagonists bind selectively to the channel in mode 0 thus maintaining the closed state of channels. However dihydropyridine agonists, such as BAY K 8644 (Schramm \textit{et al}, 1983), bind to channels in mode 2. This helps to keep the channels open resulting in vasoconstriction.

Of the \( \text{Ca}^{2+} \) channel antagonists known, the action of dihydropyridines is mainly confined to smooth muscle. The other compounds have actions on the heart, causing cardiac slowing and decreasing force of contraction. The dihydropyridines act mainly on arterial vessels, rather than on veins, to cause generalised vasodilatation (Vatner & Hintze, 1982). The main clinical uses of \( \text{Ca}^{2+} \) channel antagonists is in the treatment of hypertension or angina and in prevention of arrhythmias due to inhibition of fibrillation. In the treatment of hypertension, the drugs cause vasodilatation and a decrease in heart rate,
hence they effectively lower blood pressure. Dihydropyridines are used for the treatment of angina due to their effectiveness in causing vasodilatation.

Side effects observed with the use of calcium channel antagonists are a direct consequence of their effect on cardiac and smooth muscle. Unwanted effects include constipation, postural hypotension, headache and flushing (Rang et al, 1992).

1.8.3. Nitrovasodilators.

The usefulness of organic nitrates for angina was discovered many years ago. Amyl nitrate, synthesised in the late 1850s, was shown to cause tachycardia and a fall in blood pressure when its vapour was inhaled. The drug was used for many years before being replaced with glyceryl trinitrate (GTN). This drug is the first choice of treatment for acute attacks of angina. Pentaerythritol tetranitrate and isosorbide dinitrate are newer nitrovasodilators with longer duration of actions.

All organic nitrates were known to act by relaxing smooth muscle, however their exact mechanism of action remained elusive. Nitrovasodilators were shown to cause an increase in intracellular cGMP formation (reviewed in Waldman & Murad, 1987) and presumably stimulation of PKG. The kinase has been proposed to have multiple actions on the cell. Elevations in intracellular cGMP were shown to precede the relaxation of smooth muscle (Lincoln, 1989). Studies suggest that PKG-stimulation can lead to MLCK phosphorylation (Nishikawa et al, 1984). Other studies show that cGMP and invariably PKG affect Ca^{2+} entry and exit processes, and therefore cytosolic Ca^{2+} concentrations (Pfitzer et al, 1982; Hester, 1985; Karaki et al, 1988; Taylor & Meisher, 1986). It has also been reported, from studies using the whole cell patch clamp technique, that cGMP and its derivatives opened BK_{Ca} channels in vascular smooth muscle cells (Williams et al, 1988; Robertson et al, 1993). Other K+ channels such as K_{ATP} channels are also thought to play a role in cGMP-mediated vasodilatation in some tissues, for example pial arteries (Armstead, 1996). The discovery of the mechanism of action of organic nitrates has helped in the identification of EDRF as NO.

Although the organic nitrates have been shown to relax many kinds of smooth muscle, their main effect is on the cardiovascular system (Harrison & Bates, 1993). The effectiveness of glyceryl trinitrate and other nitrates in angina is due to their ability to cause vasodilatation of collateral coronary vessels. This leads to a more effective
distribution of blood flow away from an occluded artery to underperfused regions of the heart. As the nitrates act directly to relax the vessel wall, they are also effective against variant angina. Because of the NO-producing ability of nitrovasodilators it was proposed that they may also have an antithrombotic role (Lam et al, 1988). Indeed, experiments went onto show that GTN, at therapeutic doses, had effects on platelet aggregation (Diodati et al, 1990) and inhibited the decrease in blood flow caused by the thrombus formation (Folts et al, 1991). Therefore it appears the anti-aggregatory action of nitrovasodilators plays a part in the overall vasodilator response of these drugs (reviewed in Abrams, 1996).

The main problem seen with the use of nitrates is the development of tolerance with repeated administration. This is more clinically important in the drugs with longer duration of action. Other adverse effects include postural hypotension and headache.

1.8.4. K+ channel openers.

Electrophysiological and ion flux studies revealed that certain compounds were able to cause hyperpolarisation and increase the efflux of 86Rb across the cell membrane (Furukawa et al, 1981; Weir & Weston, 1986). These effects were blocked by K+ channel antagonists such as TEA+ ions (Wilson et al, 1988). This suggested that these compounds caused the opening of membrane K+ channels. This discovery coincided with studies demonstrating the existence of KATP channels in various cell types. Drugs such as cromakalim (a benzopyran), pinacidil (a cyanoguanidine), minoxidil (a pyrimidine), diazoxide (a benzo thiadiazine) and nicorandil (a pyridine derivative) are thought to cause vasodilatation through activation of KATP channels, since their actions could be antagonised by glibenclamide (Buckingham et al, 1989; Eltze, 1989; Winquist et al, 1989). These compounds were initially termed "K+ channel openers" rather than "agonists" or "activators" because their exact mechanism of action was unknown. The drugs were subsequently shown to cause smooth muscle relaxation by opening KATP channels and increasing K+ efflux, thus causing membrane hyperpolarisation and decreasing the excitability of the cell (reviewed in Edwards & Weston, 1996).

The prototype K+ channel opener was cromakalim. Since then other structurally dissimilar compounds have been developed. To observe if any of the compounds interacted with the glibenclamide binding site on the SUR subunit, dissociation studies were done. However the KATP channel openers did not inhibit [3H]-glibenclamide binding
(Panten et al, 1996). But recent molecular studies have shown that the SUR subunit is important for determining the sensitivity of the openers. Co-expression of Kir6.2 with SUR1 produces channels that are activated by diazoxide (Inagaki et al, 1995b) while co-expression with SUR2A induces channels that are sensitive to cromakalim and pinacidil but not diazoxide (Inagaki et al, 1996). These results suggest that the SUR receptor confers K⁺ channel opening sensitivity onto the Kₐ₅₆ channel, however the exact site of binding for these compounds is unknown.

Openers of Kₐ₅₆ channels provide new therapeutic potential for the treatment of such disorders as hypertension and angina (reviewed in Andersson, 1992). Both pinacidil and cromakalim have undergone clinical trials for use as anti-hypertensives in conjunction therapy with thiazide diuretics (Friedel & Brogden, 1990). Nicorandil is used clinically for the treatment of angina and will be discussed in greater detail below.

1.8.5. Nicorandil.

2-nicotinamidoethyl nitrate, known as nicorandil, provided the first evidence that a synthetic vasodilator caused vasorelaxation by activating K⁺ channels (Furukawa et al, 1981). Using sharp electrodes to measure membrane potential, nicorandil was shown to hyperpolarise the membrane potential in smooth muscle cells towards Eₖ by increasing K⁺ conductance (Furukawa et al, 1981). In contrast to other Kₐ₅₆ channel openers nicorandil was still able to produce some vasorelaxation in high [K⁺]₀ suggesting that other mechanisms also contribute to its action (Inoue et al, 1984). Figure 1.10. shows the chemical structure of nicorandil, which has a nitrate group that allows the compound to act as a nitrovasodilator. Nicorandil is therefore able to produce vasodilatation through the activation of Kₐ₅₆ channels and through cGMP-mediated effects due to stimulation of guanylyl cyclase (Holzmann et al, 1992). Both these mechanisms act in concert but the balance between the K⁺ channel opening and cGMP-related effects varies between different preparations (Magnon et al, 1993). The experimental evidence for nicorandil-induced relaxation is discussed in chapter 3 (section 3.1).
Animal trials with nicorandil have shown that it causes substantial dilatation of arterial vessels due to its $K_{\text{ATP}}$ channel opening ability and dilation of venous capacitance vessels because of GC stimulation (Kinoishita & Sakai, 1990). Unlike organic nitrates, the development of tolerance does not occur with nicorandil in either animal or clinical trials. Studies in dogs demonstrated that a constant intravenous infusion of nicorandil caused an increase in coronary artery diameter which was maintained for the five day period of the trial (Huckstrol & Bassenge, 1992). This was in contrast to a long-term infusion of GTN, which caused an initial coronary vasodilatation which decreased slowly with time (Stewart et al, 1987). Wagner (1992) showed that in sixteen healthy patients the effect of nicorandil on total peripheral resistance was unchanged over seven days whereas responses to GTN faded. It was initially thought that the short half life of nicorandil in the body may account for its lack of tolerance, however no cross-tolerance was observed between nicorandil and GTN. Therefore the lack of tolerance observed with nicorandil has been attributed to its $K_{\text{ATP}}$ channel opening ability (O’Rourke, 1996). Several clinical trials have demonstrated the effectiveness of nicorandil on angina pectoris (reviewed in Drugs Therapeutics Bulletin, 1995). The possible use of nicorandil for variant angina has also been investigated with favourable results (Kishida & Murao, 1978).

In pharmacological doses, the major side effect seen with nicorandil is headache. However the headache usually disappears with continuous treatment (Meeter et al, 1992). Also, compared with the calcium channel antagonists, fewer side effects from peripheral vasodilatation are noted after treatment with nicorandil (Gopalakrishnan et al, 1993).

### 1.9 Aims of the Study

There are few studies on the actions of nicorandil in resistance vessels. As an anti-anginal drug, nicorandil is likely to act on these arteries in vivo. Nicorandil has been
shown to cause vasodilatation via two mechanisms, by $K_{\text{ATP}}$ channel opening and stimulation of GC (Holzmann \textit{et al}, 1992). Studies suggest that the dominant mechanism is both tissue- and concentration-dependent (Yoneyama \textit{et al}, 1990; Kreye \textit{et al}, 1991; Kukovetz \textit{et al}, 1992). I aimed to study the mechanism of action of nicorandil in small arteries of the rat mesenteric vascular bed. I used the small vessel wire myograph to measure nicorandil-induced vasorelaxations on arterial ring segments contracted using 20 mM K$^+$ solution and BAYK 8644. In addition, whole cell recordings were obtained from acutely dissociated smooth muscle cells using the whole cell patch clamp technique.

The participation of other K$^+$ channels in nicorandil-induced hyperpolarisation cannot be excluded. Robertson \textit{et al} (1993) showed that BK$_{\text{Ca}}$ channels could be activated by PKG. Khan \textit{et al} (1993) also showed that vasodilator responses of the nitrovasodilator GTN was blocked with IbTX and ChTX, inhibitors of BK$_{\text{Ca}}$ channels. The second aim of my study was to determine if the nicorandil-induced hyperpolarisation was, in part, through the activation of BK$_{\text{Ca}}$ channels.

The opening of vascular $K_{\text{ATP}}$ channels has been shown to be enhanced by metabolic compromise (e.g. Silberberg & van Breeman, 1992; Zhang & Bolton, 1995; Ishizaka & Kuo, 1996). Furthermore, Randall & Griffith (1993) demonstrated that the vasorelaxant actions of levcromakalim, though not of pinacidil, were enhanced by inhibition of metabolism or by hypoxia. The final aim of my study was to investigate if the potency of nicorandil was increased under conditions of metabolic inhibition or in the presence of factors which occur in ischaemia. This study was done on resistance arteries of the pig coronary circulation because it is a clinically more relevant vessel.
CHAPTER 2

Methods
2.1 Preparation of tissue

2.1.1. Dissection of rat mesenteric arteries.

A rich blood supply is required by the intestinal wall for contraction and for the absorption of products of digestion. The superior mesenteric artery is the largest of the abdominal aortic branches supplying all of the small intestine with blood. Many branches run from it to different regions of the intestine, forming the mesenteric bed of blood vessels (Greene, 1963). The small arterial vessels lying in this mesenteric vascular bed were used for the experiments.

Adult male Wistar rats (greater than 300 g in weight) were killed by cervical dislocation. The abdomen was cut open and using fine forceps the intestine was laid out. Approximately 5 cms of the mesenteric vascular bed, along with the primary branch of the superior mesenteric artery supplying that region of the gut, was carefully cut out and placed in ice cold 5 mM K⁺ physiological salt solution (PSS), see the Solutions section at the end of this chapter for composition. The dissection of the mesenteric arteries was done at room temperature. The preparation was pinned out onto a Sylgard-filled petri dish in such a way that the most visible blood vessels (the mesenteric veins) were uppermost, whilst care was taken not to stretch the vessels. This was so that the main vessel lay at the top of the petri dish with the vascular "branches" lying downwards, towards the investigator. The dish was filled with normal 5 mM K⁺ PSS to cover the tissue. The preparation was illuminated with a fibre optic light source and viewed using a dissecting microscope. The vessels on top were the mesenteric veins which were dissected out using microscissors and watchmaker forceps. This dissection led to the visualisation of the arteries underneath radiating out from the larger primary branch of the superior mesenteric artery. The first branches from this artery were termed second order branches, the branches leading from these were termed third order and so forth till the distal end. For all my experiments I used second and third order branches of the artery. The segments to be used were carefully cleaned of surrounding adipose and connective tissue before being cut out. These sections were cut into 1-2 mm rings and stored on ice, in 5 mM K⁺ PSS.
2.1.2. Dissection of porcine coronary arteries.

The heart is a muscular pump responsible for blood circulation. Coronary arteries provide an extensive and plentiful blood supply to the heart in order to maintain its ability to contract and pump blood. The left coronary artery arises from the left aortic sinus behind the pulmonary trunk. The vessels split into two in between the left atrium and ventricle. One branch, the left anterior descending (LAD) coronary artery runs down the interventricular groove.

Fresh pig hearts were obtained from a local abattoir. During their transit to the laboratory, they were kept in ice cold heart collecting solution, composition of the solution was the same as the 5 mM K⁺ solution (see the Solutions section at the end). The LAD artery and surrounding cardiac tissue was cut out and washed in ice cold 5 mM K⁺ PSS solution. This piece of tissue was pinned out on Sylgard in a large petri dish and covered with 5 mM K⁺ PSS solution. The dissection was done at room temperature. The LAD artery was viewed under an fibre optic light source, and using microscissors and watchmakers forceps the artery was cleaned of surrounding adipose and muscular tissue. The vessel was then viewed under a dissecting microscope for the finer dissection. Small arterial vessels, usually second and third order branches leading away from the LAD artery, were carefully dissected out. The vessels were cut into 1-2 mm rings and kept in ice cold 5 mM K⁺ PSS solution. Some of the arterial rings were refrigerated for use the following day.
2.2 Myograph experiments

2.2.1. Principles of the use of the small vessel myograph.

This technique allows the investigation of vessels with diameters in the range of 100-1000 μM. The method is relatively atraumatic to the tissue and provides measurements of the force developed during isometric contractile responses. Isometric contractions are produced under conditions where significant shortening of the preparation is prevented. Procedures previously known for measuring the tension of blood vessels relied on the direct manipulation of the tissue. This involved the attachment of a mounting wire to the vessel and stretching the arteries longitudinally. A similar method can not be successfully used for small vessels without causing damage. The integrity of the vessels are maintained because it has been shown that the three main components of the blood vessel wall (intima, media and adventitia) can be distinguished after mounting (Mulvany et al, 1978). The characteristic of the myograph mounting procedure is such that it does not rely on the direct attachment of a wire to the vessel segment, instead a mounting wire is threaded through the vessel lumen and secured under tension on both sides therefore allowing isometric contraction to occur. The arterial ring segments are stretched horizontally, hence the shear force is the same along the length of the vessel wall.

The small size of resistance vessels discouraged investigators from undertaking mechanical experiments, hence information about these vessels was inferred from perfusion experiments and histological examination. In 1972, Bevan and Osher presented a technique for making measurements in small vessels. This was then adapted by Mulvany and Halpern (1976). Then in 1980, Mulvany & Halpern introduced a myograph for simultaneous measurements of two vessels. In the experiments described in this thesis, a Myo-interface model 500A myograph was used. This allows the testing of two vessel segments and has an automated system for adjusting the vessel extension, thereby enabling automatic normalisation, calibration and data acquisition.
2.2.2. Arrangement of the myograph.

Figure 2.1 The general arrangement of the Myograph model 500A, and components needed for experiments.
The vessels are mounted within two supports, suspended in a stainless steel chamber. Further detail of the mounting procedure is given in the next section. The supports are fixed to a force transducer and micrometer respectively. An arterial ring segment is held in place, with mounting wires, between a transducer and micrometer. The force transducer is a strain gauge transducer which is connected in a Wheatstone bridge. It is encapsulated in a housing unit for protection from saline solutions and mechanical damage. The micrometers are used to determine the extension of the vessels. On the 500A model, these are motorized, and the readings are displayed on the myograph controller. Figure 2.2 shows a diagram of the myograph chamber in greater detail. The two set of supports are referred to as "near" and "far" with respect to the operator.

![Diagram of myograph chamber.](image)

A plastic cover is placed over the chamber to reduce evaporation. Ports on the chamber cover allow the administration of drugs, aeration of the chamber with oxygen and removal of solutions. Oxygen from a cylinder is slowly bubbled into the chamber. A vacuum pump is used to empty the chamber. The myograph block also has a heater which maintains the chamber at a constant predetermined temperature. The temperature of the
solution in the chamber can be measured via a thermistor probe inserted through a port in the plastic chamber cover.

The myograph block is attached to the Myo-interface. A controller unit is also linked to the interface. It is used to maneuver the micrometers and perform functions such as normalisation and calibration procedures. The controller unit also has a screen for continuous viewing of vessel force, extension and bath temperature. Also connected to the interface is a pen recorder which is used for data acquisition.

2.2.3. Experimental procedure.

A) Calibration

Prior to performing experiments the transducers were calibrated. The principle of calibration is that when a load \( W \) is applied, the transducer is subjected to a force \( F_{\text{transducer}} \) which is equal to

\[
F_{\text{Transducer}} = W \cdot g \cdot (\text{arm ratio}) \quad (1)
\]

where \( g \) is the gravitational acceleration, i.e. \( 9.81 \, \text{m.s}^{-2} = 9.81 \, \text{mN/gm} \); \text{arm ratio} is given as the distance of the pan arm over the distance of the transducer arm.

To calibrate the myograph, a wire was mounted onto the transducer side. The holder for the calibration balance was placed on the top of the chamber. The calibration balance was placed in the holder with the tip dropping down behind the wire, so that it just touched it. For a more accurate calibration, the calibration apparatus must be at the same temperature as the myograph, hence the chamber was heated to 37 °C. A 2 g weight was placed on the top of the balance. The arm ratio was calculated to be 0.5 because the pan arm length=2 cm, and the transducer arm length=4 cm. The transducer was then calibrated to an output of 9.81 mN.

B) Mounting of tissue

The heater was switched on to maintain the chamber at 37 °C. The myograph chamber was filled with 5 mM K+ normal PSS which had been kept at 37 °C in an heated water bath. A length of 40 \( \mu \text{M} \) stainless steel wire was cut and secured under one of the fixing screws of the “near” transducer. The wire was wound around such that the tightening of the screw clockwise tended to tighten the wire. The free end of the wire was placed between the mounting supports. Using forceps, a 2 mm arterial ring segment was transferred from the ice cold 5 mM K+ PSS buffer into the myograph
chamber. The vessel was carefully threaded onto the free end of the wire. It was then fed along the wire, taking care not to stretch it, until it was situated between the supports. The wire was secured to the other fixing screw on the “near” transducer, refer to figure 2.2. A second piece of wire was then fed through the lumen of the vessel and secured onto the “near” micrometer fixing screws opposite. The tissue was now held securely between the “near” transducer and micrometer. This mounting procedure was then repeated for the “far” transducer. Such a mounting procedure ensured that the endothelium remained intact. The plastic cover was replaced above the chamber and the vessels were oxygenated. The solution in the chamber was then replaced with 0Ca²⁺ PSS (composition given in section 2.4) to prevent calcium-dependent contractions during normalisation. The pen recorder was then switched on to record the contractile responses of the vessels.

C) Normalisation The normalisation procedure was used to set the resting size of a vessel that had been mounted. The vessel size is defined as being the size when it is fully relaxed and under a transmural pressure of 100 mmHg. The procedure determined the internal circumference, denoted IC₁₀₀, of the vessel. On the 500A Myo-interface, the normalization procedure can be performed automatically, however I will briefly explain the theory.

The normalisation procedure is performed by distending the vessel in a stepwise manner, and then measuring sets of micrometer readings and force readings on a pen recorder. Each pair of recordings is used to calculate the effective pressure corresponding to each distention. The effective pressure, $P_i$, is given by the Laplace equation,

$$P_i = \frac{\text{Wall tension}}{\left(\frac{\text{internal circumference}}{2\pi}\right)}$$

(2)

The wall length of the vessel is determined using a measuring eyepiece in the dissecting microscope (the wall length is twice the segment length because there is an upper and a lower wall). The wall tension is then measured as the force, recorded on a pen recorder, divided by the wall length. The internal circumference can be calculated from the micrometer readings of the distance between the wires and the known diameter of the mounting wires.

The stepwise distension of the vessel is stopped when the effective pressure exceeds 100 mmHg, and by definition 100 mmHg=13.3 kPa. Determinants of wall tension
and internal circumference are plotted and then fitted with an exponential curve. The point on the curve corresponding to 100 mmHg (13.3 kPa), as calculated using the Laplace equation, is denoted as the IC$_{100}$. The internal circumference of the vessel is set to IC$_1$, which is 0.9IC$_{100}$. It has been shown that at IC$_1$ the active force production of the vessel is maximal (Mulvany, 1992).

D) Measurement of responses

Once the normalisation procedure was completed, the chamber was refilled with 5 mM K$^+$ normal PSS and the vessels were left to equilibrate for about 30 mins. After this time, the vessels were contracted with a combination of 20 mM K$^+$ PSS (see Solutions for composition) and 200 nM BAYK 8644, the calcium channel opener (20K and BAYK). The combination of 20K and BAYK produced a stable contraction whilst maintaining the potassium equilibrium potential sufficiently negative (~-52 mV) in order for potassium channel openers to cause hyperpolarisation and relaxation. In my experiments the endothelium was not actively removed because its removal may have caused damage to the underlying smooth muscle cells. Therefore endothelium-derived NO production was decreased with 20 μM Nω-nitro-L-arginine methyl ester (L-NAME). L-NAME inhibits endothelial NOS in a concentration-dependent manner, at concentrations of 0.1 to 100 μM (Rees et al, 1990). Investigators have shown that the inhibitor was effective at low concentrations in vascular tissue (Ralevic et al, 1991; Vo et al, 1992; Zembowicz et al, 1993; Vials & Burnstock, 1993).

Once a stable contraction had been seen to be produced on the pen recorder, after approximately 40 mins, cumulative concentration response curves to relaxants were performed. Each required concentration was added in to the bath when the response by the previous concentration had reached a stable level. On occasions, the tissues were contracted using 60 mM or 80 mM K$^+$ PSS. This made the potassium equilibrium potential more positive, thus decreasing the driving force on K$^+$ ions. Under these conditions, K$^+$ channel openers are unable to cause hyperpolarisation and relaxation. After finishing a cumulative concentration effect curve, the chamber was washed out with 5 mM K$^+$ PSS and the tissue was allowed to settle until a stable baseline was re-established. Multiple concentration response curves were performed in a day where possible. All measurements of changes in tone were then obtained from the pen recorder traces.
2.2.4. Analysis.

The decrease in tone (relaxation) was measured as a percentage of the maximal contraction generated with 20K and BAYK. The difference between the maximum tone and the baseline just prior to the contraction was measured. The reduction in tension produced by the addition of a relaxant agent was measured, and expressed as a percentage of this maximal contraction. Concentration effect curves were generated using a graphical computer package. The curves were fitted with the expression

\[
y = M \left[ \frac{1}{1 + \left( \frac{k}{x} \right)^n} \right]^{-1}
\]

where \( y \) is the % relaxation; \( x \) is the agonist concentration; \( M \) is the maximum response; \( k \) is the EC_{50} and \( n \) is the Hill coefficient, using the least squares algorithm in Sigmaplot (Jandel Scientific). EC_{50} values were obtained from individual curves as the concentration at which half maximal reduction in tone occurred.

EC_{50} values were converted to negative log EC_{50} values (pD_2) for statistical analysis. Statistical significance of the data was assessed using ANOVA and Student’s t-tests. All values are given throughout as mean pD_2±S.E.M. Student’s t-tests were used for simple comparison, whereas ANOVA tests were used for multiple comparisons.

A Student’s t-test is used to determine if two data columns are significantly different from each other, by testing the hypothesis that the means of the two columns are equal. An analysis of variance (ANOVA) is used to determine if the means of two or more groups of data are significantly different. The ANOVA test tells you if any of the group means are different, however a *post-hoc* test then needs to be done to determine exactly which groups are significantly different.


2.3 Patch clamp experiments

2.3.1. Cell dissociation.

The cell isolation method used to yield smooth muscle cells was adapted from that described by Quayle et al (1994). A 2-stage digestion was performed at 35 °C. For the dissociation of mesenteric arteries, small pieces of artery were added to an enzyme mixture containing 1.5 mgml⁻¹ papain (Sigma), 1.0 mgml⁻¹ dithioerthytiol (Sigma) and 1 mgml⁻¹ bovine serum albumin (Sigma), dissolved in low Ca²⁺ isolating buffer (see Solutions). The enzyme solution was kept in the water bath for 5 mins prior to the addition of the arterial segments. The pieces of artery were incubated in the enzyme mixture for 40 mins before being transferred to a second enzyme mixture containing 1.5 mgml⁻¹ collagenase F (Sigma) and 1.0 mgml⁻¹ hyaluronidase (Sigma). As for the first stage, the enzymes were dissolved in low Ca²⁺ isolating buffer containing 1 mgml⁻¹ albumin, and kept in the water bath for 5 mins before transferring the tissue into the mixture. The arterial segments were incubated in the second enzyme mixture for a further 20 mins. The enzyme mixtures was made up freshly every day.

After the digestion period, the tissue was carefully washed with fresh low Ca²⁺ isolating buffer solution, using a wide-bore flamed glass pipette. Single smooth muscle cells were dispersed by gentle trituration. The cells were left on ice and used over the next 4-6 hours.

2.3.2. Principles of the patch clamp technique.

The principle of the patch clamp technique is to isolate a patch of membrane, and measure current flow across the membranes of cells which are too small to be penetrated by a microelectrode. It was first introduced by Neher and Sakmann in 1976, who measured currents through single acetylcholine-activated channels of the frog skeletal muscle. The method involves the isolation of a patch of cell membrane electrically from the external solution. This is achieved by using a fire-polished patch pipette filled with an appropriate solution. Pressing the clean patch pipette against the surface of the cell and then applying light suction leads to the formation of a high resistance seal. Initial experiments, such as those by Neher and Sakmann (1976) and Neher and Steinbach (1978), only allowed the
study of channels with a high conductance due to the seal resistance being less than 100 MΩ. However in the early 1980s the ability to achieve “gigaohm seals” (Hamill et al, 1981) greatly improved the quality of recordings and also enabled investigators to study channels with much lower conductances. A high resistance seal has a number of advantages;

1. It ensures currents flow into the microelectrode rather than out under the seal, because currents will always flow through the path of least resistance, therefore electrical isolation of the cell is achieved.

2. It greatly reduces the noise of the recording, that is the background current noise ($I_n$) which can be derived from the expression

$$V_n = \sqrt{4kTRF}$$

where $V_n$ is the voltage (V) noise, $k$ is the Boltzmann constant ($1.38 \times 10^{-23} \, \text{J/°C}$), $T$ is the absolute temperature (°K) and $F$ is the bandwidth. Using Ohm's Law, current noise ($I_n$) is given by

$$I_n = \sqrt{\frac{4kTF}{R}}$$

where $R$ is the resistance (Ω). The equation can then be rewritten in the form of,

$$I_n^2 = \frac{4kTF}{R}$$

Therefore, from equation (5) the higher the resistance, the lower the current noise thus allowing investigators to observe single channel events.

Once a gigaohm seal is achieved, a number of different configurations can be used to study ion channels in the membrane, summarized in figure 2.3.
Figure 2.3  Possible configurations available to study ion channels, using the patch clamp method.

Individual channels can be studied using the cell attached (1), outside-out or inside-out patch configurations. An inside-out patch (2) is produced when a patch pipette is pulled away from the cell attached mode leaving a patch of membrane in the pipette tip whose intracellular surface is exposed to the bath solution. Further suction applied to the cell once a seal has formed leads to a breakdown of the membrane isolated in the pipette resulting in the formation of the whole cell recording configuration (3). If the patch pipette is then pulled away, some membrane comes away and reseals to give an outside-out patch (4) whose intracellular side faces the pipette solution. Use of ionophores, such as amphotericin and nystatin, in the intracellular patch solution causes permeabilisation of the membrane. The perforated whole cell configuration (5) has the advantage that it prevents the loss of high molecular weight components from with the cell. The disadvantages with this are that the ionophores are light and temperature sensitive and they prevent seal formation.

After sealing onto a cell, it is possible to voltage clamp the cell and so record currents across the membrane. In voltage clamp, the current measured is that required to maintain the membrane potential at a predetermined voltage. By clamping the membrane
potential, the investigator ensures that the whole cell current measured is linearly proportional to the ion channel activity because

\[ I = N_i P_o \]  \hspace{1cm} (7)

where \( I \) is the whole cell current; \( N \) is the number of channels; \( i \) is the single channel current; and \( P_o \) is the open probability or activity of an ion channel. Since \( N \) and \( i \) (at a given voltage) are constant, it can be said that \( I \) is proportional to \( P_o \).

The whole cell voltage clamp technique provides investigators with the versatility to control and perfuse agents into the intracellular environment of a cell. The measurement of macroscopic currents can then be used to plot current-voltage (I-V) relationships, and activation/inactivation curves, thus providing ways to characterize the ion channel under study. However with time there is a loss of important cellular components due to diffusional equilibrium being reached between the patch pipette solution and the contents of the cell, which may lead to rundown of channel activity.
2.3.3. *Arrangement of the patch clamp set up.*

*Figure 2.4* Schematic diagram of the laboratory patch clamp set up.

- 486 DX2 personal computer (Powerpaq)
- Analogue to digital Converter
- Digidata 1200 Interface
- Bessel Filter
- Oscilloscope (Tektronix)
- Digital audio tape recorder (DTC-1000ES, Sony)
- Patch clamp amplifier (Axopatch-1B, Axon Instruments Inc.)
- Headstage (CV-3[gain 1/100], Axon Instruments Inc.)
At the start of the Ph.D., I assembled the necessary components for a functional patch clamp set up so that whole cell currents could be recorded from acutely dissociated smooth muscle cells.

One or two drops of the isolating buffer containing the triturated cells was placed in a 35 mm petri dish. A perspex block was clamped onto the microscope stage, it had a hole in it to house the petri dish. The perspex block could then moved around the microscope stage. The cells were illuminated from above. An inverted microscope (Zeiss, Germany) was used to view the cells. Shiny, smooth surfaced relaxed cells were chosen for patching. A hydraulic manipulator (Narashigie Instruments Ltd., Japan) was used to position the headstage and patch pipette into the chamber. An external micromanipulator controller allowed fine movement of the patch pipette once near the cell.

It was necessary to isolate the microscope, headstage and micro manipulator both electrically and physically. The anti vibration table (Technical Manufacturing Corporation, USA) consisted of a heavy metal slab on pneumatic supports. The metal slab was supported on air-filled inner tubes thus sudden changes in air currents and vibrations were prevented. A Faraday cage was placed above the table to minimize external electrical noise. The cage was connected to the signal earth of the microelectrode amplifier. Electrical pick-up introduces noise into the system which can distort and overpower the recordings. The currents and channel openings recorded are so small that all electrical noise has to be minimized. Different types of electrical interference can be observed. Examples of radiative electrical pick-up include frequency noise from lights, power sockets and computers. Shielded BNC cables and shielding was used to reduce this type of noise. Local shielding with foil was also used to reduce noise. However if the shielding is earthed in more than one place, ground-loop noise is introduced into the system. Also if different grounds are used, a difference in potential may arise leading to a current flow and further noise. To eliminate ground-loop noise, all the shields or earthing leads are connected together and grounded at one place such as the signal ground on the microelectrode amplifier. The signal ground was then connected to a wall socket.

The headstage is securely bolted onto the micromanipulator and holds the patch pipette holder. A chlorided silver wire protrudes out of the holder onto which the patch pipette is inserted. The opening and closing of ion channels causes a current to flow ($I_m$) into the patch pipette which is measured within the headstage. A command potential
(V\textsubscript{Comm}) can also be applied to the cell via the headstage. The headstage essentially consists of two amplifiers (A\textsubscript{1} and A\textsubscript{2}) and a high resistance feedback resistor (R\textsubscript{f}), a schematic diagram of the circuitry is shown in figure 2.5. A\textsubscript{1} and A\textsubscript{2} are operational amplifiers, and they have two inputs (+ and -) and one output. An operational amplifier aims to keep both its inputs at equal potential. The flow of a membrane current (I\textsubscript{M}) will cause a change at the negative input resulting in a difference between the two inputs. A counteracting current (-I\textsubscript{M}) of equal magnitude therefore flows through the feedback resistor to maintain the negative input at the same potential as the positive input. The output from the first amplifier (V\textsubscript{1}) is therefore -I\textsubscript{M}.R\textsubscript{F}, according to Ohm's Law (equation 2, section 1.5.2.). When a command potential (V\textsubscript{Comm}) is applied to the positive input, V\textsubscript{1} is now given by the expression -I\textsubscript{M}.R\textsubscript{F}+V\textsubscript{Comm}. The differential amplifier (A\textsubscript{2}) subtracts V\textsubscript{Comm} from V\textsubscript{1}, so that the output voltage (V\textsubscript{O}) is I\textsubscript{M}.R\textsubscript{F} (Ogden, 1994).

**Figure 2.5**  The headstage circuitry, see text for meaning of the symbols.

![Headstage Circuit Diagram](image)

The feedback resistor determines the sensitivity and range of currents measured. However it also introduces noise into the system, for a high gain recording R\textsubscript{F} must be high. The higher the R\textsubscript{F}, the lower the currents measured without saturating the headstage. A feedback resistor to suit the experiment must be chosen. The headstage used for the
Axopatch 1B has a choice of two feedback resistors available. They can be switched remotely from one to another.

From the patch clamp set up shown in figure 2.4, it can be seen that the headstage is connected to the amplifier. The output voltage from the headstage was further amplified and filtered in the circuitry within the amplifier. The amplifier also allows for cell capacitance compensation which was needed for the whole cell patch clamp technique.

A) Amplification
The amplification of a signal was needed so that the signals applied to the subsequent circuitry were of an appropriate size. The patch clamp amplifier had a variable gain control which was used to produce a low noise amplification of the signal. Further amplification could also be done by an external amplification box prior to the signal being sent to the analogue to digital converter.

B) Cell Capacitance
Two types of capacitance were observed; one associated with the pipette due to currents flowing into it and another associated with cell membrane. A pipette capacitance current was seen when a potential was applied, at the start and finish of a voltage step. It was eliminated using capacitance neutralization controls. Due to the thinness of a cell membrane, it makes a very good capacitor, that is it has the ability to store charge \( Q \) when there is a potential difference \( V \) across it, given by the equation

\[
Q = CV \quad (8)
\]

where \( C \) is the capacitance. The current flow \( I \) through a capacitor is proportional to the rate of change of voltage \( \frac{dV}{dt} \) therefore,

\[
I = C \frac{dV}{dt} \quad (9)
\]

When there is a constant voltage across a membrane, the effect of the membrane capacitance on the current flowing through the channels can be ignored. However when the voltage changes transient capacitance currents are seen, evident during whole cell recordings. This is because any current applied to the cell is first required to charge the membrane capacitance before changing the cell membrane potential. The capacitance is compensated, so that the ionic currents observed are maximized without saturating the headstage.
C) Filtering  
Filtering was used to optimize the signal-to-noise of the recording. A low pass filtering circuit was commonly used to eliminate higher frequency signals whilst passing signals below the -3dB frequency. The -3dB frequency of a signal is used as a measure for filtering, it is the frequency at which the output signal of the filter falls to \( \sqrt{1/2} \) of the input signal. A simple filter can be made from one resistor and one capacitor, and is known as a single pole filter. Initial filtering of the signal was achieved using the Axopatch 1B amplifier which has a built in 4-pole Bessel filter. Bessel filters are commonly used in electrophysiological recordings because other filters, such as the Butterworth filter, produce a delay and an overshoot in response to a voltage step. An external 8-pole Bessel filter was also used (see figure 2.4) to refilter the recordings when they are sampled for digitization.

An oscilloscope is connected into the circuitry after the external Bessel filter (see figure 2.4). It was used to view the signal from the patch clamp amplifier prior to it going to the personal computer, therefore allowing subtle changes to be seen in the signal which may not be always be apparent on the computer screen.

The currents recorded were stored on a digital audio tape (DAT) recorder (shown in figure 2.4), therefore many minutes could be recorded from a cell without using up computer hard disk. The data could then be later digitized and analyzed on the computer. This method also allowed the storage of data for later reference if necessary.

The output from the Axopatch patch clamp amplifier are electrical signals, the data is such that the voltage varies continuously with time. In order to process and analyze this signal by computer it needs to be transformed into a form (binary digits) that can be handled by it. An analogue to digital converter (ADC) acquires samples at a given rate and digitizes them. The resolution of the digitized recording depends on the time interval during sampling. The sampling rate was at least twice that of the frequency of the filter. Therefore if the data is filtered at 2 kHz, a suitable sampling rate would be 4 kHz.

A personal computer was used for the acquisition and analysis of the data. The software used was written by Dr. N. Davies, whom I'd like to thank for all his help. It consisted of a suite of programs developed using the AxoBASIC library;

**STIMTOR**  
This program was used to generate holding potentials, ramps and pulses. The data was displayed on the screen and could be written to the hard disk.

**TRACAN**  
Analysis and manipulation of the data could be done using this program.
The following program was used to acquire data from the DAT recorder.

Further details of the analysis used is given later on in the chapter.

2.3.4. Electrodes and seal formation.

The construction of patch pipettes plays an important role in optimizing any recordings. The very high resistance seal produced between the glass and cell membrane suggests that some hydrophobic interaction occurs.

Filamented thin walled borosilicate glass (GC150TF, Clark Electrochemical Ltd., Berks.), with an outer diameter of 1.5 mm and an inner diameter of 0.7 mm, was used to make patch pipettes. A vertical puller (Narashigie Instruments Ltd., Tokyo, Japan) was used to perform a 2-stage gravitational pull procedure on the glass capillaries. The tip size was estimated by measuring the pressure required to expel air from the tip into methanol. An extended 10 ml syringe was connected to the patch pipette. The pipette was held in a bottle of methanol and the plunger was slowly depressed until air bubbles were seen from the pipette tip. Pipettes with a bubble number of 5 or 6 were used, which produced patch pipettes with resistances of 5-15 MΩ.

The patch pipettes were then coated with dental wax (Kemdent, UK). A stick of dental wax was lightly heated in a yellow-flame Bunsen burner and then carefully spread around the tip of the patch pipette. This thickened the walls of the pipettes whilst also forming a hydrophobic barrier which prevented the bath solution creeping up the shank of the pipette. This helped to reduce stray capacitance because both voltage and current noise increases with capacitance.

To facilitate the formation of a gigaohm seal the tip of the patch pipette was fire polished. This was done by placing the tip very close, to within a few micrometers, to a heated platinum wire coated with a glass bead. The glass bead prevented spluttering of the wire. The wire heated the tip of the pipette, causing it to smooth sharp, rough edges thereby producing blunter tips, that make better contact with the cell membrane.

The cells in the 35 mm petri dish were washed and maintained in 6 mM K⁺ extracellular solution (see Solutions section at the end of this chapter for composition). The solution was perfused into the bath from a 50 ml syringe. An angled glass tube,
attached to the rubber tubing from the syringe was used to perfuse the solution into the petri dish. The flow was controlled by a regulator placed around the rubber tubing. A angled needle attached to a vacuum pump was used to remove all the solutions from the dish. The needle was placed at such a height, using blu-tack, that it left a certain amount of solution remaining in the bath.

The patch pipettes were back-filled with 140 mM K⁺ intracellular patch solution, see Solutions at the end of this chapter of its composition. The composition of the intracellular solution was such that it was comparable to intracellular cell cytoplasm.

When a patch pipette was initially immersed into the bath, a junction potential was generated. Its magnitude depended on the concentrations and mobilities of the ions in the bath and pipette solutions. The potential was set to zero using the "Null" potential control.

Figure 2.6 Formation of the conventional whole cell configuration.
(reviewed in Ogden & Stanfield, 1994).

1) Patch pipette

OSCILLOSCOPE

1 mV step applied.

2) Depression in the step seen as the pipette touched the cell.

3) Membrane capacitance spikes appeared as a gigaohm seal was achieved.

The spikes were canceled using fast and slow capacitance compensation controls.
Formation of the whole cell configuration led to the appearance of large capacitance transients and increased noise.

The transients were compensated for using the whole cell capacitance controls.

Figure 2.6 illustrates how the whole cell configuration was accomplished. To form a seal, a voltage step of 1 mV was applied to the patch pipette (1). The size of this step gave an estimate of the resistance of the patch-pipette which was calculated using Ohm's Law. Using the coarse controls of the micromanipulator the pipette was positioned above a cell. The perfusion tip was then repositioned next to the cell. Then using the fine external manipulator controls the patch pipette was advanced towards the cell until it just touched it. This could be seen on the oscilloscope as a slight decrease in the step amplitude (2), and by the appearance of pipette capacitance current spikes. Gentle suction and a holding potential of -30 mV was applied to aid seal formation (3).

The step potential was increased to 10 mV, and the holding potential of the pipette was increased to -60 mV which was anticipated to be the resting membrane potential within the cell. Further suction was applied to the cell thus causing the rupture of the cell membrane. Formation of the whole cell configuration was indicated by the appearance of large capacitance transients and an increase in current noise (4).

Solutions were perfused directly onto the cell during an experiment. A perfusion system developed by Dr. P. Langton was used, both pressure and electrically switched solenoid valves controlled the flow of the solutions. The cell could be superfused with up to seven different solutions. Each solution was delivered from a 2 ml reservoir into a single perfusion tip. The tip was placed above the cell using a coarse-controlled motorized manipulator. The dead volume at the tip was less than 5 μl which allowed rapid changes in solution.

2.3.5. Collection and analysis of data.

After going into the whole cell configuration, a recording was started on the DAT recorder. The data was recorded on a gain of 10 and filtered at 10 KHz. Different solutions were superfused onto the cell. Voltage ramps were also applied to observe the effect of different agents on a cell.
A) Traces  A recording was digitized using the TAPE program onto the computer. Prior to the digitization, the gain was increased so that the maximal scale possible was used and the filtering was chosen to suit the scale. The sampling rate used was double that of the filtering rate. Using a function in TRACAN the data points were compressed onto the screen. Of the 1024 data points on a screen, the computer picked the highest and lowest to obtain the data points for compression from that particular length of recording. These are then displayed on a single screen. The files were stored as *.ASC files, and then imported into a graphical package for display. These files were stored as screen digitization data points, therefore in the graphical package they could be transformed to give a current against time axes.

B) Ramps  20 to 30 ramps were recorded at any one point. The ramps were then averaged in TRACAN and imported as *.TC files into Sigmaplot, the graphical package. The data was stored as current against time traces. In Sigmaplot, I-V relationship curves were constructed.
2.4 Experimental solutions

The solutions were made up in Milli-Q distilled water, and the chemicals were bought from Sigma (unless otherwise stated). All the chemicals were added in a solid form except for MgCl$_2$ (BDH Laboratories Supplies) and CaCl$_2$ (BDH Laboratories Supplies) which were added from 1 M stock solution. The solutions were made up to a pH of 7.4 (unless otherwise stated) with either KOH or NaOH. The freshly made solutions were filtered through a 0.2 μM filter and stored at 4 °C.

Table 2.1 Composition (in mM) of 5 mM K$^+$, 0Ca$^{2+}$, 20 mM K$^+$ 60 mM K$^+$ and 80 mM K$^+$ physiological salt solutions (PSS).

<table>
<thead>
<tr>
<th>CHEMICAL</th>
<th>5 mM K$^+$ PSS$^1$</th>
<th>0Ca$^{2+}$ PSS</th>
<th>20 mM K$^+$ PSS</th>
<th>60 mM K$^+$ PSS</th>
<th>80 mM K$^+$ PSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>135</td>
<td>135</td>
<td>120</td>
<td>80</td>
<td>60</td>
</tr>
<tr>
<td>KCl</td>
<td>5</td>
<td>5</td>
<td>20</td>
<td>60</td>
<td>80</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>1.8</td>
<td>-</td>
<td>1.8</td>
<td>1.8</td>
<td>1.8</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>HEPES</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>glucose</td>
<td>10</td>
<td>10</td>
<td>$10^2$</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>pH</td>
<td>NaOH</td>
<td>NaOH</td>
<td>NaOH$^3$</td>
<td>NaOH</td>
<td>NaOH</td>
</tr>
</tbody>
</table>

$^1$ This solution was also used to store the pig hearts from the abattoir.

$^2$ For a low glucose solution, the concentration of glucose was reduced to 0.5 mM or zero.

$^3$ For pH 6.8, HCl was added to the solution.
Table 2.2 Composition (in mM) of low calcium isolating solution.

<table>
<thead>
<tr>
<th>CHEMICAL</th>
<th>Low Ca^{2+} isolating solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>137</td>
</tr>
<tr>
<td>KCl</td>
<td>5.4</td>
</tr>
<tr>
<td>HEPES</td>
<td>10</td>
</tr>
<tr>
<td>MgCl(_2)</td>
<td>1</td>
</tr>
<tr>
<td>glucose</td>
<td>0.1 (^1)</td>
</tr>
<tr>
<td>CaCl(_2)</td>
<td>100(\mu)M (^1)</td>
</tr>
<tr>
<td>Na(_2)HPO(_4)</td>
<td>0.4</td>
</tr>
<tr>
<td>NaH(_2)PO(_4)</td>
<td>0.44</td>
</tr>
<tr>
<td>NaHCO(_3)</td>
<td>4.17</td>
</tr>
<tr>
<td>pH</td>
<td>NaOH</td>
</tr>
</tbody>
</table>

\(^1\) Glucose and CaCl\(_2\) were added freshly everyday.

Table 2.3 Extracellular Patch solutions.

<table>
<thead>
<tr>
<th>CHEMICAL</th>
<th>6 mM K(^+) solution</th>
<th>60 mM K(^+) solution</th>
<th>140 mM K(^+) solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>134</td>
<td>80</td>
<td>-</td>
</tr>
<tr>
<td>KCl</td>
<td>6</td>
<td>60</td>
<td>140</td>
</tr>
<tr>
<td>glucose</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>HEPES</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>CaCl(_2)</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>MgCl(_2)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>pH</td>
<td>NaOH</td>
<td>NaOH</td>
<td>NaOH</td>
</tr>
</tbody>
</table>
Table 2.4  Intracellular patch pipette solution.

<table>
<thead>
<tr>
<th>CHEMICAL</th>
<th>Intracellular patch solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>124</td>
</tr>
<tr>
<td>KOH (1M Stock solution)</td>
<td>16</td>
</tr>
<tr>
<td>EGTA</td>
<td>5</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>2.68$^{1,2}$</td>
</tr>
<tr>
<td>HEPES</td>
<td>10</td>
</tr>
<tr>
<td>glucose</td>
<td>10</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>1</td>
</tr>
<tr>
<td>pH</td>
<td>7.2 with KOH</td>
</tr>
</tbody>
</table>

$^1$ This solution gives 200 nM free intracellular Ca$^{2+}$.

$^2$ Some solutions had no added Ca$^{2+}$ to reduce BK$_{Ca}$ channel activity.

0.1 mM ATP and 0.1 mM ADP were added to the solution as required.
CHAPTER 3

Vasodilatation and $\text{K}^+$ Channel Activation by Nicorandil in Rat Mesenteric Arteries
3.1 Introduction

The aim of this study was to investigate the actions of the anti-anginal drug nicorandil in small mesenteric vessels of the rat. The chapter will be presented in two sections. The first part will concentrate on the functional actions of nicorandil, showing results obtained using the small vessel wire myograph. Section two will then describe further investigations of the channels activated by nicorandil using the whole cell patch clamp technique. The results show that nicorandil caused relaxation of pre-contracted arterial rings via at least two mechanisms; one that is inhibited by glibenclamide, a specific $K_{\text{ATP}}$ channel inhibitor, and the other which is blocked by 6-anilino-5, 8-quinolinedione (LY 83583), a GC inhibitor. Data from acutely dissociated smooth muscle cells confirms that nicorandil does indeed activate $K^+$ currents through $K_{\text{ATP}}$ channels.

3.1.1. Nicorandil.

The therapies currently used for angina have significant side effects, and are also sometimes ineffective in controlling angina to an adequate degree. The use of organic nitrates, although highly efficacious, is greatly compromised by the development of tolerance while both the calcium channel inhibitors and the β-blockers can have unpleasant side-effects (Rang et al., 1992). This has led to interest in a different class of drugs, the potassium channel openers. N-(2-hydroxylethyl)-nicotinamide nitrate, commonly known as nicorandil, was the first $K^+$ channel opener clinically available for angina (reviewed in Goldschmidt et al., 1996).

Nicorandil was launched in Japan in 1984 as a coronary vasodilator. In 1994 it was introduced in the U. K. as a new class of anti-anginal drug. Although nicorandil is classified as a potassium channel opener, its vasodilator action can not be attributed to $K^+$ channel opening alone. Vasodilatation of vessels by nicorandil was seen even in the presence of $K^+$ channel blockers. Furukawa et al (1981) demonstrated that nicorandil produced membrane hyperpolarisation in a variety of different smooth muscle tissue preparations. In 1989, Taira showed that nicorandil induced the formation of cGMP by means of its nitrate moiety.

Studies were done to further investigate and clarify the potassium channel opening mechanism of nicorandil. Kreye et al (1991) used $^{86}$Rb, as a marker for $K^+$, to show that
the hyperpolarising effect of nicorandil was due to the opening of a glibenclamide-sensitive $K_{ATP}$ channels. $K_{ATP}$ channel activation causes membrane hyperpolarisation and so reduces calcium entry through voltage operated calcium channels and possibly also IP$_3$-dependent calcium release (Nelson et al, 1990a; Nelson and Quayle, 1995). Investigators then tried to determine which of two mechanisms was the most prominent. Kreye and colleagues (1991) implied that the nitrovasodilator-like mechanism was primarily responsible for vasorelaxation, based on findings in the rabbit aorta. However, Yoneyama et al (1990) found the effect of nicorandil on the coronary circulation to be predominantly due to its $K^+$ channel opening action. Kukovetz et al (1992) went on to show that in the bovine coronary artery the relative contribution of each mechanism was also dependent on the concentration of nicorandil used. A summary of the dual mechanism of action of nicorandil is shown in figure 3.1. These studies suggest that nicorandil has both a tissue and a concentration dependent mechanism of action. Few studies have investigated the actions of nicorandil in small arterial vessels. Such a study is important because the drug is likely to act on similar sized arteries in vivo, in patients. By using the whole cell patch clamp technique it is possible to physiologically characterise the channels that underlie nicorandil-induced vasorelaxations.
Figure 3.1. The dual mechanism of action of nicorandil.

Adapted from Goldschmidt et al (1996).
MLCKase, myosin light chain kinase; VOCC, voltage operated calcium channel; SR, sarcoplasmic reticulum.

A Nitrate-like mechanism

Nicorandil

↑ Guanylyl cyclase activity

↓ cGMP

↑ In Ca²⁺ stimulated Mg²⁺ dependent ATPase extrusion pump

↓ In intracellular Ca²⁺ stores

↓ Alters MLCKase activity

RELAXATION OF SMOOTH MUSCLE

B Potassium channel opening mechanism

Nicorandil

Activation of K_ATP channels

↑ ?Ca²⁺ dependent K⁺ channels

↓ Membrane hyperpolarisation

↓ ? Interference with VOCC and release of Ca²⁺ from SR

↓ Alters MLCKase activity
3.2 Functional Myograph Experiments

Nicorandil causes vasorelaxation by opening $K_{\text{ATP}}$ channels and stimulating GC activity, however the degree of contribution by each pathway is tissue-specific. I have investigated the relaxant actions of nicorandil in small branches of the rat mesenteric artery using a small vessel wire myograph.

3.3 Methods

3.3.1. Tissue dissection.

Male adult Wistar rats were killed by cervical dislocation. Small mesenteric arteries, second and third order branches (diameters of 377±12 μM), were removed and arterial rings were mounted in a wire myograph. A detailed description of the dissection and the mounting procedure is given in chapter 2, sections 2.1.1 and 2.2.3.

3.3.2. Myograph experiments.

The rings were placed in a 10 ml bath that was continuously gassed with oxygen and maintained at 37 °C. The chemicals were added directly into the bath. All the solutions contained 20 μM Nω-nitro-L-arginine methyl ester (L-NAME) to eliminate endogenous nitric oxide activity. The vessels were contracted using 20 mM K⁺ solution (see Solutions in chapter 2, section 2.4 for composition) and 200 nM BAYK 8644 (20K and BAYK). The maximal tone achieved was about 10 mN and all relaxations were calculated as a reduction in this tone. Once a stable contraction had been produced, cumulative response curves to nicorandil (1 to 300 μM) were produced. The mechanism of action of nicorandil was investigated using glibenclamide and LY 83583. For these experiments, maximal tone was achieved with 20K and BAYK, glibenclamide was applied to the bath prior to measurement of a cumulative nicorandil concentration effect curve. Experiments were repeated in the presence of different concentrations of LY 83583, where it was added at the start of the contraction. In further experiments, I used 60 mM K⁺ solution (see chapter 2, section 2.4) to contract the tissue before applying nicorandil cumulatively. Cumulative
concentration response curves to pinacidil, another $K_{ATP}$ channel opener, were also performed on 20K and BAYK contractions.

3.3.3. Analysis.

Each response to nicorandil was normalised by expressing it as a percentage of the maximal contraction produced. The method of analysis of this data is detailed in Methods chapter 2, section 2.2.4. The concentration response curves were fitted with the expression

$$y = M \left[ \frac{1}{1 + (k/x)^n} \right]^{-1}$$  

(1)

where $y$ is the % relaxation; $x$ is the agonist concentration; $M$ is the maximum response; $k$ is the EC$_{50}$ and $n$ is the Hill coefficient. All the values are given as mean pD$_2$±S.E.M., and $n$=number of preparations and $N$=number of animals. pD$_2$ values were used for statistical analysis. Statistical significance was assessed using Student’s t-tests for simple comparisons and analysis of variance with Duncan’s post hoc test for multiple comparisons.

3.3.4. Drugs.

Nicorandil was a kind gift from Chugai Pharmaceuticals, Japan and Rhône-Poulenc Rorer, U. K. It was made up freshly every day in DMSO to give a stock concentration of 100 mM. BAYK 8644 was purchased from RBI and a stock solution of 200 µM was made up in DMSO. Glibenclamide (Sigma) was dissolved in DMSO to a stock concentration of 10 mM. LY 83583 was purchased from Eli Lilly, USA. A stock solution of 10 mM was made up in DMSO. L-NAME (Sigma) was dissolved in Milli-Q water to give a stock concentration of 20 mM. Pinacidil was purchased from RBI and dissolved in DMSO to give a stock concentration of 10 mM. A stock concentration of 28 mM of indomethacin (Calbiochem) was made up in DMSO.
3.4 Results

3.4.1. Nicorandil-induced relaxation inhibited by glibenclamide.

Nicorandil produced a concentration-dependent relaxation of the 20K and BAYK contraction, as shown in figure 3.2. The mean concentration response curve generated by nicorandil is illustrated in figure 3.3, and is described by an EC$_{50}$ of 16.3±1.1 µM, a maximum relaxation at 300 µM of 71.0±4.8 % and a Hill coefficient of 0.9±0.05 (n=26, N=13).

Glibenclamide has been shown to inhibit K$_{ATP}$ currents, with a $K_i$ of 101 nM, in smooth muscle cells from the rabbit mesenteric artery (Quayle et al, 1995). Glibenclamide is quite selective for K$_{ATP}$ channels at concentrations up to 10 µM. Figure 3.3A shows the effect of 10 µM glibenclamide on a nicorandil concentration effect curve. Glibenclamide caused a parallel rightward shift of the nicorandil curve, increasing the EC$_{50}$ to 43.1±1.2 µM (see also table 3.1).

Further evidence for the involvement of K$^+$ channel activation was obtained when the tissues were contracted with 60 mM K$^+$ solution (figure 3.3B). Under these conditions, the E$_K$ becomes less negative (~ -23 mV) thereby decreasing the driving force for K$^+$ ions, and so channel openers are unable to cause hyperpolarisation. Nicorandil was less potent against the 60 mM K$^+$ contractions. An EC$_{50}$ value of 46.9±1.2 µM was obtained, close to that measured for the effect of glibenclamide in figure 3.3A (see table 3.1). Nicorandil-induced relaxations could then be further inhibited with 1 µM LY 83583.

<table>
<thead>
<tr>
<th></th>
<th>EC$_{50}$ (µM)</th>
<th>pD$_2$±S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>16.3</td>
<td>4.8±0.05</td>
</tr>
<tr>
<td>10 µM glibenclamide</td>
<td>43.1</td>
<td>4.4±0.06 *</td>
</tr>
<tr>
<td>60 mM K$^+$ contraction</td>
<td>46.9</td>
<td>4.3±0.3 *</td>
</tr>
</tbody>
</table>

* p<0.05 when compared to control, ANOVA followed by Duncan's post hoc test.
Figure 3.2  Traces depicting cumulative nicorandil-induced relaxations in two isolated arterial ring preparations of the rat mesenteric artery. Small arterial rings were contracted using 20K and BAYK. Nicorandil was added cumulatively (1 to 300 µM) at the points indicated. The decrease in tone (relaxation) was measured as a percentage of the maximal contraction produced.
Figure 3.3  Concentration response curves for the relaxation of established tone by nicorandil in mesenteric arterial preparation of the rat.

(A) Under control conditions (○;  n=26, N=13) and in the presence of 10 μM glibenclamide (●;  n=6, N=3). (B) Concentration-response curves for nicorandil when the tone was produced with 60 mM external K⁺ solution, in the absence (□; n=8, N=4) and presence (■; n=6, N=3) of 1 μM LY 83583. n=number of preparations and N=number of animals. The points show mean±S.E.M. and curves were drawn using equation (1). Student’s t-test were done to test significance levels at the 0.05 and 0.01 significance levels. (* p<0.05 and ** p<0.01).
3.4.2. **Effect of LY 83583.**

I then went onto investigate the contribution of the guanylyl cyclase (GC) component of the nicorandil relaxation, using LY 83583. This is an inhibitor of soluble GC, and has been shown to have an IC$_{50}$ of 1 μM in the rabbit aorta (Mülsch *et al*, 1988). Figure 3.4 and table 3.2 show that LY 83583 produced a concentration-dependent rightward shift of the nicorandil relaxation curve. Therefore the activation of GC plays an important role in nicorandil-induced vasorelaxation.

### Table 3.2  Mean EC$_{50}$ values for nicorandil under control conditions and in the presence of 10 nM, 100 nM, 1 μM and 10 μM LY 83583.

The curves were all assumed to reach same maximum value, as obtained under control conditions. * p<0.05, ANOVA followed by Duncan’s multiple range test.

<table>
<thead>
<tr>
<th></th>
<th>EC$_{50}$ (μM)</th>
<th>pD$_{2}$±S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>16.3</td>
<td>4.8±0.05</td>
</tr>
<tr>
<td>10 nM LY 83583</td>
<td>24.2</td>
<td>4.6±0.09</td>
</tr>
<tr>
<td>100 nM LY 83583</td>
<td>65.9</td>
<td>4.2±0.36 *</td>
</tr>
<tr>
<td>1 μM LY 83583</td>
<td>162.0</td>
<td>3.8±0.1 *</td>
</tr>
<tr>
<td>10 μM LY 83583</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

I considered the possibility that LY 83583 might be acting as a direct blocker of K$_{ATP}$ channels in addition to inhibiting GC. Therefore, the selectivity of LY 83583 for GC over K$_{ATP}$ channels was investigated. Pinacidil is a long established potassium channel opener, which causes hyperpolarisation-induced vasodilatation via the opening of K$_{ATP}$ channels (*Cook et al*, 1988). I was therefore able to test the specificity of LY 83583 in this preparation using pinacidil. Figure 3.5 shows the mean concentration effect curve for pinacidil obtained under control conditions and in the presence of 1 μM LY 83583. The GC inhibitor had no significant effect on the pinacidil-induced relaxation, the EC$_{50}$s were 5.8±1.6 μM and 3.5±1.0 μM for control and in the presence of LY 83583 respectively.
Figure 3.4 The inhibitory effects of LY 83583 on nicorandil-induced relaxations in isolated ring preparations of the rat mesenteric artery.

The vessels were contracted using 20K and BAYK, as described in the text. Response curves to nicorandil are shown under control conditions (○, as in figure 3.3A) and in the presence of LY 83583 at 10 nM (■; n=6, N=3), 100 nM (▲; n=4, N=2), 1 μM (◆; n=6, N=3) and 10 μM (▼; n=4, N=2).

Figure 3.5 Relaxation of mesenteric arterial rings by pinacidil.

Curves shown under control conditions (□; n=4, N=2) and in the presence of 1 μM LY 83583 (■; n=4, N=2).
Thus far, the results show that both $K_{ATP}$ channel activation and stimulation of GC play a role in nicorandil-induced dilatation in rat mesenteric arteries. However, it is not possible to tell if there was any overlap of effect between the two mechanisms. This was studied with the application of both inhibitors. If the two antagonists worked synergistically, a lesser or greater inhibition would be expected than if they worked in an purely additive manner. When glibenclamide (10 µM) and LY 83583 (1 µM) were added together, the nicorandil relaxation was inhibited more than with either inhibitors alone, see figure 3.6A. The predicted $EC_{50}$ value for the nicorandil response curve increased to 286±1.2 µM, when compared to 43.1±1.1 µM and 162±1.3 µM for glibenclamide and LY 83583 respectively when applied alone. The summary barchart in figure 3.6B shows that the cumulative $pD_2$ values of the of the two inhibitors, when applied individually, is not significantly different from when they are administered together. This suggests there is little cross-talk between the two mechanisms, that is they work independently of each other to cause vasorelaxation.

3.4.3. Effect of indomethacin.

The endothelium plays an active role in the control of the contractile state of the surrounding smooth muscle cells. Endothelial cells have the ability to produce a variety of vasoactive substances such as NO and prostaglandins (PG) such as $PGI_2$, PGE, PGD which are vasodilators and endothelin which is a vasoconstrictors. The inclusion of L-NAME in all the solutions ensured that intrinsic NO activity was abolished, however, there was still the possibility however that endogenous PGs may be involved in causing vasorelaxation in my preparation. Indomethacin is a potent inhibitor of cyclooxygenase, the enzyme involved in the synthesis of prostaglandins (Lands, 1985). The effect of a supermaximal concentration of indomethacin (2.8 µM) was therefore investigated. Figure 3.7 shows that indomethacin had no significant effect on the nicorandil concentration response curve. The $EC_{50}$ values are 16.3±1.2 µM and 12.1±1.2 µM in the absence and presence of indomethacin respectively.
Figure 3.6  Cumulative effects of LY 83583 and glibenclamide.

(A) Vasorelaxation to nicorandil in the presence of 10 μM glibenclamide and 1 μM LY 83583 (▼; n=5, N=3). As in figure 3.3A, O represents the control values. The broken lines show the curves for the effect of glibenclamide (from figure 3.3A) and LY 83583 (from figure 3.4) alone. (B) Barchart showing the difference in pD₂ values from control, for glibenclamide and LY 83583 alone, and both inhibitors together.
Figure 3.7  Effect of indomethacin on nicorandil-induced vasorelaxation.

Concentration response curves to nicorandil in the absence (○, as in figure 3.3A) and in the presence of 2.8 μM indomethacin (◆; n=4, N=2). The points show mean±S.E.M. and the curves were drawn using equation (1), section 3.3.3. n=number of preparations and N=number of animals.
3.5 Whole Cell Patch Clamp Experiments

The functional investigations using the wire myograph demonstrated that both $K_{ATP}$ channel opening and nitrovasodilator actions are involved in nicorandil-induced vasorelaxation. In the following experiments I used the whole-cell voltage clamp technique to identify the current activated by nicorandil. Smooth muscle cells were isolated from rat mesenteric arterial branches, similar to those used to provide tissue for myograph experiments. Previous reports have shown that $K^+$ currents are activated by nicorandil in isolated cells of the portal vein and the proximal urethra. Kajioka et al., 1990 showed that nicorandil activated a whole cell $K^+$ current in a concentration-dependent manner, in smooth muscle cells of the rat portal vein. The current was suppressed by decreasing intracellular $Ca^{2+}$ and increasing cytosolic ATP, hence they concluded that nicorandil activated both $BK_{Ca}$ and $K_{ATP}$ channels in these cells. However, in the rabbit portal vein, nicorandil activated only $K_{ATP}$ channels in inside-out patches. Channel activation was inhibited by cellular ATP but then restored by nucleotide diphosphates (Kamouchi & Kitamura, 1994). Similarly, Teramoto and Brading (1997) demonstrated the activation of glibenclamide-sensitive $K_{ATP}$ currents in single smooth muscle cells of the pig proximal urethra.

I have used different external potassium solutions and glibenclamide to show that the current activated by nicorandil is consistent with the opening of $K_{ATP}$ channels.

3.6 Methods

3.6.1. Patch clamp experiments.

For patch clamp recordings, cells were isolated enzymatically using papain and collagenase F, as described in Methods Chapter 2 (see section 2.3.1.). Patch pipettes were pulled from thin-walled borosilicate glass tubing (o.d. 1.5 mm, Clark Electromedical), coated with dental wax (Kemdent) and fire polished. Their resistance was in the range of 2-7 MΩ and seals were in the order of 10 GΩ. Currents were measured using the whole-cell voltage clamp configuration. All the experiments were done at room temperature, 18-24 °C. The pipette solution contained 140 mM $K^+$ (see Solutions Chapter 2, section 2.4 for the composition of all the solutions). The cells were bathed in 6 mM $K^+$ solution. After going
into the whole-cell configuration the bath solution was changed to either 140 mM K⁺ or 60 mM K⁺. Changes of solution were achieved by superfusing the new solution directly onto the cell, which was maintained in the stream of solution flowing from the perfusion pipette.

3.6.2. Data collection.

Currents were recorded with an Axopatch 1B amplifier at a bandwidth of 10 kHz, and stored on a DAT tape or onto the hard disk of a personal computer after digitisation with an Axon Instruments A-D interface. Programs developed using the AxoBASIC library were used to apply command potentials and to analyse current-voltage ramps. Greater detail of the collection and analysis of data is given in Chapter 2, section 2.3.5.

3.6.3. Drugs.

The drugs used were nicorandil, glibenclamide and LY 83583. The sources of these drugs and their stock solutions were outlined in section I (3.3.4.).
3.7 Results

3.7.1. Currents activated by nicorandil.

Previous investigations have shown that nicorandil produced vasorelaxation, in part, through membrane hyperpolarisation induced by $K^+$ channel opening (Kamouchi & Kitamura, 1994; Teramoto & Brading, 1997). I have used smooth muscle cells from rat mesenteric arteries to study the current activated by nicorandil. Figure 3.8 shows the currents induced in cells, by an external perfusion of nicorandil with different $[K^+]_o$ solutions. The $[K^+]_i$ concentration was 140 mM in each case. In figure 3.8A, the cell was held at -60 mV and extracellular $[K^+]$ was 140 mM. Application of 300 μM nicorandil led to the progressive activation of a large inward current, with an amplitude of approximately 150 pA in this cell. The mean current from 12 other cells 152.5±23.6 pA. Nicorandil also caused an increase in current noise as would be expected for a channel opening action. In physiological 6 mM external $K^+$ solution, nicorandil caused the activation of a small but distinct outward current when the cell was held at 0 mV (figure 3.8B). The peak amplitude of this cell was about 40 pA. The average current evoked by nicorandil under physiological conditions was 70±10.4 pA (n=3). The current was then substantially blocked by 10 μM glibenclamide.
Figure 3.8 Whole cell currents activated by nicorandil in acutely dissociated smooth muscle cells of the rat mesenteric artery.

(A) Recording done under conditions of symmetrical 140 mM K⁺ solutions and a holding potential of -60 mV. 300 μM nicorandil was superfused directly onto the cell surface, as shown by the bar. (B) Nicorandil (300 μM) activation of a current, under conditions of physiological 6 mM external K⁺ solution and 140 mM internal K⁺ solution. The cell was held at 0 mV. 10 μM glibenclamide was superfused on to the cell as shown. The pipette solutions in both cases included 0.1 mM ATP and 0.2 mM ADP. Ramps were applied at the peak of the response to nicorandil. The dashed lines represent zero current levels.
3.7.2. Lack of voltage dependence of currents activated by nicorandil.

Voltage ramps were used to investigate the reversal potential ($E_{\text{Rev}}$) and voltage dependence of the nicorandil-activated current (figures 3.9 and 3.10). Figure 3.9A shows the current-voltage (I-V) relationship measured from a cell in 140 mM K$^+$ solution in response to voltage ramps applied from -50 to +50 mV at 2 mVms$^{-1}$, for 50 ms. Nicorandil induced an increase in both outward and inward current, with an $E_{\text{Rev}}$ in this cell of approximately -3 mV. The mean $E_{\text{Rev}}$ from 5 cells was 0.4±1.1 mV. The $E_K$, calculated using the Nernst equation (equation 1, section 1.5), is 0 mV under these conditions. Figure 3.9B illustrates the I-V relation obtained when 60 mM K$^+$ solution was used, the current reversed at -25 mV, close to the predicted $E_K$ of -20 mV. At potentials positive to +20 mV some voltage-dependent activation of BK$_{Ca}$ channels is seen to occur. When a cell was bathed in 6 mM K$^+$ solution, a ramp from 100 to 0 mV produced a current that reversed at -70 mV (figure 3.9C), again close to the predicted $E_K$ of -79 mV. The near-linear I-V relationships obtained suggest that the channel underlying the nicorandil-activated current shows little voltage dependence. And from figure 3.10, it can be seen that the measured $E_{\text{Rev}}$s closely follow the predicted $E_K$s for the different external potassium solutions. This correlation illustrates that nicorandil activates a potassium channel.
Figure 3.9  Current-voltage (I-V) relationships for the nicorandil-induced current.

(A) I-V curves when a cell was in symmetrical 140 mM K⁺ solutions, and ramps from -50 to +50 mV were applied under control conditions, in the presence of nicorandil and nicorandil + glibenclamide. (B) Voltage ramps obtained in the absence and presence of nicorandil when 60 mM [K⁺]₀ and 140 mM [K⁺]ᵢ solutions were used. Ramps run from -40 to +30 mV. (C) I-V relationships obtained when the cell was in 6 mM [K⁺]₀/140 mM [K⁺]ᵢ solutions. Ramps from -100 to 0 mV were applied in the absence and presence of nicorandil, and nicorandil + glibenclamide. The predicted equilibrium potential for K⁺ (E钾) is shown by the arrow in each case.
Figure 3.10  Correlation between $E_{Rev}$ and $E_K$.

The measured reversal potentials ($E_{Rev}$) for the nicorandil activated current are given by $\bullet$ (n=2) for 6 mM external $K^+$ solution, by $\Delta$ (n=4) for 60 mM $K^+$ extracellular solution and by $\blacksquare$ (n=7) for 140 mM $K^+$ external solution. The broken line was drawn to the Nernst equation for $K^+$, $E_K = 58mV \log_{10} \left[ K^+ \right]_o / 140mM$. 

\[ \log_{10} [K^+]_o (M) \]

Potential (mV)
3.7.3. Effect of glibenclamide.

The functional experiments had shown that nicorandil-induced vasorelaxation was inhibited with glibenclamide (figure 3.3A). Glibenclamide has been shown to be quite selective for $K_{ATP}$ channels at concentrations up to 10 $\mu$M (Nelson & Quayle, 1995). Under conditions of symmetrical 140 mM $K^+$ solutions and a holding potential of -60 mV, the application of 10 $\mu$M glibenclamide led to a rapid and substantial inhibition of the nicorandil activated current (figure 3.11). In 5 cells, glibenclamide significantly reduced ($p<0.05$) the current activated by nicorandil. An eight-fold reduction in current size was seen in the presence of glibenclamide (figure 3.13). Similarly, under physiological conditions of 6 mM $K^+$ outside and 140 mM $K^+$ patch solution, the small outward current activated by nicorandil was inhibited by glibenclamide (figure 3.8B).

3.7.4. Effect of LY 83583.

The functional experiments had further revealed that nicorandil-induced relaxations were inhibited by the GC inhibitor, LY 83583 (see figure 3.4). I therefore studied the possibility that at least part of the nicorandil-activated current could be blocked with LY 83583. The superfusion of 1 $\mu$M LY 83583 onto the cell, in the presence of nicorandil, had no effect on the $K_{ATP}$ current (figures 3.12 and 3.13), when external and internal $K^+$ concentrations were 140 mM and the cell was held at -60 mV. However the subsequent addition of 10 $\mu$M glibenclamide led to a rapid and substantial inhibition of the current. The summary barchart, presented in figure 3.13, shows normalised currents at -50 mV and +50 mV when 140 mM external and internal $K^+$ solutions were used. Voltage ramps, from -50 to +50 mV, were applied at the peak of each response and under control conditions when there were no drugs being superfused onto the cell. The values were normalised to the currents obtained at -50 and +50 mV under control conditions and these are represented as 1 and -1 respectively. Therefore under these circumstances, nicorandil has been shown to primarily open glibenclamide-sensitive $K_{ATP}$ channels.
Figure 3.11 Inhibition of the nicorandil-induced current by glibenclamide.

Whole cell recording made from a cell when symmetrical 140 mM [K⁺] solutions and a holding potential of -60 mV. 300 µM nicorandil and 10 µM glibenclamide were administered as shown by the bars. The recording was made at a bandwidth of 10 kHz and then redigitised at 2 kHz for display. The dashed line represents the zero current level.

Figure 3.12 Effect of LY 83583 on a nicorandil-induced current.

Recording from a cell held at -60 mV, and superfused with 140 mM K⁺ solution. The pipette solution was 140 mM K⁺ with 0.1 ATP and 0.2 ADP. Nicorandil (300 µM), LY 83583 (1 µM) and glibenclamide (10 µM) were applied, as indicated by the bars. The broken line represents the zero current level.
Figure 3.13  Summary barchart illustrating the effect of glibenclamide and LY 83583 on nicorandil-induced whole cell current.

Normalised nicorandil-activated currents alone and with LY 83583 or glibenclamide measured at -50 mV and +50 mV. Cells were superfused with 140 mM K⁺ solution. The pipette solution contained 140 mM K⁺. The currents were normalised as described in the text. Bars show mean currents±S.E.M. from 5-10 different cells. * indicates significant difference (p<0.05) of the effect when compared to the nicorandil response.
3.7.5. *Single channels activated by nicorandil.*

The low level of channel activity in vascular smooth muscle makes it possible to resolve single channel events from whole cell recordings (Dart & Standen, 1993). Figure 3.14A illustrates a series of records made during the onset of the nicorandil response, where single channel openings can be observed. The opening of a channel with a unitary current of approximately 1.8 pA was detected and the channel activity can be seen to increase as the response to nicorandil developed. Figure 3.14B shows a current amplitude histogram produced during the onset of the response to nicorandil in the same cell. The peaks correspond to current levels 0, 1 and 2 channels opening. The mean unitary current was found to be -1.8±0.04 pA from 6 other cells.
Figure 3.14  Detection of single channels activated by nicorandil in a whole cell recording.

(A) Segments of recording made in the early phase of channel activation by nicorandil. The records were filtered at 200 Hz and digitised at 1 kHz. The solid line indicates the zero current level, and the broken lines correspond to 1, 2 or 3 channels open. The holding potential was -60 mV and the cell was superfused with 140 mM K+ solution. (B) Histogram of current amplitude from recordings made during the onset of the response to nicorandil from the same cell as in A. The histogram was fitted with the sum of three Gaussian curves with mean and s.d. values (pA) of 0, 0.4; -1.79, 0.73 and -3.58, 0.59 respectively. I would like to thank Prof. N. Standen for his assistance with this figure.
3.8 Discussion

The conclusions from my myograph experiments are;

Nicorandil produced a concentration-dependent relaxation when the arterial rings were contracted using 20 mM K\(^+\) solution and 200 nM BAYK 8644. The mean EC\(_{50}\) was calculated to be 16.3 \(\mu\)M.

The nicorandil concentration response curve was not significantly shifted by the application of indomethacin, therefore demonstrating that production of cyclooxygenase products such as prostaglandins have no influence on the nicorandil-induced vasorelaxation.

The concentration response curve to nicorandil was shifted rightwards in the presence of the K\(_{ATP}\) channel inhibitor glibenclamide. A similar decrease in potency was observed when the nicorandil concentration response curve was performed against 60 mM K\(^+\) contractions. The mean EC\(_{50}\)s for nicorandil were significantly different from control but not from each other, the EC\(_{50}\)s were 43.1 \(\mu\)M and 46.9 \(\mu\)M for glibenclamide and 60 mM K\(^+\) respectively.

The second mechanism of action of nicorandil was investigated using LY 83583, a GC inhibitor. LY 83583 was able to inhibit the relaxant actions of nicorandil in a concentration-dependent manner (mean EC\(_{50}\) for nicorandil at 1 \(\mu\)M LY 83583 was 162 \(\mu\)M). 1 \(\mu\)M LY 83583 had no effect on concentration effect curves to the K\(_{ATP}\) channel opener pinacidil, suggesting that it does indeed block the GC-induced vasorelaxant actions of nicorandil.

Further experiments showed that 1 \(\mu\)M LY 83583 and 10 \(\mu\)M glibenclamide acted in a cumulative manner when applied together, figure 3.6B.

Thus both GC activation and K\(^+\) channel opening mechanisms contribute to the vasorelaxant action of nicorandil in small arteries of the rat mesenteric vascular bed under the experimental conditions that I used.

The main findings from the patch clamp experiments are;

In isolated smooth muscle cells, studied using the whole-cell patch clamp, nicorandil activated a large inward current at -60 mV in symmetrical 140 mM K\(^+\) solutions, and a smaller inward current at -0 mV in 6 mM \([K^+]_o\) solution.
The nicorandil-activated current was unaffected by LY 83583 (1 μM) but was substantially blocked by glibenclamide (10 μM).

The I-V relationships measured in the different external [K+] solutions showed that the nicorandil-activated currents reversed near the predicted $E_K$ for the different solutions.

During the early phase of current activation by nicorandil, openings of channels with a unitary conductance of about -1.8 pA could be detected. Assuming a reversal potential ($E_{\text{Rev}}$) of 0 mV, when the cells were superfused with 140 mM K$^+$ solution, and the membrane potential ($E_m$) to be -60 mV, the single channel conductance ($\gamma$) can be calculated by

$$\gamma = \frac{i}{E_m - E_{\text{Rev}}}$$

where $i$ is the unitary current (Hille, 1992). This gives a value of 31 pS for the single channel conductance of the nicorandil-activated channel in the rat mesenteric artery.

Drug development plays an important role in the treatment of cardiovascular diseases, because such disorders are a cause of high rate of morbidity and mortality in the Western world. Nicorandil, a recently introduced anti-anginal drug, was shown to relax small resistance vessels of the rat mesenteric artery in a concentration-dependent manner. The majority of studies have centred on large vessels, however evidence suggests that it is the small vessels that are important in the modulation of peripheral resistance (e.g. Nelson et al, 1990b; Daut et al, 1994a), hence it is necessary to assess the activity of compounds in such vessels. Decreasing peripheral resistance will lead to a fall in blood pressure and an increase in blood flow to under-perfused regions. The results obtained in my study are consistent with results from other preparations, such as the rabbit aorta (Kreye et al, 1991), canine coronary artery (Yoneyama et al, 1990) and rat aorta (Borg et al, 1991). These studies demonstrated the inhibition of nicorandil-induced relaxation by blockade of K$^+$ channels and GC activity. In my preparation, the vasodilator response to nicorandil was dissected using glibenclamide and LY 83583.

Glibenclamide is a widely used specific K$_{\text{ATP}}$ channel antagonist. It caused a parallel rightward shift of the nicorandil concentration effect curve when the arterial rings were contracted with 20K and BAYK. The contribution of potassium channel opening was then confirmed in experiments where the vessels were precontracted using 60 mM K$^+$.
solution. Under these conditions the nicorandil concentration response curve was again right-shifted. This was as expected because the membrane potential of the cell would be close to $E_K$ under these conditions, and so net $K^+$ ion flux is greatly reduced. The opening of $K^+$ channels is therefore unable to cause hyperpolarisation. The evidence for the involvement of the $K_{\text{ATP}}$ channel was strengthened with studies on acutely dissociated smooth muscle cells. Nicorandil activated a current that was dependent on extracellular $K^+$ concentration. The current reversed close to the calculated $E_K$, implying that the channels underlying the current were selective for potassium ions. The nicorandil-activated current could be inhibited with 10 $\mu$M glibenclamide. Dart and Standen (1995) measured single $K_{\text{ATP}}$ channels in the whole cell configuration, from dissociated cells of the pig coronary artery. Under conditions of symmetrical 140 mM $K^+$ solutions, a $K_{\text{ATP}}$ channel with a single channel conductance of 35 pS was found. The single channel conductance of $K_{\text{ATP}}$ channels falls broadly into two categories; small to intermediate conductance channels (15-50 pS in symmetrical high $K^+$); and large conductance channels (130 pS in high external $K^+$), reviewed in Nelson and Quayle, 1995. Intermediate conductance channels have been identified in the portal vein (Beech et al, 1993$^b$), cultured coronary arterial cells (Miyoshi & Nakaya, 1991), urinary bladder (Bonev & Nelson, 1993$^b$) and coronary artery (Dart & Standen, 1993). Large conductance channels have been described in the rat tail artery (Furspan & Webb, 1993) and canine aorta (Kovacs & Nelson, 1991). The $K_{\text{ATP}}$ channels activated by nicorandil in the rat mesenteric artery appear to fall into the former group. The single channel conductance of 31 pS for the nicorandil activated current in the rat mesenteric artery is consistent with previous reports. Kamouchi and Kitamura (1994) demonstrated the activation by 1 mM nicorandil of an [ATP]-sensitive $K^+$ channel with a single channel conductance of approximately 25 pS. Similarly, Teramoto and Brading (1997) showed that nicorandil activated a 43 pS $K^+$ channel in cells from the pig urethra.

The second part of this study involved the characterisation of the nitrate mechanism of action of nicorandil. Studies have demonstrated that stimulation of GC, leading to an increase in the levels of the second messenger cGMP, plays an important role in nicorandil-induced vasorelaxation (Meisheri et al, 1991). However its contribution to the overall effect is dependent on the concentration of nicorandil applied and on vessel diameter. Experiments on bovine coronary arterial strips showed that the contribution by cGMP to the total vasorelaxation was greater at higher concentrations of nicorandil (Kukovetz et al, 1992). Based on studies in the coronary vasculature, Akai et al (1995)
showed that the nitrovasodilator-like properties of nicorandil were primarily responsible for vasodilatation in large coronary vessels, whereas in the smaller vessels $K_{ATP}$ channel opening was the predominant mechanism. However, Yoneyama et al (1990) showed that the dilator response to nicorandil, in the canine anterior septal artery, was significantly attenuated by glibenclamide. In contrast, Satoh et al (1991) found that glibenclamide was ineffective in blocking nicorandil-induced relaxation in large canine arteries. Thus far, studies investigating the nitrate actions of nicorandil have used the GC inhibitor methylene blue (Kukovetz et al, 1991; Holzmann et al, 1992; Fujiwara & Angus, 1996). Methylene blue has been shown to have numerous side effects, for example direct inhibition of nitric oxide synthase (Luo et al, 1995). In view of this and due to its availability, we used the putative selective GC inhibitor LY 83583. However even more recently, antagonists with even greater specificity have emerged, for example compounds such as 1H-[1, 2, 4]oxadiazolo [4, 3-a] quinoxalin-1-one (ODQ) (Garthwaite et al, 1995). Using LY 83583, I was able to attenuate the nicorandil-induced vasorelaxation in the mesenteric arterial rings. The degree of inhibition increased with higher concentrations of LY 83583. I then went on to show that LY 83583 did not inhibit the whole cell $K_{ATP}$ current activated by nicorandil. The dual mechanism of action of nicorandil was also demonstrated by the application of both antagonists together. The compounds worked in a cumulative manner to greatly reduce the nicorandil-induced vasorelaxation. The effect by nicorandil was not completely abolished by the blockers because the concentration of LY 83583 used may have been insufficient to inhibit all of the cGMP-induced response. The inhibitors were less effective at high concentrations of nicorandil because at these higher concentrations the drug may have exhibited other non-specific effects on the vascular tissue. The stimulation of cGMP, due to activation of GC, does not appear to lead to activation of $K_{ATP}$ channels in my preparation, therefore other mechanisms must be used to cause the vasorelaxation. The precise way in which cGMP causes relaxation remains to be fully defined but probably involves a number of different mechanisms. One mechanism that has been proposed is the opening of $BK_{Ca}$ channels, resulting in hyperpolarisation and therefore inhibition of voltage-operated calcium channels. This is studied in the following chapter.

In conclusion, my studies on small resistance-sized vessels of the rat mesenteric artery have demonstrated that nicorandil causes relaxation via at least two mechanisms; one due to the opening of $K_{ATP}$ channels and the other due the stimulation of GC. Activation of GC leads to an increase in cGMP which is then able to cause vasorelaxation.
via a number of different cellular events. My results are consistent with previous studies on the mechanism of action nicorandil in other tissue preparations. A recent study by Fujiwara and Angus (1996) proposed a third possible mechanism of action for nicorandil in relaxing rat mesenteric arteries. In addition to block of nicorandil-induced relaxations by glibenclamide and methylene blue, they showed that the vasodilatation was also partially inhibited by the dihydropryidine antagonist of L-type calcium channels nifedipine. Therefore, Fujiwara and Angus suggested that this may be a third mechanism used by nicorandil to relax small arteries. They used the TXA$_2$ agonist U46619 as the contractile agonist. However, they needed a 10-fold greater concentration in the presence of nifedipine to obtain a similar initial contraction as in the absence of the Ca$^{2+}$ channel antagonist. Under these conditions release of Ca$^{2+}$ from intracellular stores would be expected to make a greater contribution to the overall contraction, and the role of L-type Ca$^{2+}$ channels would be correspondingly less important. Nicorandil has been shown to cause membrane hyperpolarisation (Furukawa et al, 1981), leading to inhibition of voltage operated Ca$^{2+}$ channels. Inhibition of these channels by nifedipine will decrease the number available for voltage-dependent block by nicorandil-induced hyperpolarisation. Hence the membrane hyperpolarisation caused by nicorandil will be less effective, resulting in a decrease in the drug's potency. Therefore the results obtained by Fujiwara and Angus may be consequence of the fewer voltage operated Ca$^{2+}$ channels available in the presence of nifedipine. The decreased effectiveness of nicorandil is a consequence of the reduced effect of membrane hyperpolarisation rather than a direct effect of the drug on the channels. To establish if this proposed third mechanism exists it would be necessary to perform patch clamp studies on single smooth muscle cells and directly assess the action of nicorandil on L-type Ca$^{2+}$ channels.
CHAPTER 4

Do Nitrovasodilators Activate $K^+$ Channels in Rat Mesenteric Arteries?
4.1 Introduction

The purpose of the experiments described in this chapter was to study the possible activation of $\mathbf{K^+}$ channels by three different nitrovasodilator drugs, nicorandil thought to act in part as a nitrovasodilator, and the pure NO-donors (±) S-nitroso-N-penicillamine (SNAP) and glyceryl trinitrate (GTN). Previous studies have suggested that NO-donors may produce vasodilatation, in part, via the opening of $\mathbf{K^+}$ channels (Hamaguchi et al, 1992; Ishibashi et al, 1995; Khan et al, 1993; Carrier et al, 1997).

I will first introduce this chapter by explaining the importance of nitric oxide (NO), an endothelium-derived relaxing factor. I will also introduce the evidence for the activation of $\mathbf{K^+}$ channels by NO and nitrovasodilator drugs.

4.1.1. Importance of endogenous nitric oxide.

The concept of endothelium-dependent vasodilatation was developed in 1980, after the discovery of the vasoactive agent endothelium-derived relaxant factor (EDRF) by Furchgott and Zawadzki. In 1987, Palmer and colleagues showed that EDRF could be quantitatively accounted for by endogenous NO. The biological activity of EDRF was due to the liberation of NO from endothelial cells (reviewed in Ignarro, 1993). A year later, it was shown that vascular endothelial cells synthesised NO from L-arginine (Palmer et al, 1988). Subsequent studies revealed that NO caused vascular relaxation directly or by stimulating the formation of cGMP in smooth muscle cells. NO is a small lipophilic molecule, hence it is able to permeate easily from the endothelial cells to the smooth muscle cell layer underneath. NO has a half-life of a few seconds, therefore the extent of its activity is limited. It is generated by various endogenous stimuli and its production is also enhanced by shear force, produced by the flow of blood through the vessel (reviewed in Moncada et al, 1991).

NO is synthesised from L-arginine and molecular oxygen, catalysed by the enzyme NO synthase (NOS). The reaction also produces citrulline as a co-product. Three isoforms of this enzyme exist, NOS-I, -II and -III, classified on the basis of their location and regulation by free cytosolic Ca$^{2+}$. NOS-I and -III, expressed in the brain and endothelium respectively, are the constitutive form of the enzyme present under physiological conditions. NOS-II, found in macrophages, is induced after immunological activation of
the cells by different inflammatory mediators, for example cytokines (reviewed in Schmidt et al, 1993). The constitutive form of the enzyme (termed cNOS) is responsible for the generation of NO in response to vasoactive agents and shear force. The activity of cNOS is controlled by intracellular Ca\(^{2+}\). An increase in cytosolic Ca\(^{2+}\) allows the interaction of cNOS with calmodulin. The binding of calmodulin activates the enzyme (Schmidt et al, 1993). Figure 4.1. summaries the biosynthesis of NO in endothelial cells.

**Figure 4.1. Model illustrating the biosynthesis of nitric oxide (NO) in endothelial cells.**

ACh, acetylcholine; BK, bradykinin; SubP, substance P; cNOS, constitutive nitric oxide synthase.

NO diffuses freely across the cell membrane and acts in a paracrine fashion to activate guanylyl cyclase (GC) in neighbouring smooth muscle cells. This leads to the formation of cGMP. Studies have shown that cGMP has actions on ion channels and cyclic nucleotide phosphodiesterases and on cGMP-dependent protein kinase (PKG) (reviewed in Waldman & Murad, 1987). Cyclic GMP has effects on smooth muscle Ca\(^{2+}\) homeostasis mechanisms, such as the cell membrane Ca\(^{2+}\) channels (Ishikawa et al, 1993\(^b\)), sarcoplasmic reticulum Ca\(^{2+}\) release channels and the cell membrane Ca\(^{2+}\) ATPase pump (Hester, 1985; Karaki et al, 1986; Taylor & Meisher, 1986). Recently, membrane
hyperpolarisation has also been shown to be an important mechanism of cGMP-mediated relaxation (e.g. Khan et al, 1993; Archer et al, 1994; Plane et al, 1996). These various actions result in decreasing cellular calcium levels and ultimately causes vasodilatation.

4.1.2. Activation of $K^+$ channels by NO.

One mechanism by which NO causes vasodilatation, either directly or through cGMP, is by membrane hyperpolarisation. Different studies have shown NO to cause vasorelaxation in tissue preparations, including the rabbit cerebral artery (Brayden, 1990; Tare et al, 1990), rabbit mesenteric artery (Murphy & Brayden, 1995) and rat mesenteric arteries (Garland & McPherson, 1992). The preceding hyperpolarisation of the cell membrane has been attributed to the opening of $K^+$ channels, however there is confusion about exactly what kind of channels are activated. In rat and rabbit mesenteric vessels the NO-evoked hyperpolarisation was sensitive to glibenclamide, the $K_{ATP}$ channel inhibitor (Garland & McPherson, 1992; Murphy & Brayden, 1995). Other investigations have shown that NO can stimulate large conductance Ca$^{2+}$ dependent potassium (BK$_{Ca}$) channels which are blocked with charybdotoxin (ChTX) (Bolotina et al, 1994; Weildt et al, 1997), or iberiotoxin (IbTX) (Khan et al, 1993). Recent evidence by Li et al (1997) illustrated the activation of BK$_{Ca}$ channels and delayed rectifier $K^+$ channels ($Kv$) in bovine coronary arteries by endothelial-derived vasodilators. Zhao et al (1997) have also shown the inhibition of NO-mediated hyperpolarisation in pulmonary arteries with 4-aminopyridine (4-AP), a blocker of $Kv$ channels.

4.1.3 NO liberation by nitrovasodilators.

Nitrates have been used in cardiovascular medicine for over a hundred years, however for most of this time their mechanism of action remained elusive. Nitrates are the first choice of drug therapy for the treatment of angina (reviewed in Abrams, 1996). The nitrovasodilators are a group of drugs that relax vascular smooth muscle cells by mimicking the actions of the endogenous active vasodilator, NO. Both SNAP and GTN are hypothesised to cause vasodilatation via NO-mediated increases in cGMP (Harrison & Bates, 1993).

Nitrovasodilators can be classified according to their mechanism of NO generation (reviewed in Harrison & Bates, 1993; Fung 1993). NO-donors, such as sodium
nitroprusside (SNP), SNAP and 3-morpholinosydnonimine (SIN-1) produce NO non-enzymatically. The organic nitrates, such as glyceryl trinitrate (GTN), isosorbide mononitrate and dinitrate release NO via an enzyme-dependent process.

The drug sodium nitroprusside has been used for many years, but the precise mechanism by which it releases NO has only recently been elucidated. Reports had previously stated that SNP "spontaneously" released NO (Feelisch & Noack, 1987). However in the absence of light the drug remained stable. When solutions of SNP were then exposed to reducing agents, such as dithiothreitol or glutathione, large quantities of NO were released (Fung, 1993). Such reducing side-groups are found on the intracellular surface of plasma membrane of smooth muscle cells. The drug readily diffuses across the membrane to interact with these groups, thus releasing NO. SNAP is thought to release NO in the cytosol in a similar way. SIN-1 liberates NO spontaneously in the extracellular solution, which is then able to diffuse into the cell.

The mechanism of NO liberation by organic nitrates has been elucidated using GTN. Studies using GTN showed that in homogenates of vascular smooth muscle, heated to inactivate all enzyme systems, the drug lost its capacity to release NO (Chung & Fung, 1990; Fung, 1993). The exact identity of the enzyme or enzymes involved has yet to be fully resolved. Initial studies suggested that a group of enzymes in the cytosol, called glutathione-S-transferases, could mobilise GTN (Tsuchida et al, 1990). However Chung & Fung (1990) showed that the generation of NO from organic nitrates occurred at the plasma membrane rather than in the cytosol, where glutathione-S-transferases are generally present (Chung et al, 1992).

4.1.4. Activation of $K^+$ channels by nitrovasodilators.

Nitrovasodilators are thought to mediate their effects through NO generation and the subsequent activation of GC and production of cGMP. Indeed, NO generation by GTN has been measured in bovine pulmonary arteries (Marks et al, 1992). Similarly, cGMP accumulation in smooth muscle cells, after nitrovasodilator administration, has also been measured (e.g. Hamaguchi et al, 1992; Ishibashi et al, 1995). As mentioned previously, NO has also been shown to act on $K^+$ channels directly. Bolotina et al (1994) showed that NO activated ChTX-sensitive $BK_{Ca}$ channels in excised patches of the rabbit aorta. However, the actions of nitrovasodilators are thought to be mediated through PKG rather
than by a direct action of NO on K⁺ channels. For example, SNAP-induced vasodilatation can be fully inhibited by blocking the activity of PKG with Rp-8-pCPT-cGMP (George & Shibata, 1995; Carrier et al, 1997), or blocking the activity of GC with methylene blue (Hamaguchi et al, 1992; Yamakage et al, 1996). Furthermore, K⁺ currents can be elicited by application of membrane permeant analogues of cGMP or G-kinase directly onto cells (Taniguchi et al, 1993; Robertson et al, 1993; George & Shibata, 1995; Yamakage et al, 1996).

Nitrovasodilators such as SIN-1, GTN and SNP have been shown to induce membrane hyperpolarisation in different preparations including rabbit mesenteric arteries (Khan et al, 1993), coronary arteries of the rabbit (George & Shibata, 1995), rat mesenteric arteries (Plane et al, 1996) and carotid arteries of the guinea pig (Corriou et al, 1996). As with the NO-induced hyperpolarisation the apparent nature of the K⁺ channel activated varied between different preparations and studies. Khan et al (1993) showed that GTN-induced vasodilatations of the rat mesenteric artery were blocked by ChTX and IbTX. Similar results were obtained by Plane et al (1996) in rat mesenteric vessels. However Corriou et al (1996) illustrated that hyperpolarisations induced by nitrovasodilators SIN-1 and SNP in guinea pig carotid arteries were abolished by glibenclamide.

The aim of my study was to investigate possible K⁺ channel activation by nicorandil, SNAP and GTN. Since the nitrovasodilator action of nicorandil occurs through the activation of GC, it is conceivable that the cGMP produced may mediate part of its action through K⁺ channels leading to membrane hyperpolarisation. SNAP produces NO in vascular smooth muscle cells via a non-enzymatic process, whereas GTN generates NO at the cell membrane of smooth muscle cells through an enzyme-dependent mechanism. The vasorelaxant actions of these compounds were studied using IbTX and ChTX, potent inhibitors of the BKCa channel, and glibenclamide, a KATP channel antagonist.
4.2 Methods

4.2.1. Tissue dissection.

Small arterial ring segments, second and third order branches, of the rat mesenteric artery were removed and mounted on a myograph. A detailed explanation of the dissection and mounting procedures is given in Methods (chapter 2, sections 2.1.1 and 2.2.3.)

4.2.2. Myograph experiments.

The method used for the wire myograph was previously outlined in chapter 3 (see section 3.3.2). Cumulative concentration effect curves to nicorandil were measured under control conditions and in the presence of IbTX. Concentration response curves were generated for SNAP in the presence and absence of glibenclamide, IbTX and ChTX, and for GTN in the presence and absence of glibenclamide and ChTX. Tissue densentisation was observed with the use of GTN and SNAP, therefore a partition was placed in the bath dividing it into two 5 ml baths. Concentration response curves in the absence and presence of an inhibitor were then measured concurrently. In some experiments, the vessels were contracted using 60 mM K⁺ solution, made by an eqimolar substitution of KCl for NaCl in the 20 mM K⁺ solution.

I would like to thank Ms. J. Bhosle, a third year project student, who did the experiments with SNAP in the presence and absence of 10 μM glibenclamide and 50 nM ChTX, SNAP concentration response curves on a 60 mM K⁺ contraction and GTN response curves in the absence and presence of 50 nM ChTX.

4.2.3. Patch clamp experiments.

For patch clamp recordings, the cells were isolated enzymatically using papain and collagenase as outlined in Methods chapter 2, section 2.3.1. The procedures used were previously described in chapter 3 (see section 3.6.1).

4.2.4. Data analysis.
The results were analysed as described in chapter 3 (section 3.3.3). The EC$_{50}$ values were converted to pD$_2$ values for statistical analysis. Statistical significance was assessed using Student’s t-test, and analysis of variance for multiple comparisons. N represents the number of animals and n is the number of observations used for the mean.

4.2.5. Drugs.

IbTX and ChTX were gifts from Zeneca. The other drugs were obtained from a number of companies: SNAP from Calbiochem; GTN from Lipha and BAYK 8644 from RBI. Nicorandil, glibenclamide and pinacidil were obtained as previously summarised in chapter 3 (see section 3.3.4.).

IbTX was made up in Milli Q water to give a 1 mM stock. ChTX was made up in Milli Q water to a stock of 1 mM. SNAP was made up in DMSO to give 100 mM stock concentration. A stock concentration of 4.4 mM GTN was available. Nicorandil, L-NAME, BAYK 8644 and pinacidil were made up and used as previously described in chapter 3, section 3.3.4.
4.3 Results

4.3.1. Effect of IbTX on nicorandil-induced vasorelaxation.

The myograph experiments described in chapter 3 (section 3.4) demonstrated that nicorandil produced a concentration-dependent relaxation of rat mesenteric arteries. The vasodilatation was blocked by glibenclamide and LY 83583. The precise mechanism by which the nitrovasodilator mechanism of nicorandil causes relaxation remains to be defined. One possible mechanism is through the activation of BK$_{Ca}$ channels which would lead to membrane hyperpolarisation and relaxation. Previous reports suggest that NO donors activate BK$_{Ca}$ channels in the aorta (Williams et al, 1988) and mesenteric arteries (Khan et al, 1993). Since nicorandil acts in part through activation of GC, I used IbTX to investigate the possibility that BK$_{Ca}$ channels were contributing to the nitrovasodilator mechanism of the nicorandil-induced relaxation. IbTX is a potent inhibitor of these channels, shown to have a $K_i$ of $\approx 2$ nM on BK$_{Ca}$ channels incorporated into neutral planar bilayers (Candia et al, 1992). Figure 4.2 shows the vasodilatation produced by nicorandil in mesenteric arterial rings. Application of IbTX (50 nM), had no significant effect on the nicorandil concentration effect curve. The EC$_{50}$s were $16.3\pm1.1$ $\mu$M and $14.7\pm1.2$ $\mu$M in the absence and presence of IbTX respectively. A Student's unpaired t-test gave a p value of 0.82 (see also figure 4.10A).
Figure 4.2  Effect of IbTX on nicorandil relaxation curves.

Concentration response curves for the relaxation of mesenteric arterial rings by nicorandil under control conditions (○; n=26, N=13) and in the presence of 50 nM IbTX (●; n=6, N=3). The vessels were contracted using 20K and BAYK, as described in the text and points show mean±S.E.M. The mean curves were drawn using equation (1), see chapter 3 (section 3.3.3.).
The effect of IbTX was then studied on acutely dissociated smooth muscle cells. The application of 300 μM nicorandil induced an inward current when the cell was held at -60 mV in symmetrical 140 mM K⁺ solutions (figure 4.3A). The application of 100 nM IbTX had no further effect on the current. The mean steady state current was 312.7±81.7 pA (n=11) and 304.2±141.7 pA (n=6) in the absence and presence of IbTX respectively. The K⁺ current was subsequently blocked by 10 μM glibenclamide (figure 4.3A).

Figure 4.3B shows the current-voltage (I-V) relationship obtained for the nicorandil-induced current in the presence and absence of IbTX in another cell. Nicorandil produced a near-linear current when voltage ramps of -50 to +50 mV were applied for 50 ms at the peak of the response. The I-V relationship was unaffected in the presence of IbTX. This suggests that the nitrovasodilator actions of nicorandil uses mechanisms other than the activation of BKCa channels to cause vasodilatation. Furthermore, the effectiveness of this peptide inhibitor could be assured because the IbTX used in this study was found to inhibit single BKCa channels in cerebral smooth muscle cells (Holland et al, 1996). I then went on to study whether other “pure” nitrovasodilators caused vasorelaxation via the activation of BKCa or KATP channels in small mesenteric arteries of the rat.
Figure 4.3 IbTX does not inhibit the nicorandil-activated current.

(A) The cell was in symmetrical 140 mM [K⁺]₀ and [K⁺]ᵢ, held at -60 mV. 300 µM nicorandil, 100 nM IbTX and 10 µM glibenclamide were applied as shown by the bars. The broken line represents the zero current level. I would like to thank Dr. M. Kubo for this figure. (B) A current-voltage (I-V) curve for a nicorandil-activated current in the absence and presence of 100 nM IbTX, in a different cell to that of (A). Voltage ramps were applied for 50 ms at the peak of the responses, as previously described in chapter 2 (section 2.3.5).
4.3.2. Effect of $BK_{Ca}$ channel blockers on SNAP relaxation curves.

SNAP is postulated to liberate NO in vascular smooth muscle via a non-enzymatic process (Harrison & Bates, 1993). In my experiments, endothelial derived NO was inhibited by the inclusion of L-NAME in all the solutions. Therefore any relaxations observed would be due to the exogenously applied nitrovasodilator. SNAP produced a concentration dependent response curve, with an EC$_{50}$ of 56.5±1.3 nM. 100 nM IbTX had no effect on the relaxation produced by SNAP (figure 4.4A) The EC$_{50}$ values are given in table 4.1 (see also figure 4.10B). The effect of IbTX was not statistically different from control, a Student’s t-test gave a p value of 0.46. Further studies were done using 50 nM charybdotoxin (ChTX), another highly potent inhibitor of $BK_{Ca}$ channels (reviewed in Castle et al, 1989). ChTX binds to mammalian muscle $BK_{Ca}$ channels with high affinity, with a $K_D$ value of 5 nM (Anderson & Miller, 1987). In my preparation, ChTX, like IbTX, had no significant effect on SNAP-induced vasorelaxations (see figure 4.10B and table 4.1), p=0.65.

Table 4.1 pH$_2$ and EC$_{50}$ values for SNAP under control conditions and in the presence of either 100 nM IbTX or 50 nM ChTX.

The values show mean±S.E.M. All the external solutions contained L-NAME except where indicated. Statistical significance was assessed between the different groups. * p<0.05, ANOVA followed by Duncan’s post hoc test.

<table>
<thead>
<tr>
<th></th>
<th>EC$_{50}$ (nM)</th>
<th>pH$_2$±S.E.M.</th>
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<tbody>
<tr>
<td>SNAP</td>
<td>56.5</td>
<td>7.3±0.08</td>
</tr>
<tr>
<td>100 nM IbTX</td>
<td>42.9</td>
<td>7.4±0.3</td>
</tr>
<tr>
<td>50 nM ChTX</td>
<td>57.0</td>
<td>7.2±0.1</td>
</tr>
<tr>
<td>ChTX (no L-NAME)</td>
<td>191</td>
<td>6.7±0.2 *</td>
</tr>
</tbody>
</table>
Figure 4.4 The effect of $\text{BK}_{\text{Ca}}$ channel inhibitors on SNAP-induced relaxations.

(A) Concentration response curves are shown under control conditions (□; n=21, N=21) and in the presence of 100 nM IbTX (●; n=3, N=3). (B) Relaxation curves to SNAP in the absence (□) and presence of 50 nM ChTX (▲; n=5, N=5). The rings were contracted using 20 K and BAYK. N denotes the number of animals, and n is the number of preparations from which means were taken.
4.3.3. *Effect of glibenclamide on SNAP-induced responses.*

Some studies have suggested that nitrovasodilators can activate $K_{ATP}$ channels (e.g. Corriou *et al.*, 1996). I therefore investigated the possibility of $K_{ATP}$ channel activation by SNAP. Figure 4.5A shows the mean SNAP concentration response curves in the presence and absence of glibenclamide. The blocker had no significant effect ($p=0.40$) on the SNAP-induced relaxation (see figure 4.10B and table 4.2). In further experiments, the arterial ring segments were contracted using 60 mM $K^+$ solution. Under these conditions, the SNAP relaxation curve was unchanged (figure 4.5B and table 4.2), hence implying that SNAP-induced vasodilatation does not involve the activation of $K^+$ channels.

**Table 4.2 pD$_2$ and EC$_{50}$ values for SNAP in the absence and presence of 10 μM glibenclamide.**

Also given are the mean EC$_{50}$ values obtained for SNAP when the vessels were contracted using 60 mM $K^+$ solution. The values were not significantly different.

<table>
<thead>
<tr>
<th></th>
<th>EC$_{50}$ (μM)</th>
<th>pD$_2$±S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNAP</td>
<td>56.5</td>
<td>7.3±0.08</td>
</tr>
<tr>
<td>10 μM glibenclamide</td>
<td>74.3</td>
<td>7.1±0.1</td>
</tr>
<tr>
<td>60 mM $K^+$ contraction</td>
<td>80.2</td>
<td>7.1±0.2</td>
</tr>
</tbody>
</table>

This result was also reflected in whole cell recordings from dissociated smooth muscle cells. The external application of a maximal concentration of SNAP (3 μM) had no effect, however the addition of 10 μM pinacidil resulted in the induction of a large inward $K^+$ current (figure 4.6A). Under conditions of 140 mM $[K^+]_o/[K^+]_i$ solutions and a holding potential of -60 mV, pinacidil induced a current of approximately 100 pA in this cell. The mean peak current was 97.5±19.3 pA from 4 other cells. The pinacidil-induced current was substantially blocked by 10 μM glibenclamide (figure 4.6A). Voltage ramps, applied at the peak of each response (figure 4.6B), reflected the activation of a glibenclamide-sensitive $K_{ATP}$ current by pinacidil but not SNAP.
Figure 4.5  Concentration response curves for the relaxations of established tone by SNAP.

(A) Under control conditions (☐, as in figure 4.4) and +10 μM glibenclamide (●; n=8, N=8). (B) Concentration response curve for SNAP when the maximal tension was produced with 60 mM external K⁺ solution (■; n=5, N=5), as described in the text.
Figure 4.6. Pinacidil, but not SNAP activated a $K_{ATP}$ current.

(A) The cell was held at -60 mV and symmetrical 140 mM K$^+$ solutions were used. SNAP (3 μM), pinacidil (10 μM) and glibenclamide (glib, 10 μM) were applied as indicated. The recording was made at a bandwidth of 10 kHz and then digitised at 2 kHz prior to display. (B) Currents recorded in response to a linear voltage ramp from -50 to +50 mV. The ramps were applied at the peak of the response to SNAP, pinacidil and glibenclamide. The I-V ramp performed under control conditions, in the absence of any drugs, was subtracted from each ramp. Each I-V relationship was an average of 20 identical ramps.
4.3.4. **Effect of ChTX on relaxations to GTN.**

I then went on to investigate the actions of another nitrovasodilator, GTN. Like other organic nitrates, the compound produces NO in vascular smooth muscle cells via an enzyme-dependent process (Fung, 1993). Khan *et al* (1993) demonstrated that vasorelaxations to GTN, ACh, and NO were inhibited with ChTX in the isolated mesenteric artery of the rabbit. The effects of ChTX on GTN-induced vasorelaxation were studied in this preparation. Figure 4.7 shows the mean concentration response curves for GTN in the presence and absence of 50 nM ChTX. The inhibitor had a small but insignificant effect on the GTN-induced vasorelaxation. The EC${}_{50}$s were 7.2±1.3 nM and 5.1±1.2 nM for GTN concentration effect curves under control conditions and in the presence of ChTX respectively (tabulated in table 4.3), see also figure 4.10C.

4.3.5. **Effect of glibenclamide on GTN-induced responses.**

Further experiments went on to show that GTN-induced vasorelaxation does not involve $K_{\text{ATP}}$ channel activation. Concentration response curves to GTN were changed slightly in the presence of 10 μM glibenclamide (see figure 4.8 and 4.9C). Student's t-testing for significance illustrated there was no statistical significance in GTN log EC${}_{50}$s between control and in the presence of glibenclamide ($p=0.14$). The pD${}_2$ values given in table 4.3 and summarised graphically in figure 4.10C.

**Table 4.3 EC${}_{50}$ and pD${}_2$ values for GTN under control conditions and in the presence of 10 μM glibenclamide or 50 nM ChTX.**

<table>
<thead>
<tr>
<th></th>
<th>EC${}_{50}$ (nM)</th>
<th>pD${}_2$±S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTN</td>
<td>7.2</td>
<td>8.1±0.1</td>
</tr>
<tr>
<td>10 μM glibenclamide</td>
<td>4.7</td>
<td>8.3±0.09</td>
</tr>
<tr>
<td>50 nM ChTX</td>
<td>5.1</td>
<td>8.3±0.07</td>
</tr>
</tbody>
</table>

There was no significant differences between the groups, at the 0.05 significance level.
Figure 4.7. Effect of ChTX on GTN-induced vasorelaxation.

Relaxation curves to GTN under control conditions (◇; n=8, N=8) and in the presence of 50 nM ChTX (▼; n=3, N=3). The arterial rings were contracted using 20 K and BAYK, as described in the text. The points illustrate mean±S.E.M. The curves were generated using equation (1), chapter 3 (section 3.3.3.).

Figure 4.8. Effect of glibenclamide on GTN-induced relaxation.

Concentration response curves are shown under control conditions (◇, as in figure 4.7) and in the presence of 10 μM glibenclamide (▲; n=3, N=3). n represents the number of preparations, and N is the number of animals.
4.3.5. *Effect of ChTX on SNAP-induced vasorelaxation, in the absence of L-NAME.*

The production of NO from the substrate L-arginine is catalysed by the enzyme NOS. L-NAME inhibits NO generation by inhibiting constitutive NOS (Rees *et al*, 1990). L-NAME was used in all my experiments to ensure that endogenous NO did not contribute to the vasodilatation produced by the various nitrorelaxant drugs.

A recent study by Plane *et al* (1996), on small mesenteric vessels of the rat, suggested that the vasorelaxant actions of the NO-donor SIN-1 were dependent on the presence of basal NO. Using the wire myograph, they showed that ChTX almost abolished the response to SIN-1 in endothelial intact preparations. In endothelium-denuded arterial vessels SIN-1 appeared to produce full relaxation through other pathways. Hence, the results by Plane and colleagues imply that in an intact endothelium layer, the major mechanism of vasodilatation is through BKCa channel activation. However, in the absence of the endothelium relaxation occurs via other mechanisms.

The following experiments were done to investigate the role, if any, of endothelium-derived NO on SNAP-induced vasorelaxations. As figure 4.9 shows, 50 nM ChTX has a significant effect on SNAP concentration response curve, in the absence of L-NAME. The integrity of the endothelium was assessed using methacholine (100 nM) after pre­contracting the arteries with 20K and BAYK. ChTX had an effect on SNAP response curves only in those vessels that showed a relaxation to methacholine. Table 4.1. shows the mean EC50 values for SNAP in the presence and absence of ChTX+/−L-NAME. Mean pD2 values are graphically shown in figure 4.10B.
Figure 4.9. The effect of ChTX on SNAP-induced vasorelaxation, in the absence of external L-NAME.

Concentration response curve to SNAP are shown in the absence (□, as in figure 4.4) and presence of 50 nM ChTX (▽; n=3, N=3). The vessels were contracted using 20K and BAYK, as described in the text. The points show mean±S.E.M. and curves were drawn using equation (1), chapter 3 (section 3.3.3.). Student's t-test were done to test significance levels at the 0.05 and 0.01 significance levels. (* p<0.05 and ** p<0.01).
Figure 4.10 Summary bar charts showing the $pD_2$ (negative log$_{10}$ of the EC$_{50}$) values for nicorandil, SNAP and GTN.

(A) Bar chart illustrating mean±S.E.M. $pD_2$ values for nicorandil in the absence and presence of 100 nM IbTX. (B) Bar chart illustrating the mean $pD_2$ values for SNAP in the absence and presence of 100 nM IbTX, 50 nM ChTX, 10 μM glibenclamide (glib) and when concentration response curves to SNAP were performed on a 60 mM K$^+$ contraction. Also shown is the SNAP mean $pD_2$ value in the presence of ChTX when L-NAME was excluded from the external solution (no L-NAME). (C) $pD_2$ values for GTN in the absence and presence of 50 nM ChTX and 10 μM glibenclamide. * indicates responses significantly different (p<0.05) from control.
4.4 Discussion

The experiments described in this chapter examined the contribution of BK\textsubscript{Ca} or K\textsubscript{ATP} channels in nitrovasodilator-induced vasorelaxation. In rat mesenteric vessels, the anti-anginal drug nicorandil caused vasodilatation by combining the actions of K\textsubscript{ATP} channel opening with the activation of guanylyl cyclase (GC) (Chapter 3). My results suggest that the component of nicorandil-induced vasorelaxation that is due to GC stimulation, did not involve the opening of BK\textsubscript{Ca} channels. Nicorandil-induced vasorelaxation was not significantly inhibited by IbTX, a potent BK\textsubscript{Ca} channel antagonist. Therefore to study if this effect was unique to this compound, I investigated the mechanism of action of two other pure nitrovasodilators, SNAP and GTN. The results obtained showed that vasodilator responses to GTN and SNAP were also not significantly inhibited by IbTX. My experiments then went on to show that ChTX, another potent inhibitor of BK\textsubscript{Ca} channels, also had no significant effect on concentration effect curves to SNAP and GTN. Interestingly, however, ChTX had a significant effect on SNAP-induced vasorelaxation when endothelium-dependent NO production was not inhibited by L-NAME (figure 4.9). Furthermore, pre-treatment of the arterial rings with glibenclamide, the K\textsubscript{ATP} channel blocker, had little effect on relaxation curves to SNAP and GTN. Consistent with this, SNAP was unable to elicit a K\textsubscript{ATP} current in acutely dissociated smooth muscle cells. These results suggest that in my preparation, under conditions of zero basal NO production neither BK\textsubscript{Ca} or K\textsubscript{ATP} channel activation play an important role in the vasorelaxation mediated by nitrovasodilators in rat mesenteric arteries.

At first glance the results presented in this chapter appear to be contradictory to previous studies and established ideas in the literature. A number of studies have shown that nitrovasodilators activate BK\textsubscript{Ca} channels in a variety of different tissue preparations. Khan et al (1993) and Plane et al (1996) provided evidence, based on myograph studies, for the activation of BK\textsubscript{Ca} channels by nitrovasodilator drugs in the rabbit and rat mesenteric arteries respectively. Similarly, George and Shibata (1995) showed that SNAP increased the open probability of BK\textsubscript{Ca} channels in coronary arterial smooth muscle cells from the rabbit. Other studies have shown nitrovasodilator activation of ChTX or IbTX-sensitive BK\textsubscript{Ca} channels in porcine trachea (Yamakage et al, 1996), rabbit aorta (Ishibashi et al, 1997) and rat mesenteric vessels (Carrier et al, 1997). A study has also demonstrated the activation of glibenclamide-sensitive K\textsubscript{ATP} channels. Corriou et al (1996) showed that
membrane hyperpolarisation by SNP and SIN-1 involved the opening of $K_{\text{ATP}}$ channels. In the following discussion, I will present different ideas which may go towards understanding these results.

The lack of effect of $K^+$ channel blockers in my experiments can not be easily accounted for by drug differences. All three compounds, SNAP, GTN and nicorandil, are commonly used experimental drugs. Also the concentrations of the compounds used were similar to those used by others, such as Khan et al (1993) and Carrier et al (1997).

One possibility is that tissue specificity and differences may explain the results obtained, but it is difficult to accept that there is such variability between tissues. This also seems very unlikely since Garland and McPherson (1992) and Plane et al (1996) showed membrane hyperpolarisation induced by NO and nitrovasodilators respectively in rat small mesenteric arteries, illustrating that $K^+$ channel activation can occur in this preparation.

Another possibility is that the nitrovasodilator-induced membrane hyperpolarisation may be mediated by other $K^+$ channels which were not studied, such as $K_v$ channels. NO-donors are able to activate 4-AP-sensitive $K_v$ channels in different smooth muscle preparations (e.g. Li et al, 1996; Zhao et al, 1997). Therefore using 4-AP it would have been possible to assess its role in nitrovasodilator-mediated relaxation in the rat mesenteric artery. However SNAP was just as effective in causing relaxation of 60 mM $K^+$ contractions, (figure 4.5B), suggesting that SNAP-induced vasodilatation was not due to the activation of any type of $K^+$ channel. As external $K^+$ concentration is raised, the outwardly directed electrochemical gradient for $K^+$ is reduced and at high external $K^+$ concentrations channel activators are unable to cause hyperpolarisation because the membrane potential is already very close to $E_K$.

A more plausible explanation can be obtained by comparing my results to those obtained by Plane and colleagues (1996). Using small mesenteric vessels of the rat, they showed that SIN-1 produced concentration-dependent relaxations of phenylephrine-constricted vessels in endothelium-intact and denuded preparations. ODQ, the GC inhibitor, did not alter relaxations to SIN-1 in either tissue. However, it was shown to inhibit SIN-1-stimulated generation of cGMP. In endothelium-intact arterial segments, these vasorelaxations were blocked by up to 90 % by ChTX. But when basal NO was inhibited, by L-NAME or endothelial removal, relaxations to SIN-1 were unaffected by ChTX. Under these conditions, both ODQ and ChTX were required to block SIN-1-
evoked relaxations. These results by Plane et al imply that basal NO is needed for K⁺ channel activation and that in its absence SIN-1 is able to induce vasorelaxation via other mechanisms, with or without cGMP production. Indeed, comparing this study to the earlier work of Khan et al (1993) showing the inhibition by ChTX or IbTX of GTN-induced vasorelaxation, it is clear that basal NO production was not inhibited in that study. In contrast, several investigations on isolated smooth muscle cells have shown the activation of BKCa channels by nitrovasodilators (e.g. George & Shibata, 1995; Yamakage et al, 1996; Carrier et al, 1997). In these experiments basal endothelial NO production was clearly eliminated by the absence of endothelial cells.

In my experiments, the inclusion of L-NAME would have led to inhibition of induction of endogenous NO and inhibition of the K⁺ channel activation as described by Plane and colleagues. Although the concentration of L-NAME used by Plane et al was five times greater, studies suggest that 20 μM L-NAME would have been sufficient to inhibit endothelial NOS (e.g. Ralevic et al, 1991; Vo et al, 1992). Final myograph experiments using SNAP without L-NAME (figure 4.9) showed that in the presence of basal NO, SNAP produced a relaxation that could be blocked by ChTX. However, I did not get a great an inhibition as that described by Plane et al, hence other mechanisms must still be present to cause vasorelaxation. Therefore complex mechanisms exist by which nitrovasodilators and basal NO interact to cause membrane hyperpolarisation. In addition there are other hyperpolarisation-independent mechanisms. Figure 4.11 summaries the possible mechanisms discussed above.
Figure 4.11  Putative mechanism illustrating the distinct ways in which nitrovasodilators and endogenous NO cause relaxation.

The nitrovasodilators may be unable to directly activate K\(^+\) channels. The unbroken arrows show the mechanism of nitrovasodilator-induced vasorelaxation suggested by Plane et al (1996) and my results, whereas the broken arrow illustrates the cGMP-dependent activation of K\(^+\) channels (e.g. Hamaguchi et al, 1992; Taniguchi et al, 1993; George & Shibata, 1995).

In conclusion, in my experiments I found that the nitrovasodilators SNAP and GTN did not use BK\(_{\text{Ca}}\) or KA\(_{\text{ATP}}\) channel activation to cause vasodilatation of rat small mesenteric arteries. Also the nitrovasodilator action of nicorandil did not involve BK\(_{\text{Ca}}\) channel activation. The reasons for this can not easily be explained by tissue differences or the stimulation of other K\(^+\) channels present on smooth muscle cells. In contrast to many studies, there is some evidence to suggest that basal endothelium-dependent NO is required for K\(^+\) channel activation (Plane et al, 1996). Therefore inhibition of basal endothelium-derived NO, with L-NAME in my preparation, may have prevented K\(^+\) channel opening. The cGMP, postulated to be generated by the nitrovasodilators, acts through a variety of other cellular mechanisms to decrease intracellular Ca\(^{2+}\) levels and ultimately cause vasorelaxation.
CHAPTER 5

Enhancement of the Vasorelaxant Potency of Nicorandil by Metabolic Inhibition and Adenosine
5.1 Introduction

The activity of $K_{ATP}$ channels is greatly increased under ischaemic conditions when intracellular metabolite levels change. A decrease in cytosolic ATP levels occurs whilst ADP, adenosine and $H^+$ levels increase. It has been proposed that the activity of $K_{ATP}$ channel openers may also be enhanced under such conditions (Quast & Cook, 1989). Indeed, Randall and Griffith (1993) have shown that the vasodilator action of levocromakalim, a $K_{ATP}$ channel opener, was increased in the isolated rabbit ear artery under conditions of hypoxia and impairment of oxidative phosphorylation. However, the potency of another $K_{ATP}$ channel opener, pinacidil, was unchanged under similar conditions. I was therefore interested to investigate whether the potency of the anti-anginal drug nicorandil was increased when conditions present in ischaemia were mimicked. An increase in potency under these conditions would suggest that the drug profile of nicorandil is similar to that of levocromakalim rather than that of pinacidil. This study may also have relevance for the clinical use of nicorandil, where one of the major consequences of angina is the presence of ischaemia in the myocardium.

$K_{ATP}$ channels are characterised by their sensitivity to intracellular ATP, in that high $[ATP]_i$ closes the channel (reviewed in Quayle et al., 1997). In vascular smooth muscle cells, cytosolic ATP levels remain high under physiological conditions so that the activity of $K_{ATP}$ channels is limited. A fall in intracellular ATP occurs under pathophysiological conditions, such as ischaemia and hypoxia. Some of the changes associated with ischaemia include metabolic inhibition of cellular processes such as glycolysis, changes in cellular pH and increased release of adenosine. The activation of $K_{ATP}$ channels under these situations would lead to smooth muscle cell hyperpolarisation and ultimately vessel dilatation. This would therefore improve blood flow to the ischaemic area. Investigations have since shown that $K_{ATP}$ channels in smooth muscle cells are more responsive when a cell is metabolically stressed. Fink and Luttgau (1976) noted that $K^+$ permeability increased in metabolically exhausted skeletal muscle. The pharmacological sensitivity of the increased $K^+$ permeability was characteristic of $K_{ATP}$ channels (Castle & Haylett, 1987; Standen et al., 1992). Silberberg and van Breeman (1992) were the first to demonstrate the activation of $K^+$ currents in vascular smooth muscle cells, of the rabbit mesenteric artery, under conditions of metabolic inhibition. They showed that lemakalim
(levcromakalim) also induced a similar current and both currents were sensitive to block by glibenclamide, a $K_{ATP}$ channel inhibitor.

5.1.1. **Metabolic inhibition.**

Since those initial findings, the activation of $K_{ATP}$ channels under conditions of metabolic inhibition or hypoxia has been recorded in a number of different vascular preparations (Beech *et al.*, 1993a; Zhang & Bolton, 1995; Teramoto & Brading, 1996). Conway and colleagues (1994) showed that the inhibition of glycolysis and glycogenolysis, with 2-deoxyglucose (2-DG), produced an endothelium-independent dilatation of rat pressurised coronary arteries. This dilatation was accompanied by membrane hyperpolarisation. Glibenclamide abolished the hyperpolarisation and significantly reversed the vasodilatation. Similarly, metabolic inhibition with sodium cyanide (NaCN) or 2-DG was shown to evoke glibenclamide-sensitive $K^+$ currents in on-cell patches of the rat mesenteric artery (Zhang & Bolton, 1995).

5.1.2. **Effect of acidosis.**

Over a century ago, Gaskell found that dilatation of blood vessels in frog skeletal muscle was significantly greater in external acidic solutions. Since then, several different vascular beds have been reported to vasodilate in response to acidosis (e.g. Ledingham *et al.*, 1970; Kontos *et al.*, 1977; Faraci *et al.*, 1994). A decrease in external pH has been suggested to occur during ischaemia, and is involved in blood flow regulation (Wray, 1988). Cerebral arteries are particularly sensitive to changes in pH and this effect is predominantly due to extracellular pH. External acidification has been shown to increase $K^+$ permeability and hyperpolarise smooth muscle cell membranes (Dietrich & Dacey, 1994). Ishizaka and Kuo (1996) showed that external acidosis induced vasodilatation of pressurised porcine coronary arterioles. The vasodilatation was endothelium-independent and was attenuated by glibenclamide. Conversely, increases in external and internal pH, leading to alkalisation, inhibited $K^+$ currents in coronary vascular smooth muscle resulting in depolarisation (Webster *et al.*, 1997).

These results suggest that $K_{ATP}$ channels in vascular smooth muscle contribute to arteriolar responses to pH changes. The exact mechanism by which increases in external
proton concentration affect $K_{\text{ATP}}$ channels is unknown. Evidence from skeletal muscle suggests that protons may bind directly to the $K_{\text{ATP}}$ channel, possibly at the ATP binding site, thus preventing ATP from exerting its inhibitory effect (Davies et al., 1992). However, there are also studies suggesting that protons may affect intracellular Ca$^{2+}$ levels directly by decreasing Ca$^{2+}$ entry across the membrane and Ca$^{2+}$ release from the sarcoplasmic reticulum (reviewed by Wray, 1997; see also Iwasawa et al., 1997). In contrast to these findings, alkalinisation, not acidosis, has been shown to inhibit Ca$^{2+}$ entry and causes vasodilatation in some vascular beds (Heaton et al., 1992). And so it seems that the effect of pH on vascular tone has yet to be fully elucidated.

5.1.3. Role of adenosine.

One of the major consequences of ischaemia and hypoxia is an increase in circulating adenosine. The mediator is an endogenous vasodilator which plays a major role in adjusting blood flow with respect to metabolic demand (Berne, 1980; Berne et al., 1983). Adenosine-induced vasodilatation and hyperpolarisation is partially reversed with glibenclamide (Daut et al., 1990), therefore researchers have suggested that part of the adenosine-mediated effect is through the activation of $K_{\text{ATP}}$ channels. Dart and Standen (1993) showed the activation of $K_{\text{ATP}}$ channels by exogenous adenosine in porcine coronary myocytes. However $K_{\text{ATP}}$ channels may not contribute to adenosine-induced relaxations under some conditions, since vasorelaxations in porcine and canine arterial ring segments were not blocked by glibenclamide (Makujina et al., 1994).

Adenosine mediates its effects through membrane-bound adenosine receptors. The signal transduction pathway between the receptor activation and response involves G-proteins. Initial reports showed that adenosine increased cAMP levels in rodent brain slices (Sattin & Rall, 1970). The adenosine receptors were defined by their ability to stimulate adenylyl cyclase through interactions with the G-protein, $G_\text{s}$ (reviewed in Fredholm et al., 1994). Biochemical evidence then suggested the existence of multiple adenosine receptors because adenosine analogues increased cAMP production in some preparations but decreased it in others. The receptor subtype that decreased cAMP was shown to couple to $G_\text{\gamma}$ and $G_\text{\delta}$-proteins (Freissmuth et al., 1991; Munshi et al., 1991). Because of these differing effects, the presence of two receptor subtypes termed $A_1$ and $A_2$ was proposed. $A_1$ adenosine receptor activation reduced cAMP whereas $A_2$ adenosine
receptor stimulation increased cAMP levels. Structural information, from molecular biology, revealed the presence of a third receptor subtype. This receptor shared high sequence homology with the A2 receptor in the transmembrane domain but differed in the carboxyl-terminal region (reviewed in Fredholm et al, 1994). However both receptors had essentially similar signal transduction mechanisms, therefore a reclassification of the A2 receptor to A2a and A2b nomenclature was accepted. Recently another adenosine receptor, termed A3, has been identified in the testis (Zhou et al, 1992). Unlike the others, the A3 receptor is insensitive to inhibition by methylxanthines (reviewed in Fredholm et al, 1994). Table 5.1. summarises the known agonists and antagonists for each of the adenosine receptor subtypes identified thus far.

Table 5.1. Major characteristics of adenosine receptors.
(modified from Fredholm, 1995)
2-CCPA, 2-chloro-N6-cyclopentyladenosine; CPA, N6-cyclopentyladenosine; NECA, 5'-ethylcarboxamidoadenosine; CGS-21680, 2-p-(2-carboxyethyl)pentlamino-5'-N-ethylcarboxamidoadenosine; CV-1808, 2-phenylaminoadenosine; R-PIA, R-N6-(2-phenylisopropyl)adenosine; APNEA, N6-2-2-(4-aminophenyl)-ethyladenosine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; 8-CPT, 8-cyclopentyltheophylline; ZM 241 385, 4-(2-[7-amino-2-(2-furyl)[1, 2, 4] triazolol[2, 3-a] [1, 3, 5]tiazin-5-yarno] ethyl)phenol.

<table>
<thead>
<tr>
<th>Names</th>
<th>A1</th>
<th>A2a</th>
<th>A2b</th>
<th>A3</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-protein</td>
<td>G1</td>
<td>Gs</td>
<td>Gs</td>
<td>?</td>
</tr>
<tr>
<td>coupling</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cAMP effect</td>
<td>↓</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Agonists</td>
<td>2-CCPA&gt; CPA&gt; CGS-21680&gt; NECA&gt; CGS-21680&gt; CV-1808&gt; R-PIA&gt; CPA</td>
<td>NECA&gt; NECA&gt; NECA&gt; CGS-21680</td>
<td>APNEA&gt; R-PIA&gt; NECA&gt; CGS-21680</td>
<td></td>
</tr>
<tr>
<td>Antagonists</td>
<td>DPCPX, 8-CPT</td>
<td>ZM 241 385</td>
<td>-</td>
<td>?</td>
</tr>
</tbody>
</table>

The effects of adenosine on the vasculature have been attributed to adenosine-receptor stimulation. In pig coronary arteries, the A1 adenosine receptor has been shown to be coupled to KATP channels (Merkel et al, 1992; Merkel et al, 1993). Glibenclamide antagonised vasodilatation to the A1 adenosine receptor agonist cyclopentyladenosine...
(CPA) but not to the \(A_2\) adenosine receptor agonist \(N^6\)-[2-(3, 5-dimethoxyphenyl)-ethyl] adenosine (DMPA) in these vessels. Furthermore, Dart and Standen (1993) demonstrated the activation of \(K_{\text{ATP}}\) channels in pig coronary smooth muscle cells via \(A_1\) adenosine receptor activation. \(A_1\) adenosine receptors are coupled to a pertussis toxin sensitive G-protein (\(G_i\) or \(G_o\)) which decreases AC activity, cAMP production and therefore PKA stimulation. \(K_{\text{ATP}}\) channels have been shown to be activated by the presence of the catalytic subunit of PKA in the intracellular solution (Quayle et al, 1994), hence the activation of \(K_{\text{ATP}}\) channels by \(A_1\) receptors must involve a different second messenger system. In cardiac tissue adenosine, acting through \(A_1\) receptors, appears to activate \(K_{\text{ATP}}\) channels through direct coupling of the G-protein, \(G_i\), with the channel. (Kirsch et al, 1990).

However the type of adenosine receptor coupled to vascular \(K_{\text{ATP}}\) channels appears to be tissue dependent. Several studies have shown that \(A_2\) receptor activation can also couple to \(K_{\text{ATP}}\) channels. \textit{In vivo} studies on dogs have shown that a decrease in systemic blood pressure by the \(A_2\) receptor agonist 2-(1-octynyl)-adenosine (YT-146) was attenuated by glibenclamide (Yoneyama et al, 1992). Kleppisch and Nelson (1995) then showed that stimulation of \(A_2\) receptors but not \(A_1\) receptors induced glibenclamide-sensitive \(K_{\text{ATP}}\) currents in smooth muscle cells of the rabbit mesenteric artery. Furthermore, they demonstrated that adenosine activation of \(K_{\text{ATP}}\) channels was reversed by Rp-cAMPs and H-89; both are membrane-permeant inhibitors of PKA.

To summarise, one or more adenosine receptor subtypes is coupled to \(K_{\text{ATP}}\) channels in vascular tissue. Identification of the adenosine receptors activated during ischaemia may provide a means of developing and targeting drugs to the affected areas.

5.1.4. \textit{Enhancement of channel opener activity.}

The metabolic sensitivity of \(K_{\text{ATP}}\) channels suggests that \(K^+\) channel openers might be more potent in ischaemic tissues where metabolism is compromised. Experiments by Randall and Griffith (1993) showing the increased potency of levromakalim, but not pinacidil, during metabolic inhibition suggests that the increased potency of channel openers is dependent on their exact mechanism of interaction with the channel. In contrast to the findings of Randall and Griffith, hypoxia and low external glucose had little effect on levromakalim-induced relaxations in strips of the rat aorta (Hüsken et al, 1997). The
effectiveness of the drug was in fact reduced under these conditions, implying that K\(^+\) channel openers may be more effective under conditions of metabolic compromise in small rather than large vessels.

In view of the lack of research on other K\(_{ATP}\) channel openers, and the possible clinical importance of such a study, I investigated the effects of metabolic compromise on the vasodilator responses of the channel opener, nicorandil. Nicorandil is a clinically prescribed anti-anginal drug and previous studies have shown that it causes vasodilatation of small arteries (see chapter 3). It has at least two mechanisms of action; it acts a K\(^+\) channel opener and it also acts as a nitrovasodilator, stimulating GC and increasing cGMP. Clinically, in contrast to other nitrovasodilators, tolerance does not develop on continued application of nicorandil, and this lack of tolerance seems to be due to its K\(^+\) channel opening action (Frampton et al, 1992). I investigated whether its potency in relaxing coronary arteries is increased by treatments designed to mimic those that occur in ischaemia.

The aim of the experiments described in this chapter was to investigate if the potency of nicorandil was increased by manoeuvres designed to mimic those that are thought to occur during ischaemia, and if such an effect was due to the increased activity of the drug on K\(_{ATP}\) channels rather than GC stimulation. The conditions investigated were inhibition of oxidative phosphorylation, inhibition of glycolysis, induction of external acidosis and application of external adenosine. All these different manoeuvres were found to increase the potency of nicorandil (Davie & Standen, 1997). I also investigated the adenosine receptor subtypes involved in the enhancing effect of adenosine on the potency of nicorandil in the pig coronary artery.
5.2 Methods

5.2.1. Tissue dissection.

Mesenteric arterial rings were dissected from adult Wistar rats as described before (see Chapter 2, section 2.1.1).

Fresh pig hearts from obtained from a local abattoir, after animals had been slaughtered in accordance with EEC regulations. Second or third order distal branches from the left anterior descending coronary artery were dissected as outlined in chapter 2 (see Methods, section 2.1.2.).

5.2.2. Myograph experiments.

2 mm ring segments were dissected and mounted in a wire myograph as previously described (chapter 2, section 2.2.3.). All the solutions added to the myograph chamber contained 20 μM L-NAME to remove endothelium-derived NO activity. The vessels were contracted using 20 mM K⁺ solution (see Methods, section 2.4. for composition) and 200 nM BAYK 8644 (20 K and BAYK). The 20 K and BAYK solution caused the opening of voltage operated Ca²⁺ channels by the modest depolarisation produced by 20 mM [K⁺]₀ and agonist activation. The increase in external K⁺ produced a depolarisation but it kept the K⁺ equilibrium potential sufficiently negative so that K_ATP channel activation could occur.

Once a stable contraction had been achieved, nicorandil relaxation curves were performed cumulatively, as described in chapter 2 (section 2.2.4.) In some experiments, I used 80 mM K⁺ solution for contracting the vessels rather than the 20 K and BAYK combination. The solution was made up by equimolar substitution of KCl for NaCl in the 20 mM K⁺ solution (see Methods, section 2.4 for composition).

Where used, carbonyl cyanide m-chlorophenylhydrazone (CCCP) and 2-chloroadenosine (2-CA) were added at the peak of the contraction to give bath concentrations of 100 (30 nM for rat mesenteric vessels) and 300 nM respectively. The adenosine analogues, CCPA and CGS-21680, were also added to the chamber to give concentrations of 100 nM. For 0.5 mM or zero glucose solutions, glucose was omitted as required from the 20 mM K⁺ solution. In some experiments, the 20 mM K⁺ solution
containing 0.5 mM glucose was further complemented with 4.5 mM 2-DG. The low pH solution was made acidic, to a pH of 6.8, by the addition of HCl to the 20 mM K⁺ solution. For experiments in which the K<sub>ATP</sub> channel blocker glibenclamide were used, 10 μM glibenclamide was added prior to the measurement of a nicorandil dose response curve. Similarly, the adenosine antagonists DPCPX and ZM 241385, were added to the bath to give final concentrations of 100 nM or 10 nM respectively.

5.2.3. Data analysis.

The data was collected and analysed as previously described (chapter 2, section 2.2.4). pD<sub>2</sub> values were used for statistical tests and statistical significance was assessed using Student’s t-tests for simple comparisons, and analysis of variance with Duncan’s post hoc test for multiple comparisons. n denotes the number of observations and N is the number of animals.

5.2.4. Drugs.

Nicorandil, glibenclamide, GTN, BAYK 8644 and L-NAME were obtained as outlined before in Chapters 3 and 4 (section 3.3.5 and 4.2.5). The other drugs were purchased as follows: CCCP from Sigma; 2-CA, CGS-21680 HCl, CCPA and DPCPX from RBI; ZM 241385 from Tocris.

CCCP, CGS- 21680, CCPA, DPCPX and ZM 241385 were dissolved in DMSO to give stock concentrations of 100 mM, 10 mM, 10 mM, 10 mM and 10 mM respectively. 2-CA was dissolved in Milli-Q water to give a stock concentration of 10 mM. The remaining drugs were dissolved as previously described, in chapters 3 and 4 (sections 3.3.4. and 4.2.5.).
5.3 Results

5.3.1. Metabolic inhibition in rat mesenteric arteries.

Figure 5.1 illustrates the effect of metabolic compromise on the vasorelaxant action of nicorandil in rat mesenteric arterial rings. Under control conditions, nicorandil produced a dose-dependent relaxation, with a maximum at 300 μM of 62.3±4.5 %, an EC\textsubscript{50} of 19.6±1.3 μM and a Hill coefficient of 1.2±0.13 (n=16, N=8).

Inhibition of oxidative phosphorylation and so impairment of ATP generation was achieved using CCCP which uncouples electron transfer in the respiratory chain. Randall & Griffith (1993) showed that 400 nM CCCP increased the potency of levromakalim. In my preparation, 400 nM CCCP caused the contraction to collapse and 30 nM was sufficient to significantly increase the potency of nicorandil-induced vasorelaxation (figure 5.1A). The EC\textsubscript{50} was decreased to 10.1±1.1 μM (n=8, N=4) whilst the maximum relaxation increased to 70.1±2.9 %. The K\textsubscript{ATP} channel inhibitor, glibenclamide, was able to inhibit this response and cause a rightward shift thereby increasing the EC\textsubscript{50} value to 36.7±1.1 μM.

I then investigated the effect of inhibition of glycolysis, using 2-DG which acts as a suicide substrate inhibitor. Conway \textit{et al} (1994) demonstrated that 2-DG induced vasodilatation of rat coronary arteries was reversed by glibenclamide. However I found that that decreasing the external glucose in the 20 mM K\textsuperscript{+} external solution to 0.5 mM was sufficient to cause a leftward shift in the nicorandil concentration response curve (figure 5.1B) The application then of 4.5 mM 2-DG had no further significant effect on the nicorandil-induced vasodilatation. The EC\textsubscript{50}s were 12.4±1.1 μM (n=10, N=5) and 16.2±1.0 μM (n=6, N=3) for 0.5 mM glucose and 4.5 mM 2-DG respectively. As before, 10 μM glibenclamide inhibited this response and increased the EC\textsubscript{50} value to 32.5±1.3 μM.
Figure 5.1  Effects of metabolic compromise on the vasorelaxant action of nicorandil in rat mesenteric arterial rings.

(A) Dose-response curves for the relaxation of rat mesenteric arterial segments by nicorandil under control conditions (O; n=8, N=4), in the presence of 100 nM CCCP (△; n=8, N=4) and CCCP + 10 μM glibenclamide (■; n=5, N=3). (B) Concentration response curves for nicorandil in 0.5 mM glucose solution (▼; n=10, N=5), 4.5 mM 2-DG solution (▼; n=6, N=3) and 2-DG + 10 μM glibenclamide (■; n=4, N=3). The vessels were contracted using 20K and BAYK as described in the text. The points show mean±S.E.M. * indicates responses significantly different (p<0.05) from those under control conditions. The curves were then drawn using equation (3), as given in chapter 2 (section 2.2.4.).
5.3.2. *Dose dependent relaxation by nicorandil in porcine coronary arteries.*

The preliminary results from the rat mesenteric arteries suggested that the potency of nicorandil is indeed increased during metabolic inhibition. I therefore went onto complete this study using small arteries of the pig coronary vascular bed. The mean diameter of the vessels used was 365.2±13.9 μM, and the mean maximal tension generated with 20K and BAYK was 14.5±0.9 mN. As shown in figure 5.2, nicorandil produced a concentration-dependent relaxation in porcine coronary arterial ring segments with a mean maximum relaxation at 300 μM of 93.9±0.6 %, an EC$_{50}$ of 17.6±1.1 μM and a Hill coefficient of 2.6±0.1 (n=76, N=40). Nicorandil produced an almost full relaxation of coronary vessels when compared to the mesenteric vessels.

The application of 10 μM glibenclamide inhibited the nicorandil dose response curve (figure 5.2). The nicorandil curve fitted in the presence of glibenclamide was constrained to the maximal percentage relaxation under control conditions, thereby giving a predicted EC$_{50}$ value of 142±1.3 μM. This shift is much greater than that seen in the rat mesenteric arteries, suggesting that the K$_{ATP}$ channel opening action of nicorandil is far greater in coronary vessels. Hence these vessels provided a basis for the continuation of this study.
Figure 5.2  Nicorandil dose-dependent relaxations in pig coronary arteries.

Concentration response curves for the relaxation of porcine coronary arterial rings by nicorandil in the absence (O; n=76, N=40) and presence (●; n=7, N=4) of 10 μM glibenclamide. The vessels were contracted using 20K and BAYK solution as described in the text. The curves were drawn using equation (1), given in chapter 3 (section 3.3.3.). The vertical bars represent S.E.M. * p<0.05 and ** p<0.01, indicates statistically significant difference from mean. N denotes the number of animals and n is the number of preparations used to generate mean values.
5.3.3. Effect of CCCP and zero glucose on nicorandil relaxation curves.

CCCP was used to inhibit oxidative phosphorylation. The compound caused a small (less than 20%) decrease in the maximum contractile force generated with 20K and BAYK. As shown in figure 5.3A, 100 nM CCCP increased the potency of nicorandil. Its concentration curve was shifted to the left with an EC$_{50}$ of 7.4±1.2 µM (n=8, N=4). The EC$_{50}$ values for nicorandil under control conditions, in the presence of CCCP and under other conditions is given in table 5.2. The effect of CCCP was then inhibited by glibenclamide (10 µM), causing a rightward shift and an increase in the EC$_{50}$ value to 115±1.2 (n=6, N=3). This value was not significantly different from that obtained with only glibenclamide in the bath (see figure 5.5A).

As experiments in the rat mesenteric arteries revealed, decreasing the external glucose was sufficient to increase the potency of nicorandil (figure 5.1B). Therefore, I investigated the effect of inhibition of glycolysis by removing the glucose from the external 20 mM K$^+$ solution. The mean response (n=7; N=12) is shown in figure 5.3B, zero glucose enhanced the potency of nicorandil and deceased the EC$_{50}$ value to 8.4±1.2 µM. In a similar manner to CCCP, the addition of glibenclamide inhibited this effect (EC$_{50}$=130±1.2 µM, see figure 5.5A).

5.3.4. Effect of pH on nicorandil relaxation curves.

Extracellular pH is thought to be important in regulating blood flow (Wray, 1988), and induction of acidosis has been shown to relax vessels of the cerebral, coronary and mesenteric circulations (Wray, 1988; Tian et al, 1995; Austin et al, 1996). Vasodilatation by extracellular pH in small coronary vessels of the pig was sensitive to inhibition by glibenclamide, suggesting that activation of K$_{ATP}$ channels is involved (Ishizako & Kuo, 1996). I studied if external pH affected the potency of nicorandil.

Figure 5.4A shows that decreasing the external pH from control pH 7.4 to pH 6.8 had a small but significant effect on the nicorandil response curve (see table 5.2 for EC$_{50}$s). This effect could be abolished by 10 µM glibenclamide (see figures 5.4A and 5.5A).
Figure 5.3 Nicorandil relaxation curves during metabolic inhibition.

(A) Dose response curves for nicorandil in the absence (O; n=9, N=5) and presence of 100 nM CCCP (■; n=8, N=4) and 100 nM CCCP + 10 μM glibenclamide (●; n=6, N=3). The points show mean±S.E.M. * p<0.05 and ** p<0.01 in both figures indicates the responses significantly different from those under control conditions.

(B) Dose response curves for nicorandil under control conditions (O; n=7, N=4), and during conditions of zero external glucose (▼; n=7, N=8) and zero glucose + glibenclamide (10 μM) (●; n=6, N=3). n is the number of preparations whereas N is the number of animals.
Figure 5.4  Potency of nicorandil increased by acidosis and by adenosine receptor activation.

(A) Relaxation curves to nicorandil at pH 7.4 (control, O;n=10, N=5), pH 6.8 (▲; n=9, N=5) and with the addition of 10 μM glibenclamide (●; n=6, N=3). (B) Nicorandil dose response curves in the absence (O; n=7, N=4) and presence of the adenosine analogue 2-CA (300 nM) (●; n=21; N=11) and 2-CA + 10 μM glibenclamide (●; n=7, N=4). The points show mean values±S.E.M. * p<0.05 and ** p<0.01 in both figures indicates responses significantly different from those in control.
5.3.5. **Effect of 2-CA on nicorandil relaxation curves.**

Under ischaemic conditions, there is an increased release of the hormone adenosine. Adenosine is a potent vasodilator (e.g. Daut *et al.*, 1990; Akatsuka *et al.*, 1994; von Beckerath *et al.*, 1991), and adenosine-induced hyperpolarisation or vasorelaxation is attenuated by glibenclamide, implying a role for $K_{ATP}$ channels (Daut *et al.*, 1990; von Beckerath *et al.*, 1991; Dart & Standen, 1993; Kleppisch & Nelson, 1995). I have investigated the vasodilator action of nicorandil in the presence of 2-CA, a stable adenosine analogue which acts at both $A_1$ and $A_2$ adenosine receptors. 300 nM 2-CA caused a small decrease (less than 20 %) in the maximal 20K and BAYK contraction, but it caused a significant leftward shift in the nicorandil relaxation curve (figure 5.4B). This was reflected by a decrease in the EC$_{50}$ value to 8.0±1.1 μM (table 5.2). Glibenclamide (10 μM) inhibited this response to a similar degree to that under control conditions, see figure 5.5A.

<table>
<thead>
<tr>
<th>Table 5.2</th>
<th>pD$<em>2$ and EC$</em>{50}$ values for the nicorandil concentration relaxation curves under control conditions and in the presence of CCCP (100 nM), zero external glucose, 2-CA (300 nM) and pH 6.8 external solution.</th>
</tr>
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<tbody>
<tr>
<td>Values are given as mean±S.E.M. * p&lt;0.05, when compared to control, ANOVA followed by Duncan’s multiple range test.</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Effect</th>
<th>EC$_{50}$ (μM)</th>
<th>pD$_2$±S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>17.6</td>
<td>4.7±0.04</td>
</tr>
<tr>
<td>CCCP</td>
<td>7.4</td>
<td>5.1±0.08 *</td>
</tr>
<tr>
<td>Zero glucose</td>
<td>8.4</td>
<td>5.1±0.08 *</td>
</tr>
<tr>
<td>pH 6.8</td>
<td>11.6</td>
<td>4.9±0.08 *</td>
</tr>
<tr>
<td>2-CA</td>
<td>7.9</td>
<td>5.1±0.04 *</td>
</tr>
</tbody>
</table>

5.3.6. **Relaxations in the absence of $K^+$ channel opening effects.**

The enhanced potency of nicorandil under conditions of metabolic inhibition, reduced pH and adenosine receptor activation were blocked by glibenclamide. Under control conditions nicorandil was considerably less potent in the presence of glibenclamide (figure 5.2), and this was unaffected by CCCP, zero glucose, 2-CA and acidosis (figure 5.5A).
This suggests that the $K_{ATP}$ channel opening ability of nicorandil is responsible for the increased potencies.

To further clarify whether the increased potency of nicorandil described above could be attributed to its $K^+$ channel opening action, I investigated the effects of 2-CA on nicorandil-induced relaxations of 80 mM $K^+$ contractions. Nicorandil dose response curves were performed when arterial ring segments were contracted using 80 mM $K^+$ external solution, rather than 20K and BAYK used in previous experiments. In 80 mM $K^+$ solution, the $E_K$ is calculated, using the Nernst equation (see chapter 1, section 1.5), to be approximately -15 mV. Therefore under these conditions, opening of $K^+$ channels will no longer cause hyperpolarisation and relaxation hence the $K^+$ channel opening ability of nicorandil is effectively inhibited. Any relaxation seen with nicorandil can be attributed to its nitrovasodilator action. As expected, figure 5.5B shows that the potency of nicorandil is indeed decreased in 80 mM $K^+$ solution. Nicorandil produced a dose dependent relaxation with a predicted EC$_{50}$ of 116±1.3 μM (n=4; N=3). Furthermore, 300 μM 2-CA did not affect the potency of nicorandil (EC$_{50}$=113±1.1 μM; n=5, N=3) in relaxing 80 mM $K^+$ contractions. This is in contrast to the effect of 2-CA on nicorandil-induced relaxations upon 20K and BAYK contractions, illustrated in figure 5.4B, and is consistent with the hypothesis that the action of nicorandil on $K^+$ channels is responsible for the enhancement of its potency that occurs in 2-CA.

5.3.7. Relaxations to glyceryl trinitrate (GTN).

I also studied the effect of adenosine receptor activation on vasorelaxations elicited by the nitrovasodilator GTN. The compound causes vasodilatation through stimulation of GC and subsequent increases in the secondary messenger cGMP (reviewed in Harrison & Bates, 1993). If the increased potency of nicorandil was due to its nitrovasodilator action, then the potency of GTN, which acts solely as a nitrovasodilator, should also be enhanced in a similar way. However, as figure 5.6 shows, 2-CA (300 nM) has no significant effect on the potency of GTN. The EC$_{50}$s for GTN were 1.3±1.4 μM (n=4, N=4) and 1.4±2.1 μM (n=5, N=5) in the absence and presence of 2-CA respectively. A unpaired student t-test gave a p value of 0.57.
Figure 5.5 Nicorandil-induced relaxations blocked by glibenclamide and high [K\(^+\)]\(_o\) solution.

(A) Negative log EC\(_{50}\) (pD\(_2\)) values for the inhibition of nicorandil response by glibenclamide under control conditions, + 100 nM CCCP, + zero glucose, + pH 6.8 and + 300 nM 2-CA. The data is given as mean±S.E.M. (B) Concentration response curves for nicorandil relaxations of 80 mM external K\(^+\) contractions, in the absence (△; n=6, N=3) and presence of 300 nM 2-CA (▲; n=5, N=3).
Figure 5.6  2-CA has no effect on GTN-induced vasorelaxations.

Concentration response curves for the relaxation of pig coronary arterial rings by GTN under control conditions (□; n=4, N=4) and in the presence (■; n=5, N=5) of 300 nM 2-CA. The vessels were contracted using 20 and BAYK as described in the text. The points show mean±S.E.M. The curves were drawn using equation (1), chapter 2 (section 2.2.4.).
5.3.8. $A_1$ adenosine receptor stimulation.

To further investigate the adenosine receptor(s) that mediate the effect of 2-CA on nicorandil-induced relaxation, I studied the action of $A_1$ and $A_2$ adenosine-receptor specific agonists and antagonists. My preparation provides a relatively easy way to further study and characterise the subtype of adenosine receptors present in pig coronary arteries by using the ability of adenosine receptor agonists to shift the dose response curve to nicorandil. The specific agonists and antagonists available for a particular receptor are only selective at low concentrations and at higher concentrations their selectivity cannot be completely assured. The action of different adenosine receptor agonists and antagonists on the potency of nicorandil can be investigated, and using low concentrations of the drugs ensures specificity.

Figure 5.7A illustrates the effect of the $A_1$ antagonist 8-cyclopentyl-3, 7-dihydro-1 H-purine-2, 6-dione (DPCPX) on nicorandil relaxation curves potentiated with 300 nM 2-CA. Receptor binding studies have shown that DPCPX has a $pA_2$ of 9.1 for $A_1$ adenosine receptors compared to a $pA_2$ of 6.3 for $A_2$ adenosine receptors (Haleen et al., 1987). 2-CA increased the potency of nicorandil ($EC_{50}=8.0\pm1.1$ $\mu$M, see table 5.3), and 100 nM DPCPX then fully inhibited this response. This is reflected in a decrease in the $EC_{50}$ value back to $15.4\pm1.3$ $\mu$M ($n=6$, $N=3$). I also used 10 nM DPCPX which had a similar effect on the enhancement potency of nicorandil produced by 2-CA (see table 5.4).

2-Chloro-N-cyclopentyladenosine (CCPA) is a high affinity agonist for $A_1$ adenosine receptors (Lohse et al., 1988; Klotz et al., 1989). In small arteries of the pig coronary vascular bed, 100 nM CCPA increased the potency of nicorandil to induce vasorelaxation. CCPA alone had a small effect (less than 30 %) on the maximal contraction. Nicorandil concentration effect curves were shifted to the left in the presence of CCPA, with an $EC_{50}$ of $7.4\pm1.2$ $\mu$M (see table 5.3). The effect of CCPA was abolished by 100 nM (or 10 nM ) DPCPX. Figure 5.7B illustrates the effect of 100 nM CCPA on nicorandil dose response curves and its subsequent inhibition by 100 nM DPCPX. The antagonist increased the $EC_{50}$ value to $19.2\pm1.3$ $\mu$M (table 5.4). Hence it appears that the increased potency of adenosine may be mediated through $A_1$ adenosine receptors.
Figure 5.7 Effect of A₁ adenosine receptor agonists and antagonist on nicorandil-induced vasorelaxation.

(A) Nicorandil relaxation curves in the absence (O; n=12, N=6) and presence of 300 nM 2-CA (♦, as is figure 5.4B) and 2-CA + 100 nM A₁ adenosine receptor antagonist, DPCPX (●; n=6, N=3). (B) Nicorandil concentration relaxation curves under control conditions (O; n=9, N=5), in the presence of A₁ adenosine receptor agonist 100 nM CCPA ( ■; n=8, N=4) and CCPA + 100 nM DPCPX (●; n=3, N=3). The points show mean±S.E.M. In both figures, * p<0.05 and ** p<0.01 indicates statistically significant differences from mean.
5.3.9. *A₂ adenosine receptor stimulation.*

I then went on to study the contribution, if any, of A₂ adenosine receptor stimulation to the increased potency of nicorandil, using 2-CA, 2-p-(2-carboxyethyl) phenethylamino-5'-N-ethylcarboxamidoadenosine hydrochloride (CGS-21680 HCl) an A₂ (both A₂a and A₂b) agonist, and 4-(2-[7-amino-2-(2-furyl) [1, 2, 4] triazolo [2,3-a] [1, 3, 5] triazin-5-ylamino] ethyl) phenol (ZM 241385), a A₂a antagonist.

ZM 241385 is a potent and highly selective A₂a adenosine receptor antagonist, with a pA₂ of 9.02 for A₂a receptors in the guinea pig coronary vasculature, and selectivities of 1000, 91, 500000 over A₁ and A₂b and A₃ adenosine receptors respectively (Poucher *et al.*, 1995). As previously described, 2-CA increased the potency of nicorandil. This was partially blocked by 10 nM ZM 241385 (EC₅₀=12.1±1.1 μM) (see figure 5.8A and tables 5.3 and 5.4).

The effect of the selective A₂ agonist CGS-21680 HCl on nicorandil-induced relaxation was then investigated. The compound has been shown to bind to A₂ receptors in rat brain membranes with a Kᵢ of 15 nM when compared to a Kᵢ of 2600 nM for A₁ receptors (Jarvis *et al.*, 1989). Figure 5.8B shows the mean nicorandil response curves obtained. 100 nM CGS-21680 HCl depressed the maximal contraction by less than 30 %, however it caused a leftward shift of the nicorandil concentration response curve thereby decreasing the EC₅₀ value to 8.9±1.1 μM (table 5.3). 10 nM ZM 241385 was able to completely inhibit this response, increasing the EC₅₀ value to 15.9±1.1 μM (see table 5.4). This result suggests that A₂ receptor stimulation may also play a role in adenosine-mediated actions on the potency of nicorandil.
Figure 5.8 Effect of A$_2$ adenosine receptor activation and inhibition on nicorandil relaxation curves.

(A) Dose response curves for nicorandil in the absence (O; n=8, N=4) and presence of 300 nM 2-CA (♦, as in figure 4B) and 2-CA + 10 nM A$_{2a}$ adenosine receptor antagonist ZM 241 385 (▲; n=7, N=4). (B) Dose response curves for nicorandil under control conditions (O; n=6, N=3), in the presence of 100 nM A$_2$ adenosine receptor agonist CGS-21680 HCl (■; n=6, N=3) and CGS-21680 HCl + 10 nM ZM 241 385(▲; n=6, N=3). The points show mean and the vertical bars show S.E.M. * and ** in both figures indicates significantly difference of p<0.05 and p<0.01 respectively.
Table 5.3  

Table 5.3  

Table 5.4  

Table 5.4  

5.4 Discussion

My results show that in small arteries of the rat mesenteric and porcine coronary vascular beds the potency of nicorandil was increased when oxidative phosphorylation and glycolysis were inhibited. Application of exogenous 2-chloroadenosine (2-CA) and induction of external acidosis had similar effects on vessels of the pig coronary vasculature. All these effects were subsequently blocked by glibenclamide, a K\textsubscript{ATP} channel inhibitor, to a similar degree to that under control conditions. This suggests that the mechanism responsible for the facilitation of nicorandil responses is likely to be increased activity of K\textsubscript{ATP} channels rather than increased stimulation of GC. To clarify and confirm this, I did further experiments. 2-CA had no effect on nicorandil-induced relaxations performed upon 80 mM K\textsuperscript{+} contractions, when the K\textsubscript{ATP} channel opening action of the drug would be unable to contribute to the vasorelaxation. Similarly, 2-CA had no effect on glyceryl trinitrate (GTN)-induced vasorelaxation. GTN was used because it is a "pure" nitrovasodilator that causes vasodilatation through cGMP-mediated effects, by stimulating GC. I have also gone on to study the adenosine receptor subtypes involved in mediating the enhancing actions of 2-CA, in small coronary vessels of the pig. In conclusion, nicorandil-induced vasorelaxation is increased by factors present during ischaemia and this effect appears to be mediated by increased activity of K\textsubscript{ATP} channels. Investigations to study the adenosine receptor subtype present in porcine coronary vascular smooth muscle suggests that both A\textsubscript{1} and A\textsubscript{2} receptors can lead to K\textsubscript{ATP} channel activation.

The different manoeuvres described above were significantly more effective at lower concentrations of nicorandil. This may because there would be fewer K\textsuperscript{+} channels open at low concentrations of nicorandil and so it was possible to see greater enhancement. At higher concentrations, nicorandil would have already activated the majority of K\textsubscript{ATP} channels so that it was not possible to see significant improvement by the different conditions. Also at higher concentrations the nitrovasodilator effect of nicorandil may be more significant, compared to low concentrations where the K\textsubscript{ATP} channel opening ability is predominant (Kukovetz et al, 1992). The mechanism by which this increase in potency occurred was independent of effects by endogenous NO because all the experiments were done in the presence of L-NAME to inhibit endothelial NO synthesis. Some of the manoeuvres described led to a small decrease in the maximal force generated with 20K and BAYK, but this alone does not account for the enhanced vasodilator potency of nicorandil.
Manoeuvres such as zero external glucose and external pH 6.8 did not decrease the contractile force but they still significantly increased nicorandil potency. Furthermore, 2-CA decreased maximal force and increased the potency of nicorandil in 20K and BAYK contractions, but had no effect on nicorandil relaxation curves performed upon 80 mM K⁺ contractions or on the vasodilator potency of GTN.

Nicorandil causes vasodilatation via at least two differing mechanisms, acting both as a K⁺ channel opener and as a nitrovasodilator (Holzmann et al, 1992; see also chapter 3). The evidence from my experiments suggest that the measured increases in potency of nicorandil under metabolic compromise were a result of its K⁺ channel opening action. Firstly, the enhanced potency is abolished by glibenclamide, a Kₐₜₚ channel blocker. Glibenclamide is a relatively specific blocker of these channels at 10 µM (Quayle et al., 1995). In my experiments, glibenclamide shifted the nicorandil dose response curves rightwards and rendered it insensitive to the different manoeuvres described. Secondly, 2-CA was ineffective on nicorandil concentration relaxation curves when external K⁺ was raised to 80 mM, and where the opening K⁺ channels would no longer cause hyperpolarisation. Thirdly, the potency of GTN, which acts only as a nitrovasodilator, was unaffected by 2-CA.

Finally, the results obtained from my study suggest the presence of both A₁ and A₂ populations of adenosine receptors in this tissue. Indeed, Mills and Gerwitz (1990) suggested the presence of both A₁ and A₂ adenosine receptors in cultured vascular smooth muscle cells. However, in contrast, Makujina et al (1994) showed that Kₐₜₚ channels were not involved in adenosine mediated vasodilatation in large coronary arteries of the pig. I was able to use lower concentrations of adenosine receptor agonists and antagonists compared to previous studies on single cells (e.g. Dart & Standen, 1993; Kleppisch & Nelson, 1995). Thus the specificity and selectivity of the different compounds is more certain. Previous studies have demonstrated that endogenous adenosine release plays a role in regulating blood flow under normoxic conditions (Sabouni et al, 1989; Randall, 1995). It is therefore possible that one subtype of adenosine receptor is active under basal endogenous release of adenosine, while the other subtype of receptor is activated under conditions of metabolic compromise when increased adenosine release occurs (Berne, 1980). There is some evidence to show that A₁ receptors have a greater affinity for adenosine compared to A₂ receptors (Fredholm, 1994), hence it is possible that these
receptors are stimulated by basal release of adenosine. However it was not possible to assess this fully in my preparation.

The precise mechanism by which $K_{\text{ATP}}$ channel openers act on channels is unknown, but several different mechanism have been proposed. The majority of work to date has been done in cardiac muscle cells. Experiments in cardiac muscle have shown that $K^+$ channel openers decrease the sensitivity of $K_{\text{ATP}}$ channels to inhibition by intracellular ATP (Thuringer & Escande, 1989). The actions of channel openers can then be antagonised by increasing intracellular ATP (Arena & Kass, 1989). Similar results have been reported in skeletal muscle (Forestier et al., 1996). Other lines of evidence suggest that magnesium salts of nucleotide diphosphates may be essential for the action of some openers. Nucleotide diphosphates increase the activity of $K_{\text{ATP}}$ channels and can also increase the potency of several openers (Terzic et al., 1995). Magnesium nucleotide diphosphates have also been shown to facilitate the activity of channel opening drugs (Thuringer et al., 1995).

The present study shows that metabolic compromise increases the potency of nicorandil. Hence nicorandil and other $K^+$ channel opening drugs may show selectivity for ischaemic tissue in vivo. Angersbach & Nicholson (1988) demonstrated that $K_{\text{ATP}}$ channel openers increase skeletal muscle blood flow to a greater extent in ischaemic tissue when compared to normoxic conditions. This may be because such conditions facilitate the activity of openers directly on vascular smooth muscle or as a result of release of local metabolites such as adenosine. Adenosine has been shown to activate $K_{\text{ATP}}$ channels in vascular smooth muscle (Dart & Standen, 1993), as has hypoxia (Dart & Standen, 1995). Similarly, functional experiments suggested that acidosis increased $K_{\text{ATP}}$ channel activity (Ishizaka & Kuo, 1996). In the rabbit ear artery, levcromakalim was significantly more potent at relaxing arteries under conditions of metabolic inhibition or hypoxia (Randall & Griffith, 1993). Levcromakalim potency was also increased by the adenosine analogue N$^6$-cyclohexyl adenosine (CHA) and this effect was blocked by DPCPX (Randall et al., 1994). It is therefore likely that the procedures used in this study led to an increase in the activity of $K_{\text{ATP}}$ channels. Under these conditions, a given concentration of nicorandil caused substantially more $K^+$ channel opening, hyperpolarisation and vasorelaxation then under normoxic conditions.
However the sensitivity of $K^+$ channel openers in vascular smooth muscle to enhancement by metabolic compromise may depend on the tissue and the precise mechanism of interaction between the drug and the channel. The increased potency seen with nicorandil is consistent with results obtained by Randall and Griffith (1993) using levcromakalim. In contrast, Randall and Griffith found that the vasorelaxant potency of pinacidil was unchanged by metabolic inhibition. This suggests different mechanisms of interaction between these agents and the $K_{ATP}$ channel. In terms of interaction with the channel, the opening action of nicorandil in coronary arteries resembles that of levcromakalim rather than pinacidil. Furthermore, the potency of levcromakalim is not enhanced by metabolic inhibition in all vascular tissue. Hüsken and colleagues (1997) found that neither hypoxia or low glucose produced such an increase in the potency of levcromakalim in the rat aorta, implying that the signal transduction mechanisms involved in linking the drug receptor to the channel differs between large and small arteries.

In conclusion, the $K^+$ channel opening ability of nicorandil makes a large enough contribution to the overall vasodilatation in small coronary arteries so that an increase in the potency of nicorandil was seen after metabolic inhibition. Manoeuvres to mimic factors present in ischaemia included CCCP, zero external glucose, external acidosis and $A_1$ and $A_2$ adenosine receptor activation. These findings may have significant consequences for the therapeutic actions of nicorandil in angina. Such sensitivity to the metabolic state of the tissue may result in nicorandil being effectively targeted to ischaemic regions, so that its vasodilator effect is most pronounced in those regions.
CHAPTER 6

General Discussion
6.1 Overview

Nicorandil has been prescribed for angina, in Japan, for over ten years now, and became clinically available in the United Kingdom three years ago. It was one of the first K$^+$ channel openers to be described. Nicorandil-induced relaxations in pig and guinea pig coronary and mesenteric arteries were shown to be preceded by the opening of K$^+$ channels and membrane hyperpolarisation (Furukawa et al, 1981; Karashima et al, 1982), and the compound was also shown to have nitrovasodilator-like properties (Holzmann, 1983). Both mechanisms are important in causing vasorelaxation, however the dominant mechanism is both tissue- and concentration-dependent (Yoneyama et al, 1990; Kreye et al, 1991; Kukovetz et al, 1992). The relative importance of the K$^+$ channel opening action appears to be greater in smaller vessels and at lower concentrations of nicorandil (Holzmann et al, 1992; Kukovetz et al, 1991).

The initial aim of the research described in this thesis was to investigate the mechanism of action of nicorandil in small arteries of the rat mesenteric vascular bed. Then using small coronary vessels of the pig, I studied the increase in potency of nicorandil under conditions of cellular metabolic stress.

6.2 Main Findings

The main conclusions from my research are;

I. The anti-anginal drug nicorandil acts via two mechanisms to cause vasodilatation of small vessels of the rat mesenteric artery. It appears to cause membrane hyperpolarisation by activating K$_{ATP}$ channels, an action which is blocked by glibenclamide. The drug also stimulates guanylyl cyclase (GC) which is blocked by LY 83583, an inhibitor of soluble GC.

II. Under the conditions used in my experiments, the nitrovasodilator mechanism of nicorandil-induced vasorelaxation does not appear to involve membrane hyperpolarisation due to K$^+$ channel activation. Investigations with other “pure” nitrovasodilators, GTN and SNAP, suggested that they, like nicorandil, did not activate K$^+$ channels in this preparation. Vasodilatations to SNAP and GTN were not blocked
by ChTX, IbTX or glibenclamide. However, when the production of endothelium-derived NO was permitted, SNAP-induced relaxation was significantly attenuated by ChTX.

III. Lastly, in rat mesenteric and in pig coronary arteries, the potency of nicorandil is enhanced by manoeuvres thought to mimic those changes present in ischaemia. The experimental results imply that this enhanced potency was due to an increased $K_{ATP}$ channel opening ability of nicorandil.

6.2.1. Dual mechanism of action of nicorandil.

Relatively few studies have been done on the actions of nicorandil on isolated arteries of the rat. Experiments on the rat aorta have suggested that the vasorelaxant action of nicorandil is primarily due to GC stimulation and cGMP activation (Kim et al., 1990; Borg et al., 1991). In contrast, results from the rat basilar artery suggest that nicorandil acts mainly via activation of $K^+$ channels (Ksoll et al., 1991). Experiments by Fujiwara and Angus (1996), using rat mesenteric arteries mounted in a wire myograph, suggested that nicorandil caused relaxation through three parallel pathways; stimulation of GC, $K_{ATP}$ channel opening and inhibition of voltage operated $Ca^{2+}$ channels. It is important to assess the action of nicorandil on small arteries because clinically the drug will act on similar-sized vessels in vivo. Functional studies to investigate the actions of nicorandil on rat small mesenteric vessels were done using the small vessel wire myograph. The whole cell patch clamp technique was then used to study the channels activated by nicorandil in acutely dissociated arterial smooth muscle cells.

The vessels were contracted using 20 mM external $K^+$ solution and BAY K 8644, the $Ca^{2+}$ channel agonist. This solution caused the activation of voltage-dependent $Ca^{2+}$ channels both pharmacologically and as a consequence of the modest depolarisation caused by the increase in external $K^+$ concentration. The change in $[K^+]_o$ caused an alteration in the $K^+$ equilibrium potential, however it was still sufficiently negative for channel openers to cause hyperpolarisation and ultimately relaxation.

Nicorandil was able to produce a concentration-dependent relaxation of the rat mesenteric arterial ring segments. Glibenclamide, the $K_{ATP}$ channel blocker, inhibited the nicorandil-induced relaxation. This was shown by a parallel rightward shift in the dose response curve to nicorandil. Similarly, the nicorandil relaxation curves were blocked by
LY 83583, the inhibitor of GC, in a dose-dependent manner, hence establishing that both $K_{\text{ATP}}$ channel activation and GC stimulation play a role in nicorandil-induced vasorelaxation in this tissue. This work was further complemented with whole cell voltage clamp recordings from smooth muscle cells. In 140 mM symmetrical $K^+$ concentrations, and a holding potential of -60 mV, nicorandil induced a large inward $K^+$ current. The channels underlying the current displayed the characteristics of $K_{\text{ATP}}$ channels. The $K^+$ current showed little voltage dependence and was blocked by glibenclamide. Also, the single channel amplitudes, noted at the start of the response to nicorandil, and the calculated conductances correlated with previous findings for $K_{\text{ATP}}$ channel conductances in other vascular smooth muscle cells (e.g. Dart & Standen, 1993; Clapp et al, 1994).

The observations seen are mostly consistent with results obtained in other arterial preparations, such as the rat basilar (Ksoll et al, 1991), rat coronary (Borg et al, 1991), bovine coronary (Kukovetz et al, 1992) and canine coronary arteries (Taira, 1989). In contrast to some other studies (e.g. Yoneyama et al, 1990; Kukovetz et al, 1991), using the wire myograph it was not possible to assess which of the mechanisms was dominant. However, both actions appeared to play a significant role in nicorandil-induced vasorelaxation in this tissue. I did not measure involvement of voltage operated $Ca^{2+}$ channels hence it was not possible to confirm if such a mechanism plays a role in these vessels, as suggested by Fujiwara and Angus (1996). Furthermore, nicorandil was shown to activate $K_{\text{ATP}}$ currents in arterial smooth muscle cells. This was similar to currents recorded in cells of the rabbit and rat portal vein (Kajioka et al, 1994; Kamouchi & Kitamura, 1994; Teramoto & Brading, 1997).

6.2.2. Does nitrovasodilator action involve $K^+$ channel opening?

The results thus far had shown that nicorandil produces vasodilatation of mesenteric arteries via two mechanisms. I then went on to investigate further the nitrovasodilator mechanism of nicorandil, especially in respect to $K^+$ channels and membrane hyperpolarisation. There is evidence to show that nitrovasodilators stimulate GC and increase levels of the secondary messenger cGMP (e.g. Endoh & Taira, 1983; Kukovetz et al, 1991\textsuperscript{a}). The GC/cGMP signal transduction mechanism is well established (e.g. reviewed in Schmidt et al, 1993). The secondary messenger has a wide array of cellular effects, all ultimately decreasing cytosolic $Ca^{2+}$ concentration and causing
relaxation. One proposed mechanism is the activation of K$^+$ channels, resulting in membrane hyperpolarisation, inactivation of voltage operated Ca$^{2+}$ channels and a fall in intracellular Ca$^{2+}$. Cyclic GMP has been reported to act on BK$_{Ca}$ channels, through PKG stimulation (Robertson et al, 1993; Taniguchi et al, 1993). Both Williams et al (1988) and Khan et al (1993) have demonstrated the activation of BK$_{Ca}$ channels by nitrovasodilators. Since nicorandil partially exhibits properties of a nitrovasodilator it is conceivable that it may also activate BK$_{Ca}$ channels. Such an effect by nicorandil-induced GC stimulation would complement the membrane hyperpolarisation induced by K$_{ATP}$ channel activation.

In rat small mesenteric arteries, nicorandil induced vasorelaxation was not inhibited by the BK$_{Ca}$ channel antagonist, IbTX. This finding was reinforced by whole cell recordings from isolated smooth muscle cells. The large inward K$^+$ current induced by nicorandil, in 140 mM [K$^+$_o] / 140 mM [K$^+$]i, and at a holding potential of -60 mV, was unchanged by IbTX but was substantially blocked by glibenclamide. Similarly, LY 83583 had no significant effect on nicorandil-induced K$^+$ current. This implies that BK$_{Ca}$ channel activation is not involved in nicorandil’s actions. Experiments were then done with other “pure” nitrovasodilators. SNAP and GTN produce their effect through the release of NO, stimulation of GC and increasing cytosolic cGMP levels (Fung, 1993; Harrison & Bates, 1993). In myograph experiments, the lack of effect by IbTX or glibenclamide suggests that both compounds did not activate BK$_{Ca}$ or K$_{ATP}$ channels. Furthermore, SNAP did not activate a whole cell K$_{ATP}$ current in vascular smooth muscle cells under conditions of symmetrical 140 mM K$^+$ concentrations. Yet, when endothelial NO was not inhibited by L-NAME, concentration-dependent relaxations to SNAP were significantly inhibited by ChTX.

The majority of my results are inconsistent with studies showing nitrovasodilator-induced activation of K$^+$ channels (e.g. Khan et al, 1993; George & Shibata, 1995; Yamakage et al, 1996; Carrier et al, 1997). These observations cannot be accounted for by tissue differences because other investigators have shown NO donor-induced relaxation inhibited by K$^+$ channel blockers in similar vessels (McPherson & Garland, 1992; Carrier et al, 1997). Most of my experiments were done in the presence of L-NAME, causing an inhibition of basal endothelial NO production. However, in the absence of L-NAME, SNAP-induced vasorelaxation was shown to be, in part, mediated by BK$_{Ca}$ channels. Experiments by Plane and colleagues (1996), in small mesenteric arteries of the rat,
suggest that under conditions of zero endothelium-derived NO nitrovasodilators are able to cause complete vasorelaxation through other means that do not involve K$^+$ channels and membrane hyperpolarisation. In their study, the mechanism of relaxation by the NO-donor SIN-1 was dependent on basal NO. The vasodilatation produced by SIN-1 was not significantly different between endothelium-intact and -denuded arterial segments. In endothelium-intact vessels, the vasorelaxation was notably blocked by ChTX. However, when basal NO production was attenuated, a relaxation to SIN-1 was not blocked by ChTX. Under these conditions ODQ, a GC inhibitor, was also needed to inhibit the response. This suggests that endothelium-derived NO is able to activate K$^+$ channels through a route that does not involve GC, and there is some interaction between nitrovasodilators and endothelial NO in this tissue. It appears that the interaction is such that endogenous NO is required for activation of K$^+$ channels, and SIN-1 and SNAP (and possibly GTN) are able to produce complete vasodilatation via other membrane hyperpolarisation independent mechanisms in its absence.

6.2.3. Enhancement of potency of nicorandil under conditions of metabolic stress.

The activity of vascular K$_{ATP}$ channels is increased during metabolic inhibition (Silberberg & van Breeman, 1992; Beech et al, 1993; Teramoto & Brading, 1996). Therefore it can be hypothesised that the activity of openers may also be increased under such conditions. In confirmation of this, Randall and Griffith (1993) showed that the potency of the K$^+$ channel opener levocromakalim, but not pinacidil, was enhanced by adenosine, hypoxia and impairment of oxidative phosphorylation, in small arteries of the rabbit ear. Their results also illustrate an important difference in the drug-channel interaction between levocromakalim and pinacidil. I investigated the actions of nicorandil under conditions of cellular metabolic stress. It was particularly interesting to investigate changes in the potency of nicorandil in pig small coronary arteries because of its clinical role as an anti-anginal drug. The experiments also provide clues on the pharmacology of the compound when compared to levocromakalim and pinacidil.

In contrast to pinacidil, the potency of nicorandil was increased by inhibition of oxidative phosphorylation. Initial experiments in small mesenteric arteries showed that the potency of nicorandil was increased under conditions of metabolic inhibition. Because of this successful result, experiments were continued on small vessels of the pig coronary
vascular bed. I went on to show that enhancement of nicorandil potency also occurred under other conditions, such as impairment of glycolysis, induction of external acidosis and application of external 2-chloroadenosine, a stable analogue of adenosine. $A_1$ and $A_2$ adenosine receptor analogues had similar effects on nicorandil relaxation curves. The enhanced potency of nicorandil was abolished by glibenclamide and by increasing external $K^+$ concentrations. Also, the vasodilator potency of GTN was unchanged by 2-CA. Therefore, it appears that the interaction profile of nicorandil with the $K_{ATP}$ channel is similar to that of levocromakalim rather than pinacidil.

The increased potency observed with nicorandil is consistent with the results reported by Randall and Griffith (1993). The results are consistent with an increase in the $K_{ATP}$ channel opening ability of nicorandil rather than its nitrovasodilator actions. This is as expected because $K_{ATP}$ channels are sensitive to the metabolic state of the cell (e.g. Silberberg & van Breeman, 1992).

6.3 Clinical Correlates

Nicorandil was one of the first $K^+$ channel opening drugs to be prescribed clinically. Its structure, consisting of a nitro- and nicotinamide-groups, gives rise to its dual mechanism of action. This has led to the drug being successfully introduced for the treatment of angina. When compared to existing anti-anginal treatment nicorandil has a greater efficacy (Frampton et al, 1992), and more importantly its profile is different from that of nitrovasodilators because tolerance is not observed with prolonged use. This suggests that the $K^+$ channel opening plays a significant role in nicorandil induced vasorelaxation, and is responsible for the lack of tolerance (Goldschmidt et al, 1996). Furthermore, an enhancement in potency of nicorandil appears to occur under conditions of metabolic stress due to increased activity of $K_{ATP}$ channels, hence the drug can be specifically targeted to ischaemic regions. Clinically, this means that lower concentrations of the drug can be administered to provide selective vasodilatation.

Another interesting effect of $K^+$ channel openers is their ability to protect the heart against ischaemia by mimicking the phenomenon of ischaemic pre-conditioning. This is an adaptive endogenous mechanism in which a brief episode of reversible ischaemia renders the heart more resistant to a subsequent period of sustained ischaemia. The
opening of $K_{ATP}$ channels is thought to be one of the mechanisms by which preconditioning preserves the myocardium because the phenomenon can be blocked by glibenclamide (Parratt & Kane, 1994). $K^+$ channel openers, including nicorandil, have been shown to be myocardium protective agents in several animal models of ischaemia-reperfusion injury (Gross et al, 1992; Menasche et al, 1995; Menasche et al, 1996).

### 6.1 Future Perspectives

The arterial vessels used for my experiments had an average diameter of 300 $\mu$M. The limitations of the small vessel myograph are that smaller arteries, with diameters less than 100 $\mu$M, cannot be studied. It would be useful to investigate the actions of nicorandil on smaller resistance vessels, hence other techniques such as a pressure myography and intact heart perfusion would have to be used.

The mechanism of GC-stimulation was investigated using LY 83583 (chapter 3). LY 83583 was introduced as a selective sGC inhibitor (Mülsch et al, 1988). However, since then it has been shown to have non-selective effects on free radicals (Kontos et al, 1993) including NO (Barbier et al, 1992; Luo et al, 1995). In view of this, it would be useful to repeat the experiments using a more specific blocker such as ODQ (Garthwaite et al, 1995). Measurements of intracellular cGMP levels could also be done using specific radioligand assays. Similarly, it would be possible to study the nitrovasodilator mechanism of nicorandil-induced vasorelaxation using membrane permeant analogues of cGMP, for example 8-Br-cGMP. To follow on from this, it would be interesting to determine the cellular components involved in nicorandil-induced vasorelaxation due to GC stimulation. My study showed that in this preparation $K^+$ channels were not involved therefore alternative mechanisms must exist. Such studies would help in tailoring the responses of new compounds, so that selectivity and specificity is increased whilst side effects are minimised. Overall, my research confirms that $K^+$ channel openers such as nicorandil have a mechanism of action that can be used therapeutically for the treatment of cardiovascular disorders, for example angina and hypertension.
Chapter 6

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