An investigation into the molecular identity of the K_{ATP} channel of vascular smooth muscle

Thesis submitted for the degree of
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by

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Abstract

The molecular identity of the K_{ATP} channel of vascular smooth muscle was investigated using a variety of methods. RT-PCR was used to investigate the expression of K_{ATP} subunit mRNA. Kir 6.1, 6.2, and SUR 2B transcripts were detected in all vascular smooth muscle preparations, SUR 1 was detected in mesenteric artery. Expression at the protein level was determined, in femoral artery cells, using an immunohistochemical technique, visualizing stained cells with confocal miscroscopy. Antibodies specific for Kir 6.1, 6.2 and SUR 2B, 2A, subunits were used and all resulted in staining. Using a *Xenopus* expression system cloned K_{ATP} channel currents were characterized. Differences in channel activation, via metabolic poisoning, were found to be dependent on the SUR subunit. The compound PNU-37883A was found to be selective for the Kir subunit, with channels comprising Kir 6.1 being more sensitive to inhibition by this compound than Kir 6.2. Kir 6.x chimeras were constructed to identify a possible site of modulation/binding and a region of the C-terminus was identified which was important in the channels response to the compound. The differences in sensitivity of channels with Kir 6.1 and 6.2 are similar to those reported for vascular smooth muscle and skeletal or cardiac muscle, providing further evidence that the Kir 6.1 subunit is involved in forming the K_{ATP} channel of vascular smooth muscle. However, the other evidence provided here doesn't rule out the possibility of heteromultimeric K_{ATP} channel formation in vascular smooth muscle.

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Abbreviations

$[K^+]_{ext/i}$	extracellular/intracellular potassium concentration
2P-domain	2 pore-domain
ACh	acetylcholine
ADP	adenosine diphosphate
ALD	adrenoleukodystrophy
cAMP	cyclic adenosine monophosphate
4-AP	4-amino pyridine
AP	action potential
ATP	adenosine triphosphate
BCRP	breast cancer resistance protein
bp	base pair
°C	degrees celsius
CCCP	carbonyl cyanide m-chlorophenyl hydrazone
CCD camera	charge coupled device
CFTR	cystic fibrosis transmembrane conductance regulator
cGMP	cyclic guanosine monophosphate
CIAP	calf intestinal alkaline phosphatase
C-terminus	carboxyl terminus
DEPC	diethyl pyrocarbonate
(c)DNA	(copy) deoxyribonucleic acid
DNase I	deoxyribonucease type I
dNTP	deoxy-nucleotide triphosphate
DTT	dithiothreitol
EC50	concentration at which half inhibition/activation occurs
EDTA	diaminoethanetetra acetic acid
E_K	potassium equilibrium potential
elk	ether-a-go-go like potassium channel
ER	endoplasmic reticulum
E_{rev}	reversal potential
FITC	fluorescein
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HEK	human embryonic kidney cell
HERG	human ether-a-go-go
HSP	heat shock protein
HVSM	human vascular smooth muscle
IF	initiation factor
Ig	immunoglobulin
K_{ATP} channel	adenosine triphosphate sensitive potassium channel
kb	kilobase
KCO	potassium channel opener
Ki	inhibition constant
Kir	potassium inward rectifier
Kv	voltage-gated potassium channel

LB	Luria Bertani
MΩ	megaohm
M1	membrane spanning domain 1
mg	milligrams
MiRP	minK related protein
ml	millilitre
mm	millimetres
mM	millimolar
M-MLV	Moloney Murine Leukemia Virus
MRP	multidrug resistance protein
MS	minoxidil sulphate
mV	millivolt
MXRP	mitoxantrone resistance protein
NADH	nicotinamide adenine dinucleotide (reduced)
NBD/F	nucleotide binding domain/fold
nl	nanolitres
N-terminus	amino-terminus
OD	optical densitiy
oligo dT	oligo deoxy-thymidine
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDZ	PSD-95, Drosophila discs large protein, Zona occludens protein.
PHHI	persistant hyperinsulinemic hypoglycemia of infancy
PIP_2	phosphatidyl inositol bisphosphate
PKC/A	protein kinase C/A
P_{Na+}	permeability of the sodium ion
PNACL	protein and nucleic acid chemistry laboratory
PNU-37883A	4-morpholinecarboximide-N-1-adamantyl-N'-cyclohexlhydrochloride
pS	picoSiemens
(m)RNA	(messenger) ribonucleic acid
rpm	revolutions per minute
RT-PCR	reverse transcription-polymerase chain reaction
SAP	shrimp alkaline phosphatase
SDS	sodium dodecyl sulphate
s.e.	standard error
SN	substantia nigra
SR	sarcoplasmic reticulum
SSC	standard saline citrate buffer
SUR	sulphonylurea receptor
TAE	tris acetate EDTA
TE	tris-EDTA
TEA	tetraethylammonium
UHQ	ultra high quality
µl	microlitres

UV	ultra violet

Publications

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Chapter 1

General introduction

Chapter 1. Introduction

1.1. Ion channels

The movement of ionic charge across the membrane of a cell has been attributed to the presence of ion channels and transporters. Ion channel proteins accomplish the facilitation of ion movement across the cell membrane by forming narrow hydrophilic pores through which ions are able to passively diffuse (Hille, 2001). They may be permeable to more than one ion but many are selective for one particular ion over others, i.e. relative permeabilities to various ions differ (Hille, 1975). Ion channels also differ in the mechanisms that control their gating, the transition between a conducting and non-conducting state (open and closed state).

The voltage-clamp technique, in which the membrane potential of the cell is kept constant whilst observing the change in current, enabled the direct measurement of ionic movement across the membrane. In two-microelectrode voltage-clamp two electrodes are inserted into the cell one measuring the change in voltage and the other compensating for the change by injecting current into the cell holding the membrane at a particular potential. Unfortunately the requirement for insertion of two microelectrodes or axial wires into a cell limits the size of cell that may be experimented with, and this factor explains why most of the foundations of ion channel experimentation were obtained from large systems such as the squid giant axon.

The ability to create a giga-ohm seal between a patch pipette and a membrane opened up the possibility for experimentation on small/mammalian cells that couldn't tolerate impalement with two microelectrodes. The introduction of this technique, called the patch clamp technique, by Neher and Sakmann in 1976 made possible the examination of individual ion channels (Neher and Sakmann, 1976). Briefly, a glass microelectrode with a polished tip is applied to the surface of the cell and a seal generated by applying suction through the

pipette, this isolation of a region of cell membrane then allows the activity of the individual ion channels in this patch to be recorded.

The cloning of channel subunit DNA has allowed the functional properties of recombinant channels to be examined using mammalian expression systems, such as *Xenopus laevis* oocytes, HEK cells and COSm cells. In the *Xenopus* expression system complementary mRNA is synthesised from cloned DNA, injected into the oocytes where it is then translated into protein and inserted into the cell membrane (Barnard *et al.*, 1984; Sigel, 1990). These large cells are able to transiently express many different receptors and ion channels at high levels and provide a robust system for the electrophysiological characterisation of these proteins.

1.2. Potassium channels

The potassium channel family are involved in a variety of functions within both excitable and non-excitable cells. Their regulation, which often serves as a distinguishable factor for channel definition, is governed either by the membrane potential of the cell, it's metabolic state, or by transmitters and hormones. There are three main subdivisions in this family that are divided into three main structural classes, those with 2 transmembrane domains, those with four and those with six (see fig. 1.1. for representation of potassium channel family tree and fig. 1.2. for the topology of the main family members). All the members share a conserved pore-forming domain, H5, which endows the channel with its selectivity for potassium. In 1991, Yool and Scwarz found that amino acid substitutions in this H5 region enhanced

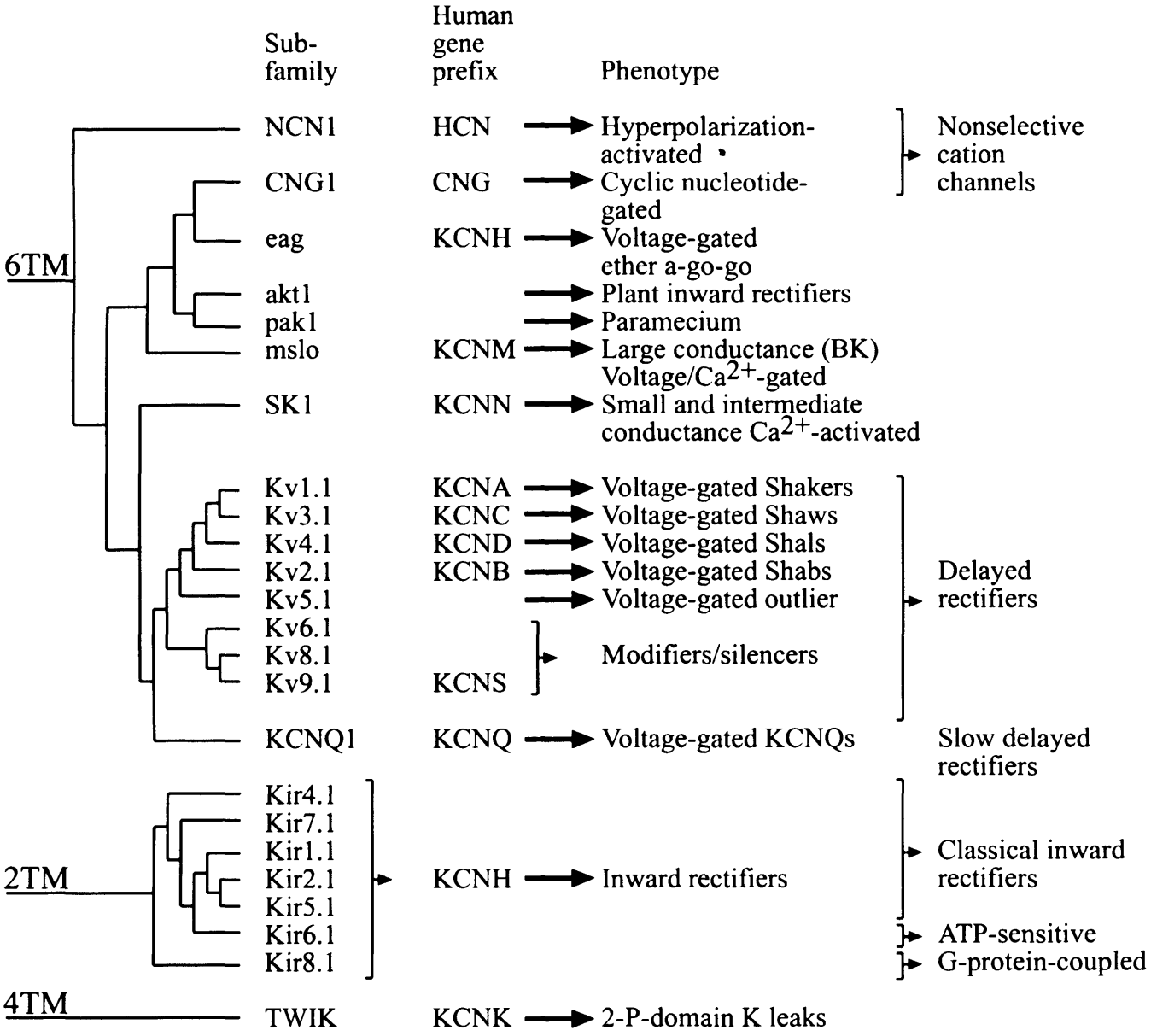


Figure. 1.1.
Potassium channel family tree (reproduced from Hille, 2001).

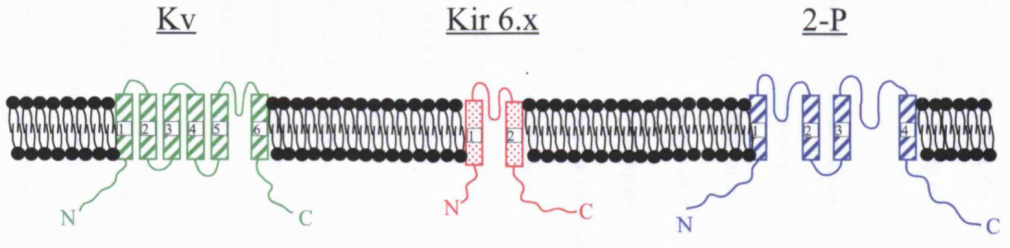


Figure. 1.2.
Topology of the main potassium channel family members. The voltage-gated (Kv) potassium channels have six transmembrane regions, the inward rectifiers (Kir) have two transmembrane regions, and the 2-P domain channels (2-P) have four transmembrane regions. Each member possesses a conserved pore forming region with the 2-P domain channels possessing two of these regions.

the passage of ions with larger diameters without affecting gating, and so postulated that this region was likely to line the pore of the potassium channel (Yool and Schwarz, 1991).

Although these channels are primarily selective for potassium over any other ion, with the delayed rectifier for instance being over 100 fold more permeable to potassium ions than sodium (Aidley and Stanfield, 1996), the family encompasses a number of subfamilies that contain channels with different gating properties.

1.2.1. Voltage sensitive potassium channel family

Containing a vast number of interrelated channels this subfamily of channels are regulated by changes in the membrane potential of the cell. They are mainly involved in control of the excitability of neurons (Rettig *et al.*, 1992; Veh *et al.*, 1995) and cardiac myocytes (Tamkun *et al.*, 1991), being associated with the repolarisation phase of action potentials. They also serve to regulate vascular smooth muscle tone (Nelson and Quayle, 1995), and have also been observed in kidney (Yao *et al.*, 1996), skeletal muscle (Abbott *et al.*, 2001), pancreas (Kalman *et al.*, 1998), cochlea (So *et al.*, 2001) and retina (Klumpp *et al.*, 1991). Differing in their kinetics of activation and inactivation, and also in sensitivity to various blocking compounds such as 4-aminopyridine (4-AP) and tetraethylammonium (TEA), they constitute the two types of current referred to as delayed rectifier current, which has a delayed activation and a slow inactivation, and transient A-type current, which activates and inactivates rapidly, though it is not clear exactly which class of Kv channel is responsible for these.

The first voltage dependent K^+ channel to be cloned was encoded by the Shaker gene of *Drosophila* (Papazian *et al.*, 1987). Since then three other Shaker related genes have also been cloned from *Drosophila*; Shal, Shab, and Shaw, which share a conserved organisation

(Butler *et al.*, 1989). Splicing of these genes gives rise to a multitude of voltage dependent potassium channels that have varying kinetics and voltage sensitivities.

1.2.1a. Shaker

The nomenclature for the voltage dependant channels varies depending on the species from which the gene was isolated. The Shaker gene is also known as, MBK1 (from mouse), RCK1 (from rat), and HK1 (from human). The resultant protein subunit is known as Kv 1. Splicing of the Shaker gene gives rise to an array of different subunits known as Kv 1.1-8 (Hille, 2001).

Channel gating by voltage is a basic mechanism underlying the electrical excitability of nerve and muscle membranes. Much effort has been put into understanding the structure and voltage dependence of the voltage gated potassium channel. The Kv 1 potassium channel is formed through the assembly of four subunits each having six transmembrane segments, S1-S6. Experimental determination has found that the voltage sensor is formed by individual charged residues lying along segment S4 (Limen *et al.*, 1991; Papazian *et al.*, 1991). During channel activation it has been shown that this segment undergoes a comformational change (Perozo *et al.*, 1994). Fluorescence/Lanthadine resonance energy transfer has been used to determine the position of the S4 region when the channel is in different gating states, and has shown that during channel activation this region twists in a helical motion apparently moving out of the membrane (Yusaf *et al.*, 1996; Cha *et al.*, 1999; Glauner *et al.*, 1999; Wang *et al.*, 1999a).

Comparison of endogenous current with cloned current has proved to be rather difficult as the alpha Kv1 subunits can coexist with function altering beta subunits in a 4:4 stoichiometry and can also form as heteromers (Po *et al.*, 1993; Rettig *et al.*, 1994; England *et al.*, 1995a; Nakahira *et al.*, 1996; Rhodes *et al.*, 1997). As well as conferring altered gating

kinetics on the alpha subunits Kv beta subunits may also act as channel chaperones promoting surface expression and stability (Shi *et al.*, 1996).

Kv1.1 has been demonstrated in brain (Verma-Kurvari *et al.*, 1997; Chung *et al.*, 2001) and mutations in the Kv1.1 gene are known to cause episodic ataxia type 1 syndrome which is an autosomal dominant neurological disorder (Browne *et al.*, 1994). Many different mutations of this have been gene characterised (Comu *et al.*, 1996; D'Adamo *et al.*, 1998; Zuberi *et al.*, 1999), with the resultant channels having altered gating properties that are thought to impair neuronal repolarisation (Adelman *et al.*, 1995; Zerr *et al.*, 1998; Bretschneider *et al.*, 1999; Zuberi *et al.*, 1999). During infancy the condition causes continuous myokymia and episodic attacks of spastic contractions of the skeletal muscle that permanently disable the afflicted.

1.2.1b. Shab

The Shab gene is referred to as DRK1 from rat, DHK1 from human and MShab from mouse. The protein subunits are known as Kv 2.1 and 2.2. Kv 2.1 is though to be the major contributing channel to the delayed rectifier current observed in hippocamal neurons (Murakoshi and Trimmer, 1999) and gastrointestinal smooth muscle (Smalz *et al.*, 1998)). Kv 2 is able to coassemble with the regulatory alpha subunits Kv 5, 6, 8 and 9 (Post *et al.*, 1996; Hugnot *et al.*, 1996; Patel *et al.*, 1997; Kramer *et al.*, 1998; Kerschensteiner and Stocker, 1999; Zhu *et al.*, 1999a; Richardson and Kaczmarek, 2000). These alpha regulatory subunits, although normally electrically silent when expressed alone, modulate the biophysical properties of the Kv 2 channel. For example in rat pulmonary artery myocytes Kv 2.1 was found to associate with Kv 9.3 with the resultant channel being able to open at resting membrane voltages, unlike Kv 2.1 alone (Patel *et al.*, 1997).

The Kv 1 and 2 family have been found to contribute to the regulation of insulin secretion in rat beta cells with Kv 2.1 and 1.4 mediating the majority of the repolarising delayed rectifier current in these cells (MacDonald *et al.*, 2001).

1.2.1c. Shaw

Kv3.1-4 (KCNC, KSh III). Kv 3 channel currents activate relatively quickly, at voltages more positive than –10 mV, and also deactivate very quickly. These properties enable the fast repolarisation of action potentials without affecting the threshold for action potential generation, and so facilitate the high frequency repetitive firing observed in fast spike neurons such as cortical GABAergic interneurons (Gan and Kaczmarek, 1998; Hernandez-Pineda *et al.*, 1999; Rudy *et al.*, 1999; Rudy and McBain, 2001).

Kv 3.1 has also been demonstrated to occur in choclea (So *et al.*, 2001), and has been found to associate with MiRP2 (KCNE3) in skeletal muscle where it is involved in setting the resting membrane potential (Abbott *et al.*, 2001). Mutations in KCNE3 are associated with periodic paralysis (Tinel *et al.*, 2000a).

1.2.1d. Shal

Kv 4.1-4.3 (KCND). The Shal gene is thought to encode channels that are responsible for the A-type potassium current, a transient outward, rapidly inactivating current described in heart and neurons (Serodio *et al.*, 1996; Fiset *et al.*, 1997; Serodio and Rudy, 1998; Zhu *et al.*, 1999b; Isbrandt *et al.*, 2000). Kv 4.2 has been shown to colocalise with filamin (Petrecca *et al.*, 2000). A family of Kv channel interacting proteins can associate with Kv 4 altering cell surface expression current density and may even endow calcium sensitivity (An *et al.*, 2000; Bahring *et al.*, 2001). The beta regulatory subunit associated with long QT syndrome, MiRP1, may also associate with Kv 4 (Zhang *et al.*, 2001)

Kv 6-9 subunits also exist but are electrically silent unless expressed in association with another Kv subunit (Hugnot *et al.*, 1996; Post *et al.*, 1996; Patel *et al.*, 1997; Salinas *et al.*, 1997; Kramer *et al.*, 1998; Kerschensteiner *et al.*, 1999; Stocker *et al.*, 1999; Zhu *et al.*, 1999a; Richardson and Kaczmarek, 2000).

1.2.1e. Calcium sensitive potassium channels

BKca channels are encoded by the slo gene (Atkinson *et al.*, 1991; Pallanck and Ganetzky, 1994) and SKca channels by the SK1,2, and 3 genes (Litt *et al.*, 1999). BKca and SKca differ in the conductance of the channels they form, with SKca forming small conductance channels that show little or no voltage dependence, and BKca forming channels with larger conductances that are voltage sensitive. Channels with intermediate conductances (IKca) have also been described (Ishii *et al.*, 1997).

BKca channels are similar in their sequence to voltage gated ion channels having a homologous S1-S6 region. Channel activation is dependent upon both direct calcium binding and membrane depolarisation. They are unique at their N and C-terminus, possessing calcium sensitive sites in the C-terminus (Schreiber and Salkoff, 1997; Schreiber *et al.*, 1999), and an additional transmembrane segment S0 (Meera *et al.*, 1997). The calcium sensitivities of these channels vary in different tissues and this has been found to be due to their association with accessory beta subunits, which also may provide binding sites for other modulatory compounds (McCobb *et al.*, 1995; Chang *et al.*, 1997; Cox and Aldrich, 2000; Nimigean and Magleby, 2000; Dick *et al.*, 2001).

1.2.1f. Ether-a-go-go related channels

Molecular analysis of mutations affecting membrane excitability in Drosophila led to the identification of the gene eag (ether-a-go-go) that encodes a polypeptide structurally

related to the Shaker K^+ channel family (Warmke and Ganetzky, 1994). When expressed the eag poplypeptide assembles into channels that conduct a voltage-activated K^+ and Ca^{2+} selective outward current modulated by internal cAMP.

A human eag related gene was found on chromosome 7 and denoted h-erg (HERG) (Warmke and Ganetzky, 1994). Although very similar to eag, HERG was found to encode an inwardly rectifying current that has an inactivation mechanism that attenuates K^+ efflux during depolarisation (Trudeau *et al.*, 1995; Schonherr and Heineman, 1996; Spector *et al.*, 1996). The HERG channel is thought to constitute the rapidly activating cardiac delayed rectifier current IKr (Sanguinetti *et al.*, 1995; Tinel *et al.*, 2000b), and the ion channel associated protein minK may regulate IKr activity by formation of a stable complex with HERG (McDonald *et al.*, 1997).

Mutations in HERG have been shown to cause a form of long QT (LQT) syndrome, an inherited cardiac disorder resulting in syncope, seizures and sudden death (Benson *et al.*, 1996; Russell and Dick, 1996). HERG is also involved in acquired LQT syndromes where the channel is inactivated by certain drugs (Mitcheson *et al.*, 2000).

Another eag related gene was also found in Drosophila that was different to both h-erg and eag, and was named elk (eag-like K^+ channel) (Warmke and Ganetzky, 1994). cDNA isolated from human brain encoded proteins termed BEC1 and 2 which were 46% identical to a rat elk (Miyake *et al.*, 1999). These proteins, exclusively expressed in the brain, formed channels that conducted a voltage gated outward current with fast inactivation and was insensitive to TEA and quinidine.

1.2.1g. KCNQ

KCNQ1 co-assembles with minK, the product of the KCNE1 gene, to form the cardiac delayed rectifier. Mutations in KCNQ1 can cause an inherited LQT disorder (Seebohm *et al.*,

2001). It is thought that KCNQ2, 3, and 5 form as heteromultimers which underlie the neuronal M-current, and that mutations in the genes encoding these channels cause an inherited form of juvenile epilepsy (Schroeder *et al.*, 1998; Selyanko *et al.*, 2000; Schwake *et al.*, 2000; Lerche *et al.*, 2000; Shapiro *et al.*, 2000; Schroeder *et al.*, 2000; Kananura *et al.*, 2000). KCNQ4 has been shown to be located in the cochlea with mutations in this gene causing an inherited deafness (Van-Hauwe *et al.*, 2000). The associated protein Mirp1 (KCNE2), which has been found to express with HERG, also associates with KCNQ1, 2 and 3, with the product of KCNE3 also associating with KCNQ1 (Tinel *et al.*, 2000a; Tinel *et al.*, 2000b).

1.2.1h. Cyclic-nucleotide gated and hyperpolarisation activated K^+ channels

These are non-selective cation channels. Cyclic-nucleotide gated channels are mainly associated with expression in retinal and olfactory neurones (Nakatani *et al.*, 1995; Leinders-Zufall *et al.*, 1997). They have a pore region that is very similar to the pore of voltage-gated K^+ channels (Heginbotham *et al.*, 1992), display little selectivity among monovalent cations, and are activated by intracellular ligands (cAMP and cGMP) rather than voltage (Kramer *et al.*, 1994). Hyperpolarisation activated K^+ channels are involved in mediating the generation of pacemaker activity in the heart and brain (Santoro *et al.*, 1998; McCloskey *et al.*, 1999; Cadetti and Belluzzi, 2001).

1.2.2. 2P-domain potassium channel family

Also known as resting or leakage channels; TWIK(1-2), TREK (1-2), TRAAK, TASK (1-3), THIK (1-2), TALK (1-2) and KCNK7 (see Patel and Honore, 2001 for review). These subunits possess four transmembrane domains and most are active as dimers (Bockenhauer *et al.*, 2000; Lopes *et al.*, 2001). They are widely expressed being found in both excitable and

non-excitable cells. Their regulation is varied with different members being sensitive to various physical and chemical stimuli; such as membrane stretch, temperature, pH, and lipids (Reyes *et al.*, 2000; Maingret *et al.*, 2000; Lesage *et al.*, 2000; Patel *et al.*, 2001). They are thought to be an important contributor to the resting membrane potential (Lesage and Lazdunski, 2000; Patel *et al.*, 2001).

1.2.3. Inwardly rectifying potassium channel family

With two transmembrane domains inwardly rectifying K^+ channels (Kir) allow the passage of K^+ in the inward direction much more readily than the outward direction regardless of extracellular potassium concentration. This inward rectification was suggested to be caused by a blocking particle (Standen and Stanfield, 1978), and later attributed to voltage and concentration dependent intracellular block by Mg^{2+} and polyamines (Vandenberg, 1987; Lopatin *et al.*, 1994, 1995; Lopatin and Nichols, 1996). Kir's show characteristic inward rectification, i.e. inward currents larger than outward currents, and can be strong or weak for different Kirs. It is thought that block by Mg^{2+} constitutes the mechanism of weak inward rectification and polyamine block that of strong rectification (Yamada and Kurachi, 1995; Fakler *et al.*, 1995).

The H5 region of Kirs differ from the homologous domains of the Kv channels in the number of charged residues present, their sign and distribution. A conserved ion pair (a glutamate and an arginine) has also been described in the H5 region of Kirs and found to be a crucial determinant of the pore structure and ion permeation properties of the channel (Yang *et al.*, 1997)

1.2.3a. Kir 1.0

The Kir 1.0 subfamily encompasses Kir 1.1, Kir 1.2, and Kir 1.3. Kir 1.1 occurs as the splice variants Kir 1.1a, b, c, d, e, and f, which are truncated forms of Kir 1.1a, although when the subunits are expressed their macroscopic currents are indistinguishable from each other (Yano *et al.*, 1994; Doupnik *et al.*, 1995; Kondo *et al.*, 1996; Shuck *et al.*, 1997). The channels of this subfamily have been localised to the kidney where they are involved in pH and K^+ homeostasis of the renal epithelia (Doi *et al.*, 1996; Xu *et al.*, 1997; Kohda *et al.*, 1998). They form in a tetrameric complex with the resulting channel being weakly inwardly rectifying and are regulated by ATP and intracellular pH, with channel closure occurring under acidification.

A weak sulphonylurea sensitivity has been found in some channels and atributed to an association with the ABC transporter CFTR (McNicholas *et al.*, 1996; Ruknudin *et al.*, 1998). Mutations in Kir 1.1 have been associated with some forms of the salt wasting disease seen in Bartter syndrome (Simon *et al.*, 1996; Derst *et al.*, 1997).

1.2.3b. Kir 2.0

A strong inward rectifier containing two transmembrane domains was isolated from a mouse macrophage cell line by Kubo and co-workers (Kubo *et al.*, 1993). The isolated cDNA encoded IRK1/Kir 2.1, and was later also isolated by Morishige and co-workers from mouse brain (Morishige *et al.*, 1993). Since this time three other Kir 2.0 members have been cloned, Kir 2.2, Kir 2.3, and Kir 2.4 (Takahashi *et al.*, 1994; Koyama *et al.*, 1994; Morishige *et al.*, 1994; Hughes *et al.*, 2000), which differ in their conductance with Kir 2.1 having the largest conductance and Kir 2.3 the smallest (Doupnik *et al.*, 1995). Kir 2.1, Kir 2.2 and Kir 2.3 are predominantly found in the brain where they're involved in the regulation of neurone excitability, they have differing distributions which may imply specific functions for these

channels in the different areas of the brain (Karschin *et al.*, 1996; Horio *et al.*, 1996; Perillan *et al.*, 2000). Apart from their presence in the brain Kir 2.0 channels have been demonstrated in other tissues; Kir 2.1 has been described in cardiac tissue (Raab-Graham *et al.*, 1994), and vascular arterial smooth muscle cells (Bradley *et al.*, 1999), and Kir 2.4 was cloned from a human retina library (Hughes *et al.*, 2000).

Kir 2.0 channels are homotetramers (Raab-Graham and Vandenberg, 1998), although Kir 2.1 and Kir 2.3 have been shown to contain a PDZ domain recognition sequence in their C-terminus which appears to mediate binding to members of the synapse-associated protein family SAP90 (Nehring *et al.*, 2000; Leonoudakis *et al.*, 2001).

1.2.3c. Kir 3.0

The G-protein-activated Kir 3.0s are widely expressed in the brain where they are activated by a number of neurotransmitters. They are also present in the heart where they underlie the ACh activated K^+ current. There are four members; Kir 3.1-3.4 (GIRK1-4) which are able to form heteromultimeric channels (Kofuji *et al.*, 1995). Kir 3.1 requires the co-expression of Kir 3.2, Kir 3.3, or Kir 3.4 to form functional channels and has been demonstrated to occur in cardiac muscle (Jelacic *et al.*, 2000) with the Kir 3.1/ Kir 3.2 combination also being observed to form channels in gastrointestinal myocytes (Bradley *et al.*, 2000), and the Kir 3.2/ Kir 3.3 channel combination found predominantly in brain (Jelacic *et al.*, 2000).

1.2.3d. Kir 4.0/ Kir 5.0

The Kir 4.1 subunit is predominantly expressed in glial cells (Horio *et al.*, 1997), and immunohistological observations have shown it to be enriched in astrocyte processes wrapping synapses and blood vessels (Higashi *et al.*, 2001). It has been shown to bind

members of the PSD-95 family at its C-terminus, a mechanism that affects the distribution of the channel (Horio *et al.*, 1997). A null mutation of the gene for this subunit has been found to cause a motor impairment in mice, caused by hypomyelination (Neusch *et al.*, 2001).

Functional channels aren't observed when Kir 5.1 is expressed alone in *Xenopus* oocytes. It is possible that this subunit may couple with another Kir to form channels in native tissue and it has been shown to associate specifically with Kir 4.1 in *Xenopus* oocyte expression studies (Pessia *et al.*, 1996; Tucker *et al.*, 2000). It is thought that the heteromerisation of Kir 4/5 sunbunits leads to the formation of channels that are involved in CO_2 chemoreception in brainstem neurones (Pearson *et al.*, 1999; Xu *et al.*, 2000; Tanemoto *et al.*, 2000; Cui *et al.*, 2001; Higashi *et al.*, 2001; Jiang *et al.*, 2001).

1.2.3e. Kir 6.0

In 1995 Inagaki and co-workers isolated a cDNA, from a rat pancreatic islet cDNA library, that encoded a novel member of the Kir family (Inagaki *et al.*, 1995a). They called this uKATP-1 and found that it formed a channel that was sensitive to ATP, and was expressed ubiquitously in rat tissues. Later they cloned the human cDNA and gene that encoded uKATP-1, the gene was mapped to chromosome 12p11.23, using fluorescence in situ hybridisation, and they designated it KCNJ8 (Inagaki *et al.*, 1995b). This inward rectifier is now known as Kir 6.1.

In the same year Inagaki and co-workers cloned a second member of this new Kir 6.x subfamily, and called it Kir 6.2 (Inagaki *et al.*, 1995c). They found that it was expressed in rat pancreatic islets and glucose-responsive insulin-secreting cell lines. When expressed with SUR 1 it was found to form channels that were sensitive to ATP, inhibited by sulphonylureas, and activated by diazoxide. They also showed that the SUR 1 and Kir 6.2 genes were clustered on human chromosome 11 at position 11p15.1. Kir 6.2 cDNA was also cloned from

an insuloma cDNA library, and its mRNA found to be strongly expressed in brain, skeletal muscle, and cardiac muscle, with a weak expression in most other tissues (Sakura *et al.*, 1995).

Kir 6.0 subunits form the pore of the K_{ATP} channel, associating in a tetramer, with each Kir 6.0 subunit requiring the association of one SUR to generate a functional channel (Clement *et al.*, 1997; Inagaki *et al.*, 1997; Shyng and Nichols, 1997).

1.2.3f. Kir 7.0

Kir 7.1 subunit expression has been observed in retinal pigment epithelium (Sgard *et al.*, 2000; Shimura *et al.*, 2001).

1.3. K_{ATP} channels in native tissue

In 1983 Noma described an inwardly rectifying potassium channel, in cardiac myocytes, which was inhibited by intracellular ATP (Noma, 1983). It was noted that this channel played an important role in the regulation of cellular metabolism and the control of membrane excitability. Although current inhibition by $[ATP]_i$ was the primary criteria for channel definition, further investigation has led to single-channel current properties, pattern of regulation by nucleotides, and distinctive pharmacology also being used in channel definition.

The channels described by Noma were located in myocardial myocytes, but have since been described in various other tissues including; pancreas (Cook and Hales, 1984; Ashcroft and Kakei, 1989), kidney (Sansom *et al.*, 1994), smooth muscle (Kajioka *et al.*, 1991; Clapp and Gurney, 1992; Beech *et al.*, 1993), skeletal muscle (Spruce *et al.*, 1987), brain (Morita *et al.*, 1984; Mourre *et al.*, 1990; Murphy and Greenfield, 1991; Ueno *et al.*, 1992; Jiang and Haddad, 1997), pituitary (Meucci *et al.*, 1992; Wu *et al.*, 2000), and mitochondria (Inoue *et*

al., 1991). Extensive investigation has implicated the K_{ATP} channel in a number of functions within these tissues.

1.3.1. Ischemic preconditioning

K_{ATP} channels are thought to be involved in the mechanism of ischemic preconditioning, in the heart (Carr *et al.*, 1997), brain (Perez-Pinzon and Born, 1999; Bajgar *et al.*, 2001), kidney (Sgard *et al.*, 2000), and retina (Ettaiche *et al.*, 2001), where repetitive brief ischemic episodes occurring before a long-term sustained ischemia induce tolerance against the ischemic cellular injury.

This natural tolerance to cellular injury can be mimicked by the activation of K_{ATP} channels by potassium channel openers (KCOs) and likewise may be blocked by the inhibition of K_{ATP} channels (Carr *et al.*, 1997). This pharmacological evidence was further supported by a study in which protection to hypoxia-reoxygenation injury was induced in K_{ATP} channel deficient cells by the introduction of cardiac K_{ATP} channel subunits (Jovanovic *et al.*, 1999).

The activation of K_{ATP} channels during myocardial ischemia, leads to the shortening of the cardiac action potential (Nichols *et al.*, 1991a), however this AP shortening is not correlated with cardioprotection suggesting a role for mitochondrial K_{ATP} channels in ischemic preconditioning (Grover, 1997). The mitochondrial membrane potential is severely compromised, in the myocardium, after ischemia reperfusion, triggering apoptotic events leading to cell death, and this can be prevented by the pharmacological activation of mitochondiral K_{ATP} channels (Akao *et al.*, 2001; Xu *et al.*, 2001a). This activation of mitochondrial K_{ATP} channels has been shown to protect against ischemic reperfusion injury as does natural ischemic preconditioning (Pomerantz *et al.*, 2000a,b; Ockaili *et al.*, 2001; Nakai *et al.*, 2001). The involvement of the mitochondrial K_{ATP} channel rather than the sarcolemmal

K_{ATP} channel in this mechanism has been investigated comparing the effects of specific K_{ATP} channel openers and inhibitors on prevention of cellular injury after ischemia reperfusion (Sato *et al.*, 2000a; Nakai *et al.*, 2001).

The opening of the channel is thought to be both directly and indirectly related to protein kinase C activation (Sato *et al.*, 1998; Wang *et al.*, 1999b). A number of endogenous substances are released from ischemic or hypoxic tissues, such as; adenosine, nitric oxide, free radicals, and bradykinin, resulting in the stimulation of protein kinase C. The phosphorylation of a conserved threonine residue in Kir 6.2 by PKC has been shown to affect the open probability of the channel (Light *et al.*, 2000). Also protein kinase C is the first element of a complex cascade leading to the phosphorylation of HSP27 a heat shock protein that controls actin filament polymerisation a factor associated with K_{ATP} channel modulation (Cohen *et al.*, 2000).

How K_{ATP} channel opening leads to preconditioning is still under discussion. One possibility is that dissipation of the mitochondrial membrane potential decreases the driving force for calcium influx through the calcium uniporter protecting the mitochondria from calcium overload (Crestanello *et al.*, 2000; Wang *et al.*, 2001). It has also been suggested that the decrease in membrane potential caused by channel opening promotes the binding of the endogenous ATPase inhibitor IF (protein synthesis initiation factor), conserving ATP during the period of ischemia (Liu *et al.*, 1998).

1.3.2. Insulin release

The role of the K_{ATP} channel in the pancreas is well understood (see fig. 1.3. for a schematic of insulin regulation in the pancreatic beta-cell). Under normal conditions the K_{ATP} channel sets the beta-cell resting membrane potential. Elevation of blood glucose concentration results in increased glucose uptake and metabolism by the beta-cell leading to

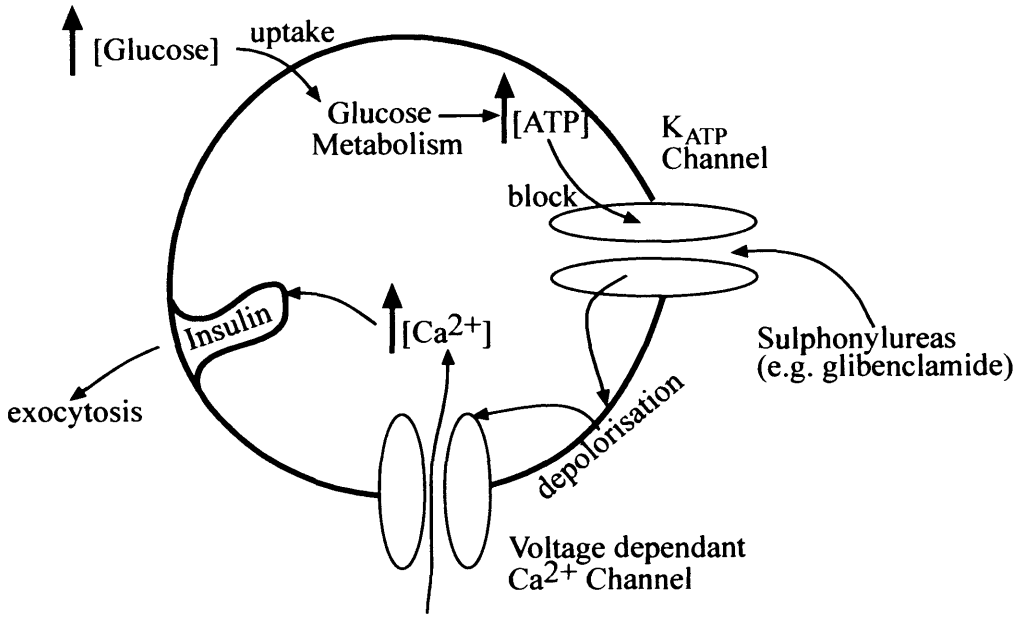


Figure. 1.3.
Insulin regulation in the pancreatic beta-cell.

an increase in the ATP/ADP ratio and subsequent closure of the K_{ATP} channel. The depolarisation of the membrane causes the opening of voltage-dependent calcium channels resulting in calcium influx triggering insulin granule exocytosis (Rajan *et al.*, 1990; Boyd, 1992; Sato *et al.*, 1992).

1.3.3. Vascular tone

In vascular smooth muscle K_{ATP} channels are involved in the regulation of muscle tone and so control of blood flow. Changes in $[ATP]_i$ directly influence vascular tone via K_{ATP} channel modulation. The inhibition of K_{ATP} channels by endogenous or synthetic channel blockers such as endothelin or glibenclamide results in an increase in vascular tone/vasoconstriction, conversely the activation of the channel by cromakalim and adenosine results in a decrease of vascular tone/vasodilation (Standen *et al.*, 1989; Quayle *et al.*, 1995).

1.4. K_{ATP} channel Pharmacology

K_{ATP} channels are the target of two main types of compound, the potassium channel openers (KCOs) a structurally heterogenous class of compounds which cause activation of the channel, and the sulphonylureas which inhibit the channel. Examples of KCOs, some of which are in use clinically, include; pinacidil, nicorandil, diazoxide, levcromakalim, and minoxidil sulphate. Pinacidil, the pyridyl cyanoguanidine P1134, was found to have an antihypertensive effect and was thought to exert its action through the opening of vascular K^+ channels, hyperpolarizing the cell at rest (Ahnfelt-Ronne, 1988; Cook *et al.*, 1988). Diazoxide is used in the treatment of hyperinsulinemia and in hypertensive emergencies, with the activation of K_{ATP} channels causing an inhibition of insulin release in pancreatic beta-cells and a decrease in vascular tone of smooth muscle. Cromakalim is another example of an antihypertensive which produces a vasodilation of smooth muscle (Quayle *et al.*, 1995).

Sulphonylureas are widely used in the treatment of type 2 diabetes, two examples being; glibenclamide, a second generation antidiabetic, and tolbutamide, a first generation antidiabetic. As well as stimulating insulin release glibenclamide also has diuretic ability (Quast, 1996). The potencies of these compounds, in the activation or inhibition of K_{ATP} channels, vary according to different tissues.

1.5. The K_{ATP} channel complex

K_{ATP} channels are composed of an inwardly rectifying K^+ channel subunit (Kir) and a sulphonylurea subunit (SUR), which is a member of the family of ATP-Binding Cassette (ABC) transporter proteins (see fig. 1.4.). They are the first example of a heteromultimeric complex assembled by an ion channel and a receptor that are structurally unrelated to each other. The physical association of the two subunits has been demonstrated by immunoprecipitation studies (Lorenz *et al.*, 1998; Lorenz and Terzic, 1999), and they are thought to associate in a 1:1 stoichiometry (Clement *et al.*, 1997) as a hetero-octamer comprising a tetramer of SUR and a tetramer of Kir which forms the pore (Inagaki *et al.*, 1997; Shyng *et al.*, 1997a).

How and why the K_{ATP} channel subunits form in an octameric stoichiometry has been a question causing much interest. A 3 amino acid trafficking sequence has been shown to be responsible for the prevention of single subunit or partial complex insertion into the cell membrane (Zerangue *et al.*, 1999). This sequence is located at the C-terminal end of Kir 6.1 and Kir 6.2, and in a cytoplasmic loop between the putative eleventh transmembrane domain and the first nucleotide binding fold of SUR1. It is thought that the sequence serves as an endoplasmic reticulum (ER) retention signal and when the channel is correctly assembled as an octameric complex this signal is masked therefore allowing free passage of the complex

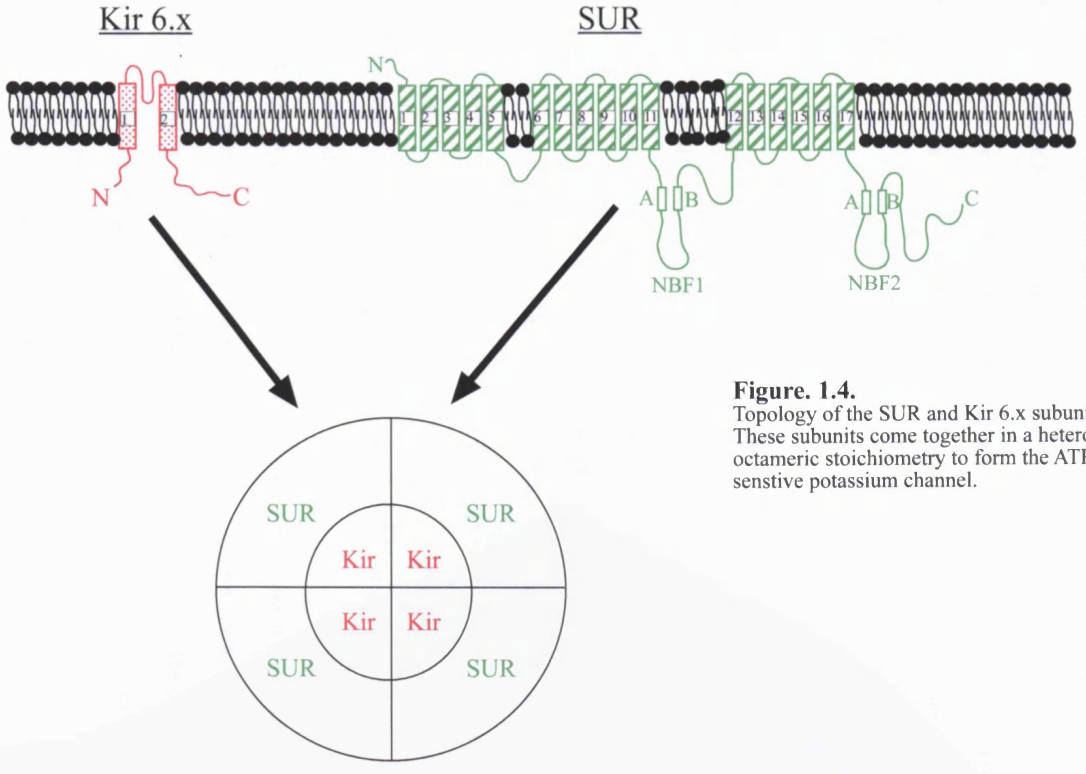


Figure. 1.4.
Topology of the SUR and Kir 6.x subunits. These subunits come together in a hetero-octameric stoichiometry to form the ATP-senstive potassium channel.

through the ER. Additionally SUR1 has been shown to possess an anterograde signal at the C-terminus, which is required for the channel to exit the ER/cis-Golgi compartments and subsequently transit to the cell surface (Sharma *et al.*, 1999). It is known that mutations of SUR1 involving the truncation of the C-terminus result in the recessive form of the disorder persistent hyperinsulinaemic hypoglycaemia of infancy (Thomas *et al.*, 1995). This may be explained by the failure of the assembled channel to traffic to the cell surface due to the loss of the anterograde signal in SUR1.

1.6. ABC transporters

SUR is a member of the ABC transporter superfamiliy. Most ABC proteins are active transporters, others are ion channels and some are involved in regulating the activity of heterologous channel proteins. There are seven ABC gene subfamilies (Dean *et al.*, 2001) that have two structural models. They either possess two, hydrophobic, multi-spanning membrane regions both followed by a hydrophilic region, known as the nucleotide-binding domain (Gileadi and Higgins, 1997; Vos *et al.*, 1999; Bungert *et al.*, 2001), or in the case of SUR and MRP an additional transmembrane region in the N-terminus (Bakos *et al.*, 1996; Tusnady *et al.*, 1997; Raab-Graham *et al.*, 1999).

ABCA (ABC1); comprises 12 full transporters that are divided into two subfamilies. The ABC1 gene has been identified as the defective gene in Tangiers disease (Bodzioch *et al.*, 1999; Brooks-Wilson *et al.*, 1999; Remaley *et al.*, 1999; Rust *et al.*, 1999). The protein is expressed by macrophages and is required for engulfing cells undergoing programmed cell death (Becq *et al.*, 1997).

ABCB (MDR/TAP); comprises four full transporters and seven half transporters. The multidrug resistance proteins provide a cell with simultaneous resistance to various unrelated anticancer drugs and include P-glycoprotein, also known as PGY1, MDR1, or ABCB1. The

products of the P-glycoprotein gene mdr1 plays an important role in the blood/tissue barrier and mdr2/3 phospholipid transport in the liver (Ling, 1997). TAP, while also involved in the multidrug resistance phenotype, is also concerned with antigen presentation, mediating peptide translocation over the membrane of the endoplasmic reticulum (Izquierdo *et al.*, 1996).

ABCC (CFTR/MRP); this group contains 12 full transporters. Another multidrug resistant gene MRP1 confers resistance by reducing drug accumulation actively exporting the drug from the cell (Payen *et al.*, 2001). Defects in the commonly known transporter CFTR are responsible for the disease cystic fibrosis (Schwiebert *et al.*, 1999), and the gene product of pfmdr is involved in conferring chloroquine resistance on malarial parasites.

ABCD (ALD). ALD encodes a peroxisomal membrane transporter that is required for the import of fatty acids into peroxisomes (Hettema and Tabak, 2000). Mutations is this gene are linked to the demyelinating disorder of the central nervous system, X-linked adrenoleukodystrophy (X-ALD) (Aubourg, 1994; Ligtenberg *et al.*, 1995; Holzinger *et al.*, 1999; Smith *et al.*, 1999).

ABCE (OABP). The organic anion binding protein, OABP, has been linked with the hepatic uptake of non-bile acid organic ions such as bilirubin by Adachi and co-workers (Adachi *et al.*, 1996).

ABCF (GCN20). Postulated to be responsible for drug export in chloroquine resistant strains of Plasmodium falciparum pfGCN20 is closely related to the yeast translational regulator Gcn20p (Bozdech *et al.*, 1996; Peel, 2001).

ABCG (White); ABCG2, also known as MXR because of the resistance it confers to mitoxantrone, or BCRP, breast cancer resistance protein, confers resistance to anthracycline anticancer drugs. It is a half transporter which is thought to produce an active transport

complex by homodimerizing or by formation of a heterodimer (Litman *et al.*, 2000; Ross, 2000).

1.7. SUR

The SUR genes are located in the subfamiliy ABCC and are known as ABCC8 and 9 (Dean *et al.*, 2001). A number of different species of SUR mRNA have so far been produced from these two different SUR genes and have been isolated and shown to yield functionally distinct K_{ATP} channels when expressed with Kir 6.x. The SUR subunit contains three hydrophobic domains, containing 17 transmembrane segments in a 5+6+6 arrangement (Tusnady *et al.*, 1997; Raab-Graham *et al.*, 1999). There are also two hydrophilic regions, following the second and third hydrophobic domains that comprise the nucleotide binding domains. These nucleotide binding domains (also referred to as nucleotide binding folds) are so called because their sequence alignment reveals the presence of two conserved nucleotide binding motifs, Walker A and B motifs, first described by Walker and co-workers (Walker *et al.*, 1982). The Walker A motif occurs as a glycine rich loop followed by an uncapped alpha-helix, it has been shown to interact with the gamma-phosphate of ATP or GTP in the presence of Mg^{2+} (Matte *et al.*, 1996), and mutations of lysine residue in either NBD of SUR 1 have been shown to affect the potentiatory effects of GTP and GDP on the K_{ATP} channel (Trapp *et al.*, 1997; Shyng *et al.*, 1997b).

The first of the SUR subunits was cloned from pancreatic tissue and seemed to confer typical beta–cell properties, it was called SUR1 (Inagaki *et al.*, 1995a). The next to be cloned, SUR2, which was also discovered by Inagaki and co-workers, resembled the skeletal and cardiac muscle type K_{ATP} channel when expressed with Kir 6.2 (Inagaki *et al.*, 1996). SUR 2 was found to occur as splice variants that differ in the usage of exons 39 and 40 at the carboxyl terminus (Isomoto *et al.*, 1996). These were called SUR2A and SUR2B. A variant of SUR2A, the cDNA of which appears to be restricted to cardiac tissue, which has a deletion of

exon 14 (sometimes called SUR2C) is also thought to exist although there is debate on whether it forms functional channels in native tissue. Chutkow and co-workers have also demonstrated that alternative splicing of exon 17, which encodes the 13 amino acids next to the Walker A motif, can occur and that this splicing alters the K_{ATP} channels sensitivity to ATP (Chutkow *et al.*, 1999). A splice variant of SUR 1, isolated from a hypothalamic cDNA library, in which exon 33 is missing has also been described (Sakura *et al.*, 1999)

1.8. Kir 6.x

Two different Kir 6.x subunit genes have been described, Kir 6.1 and 6.2, with the resultant proteins sharing a ~70% identity at the amino acid level. Kir subunits consist of two transmembrane domains , M1 and M2, and a pore forming domain called the H5 region. The H5 region has been suggested to form the outer ion selectivity filter, with the α–helical M2 segment lining the inner vestibule between the selectivity filter and the cytoplasm (Loussouarn *et al.*, 2000). K_{ATP} channels in native tissue are weakly rectifying (Light *et al.*, 1999). They differ from other more strongly rectifying Kir's in that Kir 6.x have an asparagine instead of aspartic acid in the second transmembrane domain, a site that is a crucial determinant of the rectifying activity (Shyng *et al.*, 1997a). They also differ from other Kirs in the H5 region, where a glycine-tyrosine-glycine motif which is critical for ion selectivity, is not conserved being instead glycine-phenylalanine-glycine.

The structure of the Kir 6.x pore has been modelled by homology with the known KscA structure. These studies have shown the pore to be very narrow at the inner entrance, excluding all ions, and that it may widen by a helical motion of the M2 region and rearrangement of side-chains (Loussouarn *et al.*, 2000; Loussarn *et al.*, 2001a).

Channels formed by Kir 6.2 and 6.1 have differing conductances, with that of Kir 6.2 being larger than 6.1 (Kono *et al.*, 2000). Also channels containing Kir 6.2 have been shown

to open spontaneously in the absence of $[ATP]_i$ whereas those containing Kir 6.1 don't (Okuyama *et al.*, 1998). Chimeric constructs have shown that the extracellular linker domain between the two membrane spanning regions is responsible for the differing conductances and that the N-terminus of Kir 6.2 is involved in the spontaneous channel opening (Kondo *et al.*, 1998; Repunte *et al.*, 1999).

1.9. Channelopathies: PHHI

Familial persistant hyperinsulinemic hypoglycemia of infancy (PHHI) is a disorder that is characterised by unregulated secretion of insulin, from the pancreatic beta-cell, causing profound hypoglycemia. It is an autosomal reccesssive disorder that has been linked to chromosome 11 (Thomas *et al.*, 1995), and caused by mutations in the SUR1 and/or Kir 6.2 genes (Thomas *et al.*, 1995; Thomas *et al.*, 1996). There have been many mutations observed which result in channels that have trafficking defects or an altered response to MgADP often via disruption of NBF2 (Thomas *et al.*, 1995; Thomas *et al.*, 1996; Shyng *et al.*, 1998; Matsuo *et al.*, 2000; Tanizawa *et al.*, 2000; Cartier *et al.*, 2001; Partridge *et al.*, 2001). A consequence of the lack of K_{ATP} channel activity in the membrane (either due to channel absence or a reduced response to stimulation by MgADP) is that the beta-cell is spontaneously electrically active leading to a high concentration of cytosolic calcium due to calcium influx, and resulting in unregulated insulin release (Kane *et al.*, 1996).

1.10. Molecular identity of the K_{ATP} channel

Successful cloning of the K_{ATP} channel has enabled comparisons in the pharmacology of native and cloned channels to be made. The information gained from these studies has led to an insight into the molecular identity of K_{ATP} channels in different tissues.

The pharmacological properties of SUR1 and Kir 6.2 channels have been shown to resemble those typical of native K_{ATP} channels described in pancreatic and neuronal tissues, being inhibited at similar potencies by tolbutamide and glibenclamide and in their activation by diazoxide (Gopalakrishnan *et al.*, 2000; McKay *et al.*, 2000).

A sulphonylurea sensitive but ATP-insensitive channel formed by SUR2B and Kir 6.1 is purported to form the K_{ATP} channel of vascular smooth muscle, and is similar to the native channel in its activation by pinacidil, diazoxide and nicorandil, and inhibition by glybenclamide (Yamada *et al.*, 1997).

The coexpression of SUR2A and Kir 6.2 has been shown to reconstitute the characteristic activation by pinacidil but not by diazoxide demonstrated by the cardiac K_{ATP} channel (Lorenz and Terzic, 1999)

1.11. Heteromultimerisation

RT-PCR has been used to investigate the expression of K_{ATP} channel subunits in various tissues. In some cell types both Kir 6.0 subunits and more than one SUR subunit have been detected, however, it is not known whether heteromeric channels are formed. Expression studies by Giblin and co-workers concluded that heteromultimerisation of SUR subunits did not occur, although they did not try to express tandem constructs (Giblin *et al.*, 2002). Although there is no obvious pharmacological evidence to support heteromultimerisation of SUR subunits the possibility cannot be excluded especially as tandem Kir channels have been shown to express *in vitro* (Kono *et al.*, 2000) even though dominant-negative studies by Seharaseyon and co-workers failed to show evidence for their heteromultimerisation (Seharaseyon *et al.*, 2000).

1.12. Intracellular regulation of cloned K_{ATP} channels

Comparison of the functional properties of SUR2A/Kir 6.2 and SUR2B/Kir 6.1 have shown that the single channel characteristics are determined by the Kir subunit and the pharmacological properties by the SUR subunit, while the response to intracellular nucleotides is determined by both subunits (Fujita and Kurachi, 2000)

1.12.1 ATP sensitivity

ATP is thought to stabilise the channel in the long closed state acting as an allosteric ligand, interacting with both open and closed states of the channel (Enkvetchakul *et al.*, 2000; Enkvetchakul *et al.*, 2001). It was first assumed that the SUR subunit conferred the ATP sensitivity on the K_{ATP} channel because it possessed nucleotide binding domains (NBF's) while neither Kir 6.1 or 6.2 have an identifiable ATP binding site. Mutations in the NBF's of the SUR subunit, however, failed to alter the ATP-dependent inhibition of the channel, suggesting that this subunit was not directly responsible for the channels inhibition by ATP (Drain *et al.*, 1998). This was further supported by the identification of a truncated form of Kir 6.2, in which the last 26 or 36 amino acids are deleted, that exhibited functional channel activity in the absence of SUR whilst retaining intrinsic ATP sensitivity (Tucker *et al.*, 1997). Further mutagenesis studies have also led to the identification of multiple residues, in the N- and C-termini, of Kir 6.2 that are important for endowing ATP sensitivity on the K_{ATP} channel (Proks *et al.*, 1999; Koster *et al.*, 1999). ATP has also been demonstrated to bind directly to Kir 6.2 in the absence of SUR using the ATP photoaffinity analog 8-azido-[γ-32p] (Tanabe *et al.*, 2000). However the truncated version of Kir 6.2 exhibits altered single channel kinetics and has a lower sensitivity to $[ATP]_i$ compared to Kir 6.2/SUR1, implying that the SUR subunit is involved in the modulation of the pore forming subunit or provides a link between the binding site and the gate. Comparisons between SUR1/Kir 6.2 and SUR2A/ Kir 6.2 have also demonstrated differences in burst duration, open probability, and ATP sensitivity, with

chimeras of the SURs revealing the first 5 transmembrane domains to specify the interburst kinetics and the C-terminus to determine the ATP sensitivity (Babenko *et al.*, 1999a).

K_{ATP} channels may be regulated by pH, phospholipids, and other nucleotides, allowing control of cellular excitability in conditions when the ATP concentration is normally inhibitory.

1.12.2. pH

K_{ATP} channel regulation by intracellular pH appears to be rather complicated. In 1988 Carrol and co-workers noticed that in single channel recordings, of rat cultured beta cells, lowering the extracellular pH from 7.4 to 7.0 inhibited K_{ATP} channel activity. However, in intact mouse islets, they observed that decreasing pH evoked electrical activity via the K_{ATP} channel (Carrol *et al.*, 1988). Later it was discovered that lowering the intracellular pH in rat ventricular myocytes and skeletal muscle reduced the inhibitory effect of ATP on the K_{ATP} channel, increasing the Ki for ATP from 25 μM to 50 μM when changing the pH from 7.25 to 6.25 (in ventricular myocytes) (Lederer and Nichols, 1989; Davies, 1990). Under physiological conditions the $[ATP]_i$ of cardiac and skeletal muscle is above the Ki of the K_{ATP} channel in these tissues (Noma, 1983;Spruce *et al.*, 1987). Even under metabolic stress $[ATP]_i$ is buffered, therefore the regulation of K_{ATP} channels via changes in intracellular pH is an important mechanism. *In vivo* the opening of K_{ATP} channels in vascular smooth muscle has been shown to mediate arteriolar dilation during acidosis (Ishizaka and Kuo, 1996). Recent work has provided evidence for a direct activation of the K_{ATP} channel by interaction of protons with histidine residues in the Kir 6.x subunit (Piao *et al.*, 2001; Xu *et al.*, 2001b; Xu *et al.*, 2001c).

1.12.3. Actin cytoskeleton

In 1991 Cantiello and co-workers showed, by immunolocalisation, that actin was always present in close proximity to apical sodium channels of A6 cells (Cantiello *et al.*, 1991). The application of actin filament disrupters, such as cytochalasin D, increased the activity of these sodium channels, in excised patches. Conversely, proteins that stabilsed actin filaments were found to reverse channel activation (Cantiello *et al.*, 1991; Berdiev, *et al.*, 1996). These studies provided direct evidence for the involvement of constituents of the cytoskeletal network in channel regulation. Later actin filament disruption and stabilisation was also found to regulate potassium channel activity. Disruption of the actin cytoskeleton, by cytochalasin D, in retinal neurones was found to activate voltage-gated potassium currents, and the actin filament stabiliser phalloidin to inhibit them (Maguire *et al.*, 1998). Likewise, actin filament disruption has been shown to activate cloned K_{ATP} channels by decreasing the channels sensitivity to ATP (Song and Ashcroft, 2001).

1.12.4. Phosphatidly inositol phosphates (PIP, PIP_2)

When the integrity of a cell is disrupted, as happens when a patch is excised, K_{ATP} channel activity gradually declines, this is known as run-down. It is thought that cytoskeletal elements may be important regulators of the K_{ATP} channel. Addition of Ca^{2+} to a patch of membrane expressing Kir 6.2/SUR 1 channels has been observed to accelerate this rundown, increasing the channels sensitivity to ATP (Ribalet *et al.*, 2000), while the addition of PIP_2 caused run-down to be inhibited (Furukawa *et al.*, 1996). It was proposed that this phenomenon may be due to a loss of PIP_2 from the membrane, as PIP_2 hydrolysis is mediated by Ca^{2+} dependent phospholipase C (Xie *et al.*, 1999; Ribalet *et al.*, 2000). The application of PIP_2 to the membrane has also been shown to reduce K_{ATP} channel sensitivity to ATP (Shyng and Nichols, 1998; Shyng *et al.*, 2000a,b). It has been shown to activate K_{ATP} channels in

ventricular myocytes and reactivate channels in which current had undergone run-down (Okamura *et al.*, 2001). Several cloned inward rectifiers have been shown to directly bind PIP_2 (Huang *et al.*, 1998). Baukrowitz and co-workers used studies on cloned K_{ATP} channels to show that PIP_2 acts on the Kir 6.x subunit shifting the channels sensitivity to ATP so that the channel is able to open at physiological $[ATP]_i$ (Baukrowitz *et al.*, 1998).

Actin filament disruption may not in fact directly regulate K_{ATP} channel activity, but may be linked to the amount of free phospholipid in the membrane. Alpha-actinin-2 is an actin cross-linking protein, it has also been shown to associate with PIP_2 and can be co-immuno-precipitated with human Kv 1.5 (Maruoka *et al.*, 2000). Various actin filament capping proteins are also known to bind PIP_2 (Kuhlman and Fowler, 1997), and uncapping events leading to actin filament disruption may liberate PIP_2 into the membrane where it can directly interact with the channel.

1.12.5. Phosphorylation

Hormones and neurotransmitters modulate K_{ATP} channels via protein kinases. Studies using protein kinase inhibitors have shown that the stimulation of K_{ATP} channels at low $[ATP]_i$ may be due to channel phosphorylation (Ribalet *et al.*, 1999). Phosphorylation of SUR1 by protein kinase A (PKA), at site S1571 of the C-terminus, has been shown to affect the basal properties of the K_{ATP} channel when expressed with Kir 6.2, by decreasing burst duration, interburst interval, and open probability while also increasing the number of functional channels at the cell surface (Beguin *et al.*, 1999). PKA phosphorylation of Kir 6.2 at site S372 has been shown to cause an increase in channel activity when expressed with SUR1, and phosphorylation of site T224 to alter channel gating and decrease the affinity of the channel for ATP (Beguin *et al.*, 1999; Lin *et al.*, 2000). PKC also acts via phosphorylation

of a threonine in Kir 6.2 this time at site T180, altering the open probability of the channel when in complex with SUR1 or SUR2A and also in the absence of an accessory SUR subunit (Light *et al.*, 2000).

1.13. Pharmacology of the cloned K_{ATP} channel

The differences in sensitivity of pharmacological agents for K_{ATP} channels of various tissues have been found to be due to the different subunit combinations of K_{ATP} channel occurring in these tissues. For instance, glibenclamide has been demonstrated to have over 100 fold higher affinity for SUR1 binding than to either of the SUR2 isoforms, and nicorandil has been shown to preferentially activate K_{ATP} channels containing SUR2B rather than SUR2A (Shindo *et al.*, 1998; Meyer *et al.*, 1999). Kir specificity has also been demonstrated by some compounds, with the morphilinoguanidine K_{ATP} channel blocker PNU-37883A being a more potent inhibitor of channel comprising Kir 6.1 over Kir 6.2 (Surah-Narwal *et al.*, 1999).

The SUR subunit endows the K_{ATP} channel with sensitivity to sulphonylureas (Inagaki *et al.*, 1996), K_{ATP} channel openers (Shindo *et al.*, 1998) and the stimulatory action of MgADP (Gribble *et al.*, 1997a) whilst also enhancing the channel open probability and its sensitivity to ATP (D'Hahan *et al.*, 1999, Song and Ashcroft, 2001). KCOs activate K_{ATP} channels through binding to the SUR subunit, and this binding requires a conformational change that is induced by ATP hydrolysis at NBF2 (Larsson *et al.*, 1993; Shyng *et al.*, 1997b; Matsuo *et al.*, 2000; Chutkow *et al.*, 1999). Mutations in the linker and Walker B motifs of the second NBF have been shown to abolish channel activation by diazoxide (Shyng *et al.*, 1997b), MgADP (Gribble *et al.*, 1997a) and MgATP (Gribble *et al.*, 1998), while mutations in NBF1 appear to be involved in controlling kinetics of activation. Nucleotide hydrolysis is required for channel activation and diazoxide and MgADP either stimulate hydrolysis or

stabilise the channel in a desensitised state resulting from hydrolysis (Shyng *et al.*, 1997b). The inhibitory effect of ATP on the other hand seems to occur as a result of direct binding of the nucleotide not appearing to require ATP hydrolysis since it can be mimicked by nonhydrolysable ATP analogues, AMP and ADP in the absence of Mg^{2+}.

1.14. Investigating the molecular identity of native K_{ATP} channels

The main reason behind research into the molecular identity of native K_{ATP} channels is to enable researchers to tailor specific drug therapies which will be selective for one type of K_{ATP} channel over another and so lessen possible side effects. It is now widely accepted through the strength of recent research that the pancreatic beta-cell K_{ATP} channel is composed of SUR1/ Kir 6.2 and that of cardiac myocytes is SUR2A/ Kir 6.2. However, the identity of the K_{ATP} channel of vascular smooth muscle has yet to be determined, although it is thought to be either SUR2B/ Kir 6.1 or SUR2B/ Kir 6.2. The possibility of heteromeric channel formation has made the determination of the identity of this channel even harder.

1.15. Aims

The aim of this research was to investigate the molecular identity of the K_{ATP} channel of vascular smooth muscle using different approaches. This was achieved by determining the expression of channel subunits at the mRNA level, by using RT-PCR, and at the protein level, by staining smooth muscle cells with subunit specific antibodies. The *Xenopus* expression system was used to express different Kir6.0/ SUR channel combinations and their currents characterised. Selectivity of the channel inhibitor, PNU-37883A, was also investigated and the effects compared with those elicited in native tissue.

Chapter 2

Materials and Methods

Chapter 2. Materials and Methods

2.1. Materials

All plasticware, glassware, solutions and media were sterilised by autoclaving using the series 2100 media autoclave (Prestige™ Medical) for 22 minutes at 121°C. Heat labile solutions were filter-sterilised (Nalgene®, Nalge Nunc International, USA). Sterile deionised water (Option 7, Elga, USF) was used in the manipulation of DNA, nuclease-free water (Ambion®) for mRNA, 18 MΩ molecular biology grade water (Sigma) for PCR, and UHQ (ultra high quality water, Elga) for immunocytochemistry. In PCR work aerosol resistant tips (Rainin Instrument Co. Inc., USA) were used to minimise the chances of cross contamination via aerosol formation. Chemicals were purchased from Sigma, BDH, GibcoBRL, Oxoid, and Fisons, and were of analytical grade unless otherwise stated. Restriction and modification enzymes were purchased from Life Technologies™ (GibcoBRL), New England BioLabs Inc, and Bioline. Oligonucleotide primers were designed in consultation with Dr Lodwick and supplied by the University of Leicester DNA services (PNACL), desalted and lyophilised.

2.2. RT-PCR

2.2.1. Isolation of total RNA

TRIZOL Reagent (Gibco BRL) was used in the isolation of RNA from a range of rat tissues. This reagent is a monophasic solution of phenol and guanidine isothiocyanate which works to maintain the integrity of the RNA during sample homogenisation while disrupting cells and solubilising their components. The addition of chloroform and subsequent centrifugation separates the solution into an aqueous and organic phase. The RNA which remains in the aqueous phase is then precipitated by the addition of isopropanol.

Rat tissues (Sprague-Dawley and Wistar) were dissected into RNA*later*™ preservative solution and stored at 4°C until use. Rat aorta cells grown in suspension were collected by centrifugation. Approximately 50-100 mg of tissue was homogenised in 1 ml TRIZOL™ Reagent and incubated at room temperature for 5 minutes to allow complete dissociation of nucleoprotein complexes. 0.2 ml chloroform was then added and the sample shaken vigorously for 15 seconds, incubated at room temperature for 2-3 minutes then centrifuged at 13000 rpm for 15 minutes at 4°C to separate the phases. The upper aqueous phase containing the RNA was transferred to a clean tube and 0.5 ml isopropanol added to precipitate the RNA. After incubating at room temperature for 10 minutes the samples were centrifuged at 13000 rpm for 15 minutes at 4°C and the supernatant removed. The pellet was washed with 1.5 ml 75 % ethanol and centrifuged for another 10 minutes. After being left to air dry, for 5-10 minutes, the pellet was then resuspended in 100 µl of nuclease free water, incubated for 10 minutes at 55°C, and stored at –25°C until reverse transcribed.

2.2.2. Reverse transcription of RNA

Before reverse transcription of the RNA each sample was treated with DNase I to eliminate residual genomic DNA contamination. The DNA-free™ kit (Ambion®) was used following the manufacturers protocol and briefly consisted of a DNA digestion step using DNase I and an enzyme inactivation step using DNase Inactivation Reagent. The Inactivation Reagent removed DNase and divalent cations which can catalyse heat-mediated degradation of RNA. DNase I buffer (0.1 volumes) and 1 µl (2 units) of DNase I were added to each RNA sample and, after mixing, incubated for 30 minutes at 37°C. If the sample to be treated appeared viscous or was thought to be highly contaminated with enzyme, as in the case of the liver preparation, the RNA sample was diluted in RNase-free H_2O, 2 µl of DNase I was used in the digestion and the incubation time increased to one hour. 0.1 volumes of DNase

Inactivation Reagent was then added and mixed into the sample by vortexing. After a 2 minute incubation at room temperature the samples were centrifuged to pellet the DNase Inactivation Reagent and the supernatant transferred to a clean tube.

Each sample of RNA was heated at 70°C for 10 minutes then quenched on ice for 1-2 minutes to denature any secondary structure, 0.5-2 μg of RNA was then used in an oligo dT primed reverse transcription with M-MLV reverse transcriptase. The reaction was set up in order with (in final concentrations):

1× 1st strand buffer (GIBCO BRL),

10 mM DTT (GIBCO BRL),

0.5 mM dNTP,

5 μg oligo dT primer (GIBCO BRL),

5 units of SUPERRase-in (Ambion®),

0.5-2 μg RNA,

Nuclease-Free Water (Ambion®) making the reaction volume up to 19 μl.

This was then incubated at 42°C for 2 minutes. 1 μl of M-MLV reverse transcriptase (2000 u/μl, GIBCO BRL) was then added and the reaction incubated at 42°C for a further 50 minutes finishing with a 15 minute incubation at 70°C.

The cDNAs were amplified using:

2.5 U of BIOLASE *Diamond*™ DNA Polymerase (Bioline Ltd),

3 mM $MgCl_2$,

0.2 mM dNTP

in a 50 µl reaction volume using 20 pmol of forward and reverse oligonucleotide primers specific for the different K_{ATP} channel subunit cDNAs or β-actin cDNA in the control reaction. BIOLASE Diamond DNA Polymerase was used here as it proved to be more specific than Taq DNA Polymerase, it has improved nucleotide selection and a lower rate of mis-match extension which means that only perfectly aligned primers are extended.

2.3. Immunocytochemistry

2.3.1.Enzymatic dissociation of single arterial smooth muscle cells

Rat femoral artery cells were dissociated using a two-stage enzymatic treatment followed by mechanical disruption of the tissue. The artery was dissected into dissection solution, [dissection solution: 137 mM NaCl, 5.4 mM KCl, 4.17 mM $NaHCO_3$, 0.42 mM Na_2HPO_4, 0.44 mM NaH_2PO_4, 1 mM $MgCl_2$, 10 mM HEPES, 2 mM CaCl, 10 mM glucose.], cleaned, then chopped into small (~5mm) pieces to increase the area exposed to enzyme. The arterial segments were transferred into a borosilicate vial containing isolation solution [as for dissection solution but with a calcium concentration of 0.1 mM], and incubated at 35°C in a water bath. 1.4 mg papain and 0.1 mg dithioerithritol were dissolved by triturating in 20 µl water then adding 1 ml of isolation solution, the papain was then pre-activated by incubation at 35°C for 5 minutes. The arteries were then transferred into the papain solution and incubated for 15-25 minutes at 35°C. In the second stage of digestion the arteries were transferred directly from the papain solution into a vial containing 1.4 mg collagenase and 1 mg hyaluronidase dissolved in 1 ml of isolation solution and incubated for a further 11-16 minutes at 35°C. After this second incubation the arteries were transferred into cold isolation solution and rinsed with fresh isolation solution in order to remove the enzymes. Single cells

were then obtained by trituration through a small bore, fire polished Pasteur pipette, and stored at 4°C in isolation solution.

2.3.2 Immunocytochemistry of arterial smooth muscle cells

General solutions:

10× PBS:

27 mM	KCl
15 mM	KH_2PO_4
1.37 mM	NaCl
80 mM	Na_2HPO_4

After dilution to 1× with UHQ water the pH was adjusted to 7.4.

20× Standard sodium citrate buffer (SSC, pH 7.2):

3 M	NaCl
0.3 M	Sodium citrate

Before cellular dissociation circular coverslips were cleaned using a 1:1 mixture of chloroform and methanol, then rinsed with distilled water and air dried. Coverslips were placed into the wells of a culture plate (Nunc multidish 6, Nalge Nunc International USA) and single cells in suspension were added to each coverslip then left at 4°C for 20 minutes in order for the cells to settle.

Fixation; 3 ml of fixative solution, [2% Paraformaldehyde fixative solution: 2 g paraformaldehyde was dissolved in 10 ml 10× PBS and made up to 100 ml with UHQ water.

One pellet of NaOH was added to the solution to aid in dissolving the paraformaldehyde and after it was dissolved the pH of the solution was adjusted to 7.4.], was then gently added to each well and the plate left at room temperature for 10 minutes. The paraformaldehyde contained within the fixative solution forms chemical cross-links between free amino acid groups and these links join different molecules together resulting in the stabilisation of the cell's architecture. The advantage of this technique is that the antigen, in this case the different K_{ATP} subunits, is maintained in is native locale, but a disadvantage is that antibodies which bind to primary amino groups cannot be used as the binding site will now be blocked by paraformaldehyde.

Quenching; After aspirating off the fixative 3 ml of glycine solution, [Glycine solution: 100 mM glycine was made up in UHQ water and the pH adjusted to 7.4.], was then added for 10 minutes to terminate the fixation step by chemically modifying unreacted paraformaldehyde.

Permeabilisation; The glycine solution was then removed and replaced with 3 ml of permeabilisation solution [Permeabilisation solution: 0.1% TritonX-100 in 1×PBS.]. It was essential to permeabilise the cell allowing the primary antibodies intracellular access as the antibodies used here were directed against the intracellular C-terminal domain of the subunits (table 2). The solution was left for 10 minutes, aspirated off, and the coverslips rinsed 3 times with 3 ml of 1×PBS for 5 minutes each and the cells stored in PBS at 4°C overnight.

Antibody staining; After removal of PBS the cells were incubated for 30 minutes at room temperature in antibody buffer [Antibody buffer: 2% goat serum, 1% bovine serum albumin, 0.05% TritonX-100, made up in 1×SSC and the pH adjusted to 7.2.]. Then incubated for 1 hour with the primary antibodies, at a dilution of 1 in 250, at room temperature, then washed with antibody wash solution, [antibody wash solution: 0.05% TritonX-100 made up in 1×SSC and the pH adjusted to 7.2.], 3 times for 10 minutes each. The secondary antibody, an

anti-rabbit IgG FITC conjugate (Sigma; cat# F-0382), was then added at a dilution of 1 in 1000 and the cells incubated for 1 hour, with the plate covered in foil, followed by another 3 washes.

Finally the coverslips were mounted with DABCO mounting medium, [DABCO mounting medium: 2.5% DABCO, 90% Glycerol, 0.02% NaN_3, made up in PBS.], on a slide (cell side down) and sealed with clear nail polish.

2.3.3. Imaging

The results of antibody labelling were examined using a laser scanning confocal microscope (Perkin-Elmer: Ultra-View™) and imaged using an inverted oil-immersion objective (×60). Confocal emission fluorescence was captured at wavelengths <580nm. Images were transferred to a cooled frame transfer CCD camera as full frame (1392×1040 pixels). An acquisition rate of about 1 frame per second with a 300 ms exposure, 2×2 bining, and a 4 second lapse between images, was used for all experiments. Image readout was digitised at 12 bits, with data extracted offline, using the Ultra-View™ software (Perkin-Elmer). All images were analysed offline using a background subtraction method, where the background was defined as a region of interest proximal to the cell and subtracted automatically from subsequent images. In order to represent changes in fluorescent intensity throughout the cells data was presented from a line drawn horizontally through the confocal plane. The line graph obtained represents the colour coded fluorescent profile from the cell area covered by the line.

2.4. Manipulation of cDNA and mRNA

2.4.1. Solutions used in the manipulation of DNA and mRNA

LB media (Luria-Bertani media):

10 g tryptone, 5 g yeast extract, 10 g NaCl, was made up to 1 L with dH_2O and autoclaved. 1 μl (100 mg/ml) ampicillin per ml of media was added before use.

LB Agar:

10 g tryptone, 5 g yeast extract, 5 g NaCl, 16 g agar, was made up to 1 L with dH_2O and autoclaved. After the media had cooled to approximately 55°C, 1 μl (50 mg/ml) carbenicillin was added per 1 ml of media. It was necessary to wait for the media to cool as addition of carbenicillin at higher temperatures results in its degradation and the ability to select for antibiotic resistance being lost. The media was then poured into 9cm petri dishes (Bibby Sterilin Ltd., Staffordshire, U.K.) and left to solidify. The plates were stored at 4°C and dried in a 37°C incubator before use.

SOB media:

20 g tryptone, 5 g yeast extract, 0.5 g NaCl, was dissolved in 950 ml dH_2O, and 10 ml 250 mM KCl was added, this was adjusted to pH 7 with NaOH, and made up to 1 L with dH_2O and autoclaved. Just before use 5 ml 2 M $MgCl_2$ was added.

SOC media:

20 ml 1 M glucose (filter sterilised) was added to 1 L of SOB.

TE buffer:

10 mM Tris, 1 mM EDTA, pH 8.

1× TAE buffer:

0.04 M Tris-acetate, 0.001 M EDTA.

2.4.2. Growth of *E.coli* in liquid media

All liquid cultures were grown in LB-ampicillin media. Small 5 ml (Miniprep) cultures were grown in 25 ml plastic disposable universal tubes (Bibby Sterilin Ltd., Staffordshire, U.K.), and larger 50 ml (midiprep) cultures in 250 ml glass conical flasks. All cultures were grown with aeration in an orbital incubator (Gallenkamp) at approximately 200 rpm and at 37°C.

2.4.3. Purification of plasmid DNA

Plasmid DNA was purified using an alkaline lysis technique.

Miniprep cultures were grown up in 5 ml LB-ampicillin media inoculated from single colonies after transformation. Plasmid DNA was purified using the Wizard® Plus SV miniprep kit (Promega, USA) following the manufacturers protocol. Briefly; the bacterial culture was harvested by centrifugation at 10000 rpm for 5 minutes, and the supernatant discarded. The bacterial cells were then resuspended in 250 µl Cell Resuspension Solution and lysed with 250 µl Cell Lysis Solution. 10 µl Alkaline Protease Solution was added in order to inactivate endonucleases and non-specifically degrade proteins which were released during cell lysis, reducing the amount of protein contaminants present in the cleared lysate whilst also conserving the quality of the DNA. 350 µl Neutralisation Solution was then added,

precipitating the bacterial cellular debris, and the lysate centrifuged at 13000 rpm for 10 minutes. The supernatant was then transferred to the spin column and centrifuged at 13000 rpm for 1 minute forcing the cleared bacterial lysate through leaving the plasmid DNA bound to the column. The column was washed with an ethanol wash solution, firstly with 750 µl of Column Wash Solution and centrifuging for 1 minute at 13000 rpm, and secondly with 250 µl Solution centrifuging for 2 minutes. After transferring the Spin Column to a new 1.5 ml microcentrifuge tube the DNA was then eluted by adding 100 µl Nuclease-Free Water and centrifuging at 13000 rpm for 1 minute.

Midiprep cultures were grown up in 50 ml LB-ampicillin media inoculated from frozen stocks of required clone. Plasmids were purified using the QIAfilter™ plasmid midi protocol (QIAGEN®). Briefly, after harvesting the bacterial cells by centrifugation they were resuspended in 6 ml of buffer P1 and lysed with the addition of 6 ml of buffer P2 then incubated and room temperature for 5 minutes. The lysis reaction was neutralised by the addition of 6 ml chilled buffer P3 and the cell debris filtered away from the cleared lysate using the QIAfilter Cartridge. The lysate was then passed through an anion-exchange resin, to which the plasmid DNA bound, and a medium-salt wash used to remove RNA, proteins, dyes, and low-molecular-weight impurities. The bound plasmid DNA was eluted in a high-salt buffer (QF), concentrated and desalted by isopropanol precipitation using the QIAprecipitator, and finally eluted in 1 ml TE buffer.

The DNA yield was determined by spectrophotometric analysis and quality by electrophoresing a sample of the purified product on an agarose gel.

2.4.4. Quantification of nucleic acids

The samples to be determined were diluted appropriately in 10 mM Tris pH 8 and the absorbancies read at 260 nm and 280 nm. From this reading the concentration of the sample was calculated.

1 absorbance unit (OD) at 260 nm = 50 μg/ml of double stranded DNA

33 μg/ml of oligonucleotide

40 μg/ml of mRNA

The ratio of OD260 to OD280 was also calculated in order to assess the purity of the sample (ratios usually 1.6-2.2), where a ratio of less than 1.8 suggests contamination of the sample.

2.4.5. DNA separation by agarose gel electrophoresis

All DNA samples resulting from plasmid purification or PCR were run on an agarose gel of between 1 and 2 % (see fig. 2.1.). Electrophoresis grade agarose was heated in 1× TAE until molten, cooled slightly and ethidium bromide added to a concentration of 0.5 μg ml^{-1} prior to pouring. Ethidium bromide was used to stain the gel enabling the DNA to be visualised under U.V. light (AlphaImager™version 5, Alpha Innotech Corp.). A slab gel was formed using a perspex gel former and sample wells made using a perspex comb. The solidified agarose slabs were run in TAE buffer also supplemented with ethidium bromide at a concentration of 0.5 μg ml^{-1}, at 80-120 V constant voltage (PowerPAC 300, BioRad). The DNA samples to be analysed were mixed with DNA loading buffer [10× Bluejuice: 65 % w/v sucrose, 10 mM Tris-Cl (pH 7.5), 10 mM EDTA, 0.05 % w/v bromophenol blue, made up in H_2O], and loaded onto the gel. The loading buffer served to increase the density of the sample so that the DNA fell evenly into the wells while also providing a means for visualising the progress of the sample through the gel. DNA migrated towards the anode when exposed to

Figure. 2.1.
Agarose gel photograph showing results of gel purification.
5 μl of sample was electrophoresed on a 1% agarose gel.
Lane number 1 corresponds to Bioline Hyperladder, 2; Kir6.1 minus pore region, 3; Kir6.1 pore fragment, 4; Kir6.2 minus pore region, 5; Kir6.2 pore fragment, lanes 6-9 represent the *Mfe* I to *Cla* I regions of SUR2B,
lane 6; ΔC10, 7; ΔC20, 8; ΔC30, 9; ΔC42.

an external electric field, and the size of the DNA sample estimated by comparison with a 1 Kb DNA ladder (Gibco BRL Cat#15615-016), which was loaded alongside.

2.4.6. Purification of DNA from agarose gels

In the purification of DNA from an agarose gel two methods were used: TaKaRa Recochip (Takara Shuzo co., Ltd.) and Sephaglas™ BandPrep Kit (Amersham Pharmacia Biotech Ltd.). With the first technique the DNA to be purified is electrophoresed into a dialysis membrane held on a chip which is placed into the gel. Firstly the sample was electrophoresed until the band to be purified was present as a discrete band, the RECOCHIP was then dipped into the electrophoresis buffer and pushed into the gel in front of the DNA band. The gel was again electrophoresed for the time required for the DNA to load onto the chip and after removing the chip from the gel the DNA was recovered by centrifugation for 5 seconds at 5000 rpm. In the second technique DNA fragments were excised from a 1% agarose gel and gel solubiliser was used to dissolve the agarose and provide a sodium iodide concentration sufficiently high enough to promote binding of DNA to the Sephaglas. Once the DNA had bound onto the matrix it was washed to remove gel contaminants, such as proteins, linkers, and nucleotides, and then air-dried to remove any residual ethanol from the wash stage. The DNA was then eluted from the dry matrix in a low ionic strength buffer. In both methods recovery of the DNA was then assessed by running a portion of the sample on an agarose gel and comparing this with a mass ladder (Bioline Hyperladder, Bioline).

2.4.7. Ethanol precipitation of DNA

Ethanol precipitation is used to concentrate DNA solutions and remove protein, salt, and unincorporated nucleotides. 0.1 volumes of 3 M sodium acetate (pH 5.2) and 2.5 volumes absolute ethanol were added to the sample and the tube inverted gently until the phases were

mixed. The sample was then incubated at –20°C for ≥15 minutes and centrifuged in a bench top microfuge for 15 minutes at 13000rpm. The supernatant was discarded and the pellet washed with 70% ethanol and, after air drying for 5-10 minutes, resuspended in TE buffer.

2.4.8. Phenol Chloroform Extraction

This extraction is used to remove proteins from nucleic acid solutions. An equal volume of phenol:chloroform (1:1 ratio) was added to the DNA sample to be purified and vortexed until an emulsion formed. The sample was then centrifuged in a bench top microfuge at 13000 rpm for 5-10 minutes. The aqueous phase was conserved while the organic phase discarded. A second extraction was then performed adding an equal volume of chloroform to the sample to remove traces of phenol and protein, this was vortexed and centrifuged for 2-3 minutes and the upper aqueous layer transferred to a clean tube. DNA was then precipitated from the aqueous phase by ethanol precipitation.

2.4.9. Restriction enzyme digestion of DNA

Restriction enzymes were used along with their appropriate buffer as recommended by the manufacturer, except in double digests where the buffer used was selected to be the one most amenable to both enzymes. Typically 5 units of enzyme were used for each μg of DNA included in the reaction, and typical reaction volumes were 20-100 μl. Most restriction endonucleases used required incubation at 37°C, and incubation was usually performed for one hour, though overnight incubations were performed when larger amounts of DNA were digested (see fig. 2.2. for a gel photograph of digested DNA).

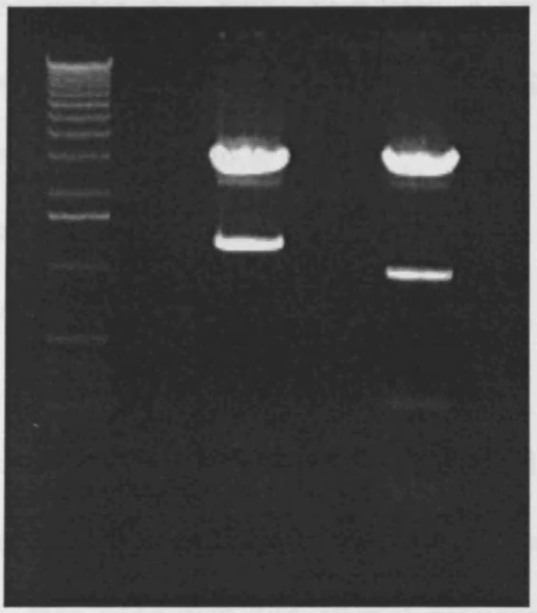


Figure. 2.2.
Agarose gel photograph of Kir6.x chimera DNA digests.
Lane 1 corresponds to 1 Kb DNA ladder, lane 2 to a Pst I digest of Kir6.1/6.2 pore, and lane 3 to a Pst I digest of Kir6.2/6.1 pore.

2.4.10. DNA ligation

After digestion the vector was dephosphorylated, a process which removes the terminal phosphate groups, thus reducing the possibility of the digested vector re-ligating to itself. A typical 20 µl digest was made up to 30 µl with:

3 µl of 10× dephosphorylation buffer,

1 µl of enzyme CIAP,

6 µl H_2O,

and incubated for 30 minutes in a 37°C water bath, then incubated at 70°C for 20 minutes to denature the enzyme. The volume of the sample was then increased to 100 µl with dH_2O followed by phenol/chloroform extraction and ethanol precipitation of the DNA. Equal volumes of both vector and insert digests were run on an agarose gel against the same volume of Hyperladder (Bioline) and the concentration of DNA present in the samples calculated. For ligation of the vector and insert DNA the Life Technologies Inc. rapid ligation protocol for plasmid cloning was followed:

Component	**Cohesive ends**	**Blunt ends**
5× ligase reaction buffer	4 µl	4 µl
vector DNA	3-30 fmol	15-60 fmol
insert DNA	9-90 fmol	45-180 fmol
autoclaved distilled water	x µl	x µl
T4 DNA ligase	1 unit	5 units
Final reaction volume	20 µl	20 µl

The reaction components were mixed and incubated at room temperature for 5 minutes. 2 µl of this ligation reaction was then used in a transformation reaction.

2.4.11. Transformation of Competent cells

50 μl aliquots of Epicurian Coli® XL-Blue [F'::Tn10proA$^+$B$^+$lanI^{q}Δ(lacZ)M15/recA1endA1gyrA96 (NaI^r) thi hsd R17 ($r_k^-M_k^+$) glnV44 relA1 lac] subcloning-grade competent cells (Strategene) and Subcloning efficiency™DH5α™[F' end A1 hsd R17 ($r_k^-M_k^+$) gln V44 thi –1 rec A1 gyrA (NaI^r) rel A1 Δ(lacZYA – arg F) U169 deo R (ϕ80dlacΔ(lacZ)M15))] competent cells (Gibco BRL, cat#18265-017), which had been stored at –80 °C, were thawed on ice and transferred to a 15 ml centrifuge tube. 0.1-50 ng of DNA was added to these cells and the mixture incubated on ice for 20 minutes then heat pulsed for 45 seconds in a 42°C water bath. Another incubation on ice was performed for 2 minutes after which 0.95 ml of SOC media was added and a further incubation for 30 minutes at 37°C with shaking at 225-250 rpm performed. ≤200 μl of the transformation mixture was then spread onto LB-carbenicillin plates using a sterile spreader and incubated at 37°C overnight. Single colonies were picked off the plate and streaked onto another LB-carbenicillin plate incubating overnight at 37°C. These single colony cultures were then screened after growing up in LB media supplemented with ampicillin.

2.4.12. DNA sequencing

Sequencing reactions consisted of 400 ng DNA, 4 pmol primer, 8 μl BigDye terminator mix (Applied Biosystems), made up to 20 μl with PCR quality H_2O and topped with 20 μl mineral oil to prevent evaporation. PCR cycling conditions used were those recommended by PNACL University of Leicester, and consisted of: a denaturing step at 94°C for 30 seconds, then 30 cycles of 96°C for 10 seconds, an annealing step at 50°C for 5 seconds and an extension step at 60°C for 4 minutes, followed by a final soak at 4°C (PCR Sprint temperature cycling system, Hybaid Ltd. UK). The reaction mix was removed from

underneath the oil layer and ethanol precipitated to remove unincorporated dyes. After air drying the pellet, it was then sent to PNACL University of Leicester DNA services for electrophoretic analysis.

2.4.13 *E.coli* storage as glycerol stocks

After screening cultures and identifying correct DNA constructs by restriction enzyme analysis and sequencing, frozen cultures were prepared by adding equal volumes of cultured clone to 2× freeze mix [6.3 g K_2HPO_4, 0.45 g sodium citrate, 0.09 g $MgSO_4.7H_2O$, 0.9 g $(NH_4)_2SO_4$, 1.8 g KH_2PO_4, 44 g glycerol, made up to 500 ml with H_2O] mixing thoroughly and storing at –80°C. The glycerol contained in the freeze mix helped prevent damage occurring to the bacterial cells when they were frozen. Frozen cultures were revived by inoculating LB-amp media with some ice, scraped with a sterile plastic pipette tip or metal loop, from the surface of the culture.

2.4.14. Mutagenesis

Site-directed mutagenesis was used to make point mutations creating unique restriction sites in the clones that were then utilised in the manipulation of the sequence, whether for swaping regions of the coding sequence as in the construction of the Kir 6.x chimeras or as a cloning site in the construction of the SUR 2B truncations. Two methods were used to create the mutations: the GeneEditor™ *in vitro* Site-Directed Mutagenesis System (Promega), and the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene), and the manufacturers protocols followed. In the GeneEditor™ method the DNA template was denatured in an alkaline denaturation step, and the reaction heated allowing the mutagenic and selection oligonucleotides to anneal to the template. DNA polymerase was then used to generate the mutant strand, and the nicked strands produced were ligated. This was followed

with a transformation into BMH 71-18 mutS [thi, supE, Δ(lac-proAB), (mutS:Tn10)(F', $proA^+B^+$, $lacI^qZ\Delta M15$)] Competent Cells, a mismatch repair minus strain of *E. coli*. Its use here prevents the repair of the newly synthesised unmethylated DNA strand resulting in a high mutation efficiency. Overnight cultures were prepared containing the GeneEditor™ Antibiotic Selection Mix and the plasmid DNA purified. A second transformation was performed into JM109 [e14$^-$(McrA$^-$)end A1 recA1 gyrA96 thi hsdR17 ($r_k^-m_k^+$) rel A1 supE44 L-Δ(lac-proAB)(F' traΔ36 $proA^+B^+$ $lacI^qA\Delta M15$)] using this purified plasmid DNA and mutants selected on LB-agar plates supplemented with the GeneEditor™ Antibiotic Selection Mix. This second transformation was performed to avoid having a mixed population of mutant and wild-type DNA in the cell. The QuikChange™ method is a far quicker method to generate mutants, having the advantage of not requiring two transformations, or the need to use one oligonucleotide to encode for antibiotic resistance, and also the parental DNA is digested before transformation avoiding transformation of a mixed population of DNA. In this method two complimentary oligonucleotides containing the desired mutation were used, and the annealing and extension steps completed using a thermalcycler (Hybaid), the cycling parameters being: 1 cycle at 95°C for 30 seconds, followed by 18 cycles of 95°C for 30 seconds, 55°C for 1 minute, and 68°C for 2 minutes/kb of plasmid length. This resulted in the generation of a mutated plasmid containing staggered nicks which was then treated with *Dpn* I. *Dpn* I digests the parental DNA as it is specific for methylated and hemimethylated DNA, therefore leaving the new mutated plasmid intact as it is not methylated. The new mutated DNA was then transformed into Epicurian Coli® XL-Blue supercompetent cells.

In both methods the resulting colonies were grown up in LB-amp media, their DNA purified and restriction digests performed followed by sequencing to identify mutants.

2.5. Generation of mRNA

2.5.1. Proteinase K treatment of DNA

Some alkaline lysis procedures may leave residual RNase A contaminating the DNA. When using the DNA as a template for generating mRNA this must be removed by digestion with Proteinase K then phenol/chloroform extracted. Proteinase K was added to the sample to obtain a final concentration of 100-200 μg/ml and SDS to a final concentration of 0.5 %. This was incubated for 30 minutes at 50°C and followed by a phenol/chloroform extraction.

2.5.2. Synthesis of capped mRNA

mRNA was transcribed from a linear DNA template using a T7 promoter. The plasmid DNA must be linearized with a restriction enzyme that lies downstream of the insert to be transcribed. In this case the enzyme *Mlu* 1 was used. Typically 20 μg of DNA was digested overnight in a volume of 100 μl using 50 units of enzyme. After linearisation the DNA was proteinase K treated to remove any contaminating RNase A which may have been carried over from DNA purification, phenol/chloroform extracted and ethanol precipitated. The DNA was then resuspended in 20 μl of nuclease-free water resulting in an approximate concentration of 0.5 μg/μl. Capped mRNA was then transcribed *in vitro* using the mMESSAGE mMACHINE™ Kit (Ambion) according to the manufacturer's instructions. The transcription reaction was assembled at room temperature and consisted of:

2 μl 10× reaction buffer,

10 μl 2× NTP/cap,

(1 μl 20 mM GTP, added in the case of SUR transcripts)

1 μg linear template DNA,

2 μl enzyme mix,

made up to 20 μl with nuclease-free water.

The constituents of this reaction were mixed thoroughly and incubated at 37°C for 1 hour. As SUR transcripts were larger than Kir transcripts extra GTP was added in these reactions; when transcribing large templates over 5 kb the amount of GTP in the reaction can become rate limiting and if insufficient is added can result in low yields of mRNA. The downside of adding additional GTP is that it results in a decrease in the amount of transcripts that are synthesised with a cap, however it also results in higher yields of full length product and a balance between these two points has to be obtained. Digestion of the DNA template was achieved by the addition of 1 µl of DNase 1, and incubating at 37°C for a further 15 minutes. The reaction was stopped by the addition of 115 µl nuclease-free water and 15 µl Ammonium Acetate Stop Solution, mixing thoroughly, and the mRNA recovered with a phenol/chloroform extraction followed by isopropanol precipitation. The mRNA was resuspended in 20 µl nuclease-free water, aliquoted, and stored at –25°C until required. The yield and quality of the mRNA generated was assessed by running a sample of product on an agarose gel (see fig. 2.3. for a representative gel) and also by checking the absorbance at 260nm. When running mRNA checking gels the gel tank and gel forming apparatus was first incubated in 2% (vol/vol) hydrogen peroxide for at least one hour then washed with DEPC treated water, and the agarose gel made up with TAE made using DEPC treated water to minimise degradation by RNAses.

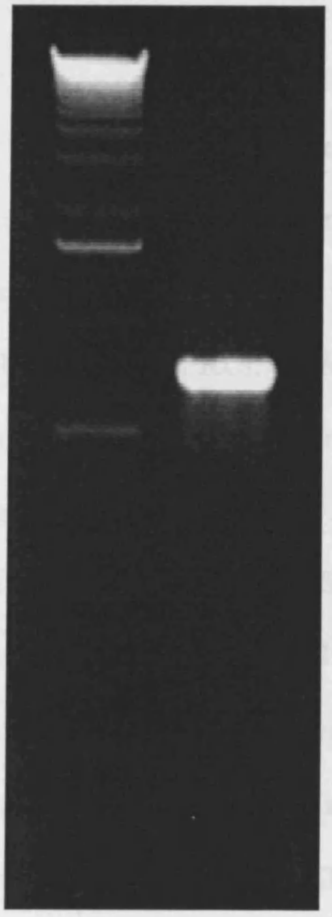


Figure. 2.3.
Agarose gel photograph of 1 μl Kir6.1 mRNA.
Lane 1 corresponds to 1 Kb DNA ladder and lane 2
to 1 μl of Kir6.1 mRNA.

2.6. The *Xenopus laevis* expression system

2.6.1. Oocyte isolation

Female *Xenopus laevis* frogs were anaesthetised through the skin with 0.3% 3-aminobenzoic acid ethyl ester, and killed by destruction of the brain and spinal cord. The ovaries were removed into a sterile 25 ml centrifuge tube (Corning Inc. USA), washing immediately in OR^{2-} [82.5 mM NaCl, 2.5 mM KCl, 1 mM $Na_2HPO_4.7H_2O$, 5 mM HEPES, 1 mM $MgCl_2$, pH 7.5, sterile filtered], cut into clumps, and washed further until the solution went clear (on average changing the solution in the tube 5 or 6 times). The oocytes were then incubated, on a tilt table (drive and platform unit STR8, Short Scientific UK) at 10 revolutions per minute, for 1 hour and 15 minutes in 1 mg ml^{-1} collagenase/OR^{2-} (Sigma, Type 1A), which had been filtered through a 0.45 μm syringe filter (Acrodisc syringe filters, Gelman sciences) to remove any undissolved material. Post digestion, the collagenase solution was poured off and the oocytes washed with $ND96^{2+}$ [96 mM NaCl, 2 mM KCl, 5 mM HEPES, 5 mM NaPyruvate, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, pH 7.5, sterile filtered, adding 1 mg L^{-1} gentamycin] another 5 or 6 times. A selection of digested oocytes were then transferred into sterile culture dishes (Sterilin) containing $ND96^{2+}$ solution where they were manually defolliculated with forceps. The defolliculated oocytes were stored in $ND96^{2+}$ at 18°C (LMC, cooled incubator) until injection.

2.6.2. Oocyte injection

Defolliculated oocytes were injected using a glass capillary (microcap 6.66 lambda, Drummond) calibrated to dispense 50nl of solution per injection. The glass capillaries were pulled into electrodes using a Flaming/Brown micropipette puller, model P-87 (Sutter Instrument Co. USA). A fine tipped permanent marker was used to indicate sections of the

electrode 1 mm apart and nuclease free water aspirated from a piece of Nescofilm (Azwell Inc. Osaka Japan) into the capillary. The water was injected (INJECT+MATIC®, Geneva) altering the time and pressure of injection until 2 injections caused the water to pass from one mark to the other, the volume of one injection then being equal to 50 nl. Mixed or single mRNAs were aspirated and injected into the oocytes in the same manner. mRNAs were diluted in nuclease free water prior to injection, if required, and SUR/Kir6.x mRNA was injected in a ratio of 20:1, with the concentration of SUR mRNA equal to 0.5 ng nl^{-1}. Oocytes were maintained in sterile culture dishes (Sterilin) containing $ND96^{2+}$ at 18°C, and electrophysiological measurements made 3-7 days post injection.

2.6.3. Two-microelectrode voltage clamp

Whole cell currents were measured using a two-electrode voltage clamp amplifier (Geneclamp 500; Axon Instruments, Inc. CA, USA) and monitored throughout the experiment with an oscilloscope (DSO 420, Gould). Recording electrodes were pulled from borosilicate glass capillaries (GC150TF-7.5, Harvard Apparatus Ltd., Kent, U.K., Pipette puller model PP-830 Narshige Japan) and had resistances of 0.2-0.8 MΩ when filled with 3 M KCl solution. Electrodes were coated with dental wax (Kemdent®, Associated Dental Products Ltd., Wiltshire, U.K.) in order to reduce capacitance. The transmembrane potential was measured differentially between the intracellular electrode and a second bath electrode, both made of Ag-$AgCl_2$. Currents were recorded using Axoscope (Axon Instruments Inc., CA, U.S.A.) and sampled at 10 KHz or Tracan (Dr N. W. Davies) and sampled at 5 KHz, filtered at 500Hz and stored on computer. All recordings were initiated in 2mM K^+ solution [96mM NaCl, 2mM KCl, 5mM HEPES, 1mM $MgCl_2$, 1.8mM $CaCl_2$, pH 7.4], which was then replaced by a high-potassium (98mM K^+) bath solution [98mM KCl, 5mM HEPES, 1mM $MgCl_2$, 1.8mM $CaCl_2$, pH 7.4], unless otherwise stated. Pinacidil was prepared from a

100mM (in DMSO) stock solution, glibenclamide, diazoxide, levcromakalim, minoxidil sulphate and CCCP were prepared from a 10mM (in DMSO) stock solution and diluted as required in 98mM K^+. PNU-37883A was prepared from a 10mM (in UHQ H_2O) stock solution and diluted as required in 98mM K^+(PNU-37883A was a gift from Upjohn Parmacia). Experiments were carried out at room temperature (18-24°C) during which the oocytes were continuously perfused with solution at a flow rate of 1 ml min^{-1}.

Chapter 3

Investigation into the expression of K_{ATP} channels of native tissue

Chapter 3. Expression of K_{ATP} channels in native tissue

3.1. RT-PCR

3.1.1. Introduction

K_{ATP} channels have been described in many different cell types including cardiac, skeletal muscle, neuronal and pancreatic β-cells (Spruce *et al.*, 1987; Ashcroft *et al.*, 1989; Inoue *et al.*, 1991; Kajioka *et al.*, 1991; Nichols *et al.*, 1991; Clapp and Gurney, 1992; Beech *et al.*, 1993; Sansom *et al.*, 1994; Jiang *et al.*, 1997). Most of our knowledge of the K_{ATP} channels in these cell types has been gained by electrophysiological techniques. Investigations into the different K_{ATP} channels in these tissues have resulted in the electrophysiological characterisation of the channel types. Channel sensitivity to potassium channel openers and sulphonylurea inhibitors have been elucidated along with single channel properties, such as conductance and rectification. The recent cloning of the subunits responsible for the formation of the K_{ATP} channel has enabled a more detailed understanding of the molecular identity of these channels to be gained. Expression studies have led to the elucidation of channel composition in some tissues through the comparison of cloned channel to native channel properties (see introduction). Recent studies have proposed that heteromultimeric channels may also be formed further complicating the number of possible subunit combinations arising.

Reverse transcriptase-polymerase chain reaction (RT-PCR) has been employed to investigate the expression of K_{ATP} genes in various tissues. In this technique the total mRNA from a single cell type or tissue is reverse transcribed and PCR used to amplify a specific region of DNA. Primers specific for the sequence of interest are used to screen for the presence of a particular channel subunit.

The aim of this investigation was to use RT-PCR (see section; 2.2.) to investigate which of the cloned K_{ATP} channel subunits are expressed in various rat tissues, specifically focusing on the different vascular smooth muscle preparations; portal vein, mesenteric artery, femoral artery and aorta , and using primers specific for; Kir 6.1, Kir 6.2, SUR 1, SUR 2A and 2B, and SUR 2Δ17. Tissues in which K_{ATP} expression had already been identified were included to validate the specificity of the primers. The tissues used were from female Sprague Dawley and Wistar rats and were dissected after the animal had been humanely killed by destruction of the spinal cord. The tissues collected were: aorta, bladder, kidney, skeletal muscle, ventricle, stomach, liver, femoral artery, mesenteric artery and portal vein. Cell cultures of rat aorta and human vascular smooth muscle (aorta, H.V.S.M.) were also used.

3.1.2. β-actin control

'Housekeeping' genes, which are ubiquitously expressed, such as β–actin are often used as internal controls in RT-PCR reactions. They are usually expressed at moderate levels making them easy to detect, and they can also be used for quantitative PCR if they are expressed at a constant level across the sample set. The β–actin gene was one of the first 'housekeeping' genes to be used as an internal standard, it encodes a ubiquitous cytoskeletal protein that is expressed at moderately abundant levels, is highly conserved in eukaryotes and is expressed in most cell types. However the level of β–actin expression does vary between tissues and for this reason it is not used here quantitatively but rather to indicate successful RNA isolation and reverse transcription, whilst also providing a means of identifying any genomic contamination.

2 μl of each reverse transcription reaction and an equivalent amount of non-reverse transcribed RNA was used in amplification with the β–actin primers. A predenaturation step at 94°C for 5 minutes was used, followed by 38 cycles consisting of three temperature steps

(denaturation step: 94°C for 15 seconds, annealing step: 58°C for 30 seconds, and an extension step: 72°C for 30 seconds). Amplified DNA fragments were analysed by agarose gel electrophoresis (see fig. 3.1.a). Primers corresponded to positions 589-608 (forward; 5' GGC TAC AGC TTC ACC ACC AC 3') and 1085-1065 (reverse; 5' TAC TCC TGG TTG CTG ATC CAC 3') accession no. D31144. Expression of the β–actin gene resulted in the amplification of a 476 bp product. All tissues investigated were found to express β–actin. Non-reverse transcribed reactions did not yield a product indicating the absence of contamination with genomic DNA.

3.1.3. Tissue distribution of Kir 6.1

The Kir 6.1 subunit is thought to be involved in the formation of smooth muscle K_{ATP} channels. The expression of Kir 6.1 mRNA appears to be quite widespread, having been demonstrated to be present, in rat pituitary gland and heart (Wulfsen *et al.*, 2000, Horinaka *et al.*, 2001), guinea pig brain, atrium, ventricle, lung, liver, kidney, spleen, stomach, pancreas, skin, and skeletal muscle (Mederos *et al.*, 2000). Here primers specific for Kir 6.1 were used to investigate the expression of this subunit in the various rat tissues. The sequences of the primers for Kir 6.1 amplification corresponded to 172 – 193 (forward; 5'AAA GGA AGA TGC TGG CCA GGA A 3') and 490 – 510 (reverse; 5' CCG TGA TGC CTT TCT CCA TGT A 3'), accession no. D42145, and resulted in an amplification product of 338 bp. Cycling conditions were as for the β–actin control experiment except the annealing temperature used was 60°C and the number of cycles was dropped to 34. Amplified products were resolved on an agarose gel (see fig. 3.1.b.) where transcripts of Kir 6.1 were detected in skeletal muscle, bladder, ventricle, kidney, stomach, femoral artery, portal vein (not shown in fig), H.V.S.M., and mesenteric artery.

3.1.4. Tissue distribution of Kir 6.2

Kir 6.2 is understood to form the pancreatic β–cell K_{ATP} channel together with SUR 1, its presence in other tissues has been demonstrated by RT-PCR examples including guinea pig skeletal muscle, skin, heart and bladder (Mederos *et al.*, 2000, Gopalakrishnan *et al.*, 1999), and rat pituitary (Wulfsen *et al.*, 2000). The primer sequences used in the detection of Kir 6.2 expression corresponded to positions 558 – 577 (forward; 5'GCA GAG GAC CCT ACA GAG CC 3') and 1102 –1121 (reverse 5'GCG GCC ATG TCG CAG GGT GA 3'), accession no. D86039, and resulted in the amplification of a 563 bp product. PCR cycling conditions were as for Kir 6.1. Amplified products were resolved on an agarose gel (see fig. 3.1.c.). Kir 6.2 transcripts were detected in cell cultured aorta, femoral artery, portal vein, mesenteric artery, bladder, kidney, skeletal muscle, ventricle, and stomach.

3.1.5. Tissue distribution of SUR1

As well as forming the pancreatic β–cell K_{ATP} channel, SUR 1 has also been implicated together with Kir 6.1 in the formation of the K_{ATP} channel of the mitochondrial inner membrane (Liu *et al.*, 2001). Transcripts of SUR 1 have also been shown in guinea pig bladder (Gopalakrishnan *et al.*, 1999), though from electrophysiological profiles, SUR 2B is the sulphonylurea receptor which is thought to form the K_{ATP} channel in this tissue. Primers specific for SUR 1 corresponded to positions 3797 – 3816 (forward; 5' CCT CCA TCT CCA ACT CCC TA 3') and 4256 – 4275 (reverse 5' GAT GAT AGA CAG GCG TGA GC 3'), accession no. X97279. Cycling conditions were as for Kir 6.1. The resulting PCR reactions were visualised by agarose gel electrophoresis and the expression of SUR 1 transcripts resulted in a product of 478 bp (see fig. 3.1.d.). Tissues expressing SUR 1 transcripts included mesenteric artery, kidney, bladder, skeletal muscle, stomach, liver and ventricle.

3.1.6. Tissue distribution of SUR 2A and B

The splice variant of SUR 2A, SUR 2B (Isomoto *et al.*, 1996) is, from electrophysological observations, thought to be involved in the K_{ATP} channel of vascular smooth muscle. A number of studies using PCR methods have shown it to be ubiquitously expressed (Mederos *et al.*, 2000, Chutkow *et al.*, 1999, Koh *et al.*, 1998, Liss *et al.*, 1999, Davis-Taber *et al.*, 2000, Isomoto *et al.*, 1996). SUR 2A expression, however, appears to be more discrete having been demonstrated in far fewer tissues, namely; heart, skeletal muscle, skin, cerebellum, eye and bladder (Mederos, *et al.*, 2000, Chutkow, *et al.*, 1999, Baron *et al.*, 1999, Isomoto *et al.*, 1996). In ventricle and skeletal muscle SUR 2A is thought to couple with Kir 6.2 (Babenko *et al.*, 1998). In this experiment the primers used corresponded to positions 4450-4490 (forward) and 4883-4865 (reverse) (Genbank accession no. AF087838). Only one primer set was used to amplify both SUR 2B and SUR 2A, as they differ in their 39/40 exon usage. By designing the reverse primer to lie in exon 40 and the forward primer to lie before exon 39 the presence of SUR 2B and 2A transcripts could be identified by the size of amplification product. As SUR 2A uses exon 39 it would result in a larger amplification product than 2B in which this exon is spliced out. 2 µl of reverse transcription reaction was used and the cycling conditions were as for Kir 6.1. The amplified products were visualised on an agarose gel (see fig. 3.1.e.). The expression of SUR 2B transcripts resulted in a product of 390 bp and SUR 2A transcripts in a product of approximately 605 bp. SUR 2B transcripts were observed in all tissues investigated whereas SUR 2A was only found in skeletal muscle and ventricle.

3.1.7. Tissue distribution of the SUR 2 splice variant Δ17

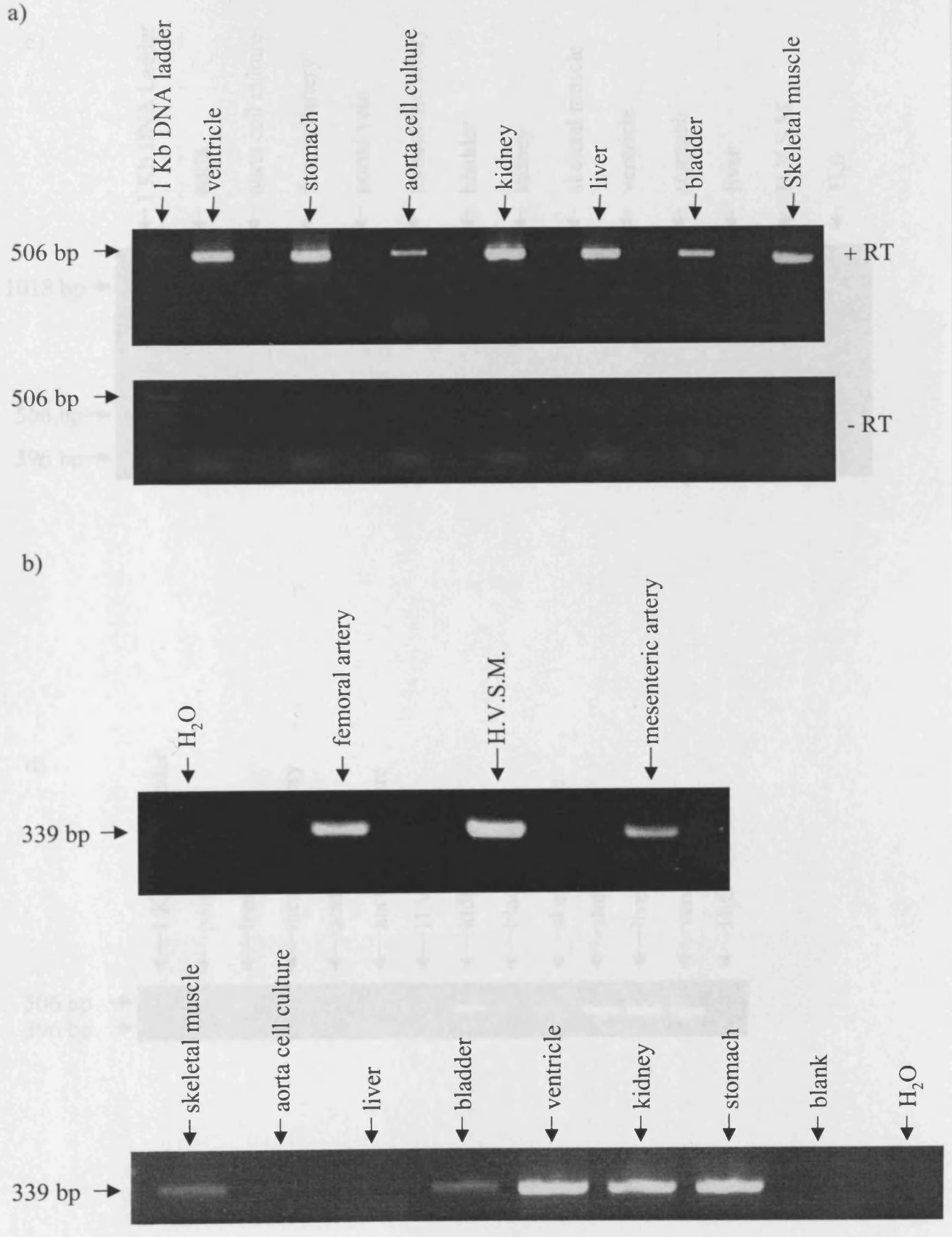
As well as the main SUR2 splice variants, named 2A and 2B, splicing of exons 14 and 17 has also been found to occur (Chutkow *et al.* 1999). The splicing of exon 17 has been

observed both in SUR 2A and 2B whereas the splicing of exon 14 has only been observed with SUR 2A. The Δ17 splice variant of SUR 2, unlike Δ14, has been shown to form functional channels when expressed with Kir 6.2 (Davis-Taber *et al.*, 2000). RT-PCR studies on human and mouse tissues have found transcripts of this splice variant to be widely expressed (Chutkow *et al.*, 1999, Davis-Taber *et al.*, 2000). Although it's expression differs between some tissues of the two species. The primers used corresponded to positions 2393 – 2417 (forward; 5' CGT CTC TCC TCC TTG CCA TCC TTG G 3') and 2656 –2682 (reverse; 5' CCT CTC TCC AAT TTC AGT TTG GTC TCC 3'), accession no. AF087838, and flanked the exon 17 region in order that one set of primers could amplify both SUR 2 and it's Δ17 variant. The exon 17 containing transcripts resolved as a 289 bp product on agarose gel electrophoresis, while the exon 17 deletion variants resolved as a 250 bp product.

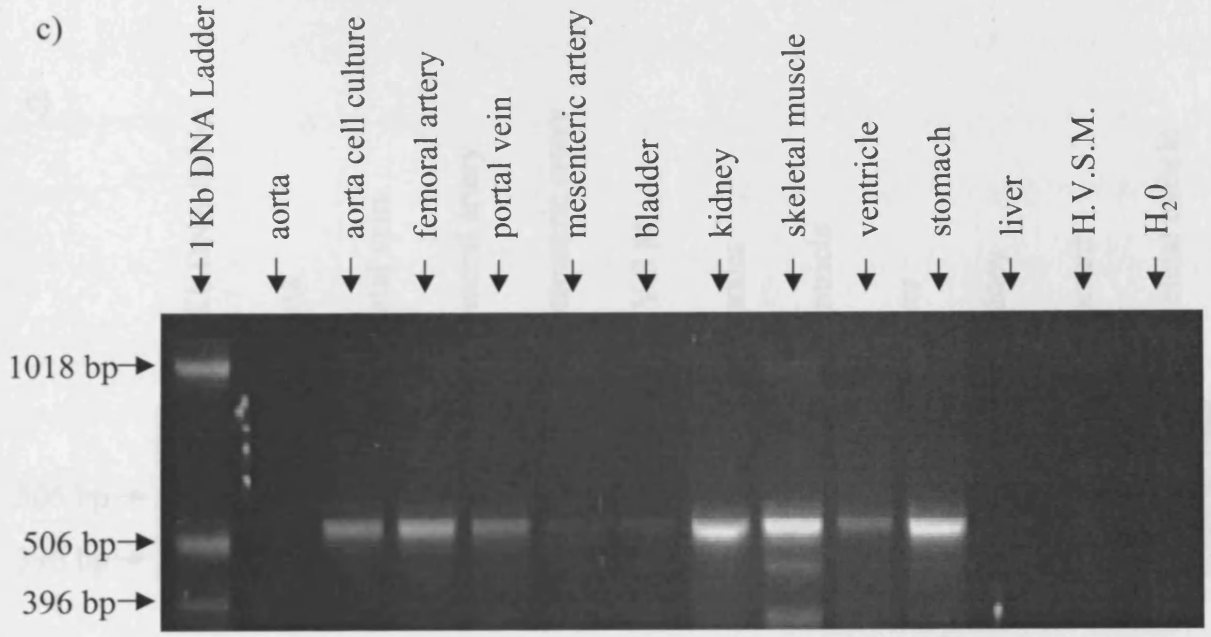
As in previous experiments 2 μl of reverse transcription reaction was used, cycling conditions were as for Kir 6.1, and the products were visualised on an agarose gel (see fig. 3.1.f.). SUR 2 Δ17 transcripts were observed in bladder, stomach, skeletal muscle, mesenteric artery, portal vein and femoral artery.

Figure. 3.1.

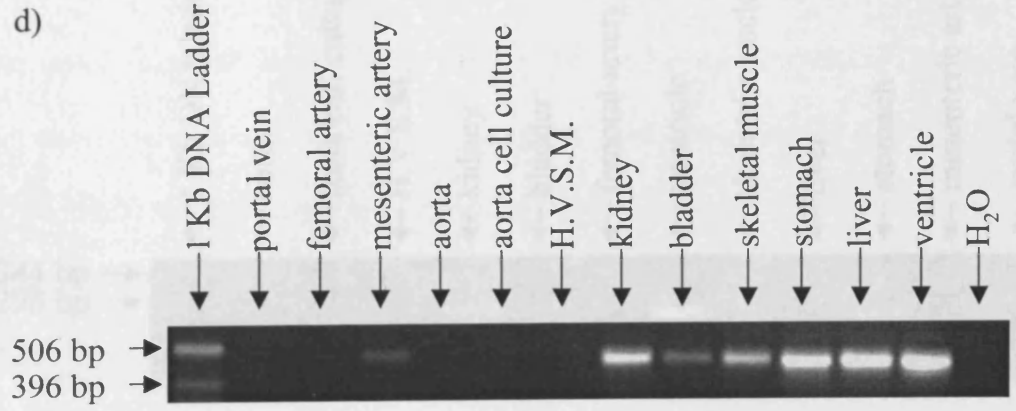
K_{ATP} subunit mRNA expression in various rat tissues. Tissues expressing K_{ATP} subunits were identified by RT-PCR analysis of total mRNA derived from various rat tissues (as indicated). Following separation by agarose gel electrophoresis, DNA bands were visualised by staining with ethidium bromide. (a) RT-PCR using specific primers for β–actin (+RT) confirmed the presence of mRNA from each tissue. The absence of reverse transcription (-RT) indicated no genomic DNA contamination. The K_{ATP} subnits screened for were [size of amplification product]: (b) Kir 6.1 [339 bp], (c) Kir 6.2 [534 bp], (d) SUR 1 [478 bp], (e) SUR 2A [605 bp], SUR 2B [390 bp], and (f) SUR 2 +17 [289 bp], SUR 2 Δ17 [250 bp]. Samples were run against a 1 Kb DNA ladder (as indicated).


a)
1 Kb DNA ladder
ventricle
stomach
aorta cell culture
kidney
liver
bladder
Skeletal muscle
506 bp
+ RT
506 bp
- RT
b)
H2O
femoral artery
H.V.S.M.
mesenteric artery
339 bp
skeletal muscle
aorta cell culture
liver
bladder
ventricle
kidney
stomach
blank
H2O
339 bp

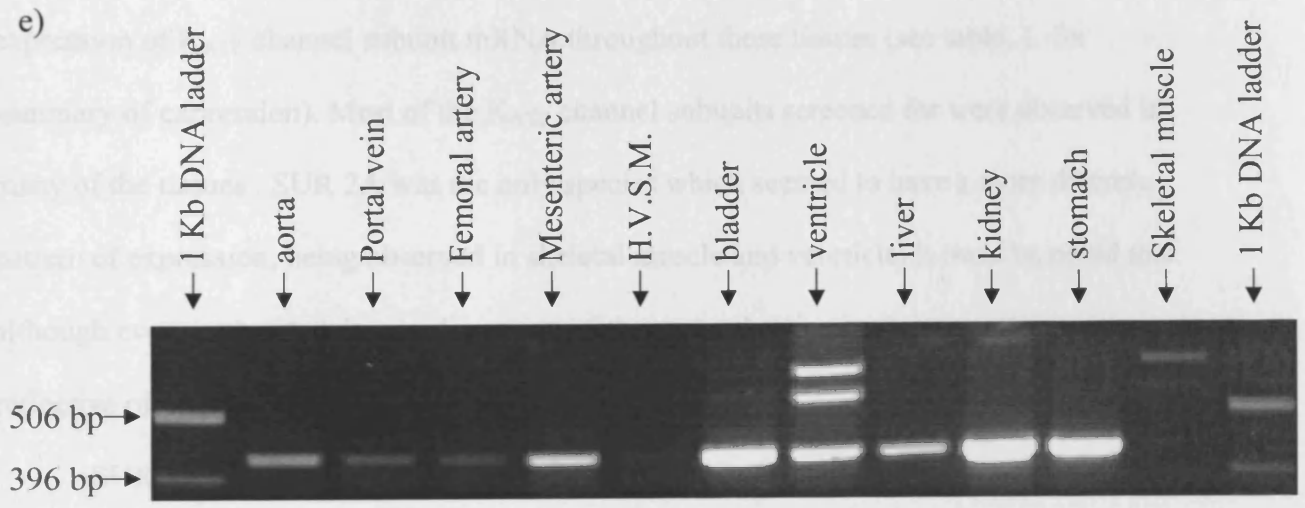
c)


1 Kb DNA Ladder
aorta
aorta cell culture
femoral artery
portal vein
mesenteric artery
bladder
kidney
skeletal muscle
ventricle
stomach
liver
H.V.S.M.
H20
1018 bp
506 bp
396 bp

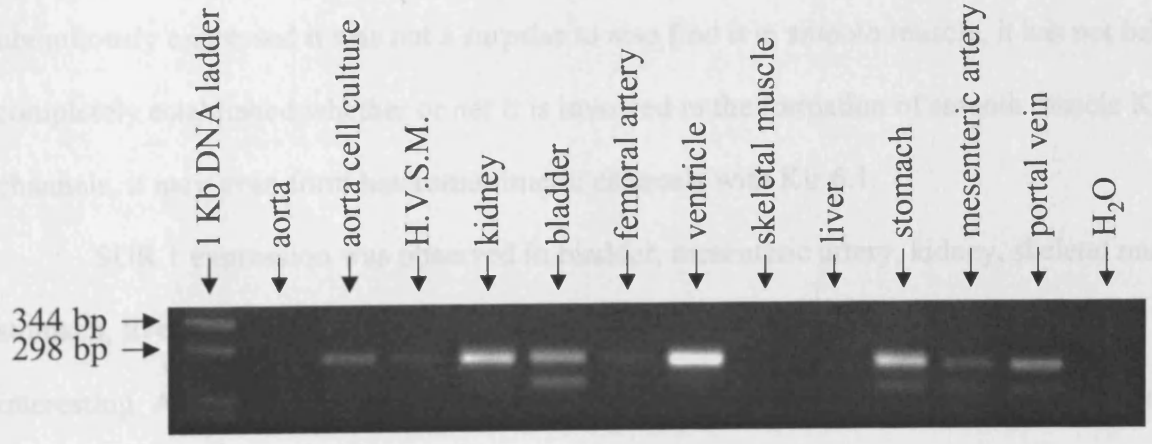
d)


1 Kb DNA Ladder
portal vein
femoral artery
mesenteric artery
aorta
aorta cell culture
H.V.S.M.
kidney
bladder
skeletal muscle
stomach
liver
ventricle
H2O
506 bp
396 bp

e)


1 Kb DNA ladder
aorta
Portal vein
Femoral artery
Mesenteric artery
H.V.S.M.
bladder
ventricle
liver
kidney
stomach
Skeletal muscle
1 Kb DNA ladder
506 bp
396 bp

f)


1 Kb DNA ladder
aorta
aorta cell culture
H.V.S.M.
kidney
bladder
femoral artery
ventricle
skeletal muscle
liver
stomach
mesenteric artery
portal vein
H_2O
344 bp
298 bp

3.1.8. Discussion

RT-PCR of the various rat tissues with subunit specific primers demonstrated the wide expression of K_{ATP} channel subunit mRNA throughout these tissues (see table. 1. for summary of expression). Most of the K_{ATP} channel subunits screened for were observed in many of the tissues . SUR 2A was the only species which seemed to have a more discrete pattern of expression, being observed in skeletal muscle and ventricle. It must be noted that although every care was taken in dissection of the particular tissues the preparations were not reflective of a single cell type.

SUR 2B, Kir 6.1 and Kir 6.2 were found in nearly every tissue, observations which support the previous studies mentioned earlier. As for the K_{ATP} channel of vascular smooth muscle, the subunits purported to form that particular channel, SUR 2B and Kir 6.1, were observed at the mRNA level in all smooth muscle preparations; mesenteric artery, femoral artery, portal vein and aorta. Kir 6.2 was also found in all the vascular smooth muscle tissues investigated, and SUR 1 was observed in mesenteric artery. As Kir 6.2 seems to be ubiquitously expressed it was not a surprise to also find it in smooth muscle, it has not been completely established whether or not it is involved in the formation of smooth muscle K_{ATP} channels, it may even form heteromultimeric channels with Kir 6.1.

SUR 1 expression was observed in bladder, mesenteric artery, kidney, skeletal muscle, stomach, liver and ventricle. The presence of SUR 1 transcripts in mesenteric artery is interesting. Although, it's presence has also been demonstrated in adipose tissue, which also surrounds the mesenteric artery, and may have been processed along with the tissue. Also, SUR 1 is thought to form the K_{ATP} channel of the mitochondrial inner membrane (Liu *et al.*, 2001), and the positive result for SUR 1 in mesentery may be due to mitochondrial K_{ATP} rather than sarcolemmal K_{ATP} message.

SUR 2 Δ17 transcripts were detected in all vascular preparations, as well as bladder, skeletal muscle, and stomach. This provides further information on the SUR subunits expressed in vascular tissue whilst also corresponding to what has been previously reported in human and mouse tissues (Davis-Taber *et al.*, 2000, Chutkow *et al.*, 1999). This SUR 2 splice variant, unlike the splice variant Δ14, has been shown to form functional channels when expressed with Kir 6.2 (Davis-Taber *et al.*, 2000). The resultant channels have altered ATP-sensitivity, with Δ17 variants being half as sensitive to ATP compared to the non-splice variant, suggesting a possible role for Δ17 in the protection of tissues that are vunerable to hypoxic damage.

The expression of Kir 6.1 mRNA has been observed to be upregulated after an ischaemic event in the kidney (Sgard *et al.*, 2000). It could be possible that the expression of the SUR 2 Δ17 variant is upregulated under hypoxic conditions suppressing non splice variant expression or forming heteromultimeric channels together with other SUR 2 subunits allowing the resultant K_{ATP} channels to open at higher [ATP]. This mechanism could be useful in the vascular system where the opening of K_{ATP} channels results in the vasodilation of blood vessels (Quayle and Standen, 1994) and may help protect tissues that are undergoing long periods of hypoxia.

SUR 2B transcript expression appears to be widespread whereas SUR 2A expression is more discrete and seems to be present mainly in ventricle and skeletal muscle. Electrophysiological studies suggest that the K_{ATP} channel of cardiac muscle is formed by the combination of SUR 2A and Kir 6.2 subunits (Okuyama *et al.*, 1998), however this investigation showed transcripts of Kir 6.1, Kir 6.2, SUR 1, SUR 2A and SUR 2B to be present in ventricle, giving rise to the possibility of many combinations of heteromultimeric channels being present in this tissue although the detection of transcripts does not necessarily equate with their expression at protein level.

Assuming that all mRNAs are translated, there is a possibility of many combinations of heteromultimeric K_{ATP} channels being formed. But in spite of this diversity of expression distinct K_{ATP} channels have been described in certain tissues where a number of mRNAs are expressed, and these channels are thought to consist of only one combination of sulphonylurea and inwardly rectifying subunit. For example Gopalakrishnan and co-workers demonstrated the presence of two types of SUR subunit to be present in guinea pig bladder smooth muscle (Gopalakrishnan *et al.*, 1999). Although they were able to detect transcripts of both SUR1 and SUR 2B by RT-PCR the electrophysiological studies provided evidence for the channels to be formed of only SUR 2B together with Kir 6.2. With a number of SUR and Kir subunit mRNAs being detected in various tissues it is not inconceivable to acknowledge that there may be heteromultimeric channels being formed. Alternatively there may be other mechanisms that, like the RKR sequence in SUR 1, control the translation of these messages and their subsequent expression in the cell membrane so that only homomultimers are formed.

As mentioned previously these experiments are not considered to be quantitative. Levels of transcript expression could not be directly compared between tissues due to differences in primer specificity and efficiency of annealing, and differences in amount of mRNA transcribed. Amounts of RNA used in the RT-PCR reaction were not always strictly quantified, due to the low amount of RNA retrieved from some tissues, mainly smooth muscle preparations. Although a positive amplification of product was assumed to indicate the expression of the specific mRNA in that particular tissue, no amplification product cannot be taken as an indication of an absence of expression. It has been shown here that many tissues contain a number of different subunit transcripts, even ones which are not thought to be involved in the formation of functional K_{ATP} channels for that particular tissue. The next step is to determine if these transcripts, detected by RT-PCR, are actually present at the protein level.

Subunit	Tissue distribution
Kir 6.1	H.V.S.M., bladder, kidney, skeletal muscle, ventricle, stomach, femoral artery, mesenteric artery.
Kir 6.2	Aorta cell culture, bladder, kidney, skeletal muscle, ventricle, stomach, femoral artery, mesenteric artery, portal vein.
SUR 1	Bladder, kidney, skeletal muscle, ventricle, stomach, liver, mesenteric artery.
SUR 2A	Skeletal muscle, ventricle.
SUR 2B	Aorta, bladder, kidney, skeletal muscle, ventricle, stomach, liver, femoral artery, mesenteric artery, portal vein.
SUR 2 Δ17	Bladder, skeletal muscle, stomach, femoral artery, mesenteric artery, portal vein.

Table. 1.

Tissue distribution of mRNA transcripts. Primers specific for the K_{ATP} channel subunits shown were used to detect mRNA transcripts in various tissues.

3.2. Immunocytochemistry of femoral artery

3.2.1. Introduction

Immunocytochemical techniques have been employed to investigate the presence of channel proteins within different tissues. Two groups used antibodies specific for a part of the ROMK potassium channel in order to localise its expression in the kidney to the apical membrane of the distal nephron (Henderson *et al.*, 1996; Kohda *et al.*, 1998). The expression of various other potassium channels has been examined in brain, for example; Kir 2.0 channel proteins (Stonehouse *et al.*, 1999), Kir 4.1 (Higashi *et al.*, 2001), and Kv 1 and 2 subunits (Bekele-Arcuri *et al.*, 1996; Shamotienko *et al.*, 1997; Rhodes *et al.*, 1997). K_{ATP} channel subunit distribution has been investigated in the pancreas by Suzuki and co-workers, who, using immunocytochemical techiniques, showed Kir 6.2 and SUR 1 subunits to be present and also observed that these two subunits co-localised providing further evidence to suggest that it is these two subunits which form the K_{ATP} channel of pancreatic beta cells (Suzuki *et al.*, 1997a, 1999). The Kir 6.1 subunit has been observed to occur in various tissues including skeletal muscle, liver, cardiac muscle, and pancreas, with antibody labelling patterns showing disperse staining throughout these tissues (Suzuki *et al.*, 1997b). Further investigation of the staining patterns using the electron microsope has shown Kir 6.1 to be localised to the mitochondria . These immunocytochemical studies are able to give an idea of the possible channel combinations which may arise in any one tissue but although they are able to show the presence of channel subunits at the protein level they are not able to determine whether channels are being functionally expressed.

Antibodies specific for the C-termini of the K_{ATP} subunits Kir 6.1, Kir 6.2, SUR 2A and SUR 2B (see table. 2. for sequence of target epitope and supplier), were used to investigate the presence of these proteins in femoral artery cells, and so suggest possible

Primary Antibody	Sequence of target epitope	Supplier
Anti- SUR 2A	^{1527}PNLLQHKNGLFSTLVMTNK^{1545} (C-terminal domain of SUR 2A)	Pepceuticals
Anti- SUR 2B	^{1528}ESLLAQEDGVFASFVRADM^{1546} (C-terminal domain of SUR 2B)	Pepceuticals
Anti- Kir 6.1	^{409}KVQFMTPEGNQCPSES^{424} (C-terminal domain of Kir 6.1)	University of Leicester
Anti- Kir 6.2	^{376}KAKPKFSISPDSLS^{390} (C-terminal domain of Kir 6.2)	Research Genetics

Table. 2.

Primary antibodies used in the detection of K_{ATP} channel subunits present in femoral artery. The sequence of the epitopes that the antibodies were raised against is shown together with the supplier, all antibodies were raised in rabbit and were targeted to the C-terminal domain of the subunit.

combinations involved in the formation of the channel in this tissue. The antibodies used have been investigated for their selectivity and cross reactivity by Mr H. Singh (University of Leicester). This involved the transfection of HEK293 cells with varying combinations of the K_{ATP} subunits mentioned, and staining of the cells with the antibodies (procedure in Methods and Materials). Antibodies specific for the transfected subunits showed fluorescent staining of the cells, which was localised to the membrane, whereas there was no significant staining observed in cells which had been treated with primary antibodies not specific for the transfected subunits (data not shown). In femoral artery cell experiments the primary antibodies were also incubated with the specific antigenic peptide to provide a control. In these cells there was a marked reduction in staining, inferring that any staining observed in other experiments was due to the specific binding of the antibody.

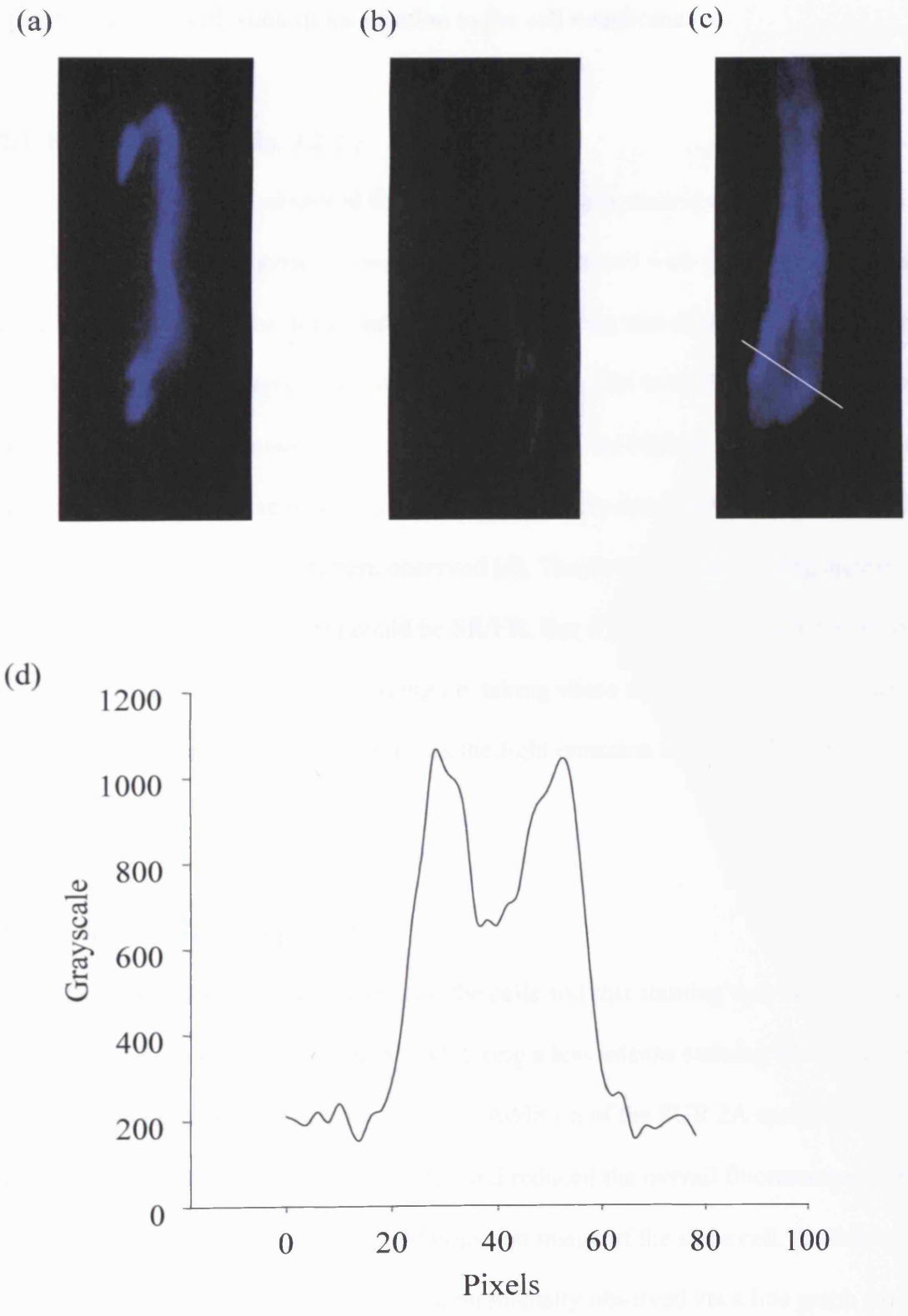
3.2.2. Kir 6.1 staining (fig. 3.2.1.)

The anti-Kir 6.1 antibody stained the femoral artery cells (see fig. 3.2.1.a and c), with high intensity fluorescence being observed compared with that of cells which had included the peptide block (see fig.3.2.1.b), showing that the staining was specific for this peptide. Two cells are shown which exhibited different regions of staining (see 3.2.1.a and c). Staining occurred throughout the cell in image (a) while image (c) showed staining that was localised to the cell membrane. This membrane staining was further examined by investigating the intensity of fluorescence in a cross section of the cell, shown by the line in (c). Graph (d) represents the change in intensity of fluorescence throughout the cell and shows two regions of high intensity fluorescence, representing the membrane, with a lower intensity region in between, maybe representing the intracellular region. The staining observed in image (a) may show membrane localisation but due to the rather thin appearance of the cell the membrane on either side may be touching and so giving the appearance of total cellular staining as any

Figure. 3.2.1.

Confocal emission fluorescent image of femoral artery cells stained with anti-Kir 6.1 antibody (a) and (c). Staining is blocked using the Kir 6.1 peptide (b), showing antibody specificity. The line graph (d) represents the change in fluorescent intensity throughout the region identified by the line marked in (a).

Figure. 3.2.1.


(a)
(b)
(c)
(d)
1200
1000
800
600
400
200
0
Grayscale
0
20
40
60
80
100
Pixels

difference in intensity would not be observed. Other cells observed here gave staining patterns as that for cell (c) with subunit localisation to the cell membrane.

3.2.3. Kir 6.2 staining (fig. 3.2.2.)

Fluorescence was observed for femoral artery cells stained with the anti-Kir 6.2 antibody, which was at a greater intensity than that observed with the peptide block (see fig. 3.2.2.a and b). Staining was localised to two main regions, that of the outside (membrane) of the cell, and a discrete loop on the inside of the cell (a). The intensity of the fluorescence throughout the cell was investigated in the plane shown by the line in (a). Four areas of high intensity fluorescence were observed which where interspersed with areas of lower intensity, corresponding to the staining pattern observed (d). The outer area of staining appears membranous while the inner area could be SR/ER. But if the cell membrane has involuted at the top, due to the mechanism of imaging i.e. taking slices through the cell, this inner area may in fact be membrane. Image (c) shows the light emission image of the peptide blocked cell.

3.2.4. SUR 2A staining (fig. 3.2.3.)

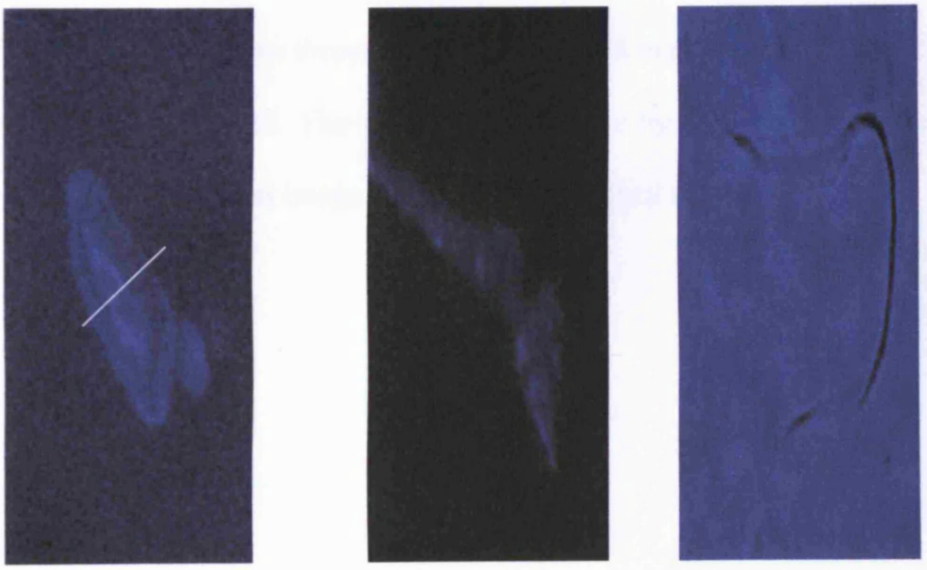
The anti-SUR 2A antibody stained the cells and this staining was localised to the cell membrane with the intracellular region exhibiting a less intense staining which was still greater than the background (see fig. 3.2.3.a). Addition of the SUR 2A specific peptide abolished membrane localised fluorescence and reduced the overall fluorescence significantly (b), with image (c) representing the light emission image of the same cell. A section was taken across the image (a) and the fluorescent intensity observed via a line graph (d). Peaks of high intensity were observed corresponding to the membrane localised fluorescence with a lesser intense region in between which was higher than the background.

Figure. 3.2.2.

(a) is the confocal emission fluorescent image of a femoral artery cell stained with the anti-Kir 6.2 antibody. (b) shows the staining when the specific peptide block is included and (c) is the light emission image of the same cell. The line graph (d) represents the change in fluorescent intensity throughout the region identified by the line marked in (a).

Figure. 3.2.2.

(a) (b) (c)



(d)

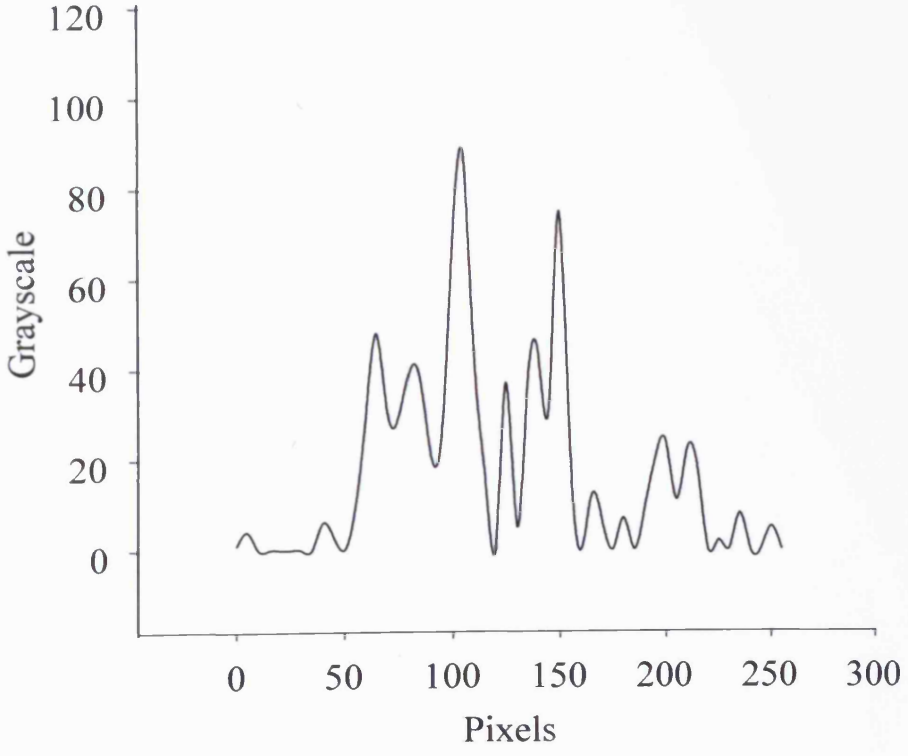
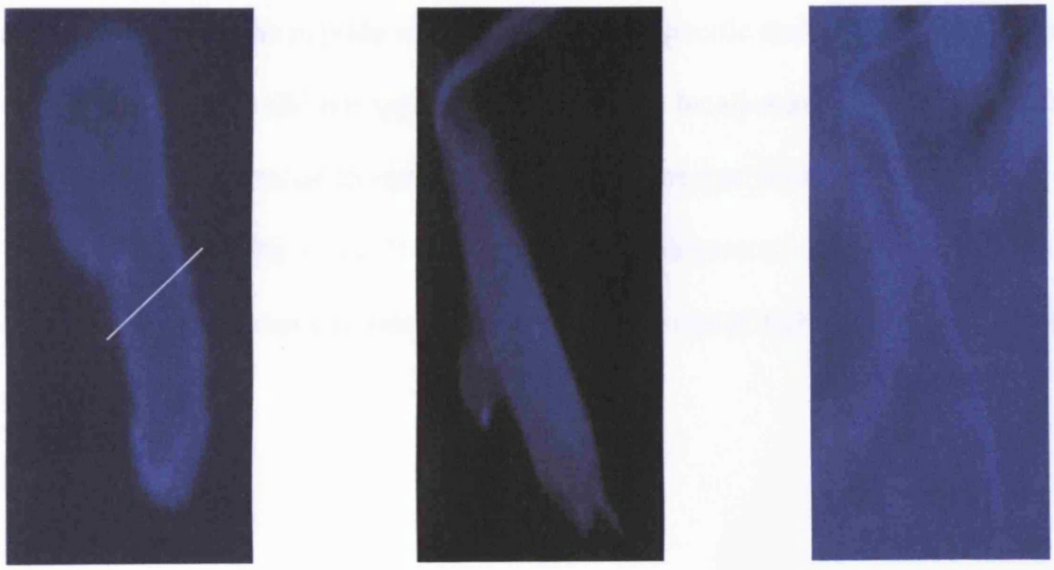


Figure. 3.2.3.

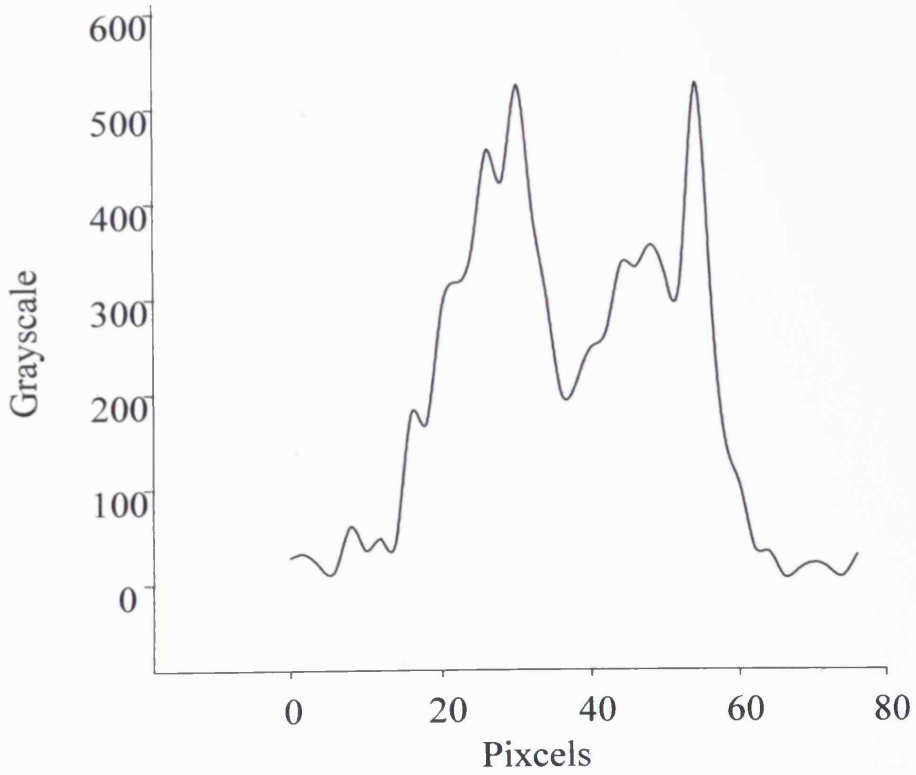
Confocal emission fluorescent image of a femoral artery cell stained with anti- SUR 2A antibody (a). The line drawn through (a) is represented in (d) as the change in fluorescent intensity throughout the cell. The staining was blocked by addition of the specific peptide (b) and (c) is the light emission image of the peptide blocked cell.

Figure. 3.2.3.

(a) (b) (c)



(d)



3.2.5. SUR 2B staining (fig. 3.2.4.)

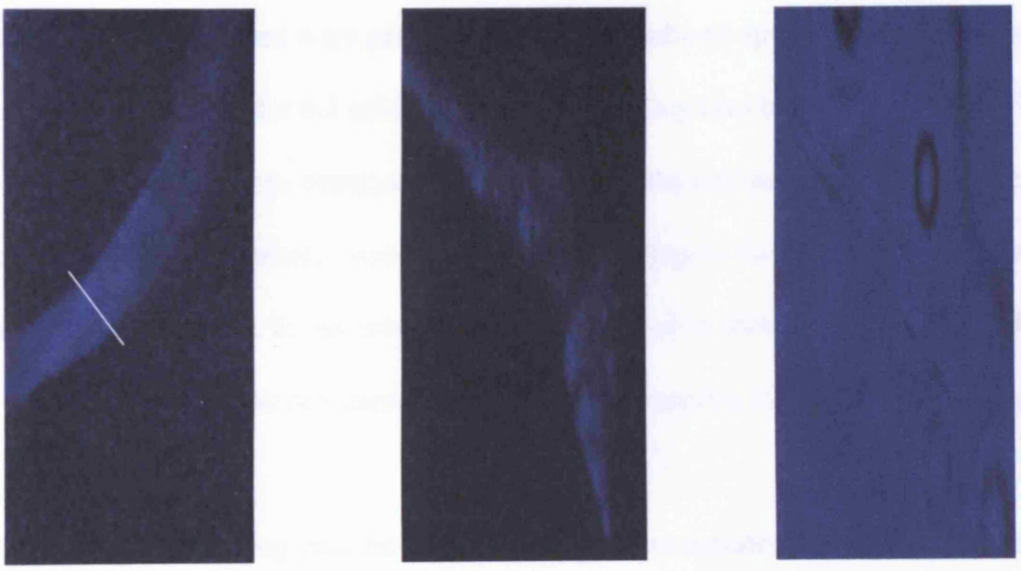
The staining observed using the anti-SUR 2B antibody was a much greater intensity than that observed when the peptide was added to block specific staining (see fig. 3.2.4.a and b). The fluorescent pattern did not appear to have discrete localisation but appeared diffuse throughout the cell. The change in intensity of fluorescence was investigated in a cross-section of the cell as defined in (a). This showed no area to have a greater intensity of fluorescence than any another (d). Image (c) was the transmitted light emission image of the peptide blocked cell.

Figure. 3.2.4.

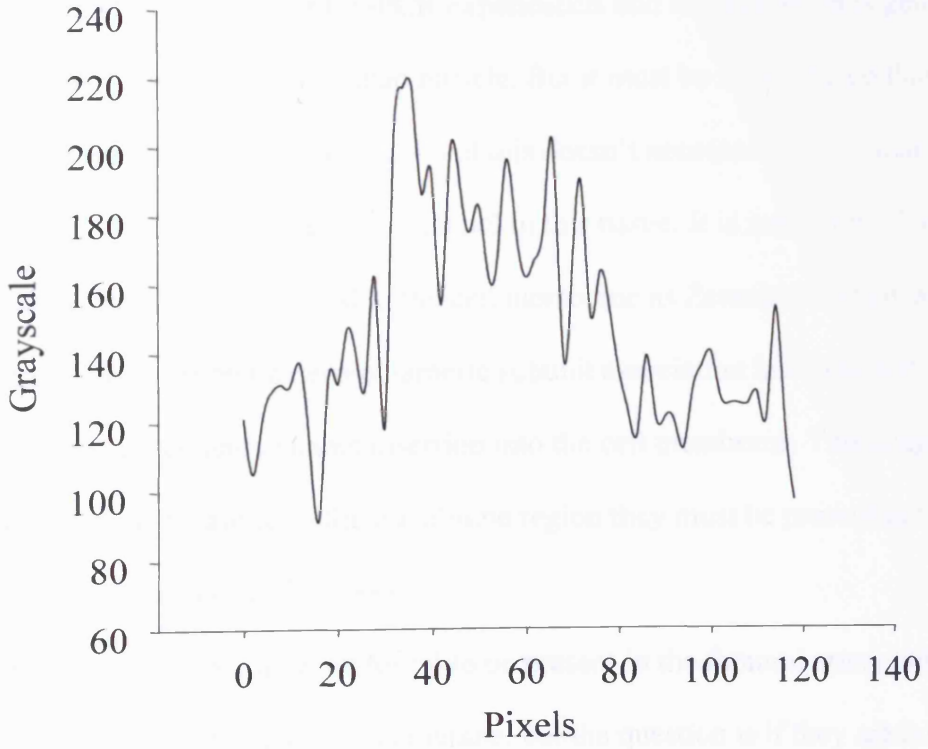
(a) is the confocal emission fluorescent image of a femoral artery cell stained with anti-SUR 2B antibody. (b) shows the reduction in staining when the specific peptide is added and (c) is the light emission image of this same cell. The fluorescent intensity of staining throughout the cell is represented by the line graph (d) the area of which is taken from the line drawn in (a).

Figure. 3.2.4.

(a) (b) (c)



(d)



3.2.6. Discussion

It appears from the staining observed in the femoral artery cells that all four K_{ATP} subunit proteins investigated were present. The Kir 6.1 subunit appeared to be localised in the cell membrane as did the Kir 6.2 subunit, although this may also be found in the ER/SR. SUR 2A has been demonstrated to occur, with localisation to the cell membrane and to a lesser extent distributed intracellularly where it may be trafficking to the cell surface. The proposed vascular smooth muscle SUR subunit, 2B, is also observed in these cells although it doesn't appear to be localised in the cell membrane but has been shown to be dispersed throughout the cell.

It is hardly surprising that the subunit mRNA demonstrated to occur in femoral artery using RT-PCR was also found to be present at the protein level. One difference, however, was the finding of SUR 2A protein and it's localisation to the cell membrane, this subunit was not found at the mRNA level in the RT-PCR experiments and its expression is generally thought to occur mainly in skeletal and cardiac muscle. But it must be remembered that although all four subunits are present at the protein level this doesn't necessarily mean that they are all involved in the formation of the K_{ATP} channel in this tissue. It is interesting, however, that the proteins have been found localised to the cell membrane as Zerangue and co-workers have demonstrated that a correct hetero-octameric subunit association has to be achieved to allow trafficking and subsequent channel insertion into the cell membrane. This suggests that for these proteins to be localised in the membrane region they must be present in this stoichiometry (Zerangue *et al.*, 1999).

Both Kir 6.x subunits were found to be present in the femoral artery cells and they appeared to be localised to the cell membrane, but the question is if they are both present do they form heteromeric channels? One means of discerning this is to look at the channel conductance. When expressed with SUR, Kir 6.2 has a conductance of about 80 pS whereas

Kir 6.1 exhibits a much lower conductance of about 30 pS (Sakura *et al.*, 1995; Inagaki *et al.*, 1995a; Okuyama *et al.*, 1998; Kondo *et al.*, 1998; Repunte *et al.*, 1999; Kono *et al.*, 2000). These values are reflected in native tissue with the K_{ATP} channel conductance of cardiac and skeletal muscle being reported to be about 70-80 pS (Kajioka *et al.*, 1991; Allard *et al.*, 1995; Deutsch *et al.*, 1994; Fujita and Kurachi, 2000) and vascular smooth muscle to be about 20-30 pS (Zhang and Bolton, 1996; Koh *et al.*, 1998; Lee *et al.*, 1999; Fujita and Kurachi, 2000). Therefore, it would appear that the channels formed in these tissues result from homomeric associations of Kir 6.x subunits. However, Kono *et al.*, 2000, have shown that Kir 6.1 and 6.2 may have the ability to form heteromeric channels, with the expression of Kir6.1-6.2 tandem channels exhibiting an intermediate conductance, between the two Kir 6.x subtypes, of 59 pS. There have in fact been K_{ATP} channels reported which exhibit this type of conductance; Zhang and Bolton found two types of ATP sensitive K^+ channel in rat portal vein which had different conductances, one channel had a conductance similar to that observed for Kir 6.1 of 22 pS, while the other had a conductance of 50 pS similar to that reported for the tandem channel (Zhang and Bolton, 1996). Also two types of ATP sensitive K^+ channel were found in human corporal smooth muscle, which exhibited differing conductances, with one having a conductance of 18 pS, similar to that for channels made up of Kir 6.1, and the other having a conductance of 59 pS, similar to that reported for the tandem channel (Lee *et al.*, 1999). This evidence suggests that there may be a possibility for heteromeric channel formation with regard to the Kir 6.x subunit, but differences in the measurement of channel conductances, ie different conductance values gained when measuring in different extracellular K^+ concentrations (Quayle and Standen, 1994), make this comparison less reliable. Perhaps standardisation of experimental conditions would allow a more reliable comparison of the different conductances of the K_{ATP} channels of different tissues to be made and so maybe

allowing the Kir 6.x subunits involved in channel formation in various tissues to be illucidated.

Unlike the Kir 6.x subunits, SUR tandem channel expression so far has not been demonstrated. The investigation of SUR heterogeneity in K_{ATP} channels of native tissue is probably impossible via pharmacological comparison. It would be reasonable to assume that heteromeric channels would resemble the most sensitive SUR subunit present, as sulphonylurea binding requires interaction with only one SUR subunit to induce channel closure (Dorschner *et al.*, 1999). Therefore at the moment it is still unknown whether the SUR subunit may form in heteromeric assemblies.

Overall femoral artery cells appear to contain all four K_{ATP} channel subunits investigated, although it is unkown whether these subunits are involved in the formation of functionally active channels at the cell membrane. However, the fluorescent staining which was localised to the cell membrane for some of these subunits does suggest that they are associating in the correct hetero-octameric stoichiometry required for channel insertion in the cell membrane. Evidence for heteromeric channel formation has been discussed and the presence of multiple SUR and Kir 6.x subunits does suggest the possibility of a number of subunit combinations occurring in femoral artery.

Chapter 4

Characterisation of cloned K_{ATP} current

Chapter 4. Characterisation of cloned K_{ATP} current

4.1. Introduction

Since Noma described a potassium channel, in cardiac myocytes, that was regulated by changes in intracellular [ATP] similar channels have been found in various other tissues (Noma, 1983). Electrophysiological studies have characterised native K_{ATP} channel regulation by intracellular nucleotides, synthetic and endogenous channel activators and inhibitors, and also described channel kinetics.

In 1992 Clapp and Gurney noted that ATP could directly modulate the resting potential of arterial smooth muscle cells by the inhibition of potassium channels (Clapp and Gurney, 1992). These smooth muscle K_{ATP} channels have been shown to be regulated by endogenous vasoconstrictors, such as serotonin and histamine which block K_{ATP} current, and vasodilators, such as calcitonin gene related peptide that activate the K_{ATP} channel (Kleppish and Nelson, 1995). K_{ATP} channels with similar properties have also been observed in portal vein and mesenteric artery. These vascular smooth muscle K_{ATP} channels are activated by UDP and GDP and unlike the classical K_{ATP} channel of cardiac myocytes do not spontaneously open on patch excision to nucleotide free solution or removal of $[ATP]_i$ (Kajioka *et al.*, 1991; Noak and Edwards, 1992; Beech *et al.*, 1993). When activated the current was found to run down on patch excision and the application of MgATP caused channel re-activation (Kamouchi and Kitamura, 1994; Zhang and Bolton, 1995, 1996). On activation these channels were found to have conductances that ranged from 10 to 24pS (Kajioka *et al.*, 1991; Noak and Edwards, 1992; Beech *et al.*, 1993; Zhang and Bolton, 1995, 1996). These channels were known as K_{NDP} channels, because of their regulation by NDPs, even though the channels are regulated by ATP and therefore are K_{ATP} channels.

The comparison of cloned K_{ATP} channel regulation and kinetics with that observed in native tissue has allowed investigators to elucidate the possible molecular identity of the K_{ATP}

channels in different tissues. Yamada and co-workers used HEK293T cells to express the subunit combination SUR 2B/ Kir 6.1 and found that the channels formed were similar in their conductance and regulation, by nucleotide diphosphates, to native vascular smooth muscle K_{ATP} channels (Yamada *et al.*, 1997). However a more thorough investigation is needed of the cloned channels before the molecular identity of the vascular smooth muscle K_{ATP} channel can be determined.

In order to validate the *Xenopus* expression system for the expression of cloned K_{ATP} channels, Kir 6.0 and SUR subunit combinations were co-injected as mRNA into *Xenopus* oocytes and the resultant K_{ATP} channels studied for their K^+ selectivity, and pharmacological properties.

4.2. Subcloning into pBF

Mouse Kir 6.2, rat SUR 1 and Kir 6.1 cDNAs were obtained from F. Ashcroft. Rat SUR 2A and SUR 2B were obtained from S. Seino, and rat Kir 6.2 from Y. Kurachi. In early experiments both rat and mouse mRNAs were used in expression, but in later experiments only rat was used to decrease the chance of any experimental differences occurring which may be due to variability between species. The cDNAs were subcloned into the vector pBF. This vector contains sequences from the 5' and 3' untranslated regions of the *Xenopus* β-globin gene. *Xenopus* β-globin mRNA has a long half-life and the presence of these sequences act to stabilise the resulting mRNA. A polyadenosine tract is also present which acts to stabilise the message. The presence of an ampicillin resistence gene allows selection of vector containing cells, and multiple cloning sites are present for subcloning of cDNAs.

The pBF vector contained an SP6 promoter but to increase the efficiency and yield of mRNA produced a T7 promoter was also added. Oligonucleotide primers, coding for the T7 promoter (top strand : agc tct aat acg act cac tat agg g, bottom strand : agc tcc cta tag tga gtc

gta tta g), were annealed together and ligated into a *Hind* III site in the vector. Successful ligation was confirmed by the absence of the Hind III site, which is destroyed on ligation, and the orientation of the promoter was confirmed by sequencing. The cDNAs of each subunit were then subcloned into this vector. For example: a blunt ended fragment encompassing the coding region of Kir 6.2 was ligated into a *Hinc* II site, SUR 2A was ligated between *Bsp* 120 I and *Hinc* II, and Kir 6.1 between *BamH* I and *Kpn* I. Ligation of cDNA into pBF was performed using the Life Technologies Rapid Ligation protocol for plasmid cloning, and the resultant reaction transformed into Epicurian Coli® XL-Blue subcloning-grade competent cells (Stratagene). The selected transformants were then sequenced to check that the cDNAs were inserted in the correct orientation.

4.3. Control experiments

Bath application of 100 μM pinacidil did not induce K_{ATP} current in uninjected oocytes, or oocytes that were injected with water. Kir 6.2 and Kir 6.1 mRNAs were injected alone (without SUR mRNA) and their ability to form functional channels investigated. The application of 100 μM pinacidil caused no change in current in 10 cells, which indicates an absence of functional K_{ATP} channels and that coinjection with SUR was required for functional channel expression.

4.4. Ion selectivity of cloned channels

The K^+ selectivity of cloned K_{ATP} channels formed by SUR 2B/Kir 6.1 (data not shown) and SUR 2B/Kir 6.2 was studied by altering the extracellular K^+ concentration ($[K^+]_{ext}$) and observing the effects on current reversal potential (see fig. 4.1.a). The reversal potential (E_{rev}) of SUR 2B/Kir 6.2 became more positive as the $[K^+]_{ext}$ was raised from 2 mM to 90 mM. A line was fitted to the data and gave an estimate of $[K^+]_{int}$ of 110mM (fig. 4.1.b).

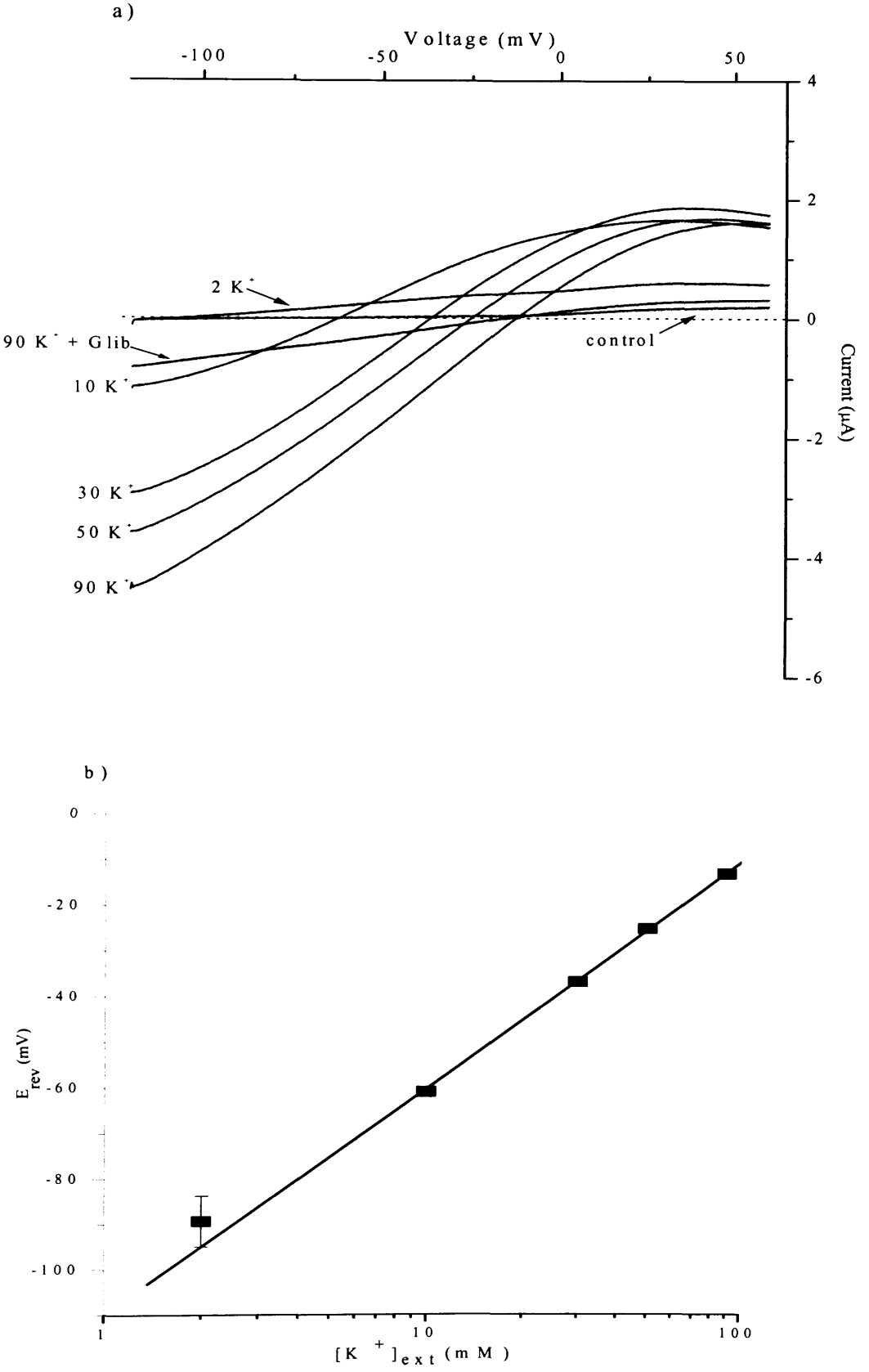
The mean change in reversal potential was found to be around 50mV for a 10-fold change in extracellular [K^+] (n=7). Similar results were observed with SUR 2B/ Kir 6.1.

Figure. 4.1.

Co-expression of Kir 6.2 and SUR 2B produces functional K^+-selective channels in *Xenopus* oocytes.

a) Current traces from an oocyte expressing SUR 2B/ Kir 6.2, each during a ramp change in voltage from –120 mV to +60 mV. The control trace was obtained in the presence of 2 mM K^+. Other traces were recorded after channel activation by 100 μM pinacidil in 2, 10, 30, 50 and 90 mM K^+, as indicated. The response to 10 μM glibenclamide in 90 mM K^+ is also shown.

b) Mean reversal potential (E_{rev}) against extracellular K^+ concentration ($[K^+]_{ext}$). Solid squares are the mean ± s.e. mean of reversal potentials for 100 μM pinacidil-induced currents in 7 oocytes expressing SUR 2B/ Kir 6.2. The line was fitted to the data with a slope of 50 mV and an intercept at $E_{rev} = 0$ of 110 mM.


a)
Voltage (mV)
-100
-50
0
50
4
2
0
-2
-4
-6
Current (μA)
2 K+
90 K+ + Glib
10 K+
30 K+
50 K+
90 K+
control
b)
0
-20
-40
-60
-80
-100
Erev (mV)
1
10
100
[K+]ext (mM)

4.5. Cloned K_{ATP} channel pharmacology

K_{ATP} channels are the target of 2 main classes of compound, the potassium channel openers (KCOs), a structurally heterogenous class of compounds which cause channel activation, and the sulphonylureas which cause channel inhibition. Examples of better known KCOs include; pinacidil, nicorandil, diazoxide, levcromakalim, and minoxidil sulphate, with some of these compounds being used clinically.

In the treatment of hyperinsulinemia and in hypertensive emergencies diazoxide activates K_{ATP} channels causing an inhibition of insulin release, from pancreatic beta-cells, and a decrease in vascular tone. Cromakalim is also an antihypertensive and produces a relaxation of vascular smooth muscle (Quayle and Standen, 1994; Nelson and Quayle, 1995).

Sulphonylureas are widely used in the treatment of type 2 diabetes, where the inhibition of K_{ATP} channels in pancreatic beta-cells stimulates the release of insulin, and some examples include; glibenclamide and tolbutamide. Glibenclamide also has diuretic ability causing the reduction of Na^+ reabsorption and K^+ secretion (Quast, 1996).

The potencies of KCOs and sulphonylureas vary in different tissues and these differences in sensitivity are related to the subunit combinations that occur in these tissues (Atwal, 1994; Isomoto *et al.*, 1996; Okuyama *et al.*, 1997; Shindo *et al.*, 1998). Pinacidil was investigated for its effect on the cloned channels SUR 2B/ Kir 6.2, SUR 2B/ Kir 6.1, SUR 2A/ Kir 6.2, and SUR 2A/ Kir 6.1, and a concentration relationship gained for the SUR 2B/ Kir 6.2 channel. The potassium channel openers diazoxide and minoxidil sulphate were also investigated for their effects on the cloned channels SUR 2B/ Kir 6.2, SUR 2B/ Kir 6.1, and SUR 2A/ Kir 6.2.

4.5.1. Pinacidil sensitivity of the cloned channel SUR 2B/ Kir 6.2

The classical potassium channel opener pinacidil induced current in the cloned channels; SUR 2B/ Kir 6.2, SUR 2B/ Kir 6.1, SUR 2A/ Kir 6.2, and SUR 2A/ Kir 6.1, which was inhibited by the sulphonylurea glibenclamide at a concentration of 10 μM (see fig.4.2.). Sensitivities of the different channels to pinacidil was dependent upon the SUR subunit, with those channels containing SUR 2B being more sensitive than SUR 2A. Channels comprising SUR 2B with either Kir 6.1 or 6.2 were maximally activated by concentrations of pinacidil in the range of 100-300 μM. However, maximal activation of channels comprising SUR 2A was not reached, even upon application of 1 mM pinacidil. A concentration-effect relationship for pinacidil activation of SUR 2B/Kir 6.2 K_{ATP} currents was generated (see fig. 4.3.), and the data fitted with the equation:

$$(I_{pin}-I_{glib})/(I_{pin}Max-I_{glib})=1/(1+[pin]^n/EC_{50}^n)$$

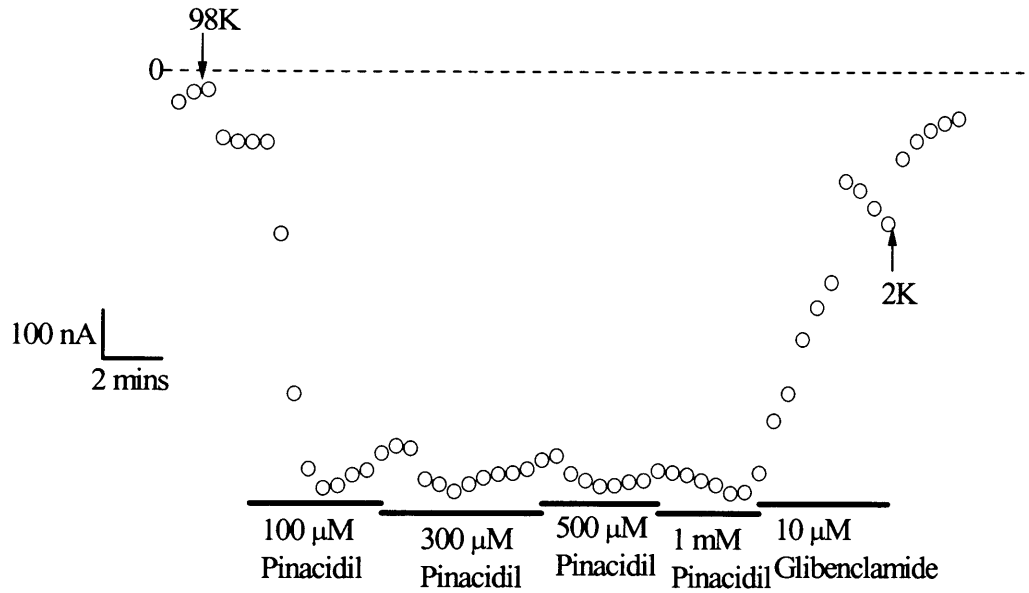
Where I_{pin} is the current in pinacidil, I_{glib} is the current in glibenclamide, and $I_{pin}Max$ is the maximum current in pinacidil. The concentration of pinacidil required for half maximal activation of SUR 2B/Kir 6.2 K_{ATP} channels was calculated to be 17.7±1.4 μM, from 7 oocytes, with a Hill coefficient of 1.4.

Figure. 4.2.

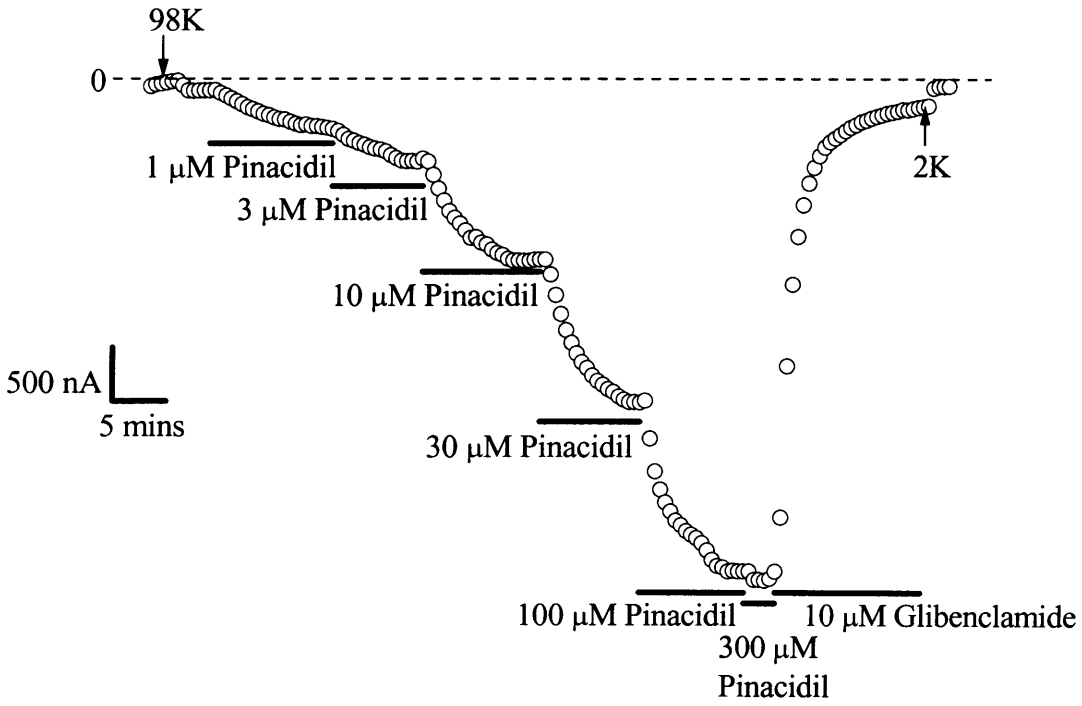
Current traces showing K_{ATP} channel activation by pinacidil.

Trace (a) depicts a representative response from an oocyte expressing SUR 2B/ Kir 6.1, (b) SUR 2B/ Kir 6.2, (c) SUR 2A/ Kir 6.1, and (d) SUR 2A/ Kir 6.2. Points represent the whole cell current, with a holding potential of –60 mV, at 30 second intervals. Various concentrations of pinacidil were applied to the cells, as indicated, and 10 μM glibenclamide was added to the highest concentration of pinacidil to block any K_{ATP} channel activation.

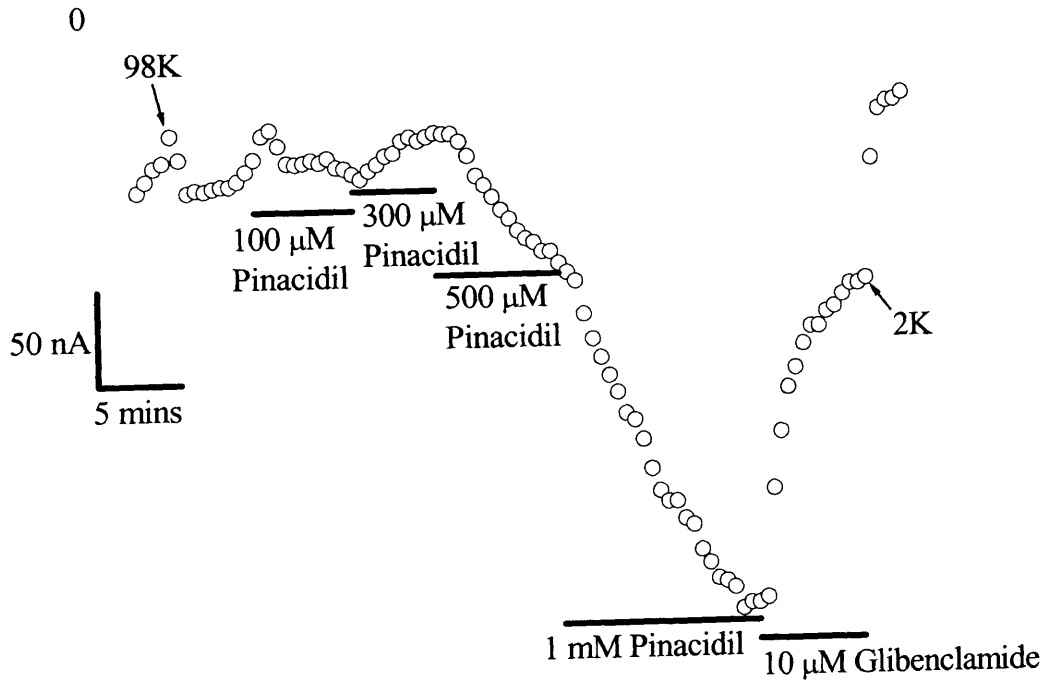
a)


98K
0
2K
100 nA
2 mins
100 μM Pinacidil
300 μM Pinacidil
500 μM Pinacidil
1 mM Pinacidil
10 μM Glibenclamide

b)


98K
0
2K
1 μM Pinacidil
3 μM Pinacidil
10 μM Pinacidil
30 μM Pinacidil
100 μM Pinacidil
300 μM Pinacidil
10 μM Glibenclamide
500 nA
5 mins

c)


0
98K
100 μM
Pinacidil
300 μM
Pinacidil
500 μM
Pinacidil
2K
50 nA
5 mins
1 mM Pinacidil
10 μM Glibenclamide

d)

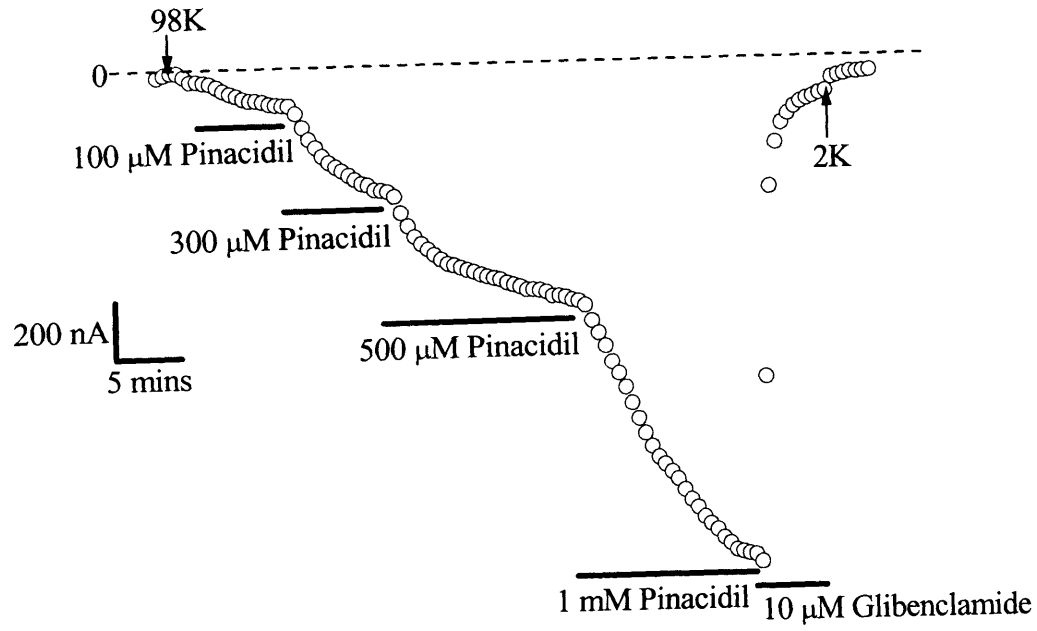

98K
0
100 μM Pinacidil
2K
300 μM Pinacidil
200 nA
5 mins
500 μM Pinacidil
1 mM Pinacidil
10 μM Glibenclamide

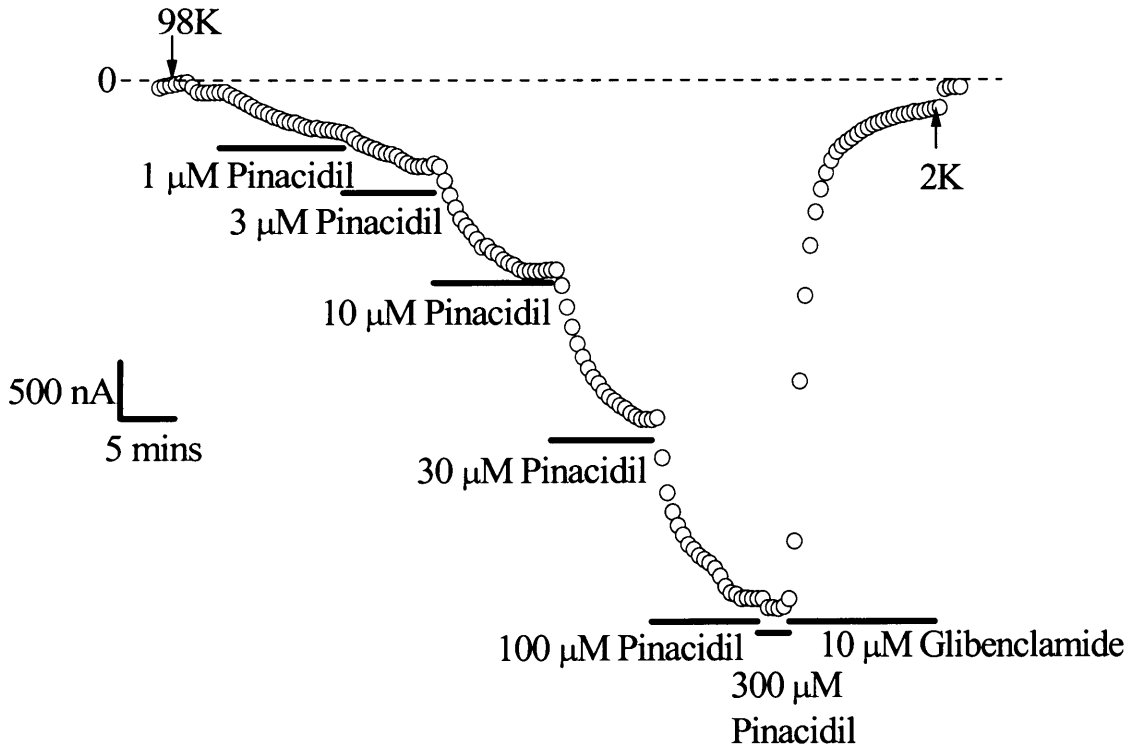
Figure. 4.3.

Co-expression of SUR 2B and Kir 6.2 induces formation of pinacidil- and glibenclamide-sensitive K_{ATP} currents.

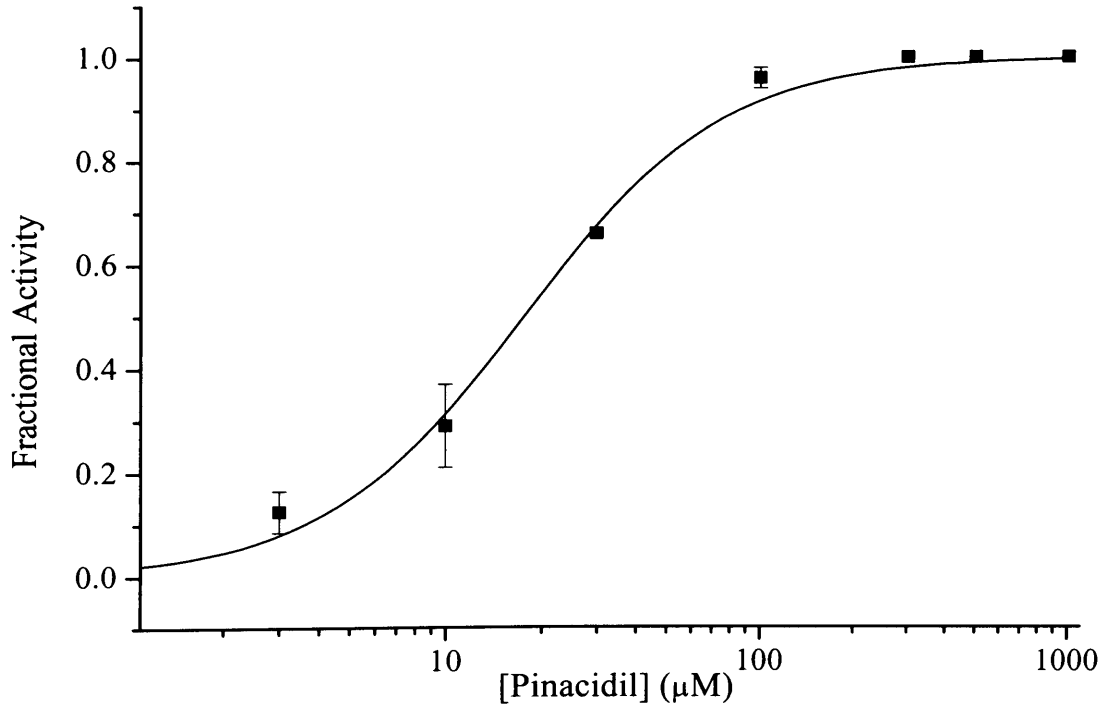
a) Current trace showing activation of SUR 2B/ Kir 6.2 by pinacidil. Pinacidil (1 μM-300 μM) was applied to the extracellular solution. The whole-cell current was measured at 30 second intervals with a holding potential of –60mV.

b) Concentration-dependent activation of SUR 2B/ Kir 6.2 by pinacidil. The current at each concentration of pinacidil was normalised to the maximal (1mM) pinacidil-induced current in the same cell. The data points are the mean ±s.e. mean for 7 cells.

a)


98K
0
1 μM Pinacidil
3 μM Pinacidil
10 μM Pinacidil
2K
500 nA
5 mins
30 μM Pinacidil
100 μM Pinacidil
10 μM Glibenclamide
300 μM
Pinacidil

b)


Fractional Activity
1.0
0.8
0.6
0.4
0.2
0.0
10
100
1000
[Pinacidil] (μM)

4.5.2. Channel activation by diazoxide

Diazoxide illicited channel activation, but was found to be far less potent than pinacidil. SUR 2B/Kir 6.2 channels were observed to be activated by 500 μM diazoxide but only at low levels, and SUR 2A /Kir 6.2 exhibited no activation by the drug. However, when K_{ATP} current had been induced by metabolic inhibition, subsequent application of diazoxide was shown to enhance this current further in oocytes expressing SUR 2B/Kir 6.1 or 6.2 (see fig. 4.4.).

4.5.3. Channel activation by minoxidil sulphate

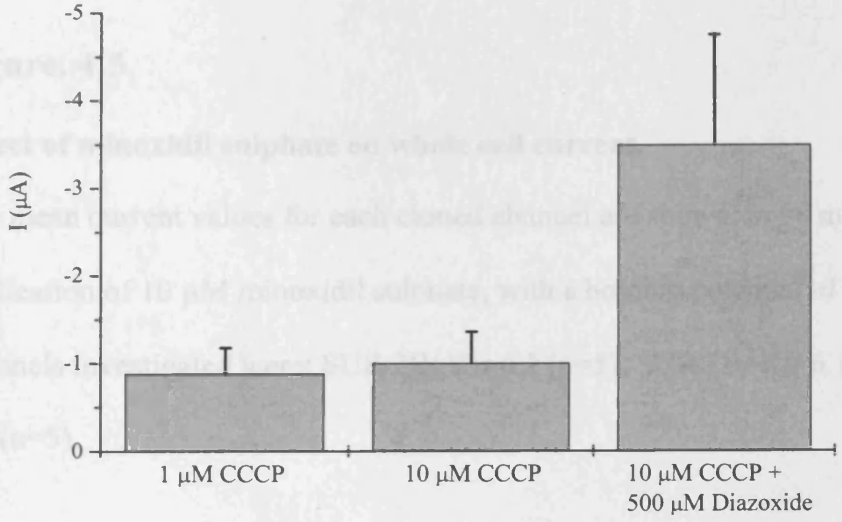
10 μM minoxidil sulphate exerted no effect on the K_{ATP} channels SUR 2A/ Kir 6.2 or SUR 2B/ Kir 6.1, and only induced a small amount of current, compared to that evoked by pinacidil, in SUR 2B/ Kir 6.2 (see fig. 4.5.).

Figure. 4.4.

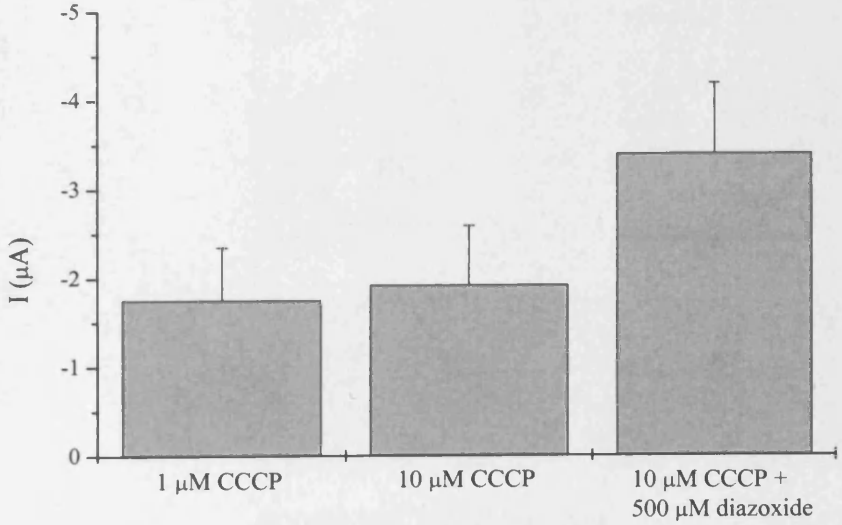
Effect of diazoxide on cloned K_{ATP} channels.

Mean K_{ATP} current in oocytes expressing; a) SUR 2B/ Kir 6.2, n=5, b) SUR 2B/ Kir 6.1, n=7, and c) SUR 2A/ Kir 6.2, n=5. Error bars represent the s.e. The external potassium concentration was 98 mM.

a)


-5
-4
-3
-2
-1
0
I (μA)
1 μM CCCP
10 μM CCCP
10 μM CCCP +
500 μM Diazoxide

b)


-5
-4
-3
-2
-1
0
I (μA)
1 μM CCCP
10 μM CCCP
10 μM CCCP +
500 μM diazoxide

c)

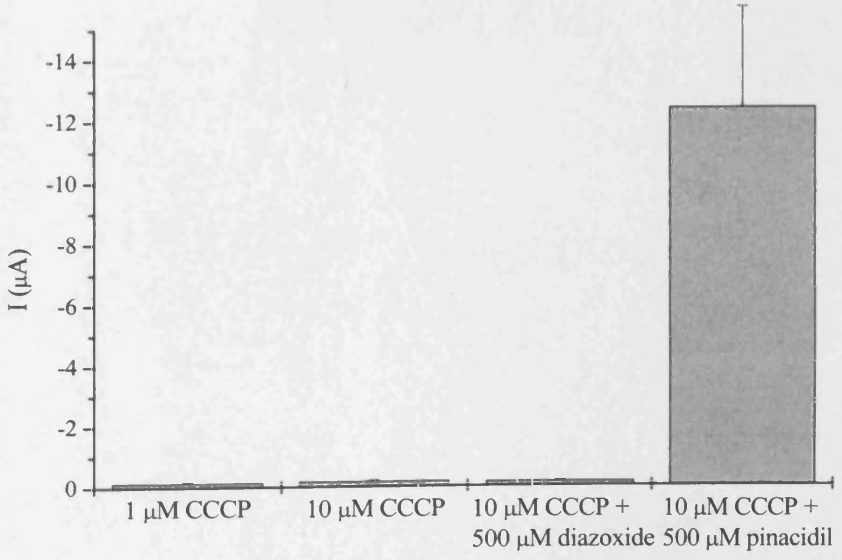
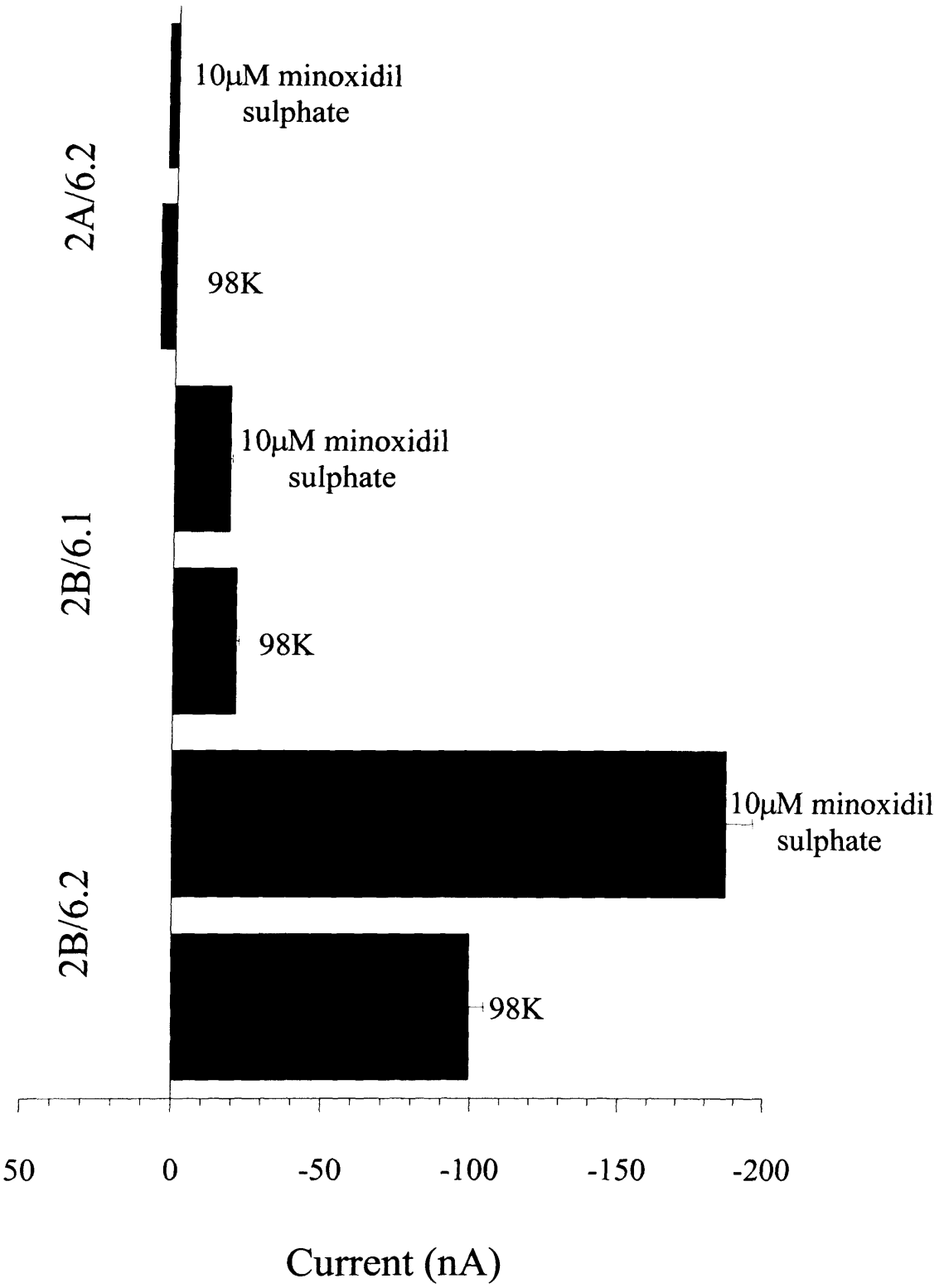

-14
-12
-10
-8
-6
-4
-2
0
I (μA)
1 μM CCCP
10 μM CCCP
10 μM CCCP +
500 μM diazoxide
10 μM CCCP +
500 μM pinacidil

Figure. 4.5.

Effect of minoxidil sulphate on whole cell current.

The mean current values for each cloned channel are shown, in 98 mM K^+ and after application of 10 μM minoxidil sulphate, with a holding potential of –60 mV. Cloned channels investigated were; SUR 2B/ Kir 6.2 (n=5), SUR 2B/ Kir 6.1 (n=6), and SUR 2A/ Kir 6.2 (n=5).

Figure. 4.5.


10μM minoxidil sulphate
2A/6.2
98K
10μM minoxidil sulphate
2B/6.1
98K
10μM minoxidil sulphate
2B/6.2
98K
50
0
-50
-100
-150
-200
Current (nA)

4.6. Discussion

4.6.1. Expression of functional K_{ATP} channels

The expression of functional K_{ATP} channels could only be obtained by the co-injection of SUR 1 or 2 and Kir 6.x mRNA. When these subunits were co-expressed, the application of pinacidil illicited currents which were inhibited by glibenclamide, indicating that these were K_{ATP} currents. The injection of these subunits alone resulted in no functional channel activity being observed. These results are similar to those reported by Gribble and co-workers (Gribble *et al.*, 1997b). No K_{ATP} channel activity was observed when SUR 1 was expressed alone in *Xenopus* oocytes (Aguilar-Bryan *et al.*, 1995). However truncations of Kir 6.2, which have lost their C-terminal 26 or 36 amino acids, have been observed to produce an ATP sensitive channel when expressed in the absence of SUR (Tucker *et al.*, 1997). It has been shown that SUR 1, Kir 6.1 and 6.2 contain a sequence, known as the RKR sequence, which prevents the surface expression of SUR 1 monomers and Kir 6.x tetramers (Zerangue *et al.*, 1999). This sequence causes the retention of partial complexes in the ER, but when the subunits come together in the correct octameric stoichiometry this signal is masked and the subunits are able to insert in the cell membrane. In Kir 6.2 the RKR sequence is located in the C-terminus, which explains why the truncated version is able to form functional channels without the need for SUR co-expression.

4.6.2. K^+ selectivity of the cloned K_{ATP} channel

Increasing the extracellular potassium concentration shifted the reversal potential to more positive values and fitting a line to this data predicted an intracellular $[K^+]$ of about 110mM. The actual intracellular $[K^+]$ of *Xenopus* oocytes is not documented but a range of values between 90 and 150mM have been quoted in the literature (Surah-Narwal *et al.*, 1999,

Drain *et al.*, 1998), and the value observed here, of 110mM, does not seem radically different. The mean values for E_{rev} deviated most from those predicted for a K^+ selective electrode at low extracellular $[K^+]$. This deviation maybe due to the K_{ATP} channels low permeability to Na^+, with P_{Na}/P_K being equal to about 0.01 (Aidley and Stanfield, 1996). This effect was not observed at high extracellular $[K^+]$ but at lower $[K^+]$ as the movement of Na^+ into the cell causes the E_{rev} to become more positive.

Inward rectifiers pass current more readily in the inward direction with outward currents being largely blocked (Standen *et al.*, 1978; Woll *et al.*, 1989). The different classes of inward rectifier exhibit differing degrees of rectification (see Baukrowitz, 2000 for review). The Kir 6.xs are weak inward rectifiers with much of the rectification being involved with block from Mg^{2+}. The current/voltage relationship shown by SUR 2B/Kir 6.2 was characteristic of an inward rectifier, exhibiting block of outward current at voltages positive to E_k. However, the degree of rectification observed here appears slightly stronger than that reported for SUR 2B/Kir 6.2 expressed in other cell lines such as HEK293T (Shindo *et al.*, 1998) and cosm6 (Babenko *et al.*, 1998), and K_{ATP} channels of native tissue (Sakura *et al.*, 1995). This difference in rectification may be due to the expression system. Previous investigation of this channel in *Xenopus* oocytes has also shown this channel to exhibit stronger rectification, and this maybe due to higher intracellular concentrations of Mg^{2+} (Gribble *et al.*, 1997b). Block by spermine and spermidine is thought to be mainly associated with strong inward rectifiers (Ficker *et al.*, 1994; Lopatin *et al.*, 1994; Fakler *et al.*, 1995) such as Kir 2 and 3, but recently a residue in the C-terminus of Kir 6.x has been identified which interacts with spermine (Baukrowitz *et al.*, 1999). The interaction is pH dependent and in alkaline conditions can change the channel from weak to strongly rectifying. These changes were observed in the physiological range of pH and so may explain the rectification observed here.

4.6.3. Sensitivity to the KCO pinacidil

As discussed previously, different isoforms of the SUR subunit confer differing KCO sensitivities on the K_{ATP} channel. It was observed that channels expressing SUR 2B exhibited a greater sensitivity to pinacidil than those expressing SUR 2A. The EC_{50} value generated for pinacidil in activation of the SUR 2B/Kir 6.2 channel was 17.7±1.4 μM which is greater than other values reported in the literature, for example; 2 μM, by Shindo *et al.*, 1998. Although, the Hill coefficient of 1.4 was found to be similar. The difference in sensitivity observed here for pinacidil to that reported for this channel expressed in other systems may primarily be due to differences between the expression systems. As these experiments were undertaken in the whole cell configuration intracellular ATP levels could not be controlled, it has been reported that changes in ATP concentration can affect the induction of K_{ATP} current by pinacidil, with the drug being less effective at higher intacellular concentrations of ATP ($[ATP]_i$) (Nakayama *et al.*, 1990; Quayle *et al.*, 1995). If the $[ATP]_i$ for Xenopus oocytes was higher than that in other cells used in expression for previously reported experiments this may explain why the EC_{50} has been shifted to a higher concentration. This could be further investigated by patching the oocytes which would allow control of the $[ATP]_i$.

An EC_{50} for SUR 2A/Kir 6.2 was not determined as 1mM did not appear to maximally activate the channel and the solubility of the drug did not allow higher concentrations to be investigated. An EC_{50} value of 10 μM has been previously reported for this channel when expressed in HEK293T cells (Okuyama *et al.*, 1998; Shindo *et al.*, 1998), again the differences observed here maybe due to the alternative expression system used. Overall the differences in sensitivity of channels containing SUR 2B and SUR 2A to pinacidil was similar to those previously observed, with SUR 2B being more sensitive than SUR 2A. Current generated by pinacidil was inhibited by the application of glibenclamide for all cloned

channels. Glibenclamide is a specific blocker of K_{ATP} channels, and so provides evidence that the expressed channels were K_{ATP} channels.

The K^+ selectivity and KCO sensitivity of the cloned channels observed here were similar to that observed in other studies and so support the use of the *Xenopus* expression system in the investigation of cloned K_{ATP} channels. However, due to the nature of K_{ATP} channel activation, ie the dependence on intracellular nucleotide concentration, whole cell voltage clamp may not be a suitable method for the investigation of concentration relationships of KCOs.

4.6.4. Diazoxide

Some KCOs have been found to exhibit tissue selectivity, for example; BMS-182264 (Atwal, 1994) and diazoxide (Weik and Neumcke, 1990), and it was thought that this may be related to the existence of different receptor subtypes. On further investigation this hypothesis has been substantiated with the cloning of a number of sulphonylurea subtypes which interact with Kir in the regulation of K_{ATP} channels (Aguilar-Bryan *et al.*, 1995; Isomoto *et al.*, 1996; Inagaki *et al.*, 1996). Inagaki and co-workers (1996) cloned an isoform of SUR1 which was found to reconstitute the major pharmacological properties of the cardiac and skeletal K_{ATP} channel. The skeletal and cardiac K_{ATP} channels are not activated by diazoxide (Weik and Neumcke, 1990) and neither were channels formed by another isoform, which they named SUR 2A. Diazoxide was found to activate SUR 2B and SUR 1 containing channels (Ammala *et al.*, 1996; Isomoto *et al.*, 1996) but not SUR 2A containing channels (Inagaki *et al.*, 1996; Okuyama *et al.*, 1998). This sulphonylurea selectivity of diazoxide was also observed here where it failed to activate SUR 2A/Kir 6.2 channels even after metabolic poisoning with CCCP, but did cause a further activation of SUR 2B/Kir 6.2 channels after metabolic poisoning. It is thought that the effect of diazoxide is dependent on the presence of MgADP

(Larsson *et al.*, 1993; Matsuoka *et al.*, 2000) and mutations in the nucleotide binding folds of the diazoxide sensitive sulphonylurea receptor, SUR 1, have been found to alter the channels response to the compound to that observed for SUR 2A (Shyng *et al.*, 1997b). As SUR 2B and 2A only differ in their last 42 amino acids it would be reasonable to assume that this region is somehow involved in the mechanism of channel activation by diazoxide. It has been suggested that the last 42 amino acids in SUR 2A somehow obstruct the binding of ADP to the second nucleotide binding fold (see chapter 5 for further discussion), and if diazoxide mediates its effect through the binding of ADP this would provide an explanation for the different sensitivities exhibited by SUR 2A and 2B.

4.6.5. Minoxidil Sulphate

The action of the potassium channel opener, minoxidil sulphate (MS), on cloned K_{ATP} channels was investigated. MS did not illicit K_{ATP} curent in *Xenopus* oocytes expressing SUR 2A/ Kir 6.2, SUR 2B/ Kir 6.1 or SUR 2B/ Kir 6.2. Reasons for this lack of effect may include; i) the binding site of the compound is intracellular and it is unable to gain entry to the oocyte to exert its effect, ii) activation may be dependent on intracellular nucleotide concentration, both of these points could be investigated by patch clamping of the membrane, controlling intracellular nucleotide concentration and applying the compound directly to the intracellular surface, iii) higher concentrations of the compound than those used here may be needed to produce channel activation, iv) the concentration used here may be inhibitory, v) the compound may not act on K_{ATP} channels. This last reason maybe the most probable as although minoxidil sulphate has been shown to activate K^+ channels studies have been unable to confirm the exact class of K^+ channel involved in mediating its effects (Leblanc *et al.*, 1989). In pancreatic beta-cells it has actually been found to cause the inhibition of K_{ATP} current, and has also been found to exert no effect on noradrenaline precontracted rat

mesenteric artery or rabbit aorta (Garrino *et al.*, 1989; Wickenden *et al.*, 1991) where you would expect to observe relaxation on K_{ATP} channel activation. Also relaxant effects produced by the compound on precontracted guinea pig airways were not antagonised by glibenclamide, inferring that this effect was not due to the activation of K_{ATP} channels (Buchheit *et al.*, 2000). This evidence does not altogether clarify the involvement of the K_{ATP} channel in mediation of the effects of minoxidil sulphate, and the lack of activation observed here cannot be taken to mean that this compound does not interact with K_{ATP} channels in native tissue.

Chapter 5

Metabolic inhibition and cloned K_{ATP} channels

Chapter 5. Metabolic inhibition and cloned K_{ATP} channels

5.1. Introduction

The classical potassium channels openers such as pinacidil and diazoxide mediate their effects by direct binding to the channel. Another way of causing channel activation is to alter the concentration of ATP and ADP making use of the channels inate sensitivity to these compounds. This can be achieved by metabolically poisoning the cell, using agents such as; azide, carbonyl cyanide m-chlorophenyl hydrazone (CCCP or FCCCP), rotenone, and nitric oxide. These compounds exert their effects on K_{ATP} channel activity by altering the functioning of the electron transport chain causing a decrease in the ATP/ADP ratio. The decreasing of ATP concentrations below that required to inhibit the channel and the increase of the stimulator ADP leads to the activation of the channel. The target of these compounds is the mitochondrial electron transport chain, where they can affect ATP production. Electron transport and oxidative phosphorylation are mediated by five multimeric complexes (I-V) that are embedded in the mitochondrial inner membrane. Rotenone and the oral antihyperglycaemic dimethylbiguanide (metformin) both inhibit respiratory complex I (NADH: Ubiquinone oxidoreductase) which catalyses electron transport from NADH to ubiquinone (El-Mir *et al.*, 2000, Barrientos and Moraes, 1999). Nitric oxide specifically inhibits cytochrome oxidase at low concentrations and other respiratory chain complexes at higher concentrations (Brown, 1999). FCCP, a proton ionophore, exerts its effects by uncoupling electron transport from ATP synthesis. Whichever approach is used the result is that ATP production is reduced or stopped and together with the consumption of the present ATP leads to an overall decrease in the cellular concentration of ATP. However not all K_{ATP} channels seem to be equally sensitive to activation by metabolic inhibition, for example; nitric oxide exhibits K_{ATP} channel selectivity, activating mitochondrial but not sarcolemmal K_{ATP} channels (Sasaki *et al.*, 2000). This is interesting as it seems to point to the SUR subunit either

contributing to the channels sensitivity to ATP or ADP or that the compounds used to metabolically inhibit the cell are activating the channel directly or via some kind of second messenger. This concept is especially interesting as the Kir subunit is thought to control ATP sensitivity of the channel.

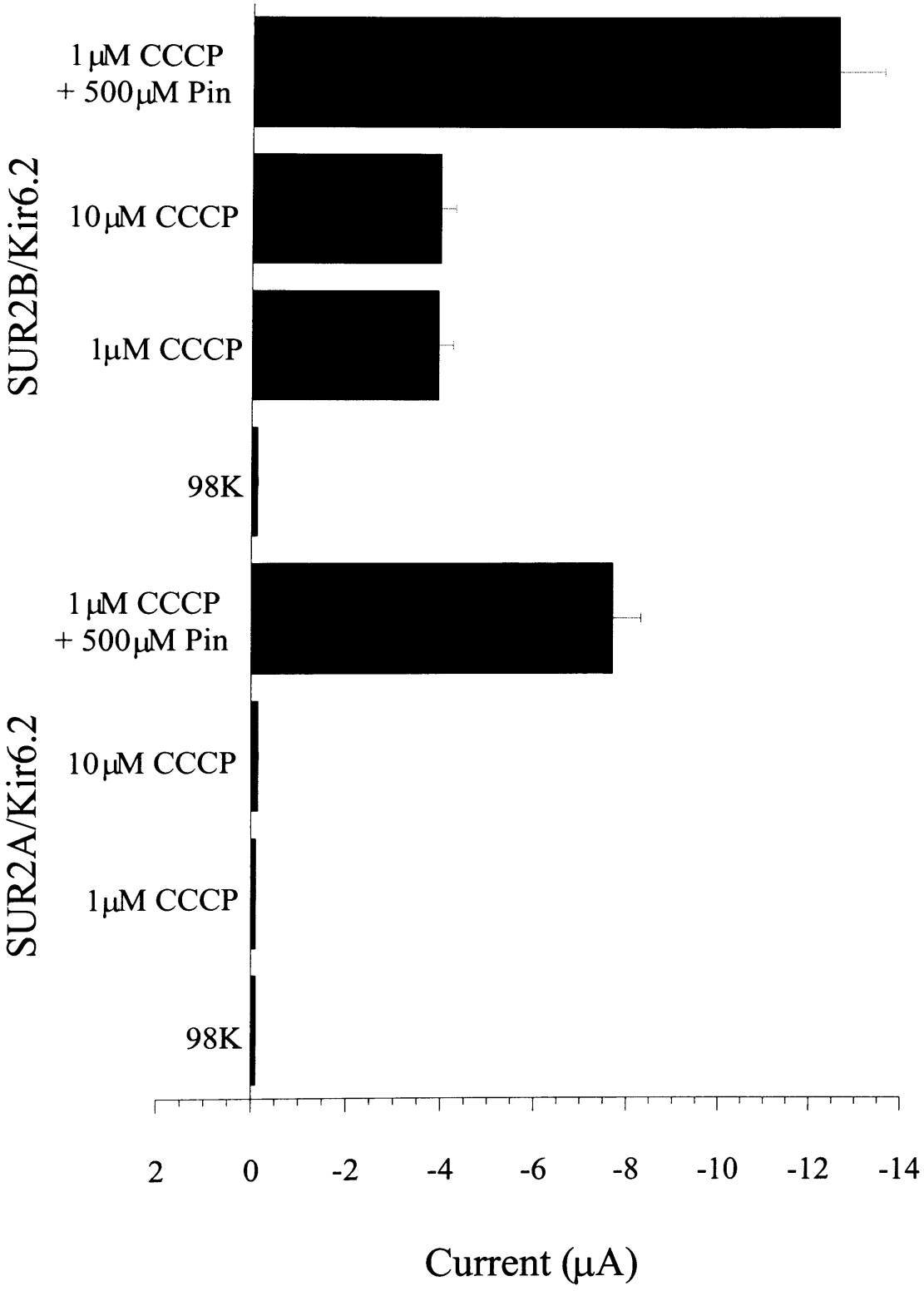
5.2. Effect of channel activation by CCCP

The subunit combinations SUR 2B/ Kir 6.1 (data not shown), SUR 2B/ Kir 6.2, and SUR 2A/ Kir 6.2 were expressed in *Xenopus* oocytes. The uncoupler of electron transport CCCP, in concentrations of 1 and 10 μM, was used as a metabolic poison. Both concentrations of CCCP caused channel activation, which was observed as an increase in current, in oocytes expressing SUR 2B. Conversely, no channel activation was observed in oocytes expressing SUR 2A. 1 μM seemed to be maximally effective for channels containing SUR 2B/ Kir 6.2, with application of 10 μM only causing a slight further increase in current. The current observed in SUR 2A expressing oocytes after application of CCCP was not significantly different from that observed in the $98K^+$ solution alone. 500 μM pinacidil was applied to the oocytes after CCCP to confirm channel expression. Pinacidil caused SUR 2A/ Kir 6.2 channel activation. In oocytes expressing SUR 2B/ Kir 6.2 the current generated by CCCP was increased more than two fold on addition of pinacidil (see fig. 5.1.).

Figure. 5.1.

Metabolic inhibition, dependence on the SUR 2 subunit.

The mean current values from whole cell recordings at a holding potential of –60 mV are shown. Oocytes expressing the cloned channels SUR 2B/ Kir 6.2 (n= 6) and SUR 2A/ Kir 6.2(n= 7) were metabolically inhibited with 1 and 10 μM CCCP. 500 μM Pinacidil was used to confirm channel expression.


1 μM CCCP
+ 500 μM Pin
10 μM CCCP
1 μM CCCP
98K
1 μM CCCP
+ 500 μM Pin
10 μM CCCP
1 μM CCCP
98K
SUR2B/Kir6.2
SUR2A/Kir6.2
2
0
-2
-4
-6
-8
-10
-12
-14
Current (μA)

5.3. Construction of SUR 2B carboxy-terminal truncations

To investigate the differences in sensitivity to metabolic inhibition shown by the two sulphonylurea receptor subtyptes, C-terminal deletions were made in SUR 2B (see fig. 5.2. for representation of SUR 2B truncations). SUR 2A and 2B differ in the last 42 amino acids of the C-terminus and it was hoped that by expression of truncated versions of SUR 2B the metabolic inhibition observed for this channel could be affected, and so indicate a region inovolved in this mechanism.

PCR was used to replicate the carboxy terminus of SUR 2B and introduce premature termination codons by inclusion in the downstream primer. These PCR fragments were then cloned into SUR 2B replacing the existing sequence and producing SUR 2B clones with varying C-terminal deletions. A unique *Mfe*1 restriction site was created, by site-directed mutagenesis using the GeneEditor™ in vitro Site-Directed Mutagenesis System from Promega, in the existing SUR 2B clone in order that the PCR fragments may be ligated into the plasmid between *Mfe*1 and *Cla*1. The resulting SUR 2B clone with a unique *Mfe*1 site was identified by restriction enzyme analysis and further confirmed by sequencing. The upstream (5') primer was positioned upstream of the *Mfe*1 site, and the downstream (3') primers incorporated a termination codon in frame with the SUR 2B sequence and included a *Cla* 1 restriction site. A series of PCR products were generated with these primers. The PCR reaction was carried out using BIOLASE Diamond™ (Bioline), and the conditions consisted of a denaturation step at 94°C for two minutes then 20 cycles consisting of a denaturation step at 94°C for 15 seconds, an annealing step at 58°C for 30 seconds and an extension step at 68°C for 40 seconds. The resulting products were then ethanol/sodium acetate precipitated overnight at -80°C, resuspended in TE buffer and digested with *Mfe*1 and *Cla*1 overnight. The relevant fragments were then gel purified and ligated into the *Mfe*1/*Cla*1 digested SUR 2B fig. 5.2.

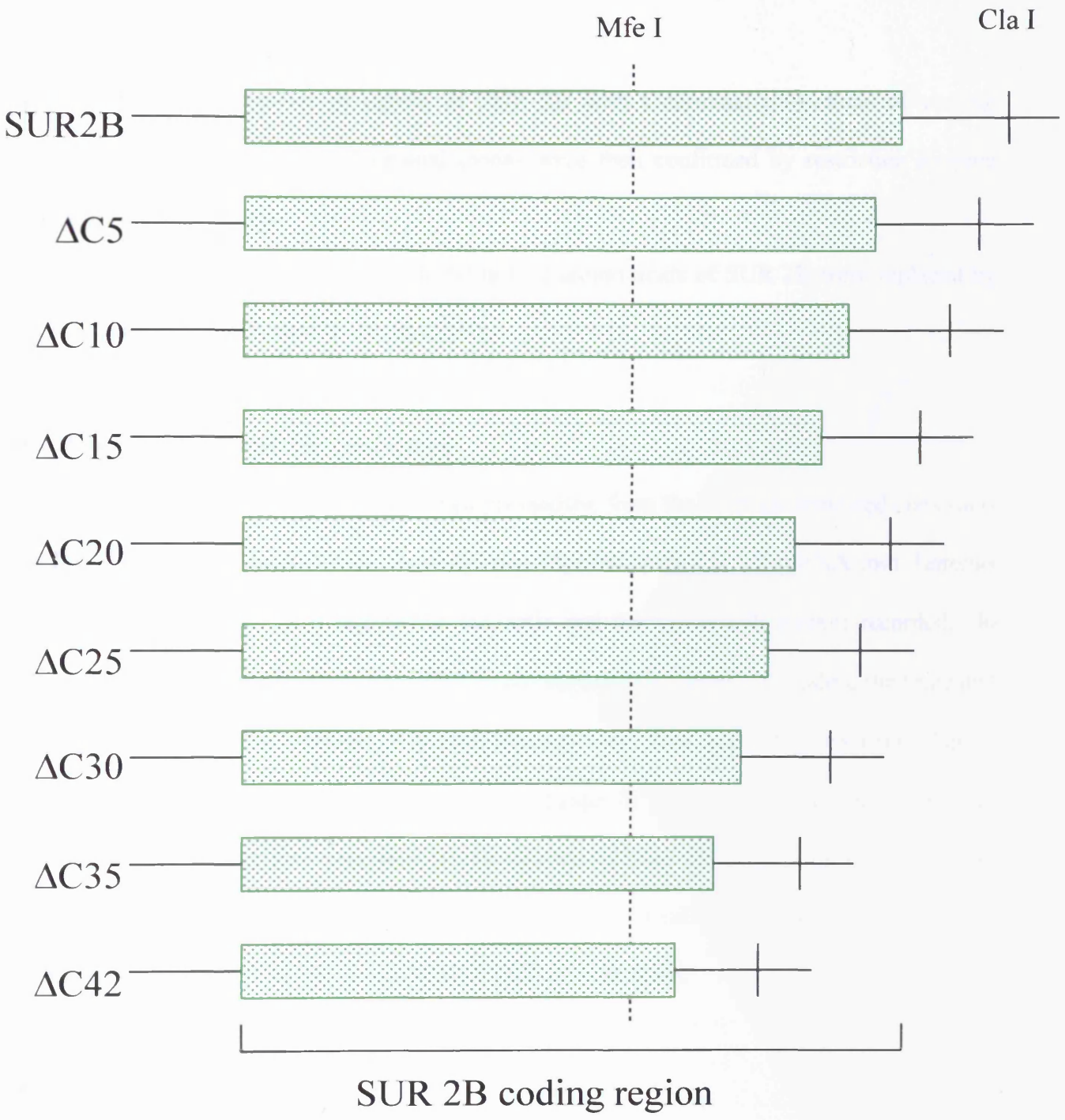


Figure. 5.2.
Nomenclature and topology of SUR2B C-terminal truncations (not to scale). The SUR coding region is indicated by a rectangular box with the construct names indicating the number of amino acid residues removed in each construct. The PCR products were cloned between the Mfe I and Cla I restriction sites as indicated.

plasmid to give a number of clones of SUR 2B with C-terminal truncations of varying lengths. The length of these truncated clones were then confirmed by restriction enzyme analysis and sequencing.

A chimeric construct in which the last 22 amino acids of SUR 2B were replaced by SUR 2A was also generated by Dr. Lodwick.

5.4. Expression of SUR 2B truncations

mRNA was generated, as described previously, from the SUR 2B truncated constructs and SUR 2A/2B chimera. These were injected together with Kir 6.2 mRNA into *Xenopus* oocytes. 10 μM CCCP was applied to the cells and the whole cell current recorded. No change in current was observed from either cells expressing channels including the truncated SUR 2B or the 2B/2A chimera. 500 μM pinacidil was then applied to confirm whether channels were being expressed, it elicited no change in current. These experiments were performed on five separate occasions taking recordings from ten oocytes on each occasion. Wild type SUR 2B/ Kir 6.2 channels were also expressed to validate the expression, these did form channels which could be activated by CCCP and pinacidil.

5.5. Discussion

Metabolic poisoning of oocytes by CCCP caused activation of SUR 2B/ Kir 6.2 and SUR 2B/ Kir 6.1 but not SUR 2A/ Kir 6.2 channels. It is the Kir 6.x subunit that is thought to confer ATP sensitivity on the K_{ATP} channel, therefore this result seems quite surprising. The differing sensitivities of the SUR subunit either indicate a direct activation of the channel by CCCP or that the SUR subunit somehow sensitises the Kir 6.x subunit to inhibition by ATP or activation by ADP. The different sensitivities of the SUR subunits to metabolic poisoning have been further confirmed by Liss and co-workers (1999), who showed that dopiminergic

SN neurones exhibited differing sensitivities to rotenone with those most sensitive expressing SUR 1 /Kir 6.2 and the least sensitive expressing SUR 2B/ Kir 6.2. As SUR 2B and SUR 2A only differ in the last 42 amino acids we investigated whether channel activation by metabolic inhibition was due to the binding of CCCP or some second messenger to this region. A number of C-terminally truncated SUR 2B clones were made and expressed with Kir 6.2. It was hoped that after the truncation of a region important for activation by CCCP a loss of function would be observed. Unfortunately no K_{ATP} current could be illicited from oocytes expressing these clones. The failure to express truncated constructs of SUR 2B may be due to the deletion of an anterograde signal when creating the constructs. An anterograde signal has since been identified in SUR 1 (Sharma *et al.*, 1999) that was required for the exit of subunits from the ER/cis-Golgi compartments and transit to the cell surface. It is likely that this signal is also present in SUR 2B and 2A. After acknowledging this problem it was decided to construct a chimera of SUR 2B/2A, replacing the last 42 amino acids of SUR 2B with SUR 2A to see if the sensitivity to metabolic inhibition could be knocked out. Hopefully by not deleting the anterograde signal this construct would be able to transit as normal to the cell membrane. Unfortunately this chimera was not expressed successfully. Tagging with green fluorescent protein would have allowed the localisation of the protein to be observed, but was not attempted here.

Since this work was undertaken a number of studies have implicated the SUR subunit in enhancing the sensitivity of Kir 6.x to ATP and ADP. The SUR subunits have been shown to have differing sensitivities to ATP and ADP, with SUR 1 and SUR 2B being more sensitive to activation by ADP and inhibition by ATP than SUR 2A (Okuyama *et al.*, 1998, Babenko *et al.*, 1999b, Matsuoka *et al.*, 2000, Matsuo *et al.*, 2000). Chimeric constructs of SUR 1 and SUR 2A in which the last 22 amino acids have been exchanged have implicated this region in endowing the different sensitivities of the SUR subunit to ADP and ATP

(Babenko *et al.*, 1999a, Matsuoka *et al.*, 2000). However, mutations of the second nucleotide binding domain in SUR 1 and SUR 2B also show properties similar to the chimeric construct SUR 1 with C42 SUR 2A, implicating the C-terminal region in inhibition of ADP binding to NBD2 (Matsuoka *et al.*, 2000). From this evidence the different sensitivities of the SURs to metabolic inhibition appears to be due to the differences in their C-terminus with the lack of activation of SUR 2A by CCCP more likely due to interference with ADP binding by the last 42 amino acids rather than the lack of a direct binding site for the metabolic inhibitor or any second messenger. Therefore K_{ATP} channel activation by metabolic inhibition does appear to be through the alteration of the intracellular ATP/ADP ratio and not through the direct activation of the channel by the metabolic inhibitor or some second messenger produced in response to cellular poisoning.

Chapter 6

Investigating the selectivity of the morpholinoguanidine PNU-37883A

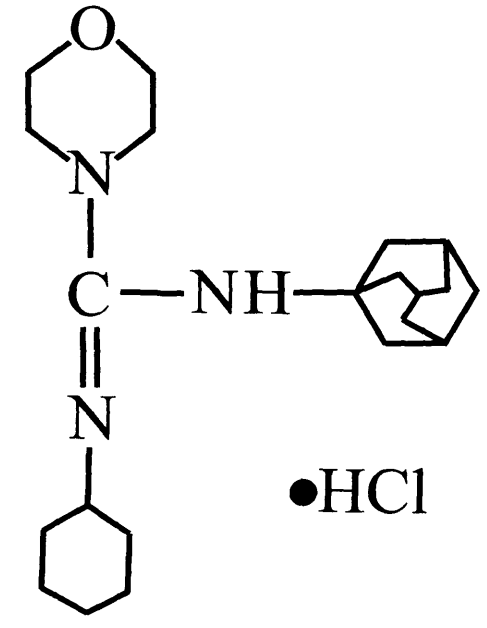
Chapter 6. Inhibition of cloned K_{ATP} channels by the morpholinoguanidine PNU-37883A

6.1. Introduction

The K_{ATP} channel blocker 4-morpholinecarboximidine-N-1-adamantyl-N'-cyclohexylhydrochloride, more commonly referred to as PNU-37883A, was the first non-sulphonylurea shown to block the pharmacological response to various potassium channel openers (see fig. 6.1. for chemical structure). *In vivo* it has been shown to reverse hypotension and *in vitro* to anatagonise vasorelaxation, produced by potassium channel openers (Meisheri *et al.*, 1993). It inhibits KCO induced K_{ATP} channel opening rather than preventing actual opening (Smith *et al.*, 1994), reducing the open probability without modification of the single-channel current amplitude (Guillemare *et al.*, 1994).

The K_{ATP} channel of the thick ascending limb of the loop of Henle plays a pivotal role in Na^+ reabsorption (Ludens *et al.*, 1995a) and clinically PNU-37883A is known as a K^+ sparing eukalemic diruetic. It also lowers plasma renin activity without affecting vascular resistance, blood flow or glomerular filtration rate (Humphrey and Ludens, 1998; Vallon *et al.*, 1998).

The compound exhibits selective inhibition of K_{ATP} channels in various tissues, for example Guillemare and co-workers observed block of *Xenopus* oocyte follicular cell K_{ATP} channels, which resemble K_{ATP} channels of vascular smooth muscle, but not those of insulinoma cells (Guillemare *et al.*, 1994). Ludens and co-workers have also shown the compound to be ineffectual at blocking K_{ATP} channels of pancreatic beta-cells while being more potent than glybenclamide on K_{ATP} channels of vascular smooth muscle and renal tubules (Ludens *et al.*, 1995b). It has been shown to potentiate the effect of glybenclamide synergistically, inferring that the two compounds act on different sites on the K_{ATP} channel (Ohrnberger *et al.*, 1993).



PNU-37883A

Figure. 6.1.
Chemical structure of PNU-37883A.

More recently a comprehensive study was undertaken by Wellman and co-workers which focused on characterising the selectivity of the compound for K_{ATP} channels of vascular smooth muscle cells over cardiac myocytes and skeletal muscle cells (Wellman *et al.*, 1999). They found that the K_{ATP} currents of cardiac and skeletal muscle were less sensitive to inhibition by PNU-37883A than those of arterial smooth muscle, with the compound having an IC_{50} of 1 μM in smooth muscle. They also showed that the compound was selective for K_{ATP} channels over Kir and Kv channels in vascular smooth muscle.

As the vascular K_{ATP} channel is proposed to be composed of SUR2B/ Kir 6.1, the cardiac channel of SUR2A/ Kir 6.2, the skeletal muscle channel of SUR2A/ Kir 6.2, and the pancreatic beta-cell channel of SUR1/ Kir 6.2, it appears that the compound may be selective for the Kir subunit, with this subunit either forming the binding site or being modulated by the compound via the SUR subunit. The molecular basis for the difference in sensitivity of the various K_{ATP} channels, to PNU-37883A, was investigated by comparing the action of the compound on K_{ATP} currents induced in oocytes expressing the K_{ATP} channel clones SUR 2B/ Kir 6.1 and SUR 2B/ Kir 6.2.

6.1.1. Control

Firstly the compound was investigated for possible effects on basal current in cells expressing SUR 2B/ Kir 6.1 and SUR 2B/ Kir 6.2. 10 μM PNU-37883A was added to the bath solution and the oocyte perfused for 5 minutes (n = 6, data not shown). No change in basal current was observed, indicating that the compound was not affecting endogenous channels or activating expressed K_{ATP} channels.

6.1.2. Differential inhibition of cloned channels by PNU-37883A, dependence on Kir 6.x expression

The effect of PNU-37883A on K_{ATP} channels formed by the co-expression of SUR 2B/ Kir 6.1 and SUR 2B/ Kir 6.2 was studied (see fig. 6.2.). 10 μM PNU-37883A, in the presence of 100 μM pinacidil, inhibited current in either K_{ATP} channel type. However, SUR 2B/ Kir 6.1 was found to be more sensitive to inhibition than SUR 2B/ Kir 6.2. For instance 10 μM PNU-37883A inhibited pinacidil-induced current by >50% for SUR 2B/Kir 6.1, with a fractional inhibition of 0.7±0.05, but <10% for SUR 2B/ Kir 6.2, with a fractional inhibition value of 0.07±0.03 (see fig. 6.3.)

Concentrations of PNU-37883A between 0.3 μM and 100 μM were used to define the concentration/fractional inhibition relationship for the SUR 2B/ Kir 6.1 channel (see fig. 6.4.). The data was fitted with an equation similar to that used in calculating pinacidil concentration dependence, assuming that in the absence of compound no inhibition occurs and at saturating concentrations complete inhibition would be observed. The IC_{50} derived from this equation was 3.3±0.97 μM with a Hill coefficient of 0.8.

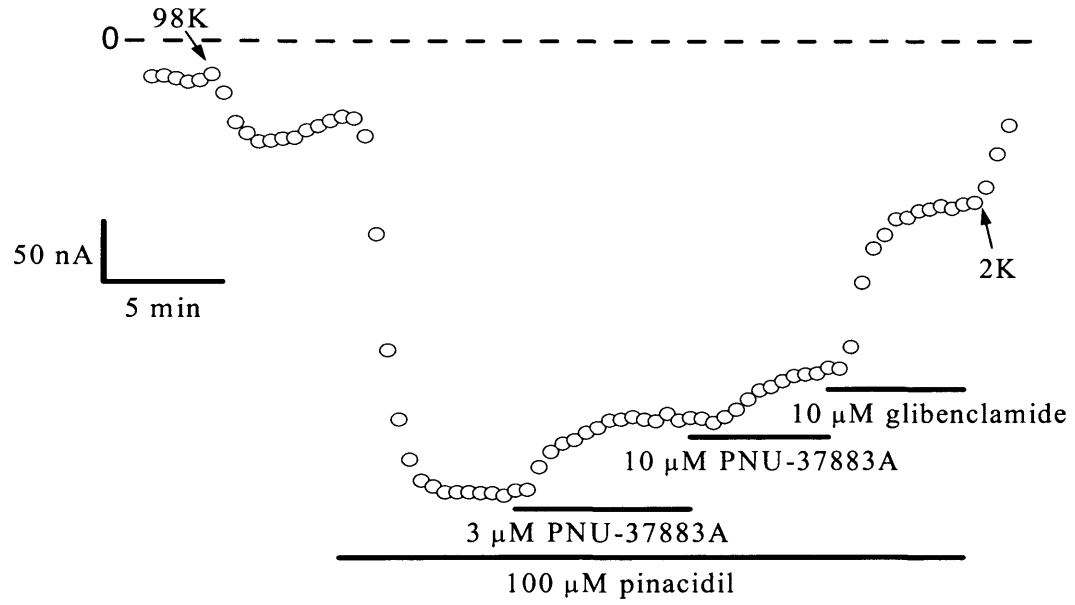
Figure. 6.2.

Effect of PNU-37883A on cloned K_{ATP} channels.

a) Effect of PNU-37883A on K_{ATP} current in an oocyte expressing SUR 2B/ Kir 6.1. The current was measured at 30 second intervals with a holding potential of –60 mV. The bath solution was changed from 2 mM K^+ to 98 mM K^+, as indicated.

b) Effect of PNU-37883A on K_{ATP} current in an oocyte expressing SUR 2B/ Kir 6.2. The current was measured at 30 second intervals at a holding potential of –60 mV.

a)


98K
0
50 nA
5 min
2K
10 μM glibenclamide
10 μM PNU-37883A
3 μM PNU-37883A
100 μM pinacidil

b)

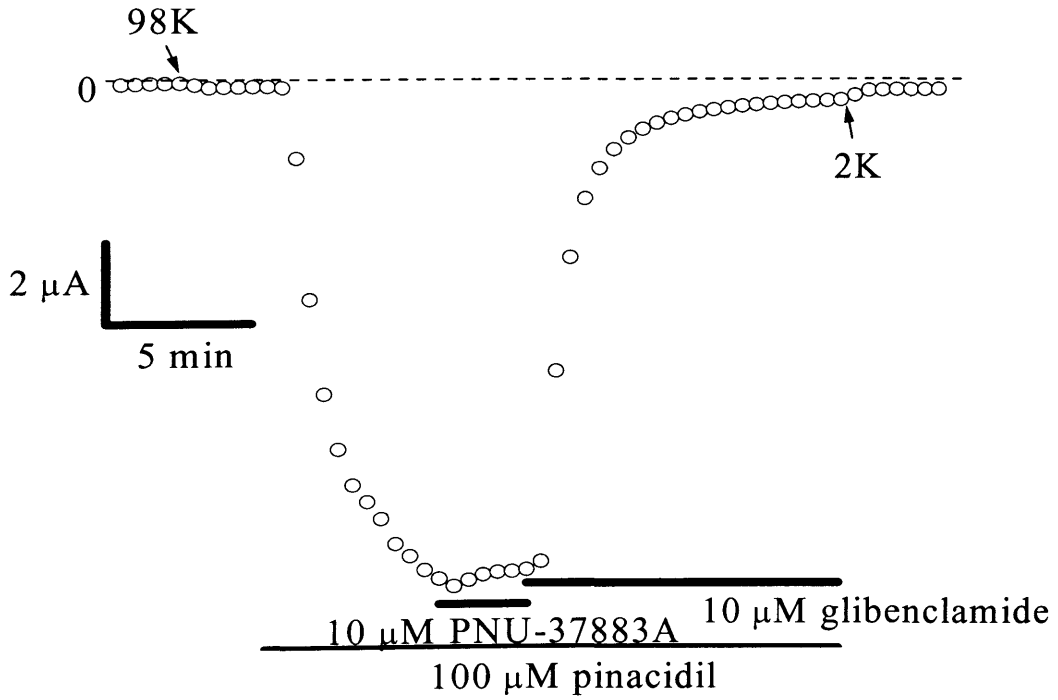
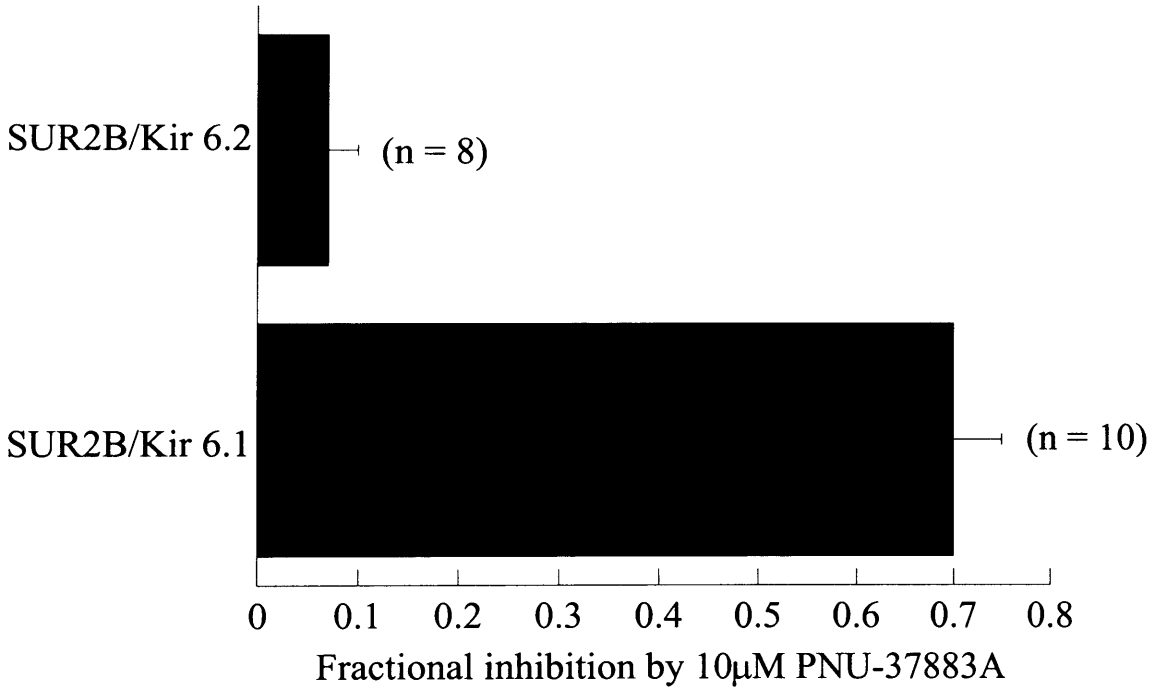

98K
0
2K
2 μA
5 min
10 μM glibenclamide
10 μM PNU-37883A
100 μM pinacidil

Figure. 6.3.

Histogram representing the fractional inhibition of glibenclamide sensitive current by 10 μM PNU-37883A. Comparison of SUR 2B/ Kir 6.2 and SUR 2B/ Kir 6.1 expressing oocytes. n= number of observations

Figure. 6.3.


SUR2B/Kir 6.2
(n = 8)
SUR2B/Kir 6.1
(n = 10)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Fractional inhibition by 10μM PNU-37883A

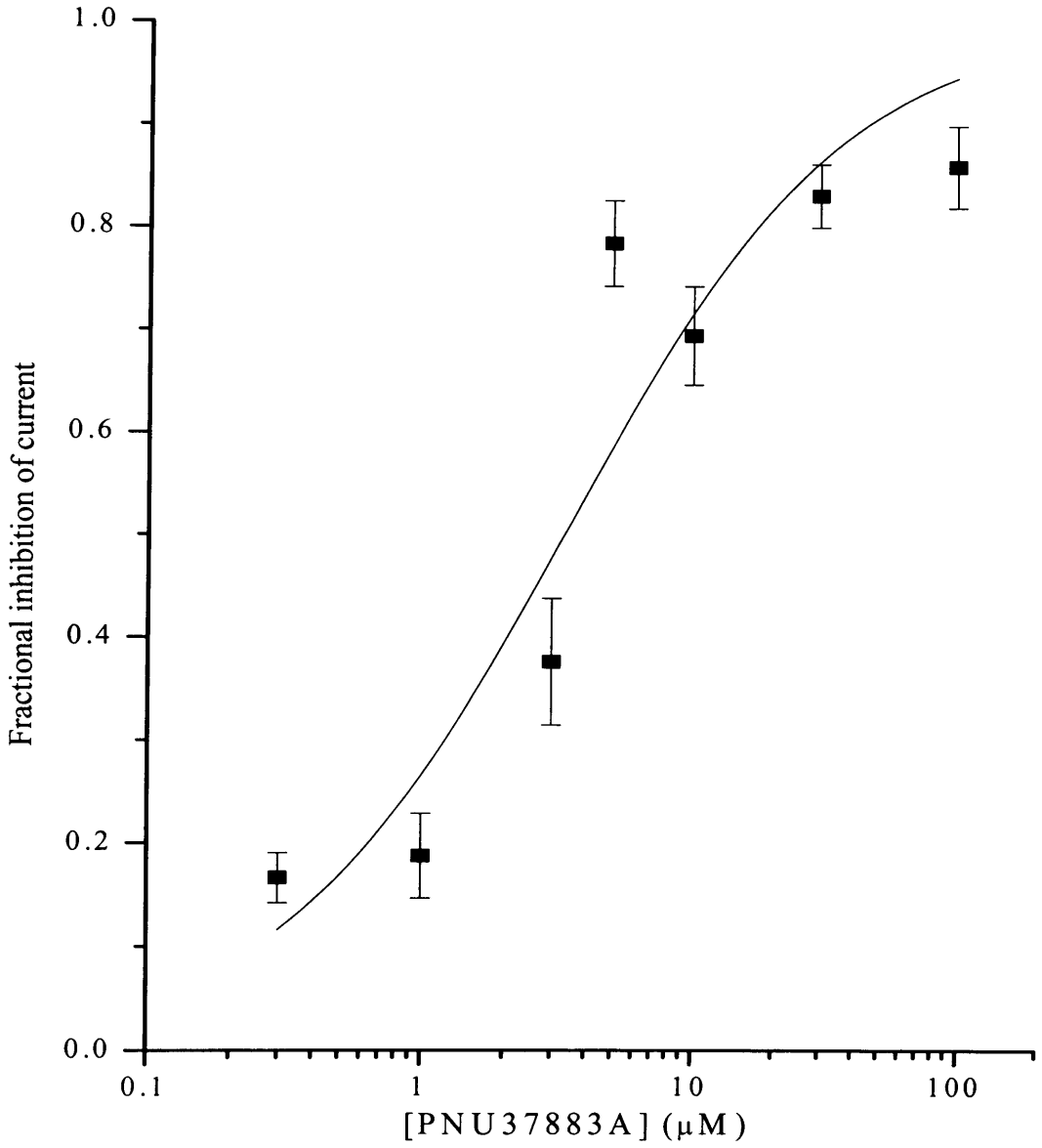


Figure. 6.4.

Data points show the fractional inhibition of 10 μM glibenclamide sensitive current induced by 100 μM pinacidil from channels formed by SUR 2B/ Kir 6.1 (n= 2 to 13 observations at each concentration) the solid line was fitted to the equation:

$$\frac{I_{U37883A}}{I_{PIN}} = \frac{1}{1 + \left[\frac{U37883A}{IC50}\right]^{n}}$$

The half inhibition constant (IC_{50}) of the fitted line was 3.3 μM, with a Hill coefficient (n) of 0.8.

6.1.3. Kir selectivity of PNU-37883A; discussion.

The morpholinoguanidine PNU-3887A has been shown here to be a selective blocker of K_{ATP} current. Unlike other better known K_{ATP} channel blockers, such as glibenclamide, which act upon the sulphonylurea subunit this compound seems to exert its effect through the Kir subunit having a higher affinity for Kir 6.1 over Kir 6.2 (see also Surah-Narwal *et al.*, 1999). As SUR 2B is though to form the vascular K_{ATP} channel together with either Kir 6.1 and/or Kir 6.2 it is interesting to compare the IC_{50} for PNU-37883A gained for SUR 2B/ Kir 6.1 with that of native vascular smooth muscle. The IC_{50} for PNU-37883A of SUR 2B/ Kir 6.1 currents was calculated to be 3.3 μM with a Hill coefficient of 0.8, which was comparable to observations made in arterial smooth muscle (Wellman *et al.*, 1999) of 1 μM. This further supports evidence that proposes the Kir 6.1 subunit to be the inwardly rectifying subunit of the vascular smooth muscle K_{ATP} channel.

6.2. Mapping regions of Kir 6.1 important in PNU-37883A inhibition

6.2.1. Introduction

The construction of chimeric channels has been used by investigators in the identification of regions responsible for certain channel properties, such as conductance or pH dependence, and to elucidate sites of modulation by certain ion channel drugs. By formation of chimeras between a sensitive and non-sensitive channel sites important in drug interactions may be mapped. These investigations may focus on the gain and loss of sensitivity observed after the exchange of particular regions. After areas of importance have been narrowed down mutagenesis of single residues can then be used to investigate single sites that may be responsible for channel modulation or in the formation of a binding site. For example two

groups have investigated the block of the K_{ATP} channel by tolbutamide using chimeric constructs (Ashfield *et al.*, 1999; Babenko *et al.*, 1999c). This drug blocks channels composed of SUR1/ Kir 6.2 with a higher affinity than those composed of SUR2A/ Kir 6.2. The investigators made chimeras of the two different SUR subunits and found that the region of SUR1 encompassing TMDs12-17 was responsible for endowing the channel with sensitivity to tolbutamide. Similar chimeras between SUR1 and SUR2A were also used by D'hahan and co-workers to investigate the interaction of the KCOs cromakalim and diazoxide (D'hahan *et al.*, 1999).

After initial investigation of cloned channel sensitivities to the compound chimeras of Kir 6.x were constructed allowing the site of modulation/binding of PNU-37883A to be investigated.

6.2.2 Construction of Kir 6.x chimeras

As Kir 6.1 appeared to be more sensitive to inhibition by PNU-37883A chimeras of Kir 6.1 and 6.2 were constructed to try and elucidate a possible site for channel modulation. The introduction of unique restriction sites into the sequences of the Kir 6.xs enabled a straight swap of three different regions (see fig.6.5. for schematic indicating restriction sites). The areas swapped were the N-termini, the C-termini, and the M1-H5-M2 regions (see fig. 6.6.)

6.2.2.1. Construction of Kir 6.1/6.2 pore swap

Site directed mutagenesis was used to create a *Sal* I site in Kir 6.2 that corresponded to one present in Kir 6.1, and a *Sph* I site in Kir 6.1 that corresponded to one present in Kir 6.2. The GeneEditor™ *in vitro* Site-Directed Mutagenesis System (Promega) was followed

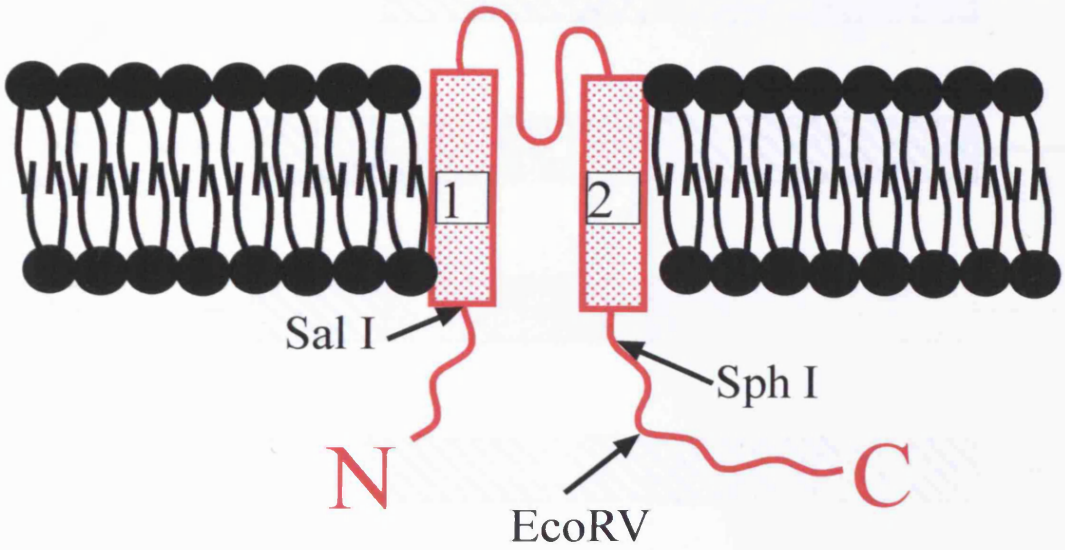


Figure. 6.5.
Schematic of the Kir 6.x subunit. Sites indicated in the diagram reflect corresponding restriction sites occurring in the coding sequence used in construction of the chimeric constructs.

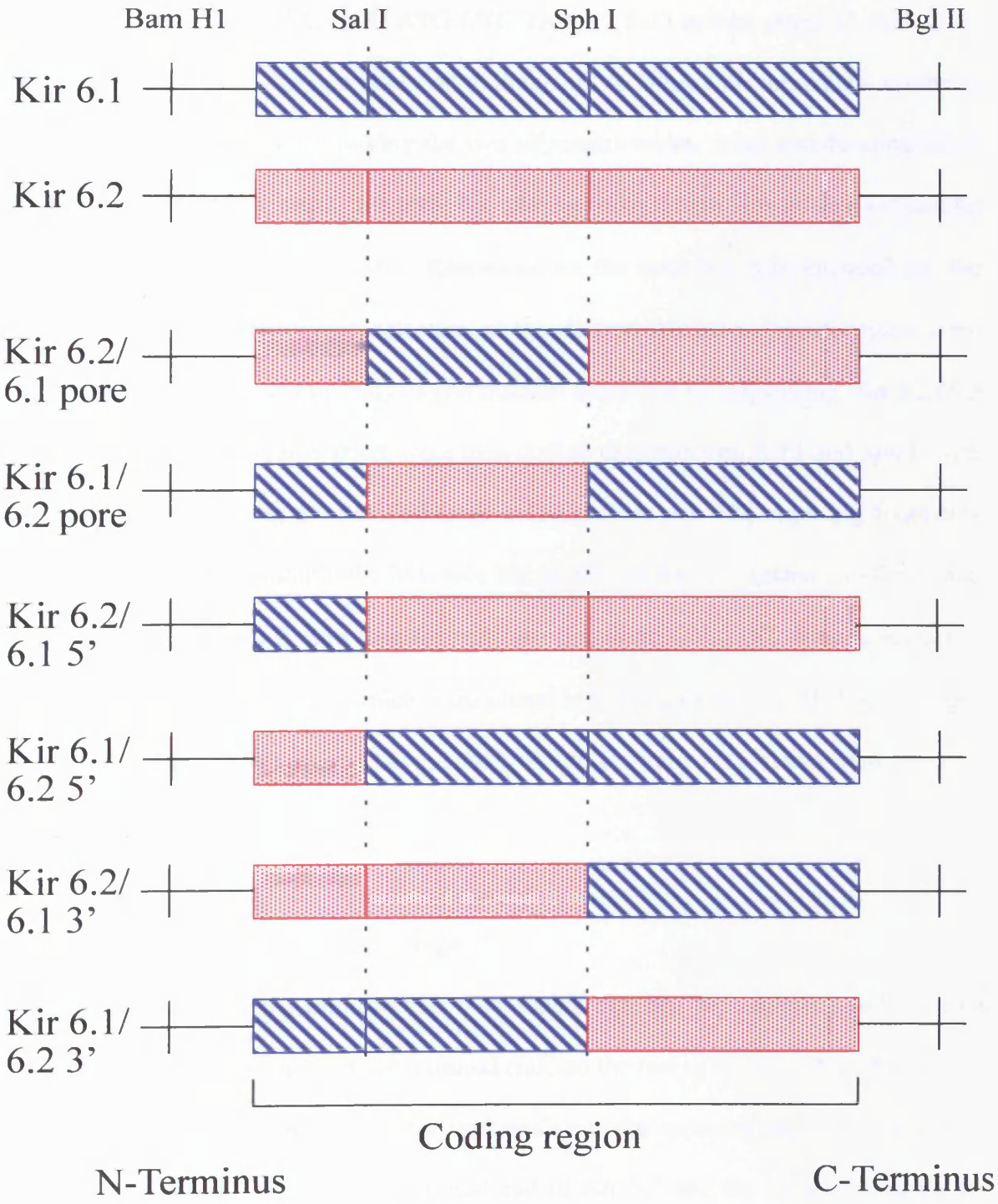


Figure. 6.6.
Nomenclature and topology of chimeric Kir 6.x constructs.
The Kir coding region is indicated by a rectangular box with the 6.1 and 6.2 subunits being presented either striped or shaded respectively.
Restriction sites used for cloning are indicated.

according to the manufacturer's instructions. Selection and mutagenic oligonucleotides (*Sph* I mutant oligo: 5' TTT TCA GCC GGC ATG CTG TAA 3', *Sal* I mutant oligo: 5' CCA CGC TGG TCG ACC TCA AG 3') were annealed to the template DNA with subsequent synthesis and ligation of the mutant strand linking the two oligonucleotides. After transforming DNA into JM109 cells mutants were selected by plating onto ampicillin plates containing GeneEditor™ Antibiotic Selection Mix. Resistance to the selection mix encoded by the mutant DNA strand then facilitated selection of the desired mutation. Transformants were screened using restriction enzyme analysis and mutants identified by sequencing. Kir 6.2 (*Sal* I) clone and Kir6.1 (*Sph* I) clone DNA were then double digested with *Sal* I and *Sph* I, with the resulting fragments being gel purified from a 1% agarose gel. The opposing fragments were ligated together (for example the M1, H5, M2 section of Kir 6.1 ligated into the N and C-terminal regions of Kir 6.2) and transformed into Epicurian Coli® XL-Blue subcloning-grade competent cells (Strategene), which were plated onto LB-carb plates. DNA from single colonies was analysed by restriction enzyme analysis and the pore swap clones confirmed by sequencing.

6.2.2.2. Construction of Kir 6.1/6.2 5' swaps

Kir 6.1 (*Sph* I) and 6.2 (*Sal* I) DNA was double digested with *BamH* I and *Sal* I to obtain fragments corresponding to the N-terminal end and the rest of Kir 6.x. These fragments were gel purified following agarose gel electrophoresis and the opposing fragments ligated to form constructs of Kir 6.1 with the N-terminal end of Kir 6.2 and the equivalent opposite construct. After transformation of the ligation reactions single colonies were screened by restriction enzyme analysis, and correct clones confirmed by sequencing.

6.2.2.3. Construction of Kir 6.1/6.2 3' swaps

Kir 6.1 (*Sph* I) and 6.2 (*Sal* I) DNA was double digested with *Sph* I and *Bgl* II to obtain fragments corresponding to the C-terminal end and the rest of Kir 6.x. These fragments were gel purified following agarose gel electrophoresis and the opposing fragments ligated to form constructs of Kir 6.1 with the C-terminal end of Kir 6.2 and the equivalent opposite construct. After transformation of the ligation reactions single colonies were screened by restriction enzyme analysis, and correct clones confirmed by sequencing.

6.2.3. Inhibition of Kir 6.1/6.2 chimeric constructs and Kir 6.2ΔC by PNU-37883A

Kir 6.x chimeras were expressed with SUR 2B or SUR 1 as indicated. K_{ATP} currents were illicited by the application of 100 μM pinacidil or 100 μM diazoxide with 10 μM CCCP in the case of SUR 2B and SUR 1 containing channels respectively. 10 μM glibenclamide was used to block channel activation. 1 μM CCCP was used in activation, and 1 mM $BaCl_2$ in blocking the Kir 6.2ΔC channel. A holding voltage of –60 mV was applied in all experiments except for those involving Kir 6.2ΔC where a holding voltage of –20 mV was used. 10 μM PNU-37883A was used to block currents generated by the agents mentioned previously. >20% of K_{ATP} current was blocked in channels containing chimeras which included the C-terminus of Kir 6.1 (see fig. 6.7. for histogram representing the fractional inhibition, see fig. 6.8. for representative recording of a chimeric construct). In experiments where channels did not contain the C-terminus of Kir 6.1, including the C-terminal truncation Kir 6.2 ΔC, 10 μM PNU-37883A caused <10% block.

Figure. 6.7.

Histogram representing the fractional inhibition of glibenclamide sensitive current by 10 μM PNU-37883A. Comparison of chimeric Kir 6.x constructs expressed with either SUR 2B (fig. 6.7a) or SUR 1 (fig. 6.7b), and the Kir 6.2 Δ26 C-terminal truncation (fig. 6.7b), in oocytes. n= number of observations.

Key:

A= SUR 2B/ Kir 6.2/6.1pore. (fractional inhibition: 0.06, n=5)

B= SUR 2B/ Kir 6.1/6.2 3'. (fractional inhibition: 0.01, n=6)

C= SUR 2B/ Kir 6.1/6.2 5'. (fractional inhibition: 0.27, n=5)

D= SUR 2B/ Kir 6.2/6.1 3'. (fractional inhibition: 0.48, n=7)

E= SUR 1/ Kir 6.2/6.1 5'. (fractional inhibition: -0.046, n=5)

F= SUR 1/ Kir 6.1/6.2pore. (fractional inhibtion: 0.23, n=6)

G= Kir 6.2ΔC. (fractional inhibtion: 0.05, n=7)

Figure. 6.7.

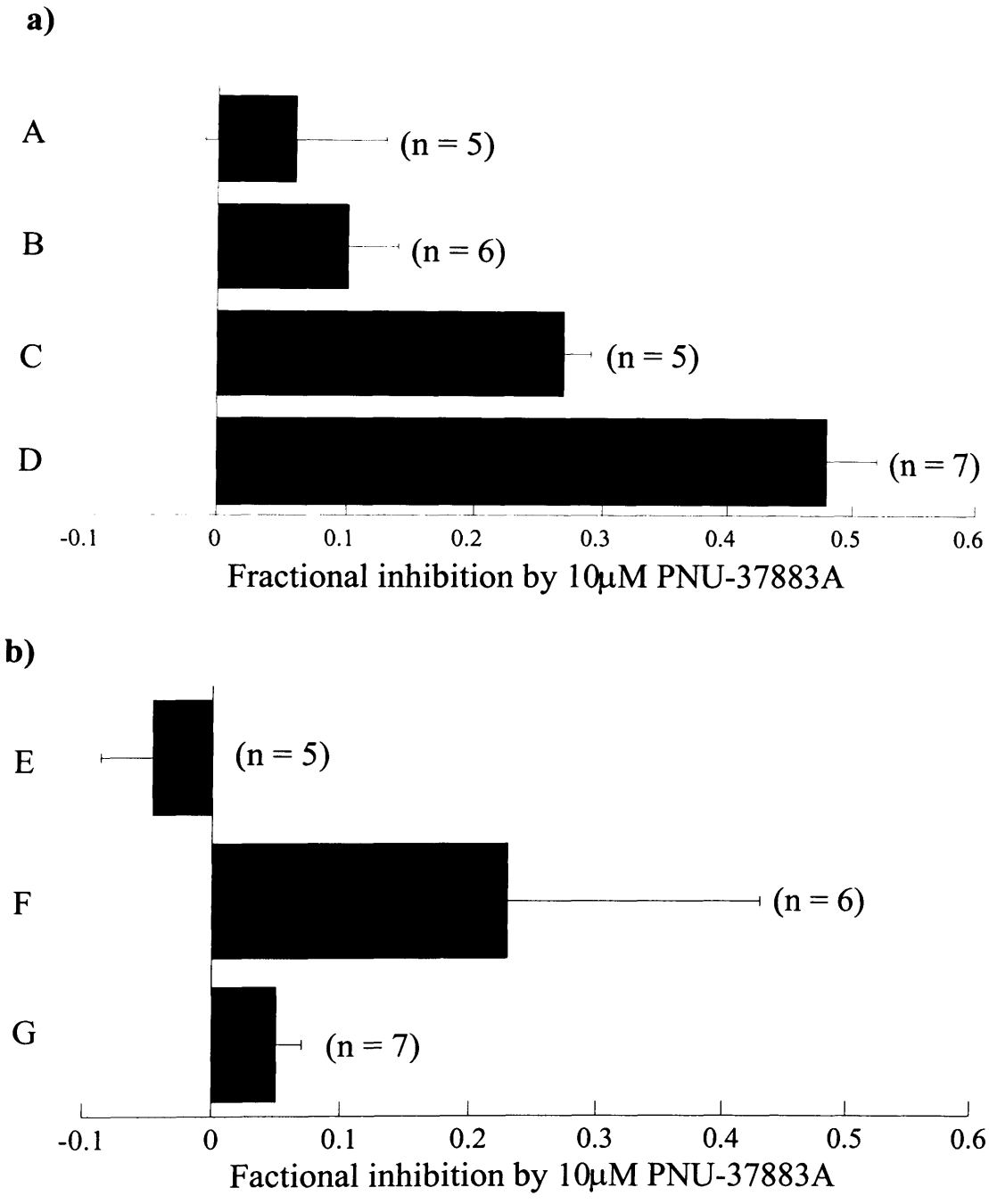
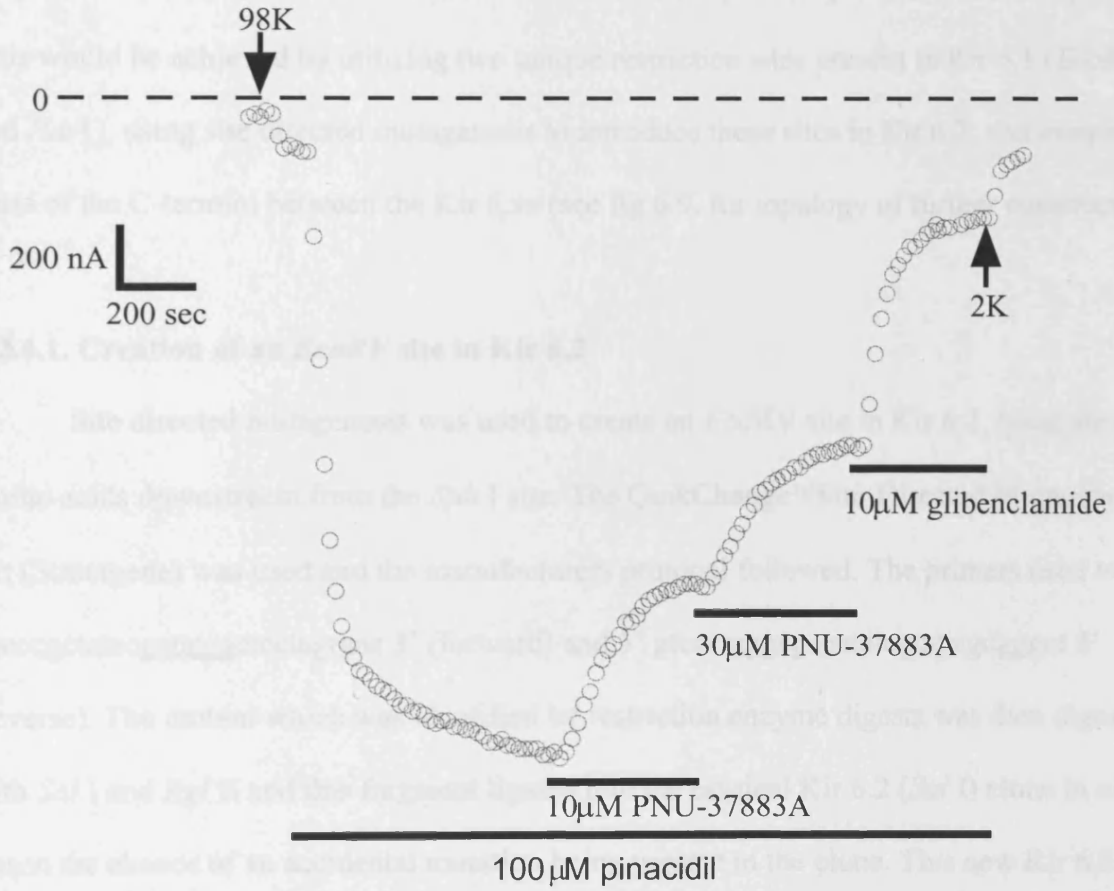

a)
A
B
C
D
(n = 5)
(n = 6)
(n = 5)
(n = 7)
-0.1
0
0.1
0.2
0.3
0.4
0.5
0.6
Fractional inhibition by 10μM PNU-37883A
b)
E
F
G
(n = 5)
(n = 6)
(n = 7)
-0.1
0
0.1
0.2
0.3
0.4
0.5
0.6
Factional inhibition by 10μM PNU-37883A

Figure. 6.8.

Current trace showing K_{ATP} channel inhibition by PNU-37883A.

The trace shows current elicited from an oocyte expressing SUR 2B/ Kir 6.1/6.2 5'. Points represent the whole cell current, at a holding potential of 60 mV, taken at 30 second intervals. 10 μM and 30 μM PNU-37883A was applied to the cell as indicated.

Figure. 6.8.


98K
0
200 nA
200 sec
2K
10μM glibenclamide
30μM PNU-37883A
10μM PNU-37883A
100 μM pinacidil

6.2.4. Localisation of PNU-37883A sensitivity on Kir 6.1: further Kir 6.1/6.2 constructs

The results of inhibition of the chimeric constructs implicated the C-terminus of Kir 6.1 in involvement in the site of action of PNU-37883A. In order to localise the site of action further it was intended to split the C-terminus (after the *Sph* I site) into three further parts. This would be achieved by utilizing two unique restriction sites present in Kir 6.1 (*Eco*RV and *Nar*1), using site directed mutagenesis to introduce these sites in Kir 6.2, and swaping parts of the C-termini between the Kir 6.xs (see fig 6.9. for topology of further constructs).

6.2.4.1. Creation of an *EcoRV* site in Kir 6.2

Site directed mutagenesis was used to create an *Eco*RV site in Kir 6.2, lying about 87 amino acids downstream from the *Sph* I site. The QuikChange™Site-Directed Mutagenesis Kit (Stratagene) was used and the manufacturers protocol followed. The primers used were 5' agcccgctctacgatatcgctcctagtgac 3' (forward) and 5' gtcactaggagcgatatcgtagagcgggct 3' (reverse). The mutant which was identified by restriction enzyme digests was then digested with *Sal* I and *Bgl* II and this fragment ligated into the original Kir 6.2 (*Sal* I) clone in order to lessen the chance of an accidental mutation being present in the clone. This new Kir 6.2 (*Sal* I, *Eco*RV) clone was then sequenced to further confirm the presence of the desired mutation and the absence of additional mutations in the transferred section.

6.2.4.2. Swapping the C-terminal regions of Kir 6.x from *EcoRV* onward

Kir 6.2 (*Sal* I, *Eco*RV) and Kir 6.1 (*Sph* I) were double digested with *Eco*RV and *Bgl*II (which occurs beyond the coding region). The resulting fragments were purified from an agarose gel and the C-terminal fragment of Kir 6.1 ligated to the rest of Kir 6.2 with the C-terminal fragment of Kir 6.2 being ligated to the rest of Kir 6.1. The ligation reactions were

transformed into DH5α™(Gibco BRL) and the transformants screened by restriction enzyme digests and candidate colonies sequenced.

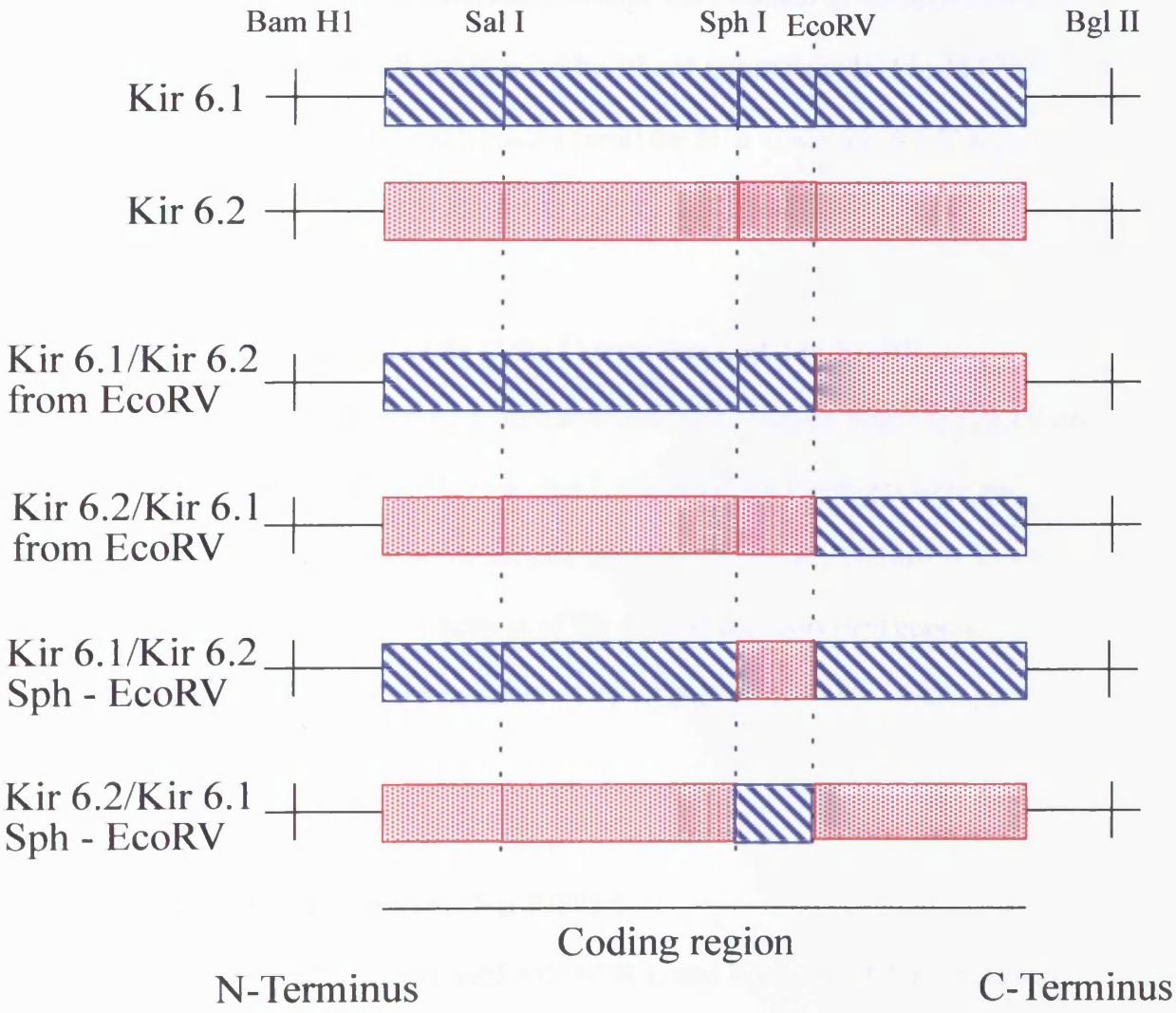


Figure. 6.9.
Nomenclature and topology of further chimeric Kir 6.x constructs.
The Kir coding region is indicated by a rectangular box with the Kir 6.1 and 6.2 subunits being presented either striped or shaded respectively.
Restriction sites used for cloning are indicated.

6.2.5. Inhibition of *Eco*RV chimeras by PNU-37883A

The 3' swaps of Kir 6.1 and 6.2 from the restriction site *Eco*RV onward were expressed with SUR 1. As previously described, currents were elicited by the application of 100 μM diazoxide with 10μM CCCP and blocked by 10 μM glibenclamide. 10 μM PNU-37883A caused a fractional inhibition of 0.06±0.1 (n=8) for SUR 1/ Kir 6.2/ 6.1 3' K_{ATP} currents and 0.35±0.1 (n=9) for SUR 1/ Kir 6.1/ 6.2 3' K_{ATP} currents (see fig. 6.10.).

6.2.6. Swapping the first 87 amino acids of the C-terminus, *Sph* I to *Eco*RV

The Kir 6.x chimeras in which the C-terminus had been swapped from the *Eco*RV site onward were double digested with *Bam*H I and *Sph* I. The resulting fragments were gel purified and ligated into the opposite construct (see fig. 6.9.), to produce constructs which were Kir 6.1 with the *Sph* I to *Eco*RV fragment of Kir 6.2 and the equivalent opposite construct. The ligation reactions were transformed into DH5α™(Gibco BRL) and single colonies sequenced.

6.2.7. Inhibition of the 87AA swaps by PNU-37883A

Kir 6.1 (87AA Kir 6.2) was expressed with SUR 1, and Kir 6.2 (87AA Kir 6.1) was expressed with SUR 2B. Currents were elicited, as described previously, by the application of 100 μM diazoxide with 10 μM CCCP or 100 μM pinacidil and blocked by the addition of 10 μM glibenclamide. 10 μM PNU-37783A caused a fractional inhibition of 0.009 (n=8), in cells expressing the Kir 6.1 (87AA Kir 6.2) construct (see fig. 6.11. for representative recording), and 0.044 (n=5), in cells expressing the Kir 6.2 (87AA Kir 6.1) construct.

Figure. 6.10.

Histogram representing the fractional inhibtion of glibenclamide sensitive current by 10 μM PNU-37883A. Comparison of Kir 6.x chimeras, in which the C-terminus had been swapped from *EcoRV* onwards, expressed with SUR 1. n= number of observations.

Figure. 6.10.

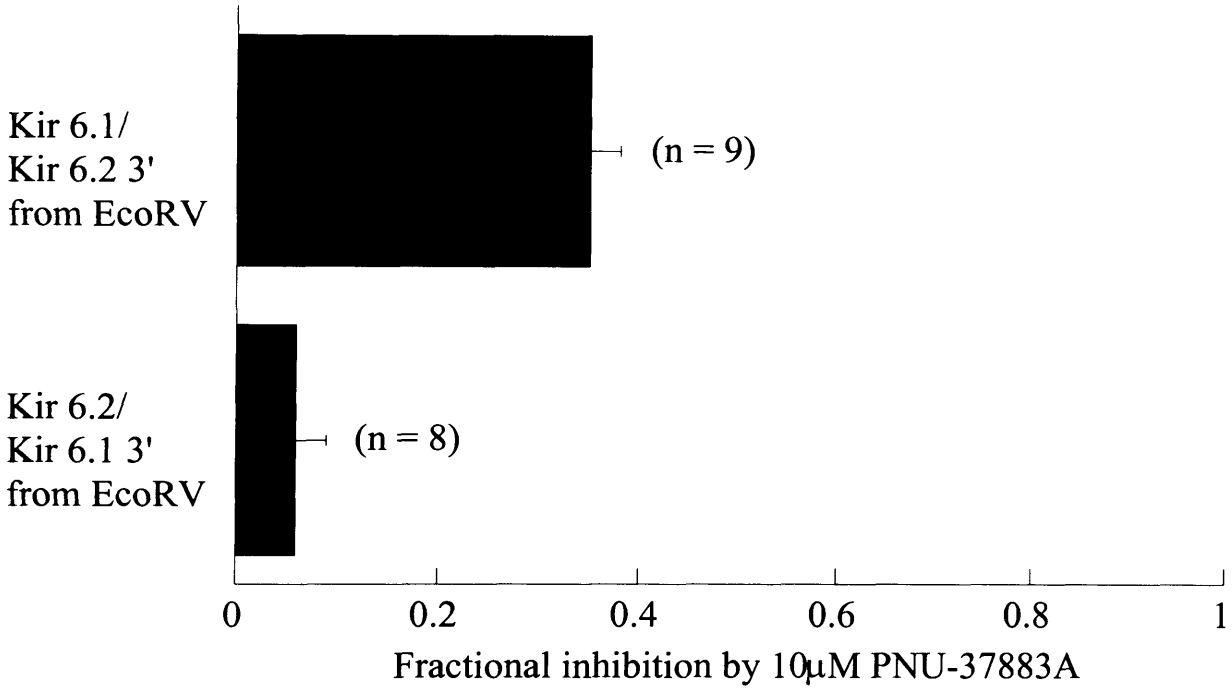

Kir 6.1/
Kir 6.2 3'
from EcoRV
(n = 9)
Kir 6.2/
Kir 6.1 3'
from EcoRV
(n = 8)
0
0.2
0.4
0.6
0.8
1
Fractional inhibition by 10μM PNU-37883A

Figure. 6.11.

Current trace showing K_{ATP} channel inhibition by PNU-37883A.

The trace shows current elicited from an oocyte expressing SUR 1/ Kir 6.2/6.1(87AA). Points represent the whole cell current, at 15 second intervals, with a holding potential of 60 mV. 10 μM PNU-37883A was applied to the cell as indicated.

Figure. 6.11.

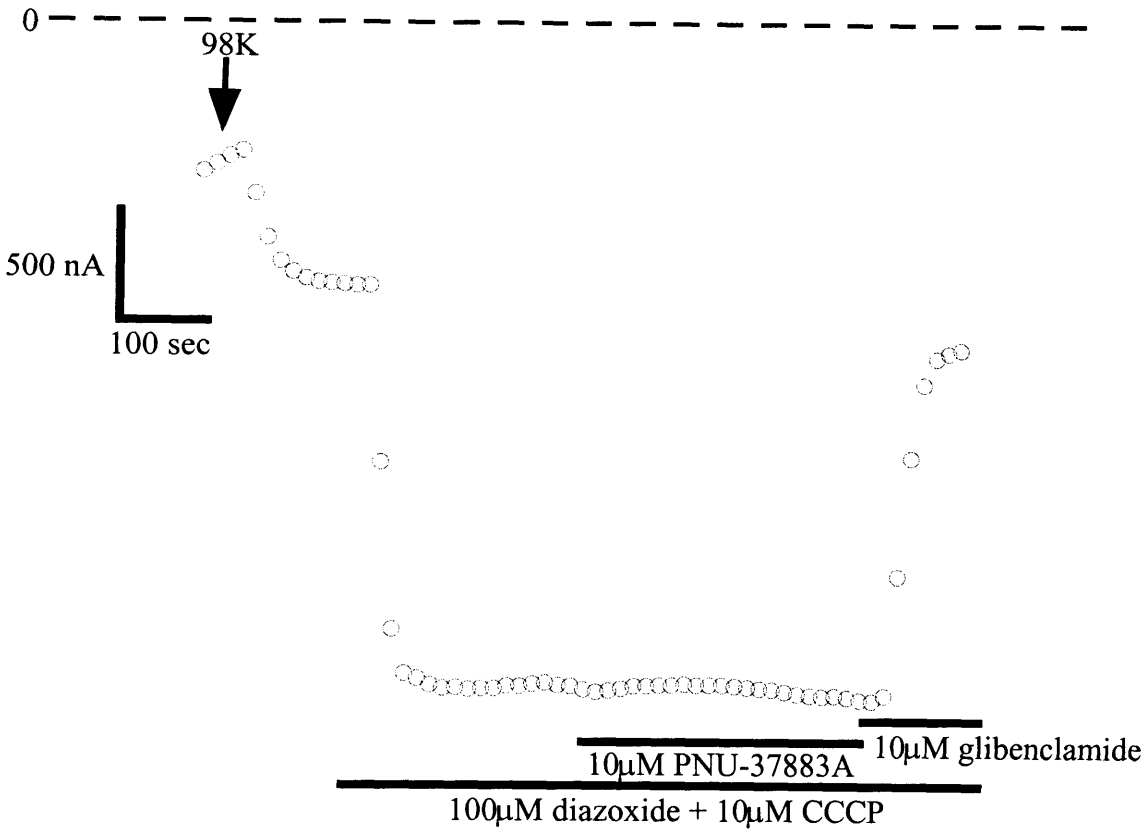

0
98K
500 nA
100 sec
10μM PNU-37883A
10μM glibenclamide
100μM diazoxide + 10μM CCCP

Table. 3.

Table showing the results of a paired T-test between the fractional inhibition observed for wild type Kir 6.2/ SUR 2B by 10 μM PNU-37883A and the chimeric constructs. Values below 0.05 are statistically significant. i.e. they are statistically different to the wild type values

Test subject	**p value**
SUR 2B/ Kir 6.2/6.1pore	0.2015
SUR 1/ Kir 6.2/6.1 5'	0.0329
SUR 2B/ Kir 6.2/6.1 3'	0.0001
SUR 1/ Kir 6.2/6.1 3' from *Eco*RV	0.7
SUR 1/ Kir 6.1/6.2pore	0.002
SUR 2B/ 6.1/6.2 5'	0.001
SUR 2B/ 6.1/6.2 3'	0.5427
SUR 1/ 6.1/6.2 3' from *Eco*RV	0.0001
Kir 6.2 ΔC	0.3916

6.3. Discussion

Chimeras were originally all expressed with SUR 2B, however after channel activation with pinacidil some constructs showed only a low level of expression. The K_{ATP} current generated was not of sufficient amplitude to continue the experiment, and these constructs were then expressed with SUR 1 and were found to exhibit a much higher level of expression. Wildtype Kir 6.1 was expressed with SUR 1 to assess the suitability of this SUR for demonstrating PNU-37883A inhibition. SUR 1 containing channels were inhibited by 10 μM PNU-37883A with a fractional inhibition of 0.42 (n=5). This is lower than what is observed when using channels expressing SUR 2B (0.7) but was considered suitable for demonstrating channel inhibition by the compound (see fig. 6.12.).

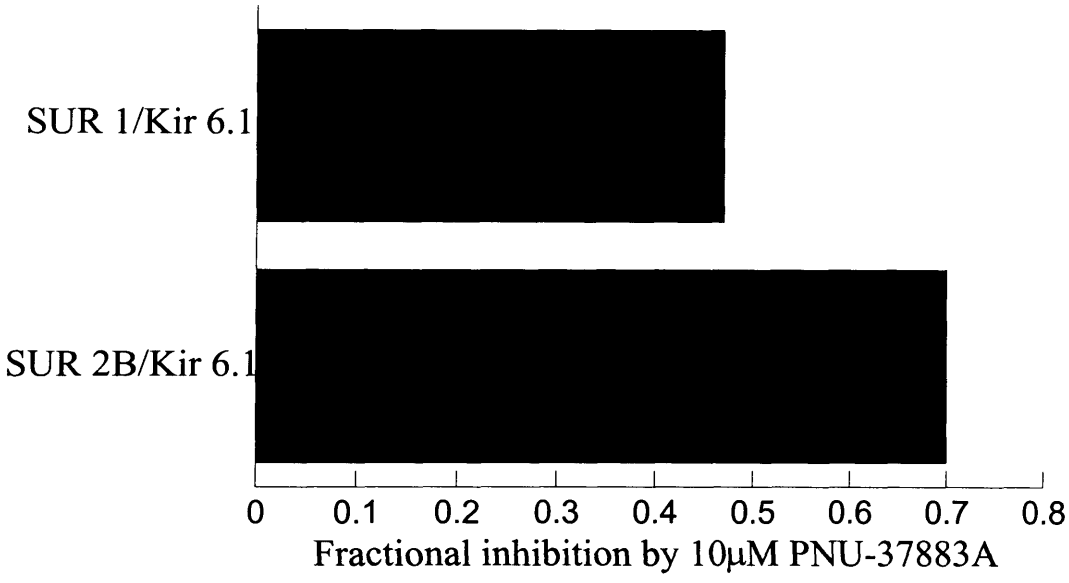
Channels containing the less sensitive Kir subunit, Kir 6.2, were rendered sensitive to PNU-37883A (demonstrating a fractional inhibition of >0.2, to 10 μM PNU-37883A) by swaping the C-terminus to that of Kir 6.1. Swapping over the pore or 5' region of Kir 6.1 alone did not render Kir 6.2 sensitive to the compound. Transferring the C-terminus of Kir 6.1 along with either the 5' region or the pore region also endowed sensitivity on Kir 6.2, although the addition of these regions did not enhance the sensitivity further from that found in the single C-terminal swap. Inhibition of the chimeric channels by 10 μM PNU-37883A was compared to that observed for the wild type Kir 6.2/ SUR 2B channel by statistical analysis (paired T-test), with a p value of >0.05 meaning the sensitivity of the channel was not statistically different to that of the wild type Kir 6.2/ SUR 2B channel (table. 3.). None of the chimeric channels obtained the level of sensitivity as observed for the wild type channel. The difference in sensitivity observed here could be due to the inability of the chimeric channel subunits to form the correct 3-D structure of the native channel and so affecting the compounds interaction with the channel.

When the C-terminus of Kir 6.1, from the *Eco*RV site onward, was replaced with Kir 6.2, sensitivity to the compound was lost indicating that the 87 amino acid region between the restrictions sites *Sph* I and *Eco*RV may be involved in the formation of a binding site or in channel modulation by the compound. To further investigate this region these 87 amino acids were swapped between Kir 6.1 and Kir 6.2. Replacing this region in Kir 6.1 with that of Kir

Figure. 6.12.

Histogram representing the fractional inhibition of glibenclamide sensitive current by 10 μM PNU-37883A. Comparison of SUR sensitivity. Kir 6.1 expressed with SUR 1 and SUR 2B. n= number of observations.

Figure. 6.12.


SUR 1/Kir 6.1
SUR 2B/Kir 6.1
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Fractional inhibition by 10μM PNU-37883A

6.2 knocked out the channels sensitivity to the compound. However, the replacement of the Kir 6.2 region with Kir 6.1 didn't endow sensitivity on these channels. The fact that sensitivity was lost on replacing the region in Kir 6.1 with Kir 6.2 does suggest that this region is important in channel modulation by the compound, but the fact that sensitivity could not be endowed to Kir 6.2 may suggest that another region in the N-terminus or pore is also necessary for channel inhibition by PNU-37883A. It is not unusual for more than one site in a channel to be important for the formation of a functional binding site, for example Hanner and co-workers identified two residues in the S5 domain and one in S6 of the voltage gated potassium channel Kv 1.3 that were all required for channel inhibition by Correolide (Hanner *et al.*, 1999). PNU-37883A has small groups of positive charge at the base of each of its rings, and each of these may require an interaction for the compound to bind, therefore a number of residues may form the binding site.

The results of this investigation suggest that the tissue selectivity demonstrated by PNU-37883A is dependent on K_{ATP} channel subunit composition and that this compound is selective for Kir 6.1 over Kir 6.2. Various K_{ATP} channel openers and inhibitors have been shown to be selective for the different SUR subunits, and it is novel to have found a K_{ATP} channel inhibitor that is selective for the Kir subunit. The evidence presented in this study has characterised PNU-37883A's selectivity for Kir 6.x using the expression of cloned K_{ATP} channels, and located an important area in the C-terminus of Kir 6.1 required for channel modulation by the compound.

Chapter 7

Summary

Summary

The aim of this investigation was to investigate the molecular identity of the K_{ATP} channel of vascular smooth muscle. This was achieved by firstly looking for subunit transcripts at the mRNA level. RT-PCR analysis of the vascular smooth muscle preparations detected the presence of Kir 6.1, Kir 6.2 and SUR 2B but not SUR 2A, mRNA. The Δ17 splice varient of SUR 2 was also present in some of the preparations and SUR 1 was found in mesenteric artery. These findings were supported by immunohistochemical observations where the three subunits, Kir 6.1, Kir 6.2, and SUR 2B, were present in femoral artery cells at the protein level. SUR 2A subunit protein was also found to be present in these cells even though it was not demonstrated at the mRNA level.

Secondly the *Xenopus* expression system was used to characterise cloned channels in their K^+ selectivity and sensitivity to certain potassium channel openers. The sensitivity to pinacidil was found to be dependent on the SUR subunit, which is comparable to other reports. A novel finding was the difference in sensitivity of the SUR 2A/ Kir 6.2 and SUR 2B/ Kir 6.2 channels to metabolic inhibition, as the sensitivity of the K_{ATP} channel to ATP is thought to be conferred by the Kir 6.0 subunit. The compound PNU-37883A was shown to be selective for channels containing Kir 6.1 over those containing Kir 6.2. The results gained for the inhibition of the cloned channel SUR 2B/ Kir 6.1 were similar to those observed for native K_{ATP} channels of vascular smooth muscle. With both cloned and native channels giving similar EC_{50} values for this compound. Although the cloned channel SUR1/ Kir 6.1 was also inhibited by the compound, the EC_{50} value was higher than that observed for the SUR 2B/ Kir 6.1 channel and the native vascular smooth muscle K_{ATP} channel. The construction of chimeric Kir 6.0's led to the idenification of an important region in Kir 6.1 required for channel inhibition by the compound.

We have shown that the compound, PNU-37883A, exhibits selectivity between the Kir 6.x's but the differences in sensitivity observed for channels comprising the SUR subunits, SUR 2B and SUR 1, further complicate the inhibition model giving the impression that this subunit may in fact contribute to the binding site. However, studies by Guillemare and co-workers have provided evidence against the compound acting through the sulphonylurea receptor. They found that glibenclamide did not displace PNU-37883A binding, and observed that the compound did not bind to RINm5F cell membranes (Guillemare *et al.*, 1994). Therefore, we propose that the Kir 6.1 subunit provides the binding site for PNU-37883A, and that this subunit is most likely to form the pore of the native vascular smooth muscle K_{ATP} channel.

In order to confirm that Kir 6.1 does form the binding site this subunit would have to be expressed independently of the sulphonylurea receptor. A similar truncation of Kir 6.1 to that of the independently expressing Kir 6.2 truncation has been engineered, by Prof. F. Ashcroft, although, it apparently does not form functional channels when expressed alone (personal communication). It would be interesting to further characterise the importance of the 87AA acid region in Kir 6.1 that is required for channel inhibition by PNU-37883A, mutagenesis could be used, perhaps targeting negative charged residues in this region to narrow down the possible interaction between compound and channel.

References.

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