Structure-function studies of inwardly rectifying potassium channels

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For Nan
Abstract

The patch clamp technique was used to investigate the effect of mutations in the P-region on permeation characteristics and ionic selectivity in members of the Kir2.0 subfamily. Kir2.2 exhibits electrophysiological characteristics similar to those of Kir2.1 though there are differences in both unitary conductance and the kinetics of Ba$^{2+}$ blockage. The P-region of these channels is virtually identical with the exception that leucine (L) at position 148 in Kir2.2 is replaced by phenylalanine (F) in Kir2.1. The effects of mutating L148 to phenylalanine in Kir2.2 and F147 to leucine in Kir2.1 on unitary conductance and channel sensitivity to Ba$^{2+}$ were investigated. Neither mutation altered unitary conductance from that seen in the wild-type channel. However, mutation L148F in Kir2.2 reduced the association rate constant for Ba$^{2+}$ blockage without affecting affinity. In contrast, mutation F147L in Kir2.1 increased channel affinity for Ba$^{2+}$ without affecting the association rate constant. Thus, residues outside the P-region are responsible for the difference in unitary conductance and some of the differences in Ba$^{2+}$ block in Kir2.2 and Kir2.1.

The effects on ionic selectivity of substituting single amino acid residues at position 143 in the P-region of Kir2.1 were also investigated. Substitution of isoleucine by hydrophobic residues such as valine (I143V) and leucine (I143L) raised the relative Rb$^+$ permeability, whilst substitution of the more hydrophilic residue threonine (I143T) enhanced K$^+$ selectivity. Two further mutants, I143C and I143S, failed to yield currents, but could be rescued by bathing cells in extracellular solution containing 10mM dithiothreitol (DTT). The permeability ratios were then similar to wild-type. The rescue of mutant channels by DTT suggests that the pore of Kir channels may undergo conformational changes. In vitro translation studies suggest that channel function is rescued through the disruption of an intra-subunit disulphide bond.
Acknowledgements

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<tr>
<td>4-AP</td>
<td>4-aminopyridine</td>
</tr>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP binding cassette</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine 5'-diphosphate</td>
</tr>
<tr>
<td>Ag⁺</td>
<td>Silver ion</td>
</tr>
<tr>
<td>AHP</td>
<td>Afterhyperpolarization</td>
</tr>
<tr>
<td>AKAP</td>
<td>The A kinase anchoring protein</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5' triphosphate</td>
</tr>
<tr>
<td>Ba²⁺</td>
<td>Barium ion</td>
</tr>
<tr>
<td>BFNC</td>
<td>Benign familial neonatal convulsions</td>
</tr>
<tr>
<td>BK⁰⁺ Ca</td>
<td>High conductance calcium-activated potassium channels</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CCD</td>
<td>Cortical collecting duct</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic fibrosis transmembrane regulator</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>Chloride ion</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>Cs⁺</td>
<td>Caesium ion</td>
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<td>CTAL</td>
<td>Cortical thick ascending limb</td>
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<td>CTX</td>
<td>Charybdotoxin</td>
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<tr>
<td>C-terminus</td>
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</tr>
<tr>
<td>D₂</td>
<td>Dopamine receptors</td>
</tr>
<tr>
<td>DCT</td>
<td>Distal convoluted tubule</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>Eᵣ</td>
<td>Resting membrane potential</td>
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<td>Eₖ</td>
<td>Potassium ion equilibrium potential</td>
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<tr>
<td>Eᵣₑᵥ</td>
<td>Reversal potential</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
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<tr>
<td>F</td>
<td>Faraday’s constant</td>
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<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
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<tr>
<td>GAGAᵦ</td>
<td>γ-aminobutyric receptors</td>
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G418 Geneticin® antibiotic
GSH Reduced glutathione
GSSG Oxidized glutathione
HEPES N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
IBX Iberiotoxin
\( I_K \) Potassium current
\( I_{K_{Ca}} \) Intermediate conductance calcium-activated potassium channels
\( I_{Na} \) Sodium current
\( K^+ \) Potassium ion
\( [K^+]_i \) Intracellular potassium ion concentration
\( [K^+]_o \) Extracellular potassium ion concentration
\( K_{ir} \) Inward rectifier potassium channel
\( K_v \) Voltage-gated potassium channel
\( K_{2P} \) Two pore potassium channel
LCR Locus control region
MAGUK Membrane associated guanylate kinase
\( Mg^{2+} \) Magnesium ion
\( Mg^{2+}_i \) Intracellular magnesium
\( Mg-ADP \) Magnesium adenosine 5' diphosphate
MEL Murine erythroleukaemia
MEM-\( \alpha \) Alpha minimal essential medium
MB-IRK2 Mouse brain IRK2
mRNA Messenger ribonucleic acid
MTAL Medullary thick ascending limb
MTSEA Ethyl-ammonium-methanethiosulphonate
MTSET 2-trimethylammoniumethyl-methanethiosulphonate bromide
\( Na^+ \) Sodium ion
nAChR Nicotinic acetylcholine receptor
\( NH^+ \) Ammonium ion
NMDG \( N\)-methyl-\( D\)-glucamine
OMCD Outer medullary collecting duct
\( I-V \) Current-voltage
\( P_{open} \) Probability of opening
\( P_X \) Permeability coefficient of ion X
PBS Phosphate buffered saline
PHHI Familial persistent hyperinsulinemic hypoglycaemia of infancy
pHᵢ Intracellular pH
PIP₂ Phosphatidylinositol-4, 5-bisphosphate
PKA Protein kinase A
PKC Protein kinase C
PSD Postsynaptic density
R Gas constant
Rb⁺ Rubidium ion
RB-IRK2 Rat brain IRK2
RNA Ribonucleic acid
SAP Synapse-associated protein
SEM Standard Error Mean
SKcₐ Low conductance calcium activated potassium channels
STX Saxitoxin
SUR Sulphonylurea receptor
T Absolute temperature
T₁ Tetramerization domain 1
τact Activation time constant
TAL Thick ascending limb
TEA⁺ Tetraethylammonium ion
TTX Tetrodotoxin
Vᵥ Membrane potential
v/v volume per volume
wv weaver
[X]ᵢ Intracellular ion concentration
[X]₀ Extracellular ion concentration
z Ion valency
Table 0.1. Abbreviations for Amino acids

<table>
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<tr>
<th>Side-chain type</th>
<th>Name</th>
<th>Triple-letter code</th>
<th>Single-letter code</th>
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<tr>
<td>Aliphatic</td>
<td>Glycine</td>
<td>Gly</td>
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<td>Sulphur-group containing</td>
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<td>Asparagine</td>
<td>Asn</td>
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The table is divided into two halves. The top half lists those residues that are hydrophobic whilst the bottom half lists those that are hydrophilic.
Chapter 1

Introduction

1.0. General introduction

In recent years it has become evident that numerous diseases are caused by mutations in a variety of genes that encode ion channels. Termed 'Channelopathies', these conditions include fatal disorders such as cystic fibrosis, the long-QT syndrome and familial persistent hyperinsulinemic hypoglycaemia of infancy (PHHI), and less serious disorders such as benign familial neonatal convulsions (BFNC). Numerous drugs and therapies exist for the treatment of such diseases. However, these medical treatments have not typically been discovered by way of molecular insight but from either clinical use for other disorders or by functional screening, and many have serious side effects (see for example Goldstein & Colatsky, 1996). Identification of the underlying molecular bases of these diseases and a more comprehensive understanding of how mutations alter the normal function of ion channels should facilitate the development of novel drugs or alternative therapies for the treatment of such conditions.
The importance of functional studies in the development of alternative therapies is well illustrated by research into cystic fibrosis (see Greger et al., 2001; Gelman & Kopito, 2002). This fatal disease, which affects 1 in 2500 people, is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR). CFTR is a member of the adenosine triphosphate (ATP)-binding cassette (ABC) protein superfamily and functions as a chloride channel involved in the regulation of chloride fluxes in the lung and exocrine tissues.

More than 1000 mutations have been identified in CFTR. However, deletion of a phenylalanine residue at position 508 (F508), located in the first ATP binding domain, accounts for the vast majority of cases of this disease (for reviews see Aidley & Stanfield, 1996; Ackerman & Clapham, 1997; Gelman & Kopito, 2002). Structure-function studies have shown that deletion of F508 leads to defects in folding, protein stability, activation kinetics and trafficking to the membrane, which in turn result in chloride transport failure (see for example Cheng et al., 1990; for review see Gelman & Kopito, 2002). The transport of chloride through CFTR channels is accompanied by the movement of water, thus the failure of chloride transport results in a reduction in the amount of fluid produced by the epithelial cells of the lungs and exocrine tissues. This leads to the accumulation of thick mucus in the lungs and pancreas, which in turn causes difficulties with breathing and digestion. Cystic fibrosis patients suffer frequent pulmonary infections, and damage to epithelial cells of the lungs and pancreas ultimately has fatal consequences.

Traditional therapies include chest percussion and postural drainage to relieve obstruction of the small airways and the use of antibiotics to treat respiratory infections, recombinant human DNase to decrease the viscosity of the secretions and anti-inflammatory drugs to reduce the inflammatory response. However, an understanding of how defects in the CFTR protein cause cystic fibrosis suggests the possibility of novel therapeutic approaches. For example, one investigation has successfully looked at relocation of ΔF508CFTR proteins at the cell surface using glycerol as a chemical chaperone (Sato et al., 1996). Thus, drugs that rescue or correct folding and/or trafficking of this CFTR mutant may be beneficial in the treatment of most forms of cystic fibrosis (see Gelman & Kopito, 2002).

Clearly, structure-function studies of ion channels are very important. However, to understand how gene mutations affect ion channels requires knowledge about how these channels function normally. In this study, I have investigated two members of the strong
inwardly rectifying potassium channel (K_{in}) family, K_{in}2.1 and K_{in}2.2. Until recently, mutations in genes encoding inward rectifier potassium channels had been linked to three disease states: Bartter's syndrome, PHHI and the weaver mouse (for reviews see Sanguinetti & Spector, 1997; Curran, 1998; Abraham et al., 1999; Reimann & Ashcroft, 1999).

*Bartter's syndrome* is a rare, autosomal recessive renal tubular disorder characterized by salt wasting and hypokalemic alkalosis. Mutations in several different ion channels and transporters have been linked with this disorder (see for example Simon et al., 1996a, b; 1997; Peters et al., 2002). Mutations in ROMK channels underlie type II Bartter's syndrome (see section 1.2.4a/1.5.1 for more detailed discussion).

Mutations in K_{i6.2} and its associated protein SUR1, which result in defective K_{ATP} channels, underlie the hereditary disorder persistent hyperinsulinemic hypoglycaemia of infancy (PHHI), a condition characterized by unregulated insulin secretion and severe hypoglycaemia (for review see Aguilar-Bryan et al., 2001; see section 1.2.4f for further discussion).

The *weaver* (wv) mutation in mice is caused by a single mutation in the pore region of K_{i3.2}, which results in the loss of K\(^+\) selectivity (see for example Patil et al., 1995; Slesinger et al., 1996; Navarro et al., 1996). This neurological disorder has been attributed to a reduced number of cerebellar granule cells during development and loss of dopaminergic cells in the substantia nigra. Owing to the degeneration of dopaminergic neurones exhibited by wv mice, these animals provide an interesting model for Parkinson's disease (see sections 1.2.4c. and 1.5.6 for further discussion).

Recently, animal knockout studies of K_{in}2.1 and K_{in}2.2 have highlighted the importance of the strong inward rectifiers in the heart and in vascular smooth muscle (see sections 1.5.2 and 1.5.3; Zaritsky et al., 2000; 2001). K_{in}2.1 knockout mice die within hours of birth from a complete cleft of the secondary palate suggesting that K_{in}2.1 also plays an important role in development. Furthermore, mutations in K_{in}2.1 have been linked with Andersen's syndrome, a rare disorder characterized by periodic paralysis, cardiac arrhythmias and dysmorphic features (Plaster et al., 2001). In their elegant study, Plaster et al. (2001) demonstrated a link between muscle and cardiac electrical phenotypes demonstrating the importance of potassium channels in modulating excitability. In addition, they also provided evidence for an important role of K_{in}2.1 in development.
In this introductory chapter I will review previous studies of structure and function in \( K_{ir} \) channels. However, my review will not focus on the \( K_{ir} \) channels alone, since studies of structure and function in voltage-gated potassium channels (\( K_v \)) have provided many clues to which structural elements of \( K_{ir} \) channels might determine their functional properties. Hence, this introductory chapter will also consider some of the previous structure-function studies undertaken in \( K_v \) channels. Particular emphasis is placed on those studies that investigated the molecular basis of \( K^+ \) selectivity. Before reviewing these studies, I will outline the earlier functional studies of inward rectification in native tissues. However, I should like to begin by introducing the concept of ion channels.

### 1.1. Ion channels

In this first section I will describe some of the major experiments that have led to the discovery of ion channels. I will then briefly consider some of the properties of ion channels and the membrane of excitable cells.

#### 1.1.1. A historical perspective of ion channels

Ion channels are generally thought to have evolved millions of years ago, around the same time that cells began to develop membranes. The cell membrane, which consists of a lipid bilayer, serves as a relatively impermeable barrier to the passage of most water-soluble molecules. To achieve transport of molecules such as ions, sugars, amino acids, nucleotides and numerous cell metabolites across the lipid membrane, cells developed specialized transmembrane proteins of which there are two main classes: carrier proteins and ion channels.

Briefly, carrier proteins (also known as ion pumps or ion transporters) are large proteins, which move ions using free energy stored in an electrochemical gradient or released by hydrolysis of ATP. Carrier proteins transport ions relatively slowly, passing \( 10^2 \) ions per second, and were initially thought to achieve transport of small molecules back and forth across the membrane via 'a ferryboat-like' mechanism (see Hille, 2001). However, carrier proteins are now considered to be like ion channels (see below), but with two gates, one external to and one internal to the ion-binding cavity. The two gates have been proposed to open alternatively, exposing the extracellular and intracellular media to the ion binding sites. These gating movements lead to the entrapment of ions within the interior of the pump and the subsequent release to the opposite side (see for example Artigas & Gadsby, 2002).
In contrast, ion channels form narrow, hydrophilic (water-filled) pores, which when open allow the passage of ions down their electrochemical gradient. Although the existence of 'membrane pores' was recognized as early as the 1840s (for review see Hille et al., 1999), the concept of discrete proteins selective for the transport of individual ions was not proposed until over a century later. Instead, early research began from a different perspective when, in the 1880s, Sydney Ringer discovered that the solution used to bathe an isolated frog heart must contain precise ratios of sodium (Na\(^+\)), potassium (K\(^+\)) and calcium (Ca\(^{2+}\)) ions in order to maintain its electrical excitability (see Hille, 2001).

At the beginning of the 20\(^{th}\) century Julius Berstein introduced the concept that the membrane of excitable cells is selectively permeable to K\(^+\) at rest, but increases its permeability to other ions during excitation. His hypothesis proposed the 'resting potential' of membrane and nerve to be a 'diffusion potential', which arises as a result of the passive movement of K\(^+\) ions from the cytoplasm to the extracellular solution down their concentration gradient. He also used the term 'membrane breakdown' to describe the transient increase in permeability to other ions during excitation (for review see Hille, 2001).

Around about the same time, Hermann proposed that the cell membrane of excitable cells was itself electrically excitable and that the propagation of a nervous impulse occurred by way of electrical stimulation of unexcited membrane by the already active regions (see Hille, 2001). Later, Hodgkin (1937a, b) confirmed that nervous impulses, commonly known as action potentials, propagate electrically as originally hypothesized by Hermann, and Cole and Curtis (1938; 1939) confirmed that membrane permeability does increase during excitation, as first proposed by Bernstein.

Following the measurement of a full action potential from an axon using an intracellular micropipette (Hodgkin & Huxley, 1939), it was suggested that the cell membrane becomes selectively permeable to sodium (Na\(^+\)) during excitation. Further experiments using low-Na\(^+\) external solutions and \(^{24}\)Na as a tracer confirmed the role of Na\(^+\) in the rising phase of the action potential (Hodgkin & Katz, 1949; Keynes, 1951).

In 1952, Hodgkin and Huxley published a series of papers (1952a, b, c, d) describing the changes in membrane Na\(^+\) and K\(^+\) permeability during action potential propagation in the squid axon, formulating equations that described the manner in which these currents varied
with membrane potential and time. Hodgkin and Huxley (1952d) proposed that sodium current ($I_{Na}$) and potassium current ($I_{K}$) were localized at particular sites in the membrane called 'active patches'.

Later, following experiments in which they measured unidirectional fluxes using isotopes of K+, Hodgkin and Keynes (1955) proposed that ions could cross the membrane through narrow tubes or channels, with several ions in the channel at any given moment but moving in single file. A picture of individual proteins responsible for the transport of ions was finally beginning to emerge.

The idea that separate pathways for Na$^+$ and K$^+$ may exist was further supported by the discovery of a number of specific blocking agents several years later. Tetrodotoxin (TTX) and Saxitoxin (STX) were shown to selectively block $I_{Na}$, leaving $I_{K}$ untouched, whilst in contrast, the tetraethylammonium ion (TEA$^+$) was shown to block $I_{K}$ but not $I_{Na}$ (reviewed in Aidley & Stanfield, 1996; Hille, 2001).

Estimates of the single channel conductance of the conventional delayed rectifier in squid axon (~12pS) and frog nerve (~4pS) from current fluctuations provided further evidence for the existence of ion channels. The single channel conductance of the delayed rectifier implied that one potassium channel could pass a K$^+$ ion at least every microsecond, a turnover number too high for a carrier mechanism (see for example Conti et al., 1976; Begenesich & Stevens, 1975; for review see Hille & Schwarz, 1978).

Perhaps the greatest breakthrough for ion channel research came in 1976 with the introduction of the patch clamp technique (Neher & Sakmann, 1976). Developed by Erwin Neher and Bert Sakmann, this technique allows the direct measurement of current flowing through individual ion channels. Following refinement (Hamill, 1981), 'patch-clamping' today remains a very useful tool for the investigation of ion channels.

More recently, the use of recombinant DNA technology has revolutionized the study of ion channels. By the early 1990s, numerous ion channels had been cloned and sequenced. Using site-directed mutagenesis combined with electrophysiological recording, many of the functionally important amino acids of ion channels have been identified. However, one drawback with such an approach is that a small change in one part of the protein may affect
the overall protein conformation, and in turn its function, even if the altered part is not directly involved in the function of interest.

By the late 1990s many hypotheses about the molecular mechanisms underlying ion channel function had been proposed, but fundamental questions remained owing to the absence of a true physical picture of channel structure (see for example Armstrong & Hille, 1998). However, the first three-dimensional structure of an ion channel was soon to be reported. Doyle et al. (1998b) used crystallographic analysis to reveal the structure of the pore of a potassium channel (KcsA) from the bacterium *Streptomyces lividans*. For the first time it was possible to understand, at a molecular level, the mechanisms that control ion selectivity and conduction in potassium channels.

1.1.2. Classification of ion channels

Since the findings of Hodgkin and Huxley in the 1950s, ion channel research has mainly focused on two channel properties: *gating* and *selectivity*.

Ion flow through channels is controlled by a process known as *gating*, during which the channel is either open or closed (see section 1.2.3a). Channel gating is usually in response to a stimulus such as a change in membrane potential or the binding of a neurotransmitter. When the channel is open, ions are free to move down their electrochemical gradient. Channel closure prevents such movement. Open channels are highly permeable to some but not all ions. This property is known as *selectivity* (see section 1.6). Pharmacological experiments have demonstrated the existence of channels selective for individual ions including K⁺, Na⁺, Ca²⁺ and chloride (Cl⁻). Together, the properties of selectivity and gating are used to classify channels. For example, a channel that is permeable to K⁺, and opens and closes in response to a change in membrane potential is classified as a voltage-gated potassium (Kᵥ) channel.

1.1.3. A role for potassium channels

As we will see later, a huge variety of potassium channels exist (see section 1.2), and potassium channel dysfunction is implicated in a number of genetic and acquired neurological disorders (Shieh et al., 2000). But, what are the functional roles of potassium channels? To answer this, let us first consider one of the essential properties of excitable cells.
All cells have a resting membrane potential \( (E_m) \), a negative electrical charge across the membrane, typically in the order of \(-70\text{mV}\) for excitable cells. The resting membrane potential plays a central role in numerous cellular responses including the excitability of nerve and muscle cells. Its maintenance is therefore crucial to cell function. How does the resting membrane potential arise?

In living cells, the concentration of \( \text{K}^+ \) inside the cell is much greater than outside, the reverse being true for \( \text{Na}^+ \). These concentration differences are maintained partly by a \( \text{Na}^+, \text{K}^+-\text{ATPase} \), which pumps two \( \text{K}^+ \) ions into the cell, for every three \( \text{Na}^+ \) ions pumped out. Although this creates a potential difference across the cell membrane, so that the inside is negative with respect to the outside, the activity of the \( \text{Na}^+-\text{K}^+ \) ATPase is not the main contributor to the resting membrane potential. Instead, it is the \( \text{K}^+ \) gradient across the plasma membrane that predominantly determines \( E_m \).

At rest the plasma membrane is selectively permeable to \( \text{K}^+ \), owing to the presence of '\( \text{K}^+ \) leak channels'. \( \text{K}^+ \) exits the cell by moving down its concentration gradient, creating a negative potential across the cell membrane, which in turn opposes any further efflux of \( \text{K}^+ \) from the cell. When the concentration gradient driving \( \text{K}^+ \) out of the cell equals the opposing electrical gradient, the net flow of \( \text{K}^+ \) ceases. The membrane potential at which this occurs is known as the equilibrium potential for \( \text{K}^+ \) \( (E_k) \). A similar balance for other major ions including \( \text{Na}^+ \) and \( \text{Cl}^- \) also exists. The equilibrium potential for an ion, \( X \), can be calculated from the Nernst equation:

\[
E_x = \frac{RT}{zF} \ln \left( \frac{[X]_o}{[X]_i} \right) \quad \text{(Equation 1.1)}
\]

where \( R \) is the gas constant, \( T \) is the absolute temperature \( (^\circ \text{K}) \), \( z \) the valency of the ion, \( F \) the Faraday constant and \([X]_o\) and \([X]_i\) are the extracellular and intracellular concentrations of the ion.

Although \( E_k \) predominantly determines \( E_m \), living cells are not exclusively permeable to \( \text{K}^+ \) but have a small resting permeability to other ions including \( \text{Na}^+ \). This is commonly reflected in an \( E_m \) slightly positive to \( E_k \).
The involvement of potassium channels in cell excitability underlies a number of their important physiological functions. Hyperpolarization of the membrane, which arises as a consequence of K⁺ moving down its concentration gradient, underlies the termination of action potentials in electrically excitable cells such as nerve and muscle. In addition, potassium channels play essential roles in, for example, the regulation of the heartbeat, the release of neurotransmitters, muscle contractility and hormone secretion (for review see Miller, 2000; Hille, 2001). Some of the physiological roles of Kᵢᵣ channels will be considered in more detail later (see section 1.5).

1.2. Potassium channel diversity

Through electrophysiological studies a large diversity of potassium channels, which differ in their biophysical properties, pharmacology, modulation and tissue distribution, have been identified. In addition to uncovering the mechanisms responsible for this functional diversity, cloning efforts have revealed new superfamilies of potassium channels. The diversity of potassium channels is well illustrated by the genome of the simple nematode *Caenorhabditis elegans* in which over 80 genes encoding potassium channels were identified (Bargmann, 1998; Wei et al., 1996). Alternative splicing, heteromeric assembly of different subunits and assembly of potassium channel subunits with various auxiliary subunits further increases this diversity.

Why is there such a huge diversity of potassium channels? It is thought that potassium channels may provide the modulatory functions that make excitable cells different from one another, whilst other channels such as calcium channels provide a function that is perhaps regulated in similar ways in all cells.

Based on structure, potassium channels are currently classified into one of three major categories (see figure 1.1): *six-transmembrane domain* potassium channels such as the Kᵥ channels, *two-transmembrane domain* potassium channels including the Kᵢᵣ channels and *two-pore* potassium channels (K₂p). Potassium channels belonging to each of these categories will be considered in more detail below. However, I would like to begin this second section of my introduction by reviewing the early studies on the cloning of ion channels, with particular emphasis on those selective for K⁺.
Figure 1.1.

Molecular architecture of potassium channels. Predicted membrane topology of 6 transmembrane (TM) domain potassium channels, which include $\text{Kv}$, $\text{Eag-like}$ and $\text{KCNQ}$ potassium channels (top panel), 2 TM domain potassium channels such as the $\text{Kir}$ channels (middle panel) and two-pore potassium channels (bottom panel). $P$ denotes the highly conserved pore region(s), $S4$ the voltage-sensor and $N$- and $C$- the amino and carboxyl termini, respectively.
1.2.1. Early potassium channel cloning studies

During the early years of ion channel studies, research was restricted to looking at the functional properties of native ion channels. However, the presence of multiple, overlapping conductances in native cells complicates the study of individual channels. In the last 20 years, the application of molecular cloning technology to ion channel research has identified the primary structure of many ion channels, and in combination with the use of heterologous expression systems, has enabled detailed analysis of the molecular basis for the function of individual channels in the absence of contaminating currents.

Biochemical purification of the nicotinic acetylcholine receptor (nAChR) channel and the voltage-gated sodium channel was relatively simple, owing to the availability of tissues containing large quantities of these proteins and specific high-affinity ligands that bind to these channels (see for example Vandlen et al., 1979; Conti-Tronconi & Raftery, 1982; Agnew et al., 1978; Barchi, 1982). The subsequent cloning of these channels quickly followed (Noda et al., 1982; Noda et al., 1984). However, the cloning of potassium channel genes required a slightly different approach since it was not possible to purify potassium channel proteins, owing to an inability to identify any tissues rich in potassium channels. Furthermore, few specific high-affinity ligands for potassium channels were known. The alternative approach, described below, made use of genetic mutations in the Shaker locus of the fruit fly Drosophila melanogaster.

Flys carrying the Shaker mutation were originally identified because they violently shake their legs when under ether anaesthesia (Kaplan & Trout, 1969). The idea that Shaker may encode a potassium channel gene was first considered in 1977 during experiments on the neuromuscular junction in Drosophila larvae (Jan et al., 1977). Following nerve stimulation, larvae carrying one of two Shaker mutations, Sh\textsuperscript{KS133} or Sh\textsuperscript{KO120}, displayed abnormal twitching and enhanced neurotransmitter release from the neuromuscular junctions. Jan et al. (1977) found that this could be mimicked in wild-type larvae following treatment with the potassium channel blockers 4-aminopyridine (4-AP) and TEA\textsuperscript{+} ions, respectively. Based on these observations, it was proposed that the two Drosophila mutations might cause defects in potassium channels, which fail to repolarize the nerve terminal properly.

Tanouye et al. (1981) provided further evidence to strengthen the hypothesis that this defect was due to an abnormal K\textsuperscript{+} conductance. A delay in the repolarization of action potentials, observed in recordings from the cervical giant fibre axons of Drosophila adults carrying...
Shaker mutation $Sh^{KS133}$, could be mimicked in wild-type fibres following application of the potassium channel blocker 4-AP.

It was later shown, using the voltage clamp technique, that a rapidly activating, inactivating K$^+$ current known as the A-type current (see section 1.2.3a), present in normal pupal and adult flight muscle, was completely absent from the flight muscle of Drosophila carrying the Shaker mutation $Sh^{KS133}$. As in previous studies, this K$^+$ current defect could be mimicked by application of 4-AP to normal pupal or adult flight muscle (see for example Salkoff & Wyman, 1981; for review see Tanouye et al., 1986). Similar results have been described in larval muscle (see for example Wu & Haugland, 1985). It was proposed that the previously reported enhanced neurotransmitter release at the neuromuscular junction, and the abnormally long delays in repolarization of the cervical giant axon action potentials in Drosophila mutants, were also likely to be the result of abnormal ‘A’ currents.

In the late 1980s several groups (Kamb et al., 1987; Papazian et al., 1987; Baumann et al., 1987) isolated and characterized genomic DNA and several complementary DNA (cDNA) clones from the Shaker locus. From the sequencing results of two cDNA clones, shA1 and shA2 (Tempel et al., 1987), it was shown that the predicted protein contained six transmembrane segments characteristic of ion channels and an arginine-rich sequence similar to a region found in voltage-gated sodium channels (see Noda et al., 1986). These results further supported the hypothesis that Shaker codes for a structural component of a voltage-gated potassium channel.

In 1988, it was confirmed that the Shaker gene does indeed encode a K$^+$-selective channel following expression studies in Xenopus oocytes (Timpe et al., 1988). Oocytes injected with Shaker mRNA displayed transient outward currents, which showed similar voltage sensitivity, inactivation kinetics, ionic selectivity and sensitivity to 4-AP with native A-current.

It is now known that the Shaker locus encodes several different protein products, a consequence of alternative splicing of a large primary transcript in which different combinations of exons are selected as the primary Shaker transcript and processed into mature messenger RNA (see for example Baumann et al., 1987; Pongs et al., 1988; Schwarz et al., 1988). These different classes of cDNA clones represent different potassium channel subtypes, which differ greatly in their kinetics and voltage-sensitivity (Butler et al., 1989; Wei et al., 1990) and are termed Shab (K,2), Shaw (K,3) and Shal (K,4). Like Shaker, Shal
encodes an A-type K\(^+\) current, whilst \textit{Shab} and \textit{Shaw} encode delayed rectifier-type K\(^+\) currents, which either inactivate very slowly or display no measurable inactivation. Mammalian homologues for each member of the \textit{Drosophila} gene family have been described (see for example Temple \textit{et al.}, 1988; for summary see Salkoff \textit{et al.}, 1992).

1.2.2. The three classes of potassium channels

To discuss the numerous types of potassium channel in detail is beyond the scope of this thesis. Therefore, members of the six-transmembrane domain and two-pore domain structural classes of potassium channels are considered only briefly. The two-transmembrane domain potassium channels, to which the inward rectifier potassium channels belong, will be discussed in more detail, though the emphasis will be on the strong inward rectifiers, which are members of the second subfamily K\(_{\text{IR}}\)2.0.

1.2.3. Six-transmembrane domain potassium channels

Potassium channels containing six transmembrane domains (S1-S6; see top panel of figure 1.1) represent by far the largest and most diverse group of potassium channels. This class comprises both voltage-gated (K\(_v\)) and calcium-activated (K\(_{\text{Ca}}\)) potassium channels, which include among their many members the \textit{Shaker}-related, \textit{ether-a-go-go}- (EAG) related and KCNQ potassium channel subunits. A functional channel comprises four \(\alpha\)-subunits (see section 1.3.1), and for some channels, four accessory \(\beta\)-subunits (see below), which may dramatically or subtly alter the physiological properties of the tetrameric \(\alpha\)-subunit complex. The region between S5 and S6, known as the P-region, constitutes part of the ion conduction pore (see section 1.6), whilst the S4 segment, in which lysine or arginine is repeated every third position, is thought to form the voltage sensor.

1.2.3a. \textit{Shaker}-related potassium channels

In addition to the four subfamilies originally isolated (K\(_{\text{IR}}\)1-4, see section 1.2.1), an additional five subfamilies (K\(_{\text{IR}}\)5-9) have been identified (for review see Coetzee \textit{et al.}, 1999; Kaczorowski & Garcia, 1999). When expressed alone K\(_{\text{IR}}\)5, K\(_{\text{IR}}\)6, K\(_{\text{IR}}\)8 and K\(_{\text{IR}}\)9 subfamily members do not generate functional channels but are capable of modulating the activity of members of the K\(_{\text{IR}}\)2 subfamily (for review see Song, 2002). Whilst some of these subfamilies contain multiple subtypes (e.g. K\(_{\text{IR}}\)1 is comprised of K\(_{\text{IR}}\)1.1-1.8), others contain only one (e.g. K\(_{\text{IR}}\)5.1). The members of this superfamily are thought to underlie the classically defined delayed-rectifier and A-type K\(^+\) currents, which are activated on depolarization but differ in
their activation and inactivation kinetics (see Mathie et al., 1998). These currents are essential for shaping action potential waveforms and their repolarization (for review see example Song, 2002).

A-type currents ($I_A$), which were first characterized in molluscan neurones (Connor & Stevens, 1971; Neher, 1971) are typically activated at subthreshold membrane potentials and rapidly inactivate upon depolarization. Five members (Kv1.4, 3.4, 4.1 - 4.3) of the Kv family exhibit similar properties to $I_A$ when heterologously expressed (for review see Song, 2002). Coexpression of the Kvβ1 subunit (see section on β-subunits below) with members of the Kv1 subfamily also yields currents with similar properties to $I_A$.

In contrast, delayed-rectifier currents ($I_{DR}$), like those originally studied by Hodgkin and Huxley (1952a-d), exhibit only mild inactivation over a 100-200ms depolarization. Members of at least five potassium channel subfamilies (Kv1, 2 and 3, eag and KCNQ) produce delayed-rectifier like currents when heterologously expressed.

Gating

The manner in which Kv channels couple changes in membrane potential to channel opening and closure has remained elusive for sometime despite being the subject of intensive investigation. The results obtained from a combination of approaches including mutagenesis studies, accessibility measurements, fluorescence labelling and electrophysiology suggest that channel gating occurs in association with the movement of charged amino acids in the S4 and S2 segments within the cell membrane (see for example Papazian et al., 1991; Aggarwal & MacKinnon, 1996; Cha & Bezanilla, 1997; Starace & Bezanilla, 2001; for reviews see Bezanilla, 2000; Fedida & Hesketh, 2001).

More recent studies in KcsA have begun to shed light on the molecular mechanism of voltage-dependent gating. In KcsA, it is thought that the gate of the channel is formed at the site where the four M2 helices intersect ('the inner helix bundle'; see Figure 1.2A) and that channel gating occurs by a rotation of the inner helices that line the pore (Perozo et al., 1999). The channel opens following a counterclockwise rotation of the M1 and M2 helices, which occurs in response to changes in pH. Gating of KcsA has also been shown to involve small movements of the C-terminus region and movement of the internal side, but not the external side, of the selectivity filter (Perozo et al., 1999). Whilst the results of Perozo et al. (1999) suggested that there is only a subtle increase in diameter at the inner helix bundle, a more
A recent study has suggested that the pore of KcsA could open to a much wider diameter (Zhou et al., 2001a).

Further clues to how potassium channels may gate have been provided by comparing the crystal structures of MthK (Jiang et al., 2002a), a channel from the archeon Methanobacterium thermoautotrophicum, and KcsA (Doyle et al., 1998b). These potassium channels were crystallized in their open and closed states, respectively. Jiang et al. (2002b) found that there were very large differences in the arrangement of the inner helices between MthK and KcsA. Whilst the helices were almost straight in KcsA, forming a bundle near the intracellular solution (i.e. the inner helix bundle), they were bent and splayed open in MthK creating a wide (12Å) entrance (see Figure 1.2B).

In MthK, the inner helices bend at a hinge point termed the gating hinge, which corresponds to a glycine residue in the amino acid sequence. Jiang et al. (2002b) proposed that opening might occur following the application of lateral force to the C-terminal half of the inner helices, causing the gating hinge to bend approximately 30° and the bundle to come apart. The glycine residue is highly conserved among potassium channels suggesting that such conformational changes may underlie gating in most potassium channels. Indeed, the results from a recent mutagenesis study in Shaker support this view (Yifrach & MacKinnon, 2002).

Inactivation

Kv channels also close spontaneously, typically in response to maintained depolarizations. Kv channels display a wide variety of inactivation time constants ranging from a few milliseconds to a few seconds, which have very different effects on the repetitive firing properties of individual neurones. Channel inactivation can be brought about in two distinct ways known as ‘N- or C-type inactivation’ (Hoshi et al., 1991; for review see Kukuljan et al., 1995).

N-type inactivation of potassium channels is typically regarded as fast inactivation and may be compared to the inactivation of sodium channels, which was proposed to occur by way of a ‘ball and chain’ type mechanism. Armstrong and Bezanilla (1977) proposed that during inactivation of sodium channels a cytoplasmic particle that forms part of the channel blocks the passage of ions when bound to the internal mouth of the pore (see Figure 1.3).
**Figure 1.2.**

*Open and closed conformations of potassium channels.*  
*A*, Schematic representation illustrating two subunits of the KcsA potassium channel. The red asterisk marks the central cavity, whilst the selectivity filter is highlighted in orange. The gate is formed by the inner helix bundle, where the M2 helices cross.  
*B*, Stereo diagrams illustrating the open and closed states of two potassium channels viewed from within the membrane (upper panel) and from the intracellular solution (lower panel). The inner helices of the closed KcsA pore (red) are almost straight, forming a bundle near the intracellular solution. In contrast, the inner helices of the open MthK pore (black) are bent and splayed open creating a wide entrance. (From Jiang *et al.*, 2002b).
Inactivation of potassium channels. Schematic representation illustrating C- and N-type inactivation of potassium channels. C-type inactivation involves a constriction of the selectivity filter, whilst N-type inactivation involves a ‘ball and chain’ type mechanism as described in the text. Adapted from Yellen (1998) according to the results of Zhou et al. (2001a), which suggested that the hydrophobic central cavity forms the receptor site for the inactivation gate in N-type inactivation.
Using mutagenesis approaches, Iverson & Rudy (1990), Hoshi et al. (1990) and Zagotta et al. (1990) demonstrated that the N-terminus was important for N-type inactivation. The first 22 amino acids are thought to form the ‘inactivation ball’, which is tethered to the rest of the channel via the remainder of the N-terminal domain (for review see Kukuljan et al., 1995). Both charged and hydrophobic amino acid residues contribute to the interaction between the inactivation ball and the channel (Murrell-Lagnado & Aldrich, 1993a, b).

In contrast, C-type inactivation can be seen as a slow decay in the current, which remains after the rapid phase of inactivation. The time constants for this slower inactivation vary among alternatively spliced Shaker channel variants that differ in their C-termini (Hoshi et al., 1991). The inhibition of C-type inactivation by external TEA⁺ (Grissmer & Cahalan, 1989; Choi et al., 1991), external K⁺ and other monovalent cations (see for example López-Bareno et al., 1993; Baukrowitz & Yellen, 1996) suggested that this type of inactivation involved a constriction or collapse of the outer mouth of the channel pore. The alteration of C-type inactivation by amino acid mutations in the pore region (position 449 in Shaker B) or the S6 transmembrane domain (position 463 in Shaker) further supported this view (Hoshi et al., 1990; López-Bareno et al., 1993; Schlief et al., 1996). Such conformational changes have been shown to involve a cooperative movement of all four subunits of the tetramer (see Panyi et al., 1995; Ogielska et al., 1995).

Additional evidence for a rearrangement of the outer mouth of the pore during C-type inactivation has been provided by cysteine substitution experiments. Shaker channels, in which threonine 449 is mutated to cysteine, are more sensitive to block by cadmium in the C-type inactivated state, suggesting that the spatial distribution of each of the four residues at position 449 changes during C-type inactivation (Yellen et al., 1994). Furthermore, substitution of cysteine for a methionine (position 448) located at the outer mouth of the Shaker channel pore resulted in the formation of disulphide bonds between subunits in the C-type inactivated state but not in the closed state (Liu et al., 1996). These studies indicated that the structural rearrangement changes the exposure pattern of at least three amino acids (448-450 in Shaker) near the outer mouth of the pore.

External cations and TEA⁺ have been proposed to compete with C-type inactivation by a ‘foot-in-the-door’ like mechanism whereby occupancy of a site within the pore by these ions prevents the closure of the channel at the outer mouth of the pore (see López-Bareno et al., 1993; Baukrowitz & Yellen, 1996). However, using Shaker B mutants, Molina et al. (1997)
have shown that TEA\(^+\) can bind to residues located at the external entryway of the pore and block the channels without altering C-type inactivation. TEA appears to compete with C-type inactivation only when it penetrates deeply into the pore. This suggests that the conformational changes associated with C-type inactivation are local, occurring at a site deep within the outer mouth of the pore close to the selectivity filter. This hypothesis is further supported by the observation that the binding of a channel toxin to a site in the outer vestibule is relatively unaffected by C-type inactivation (Liu et al., 1996). Molina et al. (1997) speculated that the residue at position 449 in Shaker is not directly involved in inactivation, but regulates the occupancy of ions in deeper regions of the channel mouth.

Later, Starkus et al. (1997) found that Shaker channels were capable of conducting Na\(^+\) and Li\(^+\) in the C-type inactivated state provided that internal K\(^+\) was completely removed, and argued that the mechanism of C-type inactivation was unlikely to involve a structural collapse affecting the conduction pathway. Instead, it was suggested that channels appear inactivated in physiological solutions because internal K\(^+\) ions prevent Na\(^+\) ions from permeating through the channels and that K\(^+\) permeability relative to Na\(^+\) permeability is greatly reduced in the C-type inactivated state. Starkus et al. (1997) proposed that the selectivity filter changes its properties during C-type inactivation and that the change in relative permeabilities is the primary mechanism of the inactivation seen in C-type inactivated states. This is likely to be a consequence of a reduction in the outer pore diameter. K\(^+\) permeability is abolished because K\(^+\) ions are too large to fit the pore whilst Na\(^+\) ions may now experience a better low energy fit.

By constructing a chimeric K\(^+\) channel that conducted Na\(^+\) in the absence of K\(^+\), Kiss and Korn (1998) found that occupancy of the selectivity filter binding-site slowed inactivation. Furthermore, occupancy of an outer site by either K\(^+\) or Na\(^+\) influenced inactivation indirectly by trapping K\(^+\) at the selectivity filter binding-site. Kiss and Korn (1998) described the selectivity filter as a ‘dynamic aperture’, which in the open state is fully open. They proposed that the inner diameter of this aperture decreases during inactivation, which results in a reduced conductance through the channel. However, such a constriction could only occur when the binding site is unoccupied.

Together, the aforementioned studies suggest that the selectivity filter region of the pore undergoes a constriction during C-type inactivation (see figure 1.3), ‘pinching off’ the permeation pathway. Further support for this hypothesis has come from experiments
investigating pore accessibility using Ba\textsuperscript{2+} as a probe (see Harris et al., 1998; Basso et al., 1998). Channel inactivation occurs with Ba\textsuperscript{2+} bound at a high affinity site in the pore, but the conformational change in the outer pore region prevents dissociation of the blocker to the external solution.

\textbf{\(\beta\)-subunits}

To date three \(\beta\) subunits, which associate with members of the \(K_v\) channel family, have been cloned (Rettig et al., 1994; for review see Robertson, 1997; Pongs et al., 1999; Hanlon & Wallace, 2002). Designated \(K_v\beta1, K_v\beta2\) and \(K_v\beta3\), these proteins share a highly conserved core sequence lacking any membrane spanning segments, but display amino termini of varying lengths. Further diversity arises from the alternative splicing of the \(K_v\beta1\) subunit (England et al., 1995). The assembly of \(\beta\) subunits with \(K_v\alpha\) subunits may serve several functions. For example, \(\beta\) subunits can increase the cell surface expression of coexpressed \(\alpha\) subunits, shift the voltage dependence of activation and confer the property of rapid inactivation to the channel (for review see Robertson, 1997; Martens et al., 1999; Korovkhina & England, 2002). X-ray crystallography studies suggest that the \(\beta\) subunit may interact either directly or indirectly with the channels voltage sensor (Gulbis et al., 1999).

\textbf{1.2.3b. Calcium-activated potassium channels (K\textsubscript{Ca})}

Gardos (1958) first reported the existence of a Ca\textsuperscript{2+}-sensitive K\textsuperscript{+} conductance in red blood cells. Subsequently, Ca\textsuperscript{2+} -activated K\textsuperscript{+} currents have been identified in many cell types including snail neurones (Meech & Strumwasser, 1970), chromaffin cells (Marty, 1981) and skeletal muscle (Pallotta et al., 1981; Romey & Lazdunski, 1984). These channels are thought to play an important role in coupling changes in intracellular Ca\textsuperscript{2+} (Ca\textsuperscript{2+\text{in}}) with membrane potential and are involved in determining the shape of action potentials and the duration of the interspike interval. On the basis of biophysical and pharmacological properties, K\textsubscript{Ca} can be divided into three main types (see Sah, 1996; Vergara et al., 1998; Sah & Faber, 2002): high- (BK\textsubscript{Ca}), intermediate- (IK\textsubscript{Ca}) and low- (SK\textsubscript{Ca}) conductance channels.

\textit{BK\textsubscript{Ca} channels}

BK\textsubscript{Ca} channels exhibit a high unitary conductance, greater than 100pS in symmetrical K\textsuperscript{+}. These voltage-dependent channels are modulated by high concentrations of Ca\textsuperscript{2+} (1-10\textmu M), and in neurones colocalize with calcium channels (for review see Toro et al., 1998; Vergara et al., 1998; Bissonnette, 2002; Choe, 2002; Orio et al., 2002). Channel opening occurs
independently of Ca$^{2+}$ but when Ca$^{2+}$ increases to the micromolar level the channel switches to a state in which less electrical energy is required to open the channel (Meera et al., 1996).

The first BK$_{Ca}$ channel to be cloned was from the Drosophila mutant Slowpoke (Atkinson et al., 1991; Adelman et al., 1992), though related channels have subsequently been isolated from other species including rat and mouse (Christie et al., 1989; Butler et al., 1993). Only one $\alpha$ subunit has been isolated but further diversity arises as a consequence of alternative splicing, phosphorylation of the $\alpha$ subunit and association with different $\beta$ subunits, four of which have been cloned to date (for review see Sah & Faber, 2002; Orio et al., 2002).

Also known as maxi-K channels, BK$_{Ca}$ channels are classified as members of the K$_v$ channel superfamily, which typically contain six transmembrane domains. However, these channels are unique in that they contain an additional membrane-spanning domain, S0, at the amino termini (for review see Toro et al., 1998; Kaczorowski & Garcia, 1999; Korovkina & England, 2002). For the purpose of this review they will be categorized in the same group as K$_v$ channels owing to similarities in their amino acid sequences.

An additional four hydrophobic segments are found in the carboxyl terminus of the BK$_{Ca}$ channel, the last of which is in close proximity to a stretch of negatively charged amino acids termed the 'calcium bowl'. This region is thought to form one of the binding sites for Ca$^{2+}$ (for review see Orio et al., 2002; Sah & Faber, 2002). A tetramerization domain (see section 1.3.1) named BK-T1 has also been identified in the hydrophilic region between the S6 and S7 hydrophobic domains (Quirk & Reinhart, 2001).

As for K$_v$ channels, BK$_{Ca}$ channels assemble as tetramers (see section 1.3.1) and associate with $\beta$ subunits, though the BK$_{Ca}$ $\beta$ subunit consists of two membrane-spanning domains (for review see Kaczorowski & Garcia, 1999). The additional transmembrane segment of the $\alpha$ subunit, S0, plays a crucial role in $\beta$-subunit modulation of the channel (Wallner et al., 1996). Although sensitivity to Ca$^{2+}$ is conferred by the $\alpha$ subunit, association of the $\alpha$ subunit with a $\beta$ subunit affects the Ca$^{2+}$ sensitivity by shifting the voltage dependence of activation to more hyperpolarized membrane potentials (see Vergara et al., 1998; Sah & Faber, 2002). Several studies have demonstrated the $\beta$ subunit to be essential for coupling localized increases in Ca$^{2+}$ to BK$_{Ca}$ currents and the repolarization of action potentials (Brenner et al., 2000; Plüger et al., 2000).
Pharmacologically, $\text{BK}_{\text{Ca}}$ channels are sensitive to block by $\text{TEA}^+$, charybdotoxin (CTX) and iberiotoxin (IBX), but are insensitive to apamin (for review see Sah & Faber, 2002). Functionally, activation of $\text{BK}_{\text{Ca}}$ channels is thought to underlie action-potential repolarization and the fast afterhyperpolarization (AHP) that follows (for review see Sah, 1996; Vergara et al., 1998).

**$\text{SK}_{\text{Ca}}$ channels**

In contrast, $\text{SK}_{\text{Ca}}$ channels display a much smaller unitary conductance (5-20pS), are voltage-insensitive and are activated by much lower concentrations of $\text{Ca}^{2+}$ (100-400nM). To date, three genes, SK1-3, which encode $\text{SK}_{\text{Ca}}$ channels have been identified (Köhler et al., 1996; Chandy et al., 1998). These subtypes differ in their tissue distribution and their sensitivity to apamin. Whilst SK2 and SK3 are blocked by apamin, SK1 is insensitive. However, all SK subtypes are insensitive to block by low concentrations of $\text{TEA}^+$, CTX and IBX (for review see Sah & Faber, 2002).

Structurally, $\text{SK}_{\text{Ca}}$ channels are similar to $\text{Kv}$ channels in that they consist of six membrane-spanning domains, though little homology between $\text{Kv}$ and $\text{SK}_{\text{Ca}}$ channels with the exception of the P-region is displayed at the amino acid level. Surprisingly, $\text{SK}_{\text{Ca}}$ channels are not gated by voltage despite the presence of an S4 domain containing three positively charged residues. Sensitivity to $\text{Ca}^{2+}$ is conferred by calmodulin to which the channel is covalently linked. The binding of $\text{Ca}^{2+}$ to calmodulin leads to a conformational change in the channel and its subsequent opening (for review see Sah & Faber, 2002).

$\text{SK}_{\text{Ca}}$ channels were thought to underlie the medium and slow AHPs (for review see Sah, 1996; Vergara et al., 1998), though recent evidence suggests that existing cloned $\text{SK}_{\text{Ca}}$ channels are not responsible for the slow component (for review see Sah & Faber, 2002). SK3 channels have been linked to the hereditary disease myotonic muscular dystrophy (for review see Wickenden, 2002).

**$\text{IK}_{\text{Ca}}$ channels**

The third type of $\text{Ca}^{2+}$-activated potassium channel, which has an intermediate conductance, exhibits limited expression having only been found in peripheral tissue. Although the first observed calcium-activated $\text{K}^+$ conductance identified by Gardos falls into this category (Christophersen, 1991), cDNA encoding an $\text{IK}_{\text{Ca}}$ channel (IK1) was not isolated until very recently (Ishii et al., 1997; Joiner et al., 1997; Logsdon et al., 1997; Ghanshani et al., 1998).
An IK_{Ca} channel is also formed on coexpression of mslo, the BK channel cloned from brain, and a newly identified potassium channel termed Slack (Joiner et al., 1998). Like SK_{Ca} channels, IK_{Ca} channels are insensitive to voltage being gated by submicromolar concentrations of Ca^{2+}, which gates the channel by way of calmodulin (Xia et al., 1998; Fanger et al., 1999; for review see Kaczorowski & Garcia, 1999). However, IK_{Ca} currents display inward rectification.

Pharmacologically, IK1 is sensitive to block by CTX and clotrimazole but is insensitive to IBX and apamin (for review see Sah & Faber, 2002). Very little is known about the function of IK_{Ca} channels. However, the biophysical and pharmacological properties of IK1 are identical to those of native IK_{Ca}s in lymphocytes (Khanna et al., 1999) and the block of native IK_{Ca} channels in red blood cells may be of therapeutic value in the treatment of sickle cell anaemia (for review see Wickenden, 2002).

1.2.3c. Eag-like potassium channels

The first member of this family, Eag, was isolated from Drosophila in the early 1990s (Warmke et al., 1991), though its locus, which carries a mutation that causes an ether-sensitive leg-shaking phenotype was originally discovered in 1969 (Kaplan & Trout, 1969). Mutations of eag cause repetitive firing and enhanced transmitter release in motor neurones (Ganetzky & Wu, 1983).

Eag contains a cytoplasmic C-terminus segment that is homologous with a cyclic nucleotide-binding domain (cNBD), and exhibits greater homology at the amino acid level with cyclic nucleotide-gated cation channels than with Shaker-related potassium channels. However, when heterologously expressed, Eag displays voltage-dependent, K^{+}-selective currents (Robertson et al., 1996; Pond & Nerbonne, 2001).

Homology screening has subsequently identified several related channels (Warmke & Ganetzky, 1994; for review see Ganetzsky et al., 1999), which are currently divided into three subfamilies (for review see Schwarz & Bauer, 1999): Eag, Elk (Eag-like potassium channel) and Erg (Eag-related gene). In Drosophila, a single gene defines each subfamily, whilst in mammals each subfamily contains multiple members with distinct kinetic properties. These potassium channels form a diverse family, which includes the cardiac delayed rectifier current I_{K(V_o)} encoded by the human ether-à-go-go related gene (HERG) and auxiliary MiRP1 (KCNE2) subunits. HERG has been implicated in the long QT syndrome, a hereditary
condition characterized by excessively long cardiac action potentials (the QT interval is prolonged on the electrocardiogram), which can cause ventricular fibrillation and may result in sudden cardiac death (for reviews see Ganetzky et al., 1999; Sanguinetti, 2000; Vandenberg et al., 2001).

Owing to similarities in current kinetics, Eag-like currents were once considered as candidates for the molecular correlates of the M-current (see for review Stansfeld et al., 1997), though members of the KCNQ family are now considered more likely candidates (see section below).

1.2.3d. KCNQ potassium channel family

The KCNQ gene family represents the newest member of the six transmembrane domain potassium channel family. To date, five genes (KCNQ1-5) have been identified (for review see Robbins, 2001). KCNQ2 and KCNQ3 heteromultimers are thought to underlie the M-current ($I_M$) (Wang et al., 1998), a slowly activating, non-inactivating $K^+$ current that is responsible for damping neuronal excitability. However, homomeric KCNQ2-5 and heteromers of KCNQ3 with KCNQ5 or KCNQ4 also form functional channels with similar biophysical and pharmacological properties to $I_M$ (see Selyanko et al., 2000; Wickenden et al., 2001; Jentsch, 2000).

Brown and Adams (1980) first discovered $I_M$ during their studies of the effects of acetylcholine (ACh) on sympathetic ganglion neurones, naming it the M-current because it is suppressed by muscarinic receptor activation. In many cell types $I_M$ is the only current active at voltages near the threshold for action potential firing (for review see Marrion, 1997). Subsequently, it has been discovered that $I_M$ is in fact suppressed by activation of many receptor types including substance P, luteinizing hormone releasing hormone (LHRH), purinergic and β-adrenergic (for review see Marrion, 1997). It is thought that a diffusible second messenger couples receptor activation to suppression of $I_M$ owing to the large number of receptor types that underlie its modulation. The mechanism by which $I_M$ is modulated remains elusive, though a recent study points to the hydrolysis of phosphatidylinositol 4, 5-bisphosphate (PIP$_2$) as a crucial determinant of M-channel modulation (Suh & Hille, 2002).

Mutations in four (KCNQ1-4) out of the five KCNQ genes underlie hereditary diseases, causing electrical hyperexcitability in cardiac arrhythmia and epilepsy, and underlying defects in transepithelial transport in congenital deafness and cell degeneration in progressive hearing loss (for review see Jentsch, 2000).
1.2.4. Two-transmembrane domain potassium channels

Until 1993 all the potassium channels that had been cloned exhibited a similar structure. The molecular features of numerous other potassium channels including inward rectifiers remained unknown. Although several studies had reported the expression of inward rectifier K⁺ currents in *Xenopus* oocytes following injection of messenger ribonucleic acid (mRNA) isolated from various sources, expressed currents were small (see Yoshii & Kurihara, 1989; Lewis *et al.*, 1991; Cui *et al.*, 1992.

Inwardly rectifying K⁺ currents are the predominant conductance in J774.1 cells, a murine macrophage cell line (McKinney & Gallin, 1988). Injection of mRNA isolated from these cells into *Xenopus* oocytes resulted in the expression of large, K⁺-selective currents (Périer *et al.*, 1992). This high level of expression of inward rectifier current and the absence of other expressed current made J774.1 mRNA highly suitable as a starting material for expression cloning of inward rectifier potassium channel cDNA. Although an inward rectifier potassium channel was eventually cloned from these cells (IRK1; Kubo *et al.*, 1993a), the first inward rectifier was cloned from rat kidney (ROMK1; Ho *et al.*, 1993).

Hydropathy analysis of ROMK1 (Ho *et al.*, 1993) and IRK1 (Kubo *et al.*, 1993a) suggested the presence of only two transmembrane domains termed M1 and M2, in contrast to the six transmembrane domains displayed by Kv channels. Although these two proteins were clearly different from Kv channels in their overall structure, they were found to contain an H5-like region (now termed the P-loop) similar to that found in Kv channels. In particular, the GYG motif (see section 1.6), which was subsequently shown to be critical for K⁺ selectivity in Kv channels (Heginbotham *et al.*, 1994), was conserved. In their structural model, Ho *et al.* (1993) proposed that the M1 and M2 segments span the membrane flanking the P-loop, which was likely to form the pore of the channel (see middle panel of figure 1.1). ROMK1 and IRK1 were proposed to resemble the 'core region' (S5, H5 and S6) of Kv channels, and were thus classified as a new superfamily (Kubo *et al.*, 1993a).

The cloning of a new member of the inward rectifier potassium channel family, GIRK1 (Kubo *et al.*, 1993b), quickly followed. GIRK1 was shown to share 43% and 39% identity in the total amino acid sequence with IRK1 and ROMK1, respectively. ROMK1, IRK1 and GIRK1 can be distinguished from each other based on their electrophysiological properties. Whilst all three clones encode inward rectifier potassium channels, ROMK1 and IRK1 are
constitutively active (see sections 1.2.4a and b) and display differences in their degree of rectification (see section 1.4.2), whilst GIRK1 currents require activation by G-proteins (see section 1.2.4c).

Since these preliminary studies, numerous cDNAs encoding inward rectifier potassium channels have been isolated (for review see Nichols & Lopatin, 1997). Because insight into the diversity of potassium channels has been gained using the combined approaches of physiology and molecular biology, a confusing array of terminology existed for the description of potassium channels. For inward rectifier potassium channels, the first channels to be cloned were named based on molecular cloning, i.e ROMK1, IRK1 and GIRK1. When Doupnik et al. (1995) divided inward rectifier potassium channels into subfamilies based on their amino acid identity and electrophysiological properties a standardized nomenclature was introduced. Members are termed Kir x.y, where ‘x’ designates the subfamily and ‘y’ designates the subtype within the subfamily. Thus, ROMK1, IRK1 and GIRK1 are now known as Kir1.1a, Kir2.1 and Kir3.1, respectively. Each of the seven subfamilies, which have been described to date, is considered further below.

1.2.4a. Kir1.0 subfamily

Shortly after the cloning of Kir1.1a from rat (Ho et al., 1993), its homologue was isolated from human (Yano et al., 1994; Shuck et al., 1994). Additional transcripts are formed by alternative splicing of Kir1.1a and are found in both rat and human (Zhou et al., 1994; Yano et al., 1994; Shuck et al., 1994; Boim et al., 1995; Kondo et al., 1996). Originally named ROMK2-6, but renamed Kir1.1b-f using standardized nomenclature, these transcripts share a common 3’ exon encoding the majority of the channel protein. However, they exhibit N-termini with variable lengths. For example, Kir1.1b lacks the first 19 amino acids of Kir1.1a, whilst Kir1.1c contains an additional 7 amino acids.

Tissue distribution of Kir1.0 subfamily channels

Kir1.1 transcripts have been detected in a wide variety of tissues including brain, heart, liver, pancreas, skeletal muscle and spleen but are most abundant in kidney (see Yano et al., 1994; Shuck et al., 1994). The Kir1.1 isoforms are differentially expressed along the nephron in the rat kidney (see Lee & Hebert, 1995; Boim et al., 1995). Kir1.1b displays the widest distribution having been detected in the medullary thick ascending limb (MTAL), cortical thick ascending limb (CTAL), cortical collecting duct (CCD) and distal convoluted tubule (DCT). In contrast, Kir1.1a is specifically expressed in the CCD and outer medullary
collecting duct (OMCD), whilst Kir1.1c is expressed in the MTAL, CTAL and the DCT (see Lee & Hebert, 1995; Boim et al., 1995; for review see Schulte & Fakler, 2000).

**Properties of Kir1.0 subfamily channels**

These low-conductance channels are characterized by a high, voltage-independent open probability \( P_{\text{open}} \), mild inward rectification and high \( K^+ \) selectivity (Ho et al., 1993). Furthermore, they exhibit a marked sensitivity to intracellular pH \( (pH_i) \), which may be important for \( K^+ \) homeostasis during different metabolic states (Fakler et al., 1996b). Small reductions in \( pH_i \) from, for example, 6.8 to 6.4 or 7.4 to 6.8 decrease the \( P_{\text{open}} \) of Kir1.0 channels (see Tsai et al., 1995; Fakler et al., 1996b; Doi et al., 1996; Choe et al., 1997; Shuck et al., 1997; McNicholas et al., 1998). The reduction in \( P_{\text{open}} \) has been shown to be a consequence of the appearance of a long closed state (Choe et al., 1997; McNicholas et al., 1998).

The steep pH sensitivity of members of the Kir1.0 subfamily is conferred by a lysine (K80 on Kir1.1a, K61 on Kir1.1b), located immediately before the first transmembrane domain M1 (Fakler et al., 1996b; Choe et al., 1997). An additional residue (T70 on Kir1.1a and T51 on Kir1.1b), located in a conserved hydrophobic region known as the ‘Q-region’ (known alternatively as M0; Ho et al., 1993), also plays an important role in the modulation of pH sensitivity (Choe et al., 1997). Furthermore, two arginines (R41 and R311) located in the N- and C-termini have also been identified as crucial determinants of gating of Kir1.0 subfamily channels by \( pH_i \) (Schulte et al., 1999; for review see Schulte & Fakler, 2000).

Kir1.0 subfamily channels are inhibited by Ba\(^{2+}\) (Ho et al., 1993; Zhou et al., 1996; Löffler & Hunter, 1997), but are insensitive to TEA\(^{+}\) (Ho et al., 1993). Kir1.1b has been shown to be modulated by ATP, low concentrations being required to prevent channel run-down, but higher concentrations resulting in channel inhibition (McNicholas et al., 1994). Kir1.1b is also modulated by protein kinase A (PKA) (McNicholas et al., 1994; Xu et al., 1996). PKA phosphorylation enhances the interaction of PIP\(_2\) with Kir1.0 subfamily channels (Liou et al., 1999), which is required for constitutive activity (see section on modulation of Kir2.0 subfamily channels for further discussion of PIP\(_2\)). Channel regulation by PKA requires the involvement of an A kinase anchoring protein, AKAP75/79 (Ali et al., 2001. See section 1.4.2b for more detailed discussion of AKAPs).
Role of $K_{ir}1.0$ subfamily channels

Members of the $K_{ir}1.0$ subfamily play an important role in the recycling of $K^+$ in the thick ascending limb (TAL) and secretion of $K^+$ across the apical membrane of mammalian cortical collecting (CCD) tubules in the kidney (see section 1.5.1. for detailed discussion; for review see Wang et al., 2002).

When expressed alone, the properties of $K_{ir}1.1$ channels are not absolutely identical to those of the native small-conductance channels expressed in the kidney (see for example Ho et al., 1993). However, co-expression with members of the ABC family of proteins such as CFTR and sulphonylurea receptors (SUR, see section 1.4.2f), which are both expressed in the distal nephron, alters channel properties (see for example McNicholas et al., 1996; Ruknudin et al., 1998). For example, co-expression of $K_{ir}1.1a$ with CFTR in oocytes increased the sulphonylurea sensitivity of $K_{ir}1.1a$ to glibenclamide to a level similar to that of the native channels (McNicholas et al., 1996).

The recycling of $K^+$ through $K_{ir}1.1$ channels is essential in the maintenance of the function of the Na$^+$.K$^+$.2Cl$^-$-cotransporter and mutations in $K_{ir}1.1$ channels causes type II Bartter’s syndrome, characterized by an impaired renal concentrating capacity (see section 1.5.1). Several mutations in $K_{ir}1.1$ have been linked with Bartter’s syndrome, which abolish channel function. These include mutations that introduce nonsense codons or frameshifts in the amino terminus (see for example Simon et al., 1996b), a mutation in the core (M1-P-loop-M2) domain that alters the ion conduction pathway (see Derst et al., 1998), and a mutation that deletes the last 60 amino acids at the extreme carboxyl terminus (see Simon et al., 1996b; Flagg et al., 1999).

1.2.4b. $K_{ir}2.0$ subfamily

The four members of this second subfamily are, like members of the $K_{ir}1.0$ subfamily, constitutively active, but in contrast exhibit strong inward rectification (see Chapter 3). The currents carried by these channels display rapid activation upon hyperpolarization and are blocked by Ba$^{2+}$ and Cs$^+$ (see section 1.4.1).

$K_{ir}2.1$

The first member, IRK1 (but now named $K_{ir}2.1$) was originally isolated from murine J774.1 cells (Kubo et al., 1993a) but homologues have subsequently been isolated from various
sources including mouse and human brain (Morishige et al., 1993; Ashen et al., 1995; Tang et al., 1995), rabbit, human, mouse, rat and guinea-pig heart (Ishii et al., 1994; Ishihara & Hiraoka, 1994; Raab-Graham et al., 1994; Ashen et al., 1995; Wood et al., 1995; Nagashima et al., 1996; Liu et al., 2001a), a rat basophilic leukaemia (RBL-2H3) cell line (Wischmeyer et al., 1995), chick cochlear sensory epithelium (Navaratnam et al., 1995), bovine aortic and corneal endothelial cells (Forsyth et al., 1997; Yang et al., 2000b), human blood eosinophils (Tare et al., 1998), rat and human arterial smooth muscle cells (Horowitz, 1997; Bradley et al., 1999), human, chick and rabbit lens epithelium (Rae & Shepard, 1998), and a HeLa cell line (Klein et al., 1999).

Kubo et al. (1993) reported the unitary conductance of the Kir2.1 clone isolated from J774.1 cells to be ~21pS with 140mM K+ in the patch pipette. The Kir2.1 clone isolated from mouse brain exhibits a similar conductance (~21pS, Morishige et al., 1993), but the Kir2.1 clones isolated from rabbit, rat and human have a slightly higher conductance at ~23pS (Nagashima et al., 1996), 25pS (Wischmeyer et al., 1995) and ~30pS, respectively despite similar recording conditions (Raab-Graham et al., 1994; Tang et al., 1995; Wood et al., 1995).

Minor differences in the amino acid sequences between species may be responsible for the variation in unitary conductance. For example, the Kir2.1 clone isolated from chick cochlear sensory epithelium, which displays a unitary conductance of ~17pS, contains a glutamine (Q125) in the M1-P-region linker, the corresponding residue of which is a negatively charged glutamate (E125) in the mouse clones. Navaratnam et al. (1995) found that mutation of this residue to a glutamate (Q125E) increased the single channel conductance of the channel to 28pS.

An alternative possibility is that species-specific regulatory proteins may associate with Kir2.1 and alter the single channel conductance. In rat, a ubiquitously expressed protein known as KCRF (for K channel regulatory factor), decreases the level of expression of Kir2.1 in oocytes (Keren-Raifman et al., 2000). However it was not tested whether this was a consequence of a reduced unitary conductance, a reduced P_{open}, or simply a reduction in the number of channels being expressed in the membrane.

Variations in the single channel conductance have also been observed within the same species. Thus, whilst the Kir2.1 clone isolated from the chick cochlear sensory epithelium has a unitary conductance of ~17pS (Navaratnam et al., 1995), the clone isolated from lens
epithelium displays a conductance of ~30pS (Rae & Sheppard, 1998), similar to the value reported for human clones. A recent study has demonstrated that on expression in *Xenopus* oocytes and in HEK293 cells, Kir2.1 channels display a broad distribution of unitary conductance ranging from 2 to 30pS (Picones *et al.*, 2001).

Kir2.1 is expressed in a wide variety of tissues, as is apparent from the numerous sources from which it has been cloned. For example, the properties of the Kir currents in arterial smooth muscle most closely resemble those of expressed Kir2.1 currents (Bradley *et al.*, 1999). These Kir currents are thought to play a pivotal role in K⁺-mediated vasodilation (see section 1.5.2. for a more detailed discussion). Several studies (Brahmajothi *et al.*, 1996; Nakamura *et al.*, 1998; Zaritsky *et al.*, 2001) have also shown that Kir2.1 is the major component of the strong inward rectifier in the heart (IKᵩ), which is involved in stabilizing the resting membrane potential and regulating the shape and duration of cardiac action potentials (see section 1.5.3 for more detailed discussion).

**Kir2.2**

The first evidence that the Kir2.0 subfamily consisted of multiple genes came in 1994 with the cloning of a novel inward rectifier potassium channel, RB-IRK2, from rat brain (Koyama *et al.*, 1994). Homologues have subsequently been cloned from mouse brain (MB-IRK2, Takahashi *et al.*, 1994), human atrium (hIRK1, Wible *et al.*, 1995), human genomic or cosmid libraries (hKir2.2, Namba *et al.*, 1996; Hugnot *et al.*, 1997) and guinea-pig heart (Liu *et al.*, 2001a). Renamed Kir2.2 according to standardized nomenclature, this second member of the Kir2.0 subfamily shares 70% homology with Kir2.1. Despite the high degree of homology that Kir2.2 shares with Kir2.1, Kir2.2 exhibits a higher single channel conductance (~34pS-41pS in 140mM K⁺; Takahashi *et al.*, 1994; Wible *et al.*, 1995) under similar recording conditions. Preliminary studies have also suggested that it may also be more sensitive to block by Ba²⁺ (Thompson *et al.*, 1999). Takahashi *et al.* (1994) have suggested that the difference in the single channel conductance between the two channels might be a consequence of a single difference in the amino acid sequences of the P-region (see Chapter 4).

A variant of Kir2.2 known as hKir2.2v, which differs by three amino acids in the P-region, does not express functional channels in *Xenopus* oocytes. Furthermore, when coexpressed with Kir2.2, hKir2.2v inhibited Kir2.2 currents (Namba *et al.*, 1996). Mutational analysis indicated that the differences in the P-region between Kir2.2 and hKir2.2v were not
responsible for the loss of conductance in hKir2.2v, suggesting a role for the intracellular C-terminal region of hKir2.2v.

In contrast to Kir2.1, Kir2.2 is found predominantly in the cerebellum, but is also expressed in many other tissues including forebrain, skeletal muscle, kidney, uterus, heart and cochlea (Koyama et al., 1994; Takahashi et al., 1994; Wible et al., 1995; Hibino et al., 1997). Kir2.1 is virtually absent from the atrium (Ishii et al., 1994; Ishihara & Hiraoka, 1994; Karschin & Karschin, 1997; Melnyk et al., 2002), whilst Kir2.2 is found in both the atrium and ventricle (Koyama et al., 1994; Wible et al., 1995; Karschin & Karschin, 1997).

Despite its location on chromosome 17p11.1, to which the Smith-Magenis syndrome has also been mapped, mutations in the Kir2.2 gene have not been implicated in any disease states (Hugnot et al., 1997).

Kir2.3

A third member of the Kir2.0 subfamily, named Kir2.3 (Morishige et al., 1994; Lesage et al., 1994), was cloned from mouse brain by several groups (see Morishige et al., 1993; Lesage et al., 1994; Bredt et al., 1995), and homologues have been isolated from human (Makhina et al., 1994; Périer et al., 1994; Tang & Yang, 1994), rat (Bond et al., 1994; Falk et al., 1995) and from hamster insulinoma and mouse collecting duct (M1) cell lines (Collins et al., 1996; Welling, 1997).

At between 10-15pS, Kir2.3 displays a much lower single channel conductance than the first two members of the Kir2.0 subfamily under similar recording conditions (Makhina et al., 1994; Morishige et al., 1994; Collins et al., 1996; Welling, 1997). Although Kir2.3 differs from Kir2.1 and Kir2.2 by only one or two residues in the P-region, it contains a distinct stretch of amino acids in the extracellular loop linking the M1 and P-regions, which may determine the lower conductance and sensitivity to Ba2+.

Kir2.3 is absent from the brain and body until embryonic day 21 (Karschin & Karschin, 1997) and thereafter displays a more restricted distribution in comparison with Kir2.1 and Kir2.2. Expression of Kir2.3 has been reported in the forebrain (see Lesage et al., 1994; Morishige et al., 1994; Bredt et al., 1995; Karschin et al., 1996; Stonehouse et al., 1999), atrium (see for

1 The phenotype of the Smith-Magenis includes midface hypoplasia, speech delay and growth retardation.
example Melynk et al., 2002), astrocytes (Perillà et al., 2000), renal epithelial cells (Welling, 1997) and dorsal root ganglion neurones (Chopra et al., 1999).

**Kir2.4**

A fourth member of the Kir2.0 subfamily, Kir2.4, has been isolated from rat brain (Töpert et al., 1998), human retina (Hughes et al., 2000) and guinea-pig heart (Liu et al., 2001a). Like the previously cloned members of this subfamily, Kir2.4 exhibits strong rectification and time-dependent kinetics, but displays a much lower affinity for the channel blocker Ba$^{2+}$. The rat brain Kir2.4 also displays a lower affinity for block by Cs$^+$, whilst human Kir2.4 exhibits an affinity similar to Kir2.1 channels. As for Kir2.3, the single channel conductance of Kir2.4 is lower (15$pS in 140mM K^+$) than for Kir$^{2,2}$ and 2.2 (Töpert et al., 1998). Sequence alignment suggests that rat Kir2.4 shares between 53-63% homology with Kir2.1-2.3 (Töpert et al., 1998) and less than 48% homology with other members of the Kir1.0, 3.0, 5.0 and 6.0 subfamilies. The human and rat Kir2.4 homologues share approximately 92% identity (Hughes et al., 2000).

Töpert et al. (1998) found that rat Kir2.4 was preferentially expressed in the large choline acetyltransferase immunopositive neurones of cell motor nuclei, but failed to detect any message for Kir2.4 in the retina. In contrast, Hughes et al. (2000) observed predominant expression of human Kir2.4 in the neural retina. The presence of Kir2.4 in the retina has been confirmed recently with its detection in Müller cells (Raap et al., 2002). Strong inwardly rectifying potassium channels such as Kir2.4 may be involved in the K$^+$ influxes through membrane domains that envelope synapses, though this remains to be confirmed. Recently, Kir2.4 has also been found in guinea-pig atrium and ventricle, though its presence is restricted to neuronal cells (Liu et al., 2001a). The localization of Kir2.4 in the brain and in neuronal cells suggests that it may play a general role in the peripheral and central nervous system.

**Kir2.0 channels interact with anchoring proteins**

Kir2.0 channel subtypes have been shown to associate with members of the membrane associated guanylate kinase (MAGUK) protein family, which are thought to function as scaffolds for molecules such as PKA, facilitating neuronal regulation. Kir channels interact with regions of these proteins known as PDZ domains by way of a PDZ binding motif (Thr/Ser-X-Val), which is located at the C-terminus of the channel (Songyang et al., 1997). The MAGUK proteins with which Kir2.0 channel subtypes interact include postsynaptic density (PSD)-95/synapse-associated protein (SAP) 90 (Cohen et al., 1996a; Nehring et al.,
In Kir2.3, phosphorylation by PKA disrupts its interaction with PSD-95 (Cohen et al., 1996a). PSD-95 family members are thought to control the cell surface expression of ion channels, influencing trafficking from the endoplasmic reticulum and inducing the clustering of channels in the plasma membrane (see for example Horio et al., 1997; Kurschner et al., 1998). However, Kir2.1 also contains a cytoplasmic C-terminal motif that is necessary for its efficient export from the endoplasmic reticulum (Stockklausner et al., 2001).

Like Kir1.0 subfamily channels, Kir2.1 has been shown to associate with another class of anchoring proteins known as AKAPs. Kir2.1 associates with AKAP5, previously known as AKAP79, by way of its amino and carboxyl termini (Dart & Leyland, 2001). AKAP5 binds several enzymes including PKA and protein kinase C (PKC). However, whilst elevations in the intracellular cAMP level result in increased Kir2.1 currents the activation of PKC has no effect.

Modulation of Kir2.0 subfamily channels

Members of the Kir2.0 subfamily are modulated by a variety of mechanisms (for review see Stanfield et al., 2002). For example, Kir2.1, Kir2.3 and Kir2.4 possess consensus sites for phosphorylation by PKA and PKC. However, downregulation of channel activity in response to stimulation by PKC has been confirmed only for Kir2.3 (Henry et al., 1996), whilst evidence for the downregulation of Kir2.1 appears to be conflicting (Fakler et al., 1994b; Henry et al., 1996; Wischmeyer & Karschin, 1996; Zhu et al., 1999b). Kir2.1 channels are, however, inhibited by tyrosine kinase phosphorylation (Wischmeyer et al., 1998). The modulation of Kir2.3 by PKC is mediated by direct phosphorylation of the channel protein and a threonine (T53) in the amino terminus has been identified as the PKC phosphorylation site (Zhu et al., 1999b).

Kir2.3 channels appear to be modulated by a variety of mechanisms that do not affect other members of the Kir2.0 subfamily. Thus, Kir2.3 is modulated by arachidonic acid (AA), a cis-polyunsaturated fatty acid present in the plasma membrane (Liu et al., 2001b). The enhancement of Kir2.3 currents by AA is thought to occur by way of a direct action. Kir2.3 is also inhibited by G-protein βγ subunits, which appears to be mediated through a direct binding of the Gβγ subunits to the N-terminus of Kir2.3 (Cohen et al., 1996b). Finally, Kir2.3 is inhibited by pH. A drop in pHo inhibits single channel conductance (see Coulter et al., 1995), whilst a reduction in pHi primarily inhibits P_open (see Zhu et al., 1999a). A short motif
in the N-terminus (in M0), which consists of approximately eight amino acids centred about threonine 53, is an important determinant of channel sensitivity to pH_1 (see Qu et al., 1999).

*Kir*2.0 subfamily channels also interact strongly with PIP_2, which when bound to the channel stabilizes the open state (see Huang et al., 1998; see also for review Stanfield et al., 2002). A stretch of positively charged amino acids (PKKR, residues 186-189 in Kir2.1) located in the C-terminus mediates channel binding of PIP_2. The arginine at position 189 is highly conserved among *K* _ir_ channels. In addition to the PKKR motif, the C-terminus of Kir2.1 possesses multiple binding sites for PIP_2, which are thought to confer direct gating of these channels by PIP_2 without the need for additional gating molecules (see Zhang et al., 1999; Soom et al., 2001; see also section 1.2.4.c). Mutations in these regions reduce binding of PIP_2 to the C-terminus, lowering channel open probability.

1.2.4c. *Kir*3.0 subfamily

This third subfamily comprises the G-protein regulated *Kir* s (GIRKS) of which four members, designated Kir3.1-3.4 (GIRKS 1-4), have been identified in mammals (see for example Kubo et al., 1993b; Dascal et al., 1993; Ashford et al., 1994; Lesage et al., 1994; Bond et al., 1995; Krapivinsky et al., 1995a; Lesage et al., 1995; Dissman et al., 1996; Spauschus et al., 1996). An additional member, Kir3.5 (GIRK5/XIR), has also been found in *Xenopus* oocytes (Hedin et al., 1996). Further diversity of the *Kir*3.0 subfamily arises from the alternative splicing of the *Kir*3.2 genes to give distinct mRNA products termed *Kir*3.2 a–d, which differ in the length and amino acid sequence of their amino and carboxyl termini (see for example Dascal et al., 1993; Lesage et al., 1995; Tsaur et al., 1995; Isomoto et al., 1996a; Nelson et al., 1997; Inanobe et al., 1999b; Zhu et al., 2001).

**Tissue distribution**

Northern analysis has shown that in the heart, mRNA for *Kir*3.1 is more abundant in the atrium than in the ventricle (Kubo et al., 1993b). *Kir*3.1, 3.2 and 3.3 are widely expressed throughout the CNS (Karschin et al., 1994, 1996; Kobayashi et al., 1995), whilst expression of *Kir*3.4 is only moderate (Ashford et al., 1994; Spauschus et al., 1996; Karschin et al., 1996; Wickman et al., 2000). However, *Kir*3.4 is abundantly expressed in the heart (see for example Ashford et al., 1994). Whilst *Kir*3.2b is ubiquitously expressed (Isomoto et al., 1996a), the other isoforms of *Kir*3.2 appear to be expressed in a tissue-specific manner. Thus, *Kir*3.2a is specifically expressed in brain (Lesage et al., 1994), *Kir*3.2c is found in both brain and
pancreas (Lesage et al., 1995; Tsauer et al., 1995) and K_{ir}3.2d is expressed in the testis (Inanobe et al., 1999b).

**K_{AC}h is a heteromultimer of K_{ir}3.1 and K_{ir}3.4.**

Although early studies demonstrated that *Xenopus* oocytes injected with K_{ir}3.1 mRNA express inward rectifier potassium channels resembling the muscarinic acetylcholine potassium channel (K_{AC}h) found in the heart (Kubo et al., 1993b; Dascal et al., 1993), subsequent studies have shown that K_{ir}3.1 cannot form functional channels by itself but must coassemble with K_{ir}s 3.2, 3.3 or 3.4 (Krapivinsky et al., 1995a; Kofuji et al., 1995; Duprat et al., 1995). In *Xenopus* oocytes, K_{ir}3.1 associates with an endogenous protein, K_{ir}3.5 (GIRK5/XIR), to form functional channels (Hedin et al., 1996).

Together, K_{ir}3.1 and K_{ir}3.4 (CIR) encode a heteromeric channel with similar electrophysiological properties to K_{AC}h (Krapivinsky et al., 1995a; Hedin et al., 1996; Corey et al., 1998), which is involved in regulating the heartbeat (see section 1.5.4). Association with K_{ir}3.4 is essential for the cell surface expression of K_{ir}3.1 (Kennedy et al., 1999), though it is thought that homomeric K_{ir}3.4 channels do not contribute significantly to the K_{AC}h current (I_{KAC}h; Bettahi et al., 2002). I_{KAC}h is severely reduced in atrial myocytes from K_{ir}3.1 knock-out mice despite normal expression levels of the K_{ir}3.4 protein. Furthermore, K_{ir}3.1 knock-out mice display a modest resting tachycardia. Residues in the carboxyl terminus of K_{ir}3.4 are, however, important for Gβγ binding and activation of I_{KAC}h (Krapivinsky et al., 1998b; see below).

**Neuronal G-protein-gated inward rectifier potassium channels**

Early studies suggested that neuronal G-protein-gated inward rectifier potassium channels might consist of K_{ir}3.1 and K_{ir}3.2 subunits (Kofuji et al., 1995; Velimirovic et al., 1996). Indeed, heteromeric channels consisting of K_{ir}3.1 and K_{ir}3.2 have been identified in the cortex, hippocampus and cerebellum (Liao et al., 1996). Furthermore, Signorini et al. (1997) have reported a reduction of K_{ir}3.1 protein levels in the brains of K_{ir}3.2 knockout mice suggesting that the majority of K_{ir}3.1 in the brain interacts with K_{ir}3.2.

K_{ir}3.2 is the only member of the K_{ir}3.0 subfamily found in dopaminergic neurones (Karschin et al., 1996; Inanobe et al., 1999a), where stimulation of dopamine (D2) or γ-aminobutyric acid (GABA_B) receptors generates slow inhibitory postsynaptic potentials by activating a G-protein-gated inward rectifier potassium channel (Innis & Aghajanian, 1987; Lacey et al., 1996).
1988). As discussed in the opening introduction, the weaver mouse phenotype, which is caused by a single point mutation (G156S) in the pore region of Kir3.2 (Patil et al., 1995), is associated with the loss of cerebellar granule cells and dopaminergic cells of the substantia nigra (see section 1.5.6 for further discussion), regions in which Kir3.2 is expressed.

Kir3.2 and Kir3.3 are also co-expressed in some brain regions (see for example Duprat et al., 1995; Lesage et al., 1995; Kofuji et al., 1995) and have been purified as a complex from native brain (Jelacic et al., 2000). Kofuji et al. (1995) reported that Kir3.3 had an inhibitory effect on Kir3.2 currents, whilst Jelacic et al. (2000) have shown that heteromeric Kir3.2/Kir3.3 channels display a smaller unitary conductance and a lower sensitivity to activation by G protein than heteromeric channels containing Kir3.1 subunits.

**Modulation of Kir3.0 subfamily channels**

Hormones and neurotransmitters regulate the activity of Kir3.0 channels. Among the G-protein-linked receptor subtypes that activate members of the Kir3.0 subfamily are muscarinic (M2), cholinergic, GABA, serotonin (5-HT1A), adenosine (P1), somatostatin, opioid (μ, κ, δ), α2-adrenoergic, and D2 receptors (see for example North, 1989; for review see Jan & Jan, 1997).

In the heart, stimulation of the muscarinic receptor leads to activation of the inward rectifier K channel (see section 1.5.4). This process occurs by way of a pertussis toxin-sensitive G protein (Pfaffinger et al., 1985; Breitweiser & Szabo, 1985), but does not involve a diffusible second messenger (Soejima & Noma, 1984). Receptor activation leads to the exchange of GTP for GDP and the subsequent dissociation of the G-protein into Ga and Gbγ subunits (for review see Yamada et al., 1998; Stanfield et al., 2002). Although both α and βγ subunits of the G-protein have been suggested to activate IKACH (see for example Logothetis et al., 1987; Codina et al., 1987; Yantani et al., 1988a, b), the majority of experimental evidence supports the hypothesis that activation of IKACH occurs by way of the βγ subunit (Logothetis et al., 1987; Kurachi et al., 1989; Wickman et al., 1994; Krapivinsky et al., 1995b; for review see Stanfield et al., 2002).

As for the native cardiac channel, members of the Kir3.0 subfamily (Reuveny et al., 1994; Kofuji et al., 1995) are gated by the βγ subunits of heterotrimeric G-proteins, which directly bind to the channel. Both the N- and C-terminal domains of GIRK1 have been demonstrated
to be important in mediating channel activation by Gβγ subunits (Reuveny et al., 1994; Takao et al., 1994; Dascal et al., 1995; Huang et al., 1995; Kunkel & Peralta, 1995; Slesinger et al., 1995).

How does the binding of Gβγ subunits lead to channel activation? Recent studies suggest that the binding of Gβγ subunits might lead to conformational rearrangements of the second transmembrane domain and the proximal C-terminal domain, which results in an increase in $P_{\text{open}}$ owing to an increase in open time kinetics and in burst duration (see for example Sadja et al., 2001; Yi et al., 2001; for review see Stanfield et al., 2002). Gβγ subunits also stabilize the interaction of G-protein gated inward rectifiers with PIP$_2$, which as described in section 1.2.4b stabilizes the open state of inward rectifier potassium channels (see for example Huang et al., 1998). Inhibition of heteromeric Kir3.1/Kir3.4 channels with a PIP$_2$ antibody suggested that the binding affinity of Kir3.0 subfamily channels for PIP$_2$ is weaker than those of Kir1.1 and Kir2.1 (Huang et al., 1998). However, the affinity of Kir3.0 channels for PIP$_2$ is enhanced by Gβγ subunits. In addition, intracellular Na$^+$ has also been shown to stabilize the channel-PIP$_2$ interaction in Kir3.0 subfamily channels (see Ho & Lagnado, 1999a; Zhang et al., 1999).

1.2.4d. Kir4.0 subfamily

The first member of the Kir4.0 subfamily was cloned from rat brain and named BIR10 (Bond et al., 1994). Also known as BIRK1 and K$_{AB}$-2 (Bredt et al., 1995; Takumi et al., 1995), this channel is now termed Kir4.1 using standardized nomenclature and has since been isolated from human kidney and mouse brain (Shuck et al., 1997; Kurschner et al., 1998). The initial classification of the Kir4.1 clones caused confusion. Because these clones displayed greatest homology with Kir1.1a, and contained both a Walker type-A motif and ATP-binding domain, Kir4.1 was initially classified into a subfamily termed K$_{AB}$, which also contained Kir1.1a (Takumi et al., 1995). Subsequently, related channels have been cloned from salmon brain (Kir4.3; Kubo et al., 1996), human kidney and skeletal muscle (Kir4.2; Ohira et al., 1997; Gosset et al., 1997; Shuck et al., 1997), guinea-pig kidney (Kir4.2; Derst et al., 1998) and mouse and rat liver (mKir4.2, Kir4.2 and Kir4.2a; Pearson et al., 1999; Hill et al., 2002).

Biophysical properties of Kir4.0 subfamily channels

Kir4.1, 4.2 and 4.3 have all been demonstrated to form functional channels when expressed in heterologous systems (see for example Bond et al., 1994; Bredt et al., 1995; Takumi et al., 1995; Tucker et al., 1996; Kubo et al., 1996; Pearson et al., 1999; Hill et al., 2002), though
the Kir4.2 clones isolated from human failed to give functional channels (Shuck et al., 1997). Kir4.1 displays a high $P_{\text{open}}$ and has a single channel conductance between 20 – 27pS in 140mM symmetrical K⁺ (Takumi et al., 1995; Yang & Jiang, 1999; Tanemoto et al., 2000). Kir4.2 also displays a high $P_{\text{open}}$ and a unitary conductance of approximately ~25pS under similar recording conditions (Derst et al., 1998; Pessia et al., 2001), whilst the conductance of Kir4.3 is higher at 37pS (Kubo et al., 1996).

Kir4.1 and Kir4.2 have been shown to be sensitive to changes in pH, though Kir4.2 is more sensitive than Kir4.1 (Shuck et al., 1997; Pearson et al., 1999; Yang & Jiang, 1999; Pessia et al., 2001). Both Kir4.1 and Kir4.2 contain a lysine residue immediately before the M1 transmembrane domain, which corresponds to a lysine previously reported to be an important determinant of pH sensitivity in Kir1.1 channels (see section 1.2.4a; see also Fakler et al., 1996b; Shuck et al., 1997; Pearson et al., 1999; Xu et al., 2000a). The increased sensitivity of Kir4.2 to pH is conferred by an additional pH-sensing mechanism involving the C-terminus (Pessia et al., 2001). In Kir4.1, intracellular protons inhibit $P_{\text{open}}$ while low pH enhances the single channel conductance (Yang & Jiang, 1999).

Members of the Kir4.0 subfamily display intermediate rectification (Bond et al., 1994; Takumi et al., 1995; Kubo et al., 1996; Shuck et al., 1997; Pearson et al., 1999; Lourdel et al., 2002), which has been linked to the presence of a serine in the carboxyl terminus. In the strong inward rectifier Kir2.1 the analogous residue is a negatively charged glutamate (E299; Kubo & Murata, 2001; see section 1.4.3).

**Distribution and role of Kir4.0 subfamily channels**

Kir4.1 was initially found to be predominantly expressed in specific brainstem nuclei (Bredt et al., 1995) and in glial cells of the brain (Takumi et al., 1995), but has also been detected in kidney (Ito et al., 1996; Shuck et al., 1997; Tanemoto et al., 2000). The function of Kir4.1 in the kidney involves the recycling of K⁺ for the maintenance of the Na⁺-K⁺-2Cl⁻ co-transporter (see also section 1.2.4e).

Kir4.1 is also present in the marginal cells of the stria vascularis in the cochlea where it is thought to be critically involved in the generation of the positive endocochlear potential, which is essential for hearing (Ito et al., 1996; Hibino et al., 1997). Hibino et al. (1997) have reported that a mutant strain of mice known as Wv/Wv, which are deaf and have an endocochlear potential of 0mV, lack Kir4.1 from their stria vascularis.
Kir4.2 has been localized in kidney, lung, pancreas, heart and brain (Ohira et al., 1997; Gosset et al., 1997; Shuck et al., 1997; Derst et al., 1998). In hepatocytes, Kir4.2 is localized to the basolateral membrane where it is thought to couple to anion secretion and transepithelial water flow during bile formation (Hill et al., 2002). In contrast, the expression of Kir4.3 appears to be limited to brain (Kubo et al., 1996), but very little is known about the function of these channels.

**Kir4.0 channels interact with anchoring proteins**

Like members of the Kir2.0 subfamily, Kir4.0 channels also interact with proteins of the PSD-95 family (Horio et al., 1997; Kurschner et al., 1998; Connors & Kofuji, 2002), which appear to increase the efficiency of channel formation. For example, in Müller cells functional expression of the dystrophin isoform Dp71 is necessary for the clustered localization of Kir4.1 channels. Dp71 is a core protein in a PDZ-containing protein complex, which spans the cell membrane to bridge the extracellular matrix with the actin cytoskeleton (Connors & Kofuji, 2002). The localized distribution of Kir4.1 channels in Müller cells is important for ‘K⁺ siphoning’, a specialized form of K⁺ spatial buffering essential for the regulation of extracellular K⁺ levels (see Newman, 1993).

Several studies have shown that interaction of Kir4.0 channels with PDZ-containing proteins leads to an increase in current density, which was found to be a consequence of an increase in the number of functional channels expressed in the membrane (Horio et al., 1997; Kurschner et al., 1998). However, whilst Connors and Kofuji (2002) found that Dp71 was critical for the clustering of Kir4.1 in mouse Müller cells, the level of cell surface expression was unaffected.

**Heteromeric Kir4.x channels display distinct biophysical properties**

Kir4.1 and Kir4.2 also form heteromultimeric channels. Kir4.1 forms heteromers with Kir1.1 (Glowatzki et al., 1995), Kir2.1 (Fakler et al., 1996a) and Kir5.1 (Pessia et al., 1996; Xu et al., 2000a), whilst Kir4.2 forms heteromers with Kir5.1 (Pearson et al., 1999; Pessia et al., 2001). Heteromeric Kir4.1-Kir5.1 or Kir4.2-Kir5.1 channels display distinct properties from their homomeric counterparts including an increase in macroscopic amplitude, a higher unitary conductance and a lower P_open (Pessia et al., 1996; Pearson et al., 1999; Tanemoto et al., 2000; Yang et al., 2000b; Pessia et al., 2001). Furthermore, heteromerization of Kir5.1 with Kir4.1 enhances the pH₇.₅-sensitivity of Kir4.1 so that the heteromultimeric channel is sensitive
to inhibition by protons within the physiological range (Tanemoto et al., 2000; Tucker et al., 2000; Xu et al., 2000a; Yang et al., 2000a; Pessia et al., 2001). This inhibition is mediated by a reduction in the $P_{\text{open}}$, which arises from a decrease in the mean open time and an increase in the mean closed time, and is modulated by PIP$_2$ (Yang et al., 2000a).

$\text{Kir}4.1$ can also form heteromers with members of the $\text{Kir}3.0$ subfamily. However, this leads to inhibition of the $\text{Kir}4.1$ current mediated by structural motifs residing in the transmembrane domains of $\text{Kir}3.0$ (Tucker et al., 1996).

### 1.2.4e. $\text{Kir}5.0$ subfamily

$\text{Kir}5.1$ resides in its own subfamily owing to significant structural differences. Originally cloned from rat brain (BIR9, Bond et al., 1994), novel sequences for mouse, rat and human homologues have also been reported (Mouri et al., 1998; Derst et al., 2001a). When expressed alone $\text{Kir}5.1$ fails to form functional channels (Bond et al., 1994; Pessia et al., 1996; Salvatore et al., 1999; Tanemoto et al., 2002). However, coexpression of $\text{Kir}5.1$ with the anchoring protein PSD-95 in HEK293T cells mediates the formation of functional homomeric channels. PSD-95 alters the intracellular location of $\text{Kir}5.1$ and increases cell surface expression by slowing the rate of internalisation (Tanemoto et al., 2002).

#### Distribution and role of $\text{Kir}5.1$/PSD-95

$\text{Kir}5.1$ has been detected in the adrenal gland, spleen, liver, kidney, testis, and brain (Bond et al., 1994; Salvatore et al., 1999; Tanemoto et al., 2000; Derst et al., 2001). Immunoprecipitation results suggest that PSD-95 and $\text{Kir}5.1$ form a protein complex in the brain (Tanemoto et al., 2002). These PSD-95/$\text{Kir}5.1$ complexes have been detected on the somato-dendritic plasma membrane in primary cultured neurons. The activity of PSD-95/$\text{Kir}5.1$ channel complexes following heterologous expression in HEK293T cells was reversibly inhibited by PKA activation. Thus, as for $\text{Kir}2.3$ channels (see section 1.2.4b), PKA activation disrupts the interaction of $\text{Kir}5.1$ with PSD-95. It has been suggested that these complexes play an important role in synaptic transmission (Tanemoto et al., 2002).

#### $\text{Kir}5.1$ heteromers

It has been suggested that $\text{Kir}5.1$ interacts exclusively with members of the $\text{Kir}4.0$ subfamily (Konstas et al., 2002). However, one study has shown that $\text{Kir}5.1$ assembles with $\text{Kir}2.1$ to give electrically silent channels (Derst et al., 2001a). Interestingly, the genes for $\text{Kir}5.1$ and
Kir2.1 are co-localized on the same chromosome, chromosomes 17q and 11 in human and mouse, respectively (Morishige et al., 1993; Mouri et al., 1998; Derst et al., 2001a). It is thought that Kir2.1 might have been copied early in the evolutionary process to encode a protein with high amino acid similarity, and that this protein, Kir5.1, acts as a negative regulator of the Kir2.1 conductance.

**Distribution and role of heteromeric Kir4.x-Kir5.1 channels**

The functional properties of heteromeric Kir4.x-Kir5.1 channels closely resemble those of potassium channels present in the distal convoluted tube (DCT) of the kidney, where the recycling of K⁺ across the basolateral membrane is essential for the reabsorption of Na⁺ by way of the Na⁺-K⁺-2Cl⁻ co-transporter (Lourdel et al., 2002). Indeed, Kir4.1, Kir4.2 and Kir5.1 are present in the DCT of kidney (Ito et al., 1996; Tucker et al., 2000; Derst et al., 2001a; Lourdel et al., 2002), and Kir4.1 co-localizes with the Na⁺-K⁺-2Cl⁻ co-transporter (Lourdel et al., 2002).

Kir5.1 and Kir4.1 are also both expressed in the brain, but their failure to coimmunoprecipitate suggests that they do not preferentially coassemble (Tanemoto et al., 2000). However, the co-localization of Kir4.1 and Kir5.1 in the brainstem and their high sensitivity to pH₅ suggest that they may be the molecular correlates of the native Kir channels involved in CO₂ chemosensitivity in brainstem neurones (Pineda & Aghajanian, 1997; Yang et al., 2000a). Inhibition of these channels results in depolarization and an increase in membrane excitability, which is thought to underlie the spread of excitation to other brainstem neurones such as those involved in cardio-respiratory control. Thus, CO₂ chemosensitivity might be coupled to a corresponding change in excitability of the cardio-respiratory system (see Yang & Jiang, 1999; Yang et al., 2000a).

Kir4.2 and Kir5.1 have been co-localized in pancreas (Pessia et al., 2001), where they may be involved in the pH-dependent secretion of K⁺ produced by the secretion of bicarbonate.

**1.2.4f. Kir6.0 subfamily**

Members of the Kir6.0 subfamily associate with sulphonylurea receptors (SURs) to form K_ATP channels, a group of weakly inwardly rectifying potassium channels that are sensitive to ATP. K_ATP channels were first described in cardiac myocytes (Noma, 1983), but have subsequently been found in numerous tissue including pancreatic beta cells (Cook & Hales, 1984), skeletal muscle (Spruce et al., 1985) and smooth muscle (Standen et al., 1989). These channels are
inhibited by millimolar concentrations of ATP and activated by Mg$^{2+}$-adenosine 5'-diphosphate (Mg-ADP; see for example Nichols et al., 1996). Channel activation occurs when the cytoplasmic ATP/ADP ratio declines, for example, in response to hypoxia or to inhibition of cell metabolism.

**Role of K$_{ATP}$ channels**

K$_{ATP}$ channels play important roles in both physiological and pathophysiological conditions by coupling cell metabolism with membrane electrical excitability. For example, they control insulin secretion from pancreatic β-cells (see section 1.5.5) and regulate vascular smooth muscle tone, but are also involved in the response to cardiac and cerebral ischaemia (for review see Ashcroft & Gribble, 1998). The diversity of K$_{ATP}$ channels arises from the assembly of different K$_{ir}$6.0 and SUR subtypes, which are distinguished by their sensitivity to inhibition by sulphonylureas such as tolbutamide and glibenclamide, and their activation by potassium channel openers such as diazoxide (see Inagaki et al., 1996; for review Aguilar-Bryan et al., 1998; Babenko et al., 1998).

**Properties of K$_{ir}$6.0 channels**

The first member of the K$_{ir}$6.0 subfamily, K$_{ir}$6.1, was isolated from a rat pancreatic islet cDNA library (Inagaki et al., 1995a), a second member (K$_{ir}$6.2) being isolated shortly after (Sakura et al., 1995). Subsequently, further homologues of K$_{ir}$6.1 and K$_{ir}$6.2 have been isolated from various human, mouse, rabbit and rat tissues (Inagaki et al., 1995b, c; Takano et al., 1996; Tokuyama et al., 1996; Yamada et al., 1997; Brochiero et al., 2002).

K$_{ir}$6.1 was originally proposed to be a K$_{ATP}$ channel, being named uK$_{ATP}$-1 accordingly (Inagaki et al., 1995a). However, it was later shown that members of the K$_{ir}$6.0 subfamily must assemble with sulphonylurea receptors (SUR) to form functional K$_{ATP}$ channels (Inagaki et al., 1995c; Sakura et al., 1995; Gribble et al., 1997). K$_{ir}$6.1 and truncated versions of K$_{ir}$6.2, in which an endoplasmic retention signal is deleted, can form functional channels in the absence of SURs (Tucker et al., 1997; Zerangue et al., 1999; Brochiero et al., 2002). However, these channels exhibit a reduced sensitivity to ATP.

K$_{ir}$6.1 and K$_{ir}$6.2 exhibit different unitary conductances (K$_{ir}$6.1/SUR ~35pS; K$_{ir}$6.2/SUR ~80pS in 150mM symmetrical K$^+$) despite that the amino acid sequences of their pore forming regions are identical (see Inagaki et al., 1995a; 1996; Isomoto et al., 1996b; Yamada et al., 1997). Residues in the extracellular linkers between the P-region and transmembrane
domains have been shown to determine this difference in unitary conductance (Kondo et al., 1998; Repunte et al., 1999).

**Sulphonylurea receptors**

Sulphonylurea receptors (SUR) are members of the ATP-binding cassette (ABC) protein superfamily to which the CFTR protein implicated in cystic fibrosis (see section 1.0) also belongs. Encoded by two genes designated SUR1 and SUR2, these receptors have a predicted topology of 17 transmembrane-spanning helices, organized into three domains termed TM0, TM1 and TM2. Furthermore, two intracytoplasmic folds (NBF1 and NBF2) contain motifs for nucleotide binding (see Aguilar-Bryan et al., 1995).

SUR1 was originally cloned from a hamster insulin secreting cell line (Aguilar-Bryan et al., 1995) and is found in pancreatic β cells, brain and heart. SUR1 exhibits a high affinity for sulphonylureas and interacts with several inward rectifier potassium channels including Kir6.2 and Kir1.1 (Ämmälä et al., 1996). Kir6.2/SUR1 complexes are thought to represent the pancreatic β-cell K\text{ATP} channel (see Inagaki et al., 1995c). A recent study has reported the existence of two novel forms of SUR1 known as SUR1A2 and SUR1BΔ31 (Gros et al., 2002). When heterologously expressed with Kir6.2, SUR1A2, which has a single amino acid change in the first nucleotide-binding domain, forms functional channels with characteristics similar to the wild-type K\text{ATP} channels of pancreatic β cells. In contrast, heteromers of Kir6.2 and SUR1BΔ31, which lacks transmembrane domains 16 and 17, fail to reach the cell membrane.

The second sulphonylurea receptor, SUR2, is ubiquitously expressed in a diverse range of tissues including the heart, skeletal muscle, ovary, brain, tongue, pancreatic islets, lung, testis, adrenal gland, stomach and colon. SUR2 is less sensitive to inhibition by both ATP and sulphonylureas, and is not activated by diazoxide (see for example Inagaki et al., 1996). Two major splice variants of SUR2, known as SUR2A and SUR2B, and a number of minor splice variants have been described (see Inagaki et al., 1996; Isomoto et al., 1996b; Chutkow et al., 1996; 1999; Bronchiero et al., 2002). Kir6.2/SUR2A displays similar properties to the K\text{ATP} channel of cardiac and skeletal muscle (see for example Inagaki et al., 1996), whilst heteromers of Kir6.1 or Kir6.2 with SUR2B are thought to represent the K\text{ATP} channel found in vascular and nonvascular smooth muscle (see Isomoto et al., 1996b; Yamada et al., 1997).
**Structure and properties of $K_{\text{ATP}}$ channels**

Functional $K_{\text{ATP}}$ channels are thought to be octameric complexes of Kir6.0 subfamily channels and SUR subunits, which combine in a 1:1 stoichiometry (Clement *et al.*, 1997; Shyng & Nichols, 1997; Inagaki *et al.*, 1997). Several lines of evidence suggest that the Kir6.1/Kir6.2 subunits form the conduction pathway of $K_{\text{ATP}}$ channels, conferring inhibition by ATP (see for example Tucker *et al.*, 1997; Proks *et al.*, 1997; for review see Ashcroft & Gribble, 1998). The SURs are thought to act as regulatory subunits, conferring sensitivity to both sulphonylureas and potassium channel openers, and enhancing ATP sensitivity (see Aguilar-Bryan, 1995; Inagaki *et al.*, 1995c; Ämmälä *et al.*, 1996).

$K_{\text{ATP}}$ channels are also modulated by PIP$_2$, which has been shown to reduce the sensitivity of $K_{\text{ATP}}$ channels to inhibition by ATP, thus increasing channel open probability (see for example Baukrowitz *et al.*, 1998; for review see Baukrowitz & Fakler, 2000). PIP$_2$ reduces channel affinity for ATP by reducing the association rate constant for ATP inhibition without affecting the dissociation rate constant. PIP$_2$ interacts with positively charged residues in the C-terminus of the Kir6.2 subunit (see also section 1.4.2b) and SUR1 further stabilizes the PIP$_2$-channel interaction (Baukrowitz *et al.*, 1998).

Mutations in both Kir6.2 and SUR1 subunits have been linked with persistent hyperinsulinemic hypoglycaemia of infancy (PHHI), an autosomal recessive disease characterized by excessive and unregulated insulin release (Thomas *et al.*, 1995; Bryan & Aguilar-Bryan, 1997). Disease-causing mutations in Kir6.2 and SUR1 subunits have been found to result in either a loss of Mg-ADP regulation or to result in a total loss of $K_{\text{ATP}}$ channel activity (see for example Thomas *et al.*, 1995; for review see Aguilar-Bryan *et al.*, 2001; Houpio *et al.*, 2002). Children born with PHHI can develop life-threatening hypoglycaemia if left untreated and individuals with severe PHHI, who fail to respond to treatment with $K_{\text{ATP}}$ channel openers such as diazoxide, typically require a partial or subtotal pancreatectomy for survival.

**1.2.4g. Kir7.0 subfamily**

Unique biophysical properties led Krapivinsky *et al.* (1998a) to propose that the inward rectifier potassium channel they had isolated from human foetal brain, which they named Kir7.1, be assigned to a new subfamily. With a hydrophobicity profile typical of Kir channel subunits, Kir7.1 displayed closest homology to human Kir1.1c, sharing 38% identity at the
amino acid level. However, lack of the putative ATP-binding site and no apparent sensitivity
to intracellular ATP distinguished Kir7.1 from members of the Kir1.0 subfamily. Kir7.1 has
subsequently been cloned from various rat, human and guinea-pig tissues (Partiseti et al.,
1998; Döring et al., 1998; Nakamura et al, 1999; Shimura et al, 2001; Derst et al., 2001b).

**Biophysical properties of Kir7.1**

The most striking features of Kir7.1 include a shallow dependence on $[K^+]_o$ (see section 1.4.1
for further discussion), a very low estimated single channel conductance (~ 50-200fS), and a
low sensitivity to block by external $Ba^{2+}$ and $Cs^+$ (see for example Krapivinsky et al., 1998a;
Döring et al., 1998). Several of these properties may be attributed to differences in the pore
region of Kir7.1. Replacement of a methionine (Met-125) in the pore of Kir7.1 with arginine,
as conserved throughout the rest of the inward rectifier potassium channel family, restored
channel dependence on $[K^+]_o$, and dramatically increased single channel conductance and
sensitivity to $Ba^{2+}$ (Krapivinsky et al., 1998a; Döring et al., 1998).

**Distribution and role of Kir7.1**

Early distribution studies (Krapivinsky et al., 1998a; Partiseti et al., 1998) showed that Kir7.1
was present in a variety of tissues including brain, kidney, small intestine and stomach.
Although such non-specific tissue distribution offered no clues to the function of the channel,
the presence of Kir7.1 in neurones but not glial cells suggested a neurone-specific role.
Krapivinsky et al. (1998a) suggested that the main role of Kir7.1 is in the reliable setting of
the resting membrane potential, a low conductance enabling significant precision in such
regulation.

In contrast, Döring et al. (1998) were unable to detect Kir7.1 in any neuronal, glial, or
connective tissue. Instead, a strong signal for Kir7.1 was detected in the secretory epithelial
cells of the choroid plexus, which forms the blood-cerebrospinal fluid barrier in the
mammalian brain. The presence of Kir7.1 in these cells, in addition to epithelia of lung,
kidney and testis, led Döring et al. (1998) to propose that Kir7.1 channels might be involved
in the regulation of transepithelial $K^+$ transport.

More recent studies have confirmed the expression of Kir7.1 in choroid plexus (Nakamura et
al., 1999) and reported a robust expression of Kir7.1 in several other epithelial cell types.
These include thyroid follicular and intestinal epithelial cells (Nakamura et al., 1999), rat
pancreatic acini (Kim et al., 2000), retinal pigmented epithelial cells (Kusaka et al., 2001;
Shimura et al., 2001) and proximal tubule epithelial cells from guinea-pig and human (Derst et al., 2001b). Such cell-specific expression provides further support for the role of Kir7.1 in the regulation of transepithelial K⁺ transport. Furthermore, it has been shown that Kir7.1 is co-localized with the Na⁺, K⁺-ATPase in several of these tissues (Nakamura et al., 1999; Kusaka et al., 2001) suggesting a close functional coupling between the channel and ion pump. It is thought that recycling of K⁺ through Kir7.1 channels is important for maintaining the activity of the Na⁺, K⁺-ATPase.

1.2.4h. Drosophila Kir s

By homology screening with conserved mammalian sequences three Kir genes designated dKirI, dKirII and dKirIII were identified in the Drosophila genome (Döring et al., 2002). Only dKirI and dKirII expressed functional channels in Drosophila S2 cells. Functional channel expression in Xenopus oocytes was also possible following introduction of mutations in the cytoplasmic domains. dKirI and dKirII are phylogenetically most closely related to the members of the human Kir2.0 channel subfamily. dKirI transcripts were absent from embryos, while dKirII and dKirIII were expressed in the embryonic hindgut and in Malpighian tubules. In the adult fly, dKirII was predominantly expressed in the head.

1.2.4i. A family of Kir channels in prokaryotes

Five prokaryotic sequences that code for proteins whose closest relatives are eukaryotic Kir s have been identified (Durell & Guy, 2001). Termed KirBacs, these prokaryotic proteins exhibit homology with eukaryotic Kir s in their C-terminus portion, whilst the sequence similarity of the P-loop and M2 transmembrane segments is intermediate between those of eukaryotic Kir s and other bacterial potassium channels.

1.2.5. Two-pore domain potassium channels

The two-pore domain potassium (K₂p) channels represent the most recently discovered class of potassium channels. Characterised by a lack of voltage- and time-dependency, with linear or near-linear current-voltage relationships in symmetrical K⁺, these channels are thought to correlate to the background (or ‘leak’) potassium channels that play essential roles in setting the resting membrane potential (see section 1.1.3).

In C.elegans, these two-pore domain channels represent the largest group of potassium channels, with approximately 50 genes encoding them (Wei et al., 1996). However, some of
these channels have unusual pore domains and may not encode functional potassium channels (Bargmann, 1998), as is the case for some of the mammalian K_{2P} channels recently isolated (e.g. TASK-5, Kim & Gnatenco, 2001; Ashmole et al., 2001; KCNK7, Salinas et al., 1999).

The first potassium channels with two P domains (P1 and P2) and either four or eight transmembrane domains were isolated from yeast (Ketchum et al., 1995; Zhou et al., 1995; Lesage et al., 1996a; Reid et al., 1996) and Drosophila (Goldstein et al., 1996). The cloning of the first mammalian K_{2P} channel quickly followed (Lesage et al., 1996b). The authors called this channel TWIK-1 (for Tandem of P domains in a Weak Inward rectifying K\(^+_\) channel) since it exhibited functional characteristics similar to a weak inward rectifier potassium channel including saturation of the current-voltage relationship at depolarized potentials in the presence of internal Mg\(^{2+}\) (Mg\(^{2+}\)\textsubscript{i}).

Based on the assumption that four P regions are required to form a K\(^+_\)-selective pore (see section 1.3) TWIK-1 was predicted to be a dimer, a functional channel arising from the homologous assembly of two subunits or from assembly with another subunit. More recent studies suggest that expression of TWIK-1 in Xenopus oocytes fails to give functional channels indicating that an unidentified accessory subunit may be required for its functional expression (Goldstein et al., 1997; Pountney et al., 1999).

Lesage et al. (1996b) proposed that the overall structural motif of TWIK-1 is similar to what one would obtain by making a tandem of two classical inward rectifier potassium channel subunits. However, unlike K\(_{ir}\) channels, TWIK-1 contains an unusually large loop of 59 amino acids between the first transmembrane domain and the first pore loop (see bottom panel of figure 1.1). A specific cysteine residue residing in this large M1-P1 loop has since been implicated in the formation of homomeric channels by extracellular disulphide bond formation (Lesage et al., 1996c). Furthermore, whilst the first pore domain contains the GYG motif typical of most other K\(^+_\)-selective channels, the second pore domain contains a leucine in place of the tyrosine residue.

TWIK-1 is expressed in a wide variety of human tissues including heart and brain. Pharmacologically, TWIK-1 is sensitive to some of the classical potassium channel blockers such as Ba\(^{2+}\), but is relatively insensitive to others including TEA and 4-AP. This lack of specific pharmacology is typical of all K\(_{2P}\) channels and has hampered the elucidation of their roles in vivo.
Subsequently, a further thirteen mammalian TWIK-1 related channels have been cloned, which can be tentatively classified into the following subgroups: the TWIK, TASK, TALK, TRAAK/TREK and THIK/KCNK7 subfamilies (for summary see Karschin et al., 2001). These K_{2P} channels are regulated by diverse physical and chemical stimuli. For example, members of the TWIK subfamily are sensitive to pH_{i}, whilst members of the TASK subfamily are regulated by pH_{o}. Other modulators of K_{2P} channel activity include free fatty acids such as arachidonic acid, volatile anaesthetics such as chloroform and halothane, and PKA and PKC (for reviews see Lesage & Lazdunski, 2000; Patel & Honoré, 2001).

1.3. Assembly and stoichiometry of potassium channels

By the end of the 1980s it was clear that the principal subunits of voltage-gated channels selective for K^{+}, Na^{+} or Ca^{2+} had closely related molecular structures (Catterall, 1988). These channels were proposed to contain four homologous transmembrane domains, each domain consisting of six membrane-spanning α-helices surrounding a central ion pore (Noda et al., 1984; Tanabe et al., 1987; Tempel et al., 1987). The α-subunit of sodium and calcium channels contains four homologous internal repeats, whilst the α-subunit of potassium channels comprises only one of these repeats. Potassium channels were therefore proposed to assemble as tetramers (Tempel et al., 1987).

1.3.1. K_{v} channels assemble as tetramers

Isacoff et al. (1990) first demonstrated, using electrophysiological analysis of covalently-linked dimers, trimers, tetramers and pentamers, that functional potassium channels contain an even number of subunits, which was later narrowed down to four (MacKinnon, 1991; Liman et al., 1992). Further evidence that K_{v} channels assemble as tetramers has been provided by studies using sucrose gradient centrifugation of in vitro translated and solubilized protein and electron-microscopy of detergent-solubilized and purified Shaker protein (Shen et al., 1993; Li et al., 1994). The latter study provided clear images of K_{v} channels, revealing a marked four-fold symmetry consistent with a tetrameric subunit composition.

Direct biochemical evidence that Shaker potassium channels contained four pore-forming subunits came in 1996 (Schulteis et al., 1996). By exposing intact cells to oxidizing conditions, disulphide bonds were generated between adjacent subunits, resulting in the formation of dimers, trimers, linear tetramers and circular tetramers of Shaker protein. More
recently, it has been shown that tetramers preferentially assemble from dimerization of dimers (Tu & Deutsch, 1999).

1.3.2. The T1 domain is required for tetramerization of K\textsubscript{v} channels

K\textsubscript{v} channels consist of either the same subunit (homomeric) or different subunits (heteromeric). However, only polypeptides in the same subfamily can form heteromeric channels (Christie \textit{et al.}, 1990; Isacoff \textit{et al.}, 1990; Ruppersburg \textit{et al.}, 1990; Covarrubias \textit{et al.}, 1991).

In \textit{Shaker} channels, a conserved cytoplasmic sequence preceding the first transmembrane domain is required for tetramerization (Li \textit{et al.}, 1992). This 114 amino-acid region known as T1, for 'Tetramerization domain 1', self-associates to form tetramers (Shen \textit{et al.}, 1993). Deletion of the T1 domain results in the failure of K\textsubscript{v} channels to form tetramers. It is thought that the T1 domain is necessary for efficient assembly and trafficking beyond the endoplasmic reticulum (Schulteis \textit{et al.}, 1998). However, K\textsubscript{v}1.3 subunits can form functional channels following its deletion (Tu \textit{et al.}, 1996). The primary role of the T1 domain in some K\textsubscript{v}1 channels is thought to be the prevention of their assembly with incompatible subunits. Indeed, the T1 domain has been shown to prevent heteromer formation of some members of the K\textsubscript{v}1 subfamily with members of other K\textsubscript{v} subfamilies (Li \textit{et al.}, 1992; Xu \textit{et al.}, 1995; for review see Papazian, 1999).

It has been suggested that two different faces of the T1 domain mediate the association of monomers to form dimers and the subsequent dimerization of dimers to give tetramers (Tu & Deutsch, 1999). The structures of the T1 domains from the K\textsubscript{v}4 and K\textsubscript{v}3 subfamilies of \textit{Aplysia californica} have been determined and have been shown to exhibit four-fold, rotational symmetry (Kreusch \textit{et al.}, 1998; Bixby \textit{et al.}, 1999). At the centre of the T1 tetramer is a water-filled cavity. This water-filled cavity does not form part of the ion conduction pore, but may represent the cytoplasmic vestibule in the ion-conduction pathway (Kreusch \textit{et al.}, 1998). The subunit interface of the T1 domain is highly polar and for all 'non-Shaker-type' K\textsubscript{v} channels, Zn\textsuperscript{2+} ions, which are present at the assembly interface, act as the main structural determinant.

In addition to the T1 domain, the first (S1) and sixth (S6) transmembrane domains of K\textsubscript{v} channels have also been shown to be important for channel assembly (Shen \textit{et al.}, 1993;
The S1 transmembrane segment enhances the binding of the T1 domain to the wild-type channel.

1.3.3. \(K_{\text{ir}}\) channels assemble as tetramers

Less is known about how \(K_{\text{ir}}\) channels assemble. Owing to the similarity between \(K_{\text{ir}}\) channels and the core region (S5-H5-S6) of \(K_{\nu}\) channels, it was proposed that \(K_{\text{ir}}\) channels also assemble as tetramers (Kubo et al., 1993a). This was soon confirmed for homomeric \(K_{\text{ir}}\)2.1 (Yang et al., 1995b), \(K_{\text{ir}}\)1.1 and \(K_{\text{ir}}\)4.1 channels (Glowatzki et al., 1995), and for the heteromERICALLY assembled \(K_{\text{ir}}\)3.1/\(K_{\text{ir}}\)3.4 channels (Silverman et al., 1996a; Corey et al., 1998). However, one study suggested that functional \(K_{\text{ir}}\)2.1 channels might consist of twelve subunits (Omori et al., 1997) and others have suggested that GIRK1, in combination with either an unknown subunit or with \(K_{\text{ir}}\)3.4, might contain between 3-5 (Inanobe et al., 1995) or 8-12 subunits (Krapivinsky et al., 1995a).

1.3.4. \(K_{\text{ir}}\) channels can form heteromers

Like \(K_{\nu}\) channels, members of the \(K_{\text{ir}}\) family also form heteromers, but in contrast, \(K_{\text{ir}}\) subunits belonging to different subfamilies can also coassemble to form functional channels. HeteromERIC assembly was first demonstrated for members of the \(K_{\text{ir}}\)3.0 subfamily (Krapivinsky et al., 1995a; Kofuji et al., 1995), and later for several other \(K_{\text{ir}}\) channels including \(K_{\text{ir}}\)4.1 and \(K_{\text{ir}}\)1.1 (Glowatzki et al., 1995), \(K_{\text{ir}}\)4.1 and \(K_{\text{ir}}\)5.1 (Pessia et al., 1996), \(K_{\text{ir}}\)2.1 and \(K_{\text{ir}}\)4.1 (Fakler et al., 1996a) and \(K_{\text{ir}}\)4.2 and \(K_{\text{ir}}\)5.1 (Pearson et al., 1999). It is likely that other members of the \(K_{\text{ir}}\) family also form functional heteromers, owing to the coexpression of different members in the same tissue. For example, \(K_{\text{ir}}\)2.1 and \(K_{\text{ir}}\)2.3 are co-localized in the same regions of the brain (Fink et al., 1996), though until recently the issue of whether members of the \(K_{\text{ir}}\)2.0 subfamily form heteromers has remained controversial.

Tinker et al. (1996) suggested that heteromerization between different \(K_{\text{ir}}\) subunits may be case specific. For example, tagged \(K_{\text{ir}}\)2.1 and \(K_{\text{ir}}\)2.2 proteins expressed in HEK293 cells do not co-precipitate suggesting that they are unable to coassemble. However, results from several recent studies have suggested that \(K_{\text{ir}}\)2.1 and \(K_{\text{ir}}\)2.2 may co-assemble to form heteromERIC channels (Zaritsky et al., 2001; Preisig-Müller et al., 2002).

Co-assembly of \(K_{\text{ir}}\)2.1 and \(K_{\text{ir}}\)2.2 has been suggested as a possible explanation for the 50% reduction of \(I_{K1}\) observed in myocytes from mice lacking the \(K_{\text{ir}}\)2.2 gene, despite evidence to
suggest that K\textsubscript{ir}2.1 accounts for the major component of this conductance (Zaritsky \textit{et al.}, 2001). These results imply that K\textsubscript{ir}2.1 is essential for the function or translocation of the heteromeric complex, whilst K\textsubscript{ir}2.2 is less essential. Thus, in the absence of K\textsubscript{ir}2.1, K\textsubscript{ir}2.2 would not be able to form homomers, but in the absence of K\textsubscript{ir}2.2, K\textsubscript{ir}2.1 forms homomeric channels that generate a current 50% smaller than that observed in wild-type, owing either to the expression of fewer channels or the smaller unitary conductance carried by homomeric K\textsubscript{ir}2.1 channels.

More direct evidence for the coassembly of members of the K\textsubscript{ir}2.0 subfamily has been presented recently (Preisig-Müller \textit{et al.}, 2002). Preisig-Müller \textit{et al.} (2002) used several approaches, including expression of concatenated K\textsubscript{ir}2.x-KIR2.y channels, coexpression of non-functional K\textsubscript{ir}2.x constructs with wild-type K\textsubscript{ir}2.x channels and co-expression of K\textsubscript{ir}2.1 mutants related to Andersen’s syndrome with wild-type K\textsubscript{ir}2.x channels to show that K\textsubscript{ir}2.1 can form heteromeric channels with K\textsubscript{ir}2.2 and K\textsubscript{ir}2.3. Co-immunoprecipitation of K\textsubscript{ir}2.1 with K\textsubscript{ir}2.3 channels provides further evidence for the formation of heteromers by members of the K\textsubscript{ir}2.0 subfamily (Preisig-Müller \textit{et al.}, 2002). The fourth member of the K\textsubscript{ir}2.0 subfamily, K\textsubscript{ir}2.4, has also been shown to form heteromers with K\textsubscript{ir}2.1 (Schram \textit{et al.}, 2002).

Although K\textsubscript{ir} channels can form heteromers between subfamilies the results from one study, which investigated coassembly of K\textsubscript{v}1.1 with K\textsubscript{ir}2.1, suggest that they do not form functional heteromers with K\textsubscript{v} channels (Tygat \textit{et al.}, 1996).

1.3.5. Structural determinants of K\textsubscript{ir} channel assembly

If heteromerization between different K\textsubscript{ir} channels is case specific, as proposed by Tinker \textit{et al.} (1996), which regions of the channel determine subunit compatibility? In contrast to K\textsubscript{v} channels, K\textsubscript{ir} channels lack a tetramerization domain. However, several regions of K\textsubscript{ir} channels have been implicated in channel assembly. These include the transmembrane domains (Tucker \textit{et al.}, 1996) and the N-terminus (Fink \textit{et al.}, 1996). However, Tinker \textit{et al.} (1996) found no effect of deletion of the N-terminus on K\textsubscript{ir} subunit assembly, but instead demonstrated the proximal C-terminus and second transmembrane domain (M2) to be important in determining the homo- and heteromeric assembly of K\textsubscript{ir} channels. For members from the same subfamily, such as K\textsubscript{ir}2.2 and K\textsubscript{ir}2.1, both the M2 and proximal C-terminus regions appear to influence assembly. In contrast, the compatibility of the C-terminus regions is the major influencing factor for heteromerization of members of different subfamilies such as K\textsubscript{ir}1.1 and K\textsubscript{ir}2.1. A recent study on the heteromeric assembly of K\textsubscript{ir}5.1 and K\textsubscript{ir}4.0
channels has confirmed the importance of the proximal C-terminus as a determinant of heteromerization (Konstas et al., 2002).

1.4. Inward rectification

The terms ‘anomalous’ or ‘inward-going’ rectification are used to describe the potassium conductance that allows the passage of large inward current but only small outward current. Katz (1949) first observed this conductance whilst studying the electrical properties of frog skeletal muscle fibres bathed in isotonic potassium sulphate solution. Katz (1949) observed a low membrane resistance for inward current but a high resistance for outward current, i.e. the membrane conductance was larger over hyperpolarizing potentials. Katz (1949) introduced the term ‘propriétés détectrices anormales’ to describe this ‘asymmetrical’ K⁺ conductance, which displayed rectification in the opposite direction to ‘delayed rectification’, previously described by Hodgkin et al. (1949). Later, anomalous rectification was also reported in cardiac Purkinje fibres (see for example Hutter & Noble, 1960; Carmeliet, 1961; Hall et al., 1963; Noble, 1965). Potassium channels that display this type of conductance are now termed ‘inward rectifier’ or ‘inwardly-rectifying’ potassium channels.

Preliminary studies of inward rectification were carried out in skeletal muscle (see for example Hodgkin & Horowicz, 1959; Adrian & Freygang, 1962a, b; Nakajima et al., 1962; Adrian, 1964; Horowicz et al., 1968; Adrian, 1969; Adrian et al., 1970a, b; Stanfield, 1970; Almers, 1972a, b). However, it was later found that egg cell membranes of tunicates (Takahashi et al., 1971; Miyazaki et al., 1974a, b) and starfish (Hagiwara & Takahashi, 1974; Miyazaki et al., 1975; Hagiwara et al., 1976) also display the property of inward rectification. Fewer complications with the technique used to measure the membrane potential and better space clamp conditions made the membrane of egg cells a more suitable preparation for studying the phenomenon of inward rectification. Furthermore, unlike muscle fibres, egg cell membranes are virtually impermeable to chloride ions, which must be eliminated if studying the effects of K⁺ on membrane potential in muscle fibres. Thus, many of the more extensive studies of the electrophysiological properties of inward rectification were carried out in egg cell membranes (see Hagiwara & Takahashi, 1974; Miyazaki et al., 1974a, 1974b, 1975; Hagiwara et al., 1976; 1977; 1978; Hagiwara & Yoshii, 1979; Hagiwara & Jaffe, 1979; Hagiwara, 1983). However, several groups continued to investigate inward rectification in skeletal muscle (see for example Standen & Stanfield, 1978a, b; 1979; 1980; Leech & Stanfield, 1981).
1.4.1. Properties of inward rectifier potassium channels

Together, the aforementioned studies revealed several characteristic features of inward rectification, which are used to distinguish inward rectifier potassium channels from other potassium channel subtypes. These properties are considered in more detail below.

1.4.1a. Inward rectification depends on $V_m$ and the external $K^+$ concentration

Hodgkin and Horowicz (1959) first suggested that inward rectification did not depend on membrane potential alone, as in $K_v$ channels, but on the difference between the membrane potential and the equilibrium potential ($V - E_K$). To explain why fairly large membrane depolarizations were initiated by a rise in $[K^+]_o$, but only small membrane hyperpolarizations were initiated by a fall in $[K^+]_o$, Hodgkin and Horowicz (1959) proposed that potassium channels in frog skeletal muscle could pass a large current in high $K^+$ if the driving force was inward (i.e. $V < E_K$), but only a small current if the driving force was outward (i.e. $V > E_K$).

To determine whether inward rectification was dependent on the electrochemical potential, as opposed to membrane potential ($V_m$) and external potassium concentration ($[K^+]_o$), Hagiwara and Yoshii (1979) studied the effects of altering intracellular potassium concentration ($[K^+]_i$). They demonstrated that channel gating depended solely on $V_m$ when $[K^+]_o$ was fixed and $[K^+]_i$ altered. Studies in frog skeletal muscle have subsequently confirmed this observation (Leech & Stanfield, 1981; Hestrin et al., 1981). It is therefore more correct to describe inward rectification as being dependent on $V_m$ and $[K^+]_o$ rather than the electrochemical potential for $K^+$.

At potentials just positive to $E_K$, outward currents flow through inward rectifier potassium channels. However, at much more positive potentials these channels switch off, which is reflected by a region of negative slope conductance in the I-V relationship. As $[K^+]_o$ increases, the outward current passing through these channels also increases causing the I-V relationships to cross (see Figure 1.4; see for example Adrian, 1969). This is often referred to as ‘cross-over’.
The 'Cross-over' effect. As $[K^+]_0$ increases, the I-V relationships of inwardly rectifying potassium channels shift to the right as $E_K$ shifts to more positive potentials. The outward current flowing through inward rectifiers at potentials just positive to $E_K$ increases with increasing $[K^+]_0$. The I-V relationships of strong inward rectifiers, including the native inward rectifiers of skeletal muscle and egg cell membranes, cross each other owing to the presence of a 'negative slope conductance' region.
1.4.1b. The conductance is proportional to the square root of [K\textsuperscript{+}]\textsubscript{0}.

The conductance of native inward rectifier potassium channels of skeletal muscle and egg cell membranes is roughly proportional to the square root of [K\textsuperscript{+}]\textsubscript{0}, a four fold increase in [K\textsuperscript{+}]\textsubscript{0} approximately doubling the slope conductance (see Horowicz et al., 1968; Hagiwara & Takahashi, 1974; Miyazaki et al., 1974; Hagiwara, 1976). Cloned K\textsubscript{ir} channels also exhibit this steep dependence of conductance on [K\textsuperscript{+}]\textsubscript{0} (see for example Ho et al., 1993; Kubo et al., 1993a; Makhina et al., 1994), which is conferred by an arginine located at the outer mouth of the pore. K\textsubscript{ir}7.1 channels, in which arginine is replaced by methionine, lack this dependence. Thus, K\textsuperscript{+}-conductance increases only slightly with varying [K\textsuperscript{+}]\textsubscript{0} in K\textsubscript{ir}7.1 channels (Döring et al., 1998; see also sections 1.2.4g and 3.4).

1.4.1c. Activation

For negative voltages below $E_K$, a step change in membrane potential elicits an instantaneous inward current through inward rectifier potassium channels, which increases in magnitude with time to reach a steady-state (see for example Hagiwara et al., 1976; Hagiwara & Jaffe, 1979; Hagiwara, 1983). The time-constant ($\tau_{\text{act}}$) for the current to reach steady-state follows a single-exponential relationship, the value of $\tau_{\text{act}}$ decreasing with increasing hyperpolarization. Like the chord conductance, activation of inward rectifiers was found to depend on both $V_m$ and [K\textsuperscript{+}]\textsubscript{0} (see for example Leech & Stanfield, 1981). It is now known that the time-dependent activation of inward current represents unblock of the channel pore by polyamines (see Lopatin et al., 1995; see section 1.4.2d for detailed discussion).

1.4.1d. Inactivation

At very negative potentials, the inward current through inward rectifier channels often declines with time after reaching its maximum amplitude. In skeletal muscle, this ‘inactivation’ at very negative membrane potentials was initially attributed to depletion of K\textsuperscript{+} in the tranverse (‘T’) tubules system (see for example Adrian & Freygang, 1962b; Adrian et al., 1970a). However, further studies (Adrian et al., 1970b; Almers, 1972a, b) suggested that the decline in conductance at very negative membrane potentials could not be explained wholly through K\textsuperscript{+} depletion. For example, Adrian et al. (1970b) found that there could be a large decrease in current without much alteration of $E_K$. Almers (1972a) found that the decline in potassium conductance in skeletal muscle fibres with hyperpolarization exhibited two components: a rapid component at very negative potentials and a slow component at less negative potentials. While the slow component was attributed to changes in tubular K\textsuperscript{+}
concentration, the kinetics of the rapid component were affected by temperature, indicating that it represented a time-dependent change in membrane permeability. Almers (1972a) proposed that membrane hyperpolarization led to the closure of a gate within the membrane, which in turn caused a reduction in the $K^+$ permeability.

Later, Standen and Stanfield (1979) reported that a voltage-dependent block of the inward current by $Na^+$, which was present in the external solution, was responsible for the permeability change under hyperpolarization. Similar effects have been reported for the inward rectifier of egg cell membranes (Ohmori, 1978, 1980; Fukushima, 1982) and for other native channels (see for example Biermans et al., 1987). However, inactivation can still occur in the absence of any $Na^+$. In skeletal muscle this was, like the decay of currents at more positive membrane potentials, attributed to $K^+$ depletion (Standen & Stanfield, 1979). However, studies in cloned $K_r$ channels have provided evidence to suggest that other mechanisms are responsible for the hyperpolarization-induced inactivation, including channel block by extracellular cations and residual hydroxyethylpiperazine (HEP) present in HEPES buffered solutions (see for example Choe et al., 1999; Guo & Lu, 2002; see section 3.4 for detailed discussion of inactivation in the absence of $Na^+)$.

1.4.1e. Block by external cations

It is well established that the inward movement of $K^+$ can be blocked by certain cations including $Cs^+$, $Ba^{2+}$ and $Rb^+$ (see for example Hagiwara & Takahashi, 1974; Hagiwara et al., 1976; 1978; Gay & Stanfield, 1977; Standen & Stanfield, 1978a; 1980). Studies of potassium channel block by, for example, $Ba^{2+}$ in starfish egg membrane (Hagiwara et al., 1978) and frog skeletal muscle (Standen & Stanfield, 1978a) have shown inward rectifier channels to be highly sensitivity to $Ba^{2+}$, which blocks in a time-, concentration- and voltage-dependent manner. Indeed, the high sensitivity of inward rectifier potassium channels to $Ba^{2+}$ is often used as a tool for identifying novel inward rectifier channels (see for example Kubo et al., 1993a; Takahashi et al., 1994; Töpert et al., 1998). $K_{ir}7.1$ appears to lack the property of high affinity blockage by $Ba^{2+}$ and $Cs^+$, but sensitivity to block by $Ba^{2+}$ is increased by the methionine to arginine mutation, which also restores the steep dependence of rectification on $[K^+]_o$ (see sections 1.2.4g and 1.4.1b; Krapivinsky et al., 1998a; Döring et al., 1998).
Although monovalent, the effective valence \( z' \) of the blocking reaction for \( \text{Cs}^+ \) in inwardly rectifying potassium channels is greater than 1. Such high values of \( z' \) result from inwardly rectifying potassium channels being multi-ion pores (see Hille & Schwarz, 1978), with voltage moving permeant ions as well as the blocking ion to bring \( \text{Cs}^+ \) to its site of action.

1.4.1. Anomalous mole fraction effects

Inwardly rectifying potassium channels display a characteristic known as anomalous mole fraction effects, where the membrane conductance and resting membrane potential become smaller in the presence of a mixture of ions than in the presence of either ion alone (see Hagiwara & Takahashi, 1974; Hagiwara et al., 1977). For example, Hagiwara & Takahashi (1974) found that the membrane conductance of starfish eggs was greater with extracellular solutions containing \( \text{TINO}_3 \) than with solutions containing \( \text{KNO}_3 \) at the same concentration. However, when the \( \text{Tl}^+ \) and \( \text{K}^+ \) solutions were mixed, the membrane conductance became smaller and the reversal potential more negative than in the presence of either of the pure salts. This characteristic of inward rectifier potassium channels supports the hypothesis that potassium channels are multi-ion pores (see Hille & Schwarz, 1978) and can be explained by the fact that \( \text{K}^+ \) binds more tightly in the pore than \( \text{Tl}^+ \).

1.4.2. Mechanism of inward rectification

By the end of the 1970s, the phenomenon of inward rectification had been studied extensively in both skeletal muscle and egg cell membranes. Although several theories to account for the asymmetrical potassium conductance displayed by inward rectifiers had been proposed, the mechanism of inward rectification remained unknown (see for example Leech & Stanfield, 1981).

The theories, which have been proposed to account for the mechanism of rectification, can be divided into two major categories. The first category includes several different models in which the gating of the channel is regulated by the binding of external \( \text{K}^+ \) to ‘sites’ on the channel (see for example Horowicz et al., 1968; Ciani et al., 1978). This has been referred to as the ‘K-activated K-channel model’ (Pennefather et al., 1992). The second category comprises models in which the channel is occluded by an internal blocking particle, which is

\[^{1}z' = \text{the effective valence of the blocking ion} \times \text{the fraction of the total potential drop through which the ion moves.}\]
driven out of the channel when K⁺ currents are driven inward (see for example Armstrong, 1969; 1975; Hille & Schwarz, 1978; Standen & Stanfield, 1978b).

1.4.2a. The K-activated K-channel model

Horowicz et al. (1968) first proposed a carrier model for the mechanism of inward rectification following a series of experiments in which they measured unidirectional K⁺ fluxes by studying tracer movement in frog skeletal muscle. They found that the 'system responsible for K⁺ movement was gradually activated by increasing [K⁺]₀'. Furthermore, it was shown that K⁺ influx was related to the square of the [K⁺]₀ at constant internal potential and [K⁺]ᵢ. These observations led Horowicz et al. (1968) to propose a model in which K⁺ ions were assumed to move through the membrane by way of association with a 'mobile carrier system', which was activated by the binding of K⁺ ions to a 'nonmobile control site'.

Later, Ciani et al. (1978) proposed a model that also involved activation by binding of K⁺ ions to a 'gating site'. They considered two mechanisms to explain the steady-state electrical properties of the egg cell membrane when K⁺ is the only permeant ion. Their first hypothesis, which they termed the 'electro-chemical gating' hypothesis, was based on a previously suggested mechanism used to explain the voltage-dependent conductance induced in artificial bilayers by various antibiotics such as alamethicin. Alamethicin molecules have been proposed to adsorb to membrane surfaces with which they have direct contact and, on application of a positive potential, orient themselves in such a manner to enable the formation of aggregates and hence conductive pores. The negative end of the molecule is thought to adsorb to the membrane so strongly that on application of a negative potential, the pore fails to conduct.

Ciani et al. (1978) noticed that the properties of inward rectification in egg cell membranes displayed similarities with the voltage-dependence and rectification of conductance in artificial bilayers following addition of antibiotics to one side of the membrane. Based on this observation, they argued that a similar model, in which charged molecules located at the inner side of the membrane are required to orient themselves and aggregate to form permeable pores, could explain the mechanism of inward rectification. However, the model for inward rectification needed to take into account the dependence of conductance on [K⁺]₀. Thus, the binding of external cations was also assumed necessary for pore formation. Both membrane potential and [K⁺]₀ were therefore assumed to affect the density of open pores.
1.4.2b. The blocking particle model

Armstrong (1969) first proposed the ‘blocking particle model’ for inward rectification following a series of experiments in which he studied the effects of TEA+ ions and their analogues on the potassium current in the giant axon of squid (see Armstrong & Binstock, 1965; Armstrong, 1966; 1969; 1971; 1975). Armstrong noticed that the TEA+ induced ‘ingoing rectification’ in squid axons and the inward rectification of skeletal muscle displayed similarities. Based on these observations he suggested a model for inward rectification in which K+ moving through the membrane displaces an internal blocking particle, which is located outside the electrical field of the membrane. The blocking particle hypothesis has since provided the basis for several other models of inward rectification (see Hille & Schwartz, 1978; Standen & Stanfield, 1978b; Urban & Hladky, 1979; Cleeman & Morad, 1979).

Hille and Schwartz (1978) simulated such a system using the Eyring rate theory. Their pore model contained three binding sites, and at least two ions were assumed to be moving in single file. The blocking ion was assumed to be a monovalent ion, which was proposed to block the channel from the inner end, but could not pass through the pore to the external solution. Their model correctly predicted the dependence of rectification on membrane potential and [K+]o, and could also account for anomalous mole fraction effects. However, the steepness of rectification predicted by their model was too shallow.

In the same year, Standen and Stanfield (1978b) used a similar blocking particle model to accurately fit their experimental measurements of potassium currents in frog sartorius muscle. Their blocking particle model assumed that the potassium conductance depends on the K+ concentration within a channel and that this conductance is reduced by a blocking particle, which is driven into the channel by depolarization. The shape of the I-V relationship could be accurately predicted based on the assumption that two monovalent blocking ions compete with K+ for binding to a site within the ion conduction pore. Interestingly, Standen and Stanfield (1978b) demonstrated that identical predictions could be made by assuming that the blocking particle was divalent, but binds at only one site, providing the dissociation constant for the blocking ion was adjusted accordingly. However, curves of the correct shape could not be predicted if only one monovalent blocking particle was assumed to bind at only one site.
1.4.2c. Mg$^{2+}$ block

Evidence to support the blocking particle model came in 1987 following three independent studies of inward rectifier potassium channels in cardiac myocytes (Horie et al., 1987; Matsuda et al., 1987; Vandenberg, 1987; for review see Matsuda, 1991). These studies indicated that rectification was a consequence of a voltage-dependent block by intracellular magnesium (Mg$^{2+}$) ions.

Horie et al. (1987) first demonstrated that physiological concentrations of Mg$^{2+}$ blocked the outward current through inwardly rectifying ATP-sensitive potassium channels of the heart. Following removal of Mg$^{2+}$, the current-voltage relationship became virtually linear. This observation was also confirmed by Matsuda et al. (1987) and Vandenberg (1987), who reported the loss of rectification in inward rectifier K$^+$ currents following excision of an inside-out patch into divalent free internal solution. On addition of Mg$^{2+}$ (~1mM) to the internal solution, rectification was restored. However, rectification was not observed following addition of either Ba$^{2+}$ or Ca$^{2+}$.

1.4.2d. Polyamine block

In addition to the voltage-dependent block of these channels by Mg$^{2+}$, Matsuda et al. (1987) also observed the rapid closure of inward rectifier potassium channels during depolarization in the absence of Mg$^{2+}$. This suggested the existence of a voltage-dependent gating mechanism, which occurs independently of Mg$^{2+}$ block.

Further studies on native (Ishihara et al., 1989; Oliva et al., 1990; Silver & DeCoursey, 1990) and cloned channels (see for example Stanfield et al., 1994a) confirmed that a time-dependent component of the inward rectifier K$^+$ current existed irrespective of the presence or absence of Mg$^{2+}$, thus supporting the idea of an 'intrinsic gating' mechanism (see Leech & Stanfield, 1981). The voltage-dependent block by Mg$^{2+}$ was proposed to be responsible for the instantaneous inward rectification on depolarization. Thus, the instantaneous current on hyperpolarization was thought to reflect relief of Mg$^{2+}$ block.

In their 1994 review, Jan and Jan compared the intrinsic gating mechanism of inward rectifier potassium channels with the 'ball-and-chain' inactivation gate of Kv channels (see section 1.2.3a). Jan and Jan (1994) proposed that inward rectifier potassium channels might have retained the pore and cytoplasmic gate of an ancestral channel from which other channels, such as the Kv channels, also arose. However, in the same year, three independent groups
published results showing that the gating mechanism believed to be intrinsic was in fact a consequence of a voltage-dependent block of the pore by polyamines (Ficker et al., 1994; Lopatin et al., 1994; Fakler et al., 1994).

In the first of these reports, Ficker et al. (1994) described the loss of rectification following the formation of inside-out patches, from Xenopus oocytes expressing Kir2.1 channels, into Mg$^{2+}$-free internal solution. However, application of spermine, spermidine and putrescine to the internal solution resulted in a block of the outward currents through Kir2.1. Lopatin et al. (1994) and Fakler et al. (1994) reported similar observations. Subsequently, several other studies have confirmed that a voltage-dependent block of inward rectifiers by polyamines underlies the 'intrinsic' rectification (see for example Fakler et al., 1995; Yamada & Kurachi, 1995; Yang et al., 1995a; Ishihara et al., 1996).

Polyamines are present in almost all cells and play important roles in protein synthesis, cell division and cell growth. Spermine, spermidine and putrescine are low molecular weight organic cations and at physiological pH the primary and secondary amine moieties of these molecules carry a charge. Lopatin et al. (1994) suggested that at potentials positive to $E_K$, these highly charged linear molecules could pass deep into the narrow pore of the channel to reach the blocking site. In a model to account for polyamine-induced inward rectification, two molecules of spermine were proposed to sequentially block the inward rectifier channel (Lopatin et al., 1995; see figure 1.5).

Lopatin et al. (1995) showed that the time constants for current activation in cell-attached patches expressing Kir2.1 closely matched the measured time constants for unblock of the channel by spermine, spermidine and putrescine. Thus, the time-dependent activation of inward current (see section 1.4.1c) represents unblock of the channel pore by polyamines. The strong voltage dependence of rectification exhibited by Kir2.1 is predominantly due to the effect of intracellular spermine, which is tetravalent. Fakler et al. (1995) suggested that the higher voltage dependence of rectification induced by spermine compared with spermidine was most likely due to its higher valency. Indeed, both the voltage-dependence and affinity of channel block by polyamines increase with the increasing valency of the polyamine molecule (Ficker et al., 1994; Lopatin et al., 1994; Fakler et al., 1994). Thus, whilst only nanomolar concentrations of spermine ($z = +4$) and spermidine ($z = +3$) are required to block Kir2.1 at positive voltages, putrescine ($z = +2$) blocks with only micromolar affinity.
Figure 1.5.

Mechanism of Inward Rectification. Schematic representation to illustrate the mechanism of polyamine block in inward rectifier potassium channels as proposed by Lopatin et al. (1995). Two molecules of spermine block the pore in a sequential manner, binding initially to a shallow site to block (B) then binding to a second site located deep (D) within the pore (see Lopatin et al., 1995; see also section 1.2.4d).
Further evidence that a voltage-dependent block of \(K_{\text{ir}}\) channels by polyamines was responsible for intrinsic rectification came from studies in which inward rectification was examined following pharmacological modification of the cellular polyamine content. Bianchi et al. (1996) studied the native inward rectifier of rat basophil leukaemia (RBL-1) cells following treatment with an inhibitor of polyamine synthesis. Similarly, Shyng et al. (1996) studied recombinant \(K_{\text{ir}}2.1\) channels in oocytes, which had also been injected with inhibitors of polyamine synthesis. Both studies reported a decrease in rectification and an increase in outward current through \(K_{\text{ir}}\) channels. Shyng et al. (1996) also expressed \(K_{\text{ir}}2.3\) in Chinese hamster ovary (CHO) cells deficient in ornithine decarboxylase, the enzyme that catalyses the synthesis of putrescine from L-ornithine. These cells require exogenous putrescine to synthesize spermidine and spermine, and to maintain normal growth. Withdrawal of exogenous putrescine from these cells led to a relief of rectification in these strong inward rectifier channels.

1.4.2e. Intrinsic gating

Together, the aforementioned studies provide substantial evidence that channel block by polyamines is responsible for the strong inward-rectification of \(K_{\text{ir}}\) channels. However, it has been proposed that there is another component of intrinsic gating that is enhanced by the action of intracellular \(Mg^{2+}\) and polyamines, but is not mediated by polyamine block.

Aleksandrov et al. (1996) incorporated \(K_{\text{ir}}2.1\) channels into lipid bilayers to test the relative contribution of various mechanisms to inward rectification. Although polyamines are presumably absent from lipid bilayers, \(K_{\text{ir}}2.1\) channels still displayed an intrinsic, voltage-dependent rectification. Aleksandrov et al. (1996) proposed that in addition to the voltage-dependent block of the channel by \(Mg^{2+}\) and polyamines, there was a distinct, fast gating process amplified by the binding of \(Mg^{2+}\) and/or polyamines to sites on the cytoplasmic face. Unlike the blocking mechanism, it was dependent on the membrane potential and the extracellular and intracellular \(K^+\) concentration. These results could not be explained by a simple pore-blocking model as originally hypothesized by Armstrong (1969), but were instead consistent with the electrochemical regulation of the gating mechanism proposed by Ciani et al. (1978).

Several other studies have since suggested the existence of an additional intrinsic gating mechanism. Shieh et al. (1996) observed a residual time-dependent inactivation of the \(K_{\text{ir}}2.1\) outward current after excising patches into \(Mg^{2+}\) and polyamine-free solution, and unlike the
long-pore plugging effect of spermine, this intrinsic gating mechanism was sensitive to pH_i.
A similar voltage-dependent deactivation of Kir2.0 currents had previously been observed,
following the prolonged washing of inside-out membrane patches with Mg^2+ - and polyamine-
free solution (Ficker et al., 1994; Lopatin et al., 1994; Fakler et al., 1994), but had been
attributed to an incomplete washout of polyamines.

In the absence of Mg^2+ and polyamines, raising the pH_i increased the inactivation rate of the
outward current, but in their presence either decreased or had no effect on the rate of
inactivation. Conversely, reducing the pH_i had no effect on channel kinetics but blocked
inward and outward currents in a voltage-dependent manner. In Kir2.1 D172N, a mutant
channel with a reduced affinity for polyamines (see section 1.4.3), raising pH_i had a similar
effect on channel kinetics as in the absence of polyamines, accelerating inactivation at
depolarized potentials. These observations led Shieh et al. (1996) to propose that the
inactivation of outward current that remained after 5 minutes in Mg^2+ and polyamine-free
solution was not due to the slow washout of these substances, but represented a third
mechanism for rectification that was likely to be intrinsic.

Shieh et al. (1996) expressed their uncertainty about whether this mechanism makes an
important contribution to the inward rectification of Kir2.1 under physiological conditions.
However, they suggested that it might play an important role in regulating the magnitude of
outward current through Kir2.1 by way of its interaction with Mg^2+ - and polyamine-bound
substrates. This could be important in, for example, the heart where the action potential is
long and inward rectifier potassium channels play a key role in regulating the onset of rapid
repolarization (see section 1.5.3). This component of gating might also be important during
ischaemia, when marked changes in pH occur. Under such circumstances, the resting K^+
conductance would decrease in response to the fall in pH and in turn contribute to cellular
depolarization, abnormal excitability and altered repolarization.

A more recent study by Lee et al. (1999) supports the idea of an intrinsic gate. By studying
interactions of the spider venom toxins, philanthotoxin and argiotoxin with spermine and
spermidine, Lee et al. (1999) provided evidence that polyamine block is more complex than a
simple direct open channel block. These toxins, which are structurally similar to spermine at
one end of the molecule, but have a bulky hydrophobic aromatic group at the other end,
interfere with the ability of spermine or spermidine to block Kir2.1 channels. This effect
cannot be readily explained by a direct open channel block mechanism, but may be accounted
for by an intrinsic gate mechanism in which high affinity block is due to a ‘spermine-intrinsic’ gate complex. It is thought that the multiple positive charges on the polyamine molecule facilitate the voltage-dependent interaction of the intrinsic gate-polyamine complex with a pore-docking site. Conversely, low affinity block may be explained by direct channel block of untethered spermine molecules.

The authors of this study recognized that they could not rule out the possibility of a compartmentalized pool of polyamines, which remained following extensive washing of excised patches. Nonetheless, their model represented an attractive alternative to the traditional direct channel block model, especially in light of the available structural information on the pore dimensions of a K⁺ channel (Doyle et al., 1998), since in this model only one spermine molecule would be required to block the pore. The distance from the internal surface to the selectivity filter for KcsA was estimated at 3nm, which could easily accommodate one spermine molecule at 2nm in length but not two stacked lengthwise on top as proposed by Lopatin et al. (1995).

In the absence of structural data showing spermine bound to the channel pore it was impossible to conclude whether either of the models described above represented the true mechanism of ‘intrinsic’ rectification in strong inward rectifier potassium channels. The drawback with the long-pore plugging model was that from available structural information, it appeared that the channel pore could not accommodate two spermine molecules stacked lengthwise on top of one another. With evidence that spermidine is unable to permeate the pore of Kᵢ2.3 (Sha et al., 1996), a model in which only one spermine blocks the pore seemed favourable at the time (Lee et al., 1999).

However, a recent study suggests that naturally occurring polyamines may act as permeant blockers (Guo & Lu, 2000b). Though Guo and Lu (2000b) acknowledged the existence of intrinsic gating, they argued that the model described by Lee et al. (1999) failed to account for the characteristic ‘hump’ in the current-voltage curve of strong inward rectifier potassium channels. Neither did this model account for the residual current remaining at positive potentials. On investigating the complex blocking nature of polyamines, they noted that block of Kᵢ2.1 by diamines tends to a non-zero level at positive membrane potentials. This is also the case for cGMP-gated channels, in which diamines act as permeant blockers (Guo & Lu, 2000a).
Furthermore, recent x-ray crystallography studies of the N- and C-termini of an inward rectifier suggest that at 30Å in length, the cytoplasmic pore of inward rectifiers could accommodate more than one polyamine molecule (Nishida & MacKinnon, 2002).

1.2.4f. Do $K_{ir}$ channels exhibit intrinsic rectification?

The results from two recent studies, described below, suggest that inward rectifier potassium channels do not exhibit intrinsic rectification.

Guo and Lu (2000c) noticed that the degree of residual rectification in the absence of Mg$^{2+}$ and polyamines, attributed in previous studies to intrinsic rectification (see section 1.4.2e), varied between studies from different laboratories. Guo and Lu (2000c) examined the effects of using different pH buffers in their experimental solutions. Interestingly, when HEPES or MOPS were used as the pH buffer, $K_{ir}2.1$ currents exhibited profound relaxation at positive membrane potentials. In contrast, when phosphate or borate solutions were used current relaxation was removed, the steady-state I-V relationship becoming virtually linear. Some inward rectification remained at very positive membrane potentials (i.e. +100mV), which became more prominent on lowering the intracellular concentration of EDTA from 5 to 1mM. Thus, the slight relaxation of current at +100mV in the presence of phosphate and 5mM EDTA appeared to be a consequence of block by intracellular cations, which was subsequently confirmed by its removal in a mutant channel ($K_{ir}2.1$ D172N; see section 1.4.3) with a reduced affinity for intracellular cations.

The degree of current relaxation at positive membrane potentials was also demonstrated to vary with different concentrations of HEPES or different commercial sources of HEPES suggesting that the apparent intrinsic rectification observed in previous studies (see Aleksandrov et al., 1996; Shieh et al., 1996; Lee et al., 1999; Guo & Lu, 2000b) may be a consequence of block by HEPES and some impurity (Guo & Lu, 2000c). It has subsequently been confirmed that the residual rectification of $K_{ir}2.1$ in the absence of intracellular Mg$^{2+}$ and polyamines can be accounted for by impurities in chemicals routinely used in recording solutions (Guo & Lu, 2002). This includes, for example, traces of hydroxyethylpiperazine (HEP) and ethylenediamine present in commercially available HEPES and EDTA, respectively. Inward rectification (and also hyperpolarization induced inactivation; see section 1.4.1d) can be completely removed in solutions using phosphate as a pH buffer and an optimal concentration of EDTA (~ 5 mM) to prevent block by intracellular cations, without introducing block by contaminating levels of ethylenediamine.
Although several complicated models have been proposed to explain the mechanism of rectification in strong inward rectifiers, the exact nature of this process remains elusive. Whilst the results of Guo and Lu (2000c; 2002) suggest that $K_{ir}$ channels do not exhibit intrinsic rectification, others disagree (see Matsuda et al., 2003). It is perhaps worthy to note, however, that the glycine residue implicated in the gating of KcsA/MthK and Shaker channels (see section 1.2.3a; Jiang et al., 2002b; Yifrach & MacKinnon, 2002) is conserved throughout the $K_{ir}$ channel family.

1.4.3. Structural determinants of inward rectification

Studies in cloned inward rectifier potassium channels have greatly advanced our knowledge of the mechanism of inward rectification and its structural determinants (for reviews see Doupnik et al., 1995; Fakler & Ruppersburg, 1996; Jan & Jan, 1997; Nichols & Lopatin, 1997). In fact, the residues that determine gating in inward rectifier potassium channels were identified before the voltage-dependent block of the pore by polyamines was implicated as the mechanism for intrinsic rectification.

Initial studies focused on a negatively charged amino acid in the M2 transmembrane domain (Stanfield et al., 1994b; Wible et al., 1994; Fakler et al., 1994a). For all cloned strong inward rectifier potassium channels this residue is an aspartate (single letter code D). In $K_{ir}2.1$, mutation of this residue to the uncharged amino acids glutamine (D172Q) or asparagine (D172N) produced channels, which displayed weaker rectification and no time-dependence on activation (Stanfield et al., 1994b; Wible et al., 1994). The affinity for Mg$^{2+}$ by the D172Q mutant channels was also reduced (Stanfield et al., 1994b), though D172N mutant channels still retained a high affinity for Mg$^{2+}$ (Wible et al., 1994).

The corresponding residue in the weak inward rectifier potassium channel $K_{ir}1.1a$ is an asparagine (N). Mutagenesis studies (Lu & MacKinnon, 1994; Wible et al., 1994) showed that substitution of aspartate for asparagine in $K_{ir}1.1a$ (N171D) converted the channel from a weak to a strong inward rectifier potassium channel with an increased affinity for Mg$^{2+}$ and ‘$K_{ir}2.1$-like’ gating. Thus, a negatively charged amino acid in M2 was shown to be essential for intrinsic gating and to form part of the binding site for Mg$^{2+}$.

Taglialatela et al. (1994) used chimeric constructs of $K_{ir}1.1a$ and $K_{ir}2.1$ to search for the molecular determinants of inward rectification. They found that exchange of the carboxyl
terminus of K_{ir}1.1a for that of K_{ir}2.1 produced a strongly rectifying channel with a higher affinity for Mg^{2+}, which suggested that the structural determinants for rectification were located in the C-terminus. A residue in the C-terminus, a glutamate (E224) in K_{ir}2.1, was later identified as being an important determinant of inward rectification (Yang et al., 1995a).

Later, other unknown sites were proposed to play a key role in determining the extent of rectification. sWIRK (see section 1.2.4d) displays weak inward rectification despite the presence of a negatively charged glutamate (E179) at the site corresponding to D172 in K_{ir}2.1 (Kubo et al., 1996). Although sWIRK contains a glycine (G231) at the analogous position to E224 of K_{ir}2.1, no differences in rectification could be distinguished when this residue was mutated to a glutamate (G231E). Thus, the differences in rectification between sWIRK and K_{ir}2.1 are not due to this difference in this C-terminus residue. Further evidence for the involvement of additional residues in determining the extent of rectification comes from the observation that a mutant of K_{ir}4.1, G210E, displays weaker inward rectification than a mutant of K_{ir}1.1a, N171E, despite the presence of identical residues at the two ‘rectification-controlling sites’ (Xu et al., 2000b).

Another cytoplasmic residue, E299 in K_{ir}2.1, was recently identified as being an important determinant of strong rectification (Kubo & Murata, 2001). The corresponding residues in all other strong inward rectifier potassium channels and in K_{ir}1.1a, which may be converted into a strong inward rectifier on mutation of the asparagine at position 171 to aspartate (see above), are negatively charged. In contrast, in sWIRK, K_{ir}4.1 and K_{ir}7.1 (which display weak inward rectification) this residue is a hydrophobic serine. The mutant channel K_{ir}2.1 E299S displayed less intense inward rectification, and the hyperpolarized-induced activation and decay of outward currents at depolarized potentials were slower. Furthermore, mutation E299S reduced both the sensitivity to block by spermine of the outward current and the single channel conductance (Kubo & Murata, 2001). Kubo and Murata (2001) proposed that E224 and E299 are located at the inner vestibule forming an intermediate site to which spermine binds but does not plug the pore. It was proposed that the purpose of this site is to facilitate the entry and exit of spermine to and from its final pore-plugging binding site, which is located deeper within the pore.

Although the three residues discussed above have been shown to be critical determinants of the intensity of inward rectification, it is thought that other residues remain to be identified. For example, preliminary studies have shown that mutation of a residue (T51E) in the Q-
region (also known as M0) affects the voltage-dependent gating of Kir1.1b (Choe *et al.*, 1997). Furthermore, the recently resolved crystal structure of the cytoplasmic pore of Kir3.1 has revealed many pore-lining residues, which remain to be altered by mutagenesis (Nishida & MacKinnon, 2002). It is likely that some of these amino acids play an important role in polyamine-induced rectification.

### 1.5. Physiological importance of Kir channels

Although Kir channels carry large inward currents, under physiological conditions these channels work at membrane potentials just positive to $E_K$ or the resting membrane potential, since the membrane potential of real cells rarely goes below $E_K$. Kir channels are involved in a number of physiological processes, some of which are considered below.

#### 1.5.1. Kir channels underlie K⁺ homeostasis in the kidney

As discussed briefly in section 1.4.2a, Kir1.1 plays an important role in the recycling of K⁺ in the thick ascending limb (TAL) and secretion of K⁺ across the apical membrane of mammalian cortical collecting (CCD) tubules in the kidney (for review see Wang *et al.*, 1992; 2002). K⁺ recycling in the TAL is essential for the maintenance of the activity of the Na⁺-K⁺-2Cl⁻ co-transporter, which is partly responsible for reabsorption of the filtered NaCl load. K⁺ secretion across the CCD tubules occurs in two steps: K⁺ is first pumped into the cell via the basolateral Na-K-ATPase, then K⁺ passively exits into the urinary space via native, small-conductance Kir₅.₁s in the apical membrane (see Wang *et al.*, 1990; Wang & Giebisch, 1991).

The role of Kir1.1 channels in K⁺ recycling in the TAL is highlighted by the renal salt-wasting disorder Bartter’s syndrome in which mutations in Kir1.1 cause loss of function (see also sections 1.0 and 1.2.4a). Mutations in Kir1.1 are thought to result in an inability to reabsorb K⁺ from the thick ascending loop to the renal tubule, causing an inhibition of the Na⁺-K⁺-2Cl⁻-transporter and salt wasting. The loss of salts causes an increase in aldosterone levels, resulting in an increased Na⁺ absorption in exchange for K⁺ and H⁺, which causes hypokalemic alkalosis (for review see Sanguinetti & Spector, 1997). Animal knockout studies provide further support for the role of Kir1.1 channels in K⁺ homeostasis in the kidney. Kir1.1-deficient mice, in which Kir1.1 expression is absent from the TAL and CCD, exhibit some of the characteristics of human Bartter’s syndrome including polyuria and salt wasting (Lorenz *et al.*, 2002; Lu *et al.*, 2002).
1.5.2. \textit{K}_{ir} channels underlie K$^+$-induced vasodilation

An increase in the activity of \textit{K}_{ir} channels has been proposed as one of the mechanisms that may contribute to K$^+$-induced hyperpolarization of vascular smooth muscle and vasodilation (see for example Edwards \textit{et al.}, 1988; Knot \textit{et al.}, 1996; for review see Sobey \& Faraci, 2000). Modest increases in [K$^+$]$_o$ in the brain or heart act as a metabolic signal causing vasodilation of small cerebral and coronary vessels, which in turn may lead to a selective increase in the perfusion of metabolically active tissue. These modest increases in [K$^+$]$_o$ cause a shift in the channel gating properties of \textit{K}_{ir} channels (see figure 1.4) and a subsequent increase in the resting outward K$^+$ current. Efflux of K$^+$ through \textit{K}_{ir} channels leads to hyperpolarization of the membrane and vasorelaxation by a mechanism involving the closure of voltage-gated calcium channels, which in turn leads to a reduction in the intracellular Ca$^{2+}$ levels (for review see Standen \& Quayle, 1998).

Native \textit{K}_{ir} channels in arterial smooth muscle display characteristics similar to members of the \textit{K}_{ir}2.0 subfamily (see for example Quayle \textit{et al.}, 1996). Bradley \textit{et al.} (1999) have identified transcripts for \textit{K}_{ir}2.1 in arterial smooth muscle and showed that the biophysical and pharmacological properties of the native \textit{K}_{ir} currents most closely resemble those of cloned \textit{K}_{ir}2.1 currents. More recently, \textit{K}_{ir}2.1 gene knockout studies have shown that cerebral arteries from mice lacking the \textit{K}_{ir}2.1 gene fail to dilate in response to modest elevations in extracellular K$^+$ (Zaritsky \textit{et al.}, 2000), thus confirming the role of \textit{K}_{ir}2.1 channels in K$^+$-induced vasodilation in cerebral arteries.

1.5.3. \textit{K}_{ir} channels regulate cardiac action potentials

$I_{K1}$, the strong inward rectifier in the heart, serves several functions. Firstly, $I_{K1}$ is responsible for maintaining the resting membrane potential of ventricular myocytes (for review see Lopatin \& Nichols, 2001). $I_{K1}$ is also involved in maintaining the long plateau phase of the cardiac action potential, owing to the decrease in membrane conductance at depolarized potentials, which helps to conserve metabolic energy (Isomoto \textit{et al.}, 1997). Finally, the outward current that flows at membrane potentials just positive to the reversal potential facilitates the final repolarization of the action potential (see for example Ibarra \textit{et al.}, 1991; Shimoni \textit{et al.}, 1992; for review see Lopatin \& Nichols, 2001).

Functional studies have revealed that $I_{K1}$ exhibits strong inward rectification and is sensitive to block by Ba$^{2+}$ and Cs$^+$ (see for example Sakmann \& Trube, 1984a, b; Imoto \textit{et al.}, 1987; Matsuda \textit{et al.}, 1989). In keeping with the functional properties of $I_{K1}$, more recent studies
have provided evidence to suggest that the molecular correlates of $I_{K1}$ are members of the Kir2.0 subfamily (Brahmajothi et al., 1996; Nakamura et al., 1998; Zaritsky et al., 2001). In situ hybridisation studies have indicated expression of Kir2.0 subfamily mRNA in ventricular myocytes, though the exact identity could not be resolved owing to the inability to design unique probes for each member of the Kir2.0 subfamily (Brahmajothi et al., 1996). However, evidence that Kir2.1 contributes to $I_{K1}$ has subsequently been provided using Kir2.1 antisense oligonucleotides, which partially reduced $I_{K1}$ in cultured rat ventricular rat myocytes (Nakamura et al., 1998). More recently, mice lacking the Kir2.1 gene have been shown to exhibit no detectable inwardly rectifying K$^+$ current (Zaritsky et al., 2001). This loss of $I_{K1}$ resulted in broader action potentials and an increase in the amount of spontaneous activity in isolated ventricular myocytes. Kir2.1 knockout mice displayed bradycardia. Thus, the Kir2.1 gene appears to encode the channels primarily responsible for the $I_{K1}$ conductance.

1.5.4. Kir$^+$ channels help regulate the heartbeat

Cardiac muscle contains a G-protein-activated inward rectifier, known as the muscarinic acetylcholine potassium channel ($K_{AC}$), which is involved in the cholinergic regulation of the heartbeat (see Kovoor & Lester, 2002 for review). Acetylcholine (ACh), released following parasympathetic stimulation of the vagus nerve, activates G$\alpha$-coupled M2 muscarinic receptors, which leads to replacement of Go-bound GDP with GTP and dissociation of G$\beta\gamma$ from G$\alpha$. As discussed in section 1.2.4c, the G$\beta\gamma$ subunits bind to $K_{AC}$ channels, causing them to open. Activation of $I_{KAC}$ leads to hyperpolarization of the myocyte membrane and, in turn, a lengthening of the pacemaker cycle. After prolonged exposure to ACh, the current desensitises owing to stimulation of M3 receptors by ACh and the subsequent activation of Goq, which in turn activates PLC$\beta$, resulting in hydrolysis of PIP$_2$ and reduction of the G-protein-activated current (for review see Jan & Jan, 2000; Kovoor & Lester, 2002).

1.5.5. Kir$^+$ channels regulate blood glucose levels

In the endocrine pancreas, $K_{ATP}$ channels formed from Kir6.2 and SUR1 (see section 1.2.4f) play a pivotal role in the regulation of blood glucose levels and insulin release (see figure 1.6). A rise in blood glucose levels leads to an increase in the uptake and metabolism of glucose, elevation of the intracellular ATP concentration, and a subsequent ATP-concentration dependent inhibition of the $K_{ATP}$ channel, which controls the cells resting membrane potential. The resulting depolarization of the plasma membrane opens voltage-dependent calcium channels and the subsequent influx of extracellular Ca$^{2+}$ triggers the fusion
**Figure 1.6.**

*K_{ATP} channels regulate blood glucose levels.* Schematic illustration of the glucose metabolism pathway in pancreatic β-cells (adapted from Baukrowitz & Fakler, 2000). Glucose metabolism decreases the ADP:ATP ratio, which, in turn, reduces the activity of $K_{ATP}$ channels leading to membrane depolarization and the opening of voltage-dependent Ca$^{2+}$ channels. Ca$^{2+}$ influx through these channels leads to an increase in [Ca$^{2+}$]$_i$, which stimulates insulin exocytosis.
of secretory granules with the plasma membrane and the release of insulin (for review see Baukrowitz & Fakler, 2000; Aguilar-Bryan et al., 2001; Houpio et al., 2002).

Sulphonylureas, such as tolbutamide and glibenclamide, which inhibit $K_{ATP}$ channels, are used in the treatment of non-insulin-dependent (type II) diabetes mellitus owing to their stimulatory effect on insulin secretion. Conversely, $K_{ATP}$ channel openers such as diazoxide are used to inhibit insulin release (for review see Baukrowitz & Fakler, 2000; Aguilar-Bryan et al., 2001; Houpio et al., 2002).

1.5.6. $K_{ir}$ channels are important in development

As discussed briefly in the opening introduction, mutations in the gene encoding $K_{ir}2.1$ channels have been shown to underlie Andersen's syndrome. This hereditary condition is characterized by periodic paralysis, cardiac arrhythmias and dysmorphic features including short stature, curvatures of the spine and changes in the shape of the face (Plaster et al., 2001), which suggests an important role for $K_{ir}2.1$ in development. This view is further supported by the observation that mice lacking the $K_{ir}2.1$ gene display a complete cleft of the secondary palate (Zaritsky et al., 2000). Furthermore, additional mutations (e.g. R67W in the N-terminus, T192A in the C-terminus) in the $K_{ir}2.1$ gene have been linked with altered cardiac and skeletal muscle phenotypes (Ai et al., 2002; Andelfinger et al., 2002). Heterologous expression of these $K_{ir}2.1$ mutants revealed loss of function and variable dominant-negative effects when co-expressed with wild-type $K_{ir}2.1$.

It is not known exactly why $K_{ir}2.1$ channel expression is so critical for normal development, though it appears to be related to the function of $K_{ir}2.1$ in setting very negative resting potentials. This is highlighted, for example, by the involvement of $K_{ir}2.1$ in the development of skeletal muscle. Skeletal muscle fibres are formed by the fusion of mononucleated myoblasts, which is essential for skeletal muscle development, growth and repair. This process is Ca$^{2+}$-dependent, requiring an influx of Ca$^{2+}$ (for review see Bernheim & Bader, 2002). It is also dependent on membrane hyperpolarization, which occurs initially through EAG currents and then through $K_{ir}$ currents (see for example Shin et al., 1997; Liu et al., 1998; Fischer-Lougheed et al., 2001; also see for review Bernheim & Bader, 2002). The $K_{ir}$ channel responsible for the hyperpolarization preceding fusion is $K_{ir}2.1$. Myoblast fusion can be prevented following block of $K_{ir}2.1$ by Ba$^{2+}$ or Cs$^+$ (Liu et al., 1998) and following inhibition of $K_{ir}2.1$ using antisense oligonucleotides (Fischer-Lougheed et al., 2001). Membrane hyperpolarization sets the resting membrane potential of 'fusion-competent'
myoblasts within a suitable voltage range for Ca\(^{2+}\) influx through T-type Ca\(^{2+}\) channels to occur.

Further evidence for the role of Kir channels in development is provided by the weaver (wv) mouse, which carries a single point mutation (G156S) in the pore region of Kir3.2. The wv mouse exhibits numerous abnormalities including ataxia, hyperactivity and male infertility (for review see Hess, 1996). These abnormalities are associated with the loss of cerebellar granule cells and the loss of dopaminergic cells in the substantia nigra (see for example Rakic & Sidman, 1973). In the wv mouse, cerebellar granule cells fail to migrate from the external to the internal granule layer, their final destination in the mature cerebellum, and as a result die in the postmitotic zone of the external granule layer (see for example Smeyne & Goldowitz, 1989).

The G156S mutation substitutes a serine for the first glycine of the highly conserved GYG motif, which is important for K\(^{+}\) selectivity (see section 1.6). Following heterologous expression, homomers or heteromers (with Kir3.1) of Kir3.2 carrying the wv mutation display a loss of K\(^{+}\) selectivity, abnormal permeability for Ca\(^{2+}\) and constitutive channel activity (see for example Slesinger et al., 1996; Navarro et al., 1996; Kofuji et al., 1996; Silverman et al., 1996b). Constitutive activation results in high basal levels of Na\(^{+}\) influx that cause chronic membrane depolarization, which in turn results in cell death (see for example Slesinger et al., 1996; Navarro et al., 1996). Similar observations have been reported in cultured granule cells from wv mice (Kofuji et al., 1996).

Why the cerebellar granule cells in wv mice fail to differentiate and subsequently die has been a topic of much debate. Kofuji et al. (1996) suggested that the Na\(^{+}\) influx through wv Kir3.2 channels was the underlying cause of the wv phenotype since block of the Na\(^{+}\) influx in mutant granule cells allowed granule cell differentiation to proceed. In contrast, Surmeier et al. (1996) suggested that the loss of Kir3.2 currents, rather than the gain of a non-specific cation conductance, was responsible for the death of cerebellar granule cell neurones. Surmeier et al. (1996) suggested that the death of cerebellar granule cells might be a consequence of a sustained membrane depolarization and excessive Ca\(^{2+}\) entry and accumulation, which results from a failure to regulate membrane potential owing to the loss of the Kir3.2 current. However, studies in Kir3.2 knockout mice suggest that the wv phenotype is not a consequence of the loss of homomeric/heteromeric Kir3.2 channel function since Kir3.2 knockout mice do not exhibit abnormalities similar to wv mice (Signorini et al., 1997),
favouring the ‘gain of function’ hypothesis. Rossi et al. (1998) argue that the loss of Kir3.2 current and its regulation of membrane potential play an important role in cerebellar granule cell degeneration in wv mice. Both constitutively active and Kir3.2 currents were absent from granule cells at the premigratory stage, the stage at which granule cell degeneration begins in wv mice.

What is the role of functional Kir3.2 channels in cerebellar development? When cerebellar granule cells become postmitotic they are exposed to elevated levels of extracellular glutamate, which activates N-methyl-D-aspartate (NMDA) receptors, leading to a rise in cytosolic Ca\(^{2+}\) levels that trigger migration. At the same time, metabotropic glutamate receptors are activated, which potentiate Ca\(^{2+}\) currents and activate Kir3.2 currents, which provide a hyperpolarizing current that is thought to counterbalance the depolarizing, ionotropic glutamate currents (see Surmeier et al., 1996).

1.6. Selectivity

All potassium channels exhibit very similar ion permeability characteristics, being at least 100 fold more permeable to K\(^{+}\) than to Na\(^{+}\) (Hille, 2001). Na\(^{+}\) ions are smaller than K\(^{+}\) ions, so how do potassium channels distinguish K\(^{+}\) ions from Na\(^{+}\) ions with such high fidelity without disrupting the flow of ions at a rate (10\(^{8}\) ions per second) near the diffusion limit? In this final section of my introduction I will discuss what is known about the molecular basis of selectivity in potassium channels. I will begin by introducing some of the early mutagenesis studies that were undertaken in Kv channels to determine which region of the channel constitutes the ion conduction pore. I will then briefly consider two of the major theories for the molecular mechanism of selectivity. Finally, I will discuss the features of ion permeation in potassium channels revealed by the crystal structure of KcsA (Doyle et al., 1998b).

1.6.1. Structural determinants of K\(^{+}\) selectivity in Kv channels

Studies of the molecular basis of selectivity in potassium channels began in the early 1990s, shortly after the cloning of Shaker and its related potassium channel genes (see section 1.2.1). Mutagenesis studies initially concentrated on the S5-S6 loop, following the finding that mutations in this region disrupted the binding of CTX, a scorpion venom toxin that inhibits several types of potassium channel (MacKinnon & Miller, 1989). Because CTX is physically large, it was concluded that the amino acids with which it interacted must lie in the outer vestibule of the pore.
Later, through the use of the smaller ion TEA\(^+\), MacKinnon and Yellen (1990) were able to identify residues lining the pore. This approach led to the identification of two positions (431 and 449) in the primary structure of the \textit{Shaker} potassium channel that seemed to be located in or near the outer mouth of the ion conduction pore.

Experiments to determine whether the S5-S6 loop of \(K_\alpha\) channels constituted part of the pore continued, and the results from several independent studies soon established that the conduction pathway was indeed located in this region. First, Yellen et al. (1991) demonstrated that internal TEA\(^+\) blockade was altered by mutation of a residue (T441 in \textit{Shaker}) at a position almost midway between the two residues (431 and 449) previously implicated in external TEA\(^+\) blockade. Next, Yool and Schwarz (1991) demonstrated that mutation of single amino acids in the corresponding region of \textit{ShB} channels, including threonine 441, altered channel selectivity, affecting NH\(_4\)\(^+\) and Rb\(^+\) permeability. Shortly after, Brown and his colleagues (Hartmann \textit{et al.}, 1991; Brown, 1994; Brown \textit{et al.}, 1994) substituted a stretch of 21 amino acids, proposed to form the pore of the \(K_\alpha 3.1\) clone for the corresponding region in a phenotypically different \(K_\alpha\) channel, \(K_\alpha 2.1\). The resulting chimera (CHM) displayed a single channel conductance and TEA blocking profile characteristic of the donor channel, whilst its voltage sensitivity resembled that of the host channel. This suggested that the ion conduction properties were specified entirely by the donor peptide module without modification by the host channel.

Subsequent studies by the same group concentrated on the individual residues within this stretch of amino acids to establish exactly which residues determined the phenotypic differences between \(K_\alpha 2.1\) and \(K_\alpha 3.1\) (Kirsch \textit{et al.}, 1992; Taglialatela \textit{et al.}, 1993). Of the 21 residues, only 12 were identical. Mutations of the remaining 9 residues were made and the valine at position 374 of \(K_\alpha 2.1\) (leucine 401 in \(K_\alpha 3.1\)) was soon identified as the residue responsible for the functional differences between \(K_\alpha 2.1\) and \(K_\alpha 3.1\).

Together, the results from the aforementioned studies implied that the stretch of amino acids between the S5 and S6 segments (now known as the P-region) formed the conduction pathway. However, if this region was \(\alpha\)-helical in structure it could not possibly span the entire membrane. Yellen \textit{et al.} (1991) considered this possible, suggesting that the channel could have an hourglass-like structure with a short, narrow pore and wider vestibules at one or both ends. However, a \(\beta\)-barrel structure for the pore was favoured at the time (Yellen \textit{et al.}, 1991).
1991; Hartmann et al., 1991; Brown et al., 1993). The residue at position 374 in Kv2.1 (valine 443 in Shaker) was considered to be positioned at an 'active site', hence its importance in determining ion conduction (Kirsch et al., 1992).

These early studies painted a somewhat oversimplified picture of the channel pore, as it soon became evident that both the S4-S5 linker and S6 segment also contribute to the pore. Mutations in the S4-S5 linker were found to alter unitary conductance (Isacoff et al., 1991), Rb⁺ selectivity and blockade of the channel by internal TEA⁺, Ba²⁺ and Mg²⁺ (Slesinger et al., 1993). Mutations in S6 also had effects on single channel conductance and block by internal TEA⁺ and Ba²⁺ (Lopez et al., 1994). Thus, the existing model of the potassium channel pore was modified from one in which the pore was comprised mainly of P-region segments to a more sophisticated model in which the long pore consisted of structural elements from the P-region, the S6 segment and the S4-S5 linker. The P-regions were thought to comprise the external region of the pore, in which the selectivity filter (see below) could be found, whilst the S6 segments and the S4-S5 loops were thought to form the inner part of the pore.

1.6.2. Theories of selectivity

Prior to the resolution of the crystal structure of KcsA, two major hypotheses had been offered to explain how potassium channels might select for K⁺ ions over Na⁺ ions. The first hypothesis, proposed by Bezanilla and Armstrong (1972) and Bertil Hille (1973), suggested that selection against Na⁺ occurred in the narrowest part of a potassium channel, which Bezanilla and Armstrong (1972) called 'the tunnel'. Approximately 3.0-3.3Å in diameter, the tunnel was considered to consist of several oxygen-containing groups fixed rigidly in position to provide a good fit for a K⁺ ion but not for a Na⁺ ion. A K⁺ ion would be required to shed its waters of hydration in order to permeate the pore. Thus, selectivity was suggested to depend on the crystal diameter of the ion. Furthermore, K⁺ ions would be kept from passing one another by mutual repulsion (Hille, 1973).

Kumpf and Dougherty (1993) proposed an alternative hypothesis for ion selectivity in potassium channels. Kumpf and Dougherty (1993) noticed that the sequence of the pore region of a number of potassium channels was surprisingly hydrophobic and contained a large number of aromatic residues. Their results from computational modelling suggested that cation-π interactions could be responsible for ionic selectivity, K⁺ in the pore being coordinated in a cage of π electrons generated at the face of a ring of aromatic residues (Kumpf & Dougherty, 1993). In support of this hypothesis, it had previously been shown that
**K^+** selectivity in *Shaker* was partly determined by the presence of the tyrosine (Y445) of the GYG motif (Heginbotham *et al.*, 1992).

A more detailed study by Heginbotham and her colleagues later demonstrated that a highly conserved stretch of eight amino acids (single letter code - TXTXGYG) known as the ‘potassium channel signature sequence’ was important for selectivity (Heginbotham *et al.*, 1994). The hydroxyl groups of the three threonines (in *Shaker* there is also a threonine (T441) at position 3 of the signature sequence) and the aromatic ring of the tyrosine seemed prime candidates for interacting with and selecting for K^+ ions. By mutating these eight amino acids and measuring permeability ratios, it was concluded that the hydroxyl groups at positions three and four of the signature sequence and the aromatic ring at position seven were not essential for K^+ selectivity. Because the aromatic group was not required for K^+ selectivity, it was suggested that K^+ selectivity was not conferred by cation-π interactions, but instead by interactions with oxygen atoms as first proposed some 20 years earlier (Bezanilla & Armstrong, 1972; Hille, 1973).

### 1.6.3. Molecular basis of selectivity

We now know from the crystal structure of the *Streptomyces lividans* potassium channel KcsA that the selectivity filter is formed by backbone carbonyl oxygen atoms from five amino acids (TVGYG), including the two conserved glycine residues in the signature GYG motif (see figure 1.7A; Doyle *et al.*, 1998b). The carbonyl oxygen atoms of the second conserved glycine residue are thought to assist in the hydration and dehydration of a K^+ ion at the extracellular entryway (Zhou *et al.*, 2001b). Once dehydrated, the partially negatively charged carbonyl oxygens of the selectivity filter mimic the waters of hydration forming four potential ion-binding sites arranged so that a dehydrated K^+ ion fits exactly, binding tightly to the pore (Doyle *et al.*, 1998b; Morais-Cabral *et al.*, 2001; Zhou *et al.*, 2001b). In contrast, a Na^+ ion would fit so loosely that it energetically favours to remain in aqueous solution.

Whilst this accounts for the high selectivity exhibited by potassium channels, such tight binding of a K^+ ion to the pore would not allow for the high rates at which K^+ moves through the channel. How is this achieved? The ion conduction pore contains three binding sites in single file, as originally hypothesized by Hille and Schwarz (1978). The hydrophobic tunnel of the selectivity filter, adjusted to a width of 3Å by a sheet of aromatic amino acids in the surrounding pore helices, accommodates two of these K^+ ions, which are located close enough (7.5Å apart) that the ions electrostatically repel each other (see figure 1.7B). This
electrostatic repulsion lowers the binding affinity for a third K\(^+\) ion residing in the inner mouth of the channel, displacing it out of the selectivity filter and into a *water-filled cavity*, the second ion then taking its place.

Remarkably, the overall structure of this potassium channel validated many of the previous findings from functional studies with Kv channels. For example, the outer vestibule possesses the right shape to bind scorpion toxins related to CTX (MacKinnon *et al.*, 1998) and, as for other potassium channels, TEA and Ba\(^{2+}\) block both the inner and outer pores (Heginbotham *et al.*, 1999). Thus, the overall architecture of the potassium channel pore may be conserved through evolution.
Figure 1.7.

Features of ion permeation in potassium channels revealed by the crystal structure of KcsA. A, Ribbon representation of two of the four subunits of the KcsA channel viewed from the side. The selectivity filter (coloured red) is formed by backbone carbonyl oxygen atoms from five amino acids including the GYG triplet. B, The selectivity filter of KcsA contains two K⁺ ions (coloured green), located at opposite ends. Mutual electrostatic repulsion between adjacent K⁺ ions underlies rapid permeation. (From Doyle et al., 1998b)
1.7. Aims

Although the atomic structure of a bacterial potassium (KcsA) channel pore has been determined using X-ray crystallography (Doyle et al., 1998), a physical picture of a Kir channel pore has yet to be resolved. With a two-transmembrane-helix topology, Kir channels are similar in their overall structure to KcsA yet share little homology at the amino acid level. In contrast, the amino acid sequence that forms the pore is well conserved between Kv channels and KcsA. It has been suggested that the ion conduction pore is conserved among all potassium channels (Lu et al., 2001b). However, the M1-P-M2 region of eukaryotic Kir channels exhibits numerous features that distinguish them from other potassium channel families (see for example Kubo et al., 1998; Durrell & Guy, 2001). Until three-dimensional pictures of Kir and Kv channels are readily available, mutagenesis studies remain a powerful tool in determining the contribution of specific amino acids to channel function. Here, by combining site-directed mutagenesis with studies of ion block, conduction and ionic selectivity, I have investigated the contribution of residues in the P-region to the permeation properties of Kir2.1 and Kir2.2.
Chapter 2

Materials & Methods

2.1. Molecular biology

2.1.1. Cloning and site-directed mutagenesis

For expression in Chinese hamster ovary (CHO) cells, EcoRI/XhoI and HindIII/XhoI fragments containing the entire coding region of the mouse brain Kir2.1 (Stanfield et al., 1994a) and Kir2.2 genes, respectively, were sub-cloned into the expression vector pcDNA3. Substitution mutations, with the exception of Kir2.1 F147L, were generated by oligonucleotide-directed in vitro mutagenesis either by the method of Taylor et al. (1985) using commercially available kits (Amersham Pharmacia Biotech), or by the method of Kunkel (1985). Mutation F147L was generated using the ‘Quik Change’ mutagenesis kit (Stratagene). For expression in murine erythroleukaemia (MEL) cells, a Hind III/XhoI fragment containing the entire coding region of the mouse brain Kir2.2 gene, ligated to the β-globin locus control region (LCR)-promoter (see section 2.2.6), was sub-cloned into the MEL
expression vector pNV3 (Shelton et al., 1993). DNA sequencing of the entire K<sub>ir</sub> cDNAs was performed to verify mutations.

2.1.2. Purification of plasmid DNA

It is important that the DNA used in transfections is not contaminated with RNA, bacterial chromosomal DNA and protein, and is generally in good condition (Ehlert et al., 1993). To reduce such contaminants, plasmid DNA encoding wild-type and mutant K<sub>ir</sub> channels was purified using either commercially available kits, such as the Wizard® Plus SV Minipreps DNA Purification System (Promega Corporation) or the Qiagen Maxi Plasmid kit (Qiagen), or by banding on a caesium chloride gradient as described by Birnboim and Doly (1979).

2.2. Cell culture

In order to minimize the risk of infection of cell populations by bacteria and fungi, all cell culture was carried out in a class II laminar flow hood (Medical Air Technology Ltd) utilizing aseptic techniques. Surfaces were swabbed with 70% alcohol prior to use and latex gloves were worn at all times.

2.2.1. Cell culture reagents and plastic-ware

All cell culture reagents were purchased from Gibco BRL. Tissue culture grade flasks, 12-well plates and 35mm petri dishes were purchased from Nunclon™. All other plasticware was purchased from Falcon. The tissue culture flasks are made of sulphonated polystyrene, which has a negatively charged surface. Adhesion between this surface and the negatively charged surface of the cell membrane occurs by way of divalent cations and basic proteins, which form a layer between the surface of the plastic and the cells (Butler, 1991; 1996).

2.2.2. Incubator

Cells were incubated at 37°C in 10% carbon dioxide (CO<sub>2</sub>) atmosphere at 95% humidity using a Galaxy CO<sub>2</sub> incubator (Scientific Lab Supplies, Ltd.). The high level of humidity in the incubator prevents excessive evaporation from the tissue culture flasks and plates whilst 10% CO<sub>2</sub> is used to maintain the cultures at their optimal pH (6.9-7.4) by way of the bicarbonate-CO<sub>2</sub> buffering system. Whilst a difference of one or two degrees below 37°C simply slows cell growth, higher temperatures result in cell death. It is essential, therefore, that the temperature of the incubator is not allowed to rise above 37°C.
2.2.3. Cell lines

From their study of the growth potential of human embryonic cells, Hayflick and Moorhead (1961) concluded that 'normal' cells have a finite lifespan. Human embryonic cells could be repeatedly sub-cultured for approximately 50 generations, beyond which they were incapable of further growth. However, some cells may display an infinite growth capacity following a process known as transformation, in which they lose sensitivity to the stimuli that control their growth. These populations of transformed cells are often described as 'continuous' cells growing well in simple growth media, which has not been supplemented with growth factors (Butler, 1991; Butler, 1996). Two different cell lines have been used in the studies described within this thesis. Outlined below are the general properties, maintenance and preparation of CHO and MEL cells, respectively.

2.2.4. Growth of cells in culture

Cells in culture follow a pattern in which they go through several different phases of growth. These are described as lag-, growth-, stationary- and decline phases. The lag phase represents a period of time during which the synthesis of cellular growth factors occurs and there appears to be no increase in cell density. The duration of this phase is dependent on the viability of the cells and the density of cells used at inoculation, the lower the initial inoculation density the longer the phase. It is thought that the cells enter the growth phase once the concentration of growth factors reach their optimum levels. The number of cells initially increases exponentially during the growth phase, following which the cells enter into a stationary phase during which there is no further increase in cell density. Although the cells are no longer growing at this stage, they may remain metabolically active. Finally, cells enter into a decline phase during which cell death occurs. This is often in response to depletion of nutrients. In order to ensure continued growth of cells in a new culture, following subculture, it is best to passage the cells whilst they are still growing. Cells split from the stationary phase will grow but the lag phase will be longer following inoculation (Butler, 1991; 1996).

2.2.5. Chinese hamster ovary (CHO) cells

CHO cells were originally derived from an ovarian biopsy of a female Chinese hamster in 1957 and have since been established as a very stable, 'continuous' cell line (for review Butler, 1991; Doyle et al., 1998a). The consistency and reproducibility of results that can be acquired by using a cell line comprised of a single cell type, such as CHO cells, in addition to
the ease with which such cells may be maintained in culture, make them highly suitable for biological studies of channels and receptors. Furthermore, only three types of voltage-dependent ionic membrane conductances, a Ca$^{2+}$-activated K$^+$ current, a voltage-activated Na$^+$ current and a voltage-activated Ca$^{2+}$ current similar to the L-type Ca$^{2+}$ current, have been recorded from untransfected CHO cells (Skryma et al., 1994). Since these epithelial-like cells appear to lack endogenous inward rectifier potassium channels, and may be easily transfected (see section 2.3) with cDNA encoding the protein of interest, they provide an ideal system for the expression of cloned Kir channels.

2.2.5a. Routine growth and maintenance of CHO cells

CHO cells were grown in 80cm$^3$ tissue culture flasks and were incubated at 37°C in 10% CO$_2$ atmosphere at 95% humidity. Growth was found to be optimal in alpha minimal essential medium (MEM-α) with Glutamax-I (L-alanyl-L-glutamine), without ribonucleotides and without deoxyribonucleosides. Standard incubation medium was supplemented with 10% (v/v) foetal bovine serum (FBS). Medium was stored at 4°C but was incubated at 37°C for 30 minutes prior to use.

2.2.5b. Subculture of CHO cells

Cells were grown to approximately 80% confluency and split twice weekly. The medium was removed and the cells washed with 10ml Ca$^{2+}$/Mg$^{2+}$-free phosphate buffered saline (PBS) to remove any traces of serum, which contains excess proteins that deactivate trypsin and glycoproteins such as fibronectin, which provide a surface coating aiding cell attachment. To dislodge cells from the substratum, approximately 1ml of (1×) trypsin-ethylenediaminetetraacetic acid (EDTA; TE) was added to the flask and left for 5 minutes. Trypsin is a proteolytic enzyme, which aids detachment of the adherent cells by breaking down the proteins that bind the cells to the substratum. In addition, the EDTA chelates any remaining divalent cations.

Once the cells had detached from the sides of the tissue culture flask, 9ml of standard incubation medium was added to inactivate the trypsin. The cell suspension was then transferred to a centrifuge tube and centrifuged for 2 minutes at 1000g at room temperature. The supernatant was discarded under sterile conditions and the pellet resuspended in 10ml standard incubation medium. In general, 200-400μl of cell suspension was transferred into 15ml of fresh medium for the maintenance of the cell line and between 10-150μl per well of a
12 well plate for transient transfection. For consistency in experiments, CHO cells were not used beyond passage 45.

2.2.5c. Preparation of CHO cells for electrophysiological recording.

In general, the transfected CHO cells were ready for electrophysiological recording 12-60 hours after transfection. To dislodge cells from the bottom of the dish, the medium was removed and the cells washed with sterile Ca\(^{2+}\)/Mg\(^{2+}\)-free PBS. 300\(\mu\)l of TE were then added and left for approximately 5 minutes. To inactivate the trypsin, 1ml of standard incubation medium was added and the cell suspension transferred to a 15ml centrifuge tube and centrifuged for 2 minutes at 1000g at room temperature. Following centrifugation, the supernatant was discarded and the cells were resuspended in approximately 1ml serum free medium. Cells were plated out into 35mm tissue culture dishes containing the appropriate bath solution. For single channel recording, dishes precoated with poly-L-lysine were used. Under sterile conditions, poly-L-lysine (Sigma) was dissolved in 50ml 'milliQ' water and 1ml was used to coat each 35mm tissue culture dish. After 5 minutes, the solution was aspirated from the dishes and the surfaces washed twice with sterile water. The dishes were left to dry overnight and stored at 4°C until required.

2.2.6. Murine erythroleukaemia (MEL) cells

MEL cells are erythroid progenitor cells, which when transformed by the Friend virus complex are arrested at the proerythroid stage of development. They were used in the initial stages of this study in conjunction with the β-LCR promoter, which is erythroid specific. Using such a system can be beneficial since expression of ion channels can be achieved independently of the position of integration of the transfected gene within the host genome. Such 'position effects' are typically responsible for variations in the gene expression level (Shelton et al., 1993; the reader is referred to this reference for a more detailed description of the use of the MEL cell expression system).

MEL cells can be induced to undergo a pattern of terminal erythroid differentiation using chemical agents such as dimethyl sulphoxide (DMSO), during which they synthesize high levels of globin proteins. In this study, the \(K_{ir}2.2\) gene was ligated to the β-LCR promoter, thus functional expression of \(K_{ir}2.2\) correlated with the DMSO-induced production of globin proteins.
As with CHO cells, the low endogenous expression of ion channels in MEL cells make them suitable for expression of \( K_r \) channels. Only three channels have been observed in untransfected MEL cells: a \( Ca^{2+} \)-activated \( K^+ \) channel, a voltage-independent, monovalent cation, stretch activated channel and a \( Cl^- \) channel (Arcangeli et al., 1987).

2.2.6a. Routine growth and maintenance of MEL cells

MEL cells were grown in 25cm\(^3\) tissue culture flasks and were incubated at 37\(^\circ\)C in 10% CO\(_2\) atmosphere at 95% humidity. Growth was found to be optimal in Dulbecco's Modified Eagle's Medium (DMEM) with Glutamax-I, with 25mM HEPES (\( N^-\)[-2-Hydroxyethyl] piperazine-\( N^-\)[-2-ethanesulphonic acid]), and without sodium pyruvate. The medium was supplemented with 10% v/v FBS, and geneticin® antibiotic (G418; Sigma) was added to give a final concentration of 0.4mg.ml\(^{-1}\) to select for transfected cells (see section 2.3.5). Under sterile conditions, G418 was dissolved in PBS to give a concentration of 200mg.ml\(^{-1}\). The G418 stock solution was then filtered using a 0.22\( \mu \)m bottle top filter (Nunclon™), divided into 1ml aliquots and stored at -20\(^\circ\)C until required. Medium was stored at 4\(^\circ\)C but was incubated at 37\(^\circ\)C for 30 minutes prior to use.

2.2.6b. Subculture of MEL cells

MEL cells were grown to approximately 90% confluency and split twice weekly. The cells were dislodged from the bottom of the flask by gently tapping the sides. In general, 30-50\( \mu \)l of cell suspension was transferred into 6ml of fresh medium for flasks to be induced 3-4 days after subculture and 60-100\( \mu \)l into flasks to maintain the cell line.

2.2.6c. Induction of MEL cells

Transfected MEL cells were induced to undergo differentiation, and hence potassium channel gene expression, by applying 2% v/v DMSO. This typically involved adding 120\( \mu \)l of DMSO to 6ml of cell suspension approximately 3-4 days after subculture when the cells were approximately 50% confluent.

2.2.6d. Preparation of MEL cells for electrophysiological recording

Typically, electrophysiological recordings can be made from MEL cells between 2-4 days post-induction. Induced cells were dislodged from the bottom of the flask by gently tapping the sides and a 200-500\( \mu \)l aliquot of cell suspension transferred into a 15ml centrifuge tube.
The cell suspension was then diluted to 5ml using extracellular recording solution (section 2.4.5) and centrifuged for 2 minutes at 1000g at room temperature. The supernatant was then discarded and the cell pellet resuspended in 1ml extracellular solution. As for CHO cells, MEL cells were plated out in 35mm tissue culture dishes containing extracellular solution.

**2.2.7. Storage of cell lines**

In order to prevent loss of an established cell line by contamination, cells were stored cryogenically frozen under liquid nitrogen. Cells can be stored indefinitely in freezing medium containing a cryoprotectant such as DMSO, which protects cells from disruption by preventing build up of electrolytes during the freezing process (Lovelock & Bishop, 1959). For maximum cell survival it was important to freeze the cells slowly and thaw rapidly.

Cells to be frozen down (either untransfected CHO or MEL cells or stably transfected MEL cells) were grown to confluency in 30ml of growth medium in 175cm$^3$ tissue culture flasks (Nunclon™). Cells were dislodged from the side of the flask as described above for each cell line. The density of cells was determined by performing a cell count using a haemocytometer (see section 2.2.8) and the appropriate volume of cell suspension containing $5 \times 10^6$ cells transferred into a sterile 15ml centrifuge tube and centrifuged for 2 minutes at 1000g at room temperature. The supernatant was poured off under sterile conditions and the pellet resuspended in 1ml of freezing medium containing 50% v/v FBS and 10% v/v DMSO. 200µl aliquots of this cell suspension were immediately transferred into previously labelled 1.25ml cryotubes and placed into a -80°C freezer overnight. The cryotubes were then transferred on dry ice to a liquid nitrogen refrigerator.

To restart a stock from frozen, a cryotube containing the required cell type or mutant was removed from the liquid nitrogen store and placed on dry ice for transport. The cell suspension was thawed by immersing the cryotube in a water bath at 37°C, and transferred to a 15ml centrifuge tube using a P200 Gilson pipette. The cells were gently resuspended by adding 10ml of prewarmed medium drop by drop and were then transferred to a 175cm$^3$ tissue culture flask containing an additional 20ml of growth medium. Safety goggles and protective gloves were worn at all times when handling frozen cryotubes in case of explosion.
2.2.8. Haemocytometer cell counts

Haemocytometer cell counts were often performed to determine cell density per ml of medium and hence, the appropriate volume of cell suspension to use when freezing cells or plating cells out prior to transfection.

A haemocytometer consists of a thick glass slide, the counting chamber and coverslip, which when placed in the correct position gives a chamber depth of 0.1mm. The glass slide is typically engraved with a nine square counting grid, each square having dimensions of 1mm², some of which are divided further into 16 or 25 smaller squares (see Doyle et al, 1998a).

Before use, the haemocytometer (Neubauer) was thoroughly cleaned using 70% IMS and the coverslip placed in the correct position on the glass slide. The appearance of Newton’s rings, ‘rainbow-like’ interference patterns, which are visible on holding the haemocytometer up to the light, indicated when the coverslip was in the correct position. The cell suspension was mixed and 50μl removed and mixed with an equal volume of Trypan blue solution, which penetrates the membrane of non-viable cells, staining them blue. The haemocytometer chamber was filled by capillary action, a Gilson tip containing the cell suspension and Trypan blue mixture being placed at the junction between the coverslip and counting chamber. The haemocytometer was then placed on a microscope, the engraved area brought into focus and, under low magnification, the number of unstained cells in each of two 1mm² areas counted. In general, cells in the left-hand and top grid markings were included whilst those in the right-hand and bottom markings were excluded (Doyle et al, 1998a). The total number of viable cells was then calculated as follows:

\[
\text{Total number of viable cells} = \text{Total cell volume (ml)} \times 2 \times \left(\text{dilution factor in Trypan blue}\right) \times \left(\frac{\text{mean number of unstained cells (unstained count} - 2\right)}{10^4}
\]

If the cell count was low, more than two 1mm² areas were counted and averaged.

2.3. Transfection methods

Transfection involves the transfer of nucleic acid into mammalian cells and can be used to produce cells yielding a large quantity of a single protein for the study of the regulation and function of genes. The incorporation of DNA into mammalian cells was first demonstrated in the 1960s. Using isolated human nuclear DNA, Szybalska and Szybalska introduced a
functional gene for the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) enzyme into human cells lacking an endogenous form of the enzyme (for reviews see Butler, 1996; MacDonald, 1991).

A variety of methods are now employed for the transfection of DNA into mammalian cells. These include: calcium phosphate coprecipitation, DEAE-dextran mediated transfection, electroporation, lipid-mediated transfection and the use of viral vectors. Whilst some of the above methods are more suited for short-term expression others are more effective for the stable expression of DNA. The transfection methods used for establishing the transient and stable expression systems used throughout this study are described below.

2.3.1. Lipid-mediated transfection

Early methods for lipid-mediated transfection involved the incorporation of DNA into mammalian cells by way of liposome vesicles (Felgner et al., 1987), formed from positively charged lipid and negatively charged DNA, which bind to the cell and are then internalised by endocytosis (for reviews see Doyle et al., 1998a; Otter-Nilsson & Nilsson, 1999). A variety of cationic lipids are commercially available including Invitrogen's PerFect Lipid™ range, with pFx™-8 giving optimum transfection efficiency in CHO cells. The incorporation of foreign DNA into mammalian cells by way of transfection is often a very inefficient process. It is, therefore, essential to have a method for selecting those cells that are expressing the gene of interest. In this study, enhanced green fluorescent protein (eGFP) was used as a marker for successful transfection. Expression of eGFP in combination with an ion channel produces a bright green fluorescent signal when viewed with conventional epi-fluorescence optics for fluorescein isothiocyanate. Fluorescent, cotransfected cells invariably express the introduced ion channel (Marshall et al., 1995).

2.3.2. Transient transfection of CHO cells using PerFect lipid™

CHO cells were plated out 24 hours prior to transfection at a density of $1 \times 10^4$ cells per well of a 12 well plate. Per reaction, 1ml of serum free medium was placed into each of two polystyrene universals (Bibby Sterilin Ltd.). Universals made from polystyrene were used because, unlike glass or polypropylene, polystyrene does not attract the concentration- and ionic strength-dependent aggregates which can form during the reaction between lipid and DNA (Doyle et al., 1998a).
Next, the lipid (pFx™-8) was removed from storage at -20°C and allowed to equilibrate to room temperature. Once warm, 12µl of the lipid were transferred into one of the two universals. Meanwhile, 1µg of eGFP DNA and 4µg of channel DNA were added to the medium in the second universal. The contents of the two universals were then mixed and set aside. The medium was aspirated and the cells washed with sterile Mg²⁺/Ca²⁺-free PBS to remove any traces of serum. 1ml of the medium containing the DNA and lipid was then added to each well (2 wells per transfection). The cells were incubated for 4 hours following which the medium was removed and replaced with 1ml standard incubation medium, and the cells returned to the incubator for 12-60 hours.

2.3.3. Transient transfection of CHO cells using FuGENE™ 6

The non-liposomal transfection reagent FuGENE 6 (Roche Molecular Biochemicals) provides an alternative method for lipid-mediated transfection of eukaryotic cells. The method offers the advantage that transfection can be performed in the presence of serum. The need to remove any traces of serum prior to transfection and to replace the serum following incubation of the cells with the transfection reagent is therefore abolished. This offers the convenience of being able to perform transfections at the end of the day, aiding experimental design.

CHO cells were plated out 24 hours prior to transfection as described above. 100µl of serum free medium was placed into a flip-top eppendorf to which 3µl of FuGENE™6 was added, being careful not to allow the lipid to come into contact with the sides of the eppendorf tube. The diluted transfection reagent was incubated at room temperature for 5 minutes. Meanwhile, 1µg of channel cDNA and 0.5µg of GFP were mixed together in a separate eppendorf and put aside. Following incubation, the diluted transfection reagent was added to the DNA in the second eppendorf, mixed and incubated for a further 15 minutes at room temperature. 50µl of the transfection mixture were removed and added to cells in a single well of a 12 well plate. This was repeated for a second well using the remaining 50µl of transfection mixture. The plates were then gently swirled to ensure even dispersal and returned to the incubator.

2.3.4. Levels of expression

Following transient transfection using PerFect lipid or FuGENE™ 6, current sizes were typically large, often in excess of 5-10nA. Consequently, large series resistance errors (see
section 2.4.14) were frequently encountered. In an attempt to reduce current size and subsequent problems with series resistance errors, the transfection protocols described above were modified. Figure 2.1 shows the average current sizes in pA/pF following various changes made to the transfection protocols (see below). Since previous studies have shown a correlation between the intensity of fluorescence and level of channel expression (Marshall et al., 1995), both bright and faint cells (i.e barely fluorescing) were patched. The following changes were made:

2.3.4a. The amount of cDNA used for PerFect Lipid transfections was reduced

The total amount of cDNA used per transfection was normally 5μg (see section 2.3.2), 4μg of which were channel cDNA and the remainder marker cDNA. To determine whether current density could be reduced, by using a lower amount of cDNA, the total cDNA used per transfection was halved, maintaining the ratio of channel to marker. Figure 2.1A shows that using lower amounts of cDNA per transfection did not significantly alter current density (students unpaired t-test, $P > 0.05$). However, there was a significant difference in current density between bright and faint cells.

2.3.4b. The incubation time for PerFect Lipid transfections was reduced

According to the manufacturers instructions, the optimal incubation period for transfection with Perfect Lipid is 4 hours. Thus, to test whether sub-optimal incubation times gave smaller currents the incubation time for transfection was reduced to 3 hours. Figure 2.1B shows that whilst reducing the incubation time for transfection to 3 hours did not significantly alter current density in bright cells ($P > 0.05$), a significant reduction in current density was seen in faint cells ($P < 0.05$).

2.3.4c. A weaker promoter was used to drive expression

Recombinant protein production in mammalian cells can be enhanced by use of the appropriate transcriptional element. The strength of any given promoter/enhancer is often cell-type dependent. In CHO cells, the immediate-early promoter and enhancer of the human cytomegalovirus (CMV) has been shown to be more effective than the Rous sarcoma virus 3’ LTR promoter (Liu et al., 1997). In general, the CMV promoter was used to drive expression of $K_{ir}$ channels throughout this study. Since this gave high current densities, expression driven by the RSV promoter was investigated to determine whether use of a weaker promoter could consistently give smaller currents. Figure 2.1C shows clearly that RSV driven
Figure 2.1.

Levels of channel expression. Graphs to show how current density changes following minor alterations to the method of transfection. A, the total amount of cDNA used was reduced from 5μg to 2.5μg. B, the incubation time for transfection was reduced from 4 to 3 hours. C, a weaker promoter (RSV) was used in place of CMV to drive expression. D, the cDNA was purified using a CsCl gradient instead of commercially available kits. E, the cells were patched at 48 rather than 24 hours post-transfection. The clear bars represent bright cells, whilst the shaded bars represent faint cells. Reductions in current density were tested for statistical significance using unpaired student t-tests (* $P < 0.05$, ** $P < 0.01$).
expression did result in significantly reduced current densities in both bright ($P < 0.05$) and faint ($P < 0.01$) cells.

2.3.4d. Different methods for the purification of cDNA were utilized

In this study, plasmid DNA was initially purified using commercially available kits as described in section 2.1.2. Such kits have become the modern standard for large-scale plasmid purification, yielding DNA with a purity equal to, or even exceeding that of DNA purified using banding on a CsCl gradient. Since the purity of the DNA can affect the efficiency of transfection (Ehlert et al., 1993), it was investigated whether current density could be reduced using CsCl purified DNA in transfections. Figure 2.1D shows that using CsCl purified DNA significantly reduced current density in bright cells ($P < 0.05$) but not in faint cells. In fact, the mean current density in faint cells, which had been transfected with CsCl purified cDNA was larger than that in faint cells that had been transfected with kit purified cDNA.

2.3.4e. Cells were patched at different intervals post transfection

Cells were typically healthy for up to 72 hours post-transfection. Thus, current density was compared at 24 and 48 hours post-transfection. Figure 2.1E shows that the average current density was significantly lower in bright ($P < 0.05$) and faint ($P < 0.01$) cells 48 hours post-transfection.

Although some of the changes to the method of transfection as described above clearly reduced current density, current levels remained high under most conditions. However, under certain conditions currents were small enough to avoid problems with series resistance. Since bright cells typically displayed very high current densities, these cells were avoided. By reducing to 1µg the amount of cDNA used per transfection, in addition to reducing the incubation time to 3 hours and patching faint cells 48-60 hours after transfection, expression levels were low enough to resolve single channels.

2.3.5. Stable transfection of MEL cells using electroporation

The stable transfection of MEL cells was performed by electroporation, which involves the exposure of a cell suspension to a brief electrical impulse resulting in the formation of transient pores in the cell membrane and DNA uptake (Doyle et al, 1998a).
1×10^7 cells in logarithmic phase growth were required per transfection. Untransfected MEL cells were grown to confluency in 30ml of G418-free medium in a 175cm³ tissue culture flask. To determine the cell density a cell count was performed using a haemocytometer (see section 2.2.8). The appropriate volume of cell suspension containing 1×10^7 cells was transferred into a sterile 15ml centrifuge tube and spun at 1000g for 2 minutes at room temperature. The supernatant was poured off under sterile conditions and the pellet washed twice by resuspending in an equal volume of PBS and centrifuging as before.

Following the second wash, the pellet was resuspended in 0.9ml of cold electroshock buffer containing (mM): NaCl, 140; HEPES, 25; NaH₂PO₄, 0.75, which was titrated to pH 7.5 with 1M NaOH, filtered through a 0.22μm bottle top filter and stored at 4°C. The cell suspension was then transferred to a sterile, pre-chilled 0.4cm path-length electroporation cuvette to which 50μl of linearized expression construct (1mg.ml⁻¹) was added. The cuvette was capped and inserted into the holder of the electroporator. The conformation of the plasmid DNA affects the efficiency of the transfection, use of linearized DNA improving the yield of stable transfectants (Potter et al., 1984; Toneguzzo et al., 1986; Chu et al., 1987). The electroporator (Bio-Rad Gene Pulser®) was set at 0.25kV and 960μF for the voltage and capacitance extender, respectively. Following electroporation, the cuvette was removed from the apparatus at which stage the cell suspension appeared frothy.

150 and 300μl aliquots of the transfected cell suspension were pipetted into 50ml centrifuge tubes containing 30ml of G418-free medium and plated out 1ml per well into separate 24 well plates. The cells were then incubated at 37°C for 24-48 hours, during which period each well was overlaid with 1ml G418 free medium supplemented with G418 at 2.0mg.ml⁻¹, giving a final concentration of 1.0mg.ml⁻¹, to select for transfectants.

In general, colonies of transfectants began to appear within 7-14 days of transfection. Using a P20 Gilson pipette the colonies were picked off and plated out into 6 well plates containing 2ml standard growth medium, containing 0.4mg.ml⁻¹ G418, per well. Following subculture and induction using 2% v/v DMSO, the individual colonies were screened for ionic currents. Those colonies that expressed high levels of current were then sub-cultured into large tissue culture flasks (175cm³) and grown to confluency for the subsequent freezing process and storage in liquid nitrogen (see section 2.2.7).
2.4. Electrophysiology

Ionic currents were measured using the patch clamp technique. Using this electrophysiological method it is possible to record both macroscopic and single-channel currents flowing across biological membranes through ion channels. Neher and Sakmann (1976) first used the patch clamp technique to record single channel currents from the membrane of denervated frog muscle fibres. The technique was later refined (Hamill et al., 1981) and today is probably the most widely used method for the recording of macroscopic and single channel currents from small cells.

2.4.1. Principles of patch-clamp

The recording of ionic currents using the patch clamp technique is achieved by 'clamping' the membrane at a pre-selected value known as the 'holding potential' using a feedback amplifier. Membrane currents are then activated by sudden changes in the command potential, or in the composition of the solution bathing the membrane.

The total membrane current \( I_m \) comprises both ionic \( I_i \) and capacitive components \( I_c \) as described by:

\[
I_m = I_i + I_c. \quad \text{(Equation 2.1)}
\]

Since

\[
I_c = \frac{dQ}{dt} \quad \text{(Equation 2.2)}
\]

where \( Q \) is the store charge and is related to voltage \( V \) and membrane capacitance \( C \) according to the relation:

\[
Q = C \times V, \quad \text{(Equation 2.3)}
\]

\[
I_c = C \left( \frac{dV}{dt} \right) \quad \text{(Equation 2.4)}
\]
Thus

\[ I_m = I_i + C \left( \frac{dV}{dt} \right) \]  

(Equation 2.5)

To eliminate capacitive currents, the voltage change must be instantaneous. During patch clamp, the membrane potential is changed from the holding potential to the desired voltage as rapidly as possible. Thus, any capacity current is restricted to the edges of the square pulse. Once the voltage change is over, any remaining current may be attributed to current flowing through ion channels. The speed at which the membrane potential is changed is dependent on two parameters, the membrane capacitance \( C_m \) of the cell and the series resistance \( R_s \), which will be considered later in more detail (see sections 2.4.13 and 2.4.14).

2.4.2. Gigaohm Seal

In order to make patch clamp recordings, it is first necessary to isolate a patch of membrane electrically from the external solution. This involves pressing a fire-polished pipette against the surface of the cell and applying gentle suction. This leads to the formation of a 'gigaohm' (GΩ) seal, with a resistance in excess of \( 10^9 \)Ω, from which it is possible to proceed onto one of several different recording configurations (sections 2.4.12; figure 2.5).

A high resistance seal is important for the following reasons:

*To reduce leak current* - the lower the seal resistance, the larger the fraction of current through the patch of the membrane that will leak out through the seal and will thus not be measured.

*To reduce thermal noise* - in any electrical recording, the thermal noise in a current 'source', termed *Johnson noise*, is determined by the source resistance according to:

\[ \sigma_n = \sqrt{\frac{4kTAf}{R}} \]  

(Equation 2.6)

where \( \sigma_n \) is the root mean square (r.m.s.) of the current flowing through the resistance \((R)\), \( k \) is the Boltzmann constant, \( T \) the absolute temperature and \(Af\) the bandwidth (Gibb, 1995). Thus, the current noise in any recording decreases with increasing source resistance. For the measurement of single channel currents, of the order of a pA, at a bandwidth of 1 kHz, \( R \)
needs to be approximately 2GΩ or higher. In a patch clamp setup, R consists of the headstage feedback resistor, the seal resistance and the patch resistance. The largest noise source tends to dominate because the noise contributions from each source add together according to the relation:

\[ \text{rms}_T = \sqrt{\text{rms}_1^2 + \text{rms}_2^2 + \ldots + \text{rms}_n^2} \]  

(Equation 2.7)

where \( \text{rms}_T \) is the total noise variance observed from \( n \) independent noise sources. Thus, both a high resistance seal and the use of a low-noise current-to-voltage converter amplifier (see section 2.4.9) are important for low noise levels and essential for good resolution of single channel events.

2.4.3. Setup and recording chamber

The patch-clamp setup used throughout this study consisted of an inverted microscope (Nikon) with fluorescent attachment for viewing eGFP-expressing cells. This was placed on the base plate of an air-suspension vibration isolation table and surrounded by a Faraday cage. Whole-cell and single channel currents were amplified and filtered using either an Axopatch 200B (Axons Instruments) or EPC-7 (List Instruments) amplifier, and digitised using a TL-1 Labmaster interface (Axon Instruments). Whole-cell and single-channel currents were viewed on a cathode-ray oscilloscope (CRO) and stored directly to a computer hard disk or to digital analogue tape (D.A.T), respectively. The amplifier headstage was mounted to a hydraulic manipulator (Narashige Instruments) by way of a metal plate, whilst the tip of the perfusion system was attached to a coarse mechanical manipulator. The recording chamber (figure 2.2) consisted of a 35mm petri dish (Nunclon™) inserted into a sheet of perspex containing a separate hole for the immersion of a silver/silver chloride reference electrode (Ag-AgCl pellet) in bath solution. The chamber containing the Ag-AgCl pellet was connected to the chamber containing the cells by way of a ‘salt bridge’ (U-shaped capillary tube) filled with the extracellular solution.

2.4.4. Pipette and reference electrodes

The pipette and reference (bath) electrodes facilitate the detection of ionic currents in solution as electrical currents by the headstage of the patch clamp amplifier. Silver/silver chloride (Ag-AgCl) electrodes, which exchange electrons for chloride ions in solution, are typically used.
Figure 2.2.

*Diagram to illustrate the recording chamber.* A 35mm Petri dish containing the cells was inserted into a sheet of perspex and connected via a 'salt bridge', filled with extracellular solution, to a separate chamber into which a silver/silver chloride pellet was inserted. This pellet was connected in turn to the circuit ground on the amplifier headstage.
The pipette electrode consisted of a silver wire coated in silver chloride, which was inserted into the pipette holder being careful to ensure it was in contact with the pin that plugs into the headstage. This wire was often scratched when exchanging patch pipettes resulting in 'drift' of the pipette potential. To prevent significant drift, the silver wire was regularly 'rechlorided' by dipping into molten AgCl.

The reference (bath) electrode consisted of a pellet made from metallic silver (30%) and powdered AgCl (~70%), compressed around a length of silver wire and connected to the headstage. The circuit from bath solution to pipette solution, to headstage, and back to bath solution was completed by way of a 'salt bridge'.

2.4.5. Solutions

All solutions used in the experiments described within this thesis were prepared by adding milliQ water to chemicals in their solid form, with the exception of CaCl2 and MgCl2, which were added in the appropriate volume from a 1M stock solution. The final pH of all solutions was adjusted to 7.2 using 1M HCl, KOH or RbOH as appropriate. Before use, all solutions were filtered using a 0.2μm acrodisc filter (Pall Gelman Sciences).

For whole cell recording, the extracellular solution contained (mM): KCl, 70; N-methyl-D-glucamine (NMDG), 70; HEPES, 10; CaCl2, 2; MgCl2, 2; pH adjusted to 7.2 with 1M HCl. In experiments examining the effects of extracellular K+ concentration [K+]o, NMDG replaced K+ when [K+]o was reduced or was omitted when the K+ concentration was increased to 140mM. For ion substitution experiments, the extracellular solution was prepared as described above but with 70mM RbCl replacing the KCl. To examine the blocking effects of Ba2+ or Cs+, BaCl2 and CsCl were added to the extracellular solution at concentrations of 5, 10, 30, 50, 100 and 300μM for Ba2+, and 50 and 500μM for Cs+.

For whole cell recording, the intracellular solution contained (mM): KCl, 105; HEPES, 10; EDTA, 10; pH adjusted to 7.2 with 1M KOH and KCl added to bring [K+] to 140mM.

For single channel recording, the intracellular solution contained (mM): KCl, 105; HEPES, 10; EDTA (free not disodium), 10; CaCl2, 2; pH adjusted to 7.2 with 1M KOH and KCl added to bring [K+] to 140mM. The extracellular solution contained (mM): KCl, 200; HEPES, 10; CaCl2, 2; MgCl2, 2; pH with 1M HCl to 7.2. To examine the blocking effects of Ba2+, BaCl2 was added to the extracellular solution at concentrations of 100, 200 and 300μM.
2.4.6. Perfusion system

Solutions were delivered from a multi-line gravity-fed perfusion system, consisting of five 5ml syringes attached via polythene tubing to a common perfusion tip positioned in the vicinity of the cell being patched. Each syringe contained different solutions, the outflow from each syringe being controlled by a one-way tap. Construction of this perfusion system is described in more detail elsewhere (McKillen, 1994; Abrams, 2000).

2.4.7. Manufacture of pipettes

For whole cell recording, the use of low resistance pipettes is more important than low noise since the use of pipettes with high resistances can result in large voltage errors. Patch pipettes were manufactured using a vertical microelectrode puller (pp-83, Narishige Instruments). Thin walled (1.5mm × 1.17mm) borosilicate glass capillaries with inner filaments (Clark Electromedical Instruments) were pulled in two stages. To promote gigaohm sealing, pipettes were then fire-polished using a Narishige fire polisher to give resistances between 2-5 megohm (MΩ). Heat polishing improves the success of sealing by smoothing the edges of the tip and removing dust particles.

Low background noise is crucial for the resolution of single channels (see section 2.4.2). Thus, pipettes for single channel recording were manufactured from thick walled (1.5mm × 0.86mm) borosilicate glass capillaries with inner filaments (Clark Electromedical Instruments), pulled in two stages using a vertical microelectrode puller as described above. To reduce stray capacitance and the noise levels of recordings, the tapered region of the patch pipettes was coated with Sylgard® (Gibb, 1995; Ogden & Stanfield, 1994; Standen & Stanfield, 1992).

Sylgard® (Dow-Corning 184 transparent encapsulating resin) is a hydrophobic substance consisting of two separate components, a resin and a curing agent, which should be mixed together in 10:1 proportions before use. It is used to coat patch pipettes for single channel recording. Coating patch pipettes with Sylgard® reduces stray capacitance by preventing creep of the bath solution up the tip of the pipette and by increasing the size of insulation between the bath and pipette solutions.
In general, a volume of 5ml was mixed together and left at room temperature for 2-3 hours. The mixture, which was usually thicker and free of air bubbles by this stage, was then aliquoted into 1ml eppendorf tubes and stored at -20°C. Prior to fire polishing, Sylgard® was applied to the tapered end of the patch pipettes using a fine syringe needle, and cured using a heated coil. Patch pipettes were then fire-polished to give resistances of 8-10MΩ.

2.4.8. Pipette diameter and resistance

Occasionally, the pipette size after pulling, or after fire polishing, was checked using a simple procedure to measure the ‘bubble number’ (see for example Standen & Stanfield, 1992). This involved connecting the back of an electrode to a polythene tube connected to a 10ml syringe with the plunger withdrawn to the 10ml mark. The tip of the pipette was dipped into methanol, contained in a vial with a dark background, and the plunger gently depressed until a stream of bubbles originating from the tip was observed. The volume at which this occurred was the ‘bubble number’. For whole cell electrodes the ideal bubble number is 6-7ml before and 5ml after fire polishing, whilst for single channel electrodes a bubble number between 4-6ml before and 2-4ml after fire polishing was desirable.

The pipette resistance was often calculated once the pipette had been inserted into the bath solution prior to seal formation. At this point, pipettes with inappropriate resistances were discarded. To calculate pipette resistance, the gain of the patch clamp amplifier was set on 10mV/pA and a 1mV command voltage of 10ms duration was applied to the amplifier to generate a current through the pipette. Pipette resistances were subsequently calculated from the size of the current trace in response to the 1mV command voltage using Ohm’s Law:

\[ V = IR \]  

(Equation 2.8)

where \( V \) is voltage, \( I \) is current and \( R \) is resistance.

2.4.9. Patch clamp amplifier

Although initial channel recordings were obtained using an EPC-7 (List Electronics) patch clamp amplifier, the majority of channel currents included in this thesis were recorded using an Axopatch 200B patch clamp amplifier (Axon Instruments). The headstage of the Axopatch 200B implements the conversion of current to voltage by way of either a ‘resistive-’ or ‘capacitive-feedback’ circuit depending on which mode (whole cell or patch, respectively) has been selected.
For whole-cell recording, the Axopatch 200B uses a high resistance-feedback circuit (figure 2.3) consisting of an operational amplifier (A1) with high input impedance, a feedback resistor ($R_f$, typically 50 or 500MΩ) and a differential amplifier (A2). The negative feedback circuit from the output of the amplifier is used to force the voltage in the patch pipette ($V_p$), detected at the inverting (-) input, to follow the command voltage ($V_{cmd}$), applied to the non-inverting input (+). Owing to the high input impedance of the operational amplifier, the current source being measured ($i_p$) is forced to flow through the feedback resistor ($R_f$). The amplifier output ($V_o$) is therefore proportional to the input current as follows:

$$-i_p = \left( \frac{V_o - V_p}{R_f} \right)$$

(Equation 2.9)

since $V_p = V_{cmd}$ then,

$$-i_p = \left( \frac{V_o - V_{cmd}}{R_f} \right)$$

(Equation 2.10)

when rearranged this gives

$$V_o = -i_p R_f + V_{cmd}$$

(Equation 2.11)

The current flow through the patch pipette is subsequently given by the output of the differential amplifier (A2), which measures the difference between the output ($V_o$) of the operational amplifier (A1) and the command voltage ($V_{cmd}$). The output from the differential amplifier is then fed into a high frequency boost circuit for correction of stray capacitance.

For single channel recording the Axopatch 200B utilises a capacitive feedback circuit (figure 2.4). Also known as the integrating headstage, this uses a small capacitor ($C_f$) in the feedback path of the current-voltage converter. In response to a constant input current, the headstage generates a ramp, the slope of which is proportional to the current (Finkel, 1991). The slope of the ramp represents the rate of charging of the voltage across the capacitor and is measured by a differentiator to give the current in the patch pipette ($i_p$).
**Figure 2.3.**

*Diagram of the resistive headstage.* The current-to-voltage converter consists of an operational amplifier, $A_1$, and its feedback resistor, $R_f$. (-) and (+) represent the inverting and non-inverting inputs at which the pipette potential ($V_p$) and command potential ($V_{cmd}$) are detected, respectively. $C_f$ is the stray capacitance across $R_f$. The differential amplifier, $A_2$, measures the difference between the output ($V_o$) of $A_1$ and $V_{cmd}$ to give the current being measured in the patch pipette ($i_p$).
Figure 2.4.

Diagram of the capacitive headstage. The current-to-voltage converter utilises a small capacitor, $C_f$, in the feedback bath. The output of amplifier A1, $V_o$ is a ramp whose slope is proportional to the current in the pipette ($i_p$). A second capacitor, $C_d$, resistor, $R_d$ and the amplifier A2 form the differentiator circuit, the output of which is proportional to the pipette current.
The output of the integrating headstage, \((V_o)\) is related to the input current according to the relation:

\[
-i_p = C_f \frac{dV_o}{dt}
\]  

(Equation 2.12)

so that the \(V_o\) is the time integral of \(i_p\) as described by:

\[
V_o(t) = -\frac{1}{C_f} \int_0^t i_p \, dt
\]  

(Equation 2.13)

Correction of the frequency response is achieved by way of a differentiator circuit formed by a capacitor \((C_d)\), resistor \((R_d)\) and amplifier \((A2)\). The final output voltage of the circuit is given by:

\[
V_o = R_d \frac{C_d}{C_f} i_p
\]  

(Equation 2.14)

and is proportional to the current measured in the pipette.

2.4.10. Procedure for obtaining a gigaohm seal

The first step when patch clamping is to obtain a high resistance or 'gigaohm' seal (section 2.4.2). In their 1981 paper, Hamill et al. (1981) described several procedures, which increased the probability of obtaining a gigaohm seal. These included precautions to keep the pipette and cell surfaces clean and the application of gentle suction to the inside of the pipette once it was in contact with the cell. By filtering all solutions as described in section 2.4.5 and applying gentle positive pressure to the pipette before immersing its tip in the bath, it is possible to keep the tip of the pipette free of debris. The resuspension of the cultured cells in serum free media or filtered extracellular solution also aids the formation of a giga-seal. Finally, once the pipette is touching the surface of the cell, gentle suction may be applied to the inside of the pipette using a syringe attached to an outlet on the pipette holder by way of a length of polythene tubing. In general, if the above conditions were adhered to, gigaohm seals could be obtained as described below.

The patch pipette was back-filled with intracellular solution and inserted into the pipette holder so that the tip of the chlorided silver wire was in contact with the solution. The patch pipette and its holder were then inserted into the input connector of the patch clamp.
headstage. To prevent damage to the headstage from static electricity, a grounded metal object, such as the Faraday cage, was touched prior to handling the headstage. Following application of slight positive pressure, the pipette was lowered into the bath solution using the coarse controls of the manipulator. At this point, any offset potentials ($V_o$) between the pipette and reference electrodes were cancelled by adjusting the 'pipette offset' control. The amplifier adds this variable offset to the command voltage ($V_{cmd}$) so that the current flow at $V_{cmd}$ is zero. However, the situation is complicated by the existence of junction potentials (see section 2.4.15) during the reference measurement, which disappear on going whole cell. With the gain of the patch clamp amplifier set on 10mV/pA, a 1mV command voltage of 10ms duration was applied to the amplifier to generate a current through the pipette. At this point, the pipette resistance was calculated (see section 2.4.8).

The formation of a gigaohm seal represents a sudden increase in seal resistance and this process may be closely monitored, by observing the size of the pulse on the oscilloscope screen. According to Ohm’s Law, an increase in resistance leads to a reduction in current. Gigaohm seal formation is therefore reflected by a rapid decrease in the size of the visible current pulse, which eventually disappears. By using a combination of the coarse and fine movements of the manipulator, the pipette was brought close to the cell to be patched. Once the pipette and cell membrane were touching, gentle suction was applied to the pipette. A negative voltage (-17mV) was applied to the interior of the pipette to encourage the formation of a gigaohm seal.

2.4.11. Capacity transients

Once a gigaohm seal had been achieved, the command voltage was increased to 10mV and the capacity transients associated with pipette capacitance ($C_p$) minimized using the pipette capacitance compensation controls. This is necessary because, for resistive headstages, even small voltage steps can lead to saturation of the voltage output during charging of the fast capacity transient. For larger voltage steps, saturation can persist for a millisecond, or more, during which time information on, for example, fast-activating currents may be lost. Cancellation of pipette capacity transients is achieved by injection of a current, equal in size to the charging current, by way of a capacitor internally connected to the input.

2.4.12. Recording configurations

Cell-attached patches were obtained by following the protocol for seal formation as described above. Although the cell remains intact, the cell-attached configuration may be used to record
single channel activity. One problem with the configuration, however, is that the cytoplasmic side of the membrane cannot be easily manipulated. Another problem associated with the cell-attached configuration is that the membrane potential is unknown. For these reasons, excised patches may be the preferred method for obtaining single channel recordings. Furthermore, by positioning the patch pipette just below the surface of solution interface, it is possible to reduce capacitance with the excised patch configurations. However, it has been documented that channel properties can change with patch excision (Trautmann & Siegelbaum, 1983; Covarrubias & Steinbach, 1990). One advantage with the cell-attached configuration is, that the biological environment of the channel remains unperturbed. As illustrated in figure 2.5, the cell-attached patch configuration is the precursor to the other patch-clamp configurations described below.

*Inside-out patches* were formed by pulling the patch pipette away from the cell, whilst in the cell-attached mode (figure 2.5). In this configuration, the membrane patch is detached from the cell with the cytoplasmic membrane surface facing the bath solution. It is, therefore, possible to change the cytosolic environment. The major problem with this configuration, and of particular relevance to inward rectifier potassium channels, is the loss of cytosolic factors involved in normal channel behaviour. Furthermore, on excising a patch the membrane often 'seals over' to form a vesicle. ‘Vesicle formation’, which was evident as a reduction in the amplitude of the single channel openings and a 'rounding' in their appearance, could occasionally be rectified on brief exposure to air by gently moving the patch through the air-solution interface, although, more often than not, this resulted in loss of the patch. In the inside-out patch configuration, positive command voltages correspond to a hyperpolarization of the patch membrane. Pipette capacitance and background noise was reduced following movement of the patch pipette near the surface of the bath solution.

The *whole-cell configuration*, as its name implies, permits the recording of ionic currents associated with the entire cell membrane. It was achieved by applying suction through the pipette following gigaohm seal formation. This resulted in the rupture of the membrane and the subsequent exposure of the cell contents to the solution inside the electrode. Rupture of the membrane was indicated by the sudden appearance of large capacity transients at the leading and trailing edges of the voltage pulse, which were minimized by adjusting the ‘whole cell capacitance’ and ‘series resistance’ controls simultaneously as described in section 2.4.13. For large currents, series resistance compensation was also used as described in section 2.4.14.
Figure 2.5.

Diagram illustrating the different configurations of patch-clamping. Once a gigaohm seal has been formed it is possible to use one of four different recording configurations. The cell-attached mode permits the recording of single-channels. However, disadvantages with this mode make the inside-out configuration more favourable for recording single-channels. The whole-cell configuration permits the recording of currents associated with the entire cell membrane. The outside-out patch, achieved by pulling the pipette away from the cell in whole-cell mode, may be advantageous if whole-cell currents are very large.
Outside-out patches were formed by slowly pulling the patch pipette away from the cell whilst in whole-cell configuration (figure 2.5) so that the outside of the membrane remains exposed to the bath solution. As with inside-out patches, the pipette was raised to just below the solution interface to reduce capacitance. Pipette capacitance was adjusted accordingly.

2.4.13. Whole cell capacitance

As described in section 2.4.12, a sudden increase in the size of the capacity transients was visualized on going whole-cell. Since any stray capacitance associated with the pipette was cancelled prior to rupturing the membrane (section 2.4.11), the remaining capacity transients could be attributed to the cell. As we saw in section 2.4.1, the total membrane current ($I_m$) has two components, one ionic ($I_i$) and the other capacitive ($I_c$). During patch clamp, the membrane potential is changed from the holding potential to the desired voltage as rapidly as possible. Thus, any capacity current is restricted to the edges of the square pulse. The speed at which the membrane potential is changed is dependent on two parameters, the membrane capacitance ($C_m$) of the cell and the series resistance ($R_s$), a resistance that exists between the cell interior and the pipette. For membrane resistances that are large in comparison with $R_s$, the time constant for charging the cell is

$$\tau = C_m R_s$$

(Equation 2.15)

The currents associated with charging the cell capacitance can be compensated using the ‘whole cell capacitance’ and ‘series resistance’ controls on the amplifier, from which it is possible to estimate values for $C_m$ and $R_s$, respectively. Occasionally, the duration of these capacity transients appeared quite long and on attempting to compensate, series resistance values were extremely high. In general, this was indicative of bad access to the cell. However, on applying additional suction access could usually be improved, indicated by an increase in the amplitude and a decrease in the duration of the capacity transients and a subsequently lower series resistance.

2.4.14. Series resistance compensation

One possible source of error during patch clamp arises as a result of the series resistance ($R_s$), which affects the accuracy with which the membrane potential can be controlled. When a current, $I$, flows across the membrane a voltage error, of size $V = IR_s$, will arise leading to a discrepancy between the actual membrane potential and the potential measured by the amplifier. Such discrepancies will be larger for larger currents or series resistances. In
studies of channel selectivity, such voltage errors have little consequence since we are interested in measuring the reversal potential of the current, at which there is no net flow of current and therefore, no voltage error. However, series resistance errors do have serious implications for the measurement of channel properties that are voltage dependent, such as block by Cs\(^+\) or Ba\(^{2+}\) and channel activation.

In CHO cells, inward rectifier currents were often in excess of 5nA. For a current of 5nA and with a typical series resistance of 5M\(\Omega\), a voltage error of 25mV will exist. Evidently, the currents activated by the command voltage will not reflect the ‘true’ currents that would be activated by the actual membrane potential. However, voltage errors due to current flow over \(R_s\) can be compensated using the amplifier series resistance cancellation circuitry, which works by adding a percentage of the voltage error to the command voltage by way of a feedback circuit. Although in theory 100% compensation is possible, in practice, only 50-80% was achieved due to the occurrence of oscillations at higher values. Thus, for a 5nA current and 5M\(\Omega\) series resistance, a voltage error of 7.5mV would still remain following 70% compensation.

2.4.15. Junction potentials

During patch clamp recording a liquid junction potential will arise at the tip of the patch pipette when solutions of different ionic composition are used to fill the pipette and bath. Liquid junction potentials are the result of an imbalance in charge, which develops as a consequence of the different rates at which ions diffuse across the concentration gradient at the interface between two solutions. This occurs because each individual ion has a different mobility, the more mobile an ion the faster it diffuses.

Using the pipette offset dial on the patch clamp amplifier it is possible to compensate for the junction potential \((V_L)\) that develops at the interface between the internal and external solutions, when the two solutions are of different ionic composition. The amplifier generates an equal and opposite potential \((V_L')\) when the pipette offset is used to zero the trace, which is added to \(V_{cmd}\) (Figure 2.6A).
**Liquid junction potentials.** A, Prior to sealing the liquid junction potential \(V_L\) that arises at the tip of the pipette is balanced by an equal and opposite 'offset potential' \(V_{L}^{1}\) using the pipette compensation on the amplifier. B, On sealing, \(V_L\) disappears, being replaced by \(V_m\) because the membrane forms a barrier between the pipette and bath solutions. The offset potential, \(V_{L}^{1}\), still exists. C, Following membrane-rupture, a new junction potential forms but disappears with dialysis of the cell contents by the pipette solution. \(V_{L}^{1}\) remains. The curly arrow represents the direction in which the circuit is traversed.
However on sealing, the junction potential between the two solutions disappears because the membrane patch forms a barrier between the pipette and the bath solutions. However, the junction potential correction applied by way of the amplifier still exists (figure 2.6B). Thus, $V_m$ becomes

$$V_m = (E_m - V_{cmd}) + V_L^1 \tag{Equation 2.16}$$

On going whole-cell, a new junction potential arises between the intracellular solution and the internal contents of the cell, but disappears on effective dialysis of the cell contents by the pipette solution (figure 2.6C). $V_m$ is then

$$V_m = V_{cmd} - V_L^1 \tag{Equation 2.17}$$

Liquid junction potentials were measured experimentally using a 3M KCl electrode as the reference electrode, which was constructed from thin walled borosilicate glass and filled with 3M KCl containing 0.5% agar. Patch pipettes were pulled as previously described and back-filled with intracellular solution. With the patch clamp amplifier in current clamp mode, both the patch pipette and reference electrodes were dipped into a petri dish containing intracellular solution. With the amplifier in ‘track’ mode, the voltage reading, or tip potential, was adjusted to zero using the pipette offset. The bath solution was then exchanged for extracellular solution. The resulting junction potential between the pipette and the bath was measured as the voltage difference between the two solutions and was noted from the LED display once the reading had stabilized.

Alternatively, the magnitude of junction potentials can be determined by calculating them using the generalized Henderson Liquid Junction Potential Equation (Barry & Lynch, 1991). For $N$ polyvalent ions, the junction potential ($V_L$) of the extracellular solution ($S$) with respect to the pipette solution ($P$), is given by:

$$V^S - V^P = \frac{RT}{F} S F \ln \left\{ \frac{\sum_{i=1}^{N} z_i^2 u_i \alpha_i^P}{\sum_{i=1}^{N} z_i^2 u_i \alpha_i^S} \right\} \tag{Equation 2.18}$$
where

\[
S_p = \frac{\sum_{i=1}^{N} \left( z_i u_i (a_i^* - a_i^p) \right)}{\sum_{i=1}^{N} z_i^2 u_i (a_i^* - a_i^p)} \tag{Equation 2.19}
\]

where \( u, a \) and \( z \) represent the mobility, activity and valency of each ion species \( (i) \), respectively, and where \( R, T \) and \( F \) have their usual meanings. In this study, the molar concentration of the ion species was used in place of its activity in junction potential calculations.

Using the flow system, the bathing solution was exchanged several times throughout the course of many of the experiments described within this study. This would have led to additional junction potentials arising between the solution immediately in contact with the cell and the original bath solution. Since the solution in the recording dish was not continuously renewed, the ionic composition of the bath solution would have changed every time a cell was perfused with a different solution. The composition of the bath solution against which the pipette was nulled was therefore unknown, as was the junction potential correction, and for this reason, no attempt was made to correct for any errors arising from liquid junction potentials in this study.

2.4.16. Data acquisition

Whole-cell and single-channel data were initially filtered at 5 and 2kHz respectively using the amplifier’s built-in 4-pole lowpass Bessel filter. Single channel data were filtered further when sampling sections of single channel recordings from D.A.T. to the computer hard drive using TAPE. Analogue Bessel filters and digital Gaussian filters add together to give a final bandwidth, \( f_c \), according to the relationship:

\[
\frac{1}{f_c} = \sqrt{\frac{1}{f_1^2} + \frac{1}{f_2^2} + \frac{1}{f_n^2}} \tag{Equation 2.20}
\]

Thus, to give a final bandwidth of 1kHz, single channel currents were digitally filtered at 1.15kHz.
Whole-cell and single-channel data were stored directly onto the computer hard disc or digital audio tape (D.A.T), respectively. However, this requires the output of the amplifier ($V_o$), which is an analogue signal, to be converted into a digital signal. This analogue-to-digital conversion (ADC) was achieved by way of an external interface, which was connected to both the amplifier and the computer (see section 2.4.3). The interface also converts the digital command voltage, generated by the computer, to an analogue signal for the amplifier (Digital-to-analogue conversion, DAC). Data were monitored on a cathode ray oscilloscope (CRO), which was connected to the amplifier and interface so that the display was synchronized with the triggering of the voltage command.

During digitisation the amplitude of the signal is measured at intervals determined by the sampling frequency, and stored as binary numbers. Data were sampled at 10kHz in accordance with the Nyquist Sampling Theorem, which states that the minimum sampling rate is twice the signal bandwidth (Ogden, 1994).

### 2.4.17. Data analysis

Electrophysiological data was acquired and analysed using AXGOX, a suite of programs written in Microsoft V.7.1.BASIC (Davies, 1993). STIMTOR was used to generate voltage commands, ramps and complex voltage sequences whilst data were manipulated and analysed in TRACAN. Selected sections of continuous, single channel recordings were transferred from D.A.T tapes to the computer hard disc by way of TAPE. Event detection was performed in TRACAN, then analysed and fitted in EVTANR. General data manipulation was undertaken using Microsoft® Excel spreadsheet, statistical analysis was performed using GraphPad Instat and graphs were created in Jandel SigmaPlot®.

For whole-cell data, preliminary analysis involved minimizing the capacity transients since a residual transient often remained following analogue capacitance compensation (section 2.4.13). This involved ‘back-subtracting’, digitally, the capacity transient at the end of the voltage pulse from the capacity transient at the beginning of the voltage pulse. In theory, this should result in the removal of any residual capacity transient. However, because the magnitude of the capacity transients was frequently asymmetrical, back-subtraction of the capacity transients was not always appropriate.

Although whole-cell current records with obvious leak components were not used for further analysis, in some experiments such as the measurement of Rb⁺/K⁺ permeability ratios in
channels exhibiting small currents, the use of records with leak components was unavoidable. Under such circumstances, leak subtraction was performed by subtracting a linear fraction obtained by fitting currents greater than 60mV and at $E_K$. This procedure assumes that the $K^+$ conductance is zero at these potentials. Further methods of analysis for whole-cell data are explained later in the relevant results chapters.

For single channel data, preliminary analysis involved producing an average leak current. Sections of continuous recording in the closed state were selected and averaged then subtracted from the file to give a zero current level for the current when in the closed state. More detailed analysis to determine the unitary current, mean open times (MOT) and probability of opening ($P_{\text{open}}$) were then carried out as described below.

Unitary current – where possible, the size of the unitary current for $K_{ir}$ channels was measured by constructing amplitude histograms from continuous recordings containing both open and closed events. Peaks corresponding to the channel closed and open levels were obtained, and fitted with gaussian distributions to give a mean channel opening. However, for single channels obtained in response to episodic stimulation, current levels were measured using cursors and a number of values averaged.

$P_{\text{open}}$ - The probability of opening was determined by measuring the times ($t_j$) spent at current levels corresponding to $j = 0, 1, 2...N$ open channels. The overall $P_{\text{open}}$ was given by:

$$P_{\text{open}} = \frac{\sum_{j=1}^{N} t_j}{TN} \quad \text{(Equation 2.21)}$$

where $T$ is the duration of the recording and $N$, which is the maximum number of channels in the patch, was set as the maximum number of simultaneous openings observed during one recording.

MOT - Mean open times were analysed using the half-amplitude threshold crossing method described by Colquhoun and Sigworth (1983) in which an event is detected when it crosses the 50% threshold between open and closed levels of the channel. Events shorter than 0.18ms were not detected since:

$$\text{deadtime} = \frac{0.179}{f_c} \quad \text{(Equation 2.22)}$$
and single channel currents filtered at an overall bandwidth of 1kHz. Events were written to an idealized record from which open- and closed-time histograms were constructed. These distributions were plotted with log time on the abscissa and the square root of occurrence on the ordinate labelled events/bin. Plotting the data as described allows exponential components with time constants varying by many orders of magnitude to be displayed on the same graph. The individual exponential components appear as peaks, which correspond to the time constant of each component, the height of the peak corresponding to the number of events for each component. The data were fit to an exponential distribution of the form:

$$f(t) = \sum_{j=1}^{n} \left( \frac{a_j}{\tau_j} \right) \exp\left(-\frac{t}{\tau_j}\right)$$  \hspace{1cm} (Equation 2.23)

where $a_j$ and $\tau_j$ are the relative area and the time constant of component $j$. The number of components, $n$, was set to the number of open or closed states and the data fit using the method of maximum likelihood (Colquhoun & Sigworth, 1983).

Mean open times were corrected for missed events (i.e short closures less than the minimum resolution, which were too brief to be detected) by multiplying the mean open time by the proportion of closed events detected, which was found by integrating the fitted closed time distribution between the minimum resolution and infinity (see Davies et al., 1992).

### 2.5. Detection methods for protein expression

Numerous methods for the detection of protein expression now exist. However, each method has its limitations. For example, whilst Western blotting is useful for determining whether expression of a certain type of protein occurs in a particular cell type or tissue, it cannot be used to determine the cellular origin of that protein (Coyler, 1999). Furthermore, immunological techniques such as Western blotting require the use of an antibody specific to the protein being investigated. In this study, two different techniques have been employed to determine the expression of wild-type and mutant channels as described below.

#### 2.5.1. Western blotting

First described by Towbin et al. (1979), Western blotting couples the separation of proteins by polyacrylamide gel electrophoresis, protein transfer to nitrocellulose sheets and subsequent binding assays to give a method, which ultimately allows the detection of proteins by autoradiography. The electrophoretic step separates the proteins according to their apparent
molecular weight whilst an exact copy of the electrophoretic gel is transferred onto the nitrocellulose membrane. The proteins are then probed with a primary antibody followed by an additional ‘detecting protein’, such as a secondary antibody, which is conjugated to a marker such as the marker enzyme horseradish peroxidase (HRP), which can be detected using an enhanced Chemi-luminescence kit. Hames (1990), Appelbaum and Shatzman (1999) and Colyer (1999) have provided excellent descriptions of Western blotting.

2.5.1a. Sample preparation

CHO cells were transfected using FuGENE™ transfection reagent as described in section 2.3.3. Forty-eight hours after transfection, the cells were collected following removal using TE as described in section 2.2.5c. At this point, cells were incubated for 30 minutes in either 10mM dithiothreitol (DTT) or PBS as appropriate, following which they were centrifuged and washed twice as described previously. 400μl homogenizing buffer (NaCl, 150mM; EDTA, 1mM; EGTA, 1mM; NEM, 20mM; Tris pH 7.4, 10; 1% Triton-X-100 [Iso-octylphenoxypolyethoxyethanol]; plus the addition of 5μl protease inhibitor cocktail) was added to each sample of cells and left on ice for 5 minutes. The samples were then triturated using a 1ml Gilson tip and aliquotted in 12.5μl samples into 1ml Eppendorf tubes and stored at -20°C until required for gel electrophoresis.

2.5.1b. Gel electrophoresis

The separation of proteins according to their molecular weight was performed using Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), using the Mini-Protean II electrophoresis system (Bio-Rad). To ensure good gel adhesion, the glass electrophoresis plates and spacers were washed prior to assembly with 70% ethanol, and allowed to air dry. The glass-plate ‘sandwich’, consisting of one large plate, one small plate and two spacers of uniform thickness, was then assembled using the gel cast apparatus. The resolving gel (acrylamide, 10%; Glycine, 213mM; Tris base, 27.5mM; SDS, 3.9mM; ammonium persulphate solution, 20μl of 10%; TEMED [N, N', N'-tetramethylethylenediamine], 20μl) was then prepared and poured between the plates, taking care to avoid air bubble formation, to a previously marked level 5cm high. Acrylamide is highly toxic, therefore disposable plastic gloves were worn at all times when handling gel mixtures in either liquid or solid form. The ammonium persulphate solution is unstable, it was therefore made fresh just before use by dissolving 0.1g ammonium persulphate in 1ml distilled water. The ammonium persulphate solution and TEMED were added just before pouring the gel mixture,
polymerisation of acrylamide being initiated by the addition of ammonium persulphate and accelerated by addition of TEMED (Hames, 1990). The resolving gel was immediately overlaid with 0.1% SDS and set aside for 20-30 minutes to allow for acrylamide polymerisation. Once polymerisation had occurred, an interface between the SDS and gel layers was clearly visible. At this point, the apparatus was tilted to discard the SDS layer and the gel rinsed with distilled water, any excess water being blotted from the gel surface using filter paper.

The stacking gel (acrylamide, 4%) was then prepared and layered on top of the resolving gel. A ten-well comb was immediately inserted into the stacking gel mixture, taking care not to trap any air bubbles beneath the comb. The stacking gel was then topped up with the remaining gel mixture and left to polymerise for 30-40 minutes.

Meanwhile, the protein samples were defrosted on ice, then mixed 1:1 with 2× laemmli denaturing sample buffer (Tris pH 6.8, 125mM; glycerol, 20% v/v; SDS, 4% w/v; bromophenol blue, 0.004% w/v) without DTT and heated at 100°C for 5 minutes. The protein samples and pre-stained markers were then centrifuged at 13,000rpm for approximately 4 minutes.

Once the stacking gel was set, the comb was carefully removed and the wells rinsed with distilled water. The glass plates were then removed from the setting frame, clipped into the reservoir frame and lowered into the electrophoresis tank. The inner and outer reservoirs of the tank were filled with running buffer (mM: glycine, 213; Tris base, 27.5; SDS, 3.9) so that the inner lower plate and the bottom of the outer plates were covered with buffer. Finally, the protein standards, pre-stained markers and samples were loaded into the appropriate wells and the gel run for approximately 1.5 hours at 200 V.

2.5.1c. Transfer of proteins

The transfer of proteins from the SDS-PAGE gel was performed by 'semi-dry electrotransfer' (for review see Colyer, 1999). On completion of electrophoresis, the SDS-PAGE gel was carefully removed from one of the glass electrophoresis plates. Using a scalpel blade, the stacking gel was removed and the upper left hand corner of the resolving gel marked to aid orientation. The gel was gently eased off the remaining glass plate into a plastic storage box containing transfer buffer (Tris base, 48mM; glycine, 39mM; SDS, 1.3mM; methanol, 20%) and left to soak for approximately 10-20 minutes.
Meanwhile, four pieces of blotting paper and one piece of nitrocellulose paper, cut to the same size as the gel, were also soaked in transfer buffer. Next, one piece of blotting paper was removed from the transfer buffer, drained and placed on the anode plate of a semi-dry electrophoretic transfer cell (Bio-Rad), being careful to remove any air bubbles using a glass rod. Using the same procedure, a second piece of blotting paper followed by the nitrocellulose membrane (Schleicher & Schuell) was superimposed upon the first piece of blotting paper. The SDS-PAGE gel was then carefully drained and positioned on top of the nitrocellulose membrane such that the first lane would transfer to the left-hand side of the membrane (i.e. marked corner to left). The stack was completed, by placing the remaining two pieces of blotting paper on top of the gel. Finally, the cathode plate was placed on top of the transfer stack and the proteins transferred for 30 minutes at 15 Volts.

The transfer of the pre-stained markers was checked after 30 minutes by carefully lifting the top layers of blotting paper and the gel away from the nitrocellulose membrane. Before discarding the gel, the nitrocellulose membrane was cut around the outline of the gel and the top left hand corner of the membrane marked.

2.5.1d. Western blotting procedure

Following overnight incubation of the nitrocellulose membrane in blocking buffer at 4°C, the membrane was washed three times for 5 minutes using Tris Buffered Saline (TBS; mM: Tris base, 50; NaCl, 150) containing 0.2% v/v Tween 20. The membrane was then incubated in primary antibody\(^1\) for at least one hour. The primary antibody was removed and retained for future use (primary antibody may be retained for up to 3 weeks after initial use) and the membrane washed a further three times for 10 minutes each wash with TBS containing 0.2% Tween 20. The membrane was then incubated in a peroxidase-conjugated secondary antibody for 1 hour followed by five washes with TBS for 10 minutes each wash. A plate shaker was used throughout the above procedures to ensure the even washing and incubation with antibody of the entire membrane.

2.5.1e. Protein detection

Protein bands were detected using the ECL Plus™ detection system (Amersham Pharmacia Biotech) according to the manufacturer instructions. The reagents were removed from the

\(^1\) Primary antibody was obtained from Dr R Norman, Dept. Medicine, University of Leicester.
fridge and allowed to equilibrate to room temperature. The solutions were mixed and poured onto the surface of a clean electrophoresis glass plate. The nitrocellulose membrane was removed from the buffer, drained of any excess solution and placed protein side down on the glass plate for 5 minutes. The nitrocellulose membrane was drained of any excess detection reagent and wrapped in cling film, being careful to remove any air bubbles. The wrapped blot was then placed, protein side up, in an x-ray film cassette. The cassette was then taken to a dark room where, under red safe lights, a sheet of autoradiography film (Hyperfilm™, Amersham) was placed on top of the membrane, the cassette closed and the film exposed for approximately 12 seconds. The film was then developed using an Amersham Hyperdeveloper. The exposure time for further pieces of film was altered according to the appearance of the preceding exposures.

2.5.2. In vitro transcription translation

Over the last decade, the synthesis of proteins in vitro has become a popular tool in research. Today, in vitro systems utilising either DNA or RNA as initial templates are readily available from commercial suppliers (Clemens, 1999). These ‘cell-free synthesizing systems’ may, for example, be used in the characterization of plasmid clones and the study of structural mutations. In this study, a ‘coupled transcription-translation system’ has been used in an attempt to determine the effects of mutations I143C and I143S in Kir2.1 on channel structure, with particular emphasis on the formation of disulphide bonds. This system combines DNA transcription and mRNA translation to give a protein product. The proteins synthesized during coupled transcription-translation were then analysed by SDS-PAGE followed by autoradiography.

It is possible that disulphide formation in Kir2.1 mutants I143C and I143S occurs after translation. Canine pancreatic microsomal membranes were therefore included in some of the protein synthesis reactions described in this study. The use of microsomal membranes, together with coupled transcription-translation, facilitates the study of cotranslational and early post-translation events.

2.5.2a. The coupled transcription-translation assay

In vitro transcription-translation was carried out using the TNT® (T7) coupled reticulocyte lysate system (Promega Corporation) according to the manufacturer instructions. The reagents were removed from storage at -70°C and immediately placed on ice. For reactions to
be carried out in the absence of canine pancreatic microsomal membranes, the following components were added, step by step, to previously labelled 0.5ml micro-centrifuge tubes: 25μl of rabbit reticulocyte lysate, 2μl reaction buffer, 1μl amino acid mixture minus methionine, 1μl ribonuclease inhibitor, 1μl RNA polymerase, 4μl (~40μCi) L-[\textsuperscript{35}S]methionine (specific activity > 1000Ci/mmol; Amersham Pharmacia Biotech), 2μl DNA (0.5μg/μl) and 14μl nuclease-free water to give a final volume of 50μl per reaction. The reagents were gently mixed and the reactions incubated in a water bath at 30°C for 90 minutes.

For reactions to be carried out in the presence of microsomal membranes the following components were added, step by step, to a previously labelled 0.5ml micro-centrifuge tube: 12.5μl lysate, 0.5μl reaction buffer, 0.5μl amino acid mixture minus methionine, 0.5μl ribonuclease inhibitor, 0.5μl RNA polymerase, 1μl (~10μCi) L-[\textsuperscript{35}S]methionine, 1μl DNA and 2 canine microsomal membranes and nuclease-free water to give a final volume of 25μl per reaction. Oxidised glutathione (GSSG) was substituted for water at concentrations of 0.5 and 1mM. Reactions were incubated as described above.

2.5.2b. Gel electrophoresis

The proteins synthesized during coupled transcription-translation were separated by SDS-PAGE using a Hoefer Scientific gel electrophoresis kit. Prior to assembly, the glass plates were cleaned with detergent and swabbed with 70% IMS. With spacers of uniform thickness at each vertical edge, the apparatus was assembled so that the glass plates were level. In initial experiments, an 8% polyacrylamide gel (0.1% w/v SDS; Tris pH 8.3, 100mM; Bicine, 100mM; ammonium persulphate, 150μl of 10%; TEMED, 150μl) was prepared and poured, being careful to avoid bubble formation. Later experiments used 10% acrylamide gels (10% SDS; mM: tris, 100; Bicine, 100). A fifteen well comb was immediately inserted into the gel mixture, being careful not to trap any air bubbles under the comb, and the gel put aside to set.

Meanwhile, the assay samples were removed from the water bath and prepared for loading. For each translation reaction, 2.5μl of translated product was mixed with 20μl loading buffer (Tris pH 6.8, 50mM; Glycerol, 25% v/v; SDS, 6%, w/v; Bromophenol blue, 0.01% w/v). β-mercaptoethanol (β-ME) was added to samples to a final concentration of 40mM. Samples were run in the presence of a reducing agent. To denature the proteins, the samples were boiled for 2 minutes at 100°C. The protein samples were then centrifuged at 13,000rpm for approximately 4 minutes.
Once the gel was set, the comb was carefully removed and the wells rinsed with distilled water. The glass plates were clipped into the reservoir frame and lowered into the electrophoresis tank. The inner and outer reservoirs of the tank were filled with running buffer (Tris pH8.3, 100mM; Bicine, 100mM; SDS, 0.1% w/v; Glycerol, 25%) so that the inner lower plate and the bottom of the outer plates were covered with buffer. Finally, the protein standards, pre-stained markers and samples were loaded into the appropriate wells and the gel run at 15mA initially then overnight at 4mA.

2.5.2c. Analysis of translation products

Following overnight electrophoresis, the gel was removed from the tank and soaked in an organic scintillant (Amersham International’s Amplify™ reagent) for fluorographic enhancement of the signals. This dramatically increases the sensitivity of detection of $^{35}$S-labeled proteins by converting the emitted energy of the isotope to visible light, thus increasing the proportion of energy that may be detected by X-ray film.

Prior to film exposure, the gel was dried under vacuum. The gel was placed on a double layer of Whatmann® 3MM filter paper, which had been dampened with distilled water, wrapped in plastic wrap and placed in a conventional gel dryer at 80°C for 1-2 hours.

When dry, the gel was exposed on X-ray film (Hyperfilm™, Ammbersham) for 24 hours at -70°C. The film was then developed using an Amgersham Hyperdeveloper. The exposure time for further pieces of film was altered according to the appearance of the preceding exposures.
3.1. Introduction

Inwardly rectifying potassium channels are currently grouped into seven subfamilies based on similarities in amino acid sequences and physiological characteristics, such as their degree of rectification (see section 1.2.4; for reviews see Doupnik et al., 1995; Fakler & Ruppersburg, 1996; Nichols & Lopatin, 1997). The second of these subfamilies, known as the $K_{ir}2.0$ subfamily, comprises the ‘strong inward rectifier potassium channels’, characterized by their steep inwardly rectifying properties and time-dependent gating kinetics (see section 1.2.4b for detailed description; for review see Stanfield et al., 2002).
To date, four members of the Kir2.0 subfamily have been isolated from various rat, mouse, rabbit and human sources (see section 1.2.4b). These channels exhibit electrophysiological properties typical of native inward rectifiers including an asymmetrical $K^+$ conductance, a shift in activation potential in accordance with $V_m$ and $[K^+]_o$, dependence of conductance on the square root of $[K^+]_o$ and block by extracellular cations such as $Ba^{2+}$ and $Cs^+$ (see section 1.4.1; see for example Hodgkin & Horowicz, 1959; Hagiwara & Takahashi, 1974; Hagiwara & Yoshii, 1979; Leech & Stanfield, 1981).

The members of the Kir2.0 subfamily can be distinguished by their different unitary conductances (see section 1.2.4b). Kir2.2 displays the highest conductance at around 34pS-41pS in 140 mM symmetrical $K^+$ (Takahashi et al., 1995; Wible et al., 1995) followed by Kir2.1 at 22pS. Kir2.3 and Kir2.4 exhibit lower conductances between 11 and 15pS under similar recording conditions (Makhina et al., 1994; Morishige et al., 1994; Collins et al., 1996; Welling et al., 1997; Topert et al, 1998).

A voltage- and $[K^+]_o$-dependent block by intracellular polyamines and Mg$^{2+}$ has been shown to be the underlying mechanism for the strong rectification exhibited by these channels (see sections 1.4.2c & d). Several independent studies have demonstrated the structural determinants of 'strong' inward rectification to be two negatively charged residues, an aspartate (D) in M2 and a glutamate (E) in the C-terminus (see section 1.4.3; see also Stanfield et al., 1994b; Wible et al., 1994; Fakler et al., 1994; Yang et al., 1995a). Both residues are conserved throughout the Kir2.0 subfamily. Recently, an additional residue (E299 in Kir2.1) also conserved amongst the strong inward rectifiers has also been shown to be important in gating by polyamines (Kubo & Murata, 2001; see section 1.2.4d).

This chapter is entirely dedicated to the properties of the second member of this subfamily, Kir2.2. Although the electrophysiological properties of Kir2.2 have been characterized previously (Takahashi et al., 1994; Koyama et al., 1994), these studies utilized expression in Xenopus oocytes. Here, I look at expression of Kir2.2 in mammalian cell lines. The content of this first chapter is intended as a reference for the electrophysiological properties of a 'wild-type' channel before presenting my results from mutagenesis studies in the outer selectivity filter of Kir2.2 and 2.1 in subsequent chapters.
3.2. Methods

The electrophysiological properties of Kir2.2 were examined following expression of the Kir2.2 gene in either murine erythroleukaemia (MEL) cells or Chinese hamster ovary (CHO) cells as described in the methods chapter. Whole cell currents were recorded with 140mM K+ in the patch pipette and 70mM K+ in the bath. For experiments investigating the effect of external K+ concentration ([K+]o), the bath solution was exchanged for solutions containing 14, 35 and 140mM K+, respectively. For the block experiments, Ba2+ and Cs+ were added to the standard bath solution (70K/70NMDG) at the concentrations indicated in figure 3.6.

Single channel currents were recorded in the inside-out or cell-attached configurations (see section 2.4.12) from CHO cells that had been transiently transfected with cDNA encoding the channel 48-60 hours before recording (see sections 2.3.2 and 2.3.4). For single channel recording, the pipette and bath solutions contained 200mM and 140mM K+, respectively.

3.3. Results

3.3.1. Kir 2.2 expresses inward rectifier potassium channels in MEL and CHO cells

Figures 3.1A and B show representative whole cell recordings from MEL and CHO cells, respectively. Currents recorded from untransfected cells are illustrated in the left panel of the figures whilst the right panels illustrate currents recorded from cells transfected with Kir2.2 cDNA. Untransfected cells exhibited no significant inwardly rectifying K+ currents. In contrast, cells in which the Kir2.2 gene had been expressed displayed prominent inward-going currents, which averaged steady-state amplitudes of -3.82 ± 0.58 nA (n = 14; Vm = -132mV) in MEL and -2.97 ± 0.38 nA (n = 17; Vm = -132mV) in CHO cells. Very little current was seen at depolarizing potentials. At extreme hyperpolarized potentials, Kir2.2 currents exhibited a time-dependent decay, or ‘inactivation’, which varied between cells.

Mean current-voltage (I-V) relationships from untransfected (triangles) and transfected (circles) MEL and CHO cells are shown in figures 3.1C and D, respectively. Steady state currents were measured 30-45ms after the onset of the voltage step. As expected for a strong inward rectifier potassium channel, the steady-state I-V relationships are steep and approximately linear for membrane potentials more negative than the K+ equilibrium potential, \(E_K\) (-17mV).
Figure 3.1.

*Kr2.2 expresses inward rectifier K\(^+\) channels in MEL and CHO Cells.* A and B show representative current recordings, obtained with 70mM [K\(^+\)]_o, from MEL and CHO cells, respectively. Currents were recorded in response to 50ms voltage steps from a holding potential of -17mV, to test potentials ranging from +63 to -132mV, in 5mV decrements. Every third record is shown for clarity. The left panel of each figure shows currents recorded from untransfected cells whilst the right panels show currents recorded from cells transfected with Kr2.2 wild-type cDNA. C and D show steady-state current-voltage relationships. The symbols represent the mean ± SEM, where larger than the symbol, for n = 11 - 17 cells. *Circles*, transfected cells; *triangles*, untransfected cells.
The deviation from this linear relationship at potentials positive to $E_K$ clearly reflects the asymmetrical conductance exhibited by these channels.

Figure 3.2 illustrates the relationship between chord conductance and membrane potential for $K_i2.2$ wild-type currents expressed in CHO cells. The chord conductance was calculated from the $I$-$V$ relationships (Figure 3.1D) using:

$$g_K = \frac{I_K}{V - E_K} \quad \text{(Equation 3.1)}$$

where $g_K$ is the chord conductance to $K^+$ and $V - E_K$ is the driving force on $K^+$. Its relation to membrane potential is best fitted with a double Boltzmann expression of the form:

$$\frac{g_K}{g_{max}} = \frac{(1-b)}{1 + \exp \left( \frac{V - V_1}{k_1} \right)} + \frac{b}{1 + \exp \left( \frac{V - V_2}{k_2} \right)} \quad \text{(Equation 3.2)}$$

where $b$ was allowed to vary between 0 and 1. $V_1$ and $V_2$ (mV) give the voltages at which the relative conductance $g_K = 0.5$ and $k_1$ and $k_2$ (mV) are the factors effecting the steepness of the relationship. In control experiments, $V_1$ and $V_2$ were $-25.1 \pm 0.8$ and $-1.8 \pm 2.2$ mV, respectively. $k_1$ and $k_2$ were $4.6 \pm 0.2$ and $9.8 \pm 0.6$ mV, respectively. In $70\text{mM} [K^+]_o$, the maximum $g_K$ expressed was $31.2 \pm 3.8 \text{nS (n = 16; mean ± SEM)}$.

### 3.3.2. Dependence of rectification on $V_m$ and $[K^+]_o$

A characteristic feature of inwardly rectifying potassium channels is their dependence of rectification on $V_m$ and $[K^+]_o$. The selectivity of $K_i2.2$ wild-type currents for $K^+$ was investigated by measuring the zero current (reversal) potential in external solutions of varying $K^+$ concentration. The reversal potential was measured by fitting a fifth order polynomial function of the form:

$$f(x) = a + bx + cx^2 + dx^3 + ex^4 + fx^5 \quad \text{(Equation 3.3)}$$

to a small region of the $I$-$V$ relationship where the current intersects the abscissa ($I = 0$).

Figure 3.3A shows the effect of changing $[K^+]_o$ on $K_i2.2$ wild-type currents. As $[K^+]_o$ was lowered from $140\text{mM}$ to 70, 35 and 14mM, external $K^+$ being substituted by N-methyl-D-glucamine, the amplitude of inward currents decreased. Lowering $[K^+]_o$ also resulted in a
Figure 3.2. 

**Representative conductance-voltage relationship for $K_{ir2.2}$ wild-type.** Plot of the normalized chord conductance ($g'_{K}$) versus membrane potential for $K_{ir2.2}$ wild-type expressed in CHO cells. The chord conductance was calculated from the $I$-$V$ relationship using equation 3.1. Values were normalized to the maximum chord conductance and are plotted against $V_m$. The data are fit with equation 3.2, and the fit shown is to voltages ranging from +63 mV to −67 mV, where the conductance starts to decline owing to inactivation of the current at negative membrane potentials. $V_1 = -19.5$ mV, $V_2 = -0.7$ mV, $k_1 = 4.7$ mV, $k_2 = 11.4$ mV and $b = 0.3$. 
parallel shift in the \(I-V\) relationship, both the reversal and rectification potentials\(^1\) shifting (figure 3.3B, C) from \(-3.1 \pm 0.7\) (140mM; \(n = 12\)) to \(-16.5 \pm 0.6\) (70mM; \(n = 14\)), \(-31.1 \pm 2.1\) (35mM; \(n = 12\)) and \(-52.1 \pm 0.7\) (14mM; \(n = 12\)). With an internal \(K^+\) concentration (\([K^+]_i\)) of 140mM, these values are in close agreement with \(K^+\) equilibrium potentials \((E_K)\) predicted from the Nernst equation \((0, -17, -35\) and \(-58\text{mV}\) for 140, 70, 35 and 14mM \([K^+]_o,\) respectively). The \(I-V\) relationships (Figure 3.3C) clearly display the ‘cross-over’ phenomenon typical of inward rectifier \(K^+\) conductances (see section 1.4.1a), where an increase in \([K^+]_o\) increases the outward current rather than decrease it as one would predict from a simple change in \(E_K\).

The relationship between \([K^+]_o\) and reversal potential is shown in figure 3.4A. As expected for a \(K^+\) selective ion channel, the reversal potential depends on \([K^+]_o\) shifting \(49.7 \pm 0.7\) mV (mean \(\pm\) SEM, \(n = 12 - 14\)) for a tenfold change in \([K^+]_o\). This is in close agreement to values obtained in previous studies on native channels (49mV), though NaCl was used to substitute for external \(K^+\) (Sakmann & Trube, 1984a). Together, these results show that \(K_{ir}2.2\) is a \(K^+\) selective channel and that rectification depends on \(V_m\) and \([K^+]_o\).

3.3.3. The conductance of \(K_{ir}2.2\) depends on \([K^+]_o^{0.38}\)

Figure 3.4B clearly illustrates that as \([K^+]_o\) is lowered, the slope conductance of \(K_{ir}2.2\) wild-type currents, measured from the straight portion of the \(I-V\) relationship, decreases. The \(K^+\) conductance of inward rectifier \(K^+\) channels in skeletal muscle (Almers, 1971), starfish egg cell membranes (Hagiwara & Takahashi, 1974), ventricular cells (Sakmann & Trube, 1984a) and of cloned inward rectifier potassium channels (for example see Ho \textit{et al.}, 1993; Kubo \textit{et al.}, 1993a; Makhina \textit{et al.}, 1994) has been shown to depend, approximately, on the square root of the \([K^+]_o\) (see also section 1.4.1b). Figure 3.4B shows a double logarithmic plot of the slope conductance as a function of the \([K^+]_o\). The data points have been fitted with a linear regression of the form:

\[
G = A\left([K^+]_o\right)^x \quad \quad \text{(Equation 3.4)}
\]

where \(A\) is a constant and \(x\) represents the slope of the fit and has the value \(0.38 \pm 0.05\) \((n = 9)\), similar to the relationships for other inward rectifier potassium channels (0.46, Pérrier \textit{et al.}\(^1\))

\(^1\)The values quoted in the text were corrected for junction potentials predicted from the Junction Potential Programme ‘JPCalc’ copyright (c) 1992-1997 PH Barry. Figure 3.3B and C do not show this correction.
Figure 3.3.

*K*$_2$*β*₂.₂ wild-type currents are sensitive to changes in external K$^+$ concentration. *A*, representative current recordings obtained with external solutions of various K$^+$ concentrations as indicated in the figure. Currents were elicited in response to 50ms voltage steps, from a holding potential of −17mV to test potentials ranging from +63 to −132mV, in 5mV decrements. Every fifth record is shown for clarity. *B* shows steady-state current-voltage relationships for *K*$_2$*β*₂.₂ wild-type currents recorded in external solutions of various K$^+$ concentrations. Steady-state current amplitudes, normalized to the maximum current amplitude at -132mV with 70mM [K$^+$]$_o$, are plotted. The symbols represent the mean ± SEM, where larger than the symbol, for n = 12 - 14 cells. *Triangles*, 14mM [K$^+$]$_o$; *squares*, 35mM [K$^+$]$_o$; *circles*, 70mM [K$^+$]$_o$; *inverted triangles*, 140mM [K$^+$]$_o$. *C*, expansion of current-voltage relationships (shown in B) about the x-axis to show ‘cross-over’ phenomenon. Individual current-voltage relationships are colour coded for clarity. Symbols as in B.
Kir 2.2 wild-type currents are dependent on $V_m$ and $[K^+]_o$.  

**A.** Semilogarithmic plot of the external K+ concentration ([K$^+$]$_o$) versus membrane potential ($V_m$). The symbols represent the mean ± SEM, where larger than the symbol, for $n = 12$ cells. The data has been fitted with a linear regression (solid line), which has a slope of 48.6 mV per decade. The dashed line represents the fit to reversal potentials predicted from the Nernst equation with a slope of 58 mV per decade.

**B.** Double logarithmic plot of the external K+ concentration ([K$^+$]$_o$) versus slope conductance ($g'_K$). Slope conductance was measured in the linear portion of the I-V curves at potentials 20 mV, and more negative, to the predicted potassium equilibrium potential ($E_K$). The symbols represent the mean ± SEM, where larger than the symbol, for $n = 9$ cells. The slope of the regression line fitting the data points is 0.39.
al., 1992; 0.47, Kubo et al., 1993a; 0.33, Makhina et al., 1994). Thus, K\textsubscript{r2.2} wild-type channel conductance is approximately proportional to the square root of the \([K^+]_o\).

### 3.3.4. Dependence of activation gating on \(V_m\) and \([K^+]_o\)

Inwardly-rectifying potassium channel currents commonly display a property known as activation whereby currents initially increase, in an exponential manner, on hyperpolarization (see section 1.4.1c). Activation of K\textsubscript{r2.2} wild-type currents, under control conditions (70K/70NMDG) is illustrated in figure 3.5A. The time constants of activation (\(\tau_{act}\)) were obtained by fitting the currents with a single exponential function of the form:

\[
f(t) = c + Ae^{\left(-\frac{t}{\tau_{act}}\right)}
\]

(Equation 3.5)

The results of the fitting procedure are shown superimposed on the activation traces in Figure 3.5A.

The relationship between the activation time constants and membrane potential are shown in figures 3.5B and C, and are fitted with a linear regression of the form:

\[
\tau_{act} = a\exp\left(\frac{V_m}{b}\right)
\]

(Equation 3.6)

where \(a\) is \(\tau_{act}\) (ms) at 0mV, and \(b\) represents the voltage-dependence of activation gating. The time constants for activation (\(\tau_{act}\)) clearly decrease (figure 3.5B, C), falling e-fold for a 17.1 ± 1.5mV hyperpolarization in MEL (n = 7) cells. A similar voltage-dependence of activation was seen in CHO cells at 70mM \([K^+]_o\); \(\tau_{act} = 16.5 \pm 1.5\)mV (n = 10), 35mM \([K^+]_o\); \(\tau_{act} = 17.2 \pm 0.2\)mV (n = 9) and 14mM \([K^+]_o\); \(\tau_{act} = 15.6 \pm 1.2\)mV (n = 12). These values agree closely with those reported by others for native inward rectifiers (e.g 18mV; Leech & Stanfield, 1981). Although the voltage-dependency of activation time constants did not change with varying \([K^+]_o\), the range over which the current activated shifted approximately with the change in \(E_k\). Thus, gating of K\textsubscript{r2.2} wild-type currents depends both on membrane potential and \([K^+]_o\) as has been previously described for native (see for example Hagiwara & Yoshii, 1979; Leech & Stanfield, 1981) and cloned (see for example Stanfield et al., 1994a) inward rectifiers.
Figure 3.5.

Activation of **Kir2.2** wild-type currents under hyperpolarization and dependence on \([K^+]_0\).  

**A**, representative current recordings elicited by 50ms voltage steps of 20, 30 and 40 mV negative from \(E_K\) (−17mV). The currents were fitted with equation 3.5 and the fits are shown superimposed on the traces. **B**, semilogarithmic plot of time constants for activation (\(\tau_{act}\)) plotted against voltage (\(V_m\)) for experiments with 70mM \([K^+]_0\). The symbols represent the mean ± SEM, where larger than the symbol, for \(n = 3-10\) cells. **Filled circles**, MEL cells; **open circles**, CHO cells. The solid and dashed lines show the best fits to the mean data for **Kir2.2** wild-type channels expressed in MEL and CHO cells, respectively. **C**, semilogarithmic plot of time constants of activation (\(\tau_{act}\)) plotted against voltage (\(V_m\)) for experiments, in CHO cells, with various external \(K^+\) concentrations. The symbols represent the mean ± SEM, where larger than the symbol, for \(n = 3-16\) cells. **Circles**, 70mM \([K^+]_0\); **triangles**, 35 mM \([K^+]_0\); **hexagons**, 14mM \([K^+]_0\). Note that the scaling of the x axis differs from that in part b. The solid line shows a linear fit to the mean data for 70mM \(K^+\), whilst the dashed lines show the mean fit for the activation time constants obtained in 70mM \([K^+]_0\) shifted −17.5mV and −40.5mV, respectively.
3.3.5. Block of Kir2.2 by extracellular cations

Another distinguishing feature of inwardly rectifying potassium channels is their susceptibility to block by extracellular cations such as Ba\(^{2+}\) and Cs\(^{+}\). Thus, the effect of Ba\(^{2+}\) and Cs\(^{+}\) on Kir2.2 wild-type currents was examined and the results are shown in figure 3.6. Kir2.2 wild-type currents were initially recorded in 70mM external K\(^{+}\) solution, then perfused with 70mM K\(^{+}\) external solution containing Ba\(^{2+}\) or Cs\(^{+}\) at the concentrations indicated in the figure. Currents were elicited in response to voltage steps, of 1000ms duration, from a holding potential of -17mV (equilibrium potential for K\(^{+}\); \(E_K\)) to test potentials ranging from +80mV to -115mV in 5mV steps. Every fifth record is shown for clarity.

As is clearly illustrated, Ba\(^{2+}\) and Cs\(^{+}\) block Kir2.2 wild-type currents in a concentration-, time-, and voltage-dependent manner, block increasing as the membrane potential is hyperpolarized. Ba\(^{2+}\) and Cs\(^{+}\) blocked both the instantaneous and steady-state currents, though inhibition by Cs\(^{+}\) exhibited less time-dependency and had little effect on currents at potentials more positive than -70mV. The block of Kir2.2 wild-type currents by extracellular Ba\(^{2+}\) and Cs\(^{+}\) is characteristic of inward rectifiers, as previously shown for RB-IRK2 (Koyama et al., 1994) and MB-IRK2 (Takahashi et al., 1994). The inhibition of Kir2.2 currents by Ba\(^{2+}\) is described in more detail in chapter 4. An extensive description of the inhibition of a strong inward rectifier by Cs\(^{+}\) is described elsewhere (see Abrams, 2000).

3.3.6. Single channel current properties of Kir2.2

Figure 3.7A shows representative, single channel current traces recorded using the cell-attached patch configuration, from CHO cells expressing Kir2.2 wild-type channels. As for macroscopic currents, single channel currents were observed predominantly in the inward-going direction thus exhibiting strong inward rectification. The patch from which example recordings are shown in figure 3.7A contained four channels. At potentials positive to -80mV events in which all four channels were closed were rare but as the membrane was hyperpolarized to -100mV and more negative, bursts of single channel openings could be resolved. Preliminary data (not shown) suggested that \(P_{\text{open}}\) decreases with hyperpolarization, \(nPo\) decreasing from 0.85 at -40mV to 0.48 at -160mV.
Figure 3.6.

Block of $K_r 2.2$ wild-type currents by $Ba^{2+}$ and $Cs^+$. A and B show representative current recordings elicited by voltage steps to +63, +38, +13, -12, -37, -62, -87 and -112mV for 1s from a holding potential of -17mV. The left panel of each figure shows records obtained under control conditions (70K+/70NMDG) whilst the right panel shows the effect of external $Ba^{2+}$ (A) or $Cs^+$ (B) at the concentrations indicated. C and D show steady-state current-voltage relationships under control conditions and in the presence of $Ba^{2+}$ (C) or $Cs^+$ (D). Steady-state current amplitudes, normalized to the maximum current amplitude at -132mV under control conditions, are plotted. The symbols represent the mean ± SEM, where larger than the symbol, for n = 5 cells. Filled circles, control; filled triangles, 5μM cation; open triangles, 50μM cation; filled squares, 500μM cation.
Figure 3.7.

Single channel current properties of Kᵢ2.2.  

A, Representative single channel recordings obtained in 140mM [K⁺]ᵢ and 200mM [K⁺]ₒ from Kᵢ2.2 wild-type in response to various membrane potentials as noted in the figure. Channel openings are represented by pulses of inward current seen as downward deflections from the baseline (dashed line). The dotted lines represent the open levels for one, two and three channels.  

B, Plot of single channel current amplitude versus membrane potential for Kᵢ2.2 wild-type. The data are fit with a linear regression of the form \( i = \gamma(V - E_{rev}) \) where \( E_{rev} \) is the predicted reversal potential, constrained to 9mV. The symbols represent the mean ± SEM, where larger than the symbol for \( n = 8 \) cells.
Figure 3.7B shows a plot of unitary conductance versus membrane potential for \( \text{K}_{\text{i2.2}} \). Slope conductances were determined by regression fits of the form:

\[
i = \gamma (V - E_{\text{rev}})
\]

(Equation 3.7)

where \( \gamma \) is the unitary current and \( E_{\text{rev}} \) is the predicted reversal potential, which in this case has been constrained to 9mV. The slope conductance (figure 3.7B) for \( \text{K}_{\text{i2.2}} \) channels, with 200mM \( \text{K}^+ \) in the pipette and 140mM \( \text{K}^+ \) in the bath, was calculated as 38.6 ± 1.1pS (mean ± SEM; \( n = 8 \)). This is in close agreement with previous measurements of unitary conductance for \( \text{K}_{\text{i2.2}} \) expressed in HEK293 cells and Xenopus oocytes (38.0 ± 0.3pS, Yamashita et al., 1996; 34.2 ± 2.1pS, Takahashi et al., 1994; 1995).

3.4. Discussion

The electrophysiological basis for identification of a strong inward rectifier potassium channel rests on characteristics such as strong rectification of the macroscopic current, voltage-dependent block by \( \text{Ba}^{2+} \) and \( \text{Cs}^+ \) and an appropriate single channel conductance. The results presented in this chapter clearly demonstrate that the whole cell and single channel properties of \( \text{K}_{\text{i2.2}} \) heterologously expressed in both MEL and CHO cells are consistent with the electrophysiological properties of native strong inward rectifier potassium channels and \( \text{K}_{\text{i2.2}} \) heterologously expressed in Xenopus oocytes (see section 1.4.1; see also Koyama et al., 1994; Takahashi et al., 1994, 1995), indicating that both MEL and CHO cells represent suitable expression systems for further mutagenesis studies of members of the \( \text{K}_{\text{i2.0}} \) channel subfamily.

As anticipated for \( \text{K}_{\text{i2.2}} \), hyperpolarization from a holding potential of -17mV revealed large, rapidly activating, inward-going currents, whilst currents in response to depolarizations were small. At very negative membrane potentials the current exhibited a time-dependent decay, or ‘inactivation’. This has been previously observed for native (see for example Biermans et al., 1987; McKinney & Gallin, 1988) and cloned channels (see for example Kubo et al., 1993a; Takahashi et al., 1994; Shieh, 2000). Whilst Na-dependent inactivation of inward-rectifying currents has been well characterized in both frog skeletal muscle (Standen & Stanfield, 1979) and tunicate egg cell membranes (Ohmori, 1978; 1980; Fukushima, 1982), the mechanism underlying inactivation in Na-free solution has, until recently, remained unclear.
In their study of MB-IRK2 (Kir2.2), Takahashi et al. (1994) also observed a prominent, voltage-dependent inactivation of currents at negative membrane potentials. Consistent with their macroscopic data, they saw a steep decline in open probability of single Kir2.2 currents as the membrane was hyperpolarized. This was attributed to an increase in the occurrence of “long closed gaps”, channel closures of 200ms duration and more. In this study, preliminary data suggested that Kir2.2 currents expressed in CHO cells display a similar decline in open probability with hyperpolarization. However, owing to high expression levels (no single channel patches were obtained) analysis to predict whether this was a consequence of an increase in the number of long closed events could not be conducted.

A decrease in open probability with hyperpolarization has also been reported for Kir1.1b (Chepilko et al., 1995; Choe et al., 1998; 1999) and Kir2.1 (Choe et al., 1999). Although the decline in open probability for Kir1.1b is less voltage dependent than for the strong inward rectifiers Kir2.1 and Kir2.2, it has been clearly associated with an increase in the number of long closures. Chepilko et al. (1995) suggested that the voltage dependence of the long closures is an intrinsic property of the channel. Although it was noted that the ‘putative blocked state’ of Kir1.1b channels in the presence of 0.5mM Ba2+ had a mean lifetime very similar to that of the long-lived closed state of the channel under control conditions, the similarity of these time constants was considered fortuitous. Further explanation for the possible mechanism underlying the decrease in open probability with hyperpolarization was not given.

Several hypotheses have been proposed to account for ‘inactivation’ of inward rectifier currents at negative membrane potentials in the absence of Na+. Early theories from studies in skeletal muscle attributed the decay of currents during hyperpolarization to depletion of K+ ions in the t-tubule system (Adrian & Freygang, 1962a; Adrian et al., 1970a). However, as described in section 1.4.1d, the decline in conductance during a maintained hyperpolarization could not be entirely attributed to depletion of tubular K+ (Adrian et al., 1970b; Almers, 1972a, b), but also involved changes in membrane permeability. Interestingly, Kir2.2 channels are co-localized with SAP97 in the t-tubules of cardiac ventricular myocytes (Leonoudakis et al., 2000). Thus, in their native environment, inactivation of Kir2.2 channels at very negative membrane potentials is likely to involve depletion of tubular K+.

Could depletion of extracellular K+ be responsible for the inactivation under the experiment conditions described herein? One study has reported that ion depletion/accumulation can
occur following overexpression of cloned channels in mammalian expression systems (Frazier et al., 2000). Following expression of Kv2.1 channels in HEK293 cells, Frazier et al. (2000) found that long depolarizations in these small cells produced current-dependent changes in [K+]i that mimicked inactivation and changes in ion selectivity (Frazier et al., 2000). We have seen that hyperpolarization activates large (>1nA), inward-going currents in mammalian cells expressing Kir2.2. In such small cells (10pF), this could lead to changes in the K+ gradient, particularly at very negative membrane potentials. If depletion of [K+]o or accumulation of [K+]i was the underlying cause for inactivation, we might expect to see the current reversing at potentials more negative than those predicted by the Nernst equation. However, this is not the case. In fact, currents are reversing at values more positive than predicted by the Nernst equation. Furthermore, [K+]o depletion is also an unlikely cause for inactivation in this study since high extracellular/intracellular [K+] has been used.

An alternative hypothesis is that block by external cations including Ca2+, Mg2+ and Ba2+ may be responsible for the hyperpolarization induced decay of inward rectifier K+ currents. Whilst the extracellular recording solution contained 2mM Ca2+ and Mg2+, Ba2+ was not added. However, it is possible that block by contaminating levels of Ba2+ present in commercially available salts could be partly responsible for the inactivation of Kir2.2 currents at hyperpolarized potentials since Kir2.2 appears to exhibit a high affinity for Ba2+ (see Chapter 4). Furthermore, several observations from previous studies on other members of the inward rectifier potassium channel family support the idea that block by trace amounts of Ba2+ may play a pivotal role in channel inactivation at very negative membrane potentials.

First, Kir2.4, which has a low affinity for Ba2+ does not exhibit any voltage dependent inactivation during a 500ms voltage-pulse in Na+-free external solution (Töpfer et al., 1998). In contrast, Kir2.2, which is highly sensitive to Ba2+ (see chapter 4), displays prominent inactivation at hyperpolarized potentials. Thus, channel sensitivity to Ba2+ appears to parallel voltage dependent inactivation.

Single channel studies of gating in Kir1.1b (ROMK2) provide further evidence that Ba2+ block may underlie channel inactivation in certain experimental situations. In the absence of divalent cations, Kir1.1b currents exhibit long closures of similar duration and voltage dependency to closures in the presence of Ba2+ (Choe et al., 1998). With 5mM EDTA, the long closed events were virtually eliminated indicating that contaminant divalent cations induce the long closed states. Further experiments using SO4 2-, which forms an insoluble
complex with Ba\textsuperscript{2+}, implied Ba\textsuperscript{2+} to be the main blocker, since long closures occurred less frequently on addition of K\textsubscript{2}SO\textsubscript{4} to the pipette solution (Choe et al., 1998).

Similar observations have also been reported for Kir\textsubscript{2.1} (Choe et al., 1999; Guo & Lu, 2002) and for other potassium channels including calcium-activated potassium channels (Diaz et al., 1996). Choe et al. (1998) proposed that a contaminating Ba\textsuperscript{2+} concentration as low as 0.1\mu M would be sufficient to promote the long closed periods observed in Kir\textsubscript{1.1b} at very negative membrane potentials.

Shieh (2000) showed that voltage-dependent processes controlled the inactivation of Kir\textsubscript{2.1} channels, which was reduced by increases in external [K\textsuperscript{+}]. This ‘protective function’ of K\textsuperscript{+} could provide further support for block of the channel by external Ba\textsuperscript{2+} at hyperpolarized potentials since K\textsuperscript{+} is known to compete with Ba\textsuperscript{2+} for the binding site in K\textsuperscript{+} channels (Standen & Stanfield, 1978a; Eaton & Brodwick, 1980; Armstrong & Taylor, 1980; Vergara & Latorre, 1983). However, Shieh (2000) carried out their experiments in the presence of 5mM EDTA, yet still observed inactivation implying that block by external Ba\textsuperscript{2+} was not the cause of inactivation. Instead, inactivation of Kir\textsubscript{2.1} currents was compared with the C-type inactivation of Kv channels and it was proposed that K\textsuperscript{+} ions may protect the channel from inactivation by binding within the outer pore of Kir\textsubscript{2.1}, which becomes obstructed during voltage change thereby preventing inactivation.

An arginine (R) at position 148 in Kir\textsubscript{2.1} was shown to influence this binding site since mutation of this residue to a tyrosine (Y) removed inactivation (Shieh, 2000). However, a previous study has shown that mutation of R148 in Kir\textsubscript{2.1} to histidine (H) alters the rate at which Ba\textsuperscript{2+} blocks the channel (Sabirov et al., 1997). Furthermore, Kir\textsubscript{7.1}, which contains a methionine at this position, does not exhibit inactivation at hyperpolarized potentials (Döring et al., 1998) and has a low affinity for Ba\textsuperscript{2+} (see Krapivinsky et al., 1998a; Döring et al., 1998).

Shieh and Lee (2001) continued to investigate whether inactivation of Kir\textsubscript{2.1} channels at negative membrane potentials is a consequence of block by permeant ions or a conformational change induced by the binding of permeant ions. Their approach involved substituting Ammonium (NH\textsubscript{4}\textsuperscript{+}) or Thalium (Tl\textsuperscript{+}) ions for K\textsuperscript{+} to examine whether other permeant ions protected against inactivation. In the presence of NH\textsubscript{4}\textsuperscript{+} or Tl\textsuperscript{+}, Kir\textsubscript{2.1} channels exhibited two types of current decay: a ‘permeant-ion-protected inactivation’ in which higher
concentrations of NH$_4^+$ or Tl$^+$ protected K$_{ir}$2.1 channels from inactivation and a ‘permeant-ion-enhanced inactivation’. At higher concentrations of NH$_4^+$, only the permeant-ion-induced inactivation was observed. Shieh and Lee (2001) speculated that NH$_4^+$ (or Tl$^+$) could be acting as a permeable blocker of K$_{ir}$2.1, or alternatively, that binds directly to the channel, inducing inactivation by destabilizing the open state. However, the rate of decay in 10mM NH$_4^+$ did not correlate well with the effective rate coefficient for ion transfer suggesting that block by NH$_4^+$ was not the cause of inactivation. In support of their hypothesis that permeant ions bind to the channel thereby causing a conformational change, Shieh and Lee (2000) found that NH$_4^+$-induced inactivation was very sensitive to temperature, inactivation increased by a factor of 2.76 with a 10°C increase in temperature. Furthermore, the R148Y mutation used in their previous study (Shieh, 2000), which alters the dimensions of the outer pore, abolished NH$_4^+$-induced inactivation.

However, a recent study suggests that in the absence of external cations and with low [K$^+$]$_o$, hyperpolarization-induced inactivation is a consequence of block by residual hydroxyethylpiperazine (HEP), present in HEPES buffered solutions (Guo & Lu, 2002). When the HEPES present in extracellular solutions was replaced with phosphate, inactivation at very negative membrane potentials was removed. Thus, it appears that the K$^+$-sensitive hyperpolarization-induced inactivation exhibited by inward rectifiers is not an intrinsic gating property of these channels.

Based on the observations described above it seems likely that block by external cations, including trace amounts of Ba$^{2+}$, and block by HEPES from the external solution may have contributed to the voltage-dependent inactivation of K$_{ir}$2.2 currents at very negative membrane potentials reported herein. However, without further experimentation it is impossible to conclude which of the above mechanisms may underlie hyperpolarization-induced inactivation of K$_{ir}$2.2 currents.

K$_{ir}$2.2 also exhibited the characteristic dependency of rectification on [K$^+$]$_o$. It is now known that these characteristic properties of K$_{ir}$ channels are conferred by an arginine, R149 in K$_{ir}$2.2, conserved in the outer pore of most K$_{ir}$ channels. K$_{ir}$7.1, which carries a methionine at this position, exhibits a shallow dependence of conductance on [K$^+$]$_o$ in addition to a non-saturating conductance-voltage relationship. Replacement with arginine, as conserved throughout the K$_{ir}$ family, restored the steep dependence of rectification on [K$^+$]$_o$ (see Döring et al., 1998).
Finally, the conductance-voltage relationship clearly follows a typical Boltzmann function with saturation, which indicates a deviation from the independence principle by ion-ion or ion-pore interactions. Furthermore, in keeping with previous studies on strong inward rectifier potassium channels (see for example Leech & Stanfield, 1981; Lopatin et al., 1995; Lopatin & Nichols, 1996), the conductance-voltage relationship exhibited both shallow and steep components. Intracellular Mg$^{2+}$ was omitted from recording solutions throughout this study, thus the shallow and steep components of the conductance-voltage relationship are likely to represent instantaneous and steady state components of polyamine block, respectively. Block of HRK1 (Kir 2.3) and Kir 2.1 by spermine (or spermidine) have previously been demonstrated to exhibit two components (Lopatin et al., 1995; Lopatin & Nichols, 1996). This has been explained by a model in which two molecules of spermine block the pore in a sequential manner, binding initially to a shallow site to block then binding to a second site located deep within the pore (see Lopatin et al., 1995; see also section 1.2.4d).

In conclusion, the whole cell and single channel properties of Kir 2.2 heterologously expressed in both MEL and CHO cells are entirely consistent with the electrophysiological properties of native strong inward rectifier potassium channels and Kir 2.2 heterologously expressed in *Xenopus* oocytes (see section 1.4.1; see also Koyama et al., 1994; Takahashi et al., 1994, 1995). Thus, both MEL and CHO cells represent suitable expression systems for further mutagenesis studies of members of the Kir 2.0 channel subfamily.
Chapter Four

A comparison of $\text{Ba}^{2+}$ blockage and unitary conductance in the murine $\text{K}^+$ channels $\text{K}_{\text{ir}2.1}$ and $\text{K}_{\text{ir}2.2}$.

4.1. Introduction

A feature of inwardly rectifying potassium channels in different cell types is their high sensitivity to block by barium ions ($\text{Ba}^{2+}$). The blocking action of $\text{Ba}^{2+}$ has been demonstrated for a large number of $\text{K}_{\text{ir}}$ channels, both native and cloned. These include $\text{K}_{\text{ir}}$ channels of frog skeletal muscle (Standen & Stanfield, 1978a), starfish eggs (Hagiwara et al., 1978), heart cell membrane (Sakmann & Trube, 1984a, b; DiFrancesco et al., 1984), rat corticotropes (Kuryshiev et al., 1997) and the cloned channels $\text{K}_{\text{ir}1.1a}$ (Löffler & Hunter, 1997) and $\text{K}_{\text{ir}1.1b}$ (Zhou et al., 1996).
Although Ba$^{2+}$ has been shown to block all members of the Kir 2.0 subfamily (Kubo et al., 1993a; Takahashi et al., 1994; Koyama et al., 1994; Morishige et al., 1994; Töpfer et al., 1998; Hughes et al., 2000), extensive characterization of Ba$^{2+}$ blockade has been described only for Kir 2.1 (Shieh et al., 1998; Alagem et al., 2001). Here, block of Kir 2.2 currents by Ba$^{2+}$ is investigated further.

The expression 'open channel block' is often used to describe the mechanism by which Ba$^{2+}$ is believed to block potassium channels. With a similar crystal radius to K$^+$, Ba$^{2+}$ can enter the pore of the open channel, but because of its divalent charge blocks current by binding to K$^+$ binding sites with a high affinity (see Standen & Stanfield, 1978a; Neyton & Miller, 1988; Sabirov et al., 1997; Jiang & MacKinnon, 2000). Previous studies in inward rectifier (Standen & Stanfield, 1978a), delayed-rectifier (Eaton & Brodwick, 1980; Armstrong & Taylor, 1980) and calcium-activated (Vergara & Latorre, 1983) potassium channels have shown that K$^+$ appears to compete with Ba$^{2+}$ for the binding site. These findings suggest that Ba$^{2+}$ ions interact with the amino acid residues lining the ion conduction pore. Thus, Ba$^{2+}$ is a very useful tool for probing the molecular nature of potassium channel pores.

In general, the strong inward rectifier Kir 2.2 exhibits electrophysiological characteristics similar to those of the closely related channel Kir 2.1 (Takahashi et al., 1994; Koyama et al., 1994; Takahashi et al., 1995). However, differences in unitary conductance (Takahashi et al., 1995) and in sensitivity to Ba$^{2+}$ (Thompson et al., 1998) have been reported. The P-regions of Kir 2.2 and Kir 2.1 are virtually identical. However, leucine (L) at position 148 of Kir 2.2 is replaced by a phenylalanine (F) in Kir 2.1, as illustrated in the partial sequence alignment of Kir 2.1 and Kir 2.2 below:

$$\begin{align*} 
\text{Kir 2.1} & \quad 139 \ldots \text{TQTTIGYFRC} \ldots 149 \\
\text{Kir 2.2} & \quad 140 \ldots \text{TQTTIGYGLRC} \ldots 150 
\end{align*}$$

A previous study has shown that a single amino acid difference between the P-regions of Kir 1.1b and Kir 2.1 accounts for the 10-fold difference in sensitivity to Ba$^{2+}$ exhibited by these two channels (Zhou et al., 1996). Furthermore, single amino acid mutations in the P-regions of potassium channels have been shown to affect unitary conductance (see for example MacKinnon & Yellen, 1990; Taglialatela et al., 1993; Choe et al., 2000). In this study, the mutants L148F in Kir 2.2 and F147L in Kir 2.1 were constructed to determine whether this
single difference in the P-region sequences of Kir2.2 and Kir2.1 accounts for the differences in unitary conductance and Ba$^{2+}$ block displayed by the two channels.

4.2. Methods

The electrophysiological properties of wild-type and mutant Kir2.2/Kir2.1 channels were examined following expression of the relevant gene in either CHO or MEL cells as described in the methods chapter. Whole cell currents were recorded from transiently transfected CHO cells, with 140mM K$^+$ in the patch pipette and 70mM K$^+$ in the bath. External barium ([Ba$^{2+}$]$_o$) was applied by changing the control bath solution for solutions containing 10 - 300µM BaCl$_2$.

For Ba$^{2+}$ block experiments, single channel currents were recorded in the cell-attached patch configuration from stably transfected MEL cells with 70mM K$^+$ in the patch pipette and 140mM K$^+$ in the bath. Ba$^{2+}$ was added to the pipette solution at either 100, 200 or 300µM. The mean open time was calculated and corrected for missed events as described in section 4.3.4.

For comparison of unitary current between wild-type and mutant Kir2.2/Kir2.1 channels, single channel currents were recorded from transiently transfected CHO cells with 200mM K$^+$ in the patch pipette and 140mM K$^+$ in the bath.

4.3. Results

4.3.1. Ba$^{2+}$ blocks Kir2.2 in a concentration-, time- and voltage-dependent manner

Figure 4.1 shows representative recordings from CHO cells expressing Kir2.2 channels in the presence of various concentrations of extracellular barium ([Ba$^{2+}$]$_o$). Currents are in response to 200ms hyperpolarizing voltage steps to -37, -57, -77, -97 and -117mV from a holding potential of -17mV. Control records are shown in figures 4.1A, whilst figures 4.1B and C show currents obtained in the presence of 30µM and 300µM Ba$^{2+}$, respectively. For clarity, figure 4.1D shows current recordings elicited in response to a single voltage step to -77mV, in the presence of 0, 30 and 300µM Ba$^{2+}$. Together, these traces clearly illustrate that Ba$^{2+}$ blocks Kir2.2 currents in a concentration-, time- and voltage-dependent manner. Ba$^{2+}$ inhibits both the instantaneous and steady-state current. However, only inhibition of the steady-state
Figure 4.1.

*Block of K\textsubscript{IR}2.2 wild-type currents by Ba\textsuperscript{2+}.* A, representative current recordings elicited in response to voltage steps to -37, -57, -77, -97 and -117mV for 200ms from a holding potential of -17mV under control conditions (70K/70NMDG). B and C, current recordings from the same cell following perfusion with 30 and 300\textmu M Ba\textsuperscript{2+}, respectively. D, current recordings elicited in response to a single voltage step of -77mV, in the presence of 0, 30 and 300\textmu M Ba\textsuperscript{2+}.
current by Ba\textsuperscript{2+} will be analysed here. For low [Ba\textsuperscript{2+}]\textsubscript{o}, voltage steps of longer duration were used to ensure that currents reached steady-state.

4.3.2. Steady-state analysis of blockade of \textit{K}_{\text{ir}2.2} by Ba\textsuperscript{2+}.

For a simple binding reaction where Ba\textsuperscript{2+} binds reversibly to the open channel, the kinetic equation can be expressed as follows:

\[
\text{C} \xrightleftharpoons[k_c]{k_o} \text{Ba}\textsuperscript{2+} + \text{O} \xrightleftharpoons[k_{ib}]{k_{ib}[\text{Ba}\textsuperscript{2+}]} \text{OBa}\textsuperscript{2+} \quad (\text{Equation 4.1})
\]

where \( k_o \) and \( k_c \) are transition rate constants for channel opening and closing, \( k_{ib} \) is the blocking rate constant and \( k_{ib} \) is the rate constant for unbinding. For such a reaction, the equilibrium dissociation constant (\( K_d \)) for binding of Ba\textsuperscript{2+} can be defined by:

\[
K_d = \frac{k_{ib}}{k_{ib}} \quad (\text{Equation 4.2})
\]

The \( K_d \) was obtained by examining the steady-state fractional current (\( I_{\text{Ba}}/I_{\text{control}} \)) at different membrane potentials (\( V_m \)). Steady-state currents obtained in the presence of Ba\textsuperscript{2+} were normalized to currents obtained in the absence of Ba\textsuperscript{2+} at the same potential. For example, at -77mV, and in the presence of 10\mu M Ba\textsuperscript{2+}, the steady-state fractional current for \textit{K}_{\text{ir}2.2} wild-type was 0.10 ± 0.01 (\( n = 6 \)).

Figure 4.2A shows a plot of steady-state fractional currents versus extracellular Ba\textsuperscript{2+} concentration for different membrane potentials. Data for concentrations of Ba\textsuperscript{2+} lower than 10\mu M were not included in the analysis because these results exhibited a large scatter, owing to series resistance errors arising as a result of large currents. Thus, the fits shown in the inset of figure 4.2A are simulated, based on fits to the data obtained at higher concentrations of [Ba\textsuperscript{2+}]\textsubscript{o}, which are shown in the main figure. The data were fit using the Hill-Langmuir equation:

\[
\frac{I_{\text{Ba}}}{I_{\text{Control}}} = \left(1 + \left(\frac{[\text{Ba}\textsuperscript{2+}]}{K_d}\right)^1\right)^{-1} \quad (\text{Equation 4.3})
\]
where the hill coefficient was constrained to 1, based on an assumption of one-to-one binding of Ba$^{2+}$ in the pore of K$\text{ir}$.2.2 as previously assumed for native strong inward rectifiers (see for example Standen & Stanfield, 1978a). At -77mV, K$\text{ir}$.2.2 was half-blocked by 1.13µM.

Figure 4.2B shows the relationship between the $K_d$ (derived from the plots in figure 4.2A) versus membrane potential ($V_m$). The data were fit to the Woodhull equation:

$$K_d(V) = K_d(0) \exp\left(\frac{zF\delta}{RT}V_m\right) \quad \text{(Equation 4.4)}$$

where $K_d(0)$ is the concentration of blocking ion causing half-maximal block at 0mV. $\delta$ is a slope factor indicating the voltage sensitivity at $K_d$, and $z$, $F$, $R$ and $T$ have their usual meanings ($RT/F = 25$mV at 20°C).

The voltage dependence of the $K_d$ for Ba$^{2+}$ binding in K$\text{ir}$.2.2 wildtype decreases e-fold for a 32.5mV hyperpolarization. $K_d(0)$ and $\delta$ were 13.68µM and 0.40, respectively. In its simplest terms, a $\delta$ value of 0.40 suggests that the binding site for Ba$^{2+}$ is located approximately 40% of the way across the membrane electrical field. However, the electrical distance is not necessarily the same as the physical distance because the membrane electric field may not be uniformly distributed along the pore. The $K_d$-$V_m$ relationship for K$\text{ir}$.2.2 became less steep at $V_m$ more negative than -107mV. This may reflect Ba$^{2+}$ dissociation into the intracellular space at very negative membrane potentials as previously described for K$\text{ir}$.2.1 heterologously expressed in Xenopus oocytes (Shieh et al., 1998) and for the high-conductance Ca$^{2+}$-activated potassium channel (see Neyton & Miller, 1988).

4.3.3. Kinetic analysis of the macroscopic K$\text{ir}$.2.2 current blockade by [Ba$^{2+}$]$_0$

The voltage-dependent inactivation of whole cell currents in the presence of Ba$^{2+}$ follows a monoexponential time course and the time constant of the decay can be found by fitting the data with a single exponential function of the form:

$$f(t) = c + A \exp\left(\frac{-t}{\tau_{\text{block}}}\right) \quad \text{(Equation 4.5)}$$

where $f(t)$ is a function of time, $t$ is the time measured from the onset of the voltage protocol, $\tau_{\text{block}}$ is the blocking time constant, $A + c$ is the current amplitude at $t = 0$ and $c$ is the steady state current. Figure 4.3A shows a semilogarithmic plot of the time constants of inactivation.
(\(\tau_{\text{block}}\)) of the whole cell currents as a function of membrane potential (\(V_m\)) in the presence of various \([\text{Ba}^{2+}]_o\). The blocking time constants for \(K_{ir}2.2\) decreased exponentially with increasing hyperpolarization, falling e-fold for a 26.9 ± 1.08 mV (\(n = 7\)) hyperpolarization in the presence of 100\(\mu\)M \([\text{Ba}^{2+}]_o\). Blockage by 100\(\mu\)M \(\text{Ba}^{2+}\) at −97mV reached equilibrium in 7.5 ± 0.3ms (\(n = 6\)).

The time constant for blockade of the channel by \(\text{Ba}^{2+}\) (\(\tau_{\text{block}}\)) may be described by:

\[
\tau_{\text{block}} = \frac{1}{k_{+b}[\text{Ba}^{2+}] + k_b}
\]

(Equation 4.6).

where \(k_{+b}\) is the blocking rate constant, \(k_b\) is the rate constant for unbinding and \([\text{Ba}^{2+}]_0\) is the extracellular \(\text{Ba}^{2+}\) concentration.

The reciprocal of the measured time constant for onset of block (1/\(\tau_{\text{block}}\)) is a linear function of the blocker concentration \([\text{Ba}^{2+}]\), and is shown in figure 4.3B as a function of \([\text{Ba}^{2+}]_0\) at negative membrane potentials ranging from −67 to −107mV. \(k_{+b}\) and \(k_b\) can be determined from the slope of the regression and from the y-axis intercept, respectively. Estimation of \(k_b\) by this method gave small negative values (not shown). However, given that \(k_{+b}\) is so much greater than \(k_b\), these negative values for \(k_b\) are within likely experimental error. Therefore, \(k_b\) values were calculated using \(k_{+b}\) and \(K_d\) according to equation 4.2 where \(K_d\) is the equilibrium dissociation constant calculated by examining the fractional current (\(I_{\text{Ba}}/I_{\text{control}}\)) at different membrane potentials as described in section 4.3.2. \(k_{+b}\) and \(k_b\), calculated in this manner, are shown in numerical form in table 4.1 below.

**Table 4.1. Rate constants for block and unblock of \(K_{ir}2.2\) by \(\text{Ba}^{2+}\)**

<table>
<thead>
<tr>
<th>(V_m) (mV)</th>
<th>Mean (k_{+b}) ((\text{M}^{-1}\text{s}^{-1}))</th>
<th>Mean (k_b) ((\text{s}^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>-67</td>
<td>(4.54 \times 10^5)</td>
<td>0.71</td>
</tr>
<tr>
<td>-77</td>
<td>(7.30 \times 10^5)</td>
<td>0.83</td>
</tr>
<tr>
<td>-87</td>
<td>(10.06 \times 10^5)</td>
<td>0.89</td>
</tr>
<tr>
<td>-97</td>
<td>(13.48 \times 10^5)</td>
<td>0.94</td>
</tr>
<tr>
<td>-107</td>
<td>(19.58 \times 10^5)</td>
<td>1.08</td>
</tr>
</tbody>
</table>
Voltage- and concentration-dependence of the steady-state block of $K_r2.2$ by $Ba^{2+}$.  

**A**, semilogarithmic plot of the steady-state current, obtained in the presence of $Ba^{2+}$, plotted as a fraction of that obtained under control conditions against $Ba^{2+}$ concentration. The voltages are -67mV (closed circles), -77mV (open squares), -87mV (closed triangles), -97mV (open inverted triangles) and -107mV (closed diamonds). The symbols represent the mean ± SEM, where larger than the symbol, for n = 1 - 9 cells. The curves were fitted to the Hill-Langmuir equation (Equation 4.3) with the hill slope constrained to 1. *Inset*, simulated fits based on fits of actual data shown in main part of figure.  

**B**, semilogarithmic plot of the voltage-dependence of $K_d(V)$. The data are fitted to the Woodhull equation (Equation 4.4).
Figure 4.3

Concentration and voltage dependence of Ba\(^{2+}\) block in Kir2.2 wild-type. **A**, dependence of the time constant for the onset of blockade, \(\tau_{\text{block}}\), upon membrane potential \((V_m)\) at different \([\text{Ba}^{2+}]_o\). The symbols represent the mean ± SEM, where larger than the symbol, for \(n = 3 - 7\) cells. *Closed circles*, \(10\mu\text{M} [\text{Ba}^{2+}]_o*; *open triangles*, \(30\mu\text{M} [\text{Ba}^{2+}]_o*; *closed squares*, \(100\mu\text{M} [\text{Ba}^{2+}]_o*; *open diamonds*, \(300\mu\text{M} [\text{Ba}^{2+}]_o*.

**B**, dependence of \(1/\tau_{\text{block}}\) upon \([\text{Ba}^{2+}]_o\) at different membrane potentials \((V_m)\). The symbols represent the mean ± SEM, where larger than the symbol, for \(n = 3 - 7\) cells. *Closed circles*, -67mV; *open squares*, -77mV; *closed triangles*, -87mV; *open inverted triangles*, -97mV; *closed diamonds*, -107mV. The data are fit with linear regressions of the form \(1/\tau_{\text{block}} = k_b[\text{Ba}^{2+}] + k_{-b}\).
Figure 4.4 shows a semilogarithmic plot of $k_+b$ and $k_-b$ versus membrane potential ($V_m$). The data is fitted with equations modified from the Woodhull model (Woodhull, 1973):

$$k_+(V) = k_+(0) \exp \left( -\frac{zF\delta_+ b}{RT} V_m \right) \quad \text{(Equation 4.7)}$$

and

$$k_-(V) = k_-(0) \exp \left( \frac{zF\delta_- b}{RT} V_m \right) \quad \text{(Equation 4.8)}$$

where $k_+(V)$ and $k_-(V)$ are the association and dissociation rate constants, $k_+(0)$ and $k_-(0)$ are the values of these rate constants at 0mV, $\delta_+ b$ is the electrical distance between the entrance of the channel pore and the energy barrier, $\delta_- b$ is the electrical distance between the Ba$^{2+}$ binding site and its rate limiting barrier for exit, $V_m$ is the membrane potential, $z$ is the valency of the ion ($z = 2$ for Ba$^{2+}$), and $F$, $R$ and $T$ have their usual meanings.

Clearly, $k_+ b$ is voltage dependent increasing e-fold for a 28.2mV hyperpolarization. $k_+(0)$ and $\delta_+ b$ were $44.81 \times 10^3 \text{ M}^{-1} \text{s}^{-1}$ and 0.45, respectively. $\delta_+ b$ is comparable to the value of $\delta$ (0.40) obtained from the analysis of steady-state fractional currents. Thus, the voltage-dependence of the $K_d$ of Ba$^{2+}$ block in $K_{ir}2.2$ is due to the voltage-dependence of the association rate constant $k_+ b$. Indeed, $k_- b$ showed little voltage dependence (figure 4.4). In fact, $k_- b$ could be well fit by assuming that it is independent of voltage (dashed line, figure 4.4) giving $k_-(0)$ as 0.89s$^{-1}$. This may be because Ba$^{2+}$ leaves the channel either to the outside, or in addition, to the inside. However, this was not investigated further.

### 4.3.4. Block of single $K_{ir}2.2$ channel by Ba$^{2+}$

The effects of Ba$^{2+}$ on $K_{ir}2.2$ currents were investigated in more detail at the single channel level. Figure 4.5 shows representative single channel recordings, obtained in the cell-attached mode at the voltages indicated, with 200$\mu$M [Ba$^{2+}$] in the patch pipette. The corresponding open-time distributions are shown to the right of the traces. Open-time distributions were fit with a single exponential as described in section 2.4.17 and of the form:

$$f(t) = \sum_{j=1}^{n} \left( \frac{a_j}{\tau_j} \right) \exp \left( -\frac{t}{\tau_j} \right) \quad \text{(Equation 4.9)}$$

where $a_j$ and $\tau_j$ are the relative area and the time constant of component $j$, respectively. The number of components ($n$) was set to the number of open or closed states and the data fit
Figure 4.4.

Voltage-dependency of the association ($k_{+b}$) and dissociation ($k_{-b}$) rate constants for block of $K_{r2.2}$ by $Ba^{2+}$. Semilogarithmic plot of $k_{+b}$ (filled symbols) and $k_{-b}$ (open symbols) versus membrane potential ($V_m$) for $K_{r2.2}$. $k_{+b}$ was obtained from $\tau_{\text{block}}$ by fitting linear regression lines to $1/\tau_{\text{block}} = k_{+b}[Ba^{2+}] + k_{-b}$, where $k_{+b}$ is the slope of the line. $k_{-b}$ was obtained from $K_d$ analysis. The data are fit with equations modified from the Woodhull model (Equations 4.7 and 4.8), where for an e-fold change in $k_{+b}$ there is a 28.2mV change in membrane potential. $k_{-b}$ displayed little voltage dependence (solid line), and could be well fit by assuming that it is independent of voltage (red dashed line).
using the method of maximum likelihood (Colquhoun & Sigworth, 1983). Mean open times were corrected for missed events (i.e. short closures less than the minimum resolution, which were too brief to be detected) by multiplying the mean open time by the proportion of closed events detected, which was found by integrating the fitted closed time distribution between the minimum resolution and infinity (see Davies et al., 1992).

As the membrane was hyperpolarized, block of Knr2.2 currents was enhanced. This is reflected in the decrease in mean open time as illustrated in figure 4.5 and recorded in numerical form in table 4.2 below.

**Table 4.2.** Voltage-dependence of the mean open time for single Knr2.2 currents in the presence of 200μM Ba\(^{2+}\).

<table>
<thead>
<tr>
<th>Membrane Potential (mV)</th>
<th>Mean Open Time (ms)*</th>
<th>*measured with 200μM [Ba(^{2+})](_o)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-57</td>
<td>5.7 ± 2.0 (n = 2)</td>
<td></td>
</tr>
<tr>
<td>-77</td>
<td>2.8 ± 0.9 (n = 3)</td>
<td></td>
</tr>
<tr>
<td>-97</td>
<td>1.8 ± 0.3 (n = 3)</td>
<td></td>
</tr>
<tr>
<td>-117</td>
<td>1.6 ± 0.3 (n = 2)</td>
<td></td>
</tr>
<tr>
<td>-137</td>
<td>0.8 (n = 1)</td>
<td></td>
</tr>
</tbody>
</table>

The concentration dependence of Ba\(^{2+}\) block at the single channel level is illustrated in figure 4.6. Single channel recordings were obtained in the cell-attached mode, at a holding potential of -97mV with 100, 200 or 300μM Ba\(^{2+}\) in the patch pipette. The mean open time decreases as the concentration of external Ba\(^{2+}\) is increased as indicated in the right hand panel of figure 4.6 and recorded in numerical form in table 4.3 below.
Block of single Kir2.2 wild-type currents by Ba$^{2+}$ is voltage-dependent. The left panel shows representative single channel recordings of Kir2.2 wild-type currents in response to 57mV (A), 77mV (B), 97mV (C), 117mV (D) and 137mV (E) hyperpolarizing steps. Data are from cell-attached patches bathed in 140K$^+$ with 70K$^+$ and 200μM Ba$^{2+}$ in the patch pipette. The arrows indicate the level at which all channels are closed. The right panel shows open time distributions for the corresponding currents shown in the left panel.
Figure 4.6.

**Block of single Kir2.2 wild-type currents by Ba\(^{2+}\) is concentration dependent.** The left panel shows representative single channel recordings of Kir2.2 wild-type currents in response to a 97mV hyperpolarizing step. Data are from cell-attached patches bathed in 140mM K\(^+\) with 70K\(^+\) and 100\(\mu\)M (A), 200\(\mu\)M (B) or 300\(\mu\)M (C) Ba\(^{2+}\) in the patch pipette. The arrows indicate the level at which all channels are closed. The right panel shows open time distributions for the corresponding currents shown in the left panel.
Table 4.3. Concentration-dependence of the mean open time for single Kir2.2 currents at -97mV.

<table>
<thead>
<tr>
<th>[Ba(^{2+})]_o (µM)</th>
<th>Mean Open Time (ms)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>3.3 ± 0.3 (n = 4)</td>
</tr>
<tr>
<td>200</td>
<td>1.8 ± 0.3 (n = 3)</td>
</tr>
<tr>
<td>300</td>
<td>2.0 ± 0.3 (n = 3)</td>
</tr>
</tbody>
</table>

*measured at -97mV

Figure 4.7A shows a plot of the corrected mean open time (cmot) of the single channel currents as a function of membrane potential (V_m) in the presence of 100µM [Ba\(^{2+}\)]_o. The mean open time for Kir2.2 channel decreased exponentially with increasing hyperpolarization, falling e-fold for a 62.3mV hyperpolarization.

The simple model given for the blocking reaction given in equation 4.1 predicts that the mean open time (t_0) in the presence of Ba\(^{2+}\) will be given by:

\[
\tau_0 = \frac{1}{k_{+b}[Ba^{2+}] + k_c}
\]

(Equation 4.10)

where k_{+b} is the association rate constant, k_c is the transition rate constant for channel closure and [Ba\(^{2+}\)] is the concentration of Ba\(^{2+}\) in the patch pipette. Thus, the reciprocal of \(\tau_0\) is a linear function of the blocker concentration as shown in figure 4.7B for -97mV. k_{+b} and k_c can be determined from the slope of the regression and the y-axis intercept, respectively. The reciprocal of k_c should predict the mean open time in the absence of Ba\(^{2+}\). At -97mV, k_c was 0.2 ms\(^{-1}\), which predicts \(\tau_0\) as 5ms in the absence of Ba\(^{2+}\). However, single channel currents were not recorded in control solutions, thus a comparison of the predicted \(\tau_0\) with \(\tau_0\) measured in the absence of Ba\(^{2+}\) cannot be made.

Figure 4.8 shows a plot of k_{+b} versus membrane potential (V_m). The data is fitted with equation 4.7 modified from the Woodhull model. Clearly, k_{+b} is voltage dependent increasing e-fold for a 30.0mV hyperpolarization. k_{+b}(0) and \(\delta_{+b}\) were 66.46 × 10^3 s\(^{-1}\) M\(^{-1}\) and 0.44, respectively. Whilst the value for the association rate at 0mV predicted from analysis of
Figure 4.7.

Concentration and voltage-dependence of mean open time. A, dependence of the mean open time (cmot) on membrane potential ($V_m$). The symbols represent the mean ± SEM for $n = 3 - 4$ cells. Data are from cell-attached patches bathed in 140K+ with 70K+ and 100µM Ba$^{2+}$ in the patch pipette. B, plot of 1/cmot versus membrane potential ($V_m$). The symbols represent the mean ± SEM for $n = 3 - 4$ cells. Data are from cell-attached patches, bathed in 140K+ with 70K+ and 100, 200 or 300µM Ba$^{2+}$ in the patch pipette, in response to a 97mV hyperpolarizing step. The data were fitted with a linear regression of the form $1/cmot = k_+\text{[Ba}^{2+}\text{]} + k_c$. 
Figure 4.8.

Voltage-dependency of the association rate constant ($k_{\text{b}}$) for block of $K_{ir.2.2}$ by Ba$^{2+}$. Semilogarithmic plot of the association rate constant obtained from analysis of microscopic currents (filled symbols) and macroscopic currents (open symbols) versus membrane potential ($V_m$). The data are fitted with equation 4.7 modified from the Woodhull model, where for an e-fold change in $k_b$, there are 30.0 and 28.2mV changes in membrane potential, respectively.
Macroscopic currents is smaller \((k_{\text{m}}(0) = 44.81 \times 10^3 \text{ s}^{-1} \text{ M}^{-1})\) than that predicted from analysis of microscopic currents, the difference is only slight and well within experimental error. Thus, the values of \(k_{\text{m}}(0)\) and \(\delta_{\text{m}}\) obtained from the analysis of single channel currents are in close agreement with those obtained from analysis of macroscopic currents (see section 4.3.3).

### 4.3.5. Effects of mutation L148F in \(K_{\text{ir}}2.2\) and mutation F147L in \(K_{\text{ir}}2.1\) on \(\text{Ba}^{2+}\) blockade.

The \(K_d(0)\) for block of \(K_{\text{ir}}2.1\) by \(\text{Ba}^{2+}\) has been reported by two separate groups as 62 \((\text{Shieh} \text{ et al., 1998})\) and 131 \(\mu\text{M}\) \((\text{Alagem} \text{ et al., 2001})\), respectively. With a \(K_d(0)\) of approximately 14\(\mu\text{M}\), \(K_{\text{ir}}2.2\) appears to have a higher affinity for \(\text{Ba}^{2+}\). However, \(\text{Ba}^{2+}\) block also depends on the extracellular \(K^+\) concentration and a meaningful comparison is possible only with identical \(K^+\) concentrations. Thus, block of \(K_{\text{ir}}2.1\) wild-type currents by \(\text{Ba}^{2+}\) was also investigated. Furthermore, block of the mutant channels \(K_{\text{ir}}2.2\) L148F and \(K_{\text{ir}}2.1\) F147L by \(\text{Ba}^{2+}\) was also investigated to determine whether the single difference in the P-region sequences of \(K_{\text{ir}}2.2\) and \(K_{\text{ir}}2.1\) accounts for the difference in affinity for \(\text{Ba}^{2+}\).

Block of wild-type and mutant \(K_{\text{ir}}2.2/K_{\text{ir}}2.1\) channels is shown in Figure 4.9. As for \(K_{\text{ir}}2.2\) wild-type, \(\text{Ba}^{2+}\) blocks \(K_{\text{ir}}2.1\) wild-type and mutant \(K_{\text{ir}}2.2/K_{\text{ir}}2.1\) channels in a concentration-, voltage- and time-dependent manner. The currents shown in figure 4.9 were obtained in the absence or presence of 100\(\mu\text{M} [\text{Ba}^{2+}]_o\) in response to a single voltage step of -97\text{mV}.

Steady-state fractional currents were examined as described in section 4.3.2. In the presence of 10\(\mu\text{M} \text{Ba}^{2+}\), the steady-state fractional currents remaining at -77\text{mV} for \(K_{\text{ir}}2.1\) (0.44 ± 0.06; \(n = 6\)) were larger than for \(K_{\text{ir}}2.2\) (0.10 ± 0.01; \(n = 6\)). Mutation F147L in \(K_{\text{ir}}2.1\) resulted in a significant reduction in the steady-state fractional currents remaining at -77\text{mV} in the presence of 10\(\mu\text{M} \text{Ba}^{2+}\) (0.14 ± 0.03, \(n = 5\); \(P < 0.05\)), while mutation L148F in \(K_{\text{ir}}2.2\) had no significant effect (0.16 ± 0.03, \(n = 3\); \(P > 0.05\)).

Figure 4.10A shows dose-response curves for \(K_{\text{ir}}2.1/K_{\text{ir}}2.2\) wild-type and mutant channels at -77\text{mV} fitted with equation 4.3. At -77\text{mV}, \(K_{\text{ir}}2.2\) and \(K_{\text{ir}}2.1\) were half-blocked by 1.13\(\mu\text{M}\) and 8.52\(\mu\text{M}\), respectively. \(K_{\text{ir}}2.2\) L148F required a similar concentration to \(K_{\text{ir}}2.2\) wild-type (2.34\(\mu\text{M} \text{Ba}^{2+}\)) for half-blockage at -77\text{mV}, whilst mutation F147L in \(K_{\text{ir}}2.1\) reduced the concentration required for half-blockage to only 1.77\(\mu\text{M}\).
Figure 4.9.

**Block of wild-type and mutant Kir2.2/Kir2.1 channels by Ba^{2+}**. Representative current recordings from Kir channels, elicited in response to a single voltage step of $-97\text{mV}$ in the absence and presence of 100$\mu$M [Ba^{2+}]_o. A, Kir2.2 wild-type. B, Kir2.1 wild-type. C, Kir2.2 L148F. D, Kir2.1 F147L.
Figure 4.10.

Voltage- and concentration-dependence of the steady-state block of $K_{v2.2}/K_{v2.1}$ wild-type and mutant currents by $Ba^{2+}$. A, semilogarithmic plot of the steady-state current, obtained in the presence of $Ba^{2+}$, plotted as a fraction of that obtained under control conditions against $Ba^{2+}$ concentration for $K_{v2.2}/K_{v2.1}$ wild-type and mutant channels at $-77mV$. The symbols represent the mean ± SEM, where larger than the symbol, for $n = 1 - 7$ cells. Solid red circles, $K_{v2.2}$ wild-type; solid blue squares, $K_{v2.1}$ wild-type; open red circles, $K_{v2.2}$ L148F; open blue squares, $K_{v2.1}$ F147L. The curves were fitted to the Hill-Langmuir equation (Equation 4.3) with the hill slope constrained to 1. Inset, simulated fits based on fits of actual data shown in main part of figure. B, semilogarithmic plot of the voltage-dependence of $K_d(V)$ for $K_{v2.2}/K_{v2.1}$ wild-type and mutant currents. Symbols as in part A. The data are fitted to the Woodhull equation (Equation 4.4).
Figure 4.10B shows the relationship between the $K_d$ (derived in plots in figure 4.10A) versus membrane potential ($V_m$). The data were fitted with equation 4.4. The equilibrium dissociation constant ($K_d(0)$) for Kir2.1 was higher at 201$\mu$M than for Kir2.2 (13.68$\mu$M) and $\delta$ was 0.52 ($\delta$ for Kir2.2 = 0.40). The values of $K_d(0)$ and $\delta$ for Kir2.1 are in close agreement with previously reported values ($K_d(0) = 165 \mu M$, $\delta = 0.54$), where block of Kir2.1 by Ba$^{2+}$ was measured under similar conditions (see Thompson et al., 2000a). Mutation F147L in Kir2.1 affected both the affinity and voltage dependence of the steady-state block by Ba$^{2+}$ reducing $K_d(0)$ and $\delta$ to 15.54$\mu$M and 0.35, respectively. In contrast, mutation L148F in Kir2.2 had no effect ($K_d(0) = 20.20\mu$M, $\delta = 0.33$).

Figure 4.11A shows a plot of $\tau_{\text{block}}$ versus membrane potential ($V_m$) in the presence of varying [Ba$^{2+}]_o$. The blocking time constants for Kir2.2/Kir2.1 wild-type and mutant channels decreased exponentially with increasing hyperpolarization having similar voltage dependencies. $\tau_{\text{block}}$ decreased e-fold for a 26.9 ± 1.08 mV (n = 7), 33.8 ± 3.3 mV (n = 13), 29.4 ± 0.5 mV (n = 7), and 29.0 ± 0.8 mV (n = 9) hyperpolarization for Kir2.2, Kir2.1, Kir2.2 L148F and Kir2.1 F147L respectively, in the presence of 100$\mu$M [Ba$^{2+}]_o$.

Blockage by 100$\mu$M external Ba$^{2+}$ at −97mV reached equilibrium faster ($P < 0.05$; ANOVA followed by Duncan’s Multiple Range Test) in Kir2.2 ($\tau_{\text{block}} = 7.5 \pm 0.3$ms; n = 6) than in Kir2.1 ($\tau_{\text{block}} = 26.0 \pm 1.9$ms; n = 12). Mutation L148F in Kir2.2 resulted in a significant increase ($P < 0.05$) in $\tau_{\text{block}}$ by 100$\mu$M Ba$^{2+}$ ($\tau_{\text{block}} = 25.9 \pm 2.6$ms; n = 7) at −97mV.

However, the reverse mutation F147L in Kir2.1 had no significant effect on $\tau_{\text{block}}$ (25.0 ± 1.8ms; n = 9).

Figure 4.11B shows a plot of $k_{+b}$ and $k_b$ versus membrane potential ($V_m$) for wild-type and mutant Kir2.2/Kir2.1 channels. The data were fit with equations 4.7 and 4.8, respectively. $k_{+b}(0)$ was 45.13 $\times 10^3$ M$^{-1}$ s$^{-1}$ for Kir2.2 wild-type and 21.17 $\times 10^3$ M$^{-1}$ s$^{-1}$ for Kir2.1 wild-type. Mutation L148F in Kir2.2 reduced $k_{+b}(0)$ to 16.99 $\times 10^3$ M$^{-1}$ s$^{-1}$, mutation F147L in Kir2.1 having no significant effect ($k_{+b} = 15.42 \times 10^3$ M$^{-1}$ s$^{-1}$). $\delta_{+b}$ was similar for both wild-type and mutant channels (Kir2.2 = 0.45; Kir2.1 = 0.41; Kir2.2 L148F = 0.37; Kir2.1 F147L = 0.42).

As for Kir2.2 wild-type, the voltage dependence of the $K_d$ of Ba$^{2+}$ block in Kir2.1 wild-type,Kir2.2 L148F and Kir2.1 F147L is predominantly due to the voltage-dependence of the
Figure 4.11

Voltage dependency of the time constant ($\tau_{\text{block}}$) and association ($k_{+b}$) and dissociation ($k_{-b}$) rate constants for block of $K_{ir}2.2/K_{ir}2.1$ wild-type and mutant channels by $\text{Ba}^{2+}$.

A, Voltage-dependency of the time constant for the onset of blockade, $\tau_{\text{block}}$, in the presence of 100$\mu$M $[\text{Ba}^{2+}]_o$. The symbols represent the mean ± SEM, where larger than the symbol, for $n = 3$ – 13 cells. **Solid red circles**, $K_{ir}2.2$ wild-type; **solid blue squares**, $K_{ir}2.1$ wild-type; **open red circles**, $K_{ir}2.2$ L148F; **open blue squares**, $K_{ir}2.1$ F147L. The data were fitted with a linear regression of the form $1/\tau_{\text{block}} = k_{+b}[\text{Ba}^{2+}] + k_{-b}$. B, Semilogarithmic plot of $k_{+b}$ and $k_{-b}$ against membrane potential ($V_m$) for $K_{ir}2.2/2.1$ wild-type and mutant channels. Symbols as in part A. The data were fitted with equations modified from the Woodhull model (equations 4.7 and 4.8), though $k_{-b}$ is fitted on the assumption that it is independent of voltage.
association rate constant $k_{+b}$. $k_b$ showed little voltage dependence and could be well fit by assuming that it is independent of voltage (see figure 4.11B). $k_{b}(0)$ was then 0.89$s^{-1}$ and 1.97$s^{-1}$ for $K_{ir}$2.2 and $K_{ir}$2.1, respectively. Mutations L148F in $K_{ir}$2.2 and FL47L in $K_{ir}$2.1 lowered $k_{b}(0)$ to 0.46$s^{-1}$ and 0.38$s^{-1}$, respectively.

5.4.6. Effect of mutation L148F in $K_{ir}$2.2 and mutation F147L in $K_{ir}$2.1 on unitary conductance

The results presented above show that the amino acid at position 148/147 in the pore of $K_{ir}$2.2 and $K_{ir}$2.1 partly controls the rate at which Ba$^{2+}$ ions enter the pore and also affects the channel affinity for Ba$^{2+}$. Single channel currents were also measured for $K_{ir}$2.2/$K_{ir}$2.1 wild-type and mutant channels to examine whether this single difference between the pores of $K_{ir}$2.2 and $K_{ir}$2.1 has a similar effect on K$^+$ permeation, and is therefore responsible for the differences in unitary conductance exhibited by the two channels.

Figure 4.12 shows representative, single channel current traces recorded using the inside-out patch configuration, from CHO cells expressing $K_{ir}$2.2 wild-type, $K_{ir}$2.1 F147L, $K_{ir}$2.2 L148F and $K_{ir}$2.1 wild-type channels at -160, -120, -80 and -40mV. As for macroscopic currents, single channel currents were observed predominantly in the inward-going direction exhibiting strong inward rectification.

Figure 4.13 shows a plot of unitary conductance versus membrane potential for wild-type and mutant $K_{ir}$2.2/$K_{ir}$2.1 channels. Slope conductances were determined by regression fits of the form:

$$i = \gamma(V - E_{rev}) \quad \text{(Equation 4.11)}$$

where $\gamma$ is the unitary current and $E_{rev}$ is the predicted reversal potential, which in this case has been constrained to 9mV.

The slope conductance (figure 4.13) for $K_{ir}$2.2 channels, with 200mM [K$^+$]o in the pipette and 140mM [K$^+$], in the bath, was higher ($\gamma = 36.4 \pm 1.2$pS; mean ± SEM; n = 3), than for $K_{ir}$2.1 wild-type channels ($\gamma = 25.0 \pm 0.3$pS; mean ± SEM; n = 3). These values were not significantly altered (P > 0.05; ANOVA followed by Duncan’s Multiple Range Test) by the mutation F147L in $K_{ir}$2.1 ($\gamma = 26.8 \pm 0.4$pS; mean ± SEM; n = 3) or by the mutation L148F in $K_{ir}$2.2 ($\gamma = 36.0 \pm 0.8$pS; mean ± SEM; n = 3).
Figure 4.12.

Comparison of unitary conductance for wild-type and mutant $K_{ir}$ channels. Single channel recordings obtained in 140mM $[K^+]_i$ and 200mM $[K^+]_o$ at -160mV (A), -120mV (B), -80mV (C) and -40mV (D) from $K_{ir}$2.2 wild-type, $K_{ir}$2.1 F147L, $K_{ir}$2.1 wild-type and $K_{ir}$2.2 L148F. Channel openings are represented by pulses of inward current seen as downward deflections from the baseline (solid line).
Figure 4.13.

**Current-voltage relationship for Kir wild-type and mutant channels.** Plot of single channel current amplitude versus membrane potential ($V_m$) for $K_{ir}$2.2 wild-type (*solid red circles*), $K_{ir}$2.1 wild-type (*solid blue squares*), $K_{ir}$2.2 L148F (*open red circles*) and $K_{ir}$2.1 F147L (*open blue squares*). The data are fit with a linear regression of the form $i = \gamma (V - E_{rev})$ where $E_{rev}$ is the predicted reversal potential, constrained to 9mV. The symbols represent the mean ± SEM, where larger than the symbol, for $n = 3$ cells.
4.4. Discussion

The results presented in this chapter clearly show that block of Kir2.2 currents, expressed in CHO cells, by Ba$^{2+}$ occurs in a concentration-, time- and voltage-dependent manner. Standen and Stanfield (1978a) were among the first to report the nature of Ba$^{2+}$ block in inwardly rectifying potassium channels. They predicted that Ba$^{2+}$ blocks the native inward rectifier in frog skeletal muscle by binding to a site within the membrane, fitting their results by assuming a one-to-one binding of Ba$^{2+}$ to the channel. Recently, X-ray crystallography studies of the KcsA potassium channel have confirmed that Ba$^{2+}$ binds at a single location just below the selectivity filter, around T141 in Kir2.1 (Jiang & MacKinnon, 2000).

For Kir2.2, data obtained at the single channel level supported observations obtained at the macroscopic level. As expected for a channel blocker, application of extracellular Ba$^{2+}$ led to a decrease in the mean open time. A similar effect in Kir1.1b channels has been reported previously, although Chepilko et al. (1995) were able to carry out more extensive characterization of Ba$^{2+}$ block in Kir1.1b at the single channel level, including analysis of closed times and open probability, because they obtained patches containing only one channel opening. They found that not only did Ba$^{2+}$ decrease the mean open time but also increased the relative number of ‘long closures’. Together, these effects resulted in a decreased open probability of the channel. They proposed that Ba$^{2+}$ reduced the open probability by introducing a new open, blocked state. However, this putative blocked state displayed similar time constants to the long closed states also seen under control conditions, and therefore could not be distinguished from such ‘intrinsic’ closed states. Subsequently, Choe et al. (1998) have proposed that the long-lived closures seen under control conditions were in fact a result of block by contaminating levels of divalent cations.

Analysis of the steady-state block of Kir2.2 and Kir2.1 by Ba$^{2+}$ confirmed that Kir2.2 exhibits a higher affinity for Ba$^{2+}$ than Kir2.1. Kir2.2 also exhibited a higher unitary conductance than Kir2.1 when recorded under similar conditions (see also Takahashi et al., 1995). The P-regions of Kir2.2 and Kir2.1 are virtually identical with the exception that leucine 148 in Kir2.2 is replaced by phenylalanine in Kir2.1. In this study, it was questioned whether this single difference could account for the differences in Ba$^{2+}$ block and unitary conductance exhibited by these two channels.

Mutation L148F in Kir2.2 resulted in an increase in the time constant for block by Ba$^{2+}$ ($\tau_{block}$) and a reduction in the association rate constant, giving values that closely resembled those
seen in Kir2.1, without affecting the affinity for Ba$^{2+}$. However, the reverse mutation F147L in Kir2.1 had no detectable effect on either $\tau_{\text{block}}$ or the association rate constant, yet increased channel affinity for Ba$^{2+}$ towards that found in Kir2.2.

The effect of mutation L148F in Kir2.2 can be accounted for if the binding of Ba$^{2+}$ is unaffected but the rate of movement between the external solution and its binding site is slowed by the increment of an energy barrier to ion movement. In contrast, the mutation F147L in Kir2.1 appears to increase channel affinity for Ba$^{2+}$ by reducing the off rate. The dissociation rate constants ($k_{\text{off}}$) could not be accurately predicted from analysis of the kinetics of Ba$^{2+}$ blockage. However, the association rate constant for Ba$^{2+}$ block in Kir2.1 F147L was similar to that in Kir2.1 wild-type suggesting that F147L increases the affinity for Ba$^{2+}$ by reducing the rate at which Ba$^{2+}$ dissociates from the channel. This would be consistent with a localized change at the binding site.

Using the blocking rate constant at 0mV, $k_{\text{on}}(0)$, and the Eyring rate theory (Hille, 2001), the energy barrier for the binding of Ba$^{2+}$ at 0mV can be estimated, as previously described by Alagem et al. (2001), and used to illustrate the effect of mutations L148F in Kir2.2 and F147L in Kir2.1. Figure 4.14 shows energy profiles for Ba$^{2+}$ passing through Kir2.2/Kir2.1 wild-type and mutant channels. The blocking rate $k_{\text{on}}$ is related to the difference between the Gibbs free energy of the state in which the ion enters the channel (which is set to be 0 at 1M concentration), and the barrier height, $\Delta G_{\text{on}}$, and can be described by the following equation:

$$k_{\text{on}} = \nu \exp \left( - \frac{\Delta G_{\text{on}}}{RT} \right)$$

(Equation 4.12)

where $\nu$ is the oscillation frequency, and $R$ and $T$ have their usual meanings. In a previous study of Ba$^{2+}$ blockade of Kir2.1, a value of $10^9$ s$^{-1}$ was assigned to $\nu$ owing to its prior use in describing the Mg$^{2+}$ block of Ca$^{2+}$ and NMDA channels (see Alagem et al., 2001). The same value has been used herein.
The depth of the energy well, $\Delta G^\circ$, was calculated for 0mV using:

$$\Delta G^\circ = RT \ln K_d$$  \hspace{1cm} (Equation 4.13)

where $K_d$ is the equilibrium dissociation constant for $\text{Ba}^{2+}$ binding at 0mV obtained from analysis of steady-state fractional currents. The barrier-well energy profile clearly illustrates how mutation L148F in Kir2.2 raises the energy barrier for $\text{Ba}^{2+}$ entry, whilst the mutation F147L in Kir2.1 has no effect on the energy barrier but appears to stabilize the binding site, since the $\Delta G^\circ$ value is more negative in this mutant channel. Since $\text{Ba}^{2+}$ binds at a site internal to the selectivity filter, around the position of T141 in Kir2.1 (Jiang & MacKinnon, 2000), it is unlikely that F147L contributes directly to the binding site. Thus, the effect of substituting leucine for phenylalanine at position 147 in Kir2.1 may be mediated allosterically, by inducing a conformational change that interferes with the binding of $\text{Ba}^{2+}$.

The F147L mutation in Kir2.1 also reduced the voltage dependence of the steady-state $\text{Ba}^{2+}$ block compared with Kir2.1 wild-type channels. There are two possible explanations for this. Firstly, mutation of phenylalanine to leucine affects the channel structure in such a way that the $\text{Ba}^{2+}$ ion experiences a different electric field. However, this seems unlikely because the voltage dependence of the association and dissociation rate constants were similar for Kir2.1 wild-type and Kir2.1 F147L. Alternatively, this mutation may allow $\text{Ba}^{2+}$ permeation. Indeed, a slight monotonic increase in $k_b$ with increasing hyperpolarization can be seen for Kir2.1 F147L, suggesting that $\text{Ba}^{2+}$ can permeate the Kir2.1 F147L channel at the voltages tested (figure 4.11B).

The different effects of mutating these residues on $\text{Ba}^{2+}$ blockage suggest the involvement of other residues aside from L148 in Kir2.2 and F147 in Kir2.1 in regulating channel block by $\text{Ba}^{2+}$. Analysis of the single channel current amplitude of Kir2.2/Kir2.1 wild-type and mutants also showed that this residue does not affect unitary conductance. Thus, it appears that other residues aside from F147/L148 also determine the single channel conductance in Kir2.2 and Kir2.1. The lack of effect of mutating leucine to phenylalanine in Kir2.2, and of the reverse mutation in Kir2.1, on single channel conductance suggests that this residue may, like the analogous residue in KcsA (Doyle et al., 1998b), orient its side chain away from the pore (figure 4.15). However, this finding contrasts with results from scanning cysteine mutagenesis studies, which have shown that the side chain of F147 in Kir2.1 is accessible to
Figure 4.15

**Schematic representation of the tetrameric structure of Kᵦ2.2 viewed from the extracellular side.** Model generated by comparative modelling using the crystal structure of KcsA (Doyle et al., 1998b) as a template and a sequence alignment based on mutational analysis of Kᵦ2.1 (Minor et al., 1999). The side chains of L148 are coloured yellow and can be seen oriented away from the pore. The pink sphere represents a K⁺ ion. Restraints were applied with MODELLER (Sali & Blundell, 1993) to represent (i) four-fold symmetry and (ii) the salt bridge between glutamate 139 and arginine 149 in adjacent sub-units (Yang et al., 1997).
block by extracellular Ag⁺, suggesting that the side chain of F147 is exposed to the pore lumen (Dart et al., 1998b; Kubo et al., 1998). There are, however, several drawbacks with the cysteine scanning mutagenesis approach to identifying pore-lining residues. For example, it is possible that any mutation may uncover a hidden reactivity at other residues. Furthermore, non-cysteine side chains may contribute to the coordination of Ag⁺ ions (see Holmgren et al., 1998). Due to the limitations of this technique it is only possible to conclude tentatively that residues that induce Ag⁺ sensitivity, when substituted by cysteine, are likely to interact directly with Ag⁺ and to face the pore.

Previous studies have shown that residues in several regions affect block of Kir channels by Ba²⁺. These include residues at the outer mouth of the pore in the M1-P-region linker (see for example Navaratnam et al., 1995; Alagem et al., 2001; Murata et al., 2002), in the P-region (see for example Zhou et al., 1996; Sabirov et al., 1997; Lancaster et al., 2000; Alagem et al., 2001) and in the M2 transmembrane domain (see Thompson et al., 2000a). Residues in the M1-P-region and P-region-M2 extracellular linkers have also been shown to determine unitary conductance in Kir channels (Navaratnam et al., 1995; Repunte et al., 1999; Choe et al., 2000), though residues in the P-region, N- and C-termini and the M2 transmembrane domains also affect unitary conductance (see for example Taglialatela et al., 1994; Yang et al., 1995a; Choe et al., 2000; Kubo & Murata, 2001).

What region of the channel is likely to determine the differences in single channel conductance and Ba²⁺ blockage in Kir2.2 and Kir2.1? Comparison of the amino acid sequences of Kir2.2 and Kir2.1 reveals several differences in the M1-P-region extracellular linker. Thus, in Kir2.2 the start of the pore helix is glycine-phenylalanine-methionine (G128-F129-M130), whilst in Kir2.1 it is serine-phenylalanine-threonine (S127-F128-T129). The M1-P-region extracellular linker in Kir2.2 also contains leucine, glutamine and histidine at positions 124, 125 and 127, respectively, which are replaced by serine, glutamate and asparagine in Kir2.1. The differences in the side-chain polarity of the residues in this region might be expected to influence ion conduction, though these residues are far from the channel entrance. However, Repunte et al. (1999) have suggested that residues in this region determine unitary conductance in Kir6.1 and Kir6.2 by modifying the conformation of the permeation pathway via interaction with an arginine, which is highly conserved in Kir channels and is crucial in stabilizing the ion conduction pore (see Yang et al., 1997). This may also be the case in Kir2.2 and Kir2.1.
Further support that residues in this region affect the ion conduction pore is provided by the difference in unitary conductance exhibited by chick and mouse homologues of Kir2.1. The chick homologue (cIRK1), which contains a non-charged glutamine at position 125 (Q125) in the M1-P-region extracellular linker, displays a reduced single channel conductance and a reduced sensitivity to block by Ba\(^{2+}\) compared with the mouse homologue (IRK1), which contains a negatively charged glutamate (E125) at the corresponding position (Navaratnam et al., 1995). Substitution of a glutamate for the glutamine in cIRK1 increased both the single channel conductance and sensitivity to Ba\(^{2+}\). Others have confirmed that the reverse mutation, E125Q, reduces channel affinity for Ba\(^{2+}\) and unitary conductance (Alagem et al., 2001; Murata et al., 2002). Given the above findings, it is perhaps surprising that Kir2.2, which displays the higher unitary conductance and a higher affinity for Ba\(^{2+}\) contains a non-charged glutamine at this position, whilst Kir2.1, which has the lower unitary conductance and is less sensitive to block by Ba\(^{2+}\), contains a negatively charged glutamate. However, it is likely that other residues in this region, which clearly differ as highlighted above, are also involved in determining the permeation properties of the ion conduction pore in Kir2.2 and Kir2.1.

Further differences in the amino acid sequences of Kir2.2 and Kir2.1 are also apparent in the M2 transmembrane domains. Thompson et al. (2000a) have found that a serine in M2 regulates the ability of Ba\(^{2+}\) to bind. Thus, the mutation S165L in Kir2.1 lowered channel affinity for Ba\(^{2+}\). This mutation has also been shown to reduce the affinity for channel blockage by Cs\(^{+}\) and abolishes Rb\(^{+}\) blockage. It has been suggested that serine 165 forms either a binding site for these ions or provides support for such sites (see Thompson et al., 2000b; see also Fujiwara & Kubo, 2002).

Residues in the M2 transmembrane domain have also been found to affect unitary conductance. In Kir4.1 and Kir1.1, the residues analogous to D172 in Kir2.1, have been shown to regulate unitary conductance (Xu et al., 2000b). In Kir4.1, which displays a single channel conductance of 24pS, this residue is a glutamate (E158). In contrast, in Kir1.1, which contains an asparagine (N171) at the analogous position, the single channel conductance is 38pS under similar recording conditions. Mutation of glutamate to asparagine (E158N) in Kir4.1 increased the single channel conductance to 35pS, whilst the reverse mutation in Kir1.1 reduced the single channel conductance to 27pS (Xu et al., 2000b).
Although the serine and aspartate are conserved in Kir2.2 and Kir2.1, these channels differ at a neighbouring position. Thus, in Kir2.2 the residue at position 164 is an alanine, whilst the analogous residue in Kir2.1 is a phenylalanine. The residues at the analogous positions in Kir2.3 and Kir2.4, which display a lower unitary conductance (~15pS under similar recording conditions) and a lower affinity for Ba\(^{2+}\) (see for example Morishige et al., 1994; Töpert et al., 1998; Liu et al., 2001b), are also different. Thus, in Kir2.3 this residue is a valine, whilst in Kir2.4 it is a leucine. As suggested for S165, this residue may also support the binding site for Ba\(^{2+}\) (see Thompson et al., 2000b) and may determine the differences in unitary conductance exhibited by the members of the Kir2.0 subfamily.

Thus, residues in both the M1-P-region extracellular linker and the M2 transmembrane domains may be important in regulating unitary conductance and Ba\(^{2+}\) blockage in Kir2.2 and Kir2.1. However, without further experimentation it is impossible to conclude which of these residues might account for these differences.

In conclusion, the residue at position 148 in Kir2.2, or at position 147 in Kir2.1, does not account for the difference in unitary conductance exhibited by these two channels. However, it does account for part, but part only, of the difference in channel block by Ba\(^{2+}\). Thus, in Kir2.2 and Kir2.1, residues in regions other than the P-region must determine the differences in unitary conductance and Ba\(^{2+}\) blockage. This is consistent with previous studies in which residues in the M1-P-region and P-region-M2 extracellular linkers have been shown to be important in determining the properties of the ion conduction pore (see for example Navaratnam et al., 1995; Repunte et al., 1999).
Chapter 5

Mutation of Isoleucine 143 affects ionic selectivity and permeation in K_{ir}2.1.

5.1. Introduction

Although K_{v} channels differ from K_{ir} channels in their overall primary structure, both contain a ‘P- region’ in which the well-conserved signature sequence resides. As discussed in section 1.6, this region is important for K^{+} selectivity.

X-ray crystallography studies have shown that in the bacterial potassium channel KcsA, the selectivity filter is formed by backbone carbonyl oxygen atoms from five (T75, V76, G77, Y78 and G79, see table 5.1) amino acids including the two, conserved glycine residues of the GYG motif (Doyle et al., 1998b). With a two-transmembrane-helix topology, KcsA is similar in its overall structure to K_{ir} channels, yet shares little homology at the amino acid level. In
contrast, the amino acid sequence that forms the pore is well conserved between Kv channels and KcsA. Despite the differences in the amino acid sequence of the pore between KcsA and Kir channels, a recent study has shown that the pore of KcsA may be substituted for that of Kir2.1, the resulting chimera retaining the functional hallmarks of an inward rectifier. Likewise, the pore of KcsA could be substituted for that of Shaker such that the resulting chimera retained the functional hallmarks of a voltage-gated potassium channel (Lu et al., 2001b). These results suggest that the ion conduction pore is conserved among potassium channels.

Previous studies with the peptide toxin blockers Lq2 and agitoxin2 have also suggested that the three dimensional structures of potassium channel pores are similar despite the lack of conservation in the P-region sequences. Thus, Lq2 has been shown to inhibit a variety of potassium channels including some members of the Kv, KCa and Kir channel families (see for example Lu & MacKinnon, 1997), whilst agitoxin2 inhibits both eukaryotic Kv channels and the prokaryotic potassium channel KcsA (MacKinnon et al., 1998). Renisio et al. (1999) have suggested that the structure of the external end of the pore serves as a frame to hold the conserved signature sequence, allowing it to form the selectivity filter.

In this chapter, I present results from experiments in which I have investigated the role of an isoleucine at position 143 of Kir2.1 (see Table 5.1) in ionic selectivity. This residue, which resides in the potassium channel signature sequence, corresponds to one of the five residues (V76) implicated in stabilizing the backbone of oxygen rings in the selectivity filter of KcsA (Doyle et al., 1998b). Mutagenesis studies in K2.1 have shown the analogous residue (V374) to be an important determinant of K⁺ and Rb⁺ selectivity (Taglialatela et al., 1993). If the molecular basis of selectivity is conserved among all potassium channels, the effects of mutating I143 in Kir2.1 on ionic selectivity should be comparable to the previously reported effects of mutating V374 in K2.1 (Taglialatela et al., 1993).

Ionic selectivity was determined by measuring permeability ratios for Rb⁺ and K⁺. Whilst Rb⁺ permeates Kv channels it blocks Kir channels including the native Kir channels of frog skeletal muscle and starfish eggs (see for example Adrian, 1964; Hagiwara & Takahashi, 1974). This block occurs in a steeply voltage-dependent manner (Standen & Stanfield, 1980), increasing initially with hyperpolarization of the membrane then falling with further hyperpolarization. Although Rb⁺ blocks Kir channels it can still be substituted as a test ion
Table 5.1.

**Alignment of the P-region sequences of Kir2.1, KcsA and several Kv channels.** Residues of the signature sequence are numbered and are marked in bold. Isoleucine 143 in Kir2.1 and its analogous residues in the other channels are highlighted in red. The single amino acid code is given in table 0.1.
when measuring the relative permeability of the channel since block of a channel does not alter its reversal potential (Hille, 2001).

### 5.2. Methods

Whole cell currents were recorded with 140mM K⁺ in the patch pipette and 70mM K⁺ or Rb⁺ as the sole external permeant cationic species. Permeability ratios (P_Rb/P_K) for each cell were calculated using a modified Goldman-Hodgkin-Katz equation (see Hille, 2001):

\[
\Delta E_{rev} = E_{rev,Rb} - E_{rev,K} = \frac{RT}{zF} \ln \frac{P_{Rb}[Rb]}{P_K[K]}_o
\]

(Equation 5.1)

which can be rearranged to give

\[
\frac{P_{Rb}[Rb]}{P_K[K]}_o = \exp \left( \frac{F \Delta E_{rev}}{RT} \right)
\]

(Equation 5.2)

where \(\Delta E_{rev}\) is the shift in reversal potential, \(E_{rev,Rb}\) and \(E_{rev,K}\) are the reversal potentials for Rb⁺ and K⁺, respectively, and \(F, R\) and \(T\) have their usual meaning. The reversal potential was measured by fitting a fifth order polynomial function of the form:

\[
f(x) = a + bx + cx^2 + dx^3 + ex^4 + fx^5
\]

(Equation 5.3)

to a region, approximately 15 – 25 mV either side of where the current intersected the abscissa, of the I-V relationship.

### 5.3. Results

#### 5.3.1. Ionic selectivity of K_ir2.1 wild-type

Figure 5.1A shows representative whole-cell recordings from CHO cells expressing K_ir2.1 bathed in equimolar K⁺ and Rb⁺ containing solutions. As expected, K_ir2.1 exhibited substantial inward currents in 70mM K⁺. As shown previously, Rb⁺ blocked K_ir2.1 currents (see for example Abrams et al., 1996; Reuveny et al., 1996).

The corresponding mean, steady-state current-voltage (I-V) relationships are shown in figure 5.1B, and in figure 5.1C are expanded for clarity. When 70mM K⁺ is replaced with equimolar
Figure 5.1.

Ionic selectivity of Kir2.1. A, Representative current recordings of Kir2.1 wild-type with 70mM [K\(^+\)]\(_o\) or 70mM [Rb\(^+\)]\(_o\). Currents were elicited in response to 50ms voltage steps from a holding potential of -17mV, to test potentials ranging from +63mV to -132mV, in 5mV decrements. Every fifth record is shown for clarity. B, normalized, steady-state current-voltage relationships. The symbols represent the mean ± SEM, where larger than the symbol, for n = 14 cells. Closed circles, 70mM [K\(^+\)]\(_o\); open triangles, 70mM [Rb\(^+\)]\(_o\). C, expansion of current-voltage relationships. Symbols as in B.
Rb+, $E_{rev}$ shifts $-17.6 \pm 1.3$ mV ($n = 14$; mean $\pm$ SEM), giving $P_{Rb}/P_K = 0.51 \pm 0.02$.

Although this permeability ratio differs somewhat from previously reported values (0.68, Abrams et al., 1996; Reuveny et al., 1996), similar values have also been reported (0.49, Choe et al., 2000; 0.57, Thompson et al., 2000b).

### 5.3.2. Ionic selectivity of I143 mutants

Next, the contribution of isoleucine 143 (I143) to ionic selectivity was investigated by mutation. Figures 5.2A, B and C illustrate representative current traces for mutants in which isoleucine 143 was replaced by valine, leucine or threonine, respectively. The corresponding $I-V$ relationships, which are expanded for clarity, are shown below. The values for the shift in reversal potential in I143V, I143L and I143T following substitution of 70mM Rb$^+$ for 70mM K$^+$ are given in Table 5.2. Substitution of isoleucine by leucine (I143L) or valine (I143V) raised the relative Rb$^+$ permeabilities, although the effect was less dramatic for I143V ($P_{Rb}/P_K = 0.62 \pm 0.03, n = 9$) than for I143L ($P_{Rb}/P_K = 0.82 \pm 0.01, n = 12$). In contrast, substitution by the polar residue threonine (I143T) enhanced K$^+$ selectivity ($P_{Rb}/P_K = 0.44 \pm 0.01, n = 11$).

### 5.3.3. I143C and I143S mutants are non-functional

On mutation of isoleucine 143 to cysteine, the mutant channels failed to yield currents ($n = 13$, not shown). However, I143C mutant channels could be rescued on bathing cells in an external solution containing 10mM dithiothreitol (DTT; figure 5.3A). When 70mM K$^+$ was replaced with equimolar Rb$^+$, $E_{rev}$ shifted $-18.9 \pm 0.07$ mV ($n = 9$; mean $\pm$ SEM; see for example figure 5.3B) giving $P_{Rb}/P_K = 0.52 \pm 0.07$, similar to that for K$_{ir}2.1$ wild-type.

The rescue of I143C mutant channels with the reducing agent DTT suggests that a disulphide bond may have formed between the thiol groups of the cysteine introduced at position 143 and another cysteine in either the same or adjacent subunits, or between two of the newly introduced cysteines in adjacent subunits. In the absence of any reducing agent this could render the channel non-conducting. This has been observed in K$_{ir}2.1$ channels following the substitution of cysteine for isoleucine 379 (Zhang et al., 1996), which is located near the outer end of the narrow ion conduction pore (the analogous residue to I379 in K$_{ir}2.1$ is R148). The K$_{ir}2.1$ mutant I379C displayed varying levels of current when expressed in Xenopus oocytes, which could be increased several fold after application of 1mM DTT. Furthermore, exposure to 0.1% H$_2$O$_2$ significantly reduced currents through K$_{ir}2.1$ I379C mutant channels.
**Figure 5.2.**

*Ionic selectivity of Kir2.1 I143V, I143L and I143T.* A, B and C show representative current recordings with 70mM [K+]₀ or 70mM [Rb⁺]₀ from Kir2.1 I143V, I143L and I143T, respectively. Currents were elicited in response to 50ms voltage steps from a holding potential of -17mV, to test potentials ranging from +63mV to -132mV, in 5mV decrements. Every fifth record is shown for clarity. D, E and F show normalized, steady-state current-voltage relationships for Kir2.1 I143V, I143L and I143T, respectively. The symbols represent the mean ± SEM, where larger than the symbol, for n = 9 - 12 cells. *Closed circles,* 70mM [K⁺]₀; *open triangles,* 70mM [Rb⁺]₀.
Mutagenesis studies and western blot analysis suggested that the cysteine introduced at position 379 could form an inter-subunit disulphide bridge with either a native cysteine at position 394 in the S6 transmembrane domain or with another cysteine 379 in an adjacent subunit (Zhang et al., 1996).

In this study, it was questioned whether a disulphide bond may have formed between the thiol group of the introduced cysteine at position 143 and another cysteine. If the formation of a disulphide bond between C143 and another cysteine underlies the loss of function in I143C mutant channels, mutation of I143 to a residue with a similar size to cysteine, but without the sulphur moiety, might be expected to give functional channels. To test this hypothesis, isoleucine 143 was mutated to serine. Surprisingly, substitution of serine for isoleucine 143 also generated mutant channels, which failed to yield currents under control conditions (n = 13, data not shown), but as for I143C mutant channels could be rescued by bathing cells in external DTT (Figure 5.3C). These results suggest that the loss of function in I143C mutant channels is related to the size of the residue substituted at position 143 (see section 5.3.7).

When 70mM K+ was replaced with equimolar Rb+, $E_{rev}$ shifted $-19.1 \pm 3.3$ mV (n = 9; mean ± SEM; see for example figure 5.3D), giving $P_{Rb}/P_K = 0.50 \pm 0.06$, again similar to that for $K_{ir}2.1$ wild-type.

### 5.3.4. Correlation between side chain properties and Rb+/K+ selectivity

In $K_{ir}2.1$, a correlation between the hydrophilicity of the side chain of the residue at position 374 and permeability has been shown (Taglialatela et al., 1993). Hydrophilicity is an index of solvent accessibility and is calculated as the equilibrium constant for partition of amino acid side chains between vapour and aqueous phases (Wolfenden et al., 1979). Whilst substitution of residues with hydrophobic side chains such as isoleucine and valine favoured Rb+, substitution of residues with hydrophilic side-chains such as serine and threonine favoured K+. Thus, the interaction between pore water and the side chains introduced at position 374 in $K_{ir}2.1$ channels were proposed to determine K+/Rb+ selectivity.

A similar correlation between the hydrophilicity of side-chains at position 143 and K+/Rb+ selectivity was sought in $K_{ir}$ channels. Figure 5.4 shows a plot of side-chain hydrophilicity versus Rb+/K+ permeability ratios. Data for $K_{ir}2.1$, taken from Taglialatela et al. (1993), is included in the graph for comparison. As for $K_{ir}2.1$, linear fits to all the data with the exception of leucine also show a statistically significant ($P < 0.05$) correlation between side-
**Figure 5.3.**

*Ionic selectivity of Kir2.1 I143S and I143C.* A and C show representative current recordings with 70mM [K+]₀ or 70mM [Rb⁺]₀ in the presence of 10mM extracellular DTT from Kir2.1 I143S and I143C, respectively. Currents were elicited in response to 50ms voltage steps from a holding potential of -17mV, to test potentials ranging from +63mV to -132mV, in 5mV decrements. Every fifth record is shown for clarity. B and D show representative membrane currents recorded in response to voltage ramps ranging from -60 to +20mV from Kir2.1 I143S and I143C, respectively. *Blue plots,* 70mM [K⁺]₀; *Red plots,* 70mM [Rb⁺]₀.
Figure 5.4.

**Correlation between $P_{RB}/P_K$ and hydrophilicity.** Plot of $P_{RB}/P_K$ against hydrophilicity of residues at position 143 in $K_{ir}2.1$. Data is compared with previous findings for mutants of the corresponding residue, valine 374, in $K_{v}2.1$ (data taken from Taglialatela et al., 1994). The symbols represent the mean ± SEM, where larger than the symbol, for n = 9 - 14 cells. *Red circles, $K_{ir}2.1$; Blue squares, $K_{v}2.1*.* For each channel, the straight line represents the linear regression fit to all the data with the exception of leucine. The curved line maps the 95% confidence limits. Hydrophilicity values were calculated as described by Wolfenden et al., 1979.
chain hydrophilicity and permeability in Kᵢᵣ2.1. One notable difference between Kᵥ and Kᵢᵣ channels is the permeability ratio obtained following substitution of the non-polar residue leucine. Whilst in Kᵥ channels substitution of a leucine gives a Rb⁺/K⁺ permeability ratio more like that expected from substitution of a polar residue, reflecting an enhancement in selectivity for K⁺, in Kᵢᵣ2.1 the Rb⁺/K⁺ permeability ratio reflects an enhancement in selectivity for Rb⁺. Nonetheless, the general correlation between side chain hydrophilicity of the residue at position 5 of the signature sequence (V374 in Kᵥ2.1 and I143 in Kᵢᵣ2.1) and selectivity for K⁺ and Rb⁺ is conserved between Kᵥ2.1 and Kᵢᵣ2.1.

5.3.5. Mutation of I143 does not alter Rb⁺ blockage

As mentioned in section 5.1, Rb⁺ blocks Kᵢᵣ channels. This property depends on residues located in the M2 transmembrane domains, which lie beyond the selectivity filter (Thompson et al., 2000b). In this study it was questioned whether mutations of I143 alter Rb⁺ blockage, since Rb⁺ must traverse the selectivity filter to reach its blocking site. In all I143 mutants, as for the wild-type channel, the current carried by Rb⁺ (I𝑅𝑏) was smaller than that carried by K⁺ (I𝐾). For example, in Kᵢᵣ2.1 wild-type I𝑅𝑏/I𝐾 = 0.058 ± 0.006 at -132mV. As shown previously for mutations in GYG (So et al., 2001), Rb⁺ blockage was little altered by mutations of I143 (see Table 5.2).

5.3.6. Mutations of I143 have little effect on channel macroscopic kinetics.

As described in chapter 1, gating in Kᵢᵣ channels involves the displacement of polyamine molecules from the channel pore by K⁺. Since permeant K⁺ activates Kᵢᵣ channels, it was questioned whether mutations of I143 affect such channel gating. Macroscopic current kinetics were investigated by measuring the relationships between voltage and both K⁺ chord conductance and rates of activation under hyperpolarization. For each mutant channel, the chord conductance, gᵣ, was calculated from the I-V relationships using:

\[ g_K = \frac{I_K}{V - E_K} \]  

(Equation 5.4)

where gᵣ is the chord conductance to K⁺ and V - Eᵣ is the driving force on K⁺. gᵣ was normalized to its maximum value and the normalized conductance, gᵣ⁻, was plotted against voltage.
Table 5.2. Properties of Isoleucine mutants

<table>
<thead>
<tr>
<th></th>
<th>H.I.$^\dagger$</th>
<th>Shift in $E_{rev}$</th>
<th>$P_{Rb}/P_{K}$</th>
<th>$I_{Rb}/I_{K}$</th>
<th>$V_1$</th>
<th>$k_1$</th>
<th>$V_2$</th>
<th>$k_2$</th>
<th>Dependence of $\tau_{act}$ on $V_m$ (e-fold)</th>
<th>$\tau_{act}$ at -52mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>I143</td>
<td>0.24</td>
<td>-17.6±1.3(14)</td>
<td>0.51±0.02(14)</td>
<td>0.058±0.006(14)</td>
<td>-25.2±1.5(6)</td>
<td>7.2±0.6(6)</td>
<td>2.0±1.0(6)</td>
<td>10.1±2.3(6)</td>
<td>22.8±3.4(8)</td>
<td>0.54±0.07(8)</td>
</tr>
<tr>
<td>I143L</td>
<td>0.11</td>
<td>-4.95±0.4(12)$^{***}$</td>
<td>0.82±0.01(12)$^{***}$</td>
<td>0.075±0.01(12)$^{***}$</td>
<td>-20.4±1.0(9)</td>
<td>6.3±0.7(9)</td>
<td>-8.7±3.5(9)</td>
<td>17.5±3.0(9)</td>
<td>25.0±3.1(10)</td>
<td>0.45±0.04(10)</td>
</tr>
<tr>
<td>I143V</td>
<td>0.4</td>
<td>-12.4±1.1(9)</td>
<td>0.62±0.03(9)</td>
<td>0.035±0.003(9)</td>
<td>-26.4±5.8(8)</td>
<td>11.7±1.8(8)</td>
<td>4.13±3.9(8)</td>
<td>9.4±1.4(8)</td>
<td>36.0±4.2(8)</td>
<td>0.53±0.04(8)</td>
</tr>
<tr>
<td>I143C</td>
<td>3.63</td>
<td>-18.9±0.1(9)</td>
<td>0.52±0.07(9)</td>
<td>0.097±0.02(9)</td>
<td>-22.8±1.0(9)</td>
<td>6.8±0.8(9)</td>
<td>1.0±3.5(9)</td>
<td>16.0±3.6(9)</td>
<td>20.5±2.0(9)</td>
<td>1.26±0.16(9)$^*$</td>
</tr>
<tr>
<td>I143T</td>
<td>7.27</td>
<td>-21.1±0.6(11)</td>
<td>0.44±0.01(11)</td>
<td>0.11±0.02(11)</td>
<td>-19.6±1.2(5)</td>
<td>10.1±1.8(5)</td>
<td>-3.0±8.8(5)</td>
<td>10.3±3.4(5)</td>
<td>36.4±3.2(10)</td>
<td>0.78±0.06(10)</td>
</tr>
<tr>
<td>I143S</td>
<td>7.45</td>
<td>-19.1±3.3(9)</td>
<td>0.50±0.06(9)</td>
<td>0.078±0.02(8)</td>
<td>-28.9±2.0(5)</td>
<td>6.9±1.4(5)</td>
<td>-10.8±4.2(5)</td>
<td>18.8±5.3(5)</td>
<td>25.7±1.9(9)</td>
<td>1.73±0.23(9)$^{**}$</td>
</tr>
</tbody>
</table>

$^\dagger$H.I. = Hydrophilicity Index.

Average values are given as means ± SEM with the number of experiments in parentheses. Results were compared using a Kruskal Wallis non-parametric ANOVA test followed by a Dunn’s multiple comparisons test. *P<0.05, **P<0.01, ***P<0.001
The relationship was best fitted with a double Boltzmann expression of the form:

$$g_K' = \frac{(1-b)}{1 + \exp\left(\frac{V - V_1}{k_1}\right)} + \frac{b}{1 + \exp\left(\frac{V - V_2}{k_2}\right)}$$  \hspace{1cm} (Equation 5.5)

where $b$ was allowed to vary between 0 and 1. $V_1$ and $V_2$ (mV) give the voltages at which the relative conductance $g_K = 0.5$ and $k_1$ and $k_2$ (mV) are the factors effecting the steepness of the relationship. The values found for the half-activation voltages and steepness factors are given in Table 5.2, from which it can be seen that mutation of I143 to valine, leucine or threonine has little effect on the relationship between chord conductance and membrane potential. The relationship between chord conductance and membrane potential was also similar for I143C and I143S mutants following rescue of channel function with 10mM DTT.

Hyperpolarization from $E_K$ of wild-type and mutant channels produced inward currents that increased with time (see figures 5.1, 5.2 and 5.3), a process that also became more rapid with increasing hyperpolarization. At $-52\text{mV}$, the time constant for activation ($\tau_{\text{act}}$) was $0.54 \pm 0.07$ ms ($n = 8$) in Kir2.1 wild-type and the rate of activation increased e-fold for a $22.8 \pm 3.4$ mV hyperpolarization. Little change in the voltage-dependence of activation was seen in any of the mutants (Table 5.2), though the time constant for activation at $-52\text{mV}$ was significantly slower ($P < 0.05$) for currents through Kir2.1 I143C ($\tau_{\text{act}}$ at $-52\text{mV} = 1.26 \pm 0.16$ ms, $n = 9$) and Kir2.1 I143S channels ($\tau_{\text{act}}$ at $-52\text{mV} = 1.73 \pm 0.23$ ms, $n = 9$) in the presence of 10mM extracellular DTT. Since the time constant for activation is also slower in wild-type channels with 10mM DTT in the extracellular solution (e.g. in Kir2.2 $\tau_{\text{act}} = 0.31 \pm 0.02$ ms in 70mM $K^+_{\text{o}}, n = 7$; $\tau_{\text{act}} = 0.60 \pm 0.1$ ms in 70mM $K^+_{\text{o}} + 10\text{mM DTT}, n = 4; P < 0.05$), the increase in $\tau_{\text{act}}$ in I143C and I143S was not investigated further.

5.3.7. Hypotheses for the mechanism underlying loss of function in I143C and I143S

In section 5.3.3, it was shown that on mutating isoleucine 143 to cysteine, channel function was lost. However, by bathing cells in extracellular solution containing the reducing agent dithiothreitol, channel function could be restored. It was initially hypothesized that the introduced cysteine might form a disulphide bond with either a native cysteine or with another C143 in an adjacent subunit, which in the absence of a reducing agent renders the channel non-conducting. However, mutation of isoleucine to serine also generated non-functional
channels, which could be rescued with 10mM extracellular DTT. These results suggest that the loss of function is related to the size of the residue at position 143.

To explain this loss of function, it was hypothesized that mutation of isoleucine to smaller residues such as cysteine or serine alters the conformation of the pore, which, in turn, prevents K\(^+\) permeation. These changes in conformation might be compared with those associated with C-type inactivation in K\(_v\) channels, where the selectivity filter region of the pore is thought to undergo a constriction that pinches off the permeation pathway (see section 1.2.3a). How might a conformational change in the pore structure lead to a loss of conduction in I143C and I143S mutant channels that can be restored following exposure to extracellular DTT?

One possibility is that a conformational change in the pore allows a native cysteine to form a disulphide bond with another residue in an adjacent subunit, which locks the channel in a non-conducting state. K\(_{ir}\)2.1 possesses several extracellular cysteine residues located at positions 122, 149 and 154. The cysteine at position 149 is located sufficiently close to the ion conduction pathway that the binding of only one Ag\(^+\) ion to one of four potential binding sites is sufficient to block channel current (Dart et al., 1998a). Previous studies in Shaker have shown that residues in this region move closer together during C-type inactivation. For example, cysteine substitution experiments have shown that a cysteine substituted for a threonine at position 449, which is analogous to C149 in Kir2.1, is more sensitive to block by cadmium in the C-type inactivated state, suggesting that the spatial distribution of each of the four residues at position 449 changes during C-type inactivation (Yellen et al., 1994).

Furthermore, substitution of cysteine for a methionine (position 448, equivalent to R148) located at the outer mouth of the Shaker channel pore resulted in the formation of disulphide bonds between subunits in the C-type inactivated state but not in the closed state (Liu et al., 1996). Thus, the formation of an inter-subunit disulphide bond between the cysteines at position 149 represents a plausible explanation for the loss of function in I143C and I143S mutant channels.

An alternative possibility is that a disulphide bond already exists in the wild-type channel, which is required for normal function or for normal folding. Indeed, the two cysteines located at positions 122 and 154, which are absolutely conserved throughout the inward rectifier potassium channel family, form an intra-subunit disulphide bond that is essential for correct channel assembly (Leyland et al., 1999; Bannister et al., 1999; Cho et al., 2000). Mutation of
these cysteines has been shown to abolish functional expression, even though mutant channels were expressed in the membrane. However, once assembled the disulphide bond is not essential for channel function since application of an extracellular reducing agent had no effect on normal channel function (Leyland et al., 1999; Bannister et al., 1999; Cho et al., 2000). It has been suggested that the disulphide bond helps to constrain and stabilize the P-loop and selectivity filter (Cho et al., 2000). Thus, an equally plausible explanation for the loss of function is that substitution of cysteine or serine for isoleucine alters the structure of the P-region so that the intra-subunit disulphide bond between C122 and C154, which is required for correct assembly, now disrupts channel function. In either case, the disulphide bond would require breaking to allow K⁺ permeance. These hypotheses were tested as described below.

5.3.8. Mutation of I143 to Alanine gives functional channels with a reduced conductance

First, to test whether substitution of other small residues at position 143 in Kir2.1 result in a loss of channel function, isoleucine was mutated to alanine. Although mutation of isoleucine 143 to alanine was not fatal (figure 5.5A), currents were considerably smaller compared with those measured from other mutant channels under similar recording conditions. I143A currents were very unstable, even in the presence of 10mM DTT in the extracellular solution. However, a substantially bigger current was seen in one cell following exposure to 10mM DTT (figure 5.5B).

5.3.9. I143C C149S and I143S C149S double mutants do not form functional channels

Next, the effects of mutating C149 to serine, in the presence of either a cysteine or serine at position 143, were tested. Expression of the double mutants I143C C149S and I143S C149S in CHO cells resulted in no detectable currents (n = 14 and 11, respectively; figure 5.6A, B). The loss of function in I143C C149S and I143S C149S mutant channels is not consistent with the hypothesis that an inter-subunit disulphide bond between the cysteines at position 149 in adjacent subunits underlies the loss of conduction in I143C and I143S mutant channels, since mutating C149 to serine would disrupt any such bond and would be expected to restore channel function. However, these results do not rule out the alternative hypothesis that the intra-subunit disulphide bond between C122 and C154 disrupts channel function in I143C and I143S mutant channels.

To test this hypothesis further, cells expressing the double mutant channels I143C C149S and I143S C149S were bathed in extracellular solution containing 10mM DTT. However,
Expression of $K_{v,2.1}$ II43A gives functional channels. $A$ and $B$, representative current recordings with 70mM $[K^+]_o$ in the absence ($A$) and presence ($B$) of 10mM extracellular DTT. Currents were recorded in response to 50ms voltage steps from a holding potential of -17mV, to test potentials ranging from +63mV to -132mV, in 5 mV decrements. Every fifth record is shown for clarity. $C$ and $D$, steady-state current-voltage relationships in the absence ($C$) and presence ($D$) of 10mM DTT. In $C$, the symbols represent the mean ± SEM for $n = 8$ cells.
Expression of \( \kappa_{2.1} \) I143C C149S, I143S C149S and \( \kappa_{2.1} \) C149S. A, B and C, Representative current recordings obtained with 70mM \([K^+]_o\) from \( \kappa_{ir}2.1 \) I143C C149S, I143S C149S and \( \kappa_{ir}2.1 \) C149S respectively. Currents were elicited in response to 50ms voltage steps from a holding potential of \(-17mV\) to test potentials ranging from \(+63mV\) to \(-132mV\), in 5mV decrements. Every fifth record is shown for clarity. D, normalized, steady-state, current-voltage relationship for \( \kappa_{ir}2.1 \) C149S. The symbols represent the mean ± SEM, where larger than the symbol, for \( n = 10 \) cells.
currents could not be detected (n = 10, not shown). This might suggest that the conserved cysteines at position 149 are essential for expression. However, mutation of C149 to serine alone generates functional channels (n = 10; figure 5.6C, D). In general, cells expressing a strong green fluorescence signal express large currents when transfected with cDNA encoding wild-type or mutant channels that are functional. Thus, it is suggested that in the double mutants I143C C149S and I143S C149S, the structure of the P-region has been altered so that even disruption of the intra-disulphide bond between C122 and C154 does not permit K⁺ permeance.

5.3.10. Evidence for an intra-subunit disulphide bond

Ideally, to determine whether the intra-subunit disulphide bond between C122 and C154 underlies the loss of function in I143C and I143S, site-directed mutagenesis would be used to disrupt this bond. However, this approach cannot be used because this bond is essential for correct channel assembly (see Leyland et al., 1999; Bannister et al., 1999; Cho et al., 2000).

However, support for this hypothesis can be provided by confirming the presence of an intra-subunit bond in these mutant channels. Thus, the expression of Kᵢr2.1 wild-type and I143C/I143S mutants was investigated at the level of the protein, under reducing and non-reducing conditions. The initial approach involved the use of western blotting (see section 2.5.1). However, although bands corresponding to Kᵢr channels were detected in samples from rat cortex and cerebellum, no bands were detected in samples from CHO cells expressing Kᵢr2.1 wild-type or mutant channels (not shown), suggesting that levels of channel expression were too low to detect using western blotting. The formation of disulphide bonds in vitro is either a cotranslational or immediate post-translational event, and can be detected using a reticulocyte lysate system (Scheel & Jacoby, 1982), which is used to detect early events. Thus, an in vitro reticulocyte lysate transcription-translation system was used in this study.

First, to determine whether Kᵢr channels were expressed in the in vitro translation system, a preliminary experiment was performed in which in vitro translation was carried out in the absence of microsomal membrane. For wild-type and mutant channels, the reticulocyte lysate system yielded proteins with a molecular weight around 48kDa, as expected for a single subunit, when run under non-reducing or reducing conditions on SDS polyacrylamide gels thus showing that Kᵢr proteins are indeed expressed using this system (see Figure 5.7). The smaller bands seen in figure 5.7, which are approximately 42kDa in size, could represent
Figure 5.7.

*In vitro* translation of \( K_{\text{ir}}2.1 \) wild-type and mutant channels using the reticulocyte system. Autoradiograph of SDS polyacrylamide gels for wild-type (WT), \( K_{\text{ir}}2.1 \) I143S, \( K_{\text{ir}}2.1 \) I143C and \( K_{\text{ir}}2.1 \) I143S C149S. *In vitro* translation was carried out in the absence of microsomal membranes and in the absence or presence of \( \beta \)-mercaptoethanol.
breakdown products where translation of the protein has temporarily stopped or started at a later codon sequence than the start codon, giving a smaller product. For example, in a previous study of Kᵢᵣ2.0 proteins a band of 43kDa was detected for Kᵢᵣ2.2, although the predicted size of the protein is 48kDa. This difference in the molecular weight could be explained by translation beginning at the third in-frame methionine codon in the Kᵢᵣ2.2 mRNA molecule (Stonehouse et al., 1999).

Since Kᵢᵣ proteins are expressed in the in vitro translation system, the next step was to determine whether an intra-subunit disulphide bond is present when the channels are incorporated into membranes. In vitro translation was therefore carried out in the presence of microsomal membranes. The protein expression of Kᵢᵣ2.1 wild-type in the presence of various concentrations of oxidised glutathione (GSSG) was investigated first (see figure 5.8) to confirm the presence of the intra-subunit disulphide bond, as previously reported (see Bannister et al., 1999; Cho et al., 2000).

Under optimal conditions GSSG would oxidize any thiols, catalyzing the formation of any disulphide bonds (see for example Scheel & Jacoby, 1982). Kᵢᵣ2.1 migrated as a monomer, even in the presence in the reaction mixture of 1mM GSSG. In fact, higher concentrations of GSSG (1mM) appeared to inhibit protein synthesis seen as a decreased intensity of the bands in lanes 5 and 8 of figure 5.8. This effect was counterbalanced in the presence of the reducing agent β-mercaptoethanol (lane 8, figure 5.8), which would have converted some of the oxidized glutathione (GSSG) to its reduced form (GSH). Bands corresponding to oligomers were not detected. However, wild-type channels migrated faster under non-reducing conditions (- β-mercaptoethanol) than under reducing conditions (+ β-mercaptoethanol) suggesting the presence of an intra-subunit disulphide bond. In the presence of intact disulphide bonds, these proteins appear as more compact units and migrate further into the polyacrylamide gel than their reduced counterparts. This is likely to be the disulphide bond between the conserved extracellular cysteines at position 122 and 154 (Leyland et al, 1999; Bannister et al., 1999; Cho et al., 2000), which is essential for channel assembly. The difference between the migrations of Kᵢᵣ2.1 wild-type when expressed in microsomal membranes compared with expression in the absence of membranes may be an indication of incorrect glycosylation of the protein. It could be possible to confirm this by pretreatment of protein samples with N-glycopeptidase and O-glycopeptidase before blotting.
Figure 5.8.

*In vitro translation of Kᵦ₂.₁ wild-type in the presence of membranes.* Autoradiograph of SDS polyacrylamide gel for Kᵦ₂.₁ wild-type in the presence of microsomal membranes. *In vitro* translation was carried out in the absence (lanes 3-5) or presence (lanes 4-6) of β-mercaptoethanol and with 0, 0.5 or 1mM GSSG. Two control lanes were run for comparison. Lane 1 shows the migration of a tandem linked dimer (TD) and lane 2 shows migration of Kᵦ₂.₁ in the absence of microsomal membranes (-M).
Figure 5.9.

**In vitro translation of Kᵢᵦ2.1 I143S in the presence of membranes.** Autoradiograph of SDS polyacrylamide gel for Kᵢᵦ2.1 I143S in the presence of microsomal membranes. *In vitro* translation was carried out in the absence (lanes 3-5) and presence (lanes 6-8) of β-mercaptoethanol and with 0, 0.5 and 1mM GSSG. Two control lanes were run for comparison. Lane 1 shows the migration of a tandem linked dimer (TD) and lane 2 shows the migration of Kᵢᵦ2.1 I143S in the absence of microsomal membranes (-M).
To verify that the system was capable of expressing dimers, a tandem linked dimer was translated and analyzed by gel electrophoresis. The system yielded a protein (lane 1, figure 5.8) with an approximate molecular weight of 100 kDa, close to that predicted for a dimer of Kir2.1 (96kDa). However, subunits were covalently linked by 10 glutamine residues (Dart et al., 1998a) rather than by disulphide bridges. This is reflected in the slightly higher molecular weight than predicted for dimers of Kir channels.

Next, the expression of Kir2.1 I143S was investigated under the same conditions as those described above for Kir2.1 wild-type. Figure 5.9 shows these results. As for wild-type channels, in the presence of microsomal membranes I143S migrated as a monomer, even in the presence in the reaction mixture of 1mM GSSG. Similarly, higher concentrations (1mM) of GSSG seemed to inhibit protein synthesis in the absence of β-mercaptoethanol (lane 5, figure 5.9). Furthermore, bands corresponding to oligomers were not detected, though a slight shift in the distance migrated is apparent between I143S channels run under non-reducing conditions (- β-mercaptoethanol), and under reducing conditions (+ β-mercaptoethanol), again suggesting the presence of an intra-subunit disulphide bond as seen in wild-type channels.

5.4. Discussion

In this chapter, the effects of mutating an isoleucine at position 143 in Kir2.1 on ionic selectivity and macroscopic gating were investigated. In Kir2.1, isoleucine 143 lies at position five of the signature sequence, a stretch of eight amino acids (TXXTXGY/FG; see table 5.1), which are highly conserved among all potassium channels.

Previous studies in Kv channels have reported various effects of mutating the corresponding residue on ionic selectivity. Thus, in Shaker, wild-type selectivity was retained when the valine at position 5 of the signature sequence was mutated to cysteine, leucine or threonine, whilst mutation to alanine, glycine, asparagine or glutamine rendered the channel non selective (Heginbotham et al., 1994). In contrast, in Kv2.1, functional expression was not obtained on mutation of the valine at position 5 of the signature sequence to alanine, glycine or glutamine. Neither was functional expression obtained on mutation of this valine to charged amino acids such as glutamate. However, mutation to leucine, isoleucine, threonine, cysteine and serine generated functional channels in which there appeared to be a correlation between Rb⁺/K⁺ permeability and the side chain hydrophilicity of the substituted amino acid at this position (Taglialatela et al., 1993).
In this study, mutation of the isoleucine at position 5 of the signature sequence to valine, leucine and threonine was tolerated, whilst mutation to cysteine and serine resulted in a loss of function. However, channel function could be rescued with 10mM extracellular DTT (see below). As for Kv\textsubscript{2.1} channels, a similar correlation between side chain hydrophilicity and Rb\textsuperscript{+}/K\textsuperscript{+} permeability was also apparent. Substitution of hydrophobic residues, such as valine, favoured Rb\textsuperscript{+} entry, whilst substitution by more hydrophilic residues, such as threonine, enhanced K\textsuperscript{+} selectivity. However, one difference between Kir\textsubscript{2.1} and Kv\textsubscript{2.1} was notable. In Kv\textsubscript{2.1} substitution of a leucine for valine enhanced K\textsuperscript{+} permeability, whilst in this study substitution of leucine for isoleucine in Kir\textsubscript{2.1} enhanced Rb\textsuperscript{+} permeability. Nonetheless, the general correlation between side chain hydrophilicity of the residue at position 5 of the signature sequence (V374 in Kv\textsubscript{2.1} and 143 in Kir\textsubscript{2.1}) and selectivity for K\textsuperscript{+} and Rb\textsuperscript{+} is conserved between Kv\textsubscript{2.1} and Kir\textsubscript{2.1}.

The correlation between $P_{Rb}/P_K$ and side-chain hydrophilicity of the substituted residue at position 5 of the signature sequence suggests that in both Kv\textsubscript{2.1} and Kir\textsubscript{2.1}, the residue at this position projects its side chain into the aqueous lumen of the pore (see Taglialatela et al., 1993; see also De Biasi et al., 1993). In support of this, cysteine-scanning mutagenesis studies have shown that when mutated to cysteine, the side chains of V374 in Kv\textsubscript{2.1} and isoleucine 143 in Kir\textsubscript{2.1} are accessible to thiol-labeling reagents such as MTSET or Ag\textsuperscript{+}, which also suggests that the side chains of these amino acids face the pore (Pascual et al., 1995; Dart et al., 1998b). Lü & Miller (1995) have reported similar findings for the analogous residue in Shaker. Mutagenesis studies of the P-region of Kir\textsubscript{1.1} and block by toxins (Jin & Lu, 1998; Lu & MacKinnon, 1997) provide further evidence that the residue at position 5 of the signature sequence is pore lining. Mutation of isoleucine 142 in Kir\textsubscript{1.1} lowers the affinity of Kir\textsubscript{1.1} for the toxins tertiapin and Lq2 by eight- and two-fold, respectively.

The results of Dart et al. (1998b) also indicated that several other residues in the P-region orient their side chains towards the pore lumen, including a phenylalanine residue at position 147 and the tyrosine of the GYG motif. Similarly, in Shaker, three of the P-region’s four aromatic side chains were suggested to point towards the pore lumen (Lü & Miller, 1995). These results provided support for the hypothesis that selectivity for K\textsuperscript{+} is conferred by cation-π interactions at the face of aromatic residues (Kumpf & Dougherty, 1993).
However, in light of the crystal structure of KcsA this hypothesis now seems unlikely. The atomic structure of KcsA shows that the selectivity filter is formed by backbone carbonyl oxygen atoms from five of the (T75, V76, G77, Y78 and G79, see table 5.1) amino acids in the signature sequence (Doyle et al., 1998b), indicating that selectivity is conferred through the interaction of K⁺ with oxygens as first proposed by Bezanilla & Armstrong (1972) and by Hille (1973; see section 1.6.2). However, this interaction requires the side chains of these residues to face away from the pore.

In contrast to the findings of cysteine scanning mutagenesis, comparative modelling of Kᵦ2.1, based on the structure of KcsA, suggests that I143 points its side chain away from the pore as shown in Figure 5.10. If isoleucine 143 in Kᵦ2.1 points away from the pore, then it is likely that its hydrophobic side chain interacts with another hydrophobic residue in close proximity. Insertion of a hydrophilic residue would disrupt such hydrophobic interactions, which in turn may alter the packing of amino acid residues in the walls of the pore. This might, in turn, be propagated to the carbonyl oxygen backbone, leading to changes in ionic selectivity. But, why does substitution of the hydrophobic residue leucine, but not valine, significantly alter channel selectivity? The side-chain of leucine differs from those of isoleucine and valine in that it branches at the gamma carbon, whilst the latter amino acid side chains have beta branches. With a leucine at position five of the signature sequence, the backbone might be expected to be more flexible, altering the effective field of the carbonyl oxygens with respect to solvating the permeating ions and resulting in the enhanced Rb⁺ permeability (see Kirsch et al., 1992).

It would be interesting to see whether substitution of more hydrophilic residues for isoleucine 143, such as glycine, asparagine or glutamine, result in non-functional channels. This might give us some further indication as to whether the side chains of this residue face the pore lumen, since one would expect pore-facing residues to be more tolerant of hydrophilic residues. In Shaker channels substitution of asparagine or glutamine for valine at this position gave functional channels, though non selective (Heginbotham et al., 1994). In contrast, in Kᵦ2.1, substitution of charged or strongly hydrophilic residues at this position resulted in channels with immeasurable currents (Taglialatela et al., 1993).

The results presented in this chapter also suggest that changes in the signature sequence can alter the conformation of the pore. This region fails to conduct when the isoleucine at position 5 of the signature sequence is replaced by cysteine or serine, and conduction is
Figure 5.10.

Schematic representation of the tetrameric structure of Kir2.1 viewed from the extracellular side. Model generated by comparative modelling (MODELLER; Sali & Blundell, 1993) using the crystal structure of KcsA (Doyle et al., 1998b) as a template and a sequence alignment based on mutational analysis of Kir2.1 (Minor et al., 1999). The side chains of I143 are coloured white and can be seen oriented away from the pore. The carbonyl oxygens of the glycine (G144) and tyrosine (Y145) of the K+ signature sequence are coloured pink whilst the pink sphere represents a K+ ion. Restraints were applied with MODELLER to represent (i) four-fold symmetry (ii) the salt bridge between glutamate 138 and arginine 148 in adjacent sub-units (Yang et al., 1997), and (iii) the interactions within Kir2.1 identified by mutagenesis (Minor et al., 1999).
reduced when an alanine is substituted at this position. However, the pore is open when the reducing agent DTT is present, which is likely to disrupt the intra-subunit disulphide bond between C122 and C154. This hypothesis cannot be tested by mutagenesis, because channels lacking this bond fail to assemble correctly (see Leyland et al., 1999; Bannister et al., 1999; Cho et al., 2000). However, the presence of an intra-subunit bond in I143S was confirmed by in vitro transcription studies using the reticulocyte system. Furthermore, these studies did not indicate the presence of any dimers, ruling out the possibility of an inter-subunit disulphide bond between native cysteines.

The conformational changes in the pore of Kir2.1 following mutation of isoleucine to cysteine or serine appear to bear some resemblance to the slow C-type inactivation process in Kv1 channels, which involves conformational changes detectable at the outer region of the pore. In Kv1 channels, the stability of the C-type inactivated state can be altered either by changing the residues surrounding the selectivity filter, or other nearby residues (see for example López-Barneo et al., 1993; see also section 1.2.3a). Similarly, the stability of the ‘non-conducting’ state in the Kir2.1 mutants I143C and I143S can also be altered by changing residues close to the P-region, in this case by disrupting the intra-subunit disulphide bond at the extracellular mouth of the pore.

The results from several other studies support the idea that the P-region can undergo conformational changes, which alter channel gating and permeation. Much of this evidence has been provided by analysis of microscopic gating in wild-type and mutant channels (see Choe et al., 1999; So et al., 2001; Lu et al., 2001a; Proks et al., 2001; Schwalbe et al., 2002).

At negative voltages, Kir channels display spontaneous single-channel gating activity. The gating kinetics of Kir1.1b can be well described by one open state and two closed states (Chepilko et al., 1995; Choe et al., 1998). The closed states are either short (~1ms) or long (~40ms) in duration, and both exhibit a biphasic voltage dependence. The long closures can be abolished by EDTA, which suggests that they are due to block by divalent cations. In contrast, the rate of entering the short closed state varies with K+ concentration and is proportional to current amplitude, which suggests that they may result from block by permeating K+ (Choe et al., 1998). Choe et al. (1998) have proposed the existence of a variable energy well in which the pore normally has a shallow energy well for K+ ions, permitting rapid conduction, but can be converted to a deep well, which traps K+ and blocks ion flow long enough to be detected as a channel closure. Since the rate of closure was
proportional to the current, Choe et al. (1998) suggested that the change in the channel is triggered when an ion passes a certain part of the pore. Thus, the short closures are thought to reflect an inactivation process, which may be associated with changes in the conformation of the P-region.

These gating properties are known to differ between Kir2.1 and Kir1.1b (see for example Choe et al., 1999). Compared to Kir1.1b, Kir2.1 channels have much longer mean open and closed times, more resolvable closed states and display a stronger voltage dependence. Using chimeras of Kir2.1 and Kir1.1b, Choe et al. (1999) have shown that the extracellular segment between the M1 and M2 transmembrane domains, which includes the P-region, is an important determinant of channel kinetics.

Recent mutagenesis studies have shown that changes in the signature sequence of Kir2.1, either from single point mutations (So et al., 2001) or backbone mutations (where the amide carbonyl of the two conserved glycines is mutated to an ester carbonyl; see Lu et al., 2001a), have a dramatic effect on single channel gating. So et al. (2001) found that channel open times were affected by mutation of the tyrosine in the GYG motif, for example, the mean open time was reduced from 102ms at -120mV in wild-type to 6ms in a wild-type-I145V dimer. Lu et al. (2001b) found that backbone mutations produced distinct subconductance levels, which were attributed to local conformational changes as well as altered ion-ion and ion-carbonyl oxygen interactions. Mutations in the selectivity filter have also been shown to disrupt the fast gating of Kir6.2/SUR1 channels (Proks et al., 2001).

Schwalbe et al. (2002) have reported the effects of several mutations in the P-region, including the mutation I143N/Y145T, on single channel gating in Kir2.1. In this study, the single channel behaviour of Kir2.1 wild-type was well described by three conductance states, corresponding to the main open state and two subconductance states. Whilst the single channel behaviour of Kir2.1 wild-type channels was dominated by two states, a closed and main open state, mutations in the P-region, including the I143N/Y145T mutation, gave rise to Kir2.1 channels that favoured various intermediate subconductances.

Finally, further evidence that the P-region can undergo conformational changes has been provided by the resolution of the crystal structure of KcsA in high and low $[K^+]_0$ (Zhou et al., 2001b). The 'conduction conformation', in which the backbone carbonyls point towards each other, is stabilized by the presence of $K^+$ ions in the selectivity filter. But, when the protein is

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partially depleted of K$^+$, the selectivity filter adopts an alternative conformation in which the backbone carbonyls twist away from the centre of the channel such that the carbonyl of valine 76, which corresponds to I143 in Kir2.1, hydrogen bonds with a water molecule outside the pore.

In summary, the results in this chapter show that mutation of isoleucine 143 affects ionic selectivity in Kir2.1. Furthermore, permeation is dependent on the residue at position 143, consistent with the involvement of the selectivity filter in the gating of inward rectifier potassium channels.
Chapter 6

6.0. Summary & Future Perspectives

The P-region of potassium channels, as for other voltage-gated channels, is typically regarded as the region primarily responsible for ionic selectivity and permeation characteristics. Recent evidence suggests that this region may also participate in the gating of some potassium channels including the inward rectifiers (see for example Lu et al., 2001a).

In this study, I have used the patch clamp technique to determine the effects of mutating residues in the P-region on the permeation properties of two members of the strong inward rectifier subfamily KIR2.1 and KIR2.2. The results presented in this study support the notion that the P-region plays an important role in determining the permeation characteristics and ionic selectivity of strong inward rectifiers. However, they also highlight that, as in Kv channels, other regions are involved in determining the properties of the ion conduction pore. Finally, the results presented here also provide further support that this region is involved in
channel gating. These studies have raised several interesting points worthy of further investigation:

i) Residues in the P-region do not determine the difference in unitary conductance exhibited by Kir2.2 and Kir2.1. Repunte et al. (1999) have shown that residues in the M1-P-region and P-region-M2 extracellular linkers determine the difference in unitary conductance between Kir6.1 and Kir6.2. Is this also the case for members of the Kir2.0 subfamily?

ii) The characteristics of Ba\(^{2+}\) blockage in Kir2.2 and Kir2.1 are not fully interchangeable following mutation of L148F in Kir2.2 and the reverse mutation in Kir2.1. Which other residues determine Ba\(^{2+}\) blockage in Kir2.0 subfamily channels?

iii) In BKCa and Shaker channels, the kinetics of Ba\(^{2+}\) binding have been shown to be greatly affected by the occupancy of three discreet K\(^{+}\) binding sites known as the 'lock-in', 'enhancement' and 'deep high affinity' sites (see Neyton & Miller, 1988; Harris et al., 1998). These sites impede the exit of the Ba\(^{2+}\) ion to the extracellular side at low concentrations, enhance the exit of Ba\(^{2+}\) in the inward direction in the presence of high K\(^{+}\) concentrations and exhibit high affinity deep binding inside the pore, respectively. It would be interesting to examine the unblocking rate of Ba\(^{2+}\) in low [K\(^{+}\)]\(_{o}\) in these channels to determine whether the residues (L148 in Kir2.2 or F147 in Kir2.1) studied here contribute to the 'lock-in' site.

iv) Mutation of isoleucine 143 to small residues such as cysteine and serine resulted in a loss of K\(^{+}\) permeation, which was proposed to be a consequence of a conformational change in the pore similar to that seen in Kv1 channels during C-type inactivation. Starkus et al. (1997) have shown that Shaker channels are capable of conducting Na\(^{+}\) and Li\(^{+}\) in the C-type inactivated state provided that internal K\(^{+}\) is completely removed. It would be interesting to determine whether I143C and I143S mutant channels conduct Na\(^{+}\) or Li\(^{+}\) under similar conditions.

v) Previous studies have shown that mutation of residues in the selectivity filter affect single channel gating behaviour (see for example So et al., 2001; Lu et al., 2001a). Mutating isoleucine to cysteine or serine clearly resulted in a loss of conduction, but what are the effects of mutating isoleucine to valine, leucine and threonine? Do these mutations cause more subtle conformational changes of the pore? It
would be interesting to examine the effects of these three mutations on single channel kinetics?

vi) The Rb⁺ currents carried by I143L mutants appear to display inactivation at very negative membrane potentials. A similar phenomenon has been observed in Kᵢ₂.1-wild-type and mutant channels with NH₄⁺ as the permeant ion and has been attributed to conformational changes of the pore, arising from the permeation and interaction of NH₄⁺ with the pore region (see for example Chang & Shieh, 2002). Does the inactivation of Rb⁺ currents in I143L represent a similar phenomenon? It would be interesting to investigate this further through analysis of macroscopic currents and single channel kinetics.
Chapter 7

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