Abstract

The voltage-gated potassium channels of the Kv1 (Shaker-type) family are proteins found in many cell types throughout the body, and are critical for regulating membrane excitability. Ion channel proteins are dynamic by nature, and undergo structural reorientations in response to voltage and other external stimuli. In this thesis, I will describe the results of experiments using the voltage clamp fluorimetry technique, which can relate movements within protein domains to associated electrical behaviour.

In Kv1.2 channels, fluorescent emissions from a fluorophore attached to the S4 helix faithfully report the movement of gating charge during depolarization. However, a second phase of fluorescence is also observed which is unique to Kv1.2. Using chimaeras where the external linkers were exchanged between Kv1 homologues, we determined that this phase tracks an interaction between the external linkers which slows channel deactivation. When fluorescence was recorded from Kv1.2 in the presence of the Kvβ1.2 subunit possessing a channel blocking N-terminus, fluorescence was unchanged during activation but slowed during deactivation, suggesting that the blocker sterically hinders activation gate closure and prevents the return of the gating charge. While Kv1.2 requires a Kvβ1 subunit to inactivate, the Drosophila homologue Shaker possesses an N-type inactivation domain on its own N-terminus. Shaker can also inactivate through conformational changes in its selectivity filter, so-called C-type inactivation. This process is structurally linked to activation gate opening, and is accelerated by N-terminal block. We have found that the conformations of the activation gate and the selectivity filter are allosterically linked, and that the N-terminus accelerates C-type inactivation by expelling potassium from a selectivity filter binding site known to inhibit its conformational
change. Acceleration of C-type inactivation was also implicated as the mechanism by which an inherited genetic mutation near the Kv1.1 activation gate causes episodic ataxia type-1. However, results from experiments using voltage clamp fluorimetry and single channel patch clamp suggest that accelerated current decay observed in those mutants is more likely due to destabilization of the open state of the activation gate. Taken together, the results of this thesis demonstrate how structural variability between channel homologues leads to their broad functional diversity.
Preface

This dissertation is made up of work that has been published, submitted for publication, or prepared for publication in peer-reviewed journals. Chapters 2 and 3 represent published articles, and are presented in their original forms, except for the reduction and modification of the introduction sections to preserve readability, as much of the background for these chapters is laid out in the broad introductory section of this thesis. Chapter 5 contains work from an article that, as of this writing, was under peer-review for publication, and similarly, it is presented in original form save for modifications to the introduction. Chapter 4 contains work that is to be submitted in the future. It is presented in the most up-to-date form at the time of completion of this thesis. Additionally, to avoid redundancy, where methods are similar between chapters, the reader will be referred back to the earliest chapter where that technique was described in detail. In experiments where oocytes were used, they were provided by the laboratory of David Fedida, and were isolated using methods approved by the UBC Animal Care Committee, certificate #A10-0074. The relative contributions of C.J. Peters to each chapter are as follows:


C.J. Peters was responsible for approximately 10% of the work in this Chapter, including designing and performing experiments using voltage clamp fluorimetry, analyzing data, and critically reviewing the manuscript, as well as in performing additional experiments as requested by journal reviewers. A.J. Horne contributed by developing the project, designing and performing the majority of the experiments, analyzing most data, and writing and revising the manuscript. T.W. Claydon was involved in developing the project and performing some experiments. D. Fedida was involved in developing the project, writing the paper and revising the paper.


C.J. Peters was responsible for approximately 80% of the work in this Chapter, including developing the project, designing and performing experiments, analyzing all data, writing the manuscript and revising it for publication. M.S. Vaid and A.J. Horne performed experiments. D. Fedida contributed by conceiving the project and revising the manuscript. E.A. Accili was involved in developing the project and writing the manuscript, and in critically revising the manuscript.

**Chapter 4**: Peters, C.J., Fedida, D., Accili, E.A. Allosteric coupling between the activation gate and the selectivity filter in Shaker channels is promoted by pore block by the channel N-terminus. Manuscript in preparation.
C.J. Peters was responsible for approximately 90% of the work in this Chapter, including conceiving and developing the project, designing and performing all experiments, analyzing data, and writing the manuscript. E.A. Accili and D. Fedida contributed to project development and manuscript revision.


C.J. Peters was responsible for approximately 70% of the work in this Chapter, including conceiving and developing the project, designing and performing the majority of experiments, analyzing data, writing and revising the manuscript. D. Werry performed experiments using single channel patch clamp, and analyzed the data therefrom. H.S. Gill assisted in performing some of the remaining experiments. E.A. Accili and D. Fedida contributed to project development and manuscript revision.
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List of Abbreviations

Amino Acid One Letter Code
A  Ala  Alanine
C  Cys  Cysteine
D  Asp  Aspartate/Aspartic Acid
E  Glu  Glutamate/Glutamic Acid
F  Phe  Phenylalanine
G  Gly  Glycine
H  His  Histidine
I  Ile  Isoleucine
K  Lys  Lysine
L  Leu  Leucine
M  Met  Methionine
N  Asn  Asparagine
P  Pro  Proline
Q  Gln  Glutamine
R  Arg  Arginine
S  Ser  Serine
T  Thr  Threonine
V  Val  Valine
W  Trp  Tryptophan
Y  Tyr  Tyrosine

Other abbreviations
4-AP  4-aminopyridine
Å  angstrom (= 100 pm)
BKCa  large-conductance, calcium-activated potassium (channel)
cDNA  complementary deoxyribonucleic acid
Ci-VSP  Ciona voltage-sensitive phosphatase
COT  concerted opening transition
cRNA  complementary ribonucleic acid
C-terminus  carboxy terminus
e₀  elementary charge equivalent ~ 1.6 x 10⁻¹⁹ Coulombs
EA-1  episodic ataxia, type 1
Eag  Ether-à-go-go (channel)
FL  full length [Shaker]
F(V)  fluorescence-voltage relationship
GFP  green fluorescence protein
G(V)  conductance-voltage relationship
HCN  hyperpolarization-activated, cyclic nucleotide-gated (channel)
HEPES  4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hERG  human Ether-à-go-go-related gene (channel)
IR  inactivation-removed
I(V)  current-voltage relationship
k  slope factor (in mV)
<table>
<thead>
<tr>
<th>Term</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCNQ</td>
<td>voltage-gated, KQT-like potassium (channel)</td>
</tr>
<tr>
<td>Kv1.x</td>
<td>voltage-gated potassium (channel), where x is an integer from 1-6</td>
</tr>
<tr>
<td>LRET</td>
<td>lanthanide-based resonance energy transfer</td>
</tr>
<tr>
<td>MEM</td>
<td>minimum essential medium</td>
</tr>
<tr>
<td>MTS</td>
<td>methanethiosulfonate</td>
</tr>
<tr>
<td>MTSET</td>
<td>2-(trimethylammonium)ethyl methanethiosulfonate</td>
</tr>
<tr>
<td>Mx</td>
<td>transmembrane helix number (1-2) in a two-helix channel subunit</td>
</tr>
<tr>
<td>NMDG</td>
<td>N-methyl-D-glucamine</td>
</tr>
<tr>
<td>N-terminus</td>
<td>amino terminus</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PIP2</td>
<td>phosphatidylinositol bisphosphate</td>
</tr>
<tr>
<td>P-loop</td>
<td>pore loop</td>
</tr>
<tr>
<td>PMT</td>
<td>photomultiplier tube</td>
</tr>
<tr>
<td>Q(V)</td>
<td>gating charge-voltage relationship</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>Sx</td>
<td>transmembrane helix number (1-6) in a six-helix channel subunit</td>
</tr>
<tr>
<td>Sx</td>
<td>selectivity filter binding site for potassium (0-4)</td>
</tr>
<tr>
<td>TBA</td>
<td>tetrabutylammonium</td>
</tr>
<tr>
<td>TEA</td>
<td>tetraethylammonium</td>
</tr>
<tr>
<td>TMRM</td>
<td>tetramethylrhodamine-5-maleimide</td>
</tr>
<tr>
<td>$V_{1/2}$</td>
<td>half-activation voltage (in mV)</td>
</tr>
<tr>
<td>VCF</td>
<td>voltage clamp fluorimetry</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
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All units are in standard S.I. format unless otherwise noted.
List of Symbols

$\alpha$  refers to membrane spanning potassium channel subunit
$\alpha$-helix  protein secondary structure, a helix with approximate period of 3.6
$\beta$  refers to auxiliary potassium channel subunit
$\gamma$  single channel conductance (in Siemens)
$\Delta$  change in
$\Delta G$  Gibbs free energy (in Joules)
$\lambda$  wavelength (in nm)
$\tau$  time constant
Acknowledgments

I would like to begin by expressing my sincere appreciation to my supervisor, Dr. Eric Accili. His creative guidance, patience, advice and mentorship throughout all phases of my graduate work have been both immensely helpful and enjoyable, and I owe him a great debt of gratitude for his contributions to this thesis. I must also mention the great assistance of Dr. David Fedida, who has been both a great collaborator and a great resource for both scientific advice and encouragement. I would also like to acknowledge the support of my supervisory committee, Drs. Ed Moore, Steven Kehl and Shernaz Bamji, as well as of Drs. Chris Ahern and Harley Kurata. I have truly enjoyed our interactions over the past 6 years, and believe I am a better scientist and person as a result.

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Chapter 1: Introduction
OverView

Excitable tissues, such as those found in the central and peripheral nervous systems and cardiac and skeletal muscles, are critical for normal functioning in human physiology. The property of excitability refers to the ability of tissues to generate, and respond to, action potentials. This property is responsible for, for example, the ability of nerves to conduct and transmit impulses, the regulation of cardiac rhythmicity, the responses of sensory organs to external stimuli, and the conversion of muscular input signals to contractile force generation. Excitability is conferred to cells by voltage-gated sodium channels and, in some cases, voltage-gated calcium channels (Hille, 2001).

Equally important to proper functioning in these cells is their ability to relax following excitation. This property is imparted by the net outward movement of potassium ions through members of the superfamily of potassium channels, including the voltage-gated potassium channels. In this introduction, I will discuss the physiological and biophysical properties of potassium currents. Firstly, I will describe the properties of currents, the cloning of the potassium channel proteins, and the structural insights gleaned from crystallography and other related methods. Secondly, I will describe the biophysical functions of potassium channels and their components, along with some of the outstanding questions in potassium channel research. Thirdly, I will outline in detail the specific experimental techniques used to perform the studies described in the body of this thesis, which I will follow with an overview of the studies themselves.
A history of voltage dependent potassium currents

Work from the laboratories of Kenneth Cole, and of Alan Hodgkin and Andrew Huxley, was responsible in large part for the identification and first descriptions of sodium and potassium currents. Most work in the field at the time was performed on a preparation of the giant axon from the *Loligo pealii* squid. The development of intracellular recording technology allowed for the recording and analysis of action potentials from the squid giant axon, and the determination that stimulation resulted in a strong drop in the impedance to ion flow (Cole and Curtis, 1939; Cole and Curtis, 1941). Researchers were soon able to measure both the resting and action potentials across the giant axon membrane, and to determine that the magnitudes of these were highly dependent on the concentrations of ions in the external medium, in particular potassium (Curtis and Cole, 1942). Electrophysiological research in the squid giant axon would eventually culminate in a unified theory of how nerve impulses are initiated and propagated (Hodgkin and Huxley, 1952b), which would earn its authors a Nobel Prize in 1963.

Hodgkin and Huxley showed that in response to positive changes in potential, the squid giant axon plasma membrane became highly permeable to the flow of sodium and potassium ions (Hodgkin and Huxley, 1952b; Hodgkin and Huxley, 1952a). A sodium conductance activated rapidly, allowing for the net inward flow of positive sodium ions down their electrical and chemical gradients. The resulting positive feedback effect caused the massive reversal in membrane potential which propagates along the nerve as an action potential (Hodgkin et al., 1952). Following the influx of sodium, two slower
processes take place to return the voltage to its resting level. Sodium conductance drops due to an inactivation process, which unlike activation, is not innately sensitive to voltage and requires only channel opening to be initiated. Simultaneously, a second ionic conductance, to outward potassium flow, becomes active (Hodgkin and Huxley, 1952b). As potassium is actively maintained at a high concentration within the cell, and the membrane potential following sodium influx is depolarized, potassium flows down electrical and chemical gradients in order to re-establish the negative resting potential of the membrane. In this way, the rapidly activating and inactivating sodium current promotes excitation of the membrane and propagation of a depolarizing wave, while potassium current acts as an inhibitory or relaxatory signal. Further research in different tissues would identify a broad functional diversity amongst currents carried by the potassium ion, a diversity not seen in sodium currents. Of particular note is the existence of transiently open potassium currents, found in crustacean walking leg axons with high frequency repetitive signals, where the currents activate in response to voltage but are rapidly attenuated following activation (Connor et al., 1977). However, consistent amongst potassium channels, and important to their function, is a high selectivity for potassium ions: in most potassium channels, potassium is preferred for conduction over sodium by a ratio greater than 100:1 (Hille, 1973). The concept that channels contained “gated” pores was clear with the demonstration that tetraethylammonium (TEA) and its derivatives could block potassium channels, but only in a state dependent way, meaning that access of TEA to its site of action required the channel to first have entered its open conformation (Armstrong, 1966; Armstrong, 1969; Armstrong, 1971). That these proposed channels contained molecular switches to regulate opening by voltage was
predicted by the Hodgkin-Huxley fourth order exponential fits of activation, where they suggested that four independent particles must move in order for currents to become active (Hodgkin and Huxley, 1952b). A physical correlate for these particles was suggested by the measurement of a small, rapid transfer of “gating” charge across the voltage field following depolarization, taking place prior to the initiation of current flow through sodium and potassium channels (Armstrong and Bezanilla, 1973; Keynes and Rojas, 1974; Keynes and Rojas, 1976; Armstrong and Bezanilla, 1977; Gilly and Armstrong, 1980).

Channels underlying ionic currents are cloned from native tissue

A seminal step towards better understanding of ionic currents in excitable tissues occurred when the channels were isolated and cloned from native tissues. Sodium channels were the first voltage-gated channels to be isolated, first biochemically by purifying the protein from the electric eel *Electrophorus electricus*, in complex with a tightly bound molecule of the blocker tetrodotoxin (Agnew et al., 1978), and later by hybridizing oligonucleotides to the messenger RNA to get complementary DNA (Noda et al., 1984). Potassium channels were first cloned three years later, using “chromosomal walk” of the potassium channel-encoding Shaker locus of the *Drosophila melanogaster* genome (Papazian et al., 1987; Tempel et al., 1987; Kamb et al., 1987). Mammalian Shaker-type potassium channels were later isolated largely thanks to their homology with the *Drosophila* channel (Chandy et al., 1990; Ramaswami et al., 1990; Tamkun et al., 1991; Roberds and Tamkun, 1991; Snyders et al., 1993; Fedida et al., 1993), and these were eventually grouped into the Kv1 family (Gutman et al., 2005).
Possession of cDNA clones provided researchers with the ability to functionally express cloned potassium channels in mammalian and *Xenopus* heterologous expression systems. Using this approach, considerable insights could be gained into channel structure and assembly. Whereas the requisite components of the sodium channel are contained within a single protein with four homologous domains (Noda et al., 1984), voltage-gated potassium channels consist of tetramers of four subunits, adopting fourfold symmetry around a central pore (MacKinnon, 1991). Tetramerization is dictated by coassembly at the intracellular T1 domain (Li et al., 1992). Since the T1 domain is present on all Kv1 subunits, the existence of heterotetramers could be predicted, and was later confirmed by isolation from bovine neurons of *in vivo* heteromeric complexes in well-defined combinations (Shamotienko et al., 1997). In addition to structural predictions, expression of Kv1 clones in heterologous systems has allowed for countless functional inferences to be made, for instance with respect to voltage gating and selectivity. Whereas the *Drosophila Shaker* clone produced a transient, rapidly decaying outward (A-type) current when expressed in oocytes (Timpe et al., 1988), enzymatic (trypsin) treatment of the intracellular aspect of these channels was able to eliminate the rapid channel block, resulting in channels displaying sustained outward potassium currents during short duration pulses (Hoshi et al., 1990), similar to those recorded from some of the mammalian *Shaker* homologues. Channel function and gating will be explored in detail in a later section of this introduction.
Crystallographic studies of potassium channel structure

Crystal structure of the KcsA potassium channel

Following ten years of structural studies in cloned potassium channels, a milestone was reached with the solving of a high resolution crystal structure of the KcsA channel (Doyle et al., 1998), providing a strong framework for further studies of ion channel structure and function. KcsA is a tetrameric potassium channel composed of only two α-helices per subunit, isolated from the gram-positive bacteria *Streptomyces lividans* (Schrempf et al., 1995), and it opens to conduct ions in response to acidic intracellular pH (Cuello et al., 1998). The closed KcsA structure is shown in Figure 1.1A.

![Figure 1.1. Crystal structure of the KcsA channel and signature sequence conservation.](image)

**Figure 1.1.** Crystal structure of the KcsA channel and signature sequence conservation.
Several important structural features of potassium channel pore architecture are visible from this diagram. Firstly, the two transmembrane α-helices most proximal to the conduction pathway, M1 and M2 in KcsA, are shaped like an “inverted teepee” (Doyle et al., 1998). The narrow internal ends allow the channel to create an enclosed central cavity within the membrane, protecting a hydrophilic milieu within an otherwise hydrophobic environment. This configuration strongly reduces the energy barrier to ions moving across the plasma membrane, and the presence of a diffuse water-filled cavity in this region establishes that potassium ions are likely stabilized at this site (Doyle et al., 1998).

Secondly, the wider external mouth of the teepee houses the channel selectivity filter, which possesses binding sites for the coordination of dehydrated permeant ions (shown in purple). The outer pore mouth architecture is made up of the M1-M2 linker (equivalent to S5-S6 in Shaker), and contains the “turret” and the re-entrant P-loop, comprising the P-helix and the narrow passageway delimited by backbone carbonyls from amino acids in the potassium channel “signature sequence” TVGYG (Heginbotham et al., 1992), highlighted in Figure 1.1A by stick representations. The signature sequence thus represents the molecular correlate of the selectivity filter, by which potassium ions are
selectively passed through the membrane. Because the selectivity filter opens internally on the inner vestibule formed by the inverted teepee, the voltage field is highly focused across the membrane at this point (Doyle et al., 1998). The signature sequence is highly conserved between KcsA and the evolutionarily distant voltage-gated potassium channels (Heginbotham et al., 1992), suggesting that the mechanism of ion conduction and selectivity is likely conserved across the potassium channel family (Figure 1.1B). The function of the selectivity filter will be explored in a subsequent section of this introduction.

Insights from the structures of KvAP and MthK

While the crystal structure of the KcsA channel provided an invaluable tool towards the investigation of potassium channel structure, some key questions could not be answered, or indeed even asked, from that information. For instance, what do other channel states look like, and how are Kv1 channels gated by voltage? Further crystallography studies from the Mackinnon laboratory were geared towards answering these questions. The crystal structure of another two-helix bacterial channel, the calcium-gated MthK channel from Methanobacterium thermoautotrophicum, provided a glimpse of a channel in its open state (Jiang et al., 2002a; Jiang et al., 2002b). In this crystal, the narrow aspect of the inverted teepee is splayed open with respect to the KcsA channel, with the M2 pore lining helices bending at a highly conserved glycine residue, and demonstrating contiguity between the inner medium and the inner vestibule potassium binding site, and suggesting that these inner helices could form the state dependent barrier to ion flow suggested by functional studies. The first voltage-gated channel to be
crystallized was the KvAP channel from the archaeon *Aeropyrum pernix* (Jiang et al., 2003). In this crystal, a tight tetramer of S5-S6 helices around the central pore was accompanied by a spatially separated voltage sensor made up of helices S1-S4, stabilized in the membrane by crystallization with Fab antibody fragments. This structure also formed the basis for the “paddle” model of voltage gating, suggesting an alternative trajectory for movement in response to voltage from the accepted dogma, where large scale translocations of S3 and S4 could take place (Jiang et al., 2003b). This model proved controversial, and was contradicted by experiments using a number of strategies, including electrophysiological studies and imaging assays performed by cysteine cross-linking and Fluorescence Resonance Energy Transfer (Laine et al., 2003; Posson et al., 2005; Chanda et al., 2005). Clearly, further structural information was required.

*Crystal structures of mammalian Kv1.2 channels to refine the position of the voltage sensor*

A new wealth of study following the availability of structures for bacterial two-helix and voltage-gated channels culminated in the solving of the first crystal structure of a mammalian potassium channel, the Kv1.2 channel from rat brain (Long et al., 2005a) (Figure 1.2).
While similar in helical topology to the KvAP structure, some important features unique to Shaker-type channels became clear in the Kv1.2 structure. Firstly, a Proline-Valine-Proline “PVP” sequence, which is highly conserved amongst Kv1 channels, had been shown to form a “bundle crossing” speculated to represent the narrowest point in the ion conduction pathway (Liu et al., 1997; del Camino et al., 2000). This was supported by the crystal structure of Kv1.2. In addition, the T1 domain involved in tetramerization and docking of auxiliary subunits had previously been crystallized independently (Gulbis et al., 2000). In the Kv1.2 structure, the T1-β subunit complex could be seen on the cytosolic aspect of the transmembrane regions (Figure 1.2A,B), forming a “hanging gondola” structure unique to the Kv1 channels. Attaching the hanging gondola to the S1
helix in each subunit is the T1-S1 linker. This linker delimits the portal windows through which potassium ions pass on their way to and from the permeation pathway.

While the Kv1.2 structure provided some insight into the configuration of the voltage sensor, interpreting this structural data remained a challenging problem. The voltage sensor is an exquisitely configured domain designed to allow charged residues to traverse a hydrophobic milieu, and, being that the methodology to generate a crystal does not permit for the channel to be in its native environment in a lipid bilayer, the model of voltage gating derived from the Kv1.2 structure continued to be questioned (Long et al., 2005b). A refined method was able to partially reconcile this. A Kv1.2-2.1 chimaera (Figure 1.3), where part of the Kv2.1 voltage sensing domain was substituted in order to increase the stability of that region, provides the current best structural interpretation of the Kv1 channel family (Long et al., 2007).

Furthermore, this structure was solved in the presence of lipids, to allow the protein to adopt a more native conformation in a crystal lattice. In this construct, in addition to consistency with previous crystals with respect to structures of non-membrane spanning and pore elements, the structural mechanism for voltage sensing has begun to closely resemble the consensus from functional studies. A mechanism by which the charged S4 helix can translocate across the membrane and articulate with S2 was later suggested by the same research group (Tao et al., 2010), and will be discussed further in a subsequent section. Thus, the Kv1.2-2.1 crystal structure represents the most recent picture of the structure of Shaker-type channels; however, as will be discussed in chapter 2, the structural homology between members of the Shaker/Kv1 family is often
overestimated, demonstrating the necessity for thorough functional characterization in addition to the insights from crystallography.

Figure 1.3. Crystal structure of the Kv1.2-2.1 paddle chimaera.
Figure 1.3. Crystal structure of the Kv1.2-2.1 paddle chimaera. Two side views of the membrane spanning regions from the crystal structure of a Kv1.2-2.1 chimaera channel (PDB ID: 2R9R), from Long et al. (2007). Two opposite subunits are shown, and the other two have been removed for clarity. The voltage sensor and S4-S5 linker are coloured in green, and the pore domain is coloured in yellow, with centres of volume of permeating cation densities shown as purple points. Top: side view highlighting the permeation pathway down the central axis of the protein. Bottom: side view showing the spatial separation on each subunit of the voltage sensing and pore domains. Regions and residues of interest throughout this thesis are shown in insets, and highlighted in red, with residue numbering as in the Shaker channel. Of note: in this crystal structure, a more accurate representation of the voltage sensor’s conformation was achieved by substituting part of the sequence encoding the S3-S4 helices of Kv1.2 with the Kv2.1 equivalent (Long et al., 2007). In this chimaera, the external aspect of the S4 remains α-helical for 9 residues longer than the equivalent region in Shaker and Kv1.2, which comprises the flexible S3-S4 linker. As a result, residues considered homologous to M356 and A359 (M288 and A291 in Kv1.2) appear to exist within an α−helical section, whereas in the Kv1.2 channel structure (PDB# 2A79), A291 is part of an α−helix but M288 is in the linker.

Summary

In this section, I have described how researchers, over the past 60 years, have gone from the first speculation into the existence of ion channels, to high-resolution images of their three-dimensional configurations. From pioneering techniques involving the electrophysiology of the squid giant axon, to the use of x-ray diffraction of protein crystal lattices, the studies mentioned above have effectively developed a strong framework upon which we can expand our understanding of the physical processes underlying channel function. However, a chief limitation of crystallography and other structural biology techniques is in their inability to accurately represent the dynamic movements within proteins that dictate their biophysical and physiological properties. In the next section, I will provide a detailed discussion of the dynamic function of Kv1 potassium channels, and our understanding of the mechanisms involved at the sub-protein level.
The principles of voltage sensing in potassium channels

A transmembrane charge displacement preceding opening

As mentioned in the preceding, early descriptions of the voltage sensitivity of opening of potassium currents in the squid giant axon demonstrated that a transmembrane transfer of charge preceded the activation of ionic current conductance (Armstrong and Bezanilla, 1974). This so-called gating current is actually recorded under voltage clamp as a non-linear component of the rapid capacitive transient observed during strong depolarizations from a negative membrane resting potential. As the presence of ionic currents, which are of much higher magnitude, can mask the recording and interpretation of gating currents, a common experimental strategy to record them is to prevent the flow of ionic current. To this end, the use of specific pore blockers, such as charybdotoxin in potassium channels, or the replacement of permeant ions with nonpermeant ones of the same valence (e.g., TEA and NMDG in potassium channels), are techniques that have been employed for this purpose (Armstrong and Bezanilla, 1973; Schoppa et al., 1992; Islas and Sigworth, 1999). Similarly, ionic current can be prevented by the introduction of the mutant (in Shaker) W434F (Perozo et al., 1993), while preserving charge movement. From gating current recordings in Shaker, the magnitude of the transferred charge in a single channel was estimated to be approximately equivalent to 13 elementary charges ($e_0$) (Schoppa et al., 1992).

By integrating the transient gating currents with time, the voltage dependence of the transfer of gating charge can be determined. This demonstrates a characteristic two state Boltzmann function fit, termed the Q(V) relationship. When the Q(V) relationship is
compared with a similar plot of channel conductance (derived from the current-voltage relationship) against voltage \([G(V)]\), the \(Q(V)\) is noticeably left-shifted on the voltage axis, suggesting that movement of charge was occurring at voltages where the channels are not yet open. This is consistent with the Hodgkin-Huxley proposition that a series of four voltage-sensitive movements, happening independently, takes place before the channel can open (Hodgkin and Huxley, 1952b). In this case, the gating element from each subunit of the channel must have entered the “ON” state for the channel to become conductive. Notably, when active channels in a depolarized membrane are given repolarizing voltage commands, the voltage dependence of \(Q(V)\) does not overlay with that recorded during depolarizations. Instead, the gating currents recorded during deactivation are strongly left-shifted with respect to those recorded during activation, meaning that much stronger voltages are required to return the voltage sensors to their resting states. This has been attributed to a stabilization of the outward position of the gating charge (Fedida et al., 1996; Olcese et al., 1997), which will be discussed further later in this section.

\emph{A positively charged S4 helix}

The displacement of elementary charges across the membrane appears to coincide nicely with the finding that each subunit of the Kv1 channel possesses a series of charged, hydrophilic amino acids within regions otherwise made up of hydrophobic residues predicted to traverse plasma membrane as \(\alpha\)-helices. In particular, the S4 helices each contain a series of 7 positively charged amino acids (five arginines and 2 lysines), occurring at a period of every third residue along the sequence (Papazian et al., 1987;
Tempel et al., 1987). Substitutions of uncharged amino acids at some of these residues reduced the size of the displaced gating charge and the voltage dependence of channel opening, indicating that they were critical to dictating voltage sensitivity in the channel (Liman et al., 1991; Logothetis et al., 1992; Lopez et al., 1991). Conversely, MTSET accessibility experiments demonstrated that the most intracellular lysine residue remains accessible at all channel states and therefore does not appear to carry any gating charge (Larsson et al., 1996). Using radio-ligand binding assays to count the number of channels, and then performing individual charge neutralizations, it was determined that the first four charged residues (all arginines) were the ones primarily responsible for generating the gating charge carried by S4 (Aggarwal and MacKinnon, 1996), which in these experiments was also measured to be approximately 13 e₀. In addition, E293, a negatively charged residue in the S2, also moves significantly in the voltage field and contributes to gating currents (Seoh et al., 1996).

**Paddles, screws and Phe-gaps: towards a model of voltage sensor movement**

The physical mechanism by which the voltage sensor moves across the membrane voltage field has been the source of considerable controversy, and accordingly, a substantial body of research has been devoted to investigating this question. The S4 helix undergoing a simple translocation perpendicular to a uniformly distributed voltage field would represent the most simple and primitive model, but several lines of experimentation have rejected this explanation. By replacing the most extracellular arginine residue with a protonatable histidine, Starace and colleagues were able to transform the S4 helix into a voltage-operated proton transporter, suggesting that this
residue may access both sides of the membrane, and indicating that the diameter of the lipid bilayer may vary considerably in the immediate vicinity of S4 (Starace et al., 1997). This was also consistent with the very small translocations of the S4 that were predicted by Lanthanide-based Resonance Energy Transfer (LRET) experiments, which supported a movement path of as little as 4 Å in length (Cha et al., 1999), thus requiring a highly focused electric field and a more complex movement pathway.

Another, separate theory for voltage sensor movement also challenged previous explanations of voltage sensor movement, the so-called paddle model (Jiang et al., 2003b). As mentioned in the first section of this introduction, the KvAP crystal structure was more consistent with large, paddle-like movements of a rigid bodied structure made up of S4 and the external end of S3. This was supported by biotin linking experiments, where components of this paddle could be modified by a tethered biotin derivative and tethered by avidin binding on either side of the membrane (Ruta et al., 2005). These experiments suggested as much as 20 Å of movement by the gating charge carrying moiety. However, further spectroscopic experiments (Posson et al., 2005; Chanda et al., 2005) and measurements of a focused electric field around the S4 segments (Ahern and Horn, 2005), as well as crosslinking between the S4 and the outer pore (Elinder et al., 2001), appeared to contradict these findings, because they appeared to rule out large transmembrane movements, and established that in its active conformation, the sensor came much closer to the pore domain than the paddle model would allow. In addition, it was demonstrated that a single amino acid substitution, near where the top of S4 meets the outer membrane leaflet, could result in formation of a transmembrane proton pore at negative membrane potentials, termed the omega current (Tombola et al., 2005; Tombola
et al., 2007). This finding further supported the notion of a thin and highly concentrated membrane field, with aqueous crevices allowing a hydrophilic environment to penetrate deep inside the bilayer. The discrepancy between these results and the paddle model appeared to arise from the use of Fab fragments in the KvAP crystal structure, which may have distorted the voltage sensor’s conformation in order to form a stable lattice. Finally, with the solving of the Kv1.2-2.1 paddle chimera, a stronger consensus on voltage sensor movement could be developed (Long et al., 2007). This crystal appeared to establish a key interacting site for the S4 residues within the voltage sensing domain. Tao and colleagues would later determine that Kv1.2 residue Phenylalanine-233 (equivalent to *Shaker* F290) represents a charge transfer site, where the charged amino acid side chains could be coordinated in sequence as they passed through the membrane (Tao et al., 2010), although it has since been suggested that only the innermost of these charged residues actually forms any measurable interaction with F233 (Lacroix and Bezanilla, 2011).

**Activation gating and coupling to voltage**

The original model of current activation suggested that it would accompany the transition of the fourth and final charge element into the “ON” conformation (Hodgkin and Huxley, 1952b). More recent modeling studies provide evidence that gating charge movement requires multiple distinct movements of the voltage sensor, and that these are followed by at least one separate and concerted movement to open the channel (Hoshi et al., 1994; Zagotta et al., 1994; Schoppa and Sigworth, 1998). These models raised two
vital questions about the activation process: where is the gate, and how is it coupled to voltage?

*The lower S6 and PVP motif form a barrier to potassium ions*

The activation gate of *Shaker* family potassium channels represents the structural element responsible for occluding or allowing ion flow in response to voltage. TEA and peptide channel blockers, when applied to the internal side of the channel, are capable of obstructing potassium current flow, but only if the channel first experiences a membrane voltage sufficiently depolarized to open the channel, suggesting that their site of action is protected from the cytosol when the channel is in its resting state (Zagotta et al., 1990; Choi et al., 1991). It was later shown that if residues in the predicted lower S6 helix in *Shaker* are mutated to cysteine, modification of residues between 470 and 478 by internally applied methanethiosulfonate (MTS) reagents was strongly dependent on the channel being in its conductive state, whereas MTS modification of residues more intracellular than residue 480 showed no state dependence (Liu et al., 1997) (see Figure 1.3). Furthermore, 470C and 474C modified channels could trap Cd$^{+}$ ions and small quaternary ammonium compounds upon repolarization, suggesting the presence of a cavity being protected by a “trap door” mechanism modulated by voltage (Liu et al., 1997; del Camino et al., 2000; Holmgren et al., 1997). Following activation, the lower S6 helix can also be crosslinked to those from neighbouring subunits by Cd$^{+}$, thereby creating a locked open pathway through the inner pore region, suggesting that a large scale reorientation takes place in this region during channel opening (Holmgren et al., 1998; Webster et al., 2004). These results are highly consistent with structural observations from crystallography experiments, which showed an inverted teepee in the
closed conformation of KcsA (Doyle et al., 1998), but a much wider aperture in the same region of the open MthK channel (Jiang et al., 2002b). Taken together, they are strong evidence that the lower S6 helices are closely packed at rest, then spread apart in response to voltage, thus widening the permeation pathway along their central axis and allowing ions to flow.

A highly conserved motif, the Pro-Val-Pro “bundle crossing” sequence, is present in all members of the Kv1 family (residues 473-475 in Shaker, see Figure 1.3). Because of the presence of two proline residues, the S6 is strongly “kinked” in the Kv1.2 crystal structure (Long et al., 2005a). While there is some evidence that the PVP residues can be shifted in location or substituted for a flexible glycine residue, removing the “kink” or “hinge” altogether strongly disrupts channel function (Labro et al., 2003). Mutation of P475 to glutamine results in constitutively open channels, further highlighting the importance of the unique proline side chain in preserving intact voltage gating (Sukhareva et al., 2003). Based on alanine and tryptophan scanning of the region and energetic analysis of resulting energetic changes in gating, the narrowest region of the pore was predicted to be between sites 478 and 481 below the bundle crossing (Hackos et al., 2002). V478 acts as the putative hydrophobic seal, representing a physical barrier which is energetically unfavourable to ion flow in the closed state of the channel (Kitaguchi et al., 2004).
Coupling between voltage and activation: a concerted opening transition represents the final step of activation

As established in the previous sections, membrane voltage leads to conformational changes within the voltage sensing domain comprised of helices S1-S4, resulting in widening of the barrier to ion permeation provided by the lower parts of the four S6 helices. But how are conformational changes transmitted from the voltage sensor to the pore? Results from a variety of techniques have improved our understanding of this process. Mutation in Shaker of S4 residues S369I, I372L and S376T (based on mutation to corresponding sites in the related “Shaw” channel, see Figure 1.3) generates the “ILT” mutant of the channel (Smith-Maxwell et al., 1998a). When Shaker ILT is subjected to depolarizing pulses, the voltage dependence of voltage sensor movement is shifted to more negative voltages (ie: requires less energy to activate), but the G(V) curves are strongly positively shifted (Ledwell and Aldrich, 1999). These data strongly suggest that the effect of the ILT mutations is to separate the independent movements of S4 from a cooperative opening step of the gate in S6, and reveals that a small fraction of voltage dependence is associated with activation gate opening (Ledwell and Aldrich, 1999). That study suggested that the lower S4 helix interacts with the gating apparatus, though the crystal structure establishes that the link is probably indirect (Long et al., 2005b).

Gating charge movement and lower S6 opening can also be functionally separated by the application of 4-aminopyridine (4-AP). 4-AP is a membrane permeable blocker of several families of voltage-gated potassium channels from the intracellular side (Kirsch et al., 1993), and requires opening of the activation gate to reach its site of action (Kirsch
and Drewe, 1993). When the binding site for 4-AP within the inner cavity is saturated, ionic current flow is blocked by the presence of the drug, but gating currents are largely unaffected (McCormack et al., 1994). Moreover, when 4-AP is present within the inner cavity, sites with state dependent accessibility in the lower S6 can no longer be modified biochemically from the inside of the membrane, showing that 4-AP isolates a distinct “activated-not-open” state (del Camino et al., 2000). A suggested model of action is that 4-AP enters the central cavity through an open activation gate, then “pulls” the aperture between the S6 helices closed, stabilizing a conformation where the voltage sensor is in an active conformation, but the activation gate remains closed around the 4-AP molecule (Armstrong and Loboda, 2001). The importance of 4-AP as a pharmacological tool is further highlighted by the observation that an inactivation process normally requiring channel opening to occur is prevented by 4-AP, confirming the ability of the drug to distinguish between processes dependent on S4 translocation, and those dependent on S6 opening (Castle et al., 1994; Claydon et al., 2007a). This will be discussed further later in this section.

**Physical interactions between the S4-S5 linker and the lower S6 helix**

The importance of the S4-S5 linker (Figure 1.3) in voltage-activation coupling is clear from the highly conserved leucine heptad repeat within that domain, which, when mutated, results in a strong rightward shift in the conductance-voltage relationship (McCormack et al., 1991). The search for a physical link between the S4-S5 linker and the activation gate has identified a cluster of amino acids in the lower parts of S5 and S6 helices that are energetically coupled to S4 movement and predicted to interact with the
S4-S5, acting as a transducer (Li-Smerin et al., 2000). Further research would identify the region of S6 below the bundle crossing as a key site of interaction for the S4-S5 linker (Lu et al., 2002; Labro et al., 2005). When either the S4-S5 linker, or the carboxy-terminal end of the S6 helix, was replaced by the equivalent structures from KcsA, the resulting channel demonstrated voltage-independent conductance (Lu et al., 2002). When the results of extensive mutagenesis studies were mapped onto the Kv1.2 crystal structure, clustering of mutations affecting the channel’s final opening step was observed in S4, S5 and the S4-S5 linker (Long et al., 2005b; Soler-Llavina et al., 2006). This suggested that following depolarization, gating charge movement precedes a cooperative movement across the S6 helices of all four subunits, which is facilitated by conformational changes in the S4-S5 linkers of adjacent subunits (Soler-Llavina et al., 2006).

*N-type inactivation, an autoinhibitory mechanism following activation*

Following the increase in current due to channel opening during activation, some Kv1 channels exhibit a rapid decrease in channel current termed N-type inactivation. This is due to the presence of a blocking peptide in the amino terminus of the channel protein, which has a compatible receptor within the open channel and acts as a voltage independent but open state dependent blocker (Hoshi et al., 1990; Demo and Yellen, 1991). The N-terminus can be cleaved from the remainder of the channel by trypsin or site-directed mutagenesis, leaving a functional channel lacking the transient inactivation (Hoshi et al., 1990), termed *Shaker “IR”* or inactivation removed, or Δ6-46, as these residues contain the requisite sequence to produce inactivation, the so-called “ball and
chain”. Addition of a synthetic peptide mimicking the N-terminus of the full length channel to Shaker IR restores the inactivation phenotype (Zagotta et al., 1990). Some associated Kvβ1 subunits can also donate a blocking peptide to the channel (Rettig et al., 1994; Heinemann et al., 1995). Block by synthetic N-terminal peptides largely imitates the action of TEA derivatives, and the two compete for a binding site within the pore, demonstrating that N-type inactivation represents entry by the peptide into a site protected by the activation gate in the lower S6 helices (Choi et al., 1991). Block in this way by the N-terminus requires only one peptide to be present per tetrameric channel, though the presence of additional subunits increases the onset rate of the block (Gomez-Lagunas and Armstrong, 1995). Peptide binding is due primarily to hydrophobic contacts between the most distal residues of the N-terminus (often referred to as the “ball”) and the residues lining the inner pore (Murrell-Lagnado and Aldrich, 1993). However, interactions between the “chain” and a S4-S5 linker segment of the channel protein outside of the pore also take place, demonstrating that the distal “ball” is not the only important moiety in N-type inactivation (Isacoff et al., 1991). The crystal structure of the “hanging gondola” of the channel, comprising the isolated tetramer of T1 domains and the associated β-subunits, establishes the windows above the T1 domains through which the ball and chain enter the central pore (Gulbis et al., 2000). Later, the Kv1.2 crystal structure would highlight a series of acidic residues along the windows thought to interact with the positively charged residues in the extended chain (Long et al., 2005a). Sequential mutagenesis of sites within the inner cavity of Kv1.4, and two mutant cycle analysis with an alanine scan of its N-terminus, shows that a series of pore lining residues form contact points for binding of the N-terminus as an extended peptide (Zhou et al.,
2001). The most critical of these are V551 and I554 (equivalent to V467 and I470 in *Shaker*), which, upon mutation to alanine, result in the reduction in binding potency by several orders of magnitude (Zhou et al., 2001). That study also established that peptide binding likely represents a multi-step reaction, suggesting a likely functional correlate of interactions within and outside of the central cavity. More recent work has separated three regions of the extended peptide: the polar region, for the initial external association with the S1-T1 portals, the flex region, to allow maximal freedom of movement of the distal peptide, and the head region, responsible for binding within the open pore (Prince-Carter and Pfaffinger, 2009).

**A selectivity filter for ion exclusion and gating**

The selectivity filter is a narrow passage with a high preference for potassium

In addition to the internally located activation gate, the potassium channel pore also possesses an externally located narrow passageway called the selectivity filter, which is housed by the wider top of the inverted tepee (Doyle et al., 1998). A sequence of residues, TXGYG (where X typically possesses an aliphatic, branched carbon side chain such as valine or isoleucine), is extremely well conserved across the potassium channel superfamily, and is termed the potassium channel signature sequence (Heginbotham et al., 1992, see also Figure 1B). Conservation of amino acid identity is essential for channel function and for selectivity for potassium ions. The molecular architecture of the selectivity filter indicates the presence of four potassium binding sites within the filter, arranged in series along the axis of ion permeation with additional sites in the outer vestibule and the central cavity (Doyle et al., 1998; Zhou et al., 2001). These sites are
provided by the backbone carbonyl groups of the signature sequence residues, and their proximity allows electrostatic ion-ion interactions between adjacent sites (Ogielska and Aldrich, 1998). A model of potassium conduction suggests that potassium ions pass through the filter by binding alternatingly at sites 1 and 3, or 2 and 4 (Morais-Cabral et al., 2001), where the negative dipoles of the carbonyl groups substitute for the normal hydration shell of the potassium ion in aqueous solution (Zhou et al., 2001). This model also helps to explain why sodium, though physically smaller than potassium, is not well conducted by the open potassium channel: coordinating the more concentrated charge of the Na\(^+\) ion strains the backbone carbonyl groups into an energetically unfavourable conformation (Noskov et al., 2004).

The selectivity filter backbone represents a highly ordered region maintaining the exquisite conducting conformation of the filter. In KcsA, residues E71 and D80 (equivalent to Shaker V438 and D447) can coordinate a water residue in the space between the selectivity filter and P-helix, and may also interact with the side chain of Y78 (Shaker Y445) of the signature sequence to stabilize the selectivity filter in a conducting conformation (Cordero-Morales et al., 2006). KcsA activation by protons - which results in conformational changes in the pore-lining helix similar to those occurring with voltage-dependent activation in Kv1 channels - causes a reorientation of E71, and appears to favour a stable interaction with W67 (Shaker W434) of the same subunit, which leads to destabilization in the selectivity filter and adoption of a collapsed, non-conducting filter conformation (Cordero-Morales et al., 2006; Cordero-Morales et al., 2007). As the KcsA and Kv1.2 crystal structures show strong similarities in structure in the selectivity filter region (Doyle et al., 1998; Long et al., 2005a), a similar backbone
structure can be inferred in voltage-gated channels. However, some key distinctions do exist. KcsA possesses a glutamate residue at position 71, which, when mutated to alanine, helps to stabilize the conducting conformation (Cordero-Morales et al., 2006). Shaker, however, possesses a valine residue at that position, and alanine mutation enhances the adoption of a non-conducting conformation (Ahern et al., 2009). Though this difference may explain why wild type KcsA has a stronger preference for the non-conducting filter conformation compared to Kv1 channels (Liu et al., 2001), a thorough understanding of the factors influencing Kv1 filter architecture remains an important problem.

**C-type inactivation: an additional gate in the selectivity filter of Kv channels**

Upon removal of the blocking peptide from the N-terminus of Shaker, a second inactivation process developing on the order of seconds (termed C-type inactivation) remained in the truncated channel (Hoshi et al., 1990; Hoshi et al., 1991). Further study of this process would demonstrate that it was highly sensitive to single mutations within the outer pore region (De Biasi et al., 1993). Classical studies had established that neuronal potassium channel closing was slowed by an elevated concentration of extracellular potassium (Swenson and Armstrong, 1981). Later findings would demonstrate that heterologously expressed channels also became less conductive in low potassium, and that high external potassium could slow C-type inactivation and accelerate its recovery (Pardo et al., 1992; Lopez-Barneo et al., 1993; Levy and Deutsch, 1996b; Levy and Deutsch, 1996a). The slow inactivation process was shown to represent a cooperative movement involving all four subunits (Ogielska et al., 1995), whereby a conformational rearrangement within the selectivity filter renders one of the selectivity
filter potassium sites non-functional (Ogielska and Aldrich, 1999; Kiss and Korn, 1998; Kiss et al., 1999), thus converting the filter from a conduit for potassium into a barrier. This is consistent with the potassium sensitivity of the inactivation process, as a variation in occupancy of that site due to a higher or lower concentration of permeant ion would be expected to antagonize or promote filter collapse, respectively. Accordingly, the crystal structure of the KcsA potassium channel in lower potassium levels shows an altered selectivity filter structure, with low electron density at potassium site 2 and a narrowing of the passage between the signature sequence peptide chains (Zhou et al., 2001; Zhou and MacKinnon, 2003). As described in the previous section, entry into this conformation can also be influenced by perturbations in the selectivity filter backbone (Cordero-Morales et al., 2006), or in KcsA, by changes in pH (Cuello et al., 1998).

In addition to being sensitive to the concentration of permeant cations, the rate of C-type inactivation can also be strongly modulated by mutations and pharmacological tools. Mutations of Shaker T449 (representing a poorly conserved site across the Kv1 family) can greatly reduce (e.g., T449V, T449Y) or increase (e.g., T449K) the rate of C-type inactivation (Lopez-Barneo et al., 1993). Site T449 is directly above the external mouth of the pore, and is close enough to the channel axis of symmetry that T449C mutants can be modified by addition of cadmium ions, resulting in block of the current (Yellen et al., 1994). Conversely, mutation at Shaker site W434F (KcsA W67) causes channels to become non-conducting (Perozo et al., 1993), due to instability in the selectivity filter backbone resulting in extremely rapid pore collapse or inactivation at rest (Yang et al., 1997). The addition of TEA to the external medium can also block the channel, but slows the onset of C-type inactivation by an alleged “foot-in-the-door”
mechanism (Liu et al., 1996), and the site T449 is known to form part of the binding site of TEA in the outer vestibule (MacKinnon and Yellen, 1990). Indeed, the potency of external TEA varies by orders of magnitude in different Kv1 channels with different T449 residue identities (Grissmer et al., 1994), and TEA can actually accelerate inactivation in the presence of mutation T449F (Ahern et al., 2009). TEA and some of its derivatives can also block from the internal side of the channel, and many of these agents can accelerate the onset of inactivation, probably by depleting the pore of permeant ions (Yellen et al., 1991; Baukrowitz and Yellen, 1996a). The mechanism of TEA block results from the ability of TEA to mimic a hydrated potassium ion owing to their similar atomic radii, causing TEA to become lodged in a potassium binding site in the permeation path (Lenaeus et al., 2005). 4-AP can also prevent C-type inactivation upon entering the pore from the internal side (McCormack et al., 1994; Armstrong and Loboda, 2001; Castle et al., 1994; Claydon et al., 2007a), though this may represent an indirect effect of inhibition of channel opening by 4-AP.

**P/C-type inactivation: two separable components of slow inactivation**

While the process by which channel conductance is reduced during C-type inactivation involves a local rearrangement within the selectivity filter, there is considerable evidence that a global protein rearrangement involving the channel turret and voltage sensors takes place concomitantly. During prolonged channel depolarizations from a negative holding potential, hysteresis is observed as the gating charge from S4 becomes immobilized in an upward position. To return the immobilized helices to their resting conformations required stronger repolarizing voltages, therefore resulting in a
negative shift in their Q(V) curves (Olcese et al., 1997). A non-inactivating mutant also does not undergo charge immobilization (Olcese et al., 1997), whereas in the enhanced inactivation mutant W434F, the ability to immobilize is preserved (Perozo et al., 1993). Furthermore, the time course of onset of charge immobilization coincides with the time course of onset of C-type inactivation (Olcese et al., 1997). Taken together, these results support an involvement of the S4 helix in the inactivation process. Studies using voltage clamp fluorimetry (VCF) to independently track movements within the pore and voltage sensor domains (a technique which will be described in detail later in this introduction) have demonstrated that during C-type inactivation, fluorescent tags bound to the S4 helix witness a secondary movement following the rapid transition that precedes opening (Cha and Bezanilla, 1998). Using VCF on sites in the sensor and the pore, the S4 was determined to move into close proximity with the outer pore during C-type inactivation (Loots and Isacoff, 1998; Loots and Isacoff, 2000; Gandhi et al., 2000). This was corroborated by the ability of the outer S4 to be cross-linked to the turret when both possess introduced cysteine residues, but only in a state-dependent way requiring depolarization (Elinder et al., 2001). As a result, C-type inactivation is sometimes branded as P/C-type inactivation, to reflect the separable but tightly associated processes of pore collapse and S4 helix reorientation which take place.

**N-terminal peptide block enhances C-type inactivation**

In channels where they co-exist, N-type inactivation accelerates the onset of P/C-type inactivation (Baukrowitz and Yellen, 1996a; Baukrowitz and Yellen, 1995). This is thought to be due to the emptying of the pore of ions by an occluding blocker
(Baukrowitz and Yellen, 1996a), thereby facilitating its collapse into the conformation observed in the low K⁺ crystal structure (Zhou et al., 2001). Not surprisingly, some derivatives of TEA are also capable of state-dependent block, and accelerate P/C-type inactivation, putatively by a similar mechanism (Baukrowitz and Yellen, 1996a). However, TEA and peptide channel block are not identical. Though spatially coincident, the binding sites for TEA and blocking N-termini require different protein contacts within the inner vestibule (Zhou et al., 2001; Yellen et al., 1991; Baukrowitz and Yellen, 1996b). Moreover, when the presence of permeant cations is factored out, TEA itself actually displays a negative allosteric coupling to pore collapse (i.e.: slowing of movement), as compared to apparent positive allostery between longer chained TEA derivatives or quinidin e and the pore (Baukrowitz and Yellen, 1996a; Bett and Rasmusson, 2004). Study of allosterism between inactivation gates may also be confounded by crosstalk between the activation gate and selectivity filter (Panyi and Deutsch, 2006), therefore the process of how N-type and P/C-type inactivation processes are mechanistically coupled remains controversial.

**Potassium channel function depends on allosteray between activation and inactivation gates**

* A pore lining residue links activation gate conformation to the selectivity filter backbone

In addition to the relationship between the N- and C-type inactivation gates, activation and inactivation gating processes are importantly functionally linked in potassium channels by allosteric coupling (Sadovsky and Yifrach, 2007). The conformation of the lower S6, which is drastically modified during activation, undergoes
an additional rearrangement during P/C-type inactivation, as evidenced by the state
dependence of the accessibility of S6 residues to the internal medium (Panyi and
Deutsch, 2006; Panyi and Deutsch, 2007). In those studies, it was shown that while the
activation gate remains open during inactivation, pore lining residue mutants I470C and
V474C are modified much more slowly by internally applied Cd$^{2+}$ (Panyi and Deutsch,
2006; Panyi and Deutsch, 2007). In KcsA channels, a mechanism of inactivation at the
selectivity filter was found to result from a widening of the activation gate similar to the
voltage dependent process occurring in Kv1 family channels (Chakrapani et al., 2007). A
series of partially open structures of KcsA establishes that the widening of the activation
gate associated with opening tends to bias the channel into the C-type inactivated state,
suggesting that the two processes are structurally linked (Cuello et al., 2010). In an
accompanying paper, the same authors demonstrated that site F103 in KcsA (equivalent
to I470 in Shaker) underwent a rotomeric reorientation in concert with the widening of
the S6 helices (Figure 1.4A,B), and suggested that this site was an allosteric transducer
between the activation and C-type inactivation gates (Cuello et al., 2010).
Figure 1.4. Single subunits of the closed and open KcsA channel. A. A single subunit of the 14.5 Å (denotes diameter of activation gate aperture at T112) partially open KcsA channel crystal structure (PDB# 3FB5), from Cuello et al. (2010b). Potassium ion volume density is shown by dot mesh. Residue F103 is highlighted by red box. B. A single subunit of the 32 Å open-inactivated KcsA channel crystal structure (PDB# 3F5W). Potassium ion density is shown in purple, and the reoriented residue F103 is highlighted in the red box.

The inner pore conformation and outer pore also appear to be coupled through multiple conformations of the voltage sensor. Though often modeled as a rigid body, the S4 helix is in fact capable of altering its secondary structure from $3_{10}$ to $\alpha$-helix, giving rise to three adoptable conformations: the resting (at negative potentials), the active (at positive potentials for short periods) and the relaxed states (at positive potentials during prolonged periods) (Villalba-Galea et al., 2008). The voltage sensing domain, in addition to the described coupling to the pore at the activation gate, is known to interact with the pore by an extracellular interface between S1 and the pore helix (Lee et al., 2009).
A family of auxiliary β–subunits to modify channel trafficking and gating

Though the membrane spanning tetramer of “α” subunits represents the minimal functional unit of voltage-gated potassium channels, \textit{in vivo} α-subunits are found in complex with a tetramer of modulatory “β” subunits (Rhodes et al., 1997; Orlova et al., 2003). The Kvβ subunits represent a family of cytosolic proteins with high homology to the aldo-keto reductase enzymes, and were first cloned thanks to their tight binding to the α subunits (Rettig et al., 1994; Heinemann et al., 1995). Structurally, the Kvβ subunits can be divided into two distinct regions: a highly conserved C-terminal core present on subunits in all three Kvβ subunit families (Kvβ1-3), and a highly variable N-terminal domain absent in the Kvβ2 subunits (Rettig et al., 1994; Heinemann et al., 1995). Accordingly, the functional effects of the Kvβ subunits can be grouped into two broad groups: effects on the protein maturation pathway due primarily to their C-terminal cores, and effects on gating due primarily to the N-termini. [Note: Use of nomenclature for Kvβ subunits will follow that proposed by England et al., (1995a)].

\textit{Kvβ subunits associate with α-subunits early in biogenesis and differentially modulate channel surface expression}

As mentioned in the first section of this introduction, a crystal structure shows that Kvβ subunits are bound tightly by their conserved core domain to the α-subunit T1 domains in equivalent tetramers on the cytosolic aspects of the channels (Long et al., 2005a; Gulbis et al., 2000; Gulbis et al., 1999). This association forms in the endoplasmic reticulum, and has a chaperone effect on Kv1.2 channels, leading to increased membrane
expression (Shi et al., 1996), and the effect is largely specific to members of the Kv1 family, though interactions with a Shal-related (Kv4) channel have also been reported (Nakahira et al., 1996). *Drosophila Shaker* channels can also associate with mammalian Kvβ subunits in the endoplasmic reticulum in heterologous cells, but trafficking of those channels is not affected by Kvβ binding, suggesting that effects on biogenesis are dependent on subunit type or species (Nagaya and Papazian, 1997), however, the *Drosophila* Kvβ homologue *Hyperkinetic* does promote membrane trafficking of *Drosophila Shaker* and *Eag1* channels (Chouinard et al., 1995; Wilson et al., 1998). Amongst mammalian Kv1 channels, Kvβ subunits upregulate Kv1.2 expression, but downregulate Kv1.5, while having no effect on expression of Kv1.4 (Accili et al., 1997b; Accili et al., 1998). These data suggest that cellular expression of Kvβ subunits exerts strong effects on the eventual composition of Kv1 channels at the plasma membrane.

**Kvβ1 family subunits introduce an N-type inactivation peptide to delayed rectifier channel complexes**

In addition to their effects on channel maturation, Kvβ1 channels possess N-termini capable of emulating the N-termini present on some α-subunits to confer rapid inactivation to the channels (Rettig et al., 1994; Heinemann et al., 1995; Accili et al., 1997b; Morales et al., 1995; Majumder et al., 1995; England et al., 1995b; Castellino et al., 1995; Heinemann et al., 1996; De Biasi et al., 1997). The Kvβ1 subunits are all splice variants of the same gene (England et al., 1995a; Wang et al., 1996), and while their C-terminal cores are identical, they demonstrate considerable variability in their N-termini. Not surprisingly, the functional characteristics of block also vary (Wang et al., 1996). The
Kvβ1.1 N-terminus is mostly homologous to the α−subunit N-termini from Shaker and Kv1.4, and blocks the channel in a similar way, entering the pore as an unstructured peptide (Zhou et al., 2001; Prince-Carter and Pfaffinger, 2009; Antz et al., 1997; Wissmann et al., 1999). However, the Kvβ1.3 subunit’s distal N-terminus forms a hairpin loop and its ability to reach its site is PIP2-dependent (Decher et al., 2008). The Kvβ1.2 subunit shows almost no sequence homology in its distal N-terminus with Kvβ1.1 and has been suggested to inhibit current by a distinct, allosteric mechanism (Morales et al., 1996). Additionally, most Kvβ1 subunit studies have identified a leftward shift in the voltage dependence of activation of channels where they are present. However, whether this represents an artefact of normalization due to open channel block (Accili et al., 1997b; De Biasi et al., 1997; Rasmusson et al., 1997), or a separate allosteric effect on channel activation (England et al., 1995b; Uebele et al., 1998), is an unresolved question. Clearly, developing a model for the mechanisms of action of different Kvβ subunits on their α−subunit binding partners, as relates to resulting changes in channel gating and expression, remains an important open problem.

Summary

In this section, I have described how the Kv1-family of voltage-gated channels function by opening to the selective passage of potassium ions. This process depends first upon independent movements in the voltage field of the voltage sensor contained with S1-S4, followed by a coupled and cooperative reorientation of the lower S6 helices to expand an internal gate to a width sufficient for potassium flow. Multiple, interrelated inactivation processes can take place to attenuate current flow following opening. I have
detailed how the functional properties stem from the known structural properties of the channel, as described in section 1. In the following section, I will describe in more detail some remaining difficult questions limiting our understanding of potassium channel function, and a key technique used in the studies comprising this thesis to explore these questions.

**POTASSIUM CHANNELS: TECHNIQUES AND QUESTIONS**

A common theme in the following chapters is the use of the voltage clamp fluorimetry technique. In this section, I will describe this technique and its application to potassium channel studies. Then, I will state the objectives of the studies described in the subsequent sections of this thesis.

**Voltage clamp fluorimetry is a combination of electrophysiology and a real-time fluorescence assay**

One of the biggest challenges facing the ion channel biophysicist is to determine the roles played by discrete, dynamic protein domains in producing experimentally measurable gating processes. Most functional studies employ voltage clamp electrophysiology to study gating, but in cases where multiple gating processes occur simultaneously, or where movements associated with gating don’t result in charge movement across a voltage field or a measurable change in ionic current, the underlying conformational changes can be difficult to infer. The development of voltage clamp fluorimetry came about largely to address this issue.
Principles of voltage clamp fluorimetry

The voltage clamp fluorimetry technique is an integration of electrophysiology and real time fluorescence imaging techniques (Cha et al., 1998). Supplementary figure A.1 (appendix) shows a simplified schematic of the voltage clamp fluorimeter. Using a two electrode voltage clamp apparatus, we can measure membrane potential from a *Xenopus* oocyte. The amplifier can then be set to clamp the voltage at a desired level, and to pass the necessary current required to hold the membrane at the desired voltage, based on the relationship:

\[
I_{\text{clamp}} = G_{\text{membrane}} (V_{\text{command}} - V_{\text{actual}})
\]

where \(G_{\text{membrane}}\) refers to the conductance of ions at the membrane and \(V_{\text{command}}\) and \(V_{\text{actual}}\) refer to the desired and actual membrane voltages, respectively. The current level is simultaneously read out to the digitizer, as it is equal to the current flowing through membrane channels, plus that flowing across the membrane itself (or through any passive diffusion pathways through the membrane), plus any linear and non-linear capacitance signals, based on the relationship:

\[
I_{\text{total}} = I_{\text{cap}} + I_{\text{leak}} + I_{\text{channels}}
\]

where \(I_{\text{total}}\) refers to the total current level, \(I_{\text{cap}}\) refers to a rapid transient capacitance signal reflecting the membrane’s charging profile, \(I_{\text{leak}}\) refers to any Ohmic conductances through leak channels or perforations in the membrane, and \(I_{\text{channels}}\) refers to currents carried by the channels themselves. In this way, the responses of ion channels to voltage are recorded. For VCF, an additional module is added: the fluorescence imaging device. Here, fluorescent light is emitted by a xenon lamp, and the appropriate wavelengths (\(\lambda\)) reflected by a dichroic mirror with an appropriate reflection threshold (for this study, a
rhodamine-based fluorophore is the most widely used, with an excitation peak $\lambda$ of 542 nm and an emission peak $\lambda$ of 574 nm in aqueous solvent, and the dichroic mirror’s threshold was 560 nm). Light at the excitatory $\lambda$ is focused through an objective lens to the surface of the oocyte, where a fluorophore bound to the channel is excited. Following a Stoke’s shift (the process by which a photon loses energy between the processes of absorption and radiation by a fluorophore), the fluorophore emits a lower $\lambda$ light, which is not reflected by the dichroic mirror, and instead traverses to the photomultiplier tube below. Here, the intensity of the fluorescence light is amplified, and the PMT generates a signal reflecting the measured intensity to the digitizer. The digitizer thus generates a fluorescence amplitude digital signal to be read into the control computer, alongside the current signal as previously mentioned. As the fluorophore changes its local environment, for instance as the protein it is attached to moves, the emission intensity can become quenched (or dequenched), changing the intensity reported by the photomultiplier. In this way, the movement of the fluorophore can be correlated to changes in channel conductance reported by the ionic currents (Cha et al., 1998).

To appropriately employ this technique, the fluorophore must be in a suitable position to accurately track movements within a protein. As such, it must be tightly bound to the desired amino acid. To accomplish this, the amino acid in question is mutated to cysteine (necessitating a characterization of the resulting mutant prior to further experimentation). For these experiments, we used the rhodamine derivative tetramethylrhodamine maleimide (TMRM). The imide group of the maleimide moiety forms a stable carbon-sulfur bond with the thiol group of a cysteine residue, allowing the fluorophore to become strongly associated with the site of interest. As necessary, native
cysteine residues elsewhere in the protein can be mutated to other amino acids to prevent contamination of fluorescence data. The fluorescent emission from TMRM bound to an ion channel can be quenched as the fluorophore comes into proximity of other parts of the protein, approaches another fluorophore or traverses a solvent interface. The latter event would result in a spectroscopic shift, which was not observed when some of the well characterized tagging sites of the protein (including ones used in this study) were subjected to spectroscopic analysis (Cha and Bezanilla, 1998), therefore, in those cases, fluorescence deflections are attributed to the spatial reorientation of discrete domains within the protein with respect to each other.

This technique has provided considerable insight into potassium channel function. By performing cysteine mutagenesis scans of regions of interest within the protein, fluorimetry has been used to gain structural insights into dynamic movements involved in gating. A scan in the S3-S4 linker and upper S4 helix has identified residues capable of tracking movements of the gating charges in the S4 helix (Cha and Bezanilla, 1998; Mannuzzu et al., 1996; Cha and Bezanilla, 1997). These studies have been instrumental in developing a complete model of potassium channel voltage sensor movement, identifying multiple conformational changes of S4 (Posson et al., 2005; Villalba-Galea et al., 2008; Sorensen et al., 2000), and calling into question the paddle movement hypothesis (Jiang et al., 2003b; Long et al., 2005b), suggesting instead a smaller range of motion involving some combination of translocation, tilt and rotation of the S4 helix, possibly combined with realignment of the S4 helix’s secondary structure, though a conclusive model has remained elusive (Long et al., 2007; Tao et al., 2010; Villalba-Galea et al., 2008; Sorensen et al., 2000; Pathak et al., 2007). Similarly, a scan of the
outer pore region has demonstrated that P/C-type inactivation involves a contribution of
the voltage sensor following activation (Loots and Isacoff, 1998; Loots and Isacoff, 2000;
Gandhi et al., 2000; Cha and Bezanilla, 1997; Mannuzzu and Isacoff, 2000), and has
demonstrated that a wide-scale cooperative conformational rearrangement in the outer
vestibule accompanies collapse within the selectivity filter during long periods of
depolarization (Gandhi et al., 2000; Pathak et al., 2005).

In addition to the approach of cysteine scanning within domains of interest of a
protein, two additional methodologies involving VCF are commonly employed. Firstly,
the use of sequence alignment software can predict homologous sites between Shaker
and other channels, allowing for fluorescence tagging sites in areas of interest to be tested in
other channels in order to compare gating in different channels. In this way, distinct
gating behaviours with properties divergent from Shaker gating have been studied in
potassium channels such as Kv1.5 (Vaid et al., 2008), spHCN (Bruening-Wright and
Larsson, 2007; Bruening-Wright et al., 2007), hERG (Smith and Yellen, 2002; Es-Salah-
Lamoureux et al., 2010), BKCa (Savalli et al., 2006; Savalli et al., 2007) and KCNQ1
(Osteen et al., 2010). Secondly, in the well-studied Shaker channel, tagging of the well-
characterized sites allows for faithful reports on principle gating transitions, such as
Shaker A359C for S4 movement (Mannuzzu et al., 1996; Cha and Bezanilla, 1997), and
Shaker S424C for outer pore movements associated with P/C-type inactivation (Loots
and Isacoff, 1998). In this way, the allosteric effects of mutations or pharmacological
treatments can be tracked while simultaneously recording ionic currents, allowing
specific effects on discrete protein domains to be ascertained and related to the effects on
protein function as a whole (Claydon et al., 2007a; Claydon et al., 2007b).
Objectives of this thesis

As mentioned in the previous section, despite their considerable homology, Kv1 type channels exhibit subtle but important structural differences that may underlie the functional diversity within the family. For instance, the extracellular linkers vary in length, with the archetypal *Drosophila Shaker* channel having a shorter S1-S2 linker but longer S3-S4 linker than the mammalian Kv1 channels. As mentioned in the introduction, the interactions of different family members with auxiliary subunits demonstrate considerable variability (Nakahira et al., 1996; Nagaya and Papazian, 1997; Accili et al., 1997b), and a critical site in the outer pore region causes potent differences in the rates of C-type inactivation (Lopez-Barneo et al., 1993). In light of these differences, a disparity exists between the use of Kv1.2 as a structural model (Long et al., 2005a), and the extensive characterization with voltage clamp fluorimetry of *Shaker* as the prototypical Kv1-type potassium channel (Mannuzzu et al., 1996; Cha and Bezanilla, 1997). Therefore, in Chapter 2 of this thesis, we employ voltage clamp fluorimetry to probe activation gating of the Kv1.2 channel, in order to investigate how variability in structure between *Shaker* and Kv1.2 can lead to the functional diversity between them.

Following characterization of Kv1.2 gating using voltage clamp fluorimetry as described in Chapter 2, we sought to apply that technique to the study of how Kv1.2 is modified by a Kvβ1.2 subunit. As discussed previously, while the pore blocking properties of Kvβ1.2 have been studied extensively (Accili et al., 1998; Castellino et al., 1995; Rasmusson et al., 1997; Accili et al., 1997a), its putative effects on activation remain equivocal (England et al., 1995b; De Biasi et al., 1997; Uebele et al., 1998). On
the one hand, β-subunit effects on potassium currents are consistent with open channel block: because the channel is only blocked when open, and conductance-voltage curves are drawn by normalization of all intermediate values to the maximum conductance, an agent selectively targeting open channels for blockade might lead to underestimation of maximum conductance, and thus artificial upward (and leftward) shift of values at intermediate voltages (De Biasi et al., 1997). However, the presence of an allosteric effect causing a separate shift cannot be ruled out by those data (England et al., 1995b; Uebele et al., 1998), largely because activation and inactivation occur on similar time scales. In Chapter 3, our objective is to resolve the controversy by applying voltage clamp fluorimetry to the study of activation gating in Kv1.2 channels in the presence of the Kvβ1.2 subunit. In this way, we can independently track the voltage dependence of activation gating (by measuring fluorescence emissions), while simultaneously recording ionic currents, allowing us to determine whether and how activation gating might be affected by the presence of a pore blocking N-terminal peptide.

Having shown that voltage clamp fluorimetry can be used to track channel gating even in the presence of additional gating mechanisms, such as N-type inactivation, we next considered the issue of the relationship between two different inactivation mechanisms. P/C-type inactivation takes place in the selectivity filter and outer pore mouth of the Shaker channel, and in the presence of inner pore blockers, such as the internal N-type inactivation gate, is strongly accelerated (Baukrowitz and Yellen, 1995). This acceleration has been attributed to pore emptying, due to repulsion of permeant cations from their binding sites by the bound N-terminus (Baukrowitz and Yellen, 1996a). However, a competing hypothesis holds that the acceleration of P/C-type
inactivation by the N-terminus involves allosteric coupling, whereby the N-terminus binds and holds the activation gate in a conformation which promotes selectivity filter collapse (Bett and Rasmusson, 2004; Li et al., 2003; Jiang et al., 2003a). Complicating the issue is the identification of an allosteric coupling pathway between the activation gate and selectivity filter (Panyi and Deutsch, 2006), which involves local side chain reorientation at Shaker site I470 (Cuello et al., 2010), an amino acid residue within the site of peptide binding (Zhou et al., 2001). The relative impacts of potassium occupancy and allosteric coupling mechanisms on the selectivity filter are hard to distinguish from electrophysiological recordings alone, largely because in the presence of pore blockers, ionic current is largely blocked, preventing a direct readout. In Chapter 4, we use voltage clamp fluorimetry, in concert with mutations at Shaker site I470 to modify allostery, to investigate how N-terminal peptides and S6-pore allostery contribute to ionic current decay when present together in an activated Shaker channel.

Where genetic pathophysiology exists in ion channels, it is sometimes found to result from mutation at an evolutionarily conserved site in gating. Such is the case for some forms of the inherited movement disorder episodic ataxia type-1. EA-1 results from the dominant negative effects of mutations in the human Shaker homologue Kv1.1 (Browne et al., 1994). Mutations at highly evolutionarily conserved Kv1.1 site valine-408 to alanine (Browne et al., 1994; Adelman et al., 1995) or leucine (Demos et al., 2009), result in accelerated decay of ionic current following channel opening, reportedly due to an enhancement of C-type inactivation (Adelman et al., 1995). However, a mechanism by which these mutations, which take place far from the inactivation gate, lead to faster current decay has never been established. In Chapter 5, we sought to determine how these
EA-1 associated mutants modify channel gating to cause the observed functional effects. After establishing that the acceleration is present in the Shaker channel, as well as hKv1.1, we used voltage clamp fluorimetry in Shaker to track movements in the voltage sensor and outer pore in order to determine how the mutations were affecting voltage dependent gating.
Chapter 2: Fast and slow voltage sensor rearrangements during activation gating in Kv1.2 channels detected using tetramethylrhodamine fluorescence.¹

INTRODUCTION

Crystallographic modeling and fluorescence based studies of protein conformation each provide distinct mechanistic insights into how ion channel structure relates to function. Crystal structures from Kv1.2 (Long et al., 2005a; Long et al., 2005b) and a Kv1.2/Kv2.1 chimera channel (Long et al., 2007) have been invaluable to our ability to visualize the plenary structure of the channel complex. Fluorescence studies, on the other hand, are ideal to study dynamic transitions within the proteins, as they provide time-resolved measurements of transmembrane domain movement, and these can be interpreted along with ionic and gating current recordings made simultaneously. Although the transmembrane domains of potassium channels are well conserved among all families in terms of primary and secondary structure (Doyle et al., 1998; Jiang et al., 2003; Long et al., 2005a; Long et al., 2007), the existing reports of secondary fluorescence amongst Kv1 channels differ (Claydon et al., 2007a; Vaid et al., 2008), suggesting that there may be dynamic differences between the pore and/or voltage sensor domain interactions of Kv1 channels arising from differences in channel structure/gating.

The high resolution structure of Kv1.2 provides an ideal model to further explore differences in Kv1.x gating. In this Chapter, we have undertaken the first detailed examination of gating current and fluorescence measurements in the Kv1.2 voltage sensor. Tetramethyl-rhodamine-5-maleimide (TMRM) labelled at the externally accessible A291C residue in S4 (Figure 2.1) exhibits a fast quenching component that correlates with the time course and voltage-dependence of gating charge movement. In addition, Kv1.2 A291C-TMRM and other residues in the S3-S4 linker, unlike all other voltage-gated channels studied, also detects a second and much slower quenching that
represents up to 60% of the total fluorescence change. Through the use of chimera channels and fluorophores placed within S1 and S2, we suggest that this slow phase may report rearrangements of voltage sensor moieties other than S4. The reversal of slow rearrangements may be required for the channel to deactivate, suggesting that they play an important role in determining the stability of the open channel.

![Alignment of S3-S4 linkers of Shaker and Kv1.2](image)

**Figure 2.1. An alignment of the S3-S4 linkers of Shaker and Kv1.2 highlights similarities in the region.** Grey boxes denote the S3 and S4 regions of the protein. Residues assayed for voltage-dependent fluorescence (Figure 2.3) are underlined, and the residue denoted by the filled circle corresponds to Kv1.2 A291C (Shaker A359C).
MATERIALS AND METHODS

Molecular biology and RNA preparation

*Shaker* and Kv1.2 constructs were expressed in *Xenopus* oocytes using a modified pBluescript SKII expression vector (pEXO) (a gift from A. Sivaprasadarao, University of Leeds, UK). An N-terminal-deleted, fast-inactivation-removed mutant Δ6-46 (*Shaker-IR*) (Hoshi et al., 1991), with the lone externally accessible endogenous cysteine removed (C245V), was utilized as the base construct for all subsequent *Shaker* mutations. Cysteine residues were introduced through site-directed mutagenesis. Oligonucleotide primers were synthesized by either Sigma-Genosys (Oakville, ON, Canada) or Integrated DNA Technologies (Coralville, IA), and mutations were generated using the Stratagene QuikChange kit (Stratagene, La Jolla, CA). Successful mutations were confirmed by sequencing the constructs using the core facility unit at the University of British Columbia. cRNA was synthesized from linear cDNA using the mMessage mMachine T7 Ultra cRNA transcription kit (Ambion, Austin, TX). Kv1.5-Kv1.2 chimera channels were made as previously described (Rezazadeh et al., 2007). Briefly, PCR amplification of the desired segment of Kv1.2 was used to introduce either a *PmlI* (Kv1.5-S12L-Kv1.2) or *ClaI* (Kv1.5-S23L-Kv1.2) restriction enzyme site. Kv1.5 cDNA was then subcloned into the Kv1.2 channel using the specified enzyme and *EcoRI*. Constructs were further subcloned into a pBluescript SK+ vector using *HindIII* and *EcoRI* restriction sites. The A291C mutation was made and sequenced as described above. For construction of the *Shaker*-Kv1.2 chimera, both channels were first subcloned into a pBluescript SK− vector (*EcoRI*) in which the *XbaI* polylinker site had been removed (Klenow). Silent mutations were made in Kv1.2 for the purposes of addition of *NsiI* and *XbaI* sites into the equivalent
positions to *Shaker*, flanking the S1-S2 linker. After removal of unwanted occurrences of these restriction sites at other locations in the channels (again through silent mutagenesis), the *Shaker* S1-S2 linker was subcloned into Kv1.2, and the A291C mutation was made as described above.

**Oocyte preparation and injection**

*Xenopus laevis* frogs were terminally anesthetized by immersion in 2 mg/mL tricaine methanesulphonate (Sigma-Aldrich, Mississauga, ON, Canada); unless otherwise stated, all chemicals were purchased from Sigma-Aldrich. Stage V-VI oocytes were isolated and defolliculation was performed through a combination of collagenase treatment, involving mild agitation in 1 mg/mL collagenase type 1a for approximately 1 hour, and manual defolliculation. Between defolliculation and injection, oocytes were incubated for 1-18 hours in Barth’s solution, which contained (in mM), 88 NaCl, 1 KCl, 2.4 NaHCO3, 0.82 MgSO4, 0.33 Ca(NO3)2, 0.41 CaCl2, 20 HEPES, titrated to pH 7.4 using NaOH. Injection of 50 nL of cRNA encoding the construct of interest was performed using a Drummond digital microdispensor (Fisher Scientific, Ottawa, ON, Canada), followed by incubation at 19°C in Barth’s solution. Currents were recorded 1-4 days after injection. All animal protocols were performed in accordance with University of British Columbia animal care guidelines.

**Two-electrode voltage clamp**

Oocytes were placed in a bath chamber that was perfused with control ND96 bath solution containing (in mM), 96 NaCl, 3 KCl, 1 MgCl2, 2 CaCl2, and 5 HEPES, titrated to
pH 7.4 with NaOH. Microelectrodes were filled with 3 M KCl and had resistances of 0.2 to 2.0 MΩ. Unless otherwise stated, data were recorded from a holding potential of -80 mV. Voltage control and data acquisition were achieved with a Warner Instruments OC-725C amplifier (Hamden, CT), and Axon Digidata 1322 A/D converter (Axon Instruments, Foster City, CA), connected to a personal computer running pClamp9 software (Molecular Devices Corp.).

**Voltage-clamp fluorimetry**

Fluorimetry was performed simultaneously with two-electrode voltage clamp. Labelling of the oocytes with 5 µM tetramethylrhodamine-5-maleimide (TMRM; Invitrogen, Carlsbad, CA) dye was performed for 30 minutes at 10°C in a depolarizing solution containing (in mM), 98 KCl, 1 MgCl₂, 2 CaCl₂, and 5 HEPES, titrated to pH 7.4 using KOH. Signals were recorded via a Nikon TE300 inverted microscope with Epi-Fluorescence attachment and a 9124b Electron Tubes photomultiplier tube (PMT) module (Cairn Research, Kent, UK). TMRM dye, exhibiting maximal light absorption at 542 nm, was excited by light from a 100 W mercury lamp filtered through a 525 nm band pass filter, reflected by a 560 nm dichroic mirror through a 20x objective lens focussed on the oocyte. Emitted light, with a maximal emission at 567 nm, was collected by the objective lens and passed through the dichroic mirror to the PMT module. Voltage signals from the PMT were digitized using an Axon Digidata 1322 analog/digital converter and passed to a computer running pClamp9 software to record fluorescence intensity. To minimize fluorophore bleaching, a Uniblitz computer-controlled shutter (Vincent Associates, Ottawa, ON, Canada) was used, and opened shortly prior to
application of voltage-clamp pulses. For 100 ms pulses, fluorescence signal sampling frequency was 20 kHz; traces were averaged, with each signal representing the average of five sweeps, and were filtered offline at 1000 Hz. To account for any photobleaching of fluorophores that may occur during shutter opening, control fluorescence data were recorded in the absence of any change in voltage, and subtracted from the voltage-dependent signal. Fluorescence records were normalized to baseline emission in the absence of a change in voltage, to control for cell-to-cell variability, and fluorescence-voltage F(V) relationships are shown normalized to maximum and minimum levels of emission over the voltage ranges tested.

**Gating currents**

Mammalian tsA201 cells were grown and maintained in Minimal Essential Medium (MEM) at 37°C in an air/5% CO₂ incubator. Media contained 10% bovine serum and 0.5 mg/ml geneticin. On the day before transfection, cells were washed with MEM, treated with trypsin/EGTA for one minute and plated on 25 mm² coverslips. WT Kv1.2-pGW1 and GFP-pcDNA3 cDNA were then transiently co-transfected using Lipofectamine 2000 (Invitrogen, Mississauga, ON, Canada). Twenty-four to forty-eight hours after transfection, coverslips with adherent cells plated on the surface were placed in a superfusion chamber (volume 300 µl) containing the control bath solution at room temperature (approximately 25°C). Whole-cell current recording and analysis were carried out using an Axopatch 200A amplifier and pClamp10 software (Axon Instruments). Patch electrodes were pulled from thin-walled borosilicate glass (World Precision Instruments, Sarasota, FL) on a horizontal micropipette puller (Sutter...
Instruments, Novato, CA). Electrodes had resistances of 1.0-1.5 MΩ when filled with control filling solution. Membrane potentials were not corrected for junction potentials that arise between the pipette and bath solution. Extracellular bath solution contained (in mM), TEA (140), HEPES (10), Dextrose (10) and MgCl₂ (1), pH adjusted to 7.4 (HCl). Intracellular pipette solution contained (in mM), NMDG (140), HEPES (10), MgCl₂ (1) and EGTA (10), pH adjusted to 7.2 (HCl). Cells were maintained at a holding potential of -100 mV, and currents were recorded during 12-ms voltage-clamp pulses from -80 mV to +80 mV in 10-mV increments. Leak subtraction was performed using a -P/6 protocol from holding potential. Data were filtered at 10 kHz and sampled at 100 kHz.

**Data analysis**

Conductance-voltage G(V) relationships were derived using normalized chord conductance, which was calculated by dividing peak current by the driving force based on the potassium equilibrium potential (internal potassium concentration was assumed to be 99 mM). F(V) relationships were calculated based on the peak change in emission of the total trace or, in some stated cases, the individual components of the signal. All G(V), gating charge-voltage Q(V), and most fluorescence-voltage F(V) relationships (see below) were fit with a single Boltzmann function:

\[
y = \frac{1}{1 + \exp \left( \frac{V_{1/2} - V}{k} \right)}
\]

*(Equation 2.1)*

where \(y\) is the conductance normalized with respect to the maximal conductance, \(V_{1/2}\) is the half-activation potential, \(V\) is the test voltage, and \(k\) is the slope factor. For analysis of
the slow phase of Kv1.2 fluorescence and S289C fluorescence data were best fit with the following double Boltzmann function:

\[
y = \frac{A_1}{1 + \exp \left( \frac{(V_{1/2})_1 - V}{k_1} \right)} + \frac{A_2}{1 + \exp \left( \frac{(V_{1/2})_2 - V}{k_2} \right)}
\]

(Equation 2.2)

Where symbols are as described above, \( A \) refers to the amplitude of the fit component, and 1 and 2 refer to the separate components of the fit.

Unless otherwise indicated, data reported throughout the text and figures are reported as mean ± SEM.

**Supplementary material**

Supplementary figures are located in the appendix at the end of this document. Figure A.2 explores the effect of TMRM on WT Kv1.2 activation and conductance. Figure A.3 shows a structural diagram of the voltage sensor domain, indicating residues in the S1, S2 and S4 regions labelled with TMRM in this study.
RESULTS

Kv1.2 WT channels do not show voltage-dependent changes in fluorescence emission

Removal of the lone potentially-labellable cysteine residue (C181) in the outer S1 greatly reduced channel expression levels, possibly due to a role of this residue in stabilizing interactions between the voltage sensing and pore domains (Lee et al., 2009). Supplemental figure A.2 shows no effects of TMRM on WT Kv1.2 gating, and no voltage-dependent changes in emission. Furthermore, fluorescence from a probe attached to A291C shows no change in the voltage-dependent characteristics with and without the WT cysteine at position C181 (data not shown). Based on these results, all studies presented here were carried out in the background of Kv1.2 C181 (WT).

Kv1.2 fluorescence at A291C exhibits differences from the Shaker A359C homologue

As shown previously (Claydon et al., 2007a), along with activation of ionic currents, TMRM fluorescence from Shaker C245V A359C shows rapid fluorescence quenching during short depolarizations (Figure 2.2A,B). During large depolarizations a rapid quenching accounts for 80-90% of the total fluorescence change and correlates with S4 translocation (Cha and Bezanilla, 1998; Mannuzzu et al., 1996; Cha and Bezanilla, 1997). At potentials negative to activation of ionic current (e.g., -60 mV), fluorescence quenching was still observed, suggesting that some S4 movement precedes pore opening; this is also shown in the comparison of peak G(V) and F(V) relationships (Figure 2.2C).
In *Shaker* C245V A359C, the $V_{1/2}$ of the F(V) relationship is left-shifted by ~27 mV compared to the G(V).

Figure 2.2. Voltage-dependent *Shaker* and Kv1.2 conductance and fluorescence deflections.
Figure 2.2. Voltage-dependent *Shaker* and Kv1.2 conductance and fluorescence deflections. Typical current traces (A and D) and fluorescence signals (B and E) are shown for *Shaker* C245V A359C and Kv1.2 A291C for 100 ms pulses between -120 mV and +60 mV, from -80 mV. The dashed line in panel (E) marks the approximate division of the two observed components of A291C fluorescence for depolarizations positive to 0 mV. (C and F) Normalized conductance-voltage (G(V), filled symbols) and total fluorescence-voltage (F(V), hollow symbols) relationships were calculated from data obtained at the end of each 100 ms pulse (mean ± SEM; n = 7-8), and data were well fit with Boltzmann equations. The V$_{1/2}$ values for *Shaker* C245V A359C were -12.9 ± 1.6 mV and -39.5 ± 1.5 mV for the G(V) and F(V), and slope factors (k) were 17.8 ± 1.1 mV and 22.3 ± 1.4 mV respectively. The Kv1.2 A291C G(V) had a V$_{1/2}$ and slope factor of -11.2 ± 1.6 mV and 22.4 ± 0.9 mV respectively, while the F(V) relationship had a V$_{1/2}$ and k of -62.9 ± 1.2 mV and 23.2 ± 1.0 mV.

Kv1.2 A291C ionic current (Figure 2.2D) had similar voltage-dependent and kinetic characteristics to both *Shaker* C245V A359C (Figure 2.2A) and Kv1.2 WT (Supplementary figure A.2) channels. In contrast, the fluorophore emissions (Figure 2.2B,E) were strikingly different. Depolarization produced an initial fast fluorescence decay similar to that observed in *Shaker*, complete within the first 2-5 ms of depolarization. This was followed by a large secondary slow component that comprised the majority of the total signal and continued for the duration of the depolarization (grey line in Figure 2.2E). Fluorescence quenching was observed at potentials quite negative to channel opening, more so than in *Shaker* (compare Figures 2.2B and 2.2E between -80 mV and -120 mV). Consequently the total F(V) relationship was found to be ~51 mV left-shifted compared to the G(V) (Figure 2.2F), much larger than the left shift in *Shaker* A359C channels (Figure 2.2C).
The slow Kv1.2 fluorescence is recapitulated more or less at other labelled sites in the S3-S4 linker

To investigate whether the differences between Kv1.2 A291C and Shaker A359C fluorescence were specific to this position, or indicative of a more general difference between the two channels, we performed a scan of seven residues within the S3-S4 linker and S4 in each channel. Three residues N-terminal to A291C (or A359C) and three C-terminal residues (Figure 2.1) were tested for voltage-dependent deflections, and typical traces for five of these are shown for Shaker (Figure 2.3A) and Kv1.2 (Figure 2.3B) for 100 ms pulses to +60 mV. Previous studies have extensively scanned the externally accessible residues of Shaker (Gandhi et al., 2000; Pathak et al., 2007), though these studies have been more concerned with changes occurring on the order of seconds rather than milliseconds. As a result, it is difficult to draw direct comparisons between these data and previous work, other than to say the directionality of the changes in fluorescence (quenching vs. dequenching) appear to be the same. We were able to obtain deflections for all 7 residues in Shaker and 6 of 7 residues in Kv1.2, with the R294C construct not expressing any discernible current or fluorescence in over 40 cells attempted. In both Shaker C245V L361C and Kv1.2 L293C, we were only able to obtain fluorescence deflections upon hyperpolarization (data not shown).
Figure 2.3. A scan of the Shaker and Kv1.2 S3-S4 linkers reveals differences in fluorescence phenotypes. A-B. Representative fluorescence traces collected in a cysteine scan of 5 consecutive homologous residues in the S3-S4 linker and NH2-terminal end of S4 in Shaker (A) and Kv1.2 (B). Cells expressing these constructs were held at -80 mV and depolarized to +60 mV for 100 ms. (C to F) Normalized G(V) (filled symbols) and F(V) (hollow symbols) relationships for four of the five Shaker (squares) and Kv1.2 (circles) constructs expressing changes in fluorescence upon depolarization, as labelled in the top left corner of each panel. For Kv1.2 S289C (Panel D), the grey circles and accompanying fit denote the Boltzmann fit to the voltage-dependent fluorescence observed between -140 mV and -20 mV. Mean half-activation and slope data obtained from the fits to all four mutant constructs for each channel can be found in Table 2.1. Data are shown as mean ± SEM, and are the average of 3-8 cells collected from each mutant.
Residues in the *Shaker* S3-S4 linker showed a predominantly rapid quenching phase, with a negligible or very small slow quenching component. In Kv1.2, M288C and S289C as well as A291C exhibited a distinct slow quenching phase in addition to the initial fast component. Conductance- and fluorescence-voltage relationships of the first four residues of the scan for both *Shaker* and Kv1.2 are shown in Figure 2.3C-F. All data are well fit with a single Boltzmann function (Table 2.1), with the exception of Kv1.2 S289C fluorescence, which can be reasonably fit with a double Boltzmann function. The first component of this relationship, scaled to its maximum value at -20 mV, is shown in grey in Figure 2.3D. Overall, the data show that the negative shift in the F(V) relationship between Kv1.2 and *Shaker* observed with A291C/A359C (Figure 2.3F) is not confined to this residue, as S289C and L290C (Figure 2.3C-E) also show hyperpolarized F(V) relations compared with *Shaker* equivalent residues. The data suggest that global rearrangements of the Kv1.2 voltage sensor domain occur at more negative potentials relative to observed gating movements in the *Shaker* channel.
<table>
<thead>
<tr>
<th>Construct</th>
<th>G(V) $V_{1/2}$ (mV)</th>
<th>G(V) $k$ (mV)</th>
<th>F(V) $V_{1/2}$ (mV)</th>
<th>F(V) $k$ (mV)</th>
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<tbody>
<tr>
<td>M288C</td>
<td>-12.4 ± 1.1</td>
<td>23.0 ± 0.4</td>
<td>-56.9 ± 3.5</td>
<td>26.2 ± 1.6</td>
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<tr>
<td>S289C (1)</td>
<td>9.8 ± 3.3</td>
<td>18.1 ± 0.7</td>
<td>-105.0 ± 2.8</td>
<td>-9.3 ± 2.6</td>
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<tr>
<td>S289C (2)</td>
<td>n/a</td>
<td>n/a</td>
<td>41.7 ± 9.2</td>
<td>-33.3 ± 2.0</td>
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<tr>
<td>L290C</td>
<td>-39.3 ± 4.7</td>
<td>26.5 ± 4.7</td>
<td>-91.9 ± 2.4</td>
<td>19.7 ± 1.7</td>
</tr>
<tr>
<td>A291C</td>
<td>-12.6 ± 1.9</td>
<td>26.1 ± 1.4</td>
<td>-62.5 ± 1.3</td>
<td>23.3 ± 1.0</td>
</tr>
</tbody>
</table>

**Shaker**

<table>
<thead>
<tr>
<th>Construct</th>
<th>G(V) $V_{1/2}$ (mV)</th>
<th>G(V) $k$ (mV)</th>
<th>F(V) $V_{1/2}$ (mV)</th>
<th>F(V) $k$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M356C</td>
<td>-0.5 ± 1.5</td>
<td>17.3 ± 0.7</td>
<td>-41.8 ± 2.6</td>
<td>20.0 ± 1.7</td>
</tr>
<tr>
<td>S357C</td>
<td>5.6 ± 2.3</td>
<td>18.6 ± 1.1</td>
<td>-42.9 ± 4.4</td>
<td>9.7 ± 1.2</td>
</tr>
<tr>
<td>-</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>L358C</td>
<td>-26.2 ± 4.2</td>
<td>18.2 ± 1.8</td>
<td>-59.5 ± 0.5</td>
<td>21.6 ± 1.7</td>
</tr>
<tr>
<td>A359C</td>
<td>-14.6 ± 1.7</td>
<td>19.8 ± 1.4</td>
<td>-38.8 ± 1.9</td>
<td>22.6 ± 1.4</td>
</tr>
</tbody>
</table>

Table 2.1. Boltzmann fits to Kv1.2 and Shaker G(V) and F(V) relationships from Figure 3. Mean half-activation ($V_{1/2}$) and slope ($k$) data were calculated from single Boltzmann fits to the data, with the exception of the F(V) relationship for S289C, which was best fit with a double Boltzmann function.
Increases in Kv1.2 fluorescence upon hyperpolarization reflect mobility of the voltage sensor

As seen in Figure 2.2, there were large differences between Shaker and Kv1.2 in the extent of fluorescence change when channels were pulsed to potentials more negative than -80 mV. Depolarizations from -120 mV still showed the same biphasic fluorescence phenotype as that seen from -80 mV, though hyperpolarizations from this voltage no longer resulted in increased fluorescence emission. When cells were held at -50 mV (Figure 2.4B), the fluorescence increase was much greater upon hyperpolarization, and fluorescence observed during depolarization was smaller and composed almost exclusively of a fast quenching component. Ionic currents (data not shown) and G(V) relationships (Figure 2.4C, filled symbols) were not any different between the three holding potentials. Furthermore, peak F(V) relationships were unchanged, when total increases in fluorescence emission at hyperpolarized potentials were taken into account (hollow circles in Figure 2.4C). Peak F(V) data from a holding potential of -120 mV (squares) and -50 mV (circles) had half-activation voltages of -69.5 ± 2.0 mV and -69.9 ± 5.8 mV respectively, compared to -69.1 ± 4.5 mV at -80 mV. These data suggest that the fluorescence unquenching at hyperpolarized potentials reflects the holding potential-dependent position of the voltage sensor domain relative to its environment, and that mobility of this region reaches one of its extreme positions at or close to -120 mV.
Figure 2.4. Holding potential affects the directionality of Kv1.2 fluorescence deflections, but does not affect the overall F(V) relationship. Representative deflections of Kv1.2 A291C held at -120 mV (A) and -50 mV (B), in response to 100 ms changes in voltage between -150 mV and +60 mV. C. Normalized G(V) (filled symbols) and total F(V) (hollow symbols) relationships for Kv1.2 C181 A291C from three different holding potentials (mean ± SEM; n = 3-5), with F(V) relationships adjusted to account for upward deflections. From -120 mV (triangles), the G(V) had a \( V_{1/2} \) of -10.7 ± 2.5 mV and a slope factor of 24.8 ± 0.9 mV, and corresponding values of -69.5 ± 2.0 mV and 22.7 ± 1.1 mV respectively for the F(V). \( V_{1/2} \) and slope factor data from -80 mV (circles) were -2.2 ± 2.3 mV and 24.6 ± 0.7 mV for the G(V) and -69.1 ± 4.5 mV and 20.8 ± 0.8 mV for the F(V). At -50 mV (squares), the \( V_{1/2} \) and slope factor for the G(V) and F(V) were 2.6 ± 0.8 mV and 24.2 ± 1.1 mV, and -69.9 ± 5.8 mV and 22.6 ± 1.5 mV respectively. D. Shaker A359C G(V) and F(V) relationships from the same three holding potentials as in (C) (mean ± SEM; n = 7-8). From -120 mV (triangles), the G(V) had a \( V_{1/2} \) of -22.2 ± 2.1 mV and a slope factor of 15.8 ± 1.6 mV, and corresponding values of -35.0 ± 4.5 mV and 27.5 ± 1.6 mV respectively for the F(V). \( V_{1/2} \) and slope factor data from -80 mV (circles) were -22.1 ± 1.9 mV and 15.9 ± 1.7 mV for the G(V) and -39.8 ± 4.0 mV and 29.1 ± 2.1 mV for the F(V). At -50 mV (squares), the \( V_{1/2} \) and slope factor for the G(V) and F(V) were -12.7 ± 1.9 mV and 15.6 ± 1.7 mV, and -79.6 ± 4.8 mV and 32.4 ± 1.5 mV respectively.
The fact that Kv1.2 F(V) relationships are similar from holding potentials between -50 mV and -120 mV also suggests that only readily reversible gating changes occur over this potential range. While it has been shown that Kv1.2 channels do not undergo significant inactivation of ionic current (Paulmichl et al., 1991; Russell et al., 1994) it is well-known that in Shaker and other Kv1 channels prolonged depolarizations associated with inactivation immobilize off-gating charge and shift the Q(V) relationship in the hyperpolarized direction due to stabilization of the S4 segment in an activated conformation, and delay its return upon hyperpolarization (Fedida et al., 1996; Olcese et al., 1997; Chen et al., 1997). In Shaker, holding the membrane potential at -50 mV resulted in a ~40 mV hyperpolarizing shift of the A359C F(V) relationship compared with those from -80 and -120 mV (Figure 2.4D, hollow squares), despite minimal differences between the corresponding G(V) relationships. Given that fluorophore labelling at this Shaker residue has been shown to track changes in S4 environment during activation, these data suggest impaired movement of the Shaker S4 helix at -50 mV, compared to -80 mV and -120 mV, consistent with holding potential-dependent modulation of S4 gating.

**The fast and slow fluorescence phases in Kv1.2 have different voltage-dependencies**

We have shown that upon depolarization, fluorescence from the S3-S4 linker of Kv1.2 A291C shows two distinct phases of quenching: a fast quenching component, followed by a slower secondary quenching component that continues for the duration of the depolarization. Figure 2.5A shows a typical fluorescence trace during a depolarization to +60 mV, best fit with a bi-exponential function (grey dotted line) at this and all other
potentials tested. Approximately 40% of the total deflection comprised a fast quenching component with a time constant of \(~1.3\) ms, while the remaining 60% of quenching occurred \(~20\) times more slowly, with a time constant of \(23.9\) ms in this particular example. The time constants of the rapid quenching component \((\tau_{F,\text{fast}})\) increased with depolarization \((3.87 \pm 0.6\) ms at \(-40\) mV, compared to \(1.2 \pm 0.3\) ms at \(+80\) mV) and were faster than the ionic current activation time constants at all potentials, while the slow quenching component \((\tau_{F,\text{slow}})\) was clearly slower than activation at depolarized potentials, and showed little voltage dependence (Figure 2.5B). These data suggest that the fast fluorescence may report on a process required for activation, while the event that the slow fluorescence change reflects is not required \(\textit{per se}\) for channel opening.

Figure 2.5. Kv1.2 A291C voltage-dependent fluorescence is well characterized by a double exponential function.
**Figure 2.5. Kv1.2 A291C voltage-dependent fluorescence is well characterized by a double exponential function.** A. Fit of the Kv1.2 A291C fluorescence signal at +60 mV to a double exponential shows that approximately 40% of the signal amplitude results from a fast movement, with a time constant of 1.3 ms. The slow phase, comprising 60% of the total signal, is slower by an order of magnitude, 23.9 ms in this example. B. Mean ± SEM time constants of the fast and slow fluorescence signal components (n = 14-20), compared to time constants of ionic current activation, fit from ~50% of maximal activation. C. Normalized F(V) relationships of the fast and slow components of Kv1.2 A291C fluorescence, normalized and plotted alongside the G(V) relationship (n = 11). The normalized fast phase, fit to a Boltzmann distribution, had a \( V_{1/2} \) and slope factor of -39.5 ± 2.0 mV and 15.6 ± 1.0 mV. The voltage-dependence of the slow phase was best fit with a double Boltzmann function, with the first component having a \( V_{1/2} \) and \( k \) of -73.9 ± 1.4 mV and 12.0 ± 0.5 mV (amplitude = 88.7 ± 2.3 %), followed by a second component (11.2 ± 2.5 %) with respective \( V_{1/2} \) and slope factors of 44.3 ± 4.2 mV and 11.6 ± 3.0 mV. D. Holding potential-dependent separation of the fast and slow fluorescence components. Representative fluorescence traces are shown for Kv1.2 A291C channels depolarized to -30 mV (grey traces) or +60 mV (black traces), from holding potentials (HP) of either -80 mV or -50 mV as labelled. The two vertical lines to the left of the fluorescence records show the contributions of fast and slow quenching components for depolarizations to +60 mV.

As a function of potential it is clear that each component of the total fluorescence signal is distinct (Figure 2.5C). The slow phase of fluorescence (hollow squares) was clearly fitted with a double Boltzmann function with widely separated \( V_{1/2} \)s of -80 mV and +44 mV. Most (89%) of the slow fluorescence corresponded to a change in voltage sensor environment that occurred at more negative potentials, based on the hyperpolarized position of the F(V) compared to either the fast quenching movement (open circles) or ionic conductance (filled circles). The second component of this slow fluorescence quenching, present only at very positive potentials, constitutes a relatively minor component (~10%) of the total slow fluorescence signal, similar to that observed presently (Figure 2.2B) and previously in Shaker A359C (Claydon et al., 2007a). The separation of the fast and slow fluorescence components is well illustrated by comparing fluorescence records from different holding potentials (Figure 2.5D). The fast
phase of fluorescence differs only slightly (~20%) during a pulse to +60 mV from either -80 or -50 mV, whereas the slow component is reduced by more than two-thirds. A similar result can be seen during a depolarization to -30 mV from the same two potentials. These data clearly support the idea that the fast and slow components of fluorescence can be modulated separately from one another, and thus likely represent different conformational changes within the channel.

**The fast phase of fluorescence correlates with S4 movement and the Q(V) relationship**

In *Shaker* A359C, rapid fluorescence quenching upon depolarization has been correlated with the translocation of S4 gating charge, based on the comparison of F(V) and Q(V) relationships (Cha and Bezanilla, 1998; Mannuzzu et al., 1996; Cha and Bezanilla, 1997). Given the speed and voltage dependence of the fast phase of Kv1.2 A291C fluorescence, it seemed appropriate to measure the charge-voltage Q(V) relationship of Kv1.2. However, simultaneous measurement of gating currents with voltage clamp fluorimetry was not possible in the oocyte due to the clamp speed limitations of two-electrode voltage clamp. Therefore, we recorded Kv1.2 gating currents from tsA201 cells using whole-cell patch clamp, as described in Methods. Figure 2.6A shows representative on-gating currents during 12 ms pulses from a holding potential of -80 mV up to +60 mV. To our knowledge these are the first reported measurements of Kv1.2 gating currents and they look much the same as other Kv1 gating current recordings from mammalian cells (Hesketh and Fedida, 1999). The Q(V) relationship during depolarization (filled triangles) matched the voltage dependence of the fast
fluorescence (Figure 2.6B) and both relationships were well fit with a Boltzmann function, with half-activation voltages of -31.5 ± 2.0 mV and -39.5 ± 2.0 mV for the Q(V) and fast F(V) relationships respectively. The time course of gating charge movement and fast fluorescence quenching are shown for a range of potentials in Figure 2.6C, and are reasonably well-matched, given the limited time resolution of the oocyte clamp. When the time course of the integrated gating charge movement was fit with a double exponential function, the slow component correlated well with the fast change in fluorescence emission (Figure 2.6D), as has been reported in Shaker channels (Cha and Bezanilla, 1997). These data support the idea that a fluorophore attached at the external end of S4 in Kv1.2 reports fast changes in fluorescence emission that correlate with the movement of gating charge, and thus S4 movement and channel activation.
Figure 2.6. Correlation of the fast component of Kv1.2 fluorescence quenching with gating charge movement. A. Representative gating currents for Kv1.2 WT, recorded from transiently transfected tsA201 cells. Data were recorded from 12 ms pulses from -80 mV to +60 mV at 10 mV increments; only every third voltage is shown here for clarity. B. Overlay of the mean normalized charge-voltage Q(V) relationship with the fast F(V) relationship from Figure 2.5C. The Q(V) relationship $V_{1/2}$ and $k$ values were -31.5 ± 2.0 mV and 11.5 ± 0.6 mV respectively. C. Superposition of fluorescence (grey) and cumulative gating charge (black) versus time in Kv1.2 channels. Data are shown for a range of depolarizations from -50 mV to +60 mV, as labelled, for the initial 8 ms of depolarization in the case of the fluorescence data, and are normalized to the respective maximum values. The fluorescence quenching at this potential is inverted in order to more closely compare with the gating charge data. D. Mean time constants of the integrated gating charge movement compared to the fast fluorescence quenching of A291C. Gating charge data were fit to a double exponential function, and the mean ± SEM data (n = 10-15) of the fast (filled squares) and slow (hollow squares) components were plotted as a function of voltage. Time constants for the fast quenching event (hollow circles) are as plotted in Figure 2.5B.
Slow changes in fluorescence recovery match the rates of Kv1.2 channel deactivation and reactivation

The major component of slow fluorescence quenching had a voltage dependence that was hyperpolarized from both gating charge movement and pore opening (Figure 2.5C). Given the voltage-dependent and kinetic properties of the fluorescence change, it is unlikely that the slow phase can be associated with pore opening. We did find, though, that the time course of slow fluorescence recovery after repolarization was strongly correlated with both channel deactivation and also re-availability after progressively longer interpulse intervals, as illustrated in Figure 2.7. After depolarizations to a range of potentials, fluorescence increased back to baseline with an identical time course to the deactivation of ionic current at -120 mV (Figure 2.7A) or a range of potentials (Figure 2.7B, 7C). For example, deactivation of ionic current to -120 mV occurred with a mean time constant of 7.6 ± 1.5 ms, similar to that observed in previous studies (Watanabe et al., 2007; Lewis et al., 2008), compared to a time constant of 9.3 ± 0.5 ms for the change of fluorescence emission.
Figure 2.7. Slow fluorescence return upon hyperpolarization correlates with deactivation of ionic current. A. Kv1.2 A291C currents (top) and fluorescence (bottom) traces at -120 mV, -60 mV, 0 mV and +60 mV, from a holding potential of -120 mV. The right panels show an enlarged view of the tail currents and off-fluorescence emissions. B. Overlay of ionic tail current (black lines) and slow off-fluorescence quenching (grey) for Kv1.2 A291C at the three potentials labelled, after a depolarization to +20 mV. Scale bars are as shown in the legend, left to right, for the corresponding holding potentials. C. Deactivation and slow off-fluorescence time constants for Kv1.2 A291C at the three holding potentials shown in (B), as a function of prepulse potential (n = 3-5). Mean data are shown every 20 mV for clarity. D. Representative current and fluorescence records from a dual pulse (P1-P2) protocol with varying interpulse recovery time. Data are shown for 100 ms pulses from -80 mV to +60 mV with interpulse intervals of 6.25 ms, 25 ms, 100 ms and 250 ms. For clarity, data are only shown up to the end of the second depolarizing pulse of intermediate records. The grey arrow shows the instantaneous level of ionic current at P1; the grey dashed line is an inverted fit of the slow component of off-fluorescence from P1. E. Overlay of the slow off-fluorescence component in (D) with the normalized initial P2 current amplitudes (black diamonds). The black line is a single exponential fit to the ionic current data. Mean time constants for the fits to individual data sets and the off-fluorescence component are shown in the panel (n = 5).

The reactivation of Kv1.2 channels during a two-pulse protocol is shown in Figure 2.7D. After short P1-P2 intervals (e.g., 6.25 ms) at -80 mV the slow fluorescence quenching during the P2 pulse to +60 mV was quite small, as expected given that little recovery of the tail had occurred in the interpulse interval. This was accompanied by
faster activating ionic currents and greater instantaneous current after a 6.25 ms or 25 ms P1-P2 interval. A scaled and inverted fit of the slow off-fluorescence (grey dashed line) matched the recovery of the initial ionic current back to P1 values (grey arrow). Figure 2.7E shows mean data for the normalized instantaneous current in the P2 pulse, compared with the slow fluorescence recovery after the first P1 pulse. The current reactivation ($\tau_{\text{ionic}} = 15.7 \pm 1.3$ ms) correlated extremely well with the time dependence of fluorescence recovery ($\tau_{\text{fl}} = 18.5 \pm 0.6$ ms), suggesting that the slow fluorescence may track a conformational change in the protein that, upon repolarization, is rate-limiting for deactivation of ionic current.

**Slow changes in fluorescence emission reflect internal rearrangement within the voltage sensor domain**

To investigate whether internal rearrangement within the voltage sensor could explain the slow quenching, we replaced portions of the voltage sensing domain of Kv1.2 with that from another mammalian *Shaker* homologue, Kv1.5. The fluorescence of Kv1.5 has been characterized in previous studies (Vaid et al., 2008; Vaid et al., 2009) and, importantly, does not have a Kv1.2-like slow quenching component in its voltage-dependent fluorescence. We made two chimeras in which Kv1.2 was replaced with Kv1.5 sequence up to the S1-S2 linker (Kv1.5-S12L-Kv1.2) and up to the S2-S3 linker (Kv1.5-S23L-Kv1.2), in the background of A291C (Figure 2.8A). Typical fluorescence records for Kv1.5-S12L-Kv1.2 are shown in Figure 2.8B. Ionic currents during depolarization were unchanged in the chimeric channels (data not shown), but there were marked differences in quenching observed compared to Kv1.2 A291C (Figure 2.2E).
Figure 2.8. Kv1.2-Kv1.5 chimera channels lack the slow fluorescence quenching at negative potentials. A. Cartoon representation of Kv1.2 A291C (top), Kv1.5 WT (bottom), and the two chimeric channels (middle cartoons). Hollow squares (and their connecting segments) originate from Kv1.2, and slashed squares are Kv1.5 segments. A291C is located approximately with a filled circle at the N-terminal S4. B. Representative fluorescence traces from the Kv1.5-S12L-Kv1.2 A291C chimera. Data were collected using the protocol outlined in Figure 2.2. C. Overlay of representative fluorescence deflections for Kv1.2 A291C and Kv1.5-S12L-Kv1.2 (S12L) A291C, for depolarizations to +30 mV, normalized to the fast fluorescence quenching components. The inset shows overlays of deactivating ionic tail currents and off-fluorescence of Kv1.5-S12L-Kv1.2 A291C (above) and Kv1.2 A291C (below), with scale bars as noted. D. Mean normalized G(V) (filled symbols) and F(V) (hollow symbols) relationships for the Kv1.5/Kv1.2 chimera channels, compared to Kv1.2 A291C, shown ± SEM (n = 10-13). Boltzmann fits to the data from Kv1.5-S12L-Kv1.2 A291C (S12L) gave $V_{1/2}$ and $k$ values of 2.6 ± 2.6 mV and 23.9 ± 0.9 mV and -40.3 ± 3.1 mV and 28.4 ± 2.6 mV for the G(V) and F(V) respectively; the fits to Kv1.5-S23L-Kv1.2 A291C (S23L) were 7.3 ± 1.9 mV and 23.9 ± 0.7 mV for the G(V) relationships and -40.9 ± 2.2 mV and 23.4 ± 2.3 mV for the F(V) relationships. Kv1.2 A291C values are as reported in Figure 2.4. E. Voltage-dependence plots of the fast fluorescence component of Kv1.5-S23L-Kv1.2 A291C with that of Kv1.2 A291C from Figure 2.5C. $V_{1/2}$ and $k$ values were -40.3 ± 2.2 mV and 19.5 ± 1.7 mV for the chimera channel (n = 15-17). F. Mean normalized F(V) relationship of the fast component of Kv1.5-S23L-Kv1.2 A291C emission, overlaid with the Kv1.2 Q(V) relationship (filled circles) from Figure 2.6B.
Replacing the first transmembrane segment, S1, and the extracellular S1-S2 linker with those from Kv1.5 dramatically reduced the slow quenching associated with Kv1.2 A291C fluorescence (Figure 2.8C). The more extensive replacement of the Kv1.2 voltage sensor with the S2 segment and intracellular S2-S3 linker did not have any further effects on the fluorescence (data not shown). The fast fluorescence quenching from these channels comprised ~80% of the total amplitude (similar to Shaker A359C over the same time course), compared to only 40% in Kv1.2 A291C. Fluorescence recorded from both chimera channels looked very similar to fluorescence recorded from Kv1.2 A291C from a holding potential of -50 mV (Figure 2.4B), a potential at which the majority of slow fluorescence change had already occurred (Figure 2.5C). A parallel loss of the slow fluorescence recovery on repolarization was observed (Figure 2.8B,C), and, importantly, the S12L chimera showed accelerated ionic current deactivation that matched the time course of fluorescence decay (Figure 2.8C, inset), further suggesting that these two processes are linked through some common or related molecular rearrangement.

In the F(V) relationships, the lack of a slow component of fluorescence at hyperpolarized potentials in the two Kv1.5-Kv1.2 constructs resulted in a depolarizing shift of the F(V) relationship (Figure 2.8D), with no change in the G(V). The fast fluorescence quenching, with time constants on the order of milliseconds, showed an identical voltage-dependence to the fast component of Kv1.2 A291C (Figure 2.8E, circles) and the Kv1.2 Q(V) relationship (Figure 2.8F), suggesting that the majority of the remaining fluorophore quenching in the chimera reports conformational changes associated with S4 translocation. The residual slow component of fluorescence in the
chimera evident at more depolarized potentials, had a \( V_{1/2} \) of \(+33.4 \pm 2.5\) mV (data not shown), very similar to the small slow component of WT Kv1.2 fluorescence.

A third chimera channel, in which only the S1-S2 linker of Kv1.2 was replaced with the equivalent segment from Shaker (Figure 2.9A), resulted in the complete abolition of slow fluorescence from A291C, similar to the Kv1.5-Kv1.2 chimeric channels, (Figure 2.9B), while ionic currents were unchanged (data not shown). The loss of fluorescence is particularly apparent at negative potentials between -120 mV and -50 mV where the slow phase of Kv1.2 was most prominent, and highlights the importance of the linker region in detecting slow reorganizations of the voltage sensor.

Figure 2.9. Effect of Shaker S1-S2 linker replacement on Kv1.2 fluorescence. A. Representative fluorescence records from a Kv1.2-Shaker S1-S2 linker A291C chimera channel (Kv1.2Sh12L). Traces are shown for depolarizations to potentials as labelled, utilizing the same protocol as outlined in Figure 2.2. B. Mean F(V) relationship for Kv1.2Sh12L (triangles), compared to Kv1.2 A291C (circles) and Shaker A359C (squares) as previously shown in Figure 2.2. For Kv1.2Sh12L, \( V_{1/2} \) and \( k \) values for the Boltzmann fit were \(-56.0 \pm 2.5\) mV and \(15.5 \pm 0.8\) mV \((n = 7)\).

The S1-S2 linker and S4 region are both intricately involved in Kv1.2 activation

The ability of a TMRM fluorophore, attached to residue C291, to report on movements of both S4 and the S1-S2 region suggested to us that these two regions are
sufficiently close in one or both of the closed and open states to alter S4 fluorophore environment in a voltage-dependent manner. To test whether S1-S2 movements alone were responsible for slow changes in Kv1.2 fluorescence, we recorded voltage-dependent fluorescence directly from cysteines inserted in the S1-S2 linker (boxed residues in Figure 2.10A), which, based on the crystal structure are somewhat more distant from S4 and the pore region, compared with A291C in the S3-S4 linker (Figure 2.11). Test residues were located near the outer end of S1 (I187) or S2 (F218, T219, D220), as well as in the middle of the linker, positioned toward adjacent subunits in the crystal structure (S208, T209) (Long et al., 2005b). Of these, only two constructs gave voltage-dependent deflections; I187C (Figure 2.10B) and T219C (Figure 2.10C), shown in bold in the alignment, and both showed only slow quenching.

Figure 2.10. TMRM attached to residues in the S1-S2 linker of Kv1.2 report only slow changes in fluorescence emission in response to voltage.
Figure 2.10. TMRM attached to residues in the S1-S2 linker of Kv1.2 report only slow changes in fluorescence emission in response to voltage A. An alignment of best fit for the amino acid residues of the S1-S2 linker region (underlined) of Shaker, Kv1.2 and Kv1.5. Residues in grey correspond to portions of the S1 and S2 helices. Residues tested for fluorescence are marked with boxes, and those giving voltage-dependent deflections are bolded. B-C. Representative fluorescence emissions recorded at +60 mV from I187C (B) and T219C (C) channels. D. Mean G(V) (filled symbols) and F(V) (hollow symbols) relationships for I187C (diamonds) and T219C (triangles) (n = 6-15) compared to Kv1.2 A291C fast and slow fluorescence components from Figure 2.5C (hollow circles and squares, respectively). Boltzmann fits to I187C data gave $V_{1/2}$ and $k$ values of $-50.6 \pm 1.4$ mV and $10.6 \pm 0.5$ mV for the F(V), and $-17.7 \pm 1.9$ mV and $27.3 \pm 1.0$ mV for the G(V). T219C half-activation potential and slope factor values were $-15.5 \pm 2.6$ mV and $22.8 \pm 0.3$ mV, and $-70.8 \pm 5.9$ mV and $18.3 \pm 2.0$ mV for the G(V) and F(V) relationships.

Labelled I187C channels showed slow voltage-dependent emission quenching with time constants similar to the slow fluorescence emission from Kv1.2 A291C (Figure 2.5), and lacked any evidence of fast fluorescence quenching. The S2 fluorophore T219C also showed only slow decreases in fluorescence emission during depolarizations, of a similar time course; this is the homologous residue to T276C in Shaker, which has previously been shown to exhibit voltage-dependent fluorescence emission left-shifted relative to the Q(V) (Cha and Bezanilla, 1997). In addition, the total F(V) relationship for T219C fluorescence (hollow triangles) correlated well with the voltage-dependence of the slow fluorescence component of A291C, and had a similar half-activation potential ($-70.8 \pm 5.9$ mV, Figure 2.10D). I187C did detect movement between -140 and -90 mV, clearly apparent in Figure 2.10D, but most change of fluorescence occurred at more positive potentials.
DISCUSSION

**Kv1.2 A291C rapid fluorescence quenching reports fast S4 displacement during channel activation**

In Kv1.2 it is only the fluorophores attached to cysteines placed at A291, L290, and M288 at the top of S4 and in the S3-S4 linker that detect rapid quenching as part of the overall fluorescence signal during depolarization (Figure 2.3B). From closed and open state models of Kv1.2 (Supplementary figure A.3, Figure 2.11), the positions of these amino acids suggest that rapid quenching is best detected by residues at the top of S4 that face either S5 (of the adjacent subunit), or the voltage sensor domain in the closed state, likely due to the positioning of the fluorescent probe in an environment subject to significant voltage-dependent change. In contrast, residues that face away from protein, S289 and I292, and fluorophores attached to sites deeper in S4, gave only smaller slow signals, or no signal at all, which suggested more limited changes in environment during depolarization.
Figure 2.11. Proposed model of Kv1.2 activation based on TMRM fluorescence. A. Closed-state structure of Kv1.2 A291C-TMRM, based on the closed-state model of Pathak et al. (2007), shown from the top. Only one voltage sensing domain (yellow) and the pore domain from an adjacent subunit (green) are shown. Residues tested are labelled and highlighted based on their ability (blue) or inability (orange) to yield voltage-dependent fluorescence; the A291C mutation is shown in red. The TMRM molecule is modeled within the external aqueous vestibule between S4 and S1-S3 as a spherical structure of carbon (green), oxygen (red) and nitrogen (blue) atoms. B. Open-state structure of Kv1.2 A291C-TMRM. The view shown, looking down upon the channel, is similar to that in (A) for the closed-state channel, relative to the pore domain, in particular S5. C-D. Side views of the closed- and open-state structures of Kv1.2 A291C-TMRM seen in A and B. Transmembrane helices of one subunit are as labelled in each panel.

The similarity of the fluorescence reports from probes attached to neighbouring residues, as well as the periodicity in the appearance of the rapid quenching component,
support some degree of secondary structure in the S3-S4 linker, in accordance with data from an alanine-scan of these residues (Li-Smerin et al., 2000; Li-Smerin and Swartz, 2001). The Kv1.2 crystal structure data is unresolved within the linker regions (Long et al., 2005a; Long et al., 2005b), but in the Kv1.2/Kv2.1 paddle chimera open-state structure there does appear to be some secondary structure (Long et al., 2007). The rapid quenching observed in Kv1.2 could report one of two possible conformational changes. It could indicate an outward movement of S4, bringing the TMRM probe into a more hydrophilic environment or altering its orientation with respect to nearby quenching amino acids. Alternatively, it could report a rotation of the S4 that, as far as the probe and its environment is concerned, leads to a rapid quenching of TMRM fluorescence. Likely, it is some combination of both, as the open state model of Kv1.2 (Figure 2.11B, D) places the A291C residue in an extruded rotated position, away from the external cavity occupied in the resting state. Recent structural studies have questioned the extent to which this extrusion away from the pore accurately reflects the open state of Kv1.2 (Lewis et al., 2008), but even a more conservative deviation of S4 away from the pore would support these findings.

In Shaker, the mainly fast fluorescence emission has been shown to have a voltage-dependence similar to (Cha and Bezanilla, 1997) or identical (Mannuzzu et al., 1996) to that of the Q(V) relationship. Here, in the first published recordings of Kv1.2 gating currents, we also observed a strong correlation between the voltage dependencies of fast fluorescence quenching and gating current measurements (Figure 2.6B). Small differences in the time course and voltage-dependence of gating charge movement and fluorescence quenching may reflect imperfect separation of the fast and slow components
of fluorescence, or simply methodological differences between mammalian and oocyte cellular models. It should be noted that the probe is also reporting on local changes in S4, while other regions of the voltage sensor, in particular S2 and S3, contain charged amino acids which may respond to changes in membrane potential (Seoh et al., 1996) and would be accounted for in a gating charge measurement, but to a lesser degree or not at all in the fluorescence signal from S4 residues.

The fast quenching Kv1.2 A291C signals from both of the Kv1.5-Kv1.2 chimeric channels also resulted in F(V) relationships identical to the Q(V) (Figure 2.8F), and interestingly, when the Shaker S1-S2 linker (7 amino acids shorter than Kv1.2) was inserted, the resulting fluorescence (Figure 2.9A), was monophasic. From the rapid fluorescence quenching seen in all constructs by A291C-TMRM we can conclude that a rapid S4 movement occurs in Kv1.2 that is responsible for the appearance of gating charge – and which eventually leads to channel opening.

**Kv1.2 A291C fluorescence also reports slower voltage-dependent rearrangements of the voltage sensor domains**

A major novel finding in this work is the description of a prominent voltage-dependent slow phase of fluorescence quenching that originates from movements of the S1-S3 voltage sensor domains of Kv1.2, and which has not been described in prior studies of Shaker or Kv1 channels (Claydon et al., 2007a; Mannuzzu et al., 1996; Cha and Bezanilla, 1997; Vaid et al., 2008). In Shaker, small slow changes in fluorescence at depolarized potentials were correlated to events in the pore underlying P/C-type inactivation (Loots and Isacoff, 1998; Loots and Isacoff, 2000), while such changes in
Kv1.5 A397C fluorescence were linked to changes in pore structure at the level of the selectivity filter (Vaid et al., 2008). Both Kv1.2 and Kv1.5-Kv1.2 chimera channels (Figure 2.8) retain a small slow quenching component at depolarized potentials, suggesting that a similar conformational change may occur in Kv1.2, in the absence of an observable inactivation process. However, none of these channels exhibited such a large slow fluorescence as Kv1.2, nor with such a negative voltage-dependence.

The experiments presented here suggest that reversal of these slow movements are obligatory for channel deactivation (Figures 2.7, 2.8). They may be coupled to the same molecular movements, or the reoriented voltage sensing domain may interact with either the activated pore or S4, preventing or altering the time course of channel closure. We cannot be sure if the slow reorientation leads to a stabilized activated/open state at positive potentials once the movement is complete, since we have not carried out experiments to investigate open-state stability directly. However, this slow reorientation process of the voltage sensor is reminiscent of S4 entry into a relaxed state, as determined by Bezanilla and colleagues in Ci-VSP (Villalba-Galea et al., 2008). In their model of activation gating, the activated state of S4 was short lived before entry to this state, though this change in state did not appear to affect the activated state of the effector portion of the protein (i.e. the coupled enzyme or pore moiety).

The S1-S2 linker of Kv1.2 is required for the slow quenching observed in A291C, as substitution of this region with that of Kv1.5 or Shaker was sufficient to either abolish or prevent detection of this slow fluorescence change (Figures 2.8, 2.9). The findings point to an integrated action of the S1-S4 elements of the voltage sensor during channel function, and there is plenty of evidence in the literature to suggest that disruption of S1
or the S1-S2 linker function alters activation gating. In Kv7.1 channels, the S1 helix has been suggested to help “steer” S4 motion through interactions occurring during gating (Haitin et al., 2008), and crosslinking S1 to the pore domain through cysteine-cysteine interactions has also been shown to interfere with function of KvAP channels, suggesting that some motion of this region may occur (Lee et al., 2009). Localized interactions within the S1-S2 linker have also been shown to modulate channel activation. In chimeric Kv2.1 channels in which the S1-S2 linker was replaced with that from Kv1.2, there was a 34 mV leftward shift in the G(V) relationship and a 2-3 fold decrease in the time constants for activation, suggesting that the S1-S2 region was important in channel stability and capable of modulating gating (Koopmann et al., 2001).

Structurally, the S1-S2 linker of Kv1.2 channels has been suggested to form a coiled loop structure near the external surface of the membrane (Zhu et al., 2003b), suggesting flexibility in this region. Differences in primary sequence of this region between Kv1.2 and Shaker or Kv1.5 (Figure 2.10A), and the S3-S4 linker (Figure 2.1), could have large effects on the positioning of this flexible segment. In Kv1.2, this may contribute to the positioning of this region with respect to the A291C S4 microenvironment. Additionally, all three channels possess extracellular glycosylation sites in the S1-S2 linker, at a similar position in the linker, although their positioning with respect to the transmembrane helices may differ based on the number of amino acids on either side of the glycosylation site (Zhu et al., 2003b) (also see Figure 2.10A). Kv1.2 glycosylation has been shown to modulate Kv1.2 activation gating (Watanabe et al., 2007), and so may interact with the voltage sensor through positioning of the S1-S2 linker, in a manner different than other Kv1 channels.
Slow voltage-dependent rearrangement of the voltage sensor domains is confirmed by fluorophores placed in S1-S2

The fluorophores attached at I187C or T219C, which would be likely to occupy a different microenvironment than at A291C, do not detect rapid movement associated with S4 displacement, but the slow movement within S1-S3 seen by A291C-TMRM can be detected by TMRM attached within the S1-S2 region (Figure 2.10). Fluorescence quenching at these residues accurately tracks the time course of slow movement of the voltage sensor, while the voltage-dependent fluorescence change of T219-TMRM overlays exactly the slow component of A291C-TMRM (Figure 2.10D). The more faithful recapitulation of the voltage-dependence of slow fluorescence from T219C compared to I187C may be in part due to the fact that fluorophores in S2 or S4 are closer to the edge of the protein, and therefore experience more significant environmental changes than a fluorophore within S1. Alternatively, the slow movement could be more prevalent within the S2 helix than in S1.

Interestingly, the fluorescence recorded from the homologous T276C residue in Shaker (Cha and Bezanilla, 1997) exhibits a considerably faster quenching component, and a somewhat smaller leftward shift relative to the Q(V) relationship (~20 mV). Cha and Bezanilla suggested that the S2 helix may undergo voltage-dependent movement prior to S4, facilitating S4 activation but carrying only a small amount of gating charge (Cha and Bezanilla, 1997). However, in Kv1.2, the much slower time course of S1-S3 rearrangement suggests that the motion cannot precede or constrain S4 movement, and indeed it continues well beyond the time required for full channel activation (Figure 2.2). While S2 does contain negatively charged side chains that appear to help stabilize S4 in
the resting and activated states (Long et al., 2005b; Long et al., 2007; Seoh et al., 1996), the minimal voltage-dependence of the S1-S3 rearrangement based on time constant data (Figure 2.5B) suggests that little net charge movement within the electric field occurs, and given the extended time course of this rearrangement it is not surprising that we were unable to resolve any associated gating charge movements.

The data from TMRM attached to A291C suggests that the most extreme inward limit of voltage-dependent movement occurs at approximately -120 mV (Figure 2.4). We also conclude that the voltage sensor domain movement, measured as a change in S3-S4 linker environment and S1-S2 rearrangements, is freely reversible between -120 mV and -50 mV (Figure 2.4C), or that any holding potential-dependent effects on mobility have reversed within 100 ms of return to more negative potentials. This is in agreement with previous work that has characterized the voltage sensor as a fluid portion of the protein (Larsson et al., 1996; Yusaf et al., 1996; Wang et al., 1999; Baker et al., 1998), but different from functional studies in Shaker channels in which prolonged depolarization leads to a hyperpolarization of the returning Q(V) relationship (Olcese et al., 1997). This hyperpolarization is recapitulated in the Shaker A359C fluorescence report of S4 movement (Figure 2.4D), but was not seen in Kv1.2. This may reflect the inclusion of rearrangements involving the entire voltage sensing domain in the Kv1.2 fluorescence record, and not just the S4 translocation measured by the rapid initial quenching.

**Conclusion**

This paper represents the first study of the Kv1.2 voltage sensor domain using fluorimetry and gating currents, and highlights at least two independent conformational
changes in this region in response to depolarization. A prominent fast change in fluorescence emission, at depolarized potentials, correlates well with channel gating currents and thus likely reports on rapid changes in S4 environment (i.e. translocation during depolarization). At more negative potentials there is a large slow quenching component reflecting fluorophore environment changes relative to the rest of the voltage sensor (particularly the S1-S2 region), that does not appear to be required for channel opening to occur, and persists well after ionic current has reached its peak. Reversal of this slow change matches the voltage-dependence and time course of channel deactivation and appears to be rate limiting for channel closure. This is the first report of such a slow protein movement within the voltage-sensing domain, and highlights both potential differences in channel gating between Kv1.2 and other Kv1 channels, and the involvement/motion of the entire Kv1.2 voltage sensing domain during activation.
Chapter 3: The molecular basis for the actions of Kvβ1.2 on the opening and closing of the Kv1.2 delayed rectifier channel.¹

INTRODUCTION

As discussed at length in the introductory chapter, the Shaker-related (Kv1) family of channels exists in vivo as heteromeric complexes between pore-forming $\alpha$-subunits and auxiliary cytosolic proteins termed Kv$\beta$-subunits (Shamotienko et al., 1997; Rhodes et al., 1997; Orlova et al., 2003; Rhodes et al., 1995; Rhodes et al., 1996; Parcej and Dolly, 1989; Scott et al., 1990; Parcej et al., 1992). To date, three mammalian Kv$\beta$ genes (Kv$\beta$1-3) have been cloned (Rettig et al., 1994; Heinemann et al., 1995; Majumder et al., 1995; England et al., 1995b; Heinemann et al., 1996; England et al., 1995a), and each encodes a protein that can be considered as two distinct regions based on primary sequence and function. The C-terminus is highly conserved among the three Kv$\beta$ genes and co-translationally forms the primary contacts with the T1 domain of the N-terminus of Kv$\alpha$1 subunits (Gulbis et al., 2000; Shi et al., 1996; Nakahira et al., 1996; Nagaya and Papazian, 1997; Wang et al., 1996; Sewing et al., 1996; Yu et al., 1996). In contrast, the N-terminus is highly variable in primary structure among the three Kv$\beta$ genes, including three splice variants of Kv$\beta$1, and does not form any long-lasting interactions with the Kv1 T1 domain (Accili et al., 1997b).

Functional interactions between Kv$\beta$1 and Kv$\alpha$1 subunits are complex. All three Kv$\beta$1 subunits convert Kv1 channels from the delayed rectifier phenotype observed with the $\alpha$-subunits alone to a rapidly decaying transient outward current, and greatly slow channel closing upon repolarization (Rettig et al., 1994; Accili et al., 1997b; Castellino et al., 1995). Fast inactivation and slowed channel closing can be abolished by the removal of the N-terminus, and the remaining Kv$\beta$1 C-termini differentially regulate the surface trafficking of Kv1 channels, with a sizable upregulation of current and cell surface
expression conferred upon Kv1.2 by Kvβ1 subunits (Shi et al., 1996; Accili et al., 1997b; Accili et al., 1998). Modeling of ionic current data suggests that open channel block by the N-terminus, in addition to causing fast inactivation, can produce saturation of activation at depolarized potentials, leading to an apparent negative shift in activation (De Biasi et al., 1997). Slowed channel deactivation is consistent with the inability of the channel gate to close while the Kvβ1 N-terminus resides in the pore, as has been demonstrated for fast inactivation produced by the N-terminus of Kv1.4 and Shaker channels, except that the extent of slowing induced by the Kvβ1 N-terminus is far greater. However, previous studies have not been able to distinguish whether or not allosteric interactions between Kvβ subunits and Kv1 channels may also contribute to the shift in channel activation (Heinemann et al., 1996; Uebele et al., 1998) or to the slowing of channel deactivation, for example, by acting directly on the S4 segment or activation gate. Conformational changes in the outer pore that are associated with slow inactivation and augmented by the Kvβ1 N-terminus (Accili et al., 1998; Morales et al., 1996) could also inhibit the closure of the activation gate as well as the return of the movement of the S4 helix to its resting position.

In Chapter 3 of this thesis, we hypothesize that the Kvβ1.2 subunit acts on Kv1.2 by an open channel block mechanism. To address the question, we have investigated whether Kvβ1 subunits modify the movement of the S4 segments. If so, how is S4 movement modified and what parts of the Kvβ subunit are responsible? To answer these questions, and thus to establish the mechanisms underlying the effects conferred by the Kvβ1.2 subunit on Kv1.2 channel activation and deactivation, movement of the S4 segment and ion flow were independently tracked by combining voltage clamp
fluorimetry and current recording. A detailed description of gating of Kv1.2 using voltage clamp fluorimetry represents the body of work described in Chapter 2. Here we use the technique to demonstrate that distinct effects on Kv1.2 channel opening and closing, and on S4 movement, result from interactions with the N-terminus of Kvβ1.2.
MATERIALS AND METHODS

Molecular biology

Human Kvβ1.2 was cloned from pcDNA3 into the vector pBluescript SK+ for expression in *Xenopus laevis* oocytes by digesting insert and vector with restriction enzymes XbaI and EcoRI (from New England Biolabs, Ipswich, MA). An N-terminal deletion mutant of Kvβ1.2 missing residues 1-77 (Kvβ1.2ΔN) was generated by simultaneous PCR amplification of the Kvβ1.2 C-terminus and introduction of an EcoRI recognition sequence at its 5’ end, after which the PCR product was digested with XbaI and EcoRI and re-inserted into pBluescript SK+. The modified pBluescript vector pEXO was used to express rat Kv1.2 in oocytes. Cysteine residues were introduced at specific sites (M288 and A291) in the S3–S4 linker for fluorophore labeling. Point mutations were introduced using the Quikchange kit (Stratagene, Cedar Creek, TX) using primers synthesized by Integrated DNA Technologies (Coralville, IA), and were sequenced at the University of British Columbia core facility. cRNA was synthesized using the mMessage mMACHINE T7 Ultra transcription kit (Ambion, Streetsville, ON) from cDNA templates linearized with SacII (Kv1.2) or NotI (Kvβ1.2 and Kvβ1.2ΔN). For co-injections of α and β subunit RNA, β-subunit RNA was pre-mixed with α-subunit RNA at a 50:1 ratio, to maximize the number of co-assembled channels, as was characterized previously (Accili et al., 1997b).

Oocyte preparation

*Xenopus laevis* oocytes were prepared and isolated as was described in Chapter 2. Following removal of the follicular layer, oocytes were injected with 50 nl (10–200 ng) of cRNA and incubated in Barth's solution, which contained (in mM) 88 NaCl, 1 KCl, 2.4
NaHCO₃, 0.82 MgSO₄, 0.33 Ca(NO₃)₂, 0.41 CaCl₂, 20 HEPES (pH 7.5), for 1–3 days at 19°C. Prior to recording, injected oocytes were labeled with a reactive fluorescent dye, 5 μM tetramethylrhodamine-5-maleimide (TMRM), in a depolarizing solution containing (in mM) 99 KCl, 1 MgCl₂, 2 CaCl₂, and 5 HEPES (pH 7.5), for 30 min at 10°C.

Two electrode voltage clamp electrophysiology and fluorimetry

Ionic currents and fluorescence signals were recorded simultaneously using two electrode voltage clamp fluorimetry as was described in Chapter 2. The bath solution contained (in mM) 96 NaCl, 3 KCl, 1 MgCl₂, 2 CaCl₂, and 5 HEPES (pH 7.5). Voltage-dependent fluorescence changes were measured from TMRM bound via stable carbon-sulfur bond to cysteine residues introduced in the S3–S4 linker. Excitation and emission light were filtered with 525-nm bandpass and 560-nm longpass filters, respectively. Emitted light was detected using a 9124b Electron Tubes PMT (ET Enterprises, Uxbridge, UK). Acquired signals (ionic current and fluorescence) were sampled at 20 kHz and low-pass filtered off-line at 1–3 kHz. Fluorescence traces recorded from TMRM represent the average of at least five sweeps. Recording microelectrodes contained 3M KCl and had resistances between 0.2–0.8 M.

Data analysis

Conductance–voltage G(V) and fluorescence–voltage F(V) relations were fitted with a single Boltzmann function,

\[ y = \frac{1}{1 + e^{(V_{50} - V)/k}} \]

(Equation 3.1)
where $y$ is the conductance or fluorescence amplitude normalized with respect to maximal conductance or fluorescence amplitude, $V_{1/2}$ is the half-activation potential, $V$ is the test voltage, and $k$ is the slope factor. Data are shown as mean ± SEM. Statistical differences were determined using the Student’s t-test. Values for “n” represent the number of oocytes considered for a given value. Where mean values for fits (Boltzmann and exponential functions) are quoted, they represent the averages of fits to data from individual oocytes in order to obtain values for SEM.
RESULTS

The Kvβ1.2 N-terminus induces fast inactivation, a negative shift of activation and slowed deactivation in Kv1.2

In order to establish the effects of the Kvβ1.2 subunit on Kv1.2 in our system, oocytes were injected with cRNA encoding Kv1.2 alone, or mixed with either full length Kvβ1.2, or a mutant of Kvβ1.2 lacking the first 77 residues, called Kvβ1.2ΔN (Figure 3.1A). The sequence of Kvβ1.2ΔN corresponds to a C-terminal region that is identical among the three Kvβ1 splice variants and thus is referred to as Kvβ1 C-terminus in previous studies from our group (e.g., Accili et al, 1997). The cysteine-substituted Kv1.2 mutants were utilized for these ionic studies, as well as for the fluorescence-tracking experiments, which were performed in parallel.

Typical delayed rectifier currents were recorded from Kv1.2-expressing oocytes and showed little to no inactivation over the 100 ms pulse duration (Figure 3.1B). In the presence of Kvβ1.2, but not the truncated Kvβ1.2ΔN, ionic currents from Kv1.2 measured at depolarized potentials were converted from delayed outward rectifying currents to transient outward currents followed by a rapid decay to a steady state value, reminiscent of N-type inactivation (Figure 3.1B). Tail current amplitudes, measured immediately following the pulse to –40 mV, were normalized to the maximum amplitude, plotted against test voltage and fitted to a Boltzmann function (Eq. 3.1; Figure 3.1C). We noted a leftward shift of the activation curve, along with markedly slower kinetics in the ionic tail currents, when Kvβ1.2 was co-injected with Kv1.2 (Figure 3.1D). When Kvβ1.2ΔN was used in place of the full length Kvβ1.2, the recorded ionic currents were kinetically indistinguishable from those recorded from Kv1.2 injected alone, though they
were significantly larger in amplitude (data not shown), consistent with previous observations of Kv1.2 current upregulation by Kvβ1 and Kvβ2 in Xenopus oocytes (Accili et al., 1997b; Accili et al., 1998). Upregulation of current has been used as functional evidence for a direct interaction, which has been demonstrated in a number of studies utilizing biochemical assays (see Introduction).

These data show that the fast inactivation, activation shift, and slowed deactivation observed when Kv1.2 was co-expressed with Kvβ1.2 were due to its N-terminus, as was shown previously (Accili et al., 1997b). Moreover, the similarity of these data with those obtained from wild type Kv1.2 channels show that the cysteine substitutions do not alter the interaction between these subunits.
Figure 3.1. Kvβ1.2 confers a spike-and-decay “fast inactivation” to Kv1.2 currents, a hyperpolarizing shift of the activation curve, and a slowing of current deactivation.
Figure 3.1. Kvβ1.2 confers a spike-and-decay “fast inactivation” to Kv1.2 currents, a hyperpolarizing shift of the activation curve, and a slowing of current deactivation. A. The Kvβ1.2 constructs used in this study. Kvβ1.2ΔN lacks the first 77 amino acids that contain the region responsible for fast inactivation. The primary sequence of the deleted amino acids of Kvβ1.2 is shown in the right panel. B. Current traces recorded from oocytes expressing Kv1.2 A291C alone, with Kvβ1.2 or with Kvβ1.2ΔN. Traces were recorded in response to depolarizing test pulses from a holding potential of -80 mV to voltages ranging from -120 mV to +60 mV in 10 mV steps, then to -40 mV to elicit outward tail currents, before being returned to the holding potential. C. A plot of normalized tail current amplitudes versus test voltage, fitted with a single order Boltzmann function (Equation 3.1). This relation (the G(V) curve) is steepened and shifted to more negative potentials significantly (p<0.05, Student’s t-test) when Kv1.2 A291C is co-injected with Kvβ1.2 (circles, dashed line; $V_{1/2} = -17.50 \pm 1.69$ mV, k = 13.30 ± 0.61 mV, n=16) as compared with Kv1.2 A291C expressed alone (squares, solid line; $V_{1/2} = -5.78 \pm 2.78$ mV, k = 15.15 ± 0.64 mV, n=23), or with Kvβ1.2ΔN (triangles, dotted line; $V_{1/2} = -8.30 \pm 3.26$ mV, k = 14.92 ± 0.44 mV, n=9). D. A plot of deactivation time constants determined from single exponential fits of tail currents recorded at -40 mV following test pulses from -20 mV to +40 mV, in 10 mV increments. The values for Kv1.2 A291C + Kvβ1.2 (circles) are considerably larger at all potentials (p<0.0001 at all potentials tested, Student’s t-test, n values as in C) compared to for Kv1.2 + Kvβ1.2ΔN (triangles) or Kv1.2 A291C alone (squares).

The voltage-dependences of fluorescence deflections upon depolarization from TMRM-labeled Kv1.2 S4 mutants are unaffected by Kvβ1.2

To track the movement of the S4 segment independently from ionic current, two sites at the extracellular end of the S4 helix were labeled with TMRM dye, and voltage clamp fluorimetry recording was performed alongside two electrode voltage clamp. As mentioned in the Experimental Procedures, cysteine residues were introduced at sites M288 and A291, as the equivalent residues (M356 and A359; Figure 3.2A) were found to faithfully track S4 movement in Shaker (Mannuzzu et al., 1996). The addition of TMRM to wild type channels produced little fluorescence quenching in response to depolarizing test pulses (Figure 3.2B), despite the presence of an external cysteine residue (C181) in wild-type Kv1.2. We generated a mutant Kv1.2 C181V A291C, and found that fluorescence signals therefrom were kinetically equivalent to Kv1.2 A291 with C181.
intact (see overlapping traces in Figure 3.2C). However, channel surface expression was reduced for the C181V A291C mutant. Therefore, C181 was left intact for these studies.

Figure 3.2. Kv1.2 current and/or fluorescence kinetics are not modified by substitutions required to record fluorescence. A. An alignment of the S3-S4 linkers of Shaker and rat Kv1.2 potassium channel α-subunits, with expected topology. Unlike the long linker present in Shaker, Kv1.2 has only a short external S3-S4 loop. Residues mutated to cysteine for voltage clamp fluorimetry, M288 and A291, are indicated by arrows. B. (Top) Current traces recorded from oocytes expressing wild-type Kv1.2 labeled with TMRM in response to depolarizing test pulses to voltages ranging from –120 mV to +60 mV in 10 mV steps, from a holding potential of -80 mV. (Bottom) Fluorescence trace recorded at +60 mV in the above protocol (see the corresponding current trace above indicated in black). No discernible changes in fluorescence signals were observed over 100 ms at +60 mV (or any other potential tested). C. Normalized fluorescence recordings from oocytes expressing Kv1.2 C181V A291C or Kv1.2 A291C during 100 ms pulses to +60 mV, which show little difference in activation and deactivation kinetics. The increased noise in Kv1.2 C181V A291C is due to the lower level of surface expression and the larger amplification of the signal with normalization.

TMRM-labeled Kv1.2 M288C and Kv1.2 A291C yielded voltage-dependent fluorescence deflections in a voltage-clamp fluorimetry configuration (Figure 3.3A). Here, oocytes were subjected to a protocol similar to the one used to generate G(V)
curves in Figure 3.1, except that after depolarization, oocytes were returned to \(-100\) mV to allow complete channel deactivation and return of the voltage sensor. As seen in traces from both M288C and A291C, robust fluorescence that varied as a function of applied voltage could be recorded under these conditions. In order to examine effects of the Kv\(\beta\)1.2 N-terminus on voltage sensor movement and on channel activation simultaneously and separately, we co-injected cRNA encoding Kv\(\beta\)1.2 (or Kv\(\beta\)1.2\(\Delta\)N) with Kv1.2 M288C or A291C, and recorded fluorescence deflections.

To compare the voltage dependencies of the fluorescence deflections associated with voltage sensor movement, signal amplitudes from Kv1.2 A291C and M288C were normalized to those at \(0\) mV, plotted against membrane potential and fit with a Boltzmann function (Equation 3.1) to generate “F(V)” curves, from which values of mid-activation voltage and slope factor were determined. For Kv1.2 A291C and Kv1.2 M288C, no differences in the voltage dependence of fluorescence were observed when Kv\(\beta\)1.2, or Kv\(\beta\)1.2\(\Delta\)N, were co-expressed with the \(\alpha\)-subunit as compared to the \(\alpha\)-subunit injected alone (Figure 3.3 B-C). In all cases, the F(V) curves span a range of voltages more negative than those of the G(V) curves (the fitted G(V) curve for Kv1.2 from Figure 3.1B is shown in gray in Figure 3.4), as would be expected for a signal correlated to voltage dependent S4 movement that precedes channel opening. Negatively shifted S4 fluorescence and gating charge as functions of test voltage have been demonstrated for the closely related Shaker channels and Kv1.5 channels (Mannuzzu et al., 1996; Cha and Bezanilla, 1997; Vaid et al., 2008; Chen et al., 1997; Bezanilla et al., 1991), as well as for Kv1.2 channels as described in Chapter 2. As mentioned previously, the F(V) curve for A291C is markedly left-shifted and less steep than that for M288C as
well as those published with the equivalent construct (A359C) in *Shaker* (Mannuzzu et al., 1996; Bezanilla et al., 1991), suggesting A291C may be additionally reporting on a separate aspect of gating from Kv1.2 M288C.

Figure 3.3. Voltage dependence of S4 movement upon depolarization in Kv1.2 is unaffected by Kvβ1.2.

Figure 3.3. Voltage dependence of S4 movement upon depolarization in Kv1.2 is unaffected by Kvβ1.2.
Figure 3.3. Voltage dependence of S4 movement upon depolarization in Kv1.2 is unaffected by Kvβ1.2. A. Fluorescence traces at indicated voltages for 100 ms, from a holding potential of -80 mV, recorded from oocytes expressing Kv1.2 M288C (above) or Kv1.2 A291C (below) alone (left), with Kvβ1.2 (middle), or with Kvβ1.2ΔN (right). Oocytes were pulsed to a series of depolarizing potentials in 10 mV steps, then returned to -80 mV, while simultaneously recording fluorescence deflections. Some traces are removed for clarity. For A291C, this protocol followed a brief –160 mV prepulse to yield directionally consistent fluorescence. B. Plots of fluorescence signals, normalized to fluorescence deflection amplitudes at 0 mV (ΔF0 mV), recorded from oocytes expressing Kv1.2 M288C versus test voltage, and fitted with a single order Boltzmann function (Equation 3.1). This relation (the F(V) curve) was unaffected (p>0.05, student’s t-test) by the full length Kvβ1.2 (circles, dashed line; $V_{1/2} = -42.12 \pm 0.77$ mV, n=7) or Kvβ1.2ΔN (triangles, dotted line; $V_{1/2} = -45.73 \pm 0.56$ mV, n=9), compared to Kv1.2 M288C alone (squares, solid line; $V_{1/2} = -46.75 \pm 1.35$ mV, n=8). C. F(V) curves generated as in B of Kv1.2 A291C alone (squares, solid line; $V_{1/2} = -60.43 \pm 0.62$ mV, n=14), are unaffected (p>0.05) by full length Kvβ1.2 (circles, dashed line; $V_{1/2} = -62.15 \pm 0.92$ mV, n=8) and Kvβ1.2ΔN (triangles, dotted line; $V_{1/2} = -63.72 \pm 0.26$ mV, n=11).

Slowing of both current deactivation, and fluorescence return to baseline levels upon channel closing, are induced by the Kvβ1.2 N-terminus and correlated to the proportion of channels undergoing fast inactivation

Notably, the fluorescence tails were considerably slowed by full length Kvβ1.2, resulting in the imposition of a second, slower phase of fluorescence at some potentials (Figure 3.3A, 3.4A). These observations are consistent with a slowed second phase of fluorescence in the full length Shaker channel (Savalli et al., 2007). To examine the voltage dependence of the onset of slowed fluorescence tails, the fluorescence tails were fitted with a double exponential function, and amplitudes for each component were determined. We reasoned that the slower component was due to the pore-localized Kvβ1.2 N-terminus and that the amplitude of this component reflects the proportion of available channels that had entered an inactivated state. When the ratios of the slower $\tau$ amplitudes to the total fluorescence tail amplitudes were normalized, and plotted against voltages of the preceding pulses (Figure 3.4B), they overlaid well with the G(V) curve of
Kv1.2 (from Figure 3.1C). This suggests that slowing depends on the fraction of open channels that are available for fast block by the Kvβ1.2 N-terminus or, alternatively, for a slower inactivation process.

Figure 3.4. The voltage dependence of the fraction of the fluorescence that is slowed upon repolarization is correlated with the Kv1.2 G(V) curve. A. Deactivation fluorescence tails from M288C with and without Kvβ1.2 at -80 mV following a test pulse to 0 mV are normalized to their respective minima and maxima, and fitted with a single (M288C alone) or a double (with Kvβ1.2) exponential function (overlaid in gray). B. Plot of amplitudes of the slow τ from Kv1.2 M288C + Kvβ1.2 fluorescence deactivation tails (from Figure 3.3A), as a fraction of the total amplitude (fast and slow) and normalized to the maximum fractional value, versus test voltage (circles, n=5). These are overlaid with a Kv1.2 G(V) curve (squares, from Figure 3.1C).
If the slower component were due to the pore-localized Kvβ1.2 N-terminus and fast block, then the channels entering the fast inactivated state should leave this state slowly and the proportion of channels inactivated would depend upon how quickly they entered this state. Therefore, depolarizing pulses of varying duration were delivered and the amplitudes of slow components of the tails as a function of time upon relaxation to negative potentials were determined. Samples of fluorescence tails at -80 mV from Kv1.2 alone, or with Kvβ1.2 or Kvβ1.2ΔN following depolarizing pulses to +60 mV for 5 ms-40 ms are shown in Figure 3.5A. As before, fluorescence tails recorded with Kvβ1.2 were fitted with a double exponential function from which amplitudes for the slow component were determined. Using a similar protocol, the amplitudes for the slow component were determined at -40 mV for ionic tail currents from Kv1.2 with Kvβ1.2 (Figure 3.5B).

These amplitudes, which likely represent the proportion of channels in the inactivated state, were plotted against prepulse duration (Figure 3.5C). Although the maximum value was larger for ionic current, single exponential fits of these data yielded almost identical values of $\tau$ of 8.5 ± 1.3 ms (n=6) for fluorescence and 8.8 ± 1.2 ms (n=8) for ionic currents. These values likely reflect the progressive entry of channels into the fast inactivated state because they are similar to the order of onset of fast current inactivation at +60 mV (6.0 ± 0.8 ms, n = 11), a sample of which is overlaid in gray in Figure 3.5C.
Figure 3.5. Current deactivation and the return of fluorescence to baseline are coupled and slowed by the Kvβ1.2 N-terminus. A. Fluorescence traces from oocytes expressing Kv1.2 M288C alone, or with Kvβ1.2 or Kvβ1.2ΔN, recorded at -80 mV following prepulses to +60 mV for variable durations between 5-40 ms (5, 20 and 40 ms traces are shown). Traces were fitted with a single (M288C alone or with Kvβ1.2ΔN) or a double (with Kvβ1.2) exponential function, which are overlaid in gray. B. Current traces elicited from oocytes expressing Kv1.2 M288C with Kvβ1.2 as for fluorescence traces in A except at -40 mV to visualize them in the outward direction. C. Plots of amplitudes of the slow τ from Kv1.2 M288C + Kvβ1.2 fluorescence (squares, n=6) and current (circles, n=8) deactivation tails (from Figure 3.5A,B), as a fraction of the total amplitude (fast and slow) versus prepulse duration. Each set of values is fitted with a single exponential function (solid, fluorescence; dashed, ionic). To better compare these fitted curves, the fit for the fluorescence data was normalized to the maximum value for the ionic current fit and overlaid in gray.
DISCUSSION

Our data demonstrate that distinct effects on Kv1.2 channel opening and closing, and on S4 movement, result from transient interactions with the N-terminus of Kvβ1.2. The voltage dependence of S4 movement was unaffected by that subunit despite the apparent shift of the G(V) curve to more negative voltages. This apparent shift is thus likely due to the progressive saturation of ionic tail currents from which channel activation is determined at depolarized membrane potentials; this is consistent with a model of open channel block to describe interactions of the Kvβ1 N-terminus with Kv1 channels (Accili et al., 1997b; De Biasi et al., 1997), and rules out a direct effect of the Kvβ1 C-terminus on the channel voltage sensor.

Along with a corresponding slowing of current deactivation, the fluorescence deflections recorded during deactivation of Kv1.2 exhibited a dramatic slowing by Kvβ1.2. This slowing correlated with the proportion of open channels entering the fast inactivated state, on a time scale too quick for a slower inactivation process. These findings compare closely with those obtained using Shaker channels, which show that gating charge is “immobilized” during the intrinsic fast inactivation invoked by its own N-terminus; this occludes the pore and prevents gate closure (Bezanilla et al., 1991). Immobilization likely results from physical associations between the activation gate and the S4 segments. Although it is possible that Kvβ1.2 N-terminus may have inhibited fluorescence return by acting directly on the S4 segment, there are three reasons to suggest that this does not occur. First, the voltage-dependence of fluorescence movement is unaffected by Kvβ1.2. Second, the observation that the slowing of ionic and fluorescence tails during deactivation are similar to the rate of onset of inactivation can
be explained most easily by open channel block by the N-terminus. Third, previous structural studies have shown that the N-terminus interacts predominantly with the pore and not with the S4 segment (Zhou et al., 2001), although it may be that such a conformation cannot be resolved by current structural methods. Therefore, although an interaction between the Kvβ1.2 N-terminus and the S4 segment is possible, it does not seem likely based on the evidence to date.

When Kvβ1.2 was coexpressed with Kv1.2, the amplitude of the slow time constant for the recovery rate of fluorescence following a depolarizing pulse of increasing length made up a smaller proportion of the total amplitude compared to that for the ionic traces: even after the longer prepulses, a significant fraction of the fluorescence recovery remained fast. This implies that the fluorescence signal is reporting on an additional or separate component of movement of the S4 segment when the channel closes that is not slowed by the Kvβ1 N-terminus. This is consistent with studies showing that the return of the S4 segment may be partitioned into several steps, some of which may not necessarily correspond temporally with activation gate closure (Wang and Fedida, 2002).

Alternatively, the Kvβ1 N-terminus may not inhibit the returning movement of all four S4 segments. Functional inequivalence in voltage sensor movement within one channel has been reported in the voltage-gated sodium channel Nav1.4, in which fast inactivation preferentially slows the return of only two of four S4 segments to their resting position during channel closure (Cha et al., 1999).

Although the influence on Kv1.2 gating behavior can be explained by open channel block by the Kvβ1.2 N-terminus, the blocking process itself may be more complex and consist of at least two separate stages (Zhou et al., 2001). The binding of the
N-terminus to the cytoplasmic surface may occur initially, placing the channel in a pre-inactivated state. This initial interaction would be followed by the movement of the N-terminus into the pore as an extended peptide, such that the channel becomes blocked and fully-inactivated. The slowed return of fluorescence and current deactivation in our studies of Kv1.2 could result from an interaction of the Kvβ1.2 N-terminus with either the cytoplasmic surface or the inner pore but, because slowing is voltage independent, the binding of the extended peptide to the cytoplasmic face, an interaction on the outside of the membrane electric field, may make the most sense intuitively. A two-step unblocking process also fits with our data showing that a fast fluorescence component remains, which may correspond to a fast exit of the Kvβ1.2 N-terminus from the inner pore. While our results are in strong accordance with the findings of Zhou et al using Kv1.4 and Kvβ1.1, the variability in primary structure within the N-termini among different Kvβ1 subunits and the involvement of other cellular elements may contribute to the complexity of the block by altering Kvβ interaction at both the outer and inner pore mouth, with potentially different consequent effects on voltage sensor movement or its coupling to channel opening. For example, the Kvβ1.3 N-terminus, unlike Kvβ1.1, may enter the pore as a hairpin and the inactivation conferred by this subunit and may be modified by the cellular metabolite PIP2 (Decher et al., 2008). So while some similarity in mechanism seems evident from our data and those of others, there are undoubtedly significant differences, which makes comparative studies among the Kvβ1 splice variants important in the future.

One ongoing challenge in constructing a cohesive picture of Kvβ subunit behaviour, and understanding the overall consequences of Kvα and β associations, is the
difficulty in performing unambiguous functional studies in native tissue. Indeed, the three Kvβ proteins and their splice variants, which lack any measurable electrical behaviour of their own, are known to interact promiscuously with known Kv1α subunit isoforms to alter trafficking and gating, as well as to enhance their sensitivity to redox environment (Wang et al., 1996; Bahring et al., 2001; Weng et al., 2006; Pan et al., 2008). Model systems, such as the oocyte expression technique used in this study, provide critical insights into possible behavioural roles by driving interactions mainly between Kvβ and Kvα proteins in a cellular context. These findings can be used to generate hypotheses about their function in native tissue but studies such as global Kvβ1 knockouts in mice have yielded, not surprisingly, data that are consistent with these subunits having a role more complex than has been observed in heterologous cells (Aimond et al., 2005; Giese et al., 1998; Murphy et al., 2004). Interactions among Kvβ subunits (Accili et al., 1997b; Accili et al., 1998) as well as between Kvβ subunits and Kv channels from other subfamilies e.g., Kv4.2 and 4.3 (Rhodes et al., 1997; Nakahira et al., 1996; Aimond et al., 2005; Yang et al., 2001; Perez-Garcia et al., 1999) further increase the potential for pleiotropism and require further analysis in both heterologous expression systems and native tissue. Approaches to better define the molecular compositions of channels, and conditional knockout experiments that target specific tissues and stages of development, and thus limit compensation of any changes induced by the deletion of genes, will also greatly aid in the understanding of the role for Kvβ subunits, and their functional interactions with Kv1 channels, in native tissue.
Chapter 4: Allosteric coupling between the activation gate and selectivity filter of *Shaker* channels is accelerated by N-type inactivation\(^1\)

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INTRODUCTION

As discussed in the previous chapter, some channel complexes possess an auto-inhibitory inactivation process conferred by a compatible N-terminal peptide, which enters the open pore and forms a series of stable contacts to block the channel (Hoshi et al., 1990; Zhou et al., 2001). In the case of Kv1.2, as discussed previously, no blocking peptide exists at the N-terminus and a Kvβ1 subunit is required to induce N-type inactivation (Rettig et al., 1994; Heinemann et al., 1995), however, in some channels, including the archetype *Shaker*, from *Drosophila*, block can be conferred by the N-terminus of the α−subunit (Hoshi et al., 1990). As described in the introductory chapter, an additional process, termed C-type inactivation, can also co-exist with N-terminal block to result in development of a non-conducting state during depolarization, which can be relieved by a repolarizing stimulus.

Despite being attributable to discrete components of the Kv1 channel protein, the onset rates of activation and both forms of inactivation are inter-related. Binding of the N-terminus within the inner vestibule of the Kv1 pore requires the channel to be in its open state (Demo and Yellen, 1991; Zhou et al., 2001), and accelerates the onset of the C-type inactivated state (Baukrowitz and Yellen, 1995; Bett et al., 2011). In Kv1 channels lacking a blocking N-terminus, opening of the activation gate favors the collapse of one of the potassium-coordinating sites in the selectivity filter (Panyi and Deutsch, 2006), which represents a local conformational change within the filter that is tantamount to C-type inactivation (Liu et al., 1996). In addition, both inactivation processes are sensitive to the extracellular concentration of permeating ions (Lopez-Barneo et al., 1993; Levy and Deutsch, 1996b; Baukrowitz and Yellen, 1995). However,
how the relationship between the activation and C-type inactivation gates is affected when an N-type inactivating peptide is present in the channel has not been well established.

Recently, a critical site in the bacterial, proton-gated channel KcsA was identified as an important interface between the inner pore lining helix and the backbone amino acids stabilizing the selectivity filter (Cuello et al., 2010). As the activation gate widens during channel opening, residue F103 in KcsA, (residue I470 in Shaker) undergoes a rotomeric reorientation and promotes the collapse of the selectivity filter to cause C-type inactivation (Cuello et al., 2010; Cuello et al., 2010) (see also Figure 1.3). Since Shaker I470 comprises part of the binding site for intracellular pore blockers such as TBA and N-type inactivation peptides (Zhou et al., 2001), this raises the question of how the acceleration of C-type inactivation in parallel with N-type block might have a structural basis in these residues. However, a challenge in answering the question of how the selectivity filter’s conformation is being affected is in being able to track the conformation of the selectivity filter independently from ionic current, on which N-type and C-type inactivation produce the same effect: a decay in amplitude.

In Chapter 4, we hypothesized that the side chain of Shaker I470 allosterically modifies C-type inactivation rate, and that the presence of an inactivating N-terminus accelerates this rate by a potassium-dependent mechanism. To test this, we used voltage clamp fluorimetry to track the conformation of the selectivity filter, while also recording membrane currents, in order to understand which components of current decay arise directly from pore block, or from selectivity filter conformational changes. These experiments showed that C-type inactivation can be controlled by the mutations at I470,
and by increasing levels of external $K^+$, and that these two manipulations act additively with each other. Furthermore, C-type inactivation taking place with an intact N-terminus is accelerated in a way retaining sensitivity to both means of modulation. These results can be explained by a mechanism by which a channel blocking N-terminus enters the inner pore and repels potassium from an intracellular coordinating site, thereby decreasing the steric resistance to selectivity filter collapse, allowing the allosteric link between I470 and the adoption of the non-conducting pore conformation to be potentiared.
MATERIALS AND METHODS

DNA and RNA preparation

A full length *Shaker* clone in pGW1 was a kind gift from Dr. Richard Horn (Thomas Jefferson University, Philadelphia, PA). *Shaker* was excised from pGW1 by PCR amplification from the 5’ and 3’ ends using Phusion polymerase (Fisher Scientific, Nepean, ON) while simultaneously engineering recognition sites for *Hind*III (5’) and *EcoR*I (3’) restriction enzymes on either end of the open reading frame. The resulting linear DNA fragment was then digested with those enzymes, and ligated into a similarly digested pBluescript II SK+ vector (Fermentas, Burlington, ON) using T4 ligase (NEB). Single point mutations were generated using the Quikchange kit (Stratagene) as described in Chapter 2. By this method, externally exposed cysteine 245 was mutated to valine, to prevent non-specific fluorescence, and site S424 was mutated to cysteine, to report upon C-type inactivation (Loots and Isacoff, 1998). All channel constructs used in this study were made in the background of these two mutations. All DNA constructs were linearized past the 3’ end of the coding sequence with *Sac*II, following which RNA was transcribed from the T7 promoter using the T7 mMessage Kit (Ambion), as described by the manufacturer.

Xenopus oocyte preparation and voltage clamp fluorimetry

For two electrode clamp and voltage clamp fluorimetry, *Xenopus* oocytes were prepared and maintained for recordings, as discussed in Chapter 2. Recordings were performed in ND96, which contained, in mM: NaCl, 96; KCl, 3; HEPES, 10; MgCl₂, 1; CaCl₂, 2; adjusted to pH 7.4 with NaOH. A modified version with elevated external K⁺
contained, in mM: KCl, 99; HEPES, 10; MgCl₂, 1; CaCl₂, 2; and was adjusted to pH 7.4 with NaOH.

Data analysis

Data was analyzed using pClamp10 (Axon), Origin 8 (OriginLabs), Microsoft Excel (Microsoft) and Graphpad Prism 4.0. All data are presented as means ± S.E.M. All comparisons employed one-way ANOVA to prevent error from multiple comparisons. Significance was assessed using the post-hoc Bonferroni test. P < 0.05 was used as a threshold of significance. Boltzmann curves and single exponential time constants were fit as described in Chapter 2. All data sets represent a minimum of 6 cells.

In silico kinetic modeling

All computer ion channel simulations were generated in IonChannelLab, version 1.0 (Santiago-Castillo et al., 2010). Activation kinetics were based on a basic Hodgkin-Huxley model for non-inactivating delayed rectifier potassium channels (Hodgkin and Huxley, 1952b). All other rate constants were constrained by experimental data for each I470 mutation, as described in the results section. All rates used to constrain in silico models, that were not voltage-dependent or sensitive to external ion concentration, are given in Table 4, whereas expressions for voltage or potassium-dependent rates are given in the text. Numerical integration was performed by the implicit Runge-Kutta method.

Supplementary figures

Supplementary figures and figure legends are shown in Appendix A.
RESULTS

Substitutions at site I470 modify C-type inactivation rate but maintain its sensitivity to extracellular potassium

In response to depolarizing voltage stimuli, Shaker IR (or inactivation removed, lacking the native blocking peptide from the N-terminus) potassium channels expressed in Xenopus oocytes open to conduct outward potassium currents. During long duration pulses, the outward current level decays as a result of conformational changes within the selectivity filter, which hinder potassium coordination (Figure 4.1A, black trace). The rate of C-type inactivation in Shaker channels is known to be regulated allosterically by a site in the central cavity, isoleucine 470, whose side chain undergoes a conformational reorientation as the pore lining helices spread apart during activation to result in a change in selectivity filter conformation (Cuello et al., 2010). Therefore, we expected to be able to modulate how activation affects C-type inactivation rate by generating mutations at that site, as shown previously (Cuello et al., 2010). Conservative mutations of I470 to leucine, phenylalanine, valine and cysteine all altered the onset rate of inactivation (Figure 4.1B-E, black traces). Although bulkier amino acid side chains generally produced faster inactivation (Darby and Creighton, 1993), R group volume and current decay rate were not strictly linked (Supplementary figure A.4A). Because C-type inactivation involves the selectivity filter’s ability to coordinate K⁺ ions, elevated concentrations of external K⁺ act to antagonize C-type inactivation (Lopez-Barneo et al., 1993; Baukrowitz and Yellen, 1995), and expectedly, when ND96 external recording solution ([K⁺]₀ = 3 mM) was replaced by a solution containing 99 mM K⁺, the rate of C-type inactivation was markedly slowed (Figure 4.1A, grey trace). Because external K⁺
and I470 mutations were both able to modify C-type inactivation rates, we examined how the channels would respond to both simultaneously. When 99 mM K⁺ recording solution was used, the time course of inactivation rates was slowed in all I470 mutant constructs as compared to recordings made in ND96 (Figure 4.1B-E, grey traces). Inactivation forward rate constants were calculated from the time constants of inactivation onset based on the formula:

\[
\alpha = \frac{1}{\tau} \left(1 - \frac{I_\infty}{I_{\text{peak}}} \right)
\]

(Equation 4.1)

where \( \alpha \) refers to the forward rate constant, \( \tau \) to the time constant of current decay, \( I_\infty \) to the steady state current at the end of the pulse, and \( I_{\text{peak}} \) to the peak current during the pulse. When inactivation rates in normal and elevated K⁺ were plotted against each other, regression analysis demonstrated a linear relationship (Figure 4.1B). Taken together, these findings are consistent with previous studies suggesting a role for site I470 in allosteric coupling between the inner S6 helix and the selectivity filter (Panyi and Deutsch, 2006; Cuello et al., 2010). Moreover, these effects retain a constant proportionality across K⁺ concentrations. The preservation of a potassium-dependent effect across the I470 mutations suggests that the influences of allostery and potassium occupancy in the selectivity filter are additive with each other, where the rate of modulation of inactivation by potassium is dictated by the properties of the residue at I470 in a fixed manner.
Figure 4.1. Control of C-type inactivation rate in Shaker IR is by site I470 mutations is dependent on the concentration of external potassium. A. Current traces from Shaker IR S424C in response to a voltage pulse to +60 mV from –80 mV, in solutions containing 3 mM (black) and 99 mM (grey) extracellular K+. Mean current τ’s in 3 and 99 mM K+, respectively, were: 1.62 ± 0.34 s and 2.73 ± 0.50 s. B-E Current traces from the same protocol in A applied to Shaker IR S424C with the indicated amino acid substitutions. Mean current τ’s in 3 and 99 mM K+, respectively, were: for I470L, 0.84 ± 0.12 s and 1.93 ± 0.51 s; for I470V, 2.48 ± 0.52 and 3.46 ± 0.54; for I470C, 4.78 ± 1.03 s and 8.48 ± 1.85 s; for I470F, 1.90 ± 0.61 s and 3.71 ± 1.18 s. F. Inactivation rates from traces in A-E in 3 mM and 99 mM K+ are calculated using Equation 4.1 and plotted against each other, and fit by linear regression analysis (slope = 0.463, R² = 0.951).

C-type inactivation is accelerated in the presence of a channel blocker, in a potassium-sensitive manner.

Because the N-terminal peptide is known to bind near I470, and also affects selectivity filter collapse, we next investigated how N-type inactivation could influence the interaction between I470 and the selectivity filter. For this purpose we made use of a wild-type Shaker construct, with the distal N-terminus intact, which we will call “Shaker FL”, or full length, to distinguish it from the inactivation removed version where that
region has been deleted (Hoshi et al., 1990). To do this, we used voltage clamp fluorimetry and recorded fluorescence from a reporter attached to site Shaker S424C. Fluorescence signals recorded from TMRM dye attached at site S424C faithfully track selectivity filter constrictions during C-type inactivation, independently from ionic currents (Loots and Isacoff, 1998; Loots and Isacoff, 2000). When given voltage pulses to +60 mV, oocytes expressing Shaker IR S424C channels dyed with TMRM showed robust fluorescence deflections whose decay rates correlated well with onset rates of C-type inactivation measured from currents (Figure 4.2A). When voltage commands were given to oocytes expressing Shaker FL S424C, an initial rapid current decay was measured, representing block by the channel N-terminus (Figure 4.2B, top). Following rapid block, a second, slower phase of current decay was observed. The fluorescence signal from Shaker FL was markedly accelerated from the one in Shaker IR, and appeared to take place on the time scale of the second phase of current decay in Shaker FL (Figure 4.2B, bottom). Finally, the mutation T449V was introduced into Shaker FL S424C. This mutation is known to strongly antagonize the onset of C-type inactivation in Shaker channels (Lopez-Barneo et al., 1993). When Shaker FL S424C T449V was depolarized, rapid N-type inactivation signal still occurs, but it is incomplete and followed by a much more slowly decaying second phase, compared to Shaker FL S424C (Figure 4.2C). Fluorescence signals recorded from Shaker FL S424C during depolarization were almost completely abolished in Shaker FL S424C T449V. Taken together, these results suggest that S424C fluorescence accurately tracks selectivity filter movement during C-type inactivation (or the absence thereof), and that the removal of C-type inactivation from Shaker channels in the presence of N-type inactivation leads to incomplete current decay.
Figure 4.2. S424C fluorescence decay rate is accelerated by the N-terminus of Shaker, and attenuated by the T449V mutation. A. Current and TMRM fluorescence traces from oocytes expressing Shaker IR S424C and pulsed from –80 mV to +60 mV. Mean $\tau_{\text{inact}}$ was 1.62 ± 0.34 s, and mean fluorescence $\tau$ was 1.55 ± 0.39 s. B. Current and fluorescence traces from oocytes expressing Shaker FL S424C and pulsed to +60 mV from –80 mV. Mean fluorescence $\tau$ was 139 ± 17.3 ms. C. Current and fluorescence traces from oocytes expressing Shaker FL S424C T449V. The fluorescence amplitude was determined by normalization to fluorescence from constructs in B.

Having established that S424C fluorescence kinetics can faithfully track selectivity filter movement during C-type inactivation, even in the presence of N-type inactivation (Figure 4.2B), we next investigated how having a blocking N-terminus present in the inner pore would affect C-type inactivation with I470 mutations in place to modify pore-filter allostery. To test this, the same series of conservative mutations were generated at site I470 in Shaker FL S424C, and the resulting constructs were expressed in oocytes and stimulated by voltage pulses to +60 mV from a –80 mV holding potential (Figure 4.3A). Current and fluorescence signals were recorded simultaneously, and fluorescence signals were fit with single exponential time constants. As in Shaker IR, fluorescence decay rates were strongly altered by I470 mutations in the Shaker FL constructs. When the same experiments were conducted with elevated levels of external K$^+$, a slowing of S424C fluorescence kinetics was observed for all constructs (Figure
As the true current peaks are obscured in these experiments by the overlap of activation with the onset of N-type inactivation, a peak to steady state approach could not be used. Instead, C-type inactivation rate constants under these conditions were calculated using off-rate constants calculated from Shaker IR constructs, which were not strongly affected by mutation or variations in K$^+$ concentration (see Supplementary figure A.4B). Forward rates were calculated using the formula:

$$\alpha = \frac{1}{\tau_{\text{Fluo}}} - \beta$$

*(Equation 4.2)*

where $\alpha$ represents the rate of C-type inactivation in the presence of the N-terminus, and $\beta$ represents the off-rate calculated from the experiments in Figure 4.1. When S424C fluorescence rates in high K$^+$ were plotted against those in regular K$^+$ for all constructs tested, regression analysis once again demonstrated linearity (Figure 4.3C), with a slope showing an enhanced sensitivity to K$^+$ across all mutants. In fact, the C-type inactivation rate in the presence of the N-terminus in high K$^+$ was still much faster than the rate of C-type inactivation without the N-terminus in any level of K$^+$, consistent with the notion that both C-type and N-type inactivation processes are sensitive to K$^+$. This suggests that the side chain at residue 470 retains its role in allosterically communicating with a potassium site in the selectivity filter, and that the effect of the N-terminus is also additive with allosteric coupling.
Figure 4.3. Rate of S424C fluorescence decay in the full length Shaker channel is modulated by I470 mutations and extracellular potassium. A. Current (top) and fluorescence (bottom) traces from Shaker FL S424C in response to a voltage pulse to +60 mV from −80 mV, in solutions containing 3 mM (black) and 99 mM (grey) extracellular K⁺. Mean fluorescence τ’s in 3 and 99 mM K⁺, respectively, were: 139.9 ± 17.3 ms and 539.2 ± 78.3 ms. B-E Current and fluorescence from the same protocol in A applied to Shaker FL S424C with the indicated amino acid substitutions. Mean current τ’s in 3 and 99 mM K⁺, respectively, were: for I470L, 61.6 ± 3.3 ms and 222.9 ± 39.1 ms; for I470V, 458.8 ± 90.1 ms and 1.71 ± 0.11 s; for I470C, 1.56 ± 0.18 s and 7.17 ± 1.00 s; for I470F, 235.2 ± 41.3 ms and 1.06 ± 0.25 s. F. Inactivation rates from fluorescence in A-E in 3 mM and 99 mM K⁺ are calculated using Equation 4.2 (substituting β values from traces in Figure 4.1), and plotted against each other, and fit by linear regression analysis (slope = 0.292, R² = 0.989).
A model of the potassium-dependent and allosteric interactions governing selectivity filter collapse

As shown in the previous sections, experiments using both full length and truncated *Shaker* demonstrated preserved potassium-dependent relationships between S6 and N-terminal blockers and the selectivity filter gate. To suggest a mechanism for these interactions, we developed a Markov scheme to describe the distinct gating states experienced by these channels (Scheme 4.1):

(Scheme 4.1)

where “C” and “O” denote the closed and open states of the activation gate, respectively, and “P” and “N” indicate states where the channel is C-type inactivated and/or N-type inactivated, respectively (“P” was used to indicate “P/C”-type inactivation to avoid repeating the use of “C”, already used to indicate the closed states). We subsequently performed further experiments to isolate which kinetic transitions of the scheme were influenced by K+ occupancy, I470 mutation, or both, and how the presence of the N-terminal peptide altered these transitions. Finally, based on the Markov model in Scheme 4.1, we developed a kinetic model *in silico* to attempt to recapitulate our data, and determined which transition rates required modification in order to simulate the effects of N-type inactivation and I470 mutation on selectivity filter constriction. For these analyses, the conservative mutations I470V and I470L were used, in addition to the wild-type I470 version of the channel.
To test and constrain various characteristics of the kinetics of inactivation in *Shaker* channels, we employed constructs with mutations at the I470 site that were also engineered to lack one of the inactivation mechanisms, in order to electrophysiologically isolate channel states of interest. As mentioned previously, the truncated “*Shaker IR*” constructs do not N-type inactivate (Hoshi et al., 1990), and therefore can be represented by a Markov model with fewer states, shown in Scheme 4.2.

We did not expect activation gating to be affected by I470 mutations, but to test this, we generated activation curves by pulsing oocytes expressing *Shaker IR* constructs to a series of depolarizing voltages and measuring the current amplitude peaks during a 50 ms pulse (Figure 4.4A). In concert with this, we initiated a kinetic model in IonChannelLab software based on a Hodgkin and Huxley model of activation gating (Hodgkin and Huxley, 1952b), representing the closed and open states shown in Scheme 4.2, with voltage dependent rate constants of:

\[
\alpha(V) = 10 \frac{(50 + V)}{1 - e^{-\frac{10}{(50+V)}}}
\]

and

\[
\beta(V) = 125e^{-\frac{(60+V)}{80}}
\]

(in s\(^{-1}\)), where V represents voltage, in mV. We then calculated the steady state conductance values at all voltages tested from simulated data as well as experimental data.
for all I470 mutations and fit these with two state Boltzmann curves, and found that simulated data and experimental data overlaid well, irrespective of I470 mutation (Figure 4.4B). This suggests that the activation transitions were not being altered by the I470 mutants, and furthermore that the voltage dependence of the simulated traces generated with this method accurately fit the experimental data. Though this model does not account for the COT, we expected that step to be fast and not rate-limiting to the inactivation processes. Therefore to simplify our model, we omitted it, as was done in simulations of the similar Kv1.4 channel (Bett et al., 2011), and found that it did not affect our ability to fit the model to the experimental data. Taken together, these data suggest that I470 mutations do not affect the voltage dependence of activation in Shaker.

Figure 4.4. I470 mutations do not affect Shaker activation gating. A. Currents are recorded from oocytes expressing Shaker IR, Shaker IR I470L and Shaker IR I470V in response to the voltage protocol shown (top). A simulated family of current traces was also generated in IonChannelLab (bottom right). Steady state conductance values from traces in (A) are plotted against voltage (B) and overlaid with a single Boltzmann fit to the data simulated from a Hodgkin-Huxley (HH) scheme in IonChannelLab (smooth curve), with a $V_{1/2}$ of $-17.7 \text{ mV}$ and $k$ of $15.1 \text{ mV}$. 

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In contrast with the *Shaker* IR DNA constructs, full length channels instead possessing the T449V mutation N-type inactivate normally, but exhibit strongly reduced rates of C-type inactivation in the absence or presence of the N-terminal blocker (see Figure 4.2C), and can therefore also be well represented by a Markov model with fewer states (Scheme 4.3).

(Scheme 4.3)

Here, we examined the N-type inactivation transition, or specifically, how the onset and recovery from block by the N-terminus were affected by I470 mutants. N-type inactivation consists of a state-dependent transition following opening, therefore strong depolarizations and hyperpolarizations were employed to test onset and offset. In addition, because the blocker binding site overlaps with an inner cavity site for potassium binding, we performed a series of protocols to determine the $K^+$ dependence of onset and recovery of N-type inactivation, in the full length *Shaker* T449V in the I470, I470V and I470L channels. To measure the rate of N-type inactivation, *Shaker* FL T449V and the I470 mutants were pulsed from a holding potential of -80 mV to +60 mV in 3 mM external $K^+$ (Figure 4.5A). We then pulsed cells to +60 mV in the presence of a series of external $K^+$ concentrations between 0 and 99 mM (Figure 4.5B), and all resulting curves were fit with single exponential time constants. To measure the dependence on $K^+$ concentration of the onset rate, we plotted $\tau$ values against the logarithm of $K^+$ concentration for all I470 constructs (Figure 4.5C). In all I470 constructs, the time constant of N-type inactivation was only minimally affected by increases in external $K^+$ concentration.
Figure 4.5. N-type inactivation gating is sensitive to extracellular potassium.
The rate of recovery from N-type inactivation was determined using a two pulse protocol as illustrated in Figure 4.5D. A pulse to +60 mV for 50 ms was applied to initiate block, then a repolarizing pulse to –80 mV was applied to close all open channels. During this time, all unblocked channels will deny their N-termini access to the blocking site, whereas N-type inactivated channels possess an obstructed activation gate which initially prevents closing but then gradually recovers from block, upon which these channels would also rapidly close to prevent blocker access. A second +60 mV pulse was then applied to determine how many channels had recovered from block during the intervening pulse. By increasing the duration between depolarizing pulses in a stepwise fashion, the amplitude of the current peak of the second pulse increases until it reaches the level of the prepulse. A single exponential time course fit to these peaks therefore represents the \( \tau \) of recovery from N-type inactivation. As with block onset rate recordings, these experiments were also performed in a series of \( K^+ \) concentrations, to determine the dependence of recovery rate on potassium (Figure 4.5D). Notably, the rate of recovery is strongly affected by I470 mutation, with a I470V causing a much accelerated offset rate as compared to I470, while I470L and I470 offset rates were
largely similar (Figure 4.5E, p > 0.05). We also noticed a strong effect of external K\(^+\) on recovery, with higher levels of extracellular K\(^+\) producing more rapid recovery rates. Offset time constants in different levels of K\(^+\) were normalized to the value in 0 mM K\(^+\), and then plotted against the logarithm of external K\(^+\) concentration, whereupon a sigmoidal concentration dependence was observed (Figure 4.5F). The potassium dependence of recovery from N-type inactivation was calculated by:

\[
\frac{\tau}{\tau_{0,K^+}} = \tau_{SS} + \frac{\tau_{0,K^+} - \tau_{SS}}{1 + 10^{[\text{LOG}[K^+] - \text{LOG}[EC50]}}} \\
(Equation 4.3)
\]

where \(\tau\) is measured time constant of recovery, \(\tau_{0,K^+}\) is the time constant in 0 mM K\(^+\) external solution, \(\tau_{SS}\) is the time constant of recovery in saturating levels of external K\(^+\), all in s, and EC50 is level of external K\(^+\) required to produce a half-maximal recovery time constant, in mM. Interestingly, the K\(^+\) concentration dependence of offset rate acceleration appeared to be unchanged across the I470 constructs, suggesting that while the affinity of the peptide for the inner pore was being changed by mutation, the ability of potassium to accelerate offset was dictated by competition with the N-terminus for the binding site, but not by the N-terminus’ own affinity for its site.

Because potassium throughput is influenced by the voltage field, and appears to compete directly with the N-terminal peptide, we investigated whether N-type inactivation may show voltage dependence as a result. As I470 and I470V showed major differences in the K\(^+\) dependence of N-type inactivation, these were compared further to assess whether they showed differences in voltage dependence. When similar protocols as in Figure 4.5D were employed, but the voltage during the repolarizing pulse between depolarizations varied, we observed that channels recovered more quickly from N-type
inactivation at negative potentials (Figure 4.6A,B). We calculated the voltage dependence of recovery rates based on the equation:

$$\ln\left(\frac{1}{\tau_{OFF}}\right) = \ln K_{OFF(0mV)} - \frac{z\delta F V}{RT}$$

(Equation 4.4)

where $\tau_{OFF}$ is the time constant of N-type recovery, $K_{OFF}$ is the rate of recovery at 0 mV, $z$ is the ionic valence (in this case = 1), $\delta$ is the fraction of the voltage field influencing the blocker, and $F$, $R$ and $T$ have their usual thermodynamic meanings. The fits of data from Shaker FL T449V and Shaker FL T449V I470V to this equation are plotted in Figure 4.6C.
Figure 4.6. Voltage dependence of recovery from N-type inactivation. A. Currents are recorded from oocytes expressing Shaker FL T449V I470 using a two pulse voltage protocol (top) with an interpulse of varying duration to the interpulse voltages shown, in 3 mM and 99 mM K⁺. B. A similar protocol to the one shown in A, but with a shorter time step interval between pulses (top), is given to oocytes expressing Shaker FL T449V I470V. C. Ln of recovery from N-type inactivation in 99 mM K⁺ are plotted against voltage, and curves are fit from Equation 4.4.
Based on the experimental data shown in Figures 4.5 and 4.6, we constrained the values for transitions between states in Scheme 4.3 by voltage and concentration. The forward rate constant, $K_{ON}$, was given by:

$$K_{ON} = 160e^{\frac{0.194FF}{RT}}$$

and the reverse rate constant, $K_{NO}$ was given by:

$$K_{NO} = \frac{20e^{\frac{-0.214FF}{RT}}}{0.3751 + \left(\frac{0.6249}{1 + 10^{(\log[K^+]_0 + 1.606)}}\right)}$$

where V refers to voltage, in V, $[K^+]$ refers to the external concentration of $K^+$, in M, and F, R and T have their usual thermodynamic meanings. When Shaker FL T449V I470V was used, the rate of recovery was multiplied by a coefficient of 4.5 to account for the observed reduction in peptide affinity. N-type inactivation protocols were then run in silico with 3 and 99 mM external $K^+$ and –80 mV and –120 mV repolarization potentials. Simulated data generated in IonChannelLab are shown in Figure 4.7A-B, and time constants of inactivation and recovery compared with experimental values in Figure 4.7C.
Figure 4.7. Data from N-type inactivation simulations from Scheme 4.2 are consistent with experimental results. A-B. Simulated channels based on Scheme 4.2 in IonChannelLab are subjected to protocols similar to those given Shaker FL T449V constructs in Figure 4.6. C. τ’s of N-type inactivation, and of recovery at –80 and –120 mV, are compared between experimental data (black bars) and simulations (grey bars). Numbers above bars indicate the concentration of external potassium, in mM.

Having experimentally constrained rate constants of activation and N-type inactivation, along with the current and fluorescence recordings used to establish C-type
rates shown in Figures 4.1-3, we considered Scheme 4.1 in its entirety (all rate constants not given in the text are shown in Table 4.1). While rates of N-type inactivation could not be measured directly, they could be calculated based on microscopic reversibility, such that if $K_{ONP}$ is assumed to be equal to $K_{ON}$, then $K_{NPOP}$ can be calculated based on the relationship:

$$K_{NPOP} = \frac{K_{ONP} \times K_{PO} \times K_{NO} \times K_{OP}}{K_{PO} \times K_{ON} \times K_{OPN}} = \frac{K_{NO} \times K_{OP}}{K_{OPN}}$$

The rate of selectivity filter conformational change during C-type inactivation as measured by the decay of S424C fluorescence is given by:

$$K_{Fluo} = \frac{d(POP + POPN)}{dt}$$

as fluorescence measured in the presence of the N-terminus would reflect C-type inactivation of both blocked and unblocked channels simultaneously. The rate of selectivity filter collapse only in channels where the central cavity is occupied by a peptide would thus be represented by $dPOPN/dt$. Values of this rate in the presence of different I470 mutations could be calculated by simulating the model of Scheme 4.1 in IonChannelLab, and are given in Table 4.1. As before, plotting these rate constants against each other in low and high $K^+$ demonstrates a linear relationship (Figure 4.8A), suggesting that C-type inactivation in the presence of the N-terminus can be explained by potassium-sensitive allosteric coupling between S6 and the selectivity filter. Simulated pulses to $+60$ mV were performed on Scheme 4.1 to generate curves tracking the onset of C-type inactivation, with only three values in the scheme (the forward rate constants of inactivation, and the coefficient of block offset representing blocker affinity) altered to
account for the I470 mutations. Simulated curves are overlaid with the appropriate experimental current or fluorescence data from Figures 4.1 and 4.3 (Figure 4.8C). When C-type rates are compared in the absence or presence of the blocker, simulated data (open circles) overlaid well with the experimental results (filled circles) (Figure 4.8B).
### Table 4.1. Rate constants used to constrain ion channel simulations in IonChannelLab. All values are given in units of s\(^{-1}\). Rates exhibiting voltage-dependence are given at 0 mV.

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<th>470 residue</th>
<th>$\alpha$</th>
<th>$\beta$</th>
<th>$K_{OPN}$</th>
<th>$K_{OP}$</th>
<th>$K_{PO}$</th>
<th>$K_{ON}$</th>
<th>$K_{ONP}$</th>
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<td>External K(^+) = 99 mM</td>
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Figure 4.8. Simulated and experimental C-type inactivation rates across I470 mutations in varying levels of extracellular potassium. A. “True” rates of C-type inactivation onset in the presence of a blocking peptide are calculated from Scheme 4.1, and plotted against each other in 3 and 99 mM K⁺. A linear relationship was fit by regression analysis (R² = 0.994). Rate values were used to constrain constant KOBI, and are shown in Table 1. B. C-type inactivation rates in the presence of a blocker are plotted against rates in the absence of a blocker, for experimental data (filled symbols) and data from IonChannelLab simulations (open symbols) in 3 mM (squares) and 99 mM K⁺ (triangles). C. Simulated data generated at +60 mV given to an in silico potassium channel based on Scheme 4.1 in IonChannelLab, and representing the time courses of C-type inactivation, are overlaid with experimental data from figures 4.1 and 4.3.
DISCUSSION

In this chapter, we have examined the relationship between N-type and C-type inactivation, and how this relationship depends upon the conformation of the activation gate. Mutations at \textit{Shaker} I470 are known to alter the C-type inactivation rate, as predicted from a structural model suggesting this site was involved in the communication to the selectivity filter backbone of the conformation of the activation gate (Cuello et al., 2010; Cuello et al., 2010), and we were able to successfully modulate inactivation by substituting different amino acids at that site. The replacement of normal extracellular solution with a high K$^+$ version, a common method used to manipulate C-type inactivation rates, resulted in a reduction in inactivation rate in all constructs. High K$^+$ inactivation rates were all proportional to the rates in regular K$^+$ with respect to the mutations, suggesting that the effect of I470 mutation and of high K$^+$ were additive, or, from a structural perspective, that reorientation of the I470 side chain and the occupancy of one of the potassium sites in the selectivity filter may counteract one another. The effects of I470 mutation and variations in external K$^+$ concentration were also examined in the full length version of the \textit{Shaker} channel, where we tracked the onset of C-type inactivation using a TMRM reporter bound to a cysteine substituted at site S424 (Loots and Isacoff, 1998), and we found inactivation to be accelerated in the presence of the intact N-terminus. Notably, the rates of inactivation across the I470 mutants followed the same trends in full length \textit{Shaker} as in \textit{Shaker} IR, suggesting that the N-terminus potentiates the allosteric coupling between S6 and the filter. When external K$^+$ is raised in full length \textit{Shaker}, C-type inactivation rate is reduced, and as before, the rates are proportional to those in regular K$^+$ with respect to the mutation, albeit with a higher
relative of effect of potassium as compared to Shaker IR. Because the effects of the N-terminus and $K^+$ are similarly additive with the effects of I470 mutations, these results suggest that the effect of the N-terminus is most likely antagonism of potassium occupancy, as has been suggested (Baukrowitz and Yellen, 1995). Accordingly, we noted that independent of potassium concentration, C-type inactivation rates with and without a blocking peptide were positively related to each other in all cases (Figure 4.8B). Notably, we noticed that recovery from N-type inactivation showed dependence on potassium concentration and voltage, despite evidence that N-type inactivation is a voltage insensitive process (Zagotta et al., 1989). We speculate that the peptide may be expelled electrostatically by ions entering the most internal potassium coordinating site, $S_4$ (Zhou et al., 2001), consistent with the calculated $\delta$ value of approximately 0.2. However, to fully separate the electrostatic and the steric effects of potassium interactions with a bound N-terminal peptide, an experimental preparation allowing potassium to be replaced by impermeant cations would be preferable. An interesting implication of the model is that N-terminus binds with greater preference to channels with a collapsed selectivity filter, consistent with previous results in a Kv1.4-Kv1.5 chimaeric channel (Kurata et al., 2004). This makes intuitive sense, as a channel where a selectivity filter site for potassium has collapsed into a non-conductive conformation would contain a barrier to $K^+$ ion flow, likely preventing ions from passing through the filter to electrostatically knock the N-terminal from its receptor in the central cavity.

Based on our results, we considered two possible scenarios to explain the effects of the N-terminus on C-type inactivation. Firstly, the N-terminus may enter the open activation gate and directly expel potassium from the central cavity and the most internal
potassium coordination site “S₄”. As the allosteric coupling mediated at I470 was shown to be sensitive to K⁺ occupancy, expulsion of K⁺ would have the effect of disinhibiting reorientation of the lower S6 and the I470 side chain, which allows for the allosteric transition to take place. Alternately, the N-terminus may enter the pore and bind to the S6 helix oriented in an allostery-promoting conformation, as has been suggested for the mode of action of quinidine block (Bett and Rasmusson, 2004). In this scenario, faster C-type inactivation would be favoured because the S6 is stabilized in a pro-allosteric conformation. To distinguish between these scenarios, we examined the relationship between C-type inactivation and N-terminal affinity: if the ability of the N-terminus to promote C-type inactivation is due to its tight association with the S6, then we would expect N-terminal off-rates, which reflect the affinity of the peptide for its binding site, to be consistently inversely related to C-type inactivation rates, however that is not the case, as I470 and I470L constructs showed nearly identical peptide behaviour but disparate effects on C-type inactivation. We then looked at the potassium dependence of off-rates, in channels where the T449V mutant was present to minimize the contribution of C-type inactivation. We reasoned that if the primary effect of the ball was to drive potassium from an inner cavity binding site, then operating in reverse, potassium should be able to drive the N-terminus from the same site, in the mode of a one site competition model, assuming that blocker recovery voltage dependence is unchanged between constructs (see Figure 4.6). If affinity between the peptide and the central cavity underlies C-type acceleration, then we’d expect the potassium dependence of any acceleration in N-terminal off rate to be reflected in the binding strengths, whereas if the effect is competition with potassium, then the changes in rates should occur with the same
potassium dependence. Because raising $K^+$ sped ball offset with the same concentration dependence, we propose that the potassium antagonism model better explains the enhancement of allosteric coupling in full length *Shaker*.

Surprisingly, a highly conservative mutation, I470L, had a very strong effect on C-type inactivation, considerably accelerating it with and without the N-terminus intact. However, the apparent potency of the peptide for the inner binding site in I470L was no different from that found in native *Shaker*. This provides some of the strongest evidence to support our theory: while potency of block by the peptide is clearly important, it is not the chief determinant of the influence on S6-filter allostery. Furthermore, the acceleration of inactivation in *Shaker* IR I470L argues against side chain volume being the sole determinant of the strength of the allosteric effect (Cuello et al., 2010), being that isoleucine and leucine have identical predicted volumes (Darby and Creighton, 1993).

In summary, in this chapter we have examined in detail how activation and two separable but interacting inactivation mechanisms are coupled. We have generated mutations at *Shaker* I470 to modulate activation gate/selectivity filter allostery and N-type inactivation, in order to ascertain the contributions of each to the C-type inactivation rate. We have proposed that the conformation of the S6 helix, and the identity and side chain orientation of site I470 within it, are key determinants of the C-type inactivation rate. Furthermore, the presence of the N-terminal peptide preserves allosteric coupling, while accelerating it in a potassium-dependent way. We have proposed as explanation that the N-terminal peptide enters the open pore, and drives potassium off an internal binding site. The emptying of potassium removes a steric barrier to C-type inactivation,
and allows C-type inactivation to take place more rapidly, at a rate strongly influenced by the preserved allosteric coupling between S6 and the selectivity filter.
Chapter 5: Mechanism of accelerated current decay caused by an episodic ataxia type-1 associated mutant in a potassium channel pore

INTRODUCTION

Episodic ataxia type-1 (EA-1) is an inherited neurological movement disorder that produces continuous myokymia and episodes of uncontrolled contractions in skeletal muscle (VanDyke et al., 1975). EA-1 is caused by mutations in the potassium channel gene \( KCNA1 \), which encodes the \textit{Shaker}-related channel Kv1.1 (Browne et al., 1994). One important mutation locus is at site V408, which is in the ion permeation pathway. When expressed in heterologous systems, V408A (Adelman et al., 1995; D'Adamo et al., 1998) and V408L (Demos et al., 2009) show accelerated decay of outward potassium current during prolonged depolarization of the membrane potential. However, the molecular mechanism by which mutations at this site lead to faster current decay is unclear.

As described in the introduction, Kv1 channel pores are gated by two distinct mechanisms, found at opposite ends of the transmembrane conduit. While the disease mutation being discussed in this chapter is found one helical turn intracellular to the PVP bundle crossing sequence of the S6 helix (Liu et al., 1997), an acceleration in constriction of the selectivity filter has been implicated as the source of pathophysiology (Adelman et al., 1995; D'Adamo et al., 1998), though the underlying physical mechanism is not clear.

In this chapter, we address the hypothesis that alanine substitution at V408 enhances current decay in Kv1.1 by accelerating C-type inactivation. To do this, we used voltage clamp fluorimetry, as well as single channel patch clamp electrophysiology, to monitor protein conformational rearrangements during membrane depolarization. We employed the \textit{Shaker} potassium channel, the \textit{Drosophila melanogaster} homologue of Kv1.1, as a model for Kv1 behaviour, due to its functional similarity to the Kv1.1
channel, and the well-established relationships of fluorescence signals from the Shaker A359C reporter site with conformational changes associated with both activation and inactivation processes (Claydon et al., 2007a). Surprisingly, the findings presented in this chapter rule out enhanced C-type inactivation by V408A and support a mechanism for V408A in which the activation gate of mutant channels can enter an “activated-not-open” conformation during depolarization, which accelerates the macroscopic rate of current decay during depolarization.
Construct cloning and oocyte preparation

A Kv1.1 clone was purchased from Origene (Rockville, MD). Kv1.1 and a Shaker IR clone (described previously) were excised from their original plasmids by PCR amplification and enzymatic treatment. Complimentary oligonucleotides to the cDNA 5’ and 3’ ends, also possessing recognition sequences for the HindIII and EcoRI restriction enzymes, were designed and synthesized by IDT (Coralville, IA). PCR amplifications were then performed, using the Phusion PCR kit (New England Biolabs, Pickering, ON), as recommended by the manufacturer. Linear PCR products were then digested using HindIII and EcoRI enzymes (NEB), and ligated into a similarly digested pBluescriptII SK+ vector, from Fermentas (Burlington, ON) using T4 ligase (NEB). Single site mutations were generated, and RNA transcribed, as described in Chapter 2.

For two electrode clamp and voltage clamp fluorimetry, Xenopus oocytes were prepared, maintained, and recorded from as discussed in Chapter 2. For 4-aminopyridine recordings, ND96 was supplemented with the indicated concentration of 4-AP. Recording pipettes contained 3 M KCl. Tetramethylrhodamine maleimide (TMRM) was used as a fluorophore for all experiments in this chapter, and was applied to oocytes immediately prior to recording, as described in Claydon et al. (2007).

Single channel patch clamp recordings

Mouse Itk- cells were transiently transfected with 0.5 μg Shaker IR or Shaker IR V478A DNA along with 1 μg GFP DNA using LipofectAMINE 2000 (Gibco-BRL). All recordings were made at room temperature 24-48 hours after transfection. Single-channel
currents were recorded in the cell-attached patch configuration with an Axopatch 200B patch clamp amplifier and pClamp8 software (Molecular Devices Inc., Sunnyvale, CA). Patch electrodes were fabricated using thin-walled borosilicate glass (World Precision Instruments, Sarasota, FL) and coated with Sylgard (Dow Corning, Midland, MI). Electrodes resistances were between 10-25 MΩ. The single channel currents were low-pass filtered at 2 kHz (-3dB, 4-pole Bessel filter) and sampled at 10 kHz. No junction potential correction was done on data acquired in the cell-attached recording.

For single channel patch clamp recordings, the patch pipettes contained (in mM): NaCl, 135; KCl, 5; HEPES, 10; MgCl₂, 1; CaCl₂, 1; and was adjusted to pH 7.4 with NaOH. The bath solution contained (in mM): KCl, 135; MgCl₂, 1; CaCl₂, 1; HEPES, 10; and was adjusted to pH 7.4 with KOH.

Data analysis

Single channel records were analyzed with pClamp10 software (Molecular Devices Inc.) after digital filtering at 1 kHz unless stated otherwise. Given a combined analogue and digital filter frequency of 894 Hz, the rise time (T_r) of the system was calculated to be 0.37 ms using the formula 0.3321/f_c, where f_c represents the overall combined analogue and digital filter cut-off frequency in Hz (Colquhoun and Sakmann, 1985). Half-amplitude threshold analysis (Colquhoun and Sakmann, 1985) was used to detect events longer than 2 T_r and generate idealized records from which dwell time histograms and ensemble time courses were constructed. Histograms were plotted with a logarithmic time axis. Events were logarithmically binned and subject to square root transformation (Sigworth and Sine, 1987) and normalization for comparison. Open dwell
time and burst duration histograms were fit with Gaussian distributions to yield two or
three mean time constants, calculated using the method outlined by Colquhoun and
Sakmann (1985). n=3 for Shaker IR and Shaker IR V478A.

Off-line data analysis was performed using pClamp10 (Molecular Devices),
Origin 8.0 (OriginLab Corporation, Northampton, MA), and GraphPad Prism 4.0
(GraphPad Software, Inc., La Jolla, CA). Boltzmann curves were fit as in Chapter 2,
using equation 2.1.

All data are presented as means ± S.E.M. All comparisons employed one-way
ANOVA to prevent error from multiple comparisons. Statistical significance was
assessed using the post-hoc Bonferroni test. P < 0.05 was used as a threshold of
significance. To facilitate comparisons of A359C fluorescence to track voltage sensor
return (which decayed as double exponentials), weighted time constants were generated
following the method of Lacroix et al. (2011). Following fitting of double exponential
curves using pClamp10, a weighted average was generated for each data set by
multiplying each τ value by its corresponding amplitude, then dividing by the sum of
both amplitudes. All data sets represent results of at least 6 cells from 3 separate
experiments, except single channel records, where n=3 cells. Figure 5.9 was generated
using the PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC.
RESULTS

A mutation in the lower S6 accelerates current decay in \textit{Shaker} IR and hKv1.1

In order to investigate the functional effects of a known disease mutation at Kv1.1 V408, we injected \textit{Xenopus} oocytes with mRNA encoding wild-type Kv1.1 or the mutant Kv1.1 V408A. As mentioned in the introduction, we also injected the \textit{Drosophila melanogaster} Kv1.1 homologue, \textit{Shaker} IR, and \textit{Shaker} IR V478A, equivalent to V408A in Kv1.1 (Figure 5.1A, *). When 35 second depolarizing pulses to $+20 \text{ mV}$ were applied to these oocytes, channels activated rapidly, then underwent a slow, time-dependent decay in current, which is usually attributed to C-type inactivation (Figure 5.1B,C). These traces were fit with single exponentials for comparison of the time constants of current decay (Figure 5.1E). In V408A mutants, the Kv1.1 channel currents decayed significantly faster compared to wild-type. Current decay in \textit{Shaker} IR V478A was also accelerated, suggesting a likely shared mechanism between Kv1.1 and \textit{Shaker} IR, though acceleration was to a lesser extent, possibly because C-type inactivation occurs more quickly in \textit{Shaker} IR due to a more inactivation compatible residue at site T449 (Lopez-Barneo et al., 1993). To examine this possibility, T449 in \textit{Shaker} IR was substituted with tyrosine, which specifically slows C-type inactivation (Lopez-Barneo et al., 1993), and also matches the identity in Kv1.1 (Figure 5.1A, +). Current decay in \textit{Shaker} IR T449Y was slowed (Figure 5.1D) compared to \textit{Shaker} IR, whereas in the V478A version of the T449Y \textit{Shaker} IR channel, current decay remained fast and comparable to \textit{Shaker} IR V478A and Kv1.1 V408A (Figure 5.1E). These data establish that \textit{Shaker} IR is a suitable channel in which to study EA-1 mutations. The absence of modulation of C-type inactivation rate by T449Y in the background of the V478A mutant also suggests that the
current decay rate following channel opening in V478A mutants may not be related to modulation of C-type inactivation, but may in fact reflect a separate underlying mechanism.

Figure 5.1. A valine to alanine mutation accelerates current decay in Kv1.1 and Shaker channels.
Figure 5.1. A valine to alanine mutation accelerates current decay in Kv1.1 and Shaker channels. A. An alignment of the selectivity filter and S6 pore lining regions in Shaker and Kv1.1 demonstrates 93% amino acid conservation across the region shown. * denotes site of EA-1 mutant V408A (in Kv1.1) and the equivalent mutant V478A in Shaker. + denotes a Shaker site “T449” (Kv1.1 Y379), a poorly conserved site involved in modulation of C-type inactivation. Currents during pulses to +60 mV from a holding potential of -80 mV are shown for Kv1.1 and Kv1.1 V408A (B), Shaker IR and Shaker IR V478A (C), and Shaker IR T449Y and Shaker IR T449Y V478A (D). E. Mean time constants from fits to traces in B, C and D. Measured mean $\tau$ values were: 24.1 ± 0.6 s (Kv1.1 w.t.), 4.89 ± 0.42 s (Kv1.1 V408A); 9.52 ± 0.76 s (Shaker IR), 6.30 ± 0.30 s (Shaker IR V478A); 13.7 ± 1.3 s (Shaker IR T449Y), 5.08 ± 0.35 s (Shaker IR T449Y V478A). Comparisons were made using one-way ANOVA with Bonferroni post-hoc tests for significance, $p < 0.01$ (** or $p<0.001$ (***)]. Horizontal scale bars denote 5 s.

Pore opening, but not voltage-sensing by the S4 helix, is shifted to depolarized membrane potentials by the V478A mutation.

Being that channel current decay in both mutant and wild-type channels is dependent on the channel being in its open state, we also investigated the voltage dependence of activation and of movement in the S4 helix, representing the primary charge carrying component of the voltage sensor. Previous work has demonstrated the sensitivity of activation gate stability to mutations at site V478 (Hackos et al., 2002; Kitaguchi et al., 2004; Maylie et al., 2002; Yifrach and MacKinnon, 2002), so we considered that functional effects of mutation on channel behaviour may be linked to disruptions in structure in the lower S6 activation gate region. When oocytes expressing Kv1.1 or Kv1.1 V408A (Figure 5.2A) or Shaker IR and Shaker IR V478A (Figure 5.2C-D, upper panels) were pulsed to a series of depolarizing voltages, in 10 mV steps from a -80 holding potential, and the resulting current amplitudes were divided by potential gradient and normalized to generate conductance-voltage relationships $[G(V)]$, a significant rightward shift could be observed in both Kv1.1 (Figure 5.2B), and Shaker IR (Figure 5.2E, filled symbols) from Boltzmann curves fit with equation 5.1.
While we were unable to record fluorescence traces from Kv1.1 channels, possibly due to the absence of requisite residues within the external linkers (Sorensen et al., 2000), the similarity between the effects of V to A mutants on Shaker IR and Kv1.1 voltage- and time-dependent gating events suggested that Shaker IR could be an adequate model channel for use in voltage clamp fluorimetry. We chose to use the well-characterized Shaker IR A359C channels (whose G(V) relationships are unchanged from channels without the fluorescence reporter mutant - data not shown). When these channels are tagged with TMRM, and subjected to voltage clamp fluorimetry, a series of voltage dependent deflections can be recorded (Figure 5.2C). The faster of these represents fast S4 movements and is the functional correlate of activation gating charge transfer “q_{ON}” (Cha and Bezanilla, 1997), which primarily takes place at potentials negative to 0 mV, the point to which these values were normalized (Figure 5.2E, open squares). The Boltzmann curve fit to the voltage dependence of these fluorescence deflections F(V), in keeping with properties of Kv channel gating currents, covers voltages at more negative potentials with respect to the conductance (Claydon et al., 2007a; Cha and Bezanilla, 1997). When the same technique was applied to Shaker IR A359C V478A (Figure 5.2D), the Boltzmann fit of the generated F(V) relationship using Equation 5.1 overlaid well with the curve from Shaker IR A359C (Figure 5.2E, open circles), suggesting that changes in voltage sensing are not responsible for the positive shift in G(V) observed with the V478A mutation.
Figure 5.2. EA-1 mutants shift the voltage dependence of activation, but not of voltage sensor movement.
Figure 5.2. EA-1 mutants shift the voltage dependence of activation, but not of voltage sensor movement. A. Currents recorded in response to pulses from -80 to +60 mV, in 10 mV steps, in Kv1.1 and Kv1.1V408A. B. Steady state conductance values calculated from currents in A are normalized and plotted against voltage, then fit with two state Boltzmann curves. Values of $V_{1/2}$ and $k$ for the G(V) curve of Kv1.1 were $-12.7 \pm 2.3$ mV and $10.3 \pm 0.6$ mV respectively, while for Kv1.1 V408A they were $+5.49 \pm 4.62$ mV and $+12.1 \pm 1.1$ mV. A similar protocol to the one shown in A is used to elicit outward currents from Shaker IR A359C (C), and Shaker IR A359C V478A (D), while concurrently recording fluorescence from A359C modified by TMRM dye. For clarity, 20 mV steps are shown, for potentials from $-100$ through 60 mV. E. Steady state conductance values and fluorescence signals from C and D are normalized and plotted against voltage. Conductance-voltage G(V) curves are fit with single Boltzmann functions from -80 to +60 mV, whereas F(V) curves are fit from $-120$ mV to 0 mV. Values of $V_{1/2}$ and $k$ for the F(V) curve of Shaker IR A359C were $-53.7 \pm 1.1$ mV and $15.0 \pm 0.6$ mV respectively, while for F(V) of Shaker IR A359C V478A they were $-51.6 \pm 1.4$ mV and $14.7 \pm 0.9$ mV, respectively. Values of $V_{1/2}$ and $k$ for the G(V) curve of Shaker IR A359C were $-7.07 \pm 2.57$ mV and $13.7 \pm 1.1$ mV respectively, while for G(V) of Shaker IR A359C V478A they were $+9.60 \pm 2.76$ mV and $21.6 \pm 0.7$ mV, respectively. Where scale bars are invisible they are contained within the data points.

Slower movements of the voltage sensor associated with outer pore constriction in Shaker IR A359C are attenuated by V478A mutation

Being that C-type inactivation is correlated with slower movements of the voltage sensor, which can be observed during longer periods of depolarization (Claydon et al., 2007a), we next examined fluorescence dynamics during longer pulses, in the absence and presence of V478A. We used fluorescence constructs possessing the Kv1.1 residue tyrosine at the T449 site (see Figure 5.1). Shaker IR A359C T449Y and Shaker IR A359C T449Y V478A were pulsed to a series of depolarizing potentials from a holding potential of -80 mV (Figure 5.3A,B). A second component of A359C fluorescence, developing during long periods of channel opening, is known to be associated with the onset of C-type inactivation (Claydon et al., 2007a), though it appears to occur on a faster time scale than current decay in non-mutant channels, perhaps indicating that it reports on a voltage sensor re-orientation preceding selectivity filter constriction (see discussion).
We noticed that, while the rate of current decay was appreciably accelerated at all potentials by V478A (Figure 5.3B,C), the secondary component of fluorescence was unexpectedly slowed (Figure 5.3B,D) and strongly reduced in amplitude. This is inconsistent with an acceleration of secondary voltage sensor movement or C-type inactivation, as the amplitude of this slow component is expected to reflect the contribution of C-type inactivation to current decay. The reduction in fluorescence decay rate and amplitude more likely implied that a process distinct from C-type inactivation may underlie the effects of V478A mutants on current decay.

Figure 5.3. Opposite effects on kinetics of Shaker IR current decay and A359C secondary fluorescence quenching resulting from mutation V478A.
Figure 5.3. Opposite effects on kinetics of Shaker IR current decay and A359C secondary fluorescence quenching resulting from mutation V478A. A. Outward currents recorded in response to a series of 20 s pulses to voltages from –20 to +40 mV, in 20 mV steps, in Shaker IR A359C T449Y, and a sample fluorescence trace recorded simultaneously with current at +40 mV. B. Currents and fluorescence recorded from Shaker IR A359C T449Y V478A. Grey lines in panels A & B represent single exponential fits. Fluorescence traces are normalized to fast amplitudes for comparison of relative slow fluorescence amplitudes. C. Mean time constants for current traces in A and B. D. Mean time constants fit to current and fluorescence at +40 mV for constructs in panels A and B.

V478 mutation and 4-aminopyridine similarly reduce the amplitude of slow movements of the voltage sensor

We next considered the possibility that disruptions in the final transition of the channel activation pathway, or in coupling of the voltage sensor to activation, rather than acceleration of C-type inactivation, may be responsible for the faster current decay in V478A mutants, and could explain the unexpected decrease of slow fluorescence amplitude in Figure 5.3B. Such an effect might also account for the shift in activation voltage dependence and the apparent uncoupling from voltage sensor movement as tracked by the F(V) of A359C fluorescence, as shown in Figure 5.2. Normally, the concerted opening transition (COT) from the “activated-not-open” state to the open state is normally tightly coupled to S4 movement (Ledwell and Aldrich, 1999; Smith-Maxwell et al., 1998b). As before, we examined the slow component of Shaker IR A359C fluorescence, as emission spectra from this construct can report on a slow conformational change of the S4 helix that accompanies C-type inactivation. Furthermore, we introduced 4-AP as an additional tool to study activation gate dynamics. 4-AP is known to enter the inner pore of open channels and bind within the inner vestibule, blocking the permeation pathway and “pulling” the activation gate closed (McCormack et al., 1994; Armstrong
and Loboda, 2001). This has the effect of trapping 4-AP inside the activation gate and antagonizing the COT, thereby maintaining the channel in the “activated-not-open” state (del Camino et al., 2005). As a consequence, C-type inactivation, which takes place primarily from the open state, is largely prevented.

When Shaker IR A359C was subjected to 10 s pulses to +60 mV (Figure 5.4A), the slowly inactivating ionic current was accompanied by a slow decay in fluorescence intensity after the initial rapid fluorescence signal, as observed previously (see Figure 5.3). When oocytes were bathed in 10 mM 4-AP, potassium current was significantly blocked, C-type inactivation was prevented, and the normalized amplitude of the second fluorescence component was considerably diminished (Figure 5.4C). When the same experiment was carried out on Shaker IR A359C V478A, a decaying outward current was observed, but the slow fluorescence signal was already small (Figure 5.4B), so that when 4-AP was applied, although current amplitude was strongly decreased, the slow fluorescence amplitude was unchanged (Figure 5.4C). When the mutation T449Y was introduced to Shaker IR A359C, slow fluorescence amplitude was diminished from Shaker IR A359C (Figure 5.4C), and following 4-AP application, fluorescence amplitude resembled that from Shaker IR A359C with 4-AP or Shaker IR A359C V478A (Figure 5.4C). Finally, when both T449Y and V478A were present together, slow fluorescence amplitude was even further reduced, but unchanged following addition of 4-AP (Figure 5.4C). Because the effects of V478A mutation and 4-AP are similar and non-additive, it appears that the mutation may in fact act by locally destabilizing the activation gate region and promoting bundle crossing closing during depolarization. Consistently, 4-AP, which is known to associate with greatest preference to the activated-not-open state of the
channel (Armstrong and Loboda, 2001; del Camino et al., 2005), binds with an
approximately 20 times greater potency to Shaker IR A359C V478A as compared to
Shaker IR A359C when subjected to repetitive pulse protocols. Shaker IR A359C V478A
had an IC50 of 48 μM in 4-AP, compared to Shaker IR A359C with an IC50 of 868 μM
(Figure 5.5). The increased potency is consistent with a reduction in exposure of the open
state following 4-AP binding in the presence of V478A, leading to a stronger 4-AP
trapping effect (McCormack et al., 1994; Armstrong and Loboda, 2001). In concert with
results using voltage clamp fluorimetry, this finding suggests that local perturbations in
the lower S6 may underlie the changes in current decay rate.

As raising the concentration of extracellular K+ is known to be a potent way of
inhibiting C-type inactivation (Lopez-Barneo et al., 1993; Levy and Deutsch, 1996b),
therefore we examined its effects on the slow component of fluorescence in Shaker IR
channels with and without the V478A mutations. As seen previously in the 4-AP
experiments, long pulses in Shaker IR A359C demonstrated a large, slow, secondary
component of fluorescence, which can be greatly reduced by increasing external K+
concentration from 3 mM to 99 mM (Figure 5.4D). However, no effect of increasing
external K+ concentration was observed on the slow ΔF signals from V478A mutant
channels (Figure 5.4E). This suggests that the secondary voltage sensor environmental
changes in the outer pore region that accompany C-type inactivation during longer
periods of depolarization are absent in the V478A mutation (Figure 5.4F), and may in
fact suggest that the extent of C-type inactivation is being reduced in these channels.
Figure 5.4. V478A mutants and 4-aminopyridine similarly prevent C-type inactivation by destabilization of the channel open state. Currents (top) and fluorescence from \textit{Shaker IR A359C} (A) and \textit{Shaker IR A359C V478A} (B), in ND96 (black) and in ND96 + 10 mM 4-aminopyridine (grey), in response to pulses from +60 mV from -80 mV. Slow fluorescence deflection amplitudes (denoted by arrows) are normalized to the amplitudes of the rapid fluorescence components of each signal. C. Normalized amplitudes of slow fluorescence signals from \textit{Shaker IR A359C} and A359C V478A, and \textit{Shaker IR A359C T449Y} and A359C T449Y V478A. Fluorescence amplitudes of the fast component were normalized to a value of 1, then slow amplitudes were quantified by normalization to the fast components from the same traces, in ND96 (dark grey) and 10 mM 4-AP (light grey). D. Fluorescence traces from \textit{Shaker IR A359C} in the presence of ND96 (black) and ND96 containing 99 mM external K$^+$ (grey). E. Fluorescence traces from \textit{Shaker IR A359C V478A} in the presence of ND96 (black) and ND96 containing 99 mM external K$^+$ (grey). F. Slow fluorescence amplitudes recorded from channels in the presence of ND96 and 99 mM potassium are normalized to fast amplitudes within the same traces, and then plotted against each other for comparison.
Figure 5.5. V478A mutation increases potency of 4-aminopyridine block.
Figure 5.5. V478A mutation increases potency of 4-aminopyridine block. Oocytes expressing Shaker IR A359C (left) or Shaker IR A359C V478A (right) are given repeated 200 ms pulses to +60 mV from a holding potential of –80 mV. A. Following recording of 200 ms control traces in the absence of 4-AP (shown in black in the top panels, and in grey in bottom panels), oocytes are exposed to increasing concentrations of 4-AP, and given 200 ms pulses until the block at the end of each pulse reaches a consistent steady state. For clarity, only the first, second and final pulses are shown. B. Final pulses are shown for each construct in the presence of ND96 containing the indicated concentration of 4-AP. C. Steady state current values for both constructs in (B) are normalized to the values in the absence of 4-AP, and plotted against 4-AP concentration (on a Log scale), and single site concentration response curves are fit to the resulting data points. Shaker IR A359C had an IC50 of 868 ± 220 μM, while Shaker IR A359C V478A had an IC50 of 48 ± 8 μM.

Facilitation of S4 segment re-mobilization by the pore mutant and 4-AP during membrane potential repolarization

To further understand the behaviour of the activation gate and voltage sensors of Shaker IR V478A during long depolarizing stimuli, we examined voltage sensor return during repolarization. The process of C-type inactivation is functionally associated with an energetic stabilization of the gating charges of S4 in an outward position, known as charge immobilization (Fedida et al., 1996; Olcese et al., 1997) or relaxation (Villalba-Galea et al., 2008), such that, upon repolarization of the membrane potential, the return of the voltage sensor to its resting position within the closed channel is slowed. If, as we suspect, C-type inactivation is reduced by V478A, then we would expect that charge immobilization would occur to a lesser extent compared to the channel lacking the pore mutation.

To examine charge immobilization, channels were subjected to long pulses to +60 mV, then returned to a series of voltages from 0 to –120 mV in 10 mV steps (Figure 5.6A). Fluorescence amplitudes were measured 50 ms following the start of repolarization pulses, when any fast S4 movements have likely occurred, but slower ones
reflecting charge immobilization mostly have not. Energetic stabilization of S4 in its outward position would be expected to negatively shift the voltage dependence of S4 to its resting conformation (Olcese et al., 1997) compared to the F(V) relationship observed during channel activation (see Figure 5.2E). The voltage dependence of the changes in Shaker IR A359C fluorescence intensity, when measured in this way, was hyperpolarized by approximately 40 mV (Figure 5.6B, D), and, expectedly, the addition of 10 mM 4-AP largely attenuated the shift. When Shaker IR A359C V478A was subjected to the same experimental protocol, the voltage dependence of A359C fluorescence during repolarization overlaid well with the trace of Shaker IR A359C in the presence of 4-AP. This is consistent with the idea that the mutation and 4-AP both antagonize the stability of the open state (Figure 5.6C, D). When 4-AP was subsequently added to Shaker IR A359C V478A, a slight additional rightward shift of the Boltzmann curve was seen, aligning it with the F(V) for Shaker IR A359C during activation, and indicating that charge immobilization had been fully prevented by this combination.

In order to chart the time dependence of voltage sensor return, the fluorescence traces were fit to weighted time constants, in a method similar to one employed previously with gating currents (Lacroix et al., 2011). We observed that at all potentials, the time constant of fluorescence return was accelerated by V478A mutants as compared to Shaker IR A359C (Figure 5.6E), further consistent with an attenuation of conformational changes in S4 in the presence of V478A.
Figure 5.6. V478A mutation prevents S4 charge immobilization in *Shaker IR*. 
Figure 5.6. V478A mutation prevents S4 charge immobilization in Shaker IR. A. Sample current (top) from a voltage protocol (bottom) to measure fluorescence immobilization (for explanation see text). Arrows indicate the period over which fluorescence signals in (B) and (C) were recorded. B. Fluorescence traces recorded during repolarization to the indicated potentials in Shaker IR A359C, in ND96 (top) and in ND96 with 10 mM 4-AP (bottom). Arrows indicate where ΔF/FMAX was measured, for panel (D). C. Fluorescence traces recorded during repolarization for Shaker IR A359C V478A. D. Fluorescence amplitudes 50 ms after repolarizing voltage pulses in (B) and (C) plotted against voltage and fit with single Boltzmann functions, in ND96 (filled symbols) and 10 mM 4-AP (open symbols). V1/2 values of Boltzmann fits for Shaker IR A359C fluorescence were -98.9 ± 1.5 mV with 0 mM 4-AP and −60.7 ± 1.5 mV with 10 mM 4-AP. V1/2 values of Boltzmann fits for Shaker IR A359C V478A fluorescence were −65.6 ± 1.4 mV with 0 mM 4-AP and −55.1 ± 2.1 mV with 10 mM 4-AP. A Boltzmann function fit to Shaker IR A359C fluorescence during activation pulses (from Figure 5.2) is overlaid in grey (V1/2 = -53.7 ± 1.1 mV). E. Time constants fit at all potentials to repolarization fluorescence traces for Shaker IR A359C and A359C V478A. τ values were significantly different at all displayed potentials where they could be fit, p<0.05.

A stable channel open state is prevented by V478A mutation

Data acquired using voltage clamp fluorimetry suggest that a destabilization of the open state at depolarized potentials is responsible for the acceleration of current decay in Shaker IR V478A. A possible explanation is that channels do not reach a stabilized conducting state following opening, in which the voltage sensors have entered their relaxed states, instead allowing the activation gate to “slip” closed even while the voltage sensors are in their activated conformation. If this were the case, we would expect that in measurements of currents from single channels, the open dwell time during depolarization, a measure of open state stability, would be affected by V478A. To investigate this, we expressed wild-type and mutant channels in ltk- cells, and studied them using single channel cell-attached patch clamp. Patches expressing a single Shaker IR channel or single Shaker IR V478A channel were subjected to repeated 8 second pulses to +60 mV from a holding potential of −80 mV (Figure 5.7A,B). Inset views allow for closer examination of flickering (lower panels), which was further subjected to burst
analysis. It appears that the V478A mutant shows extensive rapid closing events during bursts such that the full open level is rarely observed, at the sampling rate and filter frequency that were used.

**Figure 5.7. Shaker IR V478A has altered cell-attached single channel properties.** A. Representative sweeps of Shaker IR currents elicited from 8 s pulses to +60 mV from a holding potential of –80 mV, as shown. Bottom panel shows a higher time resolution view of the area indicated by the dotted lines. B. Representative sweeps of Shaker IR V478A currents with higher time resolution in the bottom panel.

Dwell times of individual opening events were measured, and plotted against occurrence frequencies normalized to the maximum value. A significant reduction in open dwell time was found in Shaker IR V478A as compared to Shaker IR (Figure 5.8A). While Shaker IR could be fit with three Gaussian functions, the fit corresponding to the longest time constant, $\tau_3$, was abolished by V478A. Thus, sustained openings longer than 10 ms were removed in the presence of V478A. In addition, burst durations (rapid
opening behaviour between closings longer than 5 ms) were binned and plotted against normalized frequency, then fit with Gaussian functions to determine mean burst durations (Figure 5.8B). Here, the distribution was also fit as the sum of three Gaussian distributions, and the longest of these was not seen with V478A mutation. These data suggest a less stable open state in the presence of V478A. Conversely, no significant effects of V478A were observed in closed times between high frequency opening events (Figure 5.8C) or in relative frequency of closing events longer than 5 ms (Figure 5.8D). Taken together, these findings are consistent with fluorescence data, which suggest that a stabilized open conformation of the channel, associated with transfer of the S4 helix into its relaxed position and longer periods of activation gate opening, is being prevented by the EA-1 associated mutation V478A.

Figure 5.8. *Shaker IR* V478A does not enter a long-lasting open state and has shorter bursts than *Shaker IR.*
Figure 5.8. *Shaker* IR V478A does not enter a long-lasting open state and has shorter bursts than *Shaker* IR. A. Frequency histogram of opening event durations normalized to maximum frequency and binned logarithmically by duration for currents from *Shaker* IR (black) and *Shaker* IR V478A (grey). Gaussian distributions fit to the peaks had mean time constants, for *Shaker* IR, of $\tau_1 = 0.8 \pm 0.1$ ms, $\tau_2 = 1.3 \pm 0.1$ ms, $\tau_3 = 4.7 \pm 0.9$ ms, and for *Shaker* IR V478A, of $\tau_1 = 0.8 \pm 0.1$ ms and $\tau_2 = 1.4 \pm 0.3$ ms. B. Normalized frequency histogram of burst duration (single bursts delimited by closed intervals longer than 5 ms). Gaussian distributions fit to the peaks had mean time constants, for *Shaker* IR, of $\tau_1 = 3.4 \pm 0.1$ ms, $\tau_2 = 25.2 \pm 4.0$ ms, $\tau_3 = 169.0 \pm 15.5$ ms, and for *Shaker* IR V478A, of $\tau_1 = 9.9 \pm 0.6$ ms and $\tau_2 = 98.3 \pm 16.4$ ms. C. Normalized frequency histogram of intraburst durations. Single Gaussian fits (not shown) had mean time constants of $1.1 \pm 0.1$ ms and $1.3 \pm 0.1$ ms for *Shaker* IR and *Shaker* IR V478A, respectively. D. Normalized frequency histogram of interburst durations (denoted as closing events longer than 5 ms). These curves were not fit with Gaussian functions.
DISCUSSION

Insights into atypical gating in an EA-1 mutant Kv1 channel from voltage clamp fluorimetry and single channel patch clamp

In this paper, we have investigated how EA-1 associated mutations in the lower S6 of a Shaker family channel can lead to the increased rate of decay of outward current amplitude, as a reduction in Kv1.1 current amplitude in cerebellar basket cells is known to underlie the onset of disease (Herson et al., 2003). We have established using voltage clamp fluorimetry and single channel patch clamp electrophysiology that this decay likely results from destabilization of the open state accelerating closing of the activation gate at depolarized potentials. Several lines of evidence support this conclusion. While current decay is significantly faster in Shaker IR V478A and Kv1.1 V408A as compared to their non-mutant forms, a slowly decaying fluorescence signal from Shaker IR A359C, which is known to accompany C-type inactivation (Claydon et al., 2007a), is slowed and greatly reduced by the mutation V478A. Furthermore, two known antagonists of C-type inactivation, mutation at site T449 (Lopez-Barneo et al., 1993), and elevated external K⁺ (Levy and Deutsch, 1996b), while producing significant effects on current decay and slow fluorescence amplitude in non-mutant channels, had no discernible effects on V478A mutant current or fluorescence.

In addition, the potency of 4-aminopyridine was greatly increased in the presence of V478A, consistent with a local disruption in the lower S6. Slow fluorescence signals from Shaker IR A359C were attenuated by 4-AP, and this effect was mimicked by V478A, with no additional effect of 4-AP, suggesting that both effects likely take place in the activation gate. Finally, the strong reduction in gating charge immobilization with
V478A, which normally occurs during prolonged depolarization and is linked with the development of C-type inactivation (Olcese et al., 1997), indicates that the accelerated current decay seen with V478A cannot be explained by an increased rate and contribution of slow C-type inactivation.

In concert with fluorescence experiments, we have used patch clamp electrophysiology to probe the effects of V478A on single channel opening behaviour. We observed a strong reduction in mean open time during flickering, as well as shortening of burst durations themselves. These findings most likely reflect a destabilization of the open state, causing local perturbations in conformation that could allow channels to close during depolarizations. This finding is similar to a proposed mechanism of current decay during long periods of activation proposed in spHCN channels (Shin et al., 2004) and in Kv4.2 (Dougherty et al., 2008), and is consistent with the finding that multiple pathways of current decay exist in other Kv1 channels, as was shown in Kv1.3 (Marom and Levitan, 1994). Though it represents a different conclusion, this result also appears to be consistent with observations of a shorter mean open time in single channel analysis of homotetramers of Kv1.1 V408A as well as Kv1.1-Kv1.2 heterotetramers possessing the same mutation (D'Adamo et al., 1998).

Interestingly, the valine examined in this study is known to form part of the binding site for compatible N-termini involved in fast inactivation (Zhou et al., 2001). Indeed, impaired binding has been observed in Kv1.1-Kv1.4 tetramers (Imbrici et al., 2006), as well as in Kv1.1 co-expressed with a Kvβ1.1 subunit (Maylie et al., 2002), and entry into the open state of the channel activation gate is a known requirement for peptide binding (Hoshi et al., 1990; Demo and Yellen, 1991). An intriguing implication of our
findings is that reduction in exposure of the open state of the activation gate, resulting from destabilization by the V to A mutation, may in fact underlie the decrease in the extent of N-type inactivation observed in mutants at this site in Kv1.1.

**A proposed mechanism for normal and disease-causing channel gating in *Shaker* channels**

We have used slow secondary quenching of the *Shaker* A359C fluorophore to track secondary movements of the S4 helix following opening. A plausible explanation for the slow quenching phenomenon observed in A359C during long pulses is that it reflects a reorientation of the S4 with respect to the outer pore leading to its increased stability in that position (Loots and Isacoff, 1998; Loots and Isacoff, 2000), which may represent a mode shift in channel gating (Batulan et al., 2010; Haddad and Blunck, 2011), and favour the onset of C-type inactivation. Recent work suggests this is likely due to a relaxation of the voltage sensor, which is associated with, but not obligatorily linked to C-type inactivation (Villalba-Galea et al., 2008; Lacroix et al., 2011; Batulan et al., 2010). Consistent with this, our data using the T449Y mutant in *Shaker* IR A359C to antagonize inactivation suggests that, while it is likely that charge immobilization and C-type inactivation are in fact tightly linked, the former appears capable in some conditions of taking place at a faster rate than the latter (see Figure 5.3). As such, the S4 helix may enter into a stable outward conformation with respect to the outer pore (i.e.: the relaxed state), leading to longer duration opening events in wild-type channels but not those possessing the EA-1 mutation. As C-type inactivation occurs preferably from the open state under normal conditions, stabilization would probably favour the onset of C-type
inactivation, whereas disruption of stability could lead to closing during periods of depolarization, and actually reduce the extent to which C-type inactivation can occur.

Based on the findings of this study, we propose a mechanism by which prolonged depolarizations may lead to conformational changes within the pore and voltage sensor and lead to C-type inactivation, and how the EA-1 mutation Kv1.1 V408A and its Shaker homologue V478A may contribute to the process. When the membrane becomes depolarized at positive potentials, the voltage sensors move to their active positions, and the lower S6 is pulled to its open position. Normally in Kv1 channels, the lower S6 can then transition between the first open and the stabilized open state. In the Kv1.2-2.1 crystal structure (Long et al., 2007), an apparent hydrophobic pocket exists in this region which we propose may allow contacts between the side chains of V478, and A471, L472 and P475 of an adjacent subunit to stabilize the lower S6 (Figure 5.9A), and reduce the mechanical load on the S4 helix allowing it to undergo a mode shift to a relaxed position (Haddad and Blunck, 2011). This conformation then facilitates entry into a stable quiescent state where the selectivity filter has become constricted (C-type inactivation).

Conversely, when the mutation V478A is present, the entry to a stabilized open state is prevented as evidenced by single channel records, which may be due to the absence of stabilizing hydrophobic interactions between S6 helices of adjacent subunits (Figure 5.9B). Under these conditions, the “mechanical load” of the open activation gate can no longer be transferred from the S4 to the pore. As such, as the S4 attempts to adopt its relaxed state, the S6 is able to slip into its closed conformation, blocking the flow of current. Mutational analysis of hydrophobic interactions behind the activation gate would help to establish the properties of a putative “stabilized open state”.

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Figure 5.9. **EA-1 mutations may weaken channel open state stability.** A. Intracellular regions of the S5 and S6 helices from two adjacent subunits in the Kv1.2-2.1 chimera (PDB ID: 2R9R) are shown (red and green coloration denote separate subunits), from Long et al. (2007). Key residues (all within the S6 helices) are highlighted in spherical representation, including V478 from the first subunit and A471, L472 and P475 from the second subunit (residue numbering as in *Shaker* IR). B. Substitution of V478 for alanine results in absence of both γ-carbons normally present with a valine side chain.

**Summary**

In summary, we have established a mechanism by which mutations at Kv1.1 V408A leads to an increase in the rate of current decay. Our results suggest that the phenotype results from an instability introduced in the open state, causing the activation gate to close at depolarized potentials, with implications in future strategies for development of treatments. These results also demonstrate the utility of the voltage clamp fluorimetry methodology to determine the diverse biophysical mechanisms underlying pathophysiology caused by genetic mutations. Using a combination of techniques, we have demonstrated that the established phenotype of neurological genetic disease episodic ataxia type-1 can result from a distinct closing phenomenon in the channel.
activation gate, introduced or accelerated by mutations within the permeation pore, rather than modulation of existing mechanisms.
Chapter 6: Discussion
**Contribution of external linkers to channel gating**

While crystallography studies have been critical to the development of a clear image of Kv1-type channel structure (Long et al., 2005a; Long et al., 2007), a limitation of such techniques lies in the fact that they are not able to inform about the flexible linkers between transmembrane helices, as the flexibility in those regions makes them unwilling to form a stable crystal lattice. While linkers may not contribute heavily to the process of rapid activation gating - indeed, this much was clear from the persistence of the paddle model of gating, wherein S3 and S4 were predicted to move as a fixed unit with no functional importance whatsoever of the S3-S4 linker (Long et al., 2005b) - the external linkers are nonetheless known to come into close proximity with the upper parts of voltage sensor helices during depolarization (Sorensen et al., 2000). Because the linkers represent regions of surprisingly low homology between the thoroughly characterized *Drosophila Shaker* channel and the crystallized mammalian Kv1.2 channel, any attempt to contrast their biophysical properties to develop a full understanding of the biophysical distinctions amongst *Shaker* family channels would likely necessitate a consideration of external linker dynamics. For this reason, we used the technique of voltage clamp fluorimetry, with tagging sites mostly in the S3-S4 linker, as well as in the S1-S2 linker.

Analysis of rapid fluorescence quenching from Kv1.2 A291C (equivalent to the widely used *Shaker* A359C) showed the expected result that it changed with a voltage dependence negatively shifted with respect to channel conductance. As *Shaker* A359C is known to faithfully track gating charge movement in advance of channel opening
(Mannuzzu et al., 1996), we inferred that the same was likely taking place in Kv1.2 A291C, and when Kv1.2 gating currents were recorded independently, the resulting Q(V) curve overlaid well with the F(V) from A291C (see Figure 2.6). However, following this rapid quenching, Kv1.2 A291C TMRM fluorescence shows a second, slower fluorescence signal. While there is a secondary component present in traces from Shaker A359C, that fluorescence is linked to the C-type inactivation process (Claydon et al., 2007a) and correlates well with decay in the ionic current. In Kv1.2, secondary fluorescence is much more rapid compared to Shaker, but is not accompanied by any appreciable current decay, which is consistent with the presence of a valine at residue 377, known to strongly inhibit C-type inactivation (Lopez-Barneo et al., 1993). Therefore, movements in the environment of the S3-S4 linker of Kv1.2 following activation are distinct from those in Shaker. Consistent with this, a fluorescence scan of neighbouring residues showed similarity between rapid quenching between Shaker and Kv1.2, but a pronounced slow component in most residues in the latter. Using a two pulse protocol with a repolarizing interpulse of varying duration, we determined that the slow phase recovers at the same rate as the channel depolarizes, suggesting that voltage sensor return is a rate-limiting step for deactivation. As a corollary, these data also imply that S4 and S6 movements are tightly coupled following depolarization in Kv1.2 channels, as deactivation and S4 return must happen simultaneously. This stands in contrast to Kv1.5 channels, where gating charge and activation gate closing can be uncoupled by depolarizing pulses (Wang and Fedida, 2002). Finally, Kv1.2 fluorescence signals recorded using tagged sites in the S1-S2 linker showed slow fluorescence similar to that from A291C, and the latter could be abolished in chimaeras generated with S1-S2 linkers.
from other channels. Taken together, these data suggest that following activation, an interaction between S1-S2 and S3-S4 develops to stabilize an outward position of S4 helix, but without triggering C-type inactivation as is observed in other channels. This represents a novel finding in Kv1.2 channels, and highlights that considerable functional variability amongst channels of the *Shaker* family does exist, which will be discussed further later in this discussion.

A clear limitation in our approach of using TMRM fluorescence in this study is that the fluorophore itself may re-orient and prevent development of a faithful model of protein movement. The TMRM molecule consists of a bulky aromatic moiety (providing the active fluorescent component) attached with a flexible linker to the maleimide group, meaning that unexpected reorientation at groups within the linker could alter the orientation of the fluorophore in unpredictable ways. Development of more compact fluorophores is critical to improving the spatial resolution of any structural inferences made by the VCF technique (Cohen et al., 2002; Cohen et al., 2005), in particular as concerns the use of the technique to infer structure by performing cysteine scans within discrete protein domains (as discussed in the introduction). However, as our studies largely employed fluorescence as a readout of well-characterized and attributed protein re-orientations at commonly employed sites, rather than to probe poorly studied areas of the protein, use of the TMRM seemed to be a prudent approach.

**N-type inactivation effects on activation and C-type inactivation**

Additional studies on the Kv1.2 channel focused on the interaction with the Kvβ1.2 subunit. Most notably, while the voltage dependence of activation of Kv1.2
appeared to be left-shifted by the presence of the Kvβ1.2 N-terminus, a similar shift in the voltage dependence of F(V) curves reflecting upward S4 movement, which would be expected if interactions between Kvβ1.2 and Kv1.2 were affecting activation, were not observed. Co-expression of Kv1.2 with the Kvβ1 subunit with a truncated N-terminus resulted in currents indistinguishable from Kv1.2 expressed alone, save for the expected strong upregulation of current amplitude known to occur in co-assembled channels (Accili et al., 1997b), suggesting that the distal N-terminus is the necessary component to produce the observed gating effects. For these reasons, we suggest that a model of open channel block can suitably explain the effects of Kvβ1.2 on Kv1.2 activation (De Biasi et al., 1997). In the model, the blocker is only capable of entering open channels, with kinetics similar to or slower than those for activation. At strongly depolarized potentials, channels are primarily open, and in this voltage range the macroscopic currents increase in amplitude linearly with a slope dictated by the maximum conductance, as per Ohm’s law. At these potentials, block by the N-terminus of Kvβ1.2 takes place most readily, dictated by the affinity for the blocker for the open pore, and the recorded steady state currents are partially reduced by the current obstruction. N-type inactivation takes place from the open state, and its onset is rapid compared to recovery. At strongly depolarized voltages where the open state is largely favored over the closed state, the high open probability also means a high probability of channels entering the N-type inactivated state. Conversely, at potentials where channels open more reluctantly, and for shorter durations (ie: at voltage levels near the half-activation potential \(V_{1/2}\)), the lowered open probability also means a reduced number of channels available to N-type inactivate. When activation curves are generated, normalization of conductance is to steady state
values where channels are normally predominantly closed or open, therefore, open channel block at depolarized potentials will result in normalization to reduced maxima, causing the data at intermediate potentials to appear to represent larger current amplitudes than would actually reflect the true open probability of the activation gates, thus causing the observed upward (or leftward) shift in the activation curve.

Another observation from studies of Kv1.2 interactions with Kvβ1.2 was that the return of the gating charge, as reflected by fluorescence decay rates from TMRM attached at M288C at the external end of S4, was slowed by the peptide. As the rate by which the fluorescence decayed took place on the timescale of channel block, we suggested that it represented a charge immobilization phenomenon of the S4 helices, similar to what had been observed previously in N-type inactivating channels (Bezanilla et al., 1991). This suggests that the activation gate in Kv1.2 is tightly coupled to the S4 helix, such that as the blocker enters, the S6 is obstructed from closing by the bound peptide, and the S4 helix remains in an activated position until the blocker is cleared. Being that the ionic current tails during deactivation are slowed, the S4 may actually also remain immobilized for some time afterwards. This appears to represent a mechanism of charge immobilization distinct from the one observed from gating charge hysteresis (Olcese et al., 1997), and from fluorescence records from Chapter 5 in non N-type inactivating channels such as Shaker IR. In the presence of a blocking N-terminus, S4 fluorescence returns to its resting fluorescence level during repolarization with a double exponential time course, and the relative contribution of the fast fluorescence to the total fluorescence amplitude decays exponentially on the time scale of N-type block (Figure 6.1A), even in the presence of the C-type inactivation-antagonizing T449V mutation.
Importantly, comparisons between Kv1.2 and Shaker show that both gates can be rapidly immobilized by the Kvβ1.2 and Shaker N-terminal peptides, respectively. Deactivation in Shaker shows slowing of current tails, on the time scale of S4 fluorescence immobilization by the N-terminus (Figure 6.1B), suggesting that full length Shaker channels are being immobilized by a similar mechanism to Kv1.2 with the Kvβ1.2 subunit, despite the lack of N-terminal peptide identity between Shaker and Kvβ1.2.

When the rate of recovery from N-type inactivation was recorded using two pulse protocols (Figure 6.1C), it was found to match the simultaneously recorded rate of fluorescence decay from site A359C (Figure 6.1D). These results are consistent with our hypothesis from Chapter 3 that the N-termini hold open the activation gate by a steric mechanism, and that S4 remains tightly coupled to the position of S6, prevented in this case from returning to its resting conformation until the pore blocker is cleared. Interestingly, when time constants of fluorescence recovery following immobilization are recorded at different voltages (Figure 6.1E) and compared to those recorded in Chapter 5 from Shaker IR channels given long depolarizing pulses, we noticed a much faster recovery in N-terminally immobilized channels than C-type inactivated Shaker IR channels (Figure 6.1F). This appears to suggest that the immobilization of S4 charge movement can result from more than one mechanism, depending on whether it happens as a consequence of rapid N-type inactivation, or alongside current decay during prolonged pulses. This will be discussed further later in this discussion.
Figure 6.1. Effects on current and A359C fluorescence of N-type block in full-length *Shaker* T449V.
Figure 6.1. Effects on current and A359C fluorescence of N-type block in full-length \textit{Shaker} T449V. A. Sample ionic tails at –40 mV (top) and fluorescence deflections at –80 mV (bottom), from oocytes expressing \textit{Shaker} FL (full length) A359C T449V, following pulses to +60 mV for the indicated time (in ms). B. The amplitudes of slow components from double exponentials, fit to the current (triangles) and fluorescence traces (squares) in A, normalized to their total amplitudes and plotted against prepulse times. Single exponentials of the development of the slow $\tau$ amplitudes had mean time constants of 13.4 ± 1.7 (for current) and 11.1 ± 1.0 (for fluorescence). Grey circles show normalized $\tau$ amplitudes from currents recorded in a similar experiment for Kv1.2 and Kv$\beta$1.2, from Figure 3.5. Also shown in grey is an (inverted) sample trace of N-type inactivation onset from \textit{Shaker} FL A359C T449V. C. Currents from \textit{Shaker} FL A359C T449V in response to a protocol where +60 mV voltage pulses are separated by a pulse to –80 mV of variable duration, between 10 and 200 ms. Fluorescence recorded simultaneously with the 200 ms interval is overlain in grey. D. $\tau$ values from single exponentials fit to the current peak amplitudes and fluorescence decay in C. E. Sample fluorescence deflections from \textit{Shaker} FL A359C T449V in response to voltage pulses to the indicated voltages, following 100 ms pulses to +60 mV. F. Weighted time constants fit to traces in E across the indicated range of voltages are shown (triangles), for comparison, $\tau$ values from a similar experiment using \textit{Shaker} IR A359C (with 8 s prepulses) are also shown (squares).

In Chapter 4, the N-terminus of \textit{Shaker}, in addition to blocking the pore and presumably directly inhibiting voltage sensor return (see Figure 6.1), was shown to exert an additional effect on an allosteric pathway linking the S6 and the selectivity filter, having the effect of accelerating C-type inactivation. This effect clearly requires the selectivity filter to be locally capable of inactivation, as a mutation in the outer pore that normally inhibits C-type inactivation prevents it from occurring even in the presence of an intact N-terminus (see Figure 4.2). While it has been long known that N-type inactivation speeds the onset of C-type inactivation, the mechanism by which this takes place has remained controversial, as competing theories posit that either evacuation of the potassium sites, or an additional allosteric effect, is the underlying cause (Baukrowitz and Yellen, 1996a; Baukrowitz and Yellen, 1995; Bett and Rasmusson, 2004; Li et al., 2003). An additional level of complexity is added by the discovery that a site within the blocker binding site is involved in allosteric coupling of the S6 to the selectivity filter backbone
(Cuello et al., 2010), allowing the orientation of the activation gate to be communicated to the filter. In this thesis, I have presented evidence that an allosteric effect of the N-terminus’ binding within the inner pore cavity is likely due to an accelerated expulsion of potassium ions from the selectivity filter, combined with a coupling between activation gate opening and filter collapse which is sensitive to the presence of potassium.

Our results presented in Chapter 4 are perhaps best interpreted in the context of the known structural basis of potassium conduction across the selectivity filter. In the crystal structure of the KcsA channel, four potassium-binding sites within the filter were seen, named S$_1$-S$_4$ from external to internal, with additional sites of electron density representing additional potassium ions in the external vestibule (S$_0$) and the inner cavity (Zhou et al., 2001). Potassium ions are bound alternatingly at sites S$_0$-S$_2$-S$_4$, or at S$_1$-S$_3$, and repel preceding ions across the voltage field by electrostatic interactions between adjacent sites (Morais-Cabral et al., 2001). Molecular dynamic simulations of the occupied selectivity filter have suggested that while the S$_2$ and S$_4$ sites are occupied, a local reorientation to a non-conducting filter conformation (i.e., C-type inactivation) is energetically prohibitive, whereas when S$_1$ and S$_3$ are occupied by potassium, the energy barrier to local conformational changes is much lower (Berneche and Roux, 2005). Because S$_0$ is the most external site and is directly exposed to the extracellular media, higher concentrations of potassium would be expected to favour occupancy of S$_0$, and by association would also favour the occupancy of sites S$_2$ and S$_4$, consistent with the foot-in-the-door mechanism of inhibition of C-type inactivation by K$^+$ ions or TEA (Lopez-Barneo et al., 1993). When the mutation A463C is introduced to Shaker, the affinity for K$^+$ of a selectivity filter potassium-binding site [positionally equivalent to S$_3$ in the KcsA
crystal structure, Doyle et al., (1998)] was strongly reduced (Ogielska and Aldrich, 1998; Ogielska and Aldrich, 1999). Consistent with this finding, C-type inactivation rates were sharply reduced in those constructs, as would be expected if the S₂ and S₄ occupied conformation had become relatively more favourable (Ogielska and Aldrich, 1999). Furthermore, crystal structures of the selectivity filter in low potassium concentrations, when it is able to adopt its non-conducting conformation, showed potassium ions spatially localized at the S₁ site but not at S₂ (Zhou et al., 2001), suggesting that C-type inactivation probably takes place when S₁ is occupied and when S₂ is empty. When the crystal structures of the partially open KcsA channels were solved, the occupancy of the S₂ was inversely related to the degree of opening of the activation gate, where activation gate opening favoured the depletion of potassium from S₂ (Cuello et al., 2010). Accompanying opening of the activation gate was a rotomeric reorientation at F103 (the positional equivalent of Shaker I₄₇₀), such that when the activation gate was closed, the side chain of F103 was in a “resting” orientation, and the four selectivity filter potassium binding sites were equally likely to be occupied in a conducting conformation, whereas as the activation gate opened, the side chain of F103 rotated into an “active” orientation, and the pore adopted a non-conducting conformation with no K⁺ ion at S₂ (Cuello et al., 2010; Cuello et al., 2010). Local interactions of F103 with neighbouring residues of the pore helix forming the selectivity backbone were perturbed when F103 entered its active conformation, and this was predicted to destabilize the open filter and promote its entry into a non-conducting conformation (Cuello et al., 2010; Cuello et al., 2010). It was for these reasons that those authors suggested that KcsA F103 and Shaker I₄₇₀ act
allosterically as a structural link between activation gate opening and conformational changes in the selectivity filter (Cuello et al., 2010; Cuello et al., 2010).

In our experiments, we extended the study of the relationship between the activation gate and selectivity filter to examine the additional effect of N-type inactivation, in the form of an intact Shaker N-terminus. When Shaker I470 was mutated to a series of different residues, the rate of C-type inactivation was strongly affected (Figure 4.1), whereas no effect was observed on voltage dependence of activation (Figure 4.4), consistent with the notion that the identity of the side chain at that site does influence the conformation of the selectivity filter when the channel is open (Cuello et al., 2010). As the reorientation of the selectivity filter to its non-conducting conformation was contingent on the depletion of S2 of potassium ions (Cuello et al., 2010), we expected that raising the external concentration of potassium would increase the occupancy of potassium at S2, and therefore slow the onset of C-type inactivation. We observed that C-type inactivation was much slower in high external potassium and that its effects on inactivation rate were additive with the effects of mutation at I470, consistent with the suggestion that both effects were directly or indirectly affecting the ion occupancy of S2.

When Shaker FL was used in the place of Shaker IR, C-type inactivation rates were strongly accelerated. The C-type inactivation accelerating effect of the N-terminus has been attributed previously to depletion of the selectivity filter of potassium ions (Baukrowitz and Yellen, 1995), and it seems likely that this could in fact represent the depletion of K⁺ ions from the S₀-S₂-S₄ sites in favour of occupancy of C-type inactivation-friendly sites S₁-S₃. If this were the case, we would expect elevated levels of external potassium to antagonize N-type inactivation, and this was indeed observed
(Figure 4.5). Furthermore, block from the N-terminus, though described as being voltage independent (Zagotta et al., 1989), exhibited clear voltage dependence equivalent to a depth of approximately 20% of the voltage field (Figure 4.6), consistent with the N-terminus likely sterically or electrostatically interacting with potassium occupying binding site S₄ in the selectivity filter (Zhou et al., 2001). If the N-terminus was able to expel potassium from the S₂-S₄ sites, we would expect the allosteric effects of I₄₇₀ to be accelerated, and that the effects of mutation at that site to act additively with N-type inactivation, and this was observed for all I₄₇₀ mutations tested (Figure 4.3). For these reasons, it is reasonable to suggest that the effect of N-type inactivation on C-type inactivation is to potentiate the allosteric coupling of the activation gate with the selectivity filter by emptying the pore of potassium ions.

**Shaker channels can enter a stable activated state, which is prevented by V478A**

In Chapter 5, we demonstrated that entry into the C-type inactivated state in Shaker IR may depend first upon entry into a compatible open state. This was demonstrated using single channel recordings in Shaker IR, which demonstrated flickery openings of three kinetically distinguishable durations. When episodic ataxia type-1 mutation V478A, known to accelerate current decay, is introduced, the longest lasting open state is no longer entered, consistent with a suggested destabilization of an open state by this mutant (D'Adamo et al., 1998; Maylie et al., 2002). Coincident with this, VCF recordings demonstrate that the secondary phase of Shaker A359C fluorescence is no longer observed in V478A channels, and the immobilization of S4, normally observed in Shaker gating charge (Olcese et al., 1997) and fluorescence traces, is largely prevented
by the V478A mutation. This represents an unexpected but important finding in these channels, as previous work had attributed the accelerated current decay in this mutant to an acceleration of C-type inactivation (Adelman et al., 1995; D'Adamo et al., 1998). We found that C-type inactivation unfriendly residue T449Y (Figure 5.1), and elevated external potassium (not shown) had no effects on V478A current decay, while slowing the rate of inactivation in the wild-type channel, suggesting an alternate mechanism may be involved. Our results more likely suggest instead that the channel voltage sensors fail to “relax”, a process of reorientation within the activated S4 helix to stabilizes its outward conformation (Villalba-Galea et al., 2008).

**New insights into voltage sensor relaxation and mode shifts**

Although both charge immobilization following long depolarizations (Olcese et al., 1997), and secondary voltage sensor fluorescence from A359C (Claydon et al., 2007a), are known to be related to the development of C-type inactivation, recent studies have demonstrated that the two processes are separable (Batulan et al., 2010; Haddad and Blunck, 2011), and this is consistent with the faster time scale of secondary fluorescence decay as compared to current decay in a *Shaker* IR T449Y mutant, where A359C fluorescence remains faster than the attenuated rate of C-type inactivation (Figure 5.3). Those studies, and others from the Ci-VSP protein, where a voltage sensing domain can undergo relaxation in its ON conformation despite the absence of an associated pore (Villalba-Galea et al., 2008), suggested that the process of relaxation may be independent of interactions between the voltage sensor and the pore. However, our data appear to show that in *Shaker*, that may not be the case. When *Shaker* IR A359C fluorescence is
recorded in the presence of 10 mM 4-AP, the secondary fluorescence is abolished, but the fast fluorescence correlating with the Q(V) curve is preserved. This suggests that translocation of gating charge is insufficient to allow the S4 to enter its relaxed state, instead also requiring the concerted open transition to take place, which is known to carry a small fraction of the total gating charge and be antagonized by 4-AP (Armstrong and Loboda, 2001; Loboda and Armstrong, 2001). However, the COT must occur prior to channel opening (Ledwell and Aldrich, 1999), therefore while it is not what’s being reported upon by slow $\Delta F$ in A359C, it is necessary for a subsequent reorientation to take place. In Ci-VSP, no pore is present, therefore no COT takes place (Villalba-Galea et al., 2008), so either the linker with the phosphatase moiety undergoes an equivalent reorientation as the S6 to allow relaxation, or the processes are subtly different between the two proteins. In Shaker channels, this can be explained by the mode shift hypothesis, where the coupling energy to maintain the S4 helix and activation gate in their activated conformation is transferred to an interface between the S4 and the outer pore (Haddad and Blunck, 2011), which is also in line with the finding that an anchor point exists there between the voltage sensor and turret (Lee et al., 2009). For this to be true, the strong coupling between the open S6 and S4-S5 linker must be altered during long depolarizations. Inactivated (and relaxed) channels are known to have open and accessible inner pore cavities (Wang and Fedida, 2002; Starkus et al., 1997; Wang et al., 2000), evidenced by the ability of sodium currents to pass through in activated channels, and by direct accessibility measurements of residues within the activation gate of inactivated channels (Panyi and Deutsch, 2006; Panyi and Deutsch, 2007). Therefore, if
S4 becomes uncoupled from S6 during the mode shift, the S6 must therefore become otherwise stabilized during the inactivation process.

When considering the V478A mutant, four critical observations demonstrate that a normally occurring charge transfer process to unload the inner coupling interface may be prevented: i. the secondary decay in the fluorescence is prevented by V478A without 4-AP, but ionic current traces establish that opening does occur, suggesting that a transition between open states, following the COT, is being prevented; ii. 4-AP has no additional effect on V478A slow fluorescence, suggesting that the secondary change prevented by 4-AP by antagonizing the COT is similar to the one occurring following opening in V478A; iii. the voltage dependence of fast fluorescence return in V478A mutants undergoes a much reduced negative shift during long pulses, suggesting the S4 has not become outwardly immobilized under those conditions, and iv. single channel kinetics establish that a longer duration open state does not occur in V478A channels. Taken together, these data are consistent with the existence of a “stabilized open” conformation, which, when reached, would provide sufficient stability for the activation gate to allow the S4 to reorient, or undergo mode shift (Haddad and Blunck, 2011). An implication of this reasoning is also that S4 coupling to S6 is likely stronger in the closed position than the open position: while the COT is known to reliably follow S4 translocation after depolarization, various perturbations appear to allow the S4 return and activation gate closing to become dissociated and temporally distinguishable (Wang and Fedida, 2002; Haddad and Blunck, 2011).

To more clearly explain our data, we proposed a simplified gating scheme as illustrated in Figure 6.2. Following depolarization, channels move through the activation
pathway representing independent S4 movement, and summarized by dashed lines between C₀ and Cₐ (closed activated). The Cₐ state rapidly moves to the O (open) state, representing the COT (which is prevented in saturating 4-AP). In wild-type channels, the channel can then move into a stable state (Oₛ), which favours relaxation of the voltage sensor and collapse of the selectivity filter into the inactivated state (I). The ability of the channel to become stabilized and “relaxed” likely favours the coupling of the inner S6 to the selectivity filter, as discussed in Chapter 4, because any key residues to this interaction would be able to stably assume their inactivation-promoting orientation. Recovery from inactivation requires long hyperpolarizations, though this process was not examined in the studies of Chapter 5. In Shaker IR V478A, the transition to Oₛ is inhibited, and perhaps replaced with a transition to C₅ (closed desensitized), where the activation gate, unable to reach a stable open conformation, instead “slips” its coupling and enters a conformation with a closed activation gate. This may occur in a similar mechanism to the current decay observed in spHCN (Shin et al., 2004) and Kv4.2 (Dougherty et al., 2008). Consistent with this hypothesis, we observed a strongly enhanced potency of 4-AP for its internal binding site (Figure 5.5). 4-AP is known to bias channels into the activated-not-open conformation, to which it binds tightly (McCormack et al., 1994; Armstrong and Loboda, 2001), whereas entry into the C-type inactivated state reduces the ability of 4-AP to bind (Castle et al., 1994). Therefore, the enhanced potency of 4-AP with V478A channels is consistent with them opening and binding the drug, and willingly entering the closed state following depolarization. As wild type Shaker A359C fluorescence decay appears to correlate well with V478A current decay, an intriguing possibility is that the same reorientation of S4 that leads to relaxation could
be attempted, but unsuccessful, in the V478A channel, which then enters the stable deactivated state, as a result of a weakened coupling in the mutant; however, this hypothesis would require further study. Validation of the proposed gating scheme through future experiments will require development of a structural model to explain how the S4 and S6 helix interactions are altered when the V478A activation gate slips into the stable closed conformation. At present, a re-closure of the activation gate to a conformation reluctant to re-open during the same pulse remains the most likely explanation for the acceleration of current decay in the absence of voltage sensor relaxation and C-type inactivation. However, the deviation in structure underlying the resulting conformation has not been defined. As mentioned in Chapter 5 discussion, generation of mutations in the proposed hydrophobic pocket allowing for activation gate stabilization (Figure 5.9) would allow for a better validation of the proposed model.

![Diagram](Image)

Figure 6.2. Proposed Markov scheme to describe gating events in Shaker IR A359C and the perturbations caused by V478A. C0 represents the deepest closed state of the channel, for simplicity all voltage dependent gating transitions are summarized by rate constants $\alpha$ and $\beta$. CA denotes the activated-not-open state, and the concerted opening transition allows the channel to enter the Open (O) state. A stabilized open state (OS) and open-inactivated state (OI) are entered following channel opening. A closed desensitized (CD) state represents a conformation entered by V478A channel mutants. Transitions or states that are affected by V478A mutation are denoted in red.

The finding from Chapter 5 that current decay results from the inability of the channels to reach their stabilized open conformation also relates to our understanding of
N-type inactivation, as described previously. The valine examined in this study is known to form part of the binding site for compatible N-termini involved in fast inactivation (Zhou et al., 2001). Indeed, impaired binding has been observed in Kv1.1 co-expressed with a Kvβ1.1 subunit (Maylie et al., 2002), as well as in Kv1.1-Kv1.4 heterotetramers (Imbrici et al., 2006). However, Kv1.4 subunits have not been found in the cerebellar basket cells underlying episodic ataxia type-1 (Rhodes et al., 1997), and Kv1.1-Kv1.2 α subunit tetramers are seldom found in complex with inactivating Kvβ1 subunits in basket cells (Rhodes et al., 1997; Rhodes et al., 1996). Furthermore, in our current understanding of the molecular etiology of episodic ataxia type-1 (Herson et al., 2003), impairment of N-type inactivation does not appear to be involved, though it is certainly possible that other brain functions, in cells where N-type inactivation does modulate signalling, may be affected by V408A. An intriguing implication of our findings is that a decreased mean open time, required for peptide binding (Hoshi et al., 1990; Demo and Yellen, 1991), may in fact be the underlying cause for a decrease in N-type inactivation observed in these mutants. This is consistent with the previous finding that N-type inactivation was both slowed and diminished in amplitude by V408A, which previous authors have attributed, as we do, to a destabilization of opening (Maylie et al., 2002). Exactly how V408A/V478A influences the interplay between N-type inactivation with the onset of C-type inactivation (which is largely attenuated by V408A/V478A), in channels where they both occur, is not clear, and would represent an interesting future direction of research.
Diversity of channel behaviour amongst Kv1 subtypes

One of the more persistent themes highlighted in the previous section of this introduction is the diversity in gating properties amongst channels of the Shaker-related Kv1 family. While the Kv1.2 crystal structure is often used as a homology model (Long et al., 2005a), activation, deactivation and inactivation all show distinct behaviours between different Kv1-family members. Furthermore, experiments examining Kvβ subunit effects show that the structural diversity between the N-terminus of Shaker and that of Kvβ1.2 also translates into different functional effects: Kvβ1.2 appears to rapidly immobilize gating charge in the Kv1.2 channel and slows deactivation, whereas the effects of the Shaker N-terminus also include prominent effects on allosteric communication with the selectivity filter. When C-type inactivation is attenuated by mutation in Shaker FL, slow tail currents and fluorescence persist, indicating a possible steric inhibition of deactivation; however, the rates of recovery were much faster for blocked channels than they were for C-type inactivated Shaker IR channels (Figure 6.1F). This is strong evidence that at least two mechanisms of charge immobilization are possible in Kv1 channels. Steric interference of activation gate closure requires an inactivating N-terminus to be present but takes place regardless of the channel’s ability to C-type inactivate (Bezanilla et al., 1991). Conversely, the development of a stable, outward conformation of the S4 helix takes place without an inactivating N-terminus, but requires a stabilization of the open conformation, or relaxation, and is associated with the onset of C-type inactivation (Olcese et al., 1997).

The physiological relevance of diversity amongst Kv1 channels is exemplified by the contributions of both Kv1.1 and Kv1.2 to proper functioning of cerebellar basket
cells, which provide inhibitory input to the Purkinje cells and malfunction during episodic ataxia type-1 (Herson et al., 2003). In normal mice, Kv1.1 and Kv1.2 are expressed together, and colocalize, at synaptic boutons, along with the Kvβ2 subunit (Rhodes et al., 1997). When Kv1.1 is knocked out in mice, an epileptic phenotype ensues, though this was noted to be distinct from ataxia (Smart et al., 1998). However, if heterologous mice possessing a single, V408A mutant allele of Kcna1 are generated in the knockout background, the EA-1 phenotype is re-established (Herson et al., 2003), suggesting that the V408A mutant subunits have a dominant negative effect. Furthermore, as Kv1.2 expression is not being targeted in the knockouts, it is clear that Kv1.2 channels are unable to functionally substitute for Kv1.1. Conversely, homomeric Kv1.1 channels are known to traffic very poorly to the plasma membrane, due to an ER-retention signal in their external linkers and turret region (Vacher et al., 2007; Zhu et al., 2003a). Therefore, it is possible that the Kv1 heterotetramers found in vivo (Shamotienko et al., 1997; Parcej and Dolly, 1989) represent a marriage of necessity: Kv1.1 channels are reluctant to traffic to the membrane, whereas Kv1.2 channel gating is not ideally tuned to their function in neural tissue.

The separable physiological contributions of different members of the Kv1 family highlight an important limitation of the research from this thesis. In chapters 4 and 5, in order to gain insight into how N-terminal peptides or inner pore disease mutants affect normal channel gating, the Shaker channel was used as a model. However, results from the 2nd and 3rd chapters point to subtle but significant variability between Shaker gating and Kv1.2, differences which, in all likelihood, are also present in comparison with the Kv1.1 channel.
In Chapter 4, the N-terminus of the *Shaker* channel was shown to strongly accelerate an inner pore-selectivity filter allosteric pathway by a potassium-dependent pore-emptying effect. However, the ability of the channel to C-type inactivate was strongly abrogated by the T449V mutant, as has been shown previously (Lopez-Barneo et al., 1993), irrespective of the presence of the N-terminal blocker or of the identity of site I470. As the equivalent site to T449V in Kv1.2 is a valine, it seems likely that that channel would demonstrate less pronounced allosteric effects on C-type inactivation by an N-terminal peptide. Consistently, the Kvβ1.2 subunit did not appear to influence C-type inactivation in Kv1.2, as channel block by that subunit’s N-terminus was not followed by any appreciable slow current decay. However, that may also be due to distinct binding or allosteric properties of the Kvβ1.2 subunit (Morales et al., 1996), and our data do not allow us to predict how different N-termini might affect different channels’ inactivation or deactivation properties, nor indeed how mutations of the I470 site in Kv1 homologues also affect them. For instance, a recent paper identified an ataxia-related mutation I402T in Kv1.2, which demonstrated small but appreciable effects on gating in that channel (Xie et al., 2010). Therefore, an interesting future direction would be to compare the effects of the non-homologous inactivating N-termini from Kvβ1 subunits on S6 immobilization and C-type inactivation in *Shaker*, as well as in other Kv1 type channels, and to examine the importance of inner pore allosteric coupling to C-type inactivation across the Kv1 family. However, our data, together with the strict conservation of the positional equivalent of *Shaker* I470 across the Kv1 family, and the consistency of mutational effects at that site on selectivity filter collapse between Kv1 channels and the distantly related KcsA channel (Cuello et al., 2010; Cuello et al., 2010),
do allow us to suggest that in channels where C-type inactivation takes place, I470 or its equivalent is probably a critical site for communication between the inner pore and selectivity filter.

In Chapter 5, voltage clamp fluorimetry of the Shaker channel was used to examine how mutations in the inner pore lining of the permeation path can accelerate decay of the outward potassium currents. These mutations were found in patients suffering from episodic ataxia type-1, in the KCNA1 gene encoding Kv1.1 (Browne et al., 1994). However, attempts to record fluorescence from Kv1.1 were fruitless, possibly due to a S3-S4 linker which was incompatible with recording fluorescence (Sorensen et al., 2000). Fluorescence mutations also strongly depressed channel expression (data not shown), an effect not observed in our studies on Shaker or Kv1.2, or in Kv1.5 (Vaid et al., 2008), and a low number of channels at the surface may explain an inability to resolve any small fluorescence deflections from background noise. As the Shaker IR channel is a very well-characterized homologue, it was used as a surrogate for Kv1.1, however, it is feasible that different gating properties are present in these two channels, particularly as distinct functioning between Shaker and Kv1.2 was demonstrated in Chapter 2. However, upon introduction of Shaker IR mutation V478A (equivalent to Kv1.1 V408A), which is highly conserved site across Kv1 channels, and harmonization of the T449 equivalent residues to the tyrosine found at Kv1.1 site 379, a consistent effect on both current decay and voltage dependence of activation was observed between the two channels. Therefore, it appeared that the effect of EA-1 equivalent mutations would be unchanged across family members, suggesting that Shaker could be an adequate model for Kv1.1 in this instance.
As mentioned in the previous section, an often overlooked element in the understanding of Kv1 channel function is the consideration of how observed functional effects in vitro may translate to physiological dysfunction in vivo. Complicating this is the fact that many channels exist as heterotetramers in native tissues (Shamotienko et al., 1997; Parcej and Dolly, 1989), and any functional disease mutant subunit would become part of a channel complex also containing presumably normal subunits of a different Kv1 family member, thus generating a complex phenotype (Akhtar et al., 2002). In the case of Kv1.1, V408A mutants are known to confer an altered channel phenotype to Kv1.1-Kv1.2 tetramers, resulting in that case in accelerated current decay (D'Adamo et al., 1999). This presents an interesting avenue of research for understanding how structural alterations underlie pathophysiology, as the mutant phenotype must be having a dominant negative effect on gating through an unknown mechanism. As alluded to in the previous section, Kv1.1 and Kv1.2 appear to have different roles in these complexes, since an abundance of Kv1.2 in Kv1.1 knockout mice cannot rescue them from an ataxic or epileptic phenotype (Smart et al., 1998). Similarly, in channels where different C-type inactivation properties exist, the influence of allosteric coupling pathways from the inner S6 to the selectivity filter, and the role of permeating potassium in antagonizing filter collapse, are also important future questions towards translating knowledge of channel biophysical functioning into a clear understanding of in vivo channel physiology. Use of VCF techniques, like the one described in this thesis, represents a promising avenue of research towards identifying the contributions of individual subunits to overall gating within heteromeric channels or forced concatamers. As stoichiometrically defined
channel complexes generated using concatamerization resemble voltage-gated sodium channels, such a methodology may exploit an approach used in studies of sodium channel voltage sensing, where labelling of S4 helices of individual domains within the protein has allowed asymmetric subunit contributions to activation gating to be analyzed (Cha et al., 1999; Muroi et al., 2010).

SUMMARY

The experiments presented within this study highlight and address some of the major existing questions surrounding the study of voltage-gated potassium channels. The functional diversity within ion channel families, and how this diversity allows these channels to fulfill their unique roles within excitable tissues, remains an intriguing question. The technique of voltage clamp fluorimetry has proven instrumental in investigating a number of difficult questions of this nature: it provides a powerful tool for directly correlating structure with function. In this thesis, I have described the results from four studies performed using this method. A common theme amongst these studies has been that while they are ostensibly structurally and functionally similar, Shaker-type channels can be tuned to a wide range of physiological roles by subtle (such as in episodic ataxia type-1 mutations) or drastic (such as in co-assembly with a cytosolic β-subunit) modulations of channel gating elements. Future directions in this area of research will benefit greatly from the adaptability of the voltage clamp fluorimetry technique to probe and dynamically track conformational rearrrangements within an ever growing number of complex and interesting membrane proteins.
References


potassium channels Kv1.4-1.1/Kvβ1.1 and Kv1.4-1.1/Kvβ1.2. Eur J Neurosci 24:3073-3083.


Zagotta WN, Hoshi T, Aldrich RW (1990) Restoration of inactivation in mutants of Shaker potassium channels by a peptide derived from ShB. Science 250:568-571.


Appendix A: Supplementary Figures
Figure A.1. Schematic representation of the voltage clamp fluorimetry recording method. Simultaneous recording of current and fluorescence quenching from a voltage clamped channel allows electrical signals to be correlated to molecular movements. Membrane voltage is measured by the voltage electrode and communicated to the voltage clamp amplifier, which delivers the current necessary to clamp the membrane voltage at the desired level through the current electrode, which is also displayed to the user. Simultaneously, the oocyte membrane, within which are expressed the channels of interest tagged with a fluorescent dye, is excited by the fluorescent beam reflected through the objective by the dichroic mirror. Emitted fluorescence from the channels is transmitted through the dichroic mirror to a photomultiplier, whose signal is then digitized and read out simultaneously with the current.
Figure A.2. TMRM labelling of WT Kv1.2 channels does not alter activation kinetics, or give voltage-dependent fluorescence deflections. A-B. Representative current traces recorded from unlabeled control (A) and TMRM-labelled (B) Kv1.2 WT channels (Kv1.2 WT-TMRM) are shown for 100 ms pulses from -120 mV to +60 mV, from a holding potential of -80 mV. Although traces were recorded at 10 mV increments, only every third trace is shown for clarity. C. Conductance-voltage relationships were calculated from peak current at the end of each pulse, and are shown as mean ± SEM for Kv1.2 WT (filled circles) and Kv1.2 WT-TMRM (hollow circles) channels. Boltzmann fits to the data give a $V_{1/2}$ of 1.2 ± 1.6 mV and -1.7 ± 1.5 mV and slope factors of 18.0 ± 0.9 mV and 17.1 ± 0.8 mV for Kv1.2 WT and Kv1.2 WT-TMRM respectively ($n = 7-8$). D. Mean activation time constants and standard error were calculated from -50 mV to +100 mV, for Kv1.2 WT and Kv1.2 WT-TMRM channels ($n = 7-10$). E-F. Voltage-dependent fluorescence deflections were absent from cells expressing TMRM-labelled Kv1.2 WT channels at 100 ms (E), only surfacing as a minor deflection with long depolarizations (F), shown here with a 6.3 s depolarizing pulse. Dotted lines refer to the baseline level of fluorescence emission.
Figure A.3. Mapping of the S3-S4 linker scan residues onto the Kv1.2 closed state. Cartoon of the closed state Kv1.2 model generated by Pathak et al. (2007), with residues M288 (orange), S289 (cyan), L290 (magenta), A291 (red) and I292 (dark green) labelled and showing side chain residues. Blue residues correspond to I187 and T219, residues also shown to give voltage-dependent deflections upon TMRM labeling.
Figure A.4. I470 amino acid volume is not strictly linked with C-type inactivation, and recovery from inactivation is not strongly affected by I470 mutation. A. C-type inactivation rates in the presence of different I470 mutants are plotted against the predicted volumes of the substituted amino acid side chains (Darby and Creighton, 1993). B. C-type inactivation off-rates in the presence of different I470 mutants are calculated using Equation 4.1 in 3 and 99 mM external K⁺. With the exception of I470F, these rates were largely insensitive to mutation and to changes in external potassium concentration. A mean value of 0.064 s⁻¹ was calculated and used for in silico modeling (see Table 4.1).