REGULATION OF SLOW INACTIVATION IN Kv1.5 BY THE TURRET

by

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ABSTRACT

In Kv channels, slow, P/C-type, inactivation is thought to eliminate ionic current via a concerted structural rearrangement of the outer pore mouth. However, little is known regarding the contributions of other pore regions to the regulation of slow inactivation. Our experiments now indicate that the turret region of Kv1.5 can be an important determinant of slow inactivation. Application of extracellular protons, or divalent cations such as Zn\(^{2+}\) or Ni\(^{2+}\), was found to reduce the peak current amplitude, and with protons, also increase the rate of depolarization-induced inactivation. These effects were alleviated by the substitution of residue H463 in the turret with a glutamine, indicating that the interaction of this turret residue with extracellular ligands facilitated the inhibition of current. Based on macroscopic, unitary and gating current analyses, we have attributed the effects of protonation and divalent cations to an enhancement of depolarization-induced and closed-state inactivation. These findings provide strong evidence that the turret region contributes to the regulation of slow inactivation in Kv1.5. To determine which properties of position 463 were important in modulating inactivation, various side chain substitutions of this position were examined and several were found to mimic the effects of proton or divalent cation binding. However, the properties of the substituted side chains were not well correlated with the effects on channel function, and it appeared that the turret and inactivation gate did not interact strictly via an electrostatic interaction, as suggested by others. A SCAM analysis was subsequently conducted by substituting cysteine residues into each turret position to determine which regions of the turret were important for regulating slow inactivation. Modification of substituted cysteine residues in the distal turret by MTS reagents produced an inhibition that could also be related to an enhancement of slow inactivation and demonstrated that position
463 was contained within a distinct locus of turret residues that could influence slow inactivation. These results indicate that in Kv1.5 a specific region of the turret modulates the state of the inactivation gate and provide constraints on the mechanisms of interaction between the turret and inactivation gate.
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INTRODUCTION

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Recording solutions
Signal recording and data analysis
Expression of cysteine mutants and Proteinase-K experiments

RESULTS

The addition of a positive or negative charge or a decrease of volume of the side chain at position 463 increases the rate of slow inactivation and induces 

\[ [K^+]_0 \] dependence

Cysteine substitutions have modest effects on macroscopic properties of Kv1.5

MTSET and MTSES irreversibly inhibit currents of cysteine-substituted mutants in the distal turret

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<tr>
<th>Amino Acid</th>
<th>3 Letter Code</th>
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a.a. – amino acid  
AT₁ – Agitoxin₁  
C₉ – nonyltriethylammonium  
C₁₀ – decyltriethylammonium  
Cav – voltage-gated calcium channel  
CHO – chinese hamster ovary  
CNG – cyclic-nucleotide gated channel  
COVG – cut-open oocyte vaseline gap  
CTX – charybdotoxin  
DPA – dipicrylamine  
DTT – dithiothreitol  
e₀ – elementary charge  
FRET – fluorescence resonance energy transfer  
g – conductance  
gₖ – potassium conductance  
gₙₐ – sodium conductance  
HCN – hyperpolarization-activated cyclic nucleotide-gated  
HEK – human embryonic kidney  
hEAG – human ether-à-go-go channel  
HERG – human ether-à-go-go related gene channel  
HH – Hodgkin-Huxley  
I – current  
Iₔ – gating current
$I_{g_{off}}$ – off-gating current
$I_{g_{on}}$ – on-gating current
$K_d$ – equilibrium dissociation constant
$K_{ir}$ – inward rectifier potassium channel
$K_v$ – voltage-gated potassium channel
LRET – lanthanide resonance energy transfer
ms – milliseconds
MTS – methanethiosulfonate
MTSES – sodium (2-sulfonatoethyl) methanethiosulfonate
MTSET – [2-(trimethylammonium) ethyl] methanethiosulfonate bromide
mV – millivolts
nA – nanoamperes
Nav – voltage-gated sodium
$Q$ – charge
$Q_{max}$ – maximum gating charge
$Q_{off}$ – off-gating charge
$Q_{on}$ – on-gating charge
QA – quaternary ammonium
rELK – rat ether-a-go-go-like potassium channel
s – seconds
SA – sinoatrial
SCAM – substituted cysteine accessibility method
SGA – squid giant axon
ShA – Shaker-A
ShB – Shaker-B
TEA$^+$ – tetraethylammonium
TM – transmembrane
TMRM – tetramethylrhodamine maleimide
TRPV – vanillloid-related transient receptor potential
TTX – tetrodotoxin
$V_{1/2}$ – half-activation voltage
VCF – voltage-clamp fluorimetry
$V_m$ – membrane voltage
wt – wild type
ACKNOWLEDGEMENTS

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CO-AUTHORSHIP STATEMENT


Cyrus Eduljee was responsible for i) creating the mutants studied (except the R487V mutation); ii) performing the experiments and the analyses of the zinc- and pH-sensitivities of the H463Q mutant; iii) performing the experiments and analysis of the zinc-sensitivity of the H463Q/R487V mutation; iv) examining the [K$^+$]$_o$-sensitivity of the H463G mutant; v) creating and maintaining the mutant HEK stable cell lines; and vi) constructing Figure 2.5 (30% contribution). Daniel Kwan performed the experiments on the R487V mutant. Shetuan Zhang examined the effects of pH on gating currents. Steven Kehl conducted the remainder of experiments and wrote the manuscript.


Cyrus Eduljee was responsible for i) creating the H463Q mutant; ii) performing experiments and data analysis regarding the effect of [K$^+$]$_o$ on the nickel-sensitivity of the H463Q mutant (15%). Shetuan Zhang conducted the gating current experiments. Daniel Kwan examined the nickel-sensitivity of the R487V mutant and performed the single channel experiments. Logan Lee assisted with some of the experiments and the remainder of data was collected and analyzed by Steven Kehl. S. Kehl was the author of the manuscript.


Cyrus Eduljee was responsible for i) the planning of the experiments and project management; ii) creating the majority (7/13) of the cysteine mutants; iii) creating all of the H463 mutants; iv) assessing the activation properties of many of the cysteine mutants; v) characterizing the MTS-inhibition; vi) creating the cysteine/R487V double mutants; vii) examining the effects of [K$^+$]$_o$ and the R487V mutation on the MTS-inhibition; viii) writing the manuscript (85% contribution). Thomas Claydon constructed some of the mutants and analyzed the inactivation, deactivation and recovery kinetics. T. Claydon also examined the activation properties of some of the cysteine mutants and assisted in editing the manuscript. Vijay Viswanathan conducted the proteinase-K assay. Steven Kehl assisted in editing the manuscript.
CHAPTER 1: INTRODUCTION
I: Overview

Voltage-gated potassium (Kv) channels are membrane spanning proteins present in many electrically excitable tissues, including muscles and nerves. As a result of their widespread distribution and diversity, Kv channels are involved in many aspects of cell physiology. The major function of Kv channels is to selectively conduct potassium (K\textsuperscript{+}) ions across the membrane bilayer in response to changes in the membrane voltage. Due to the high intracellular concentration of potassium in the majority of cells, relative to the extracellular concentration, the resultant outward K\textsuperscript{+} flux hyperpolarizes the cell membrane. To properly function, voltage-gated potassium channels must be able to sense the voltage across the cell membrane, open and close in response to changes in the voltage (a process referred to as gating), and selectively conduct K\textsuperscript{+} ions at a high rate while excluding other ions.

The recent crystal structures of the bacterial KcsA and the human Kv1.2 channels have illuminated our understanding of how potassium selectivity is achieved, but many other aspects of channel function remain controversial. In particular, although we know that potassium channels move through various states during the gating process, the exact details of activation and deactivation (the opening and closing in response to changes in voltage) are still unknown. In addition, several processes lead to a loss of current and are collectively known as inactivation. Inactivation appears macroscopically to be regulated by changes in membrane voltage, due to the coupling of inactivation to activation, but is not thought to be intrinsically voltage-sensitive per se. While many of the mechanistic details of N-type inactivation are known, the mechanism of slow, or P/C-type, inactivation is poorly understood. This introduction will describe the historical development of our understanding
of potassium channel structure and function and will also examine many of the less well-understood areas of channel biophysics, with a particular emphasis on the inactivation process, which is the focus of this Dissertation.

II: Classical ion channel description

The properties of a $\text{K}^+$ current were first described in a landmark series of papers by Hodgkin and Huxley in 1952 (Hodgkin & Huxley, 1952a; Hodgkin & Huxley, 1952b; Hodgkin & Huxley, 1952c; Hodgkin & Huxley, 1952d). In these studies, the authors used a preparation of the squid giant axon (SGA) to perform voltage-clamp experiments (Hodgkin et al., 1952), whereby they held the voltage across the axonal membrane constant using a feedback amplifier system, and examined the resultant currents across the membrane. The voltage clamp technique still forms the basis for much ion channel research. Using this technique the authors deduced that the current elicited across the SGA membrane was likely a combination of separate sodium ($I_{\text{Na}}$) and potassium ($I_{\text{K}}$) currents. The sodium current was activated by depolarization and decayed quickly, while the potassium current activated more slowly (hence, it was named the delayed rectifier) and did not appear to inactivate. At the time, the authors did not know the physical mediators of the observed currents, but instead described only the changes in macroscopic conductance for sodium and potassium. By analyzing the sigmoidal time course of the activating currents, Hodgkin and Huxley determined that, whatever the physical carriers of the current were, they likely progressed through several closed states, in response to voltage changes, before opening. Based on the empirical equations that were derived from their observations, they envisioned three activating gates or ‘particles” controlling the sodium conductance ($g_{\text{Na}}$), each of which had to
enter a permissive state before the channel would open, and an inactivation particle, or gate, to account for the inactivation of the current. In contrast, the potassium conductance ($g_K$) was hypothesized to contain only 4 activation particles.

In addition, the authors constructed an empirical description, known as the Hodgkin-Huxley (HH) model, for each of the conductances. This is shown in Figure 1.1 for $g_K$. In this model, what we now call a potassium channel begins at hyperpolarized potentials in a closed state furthest from the open state. Upon depolarization, the channel responds to the changes in the membrane voltage by moving through a series of closed states until it reaches the open state, through which the potassium current is conducted. This is called activation, while the reverse process, in response to a hyperpolarization (the term repolarization is also used in the literature), is called deactivation. These early quantitative studies provided the first basis for the ionic fluxes that regulate the membrane voltage and a model with which to predict their behaviour. With the eventual identification of the genes that encode for ion channels, the proteins that form these channels could be expressed at high levels to better study their currents and a structural understanding of their behaviour was developed.

III: Kv channel classification

The first potassium channel to be cloned was the ~70kDa Shaker channel isolated from the fruit fly Drosophila melanogaster (Tempel et al., 1987; Papazian et al., 1987). Three Shaker-related genes known as Shal, Shab and Shaw were also discovered in Drosophila and were found to exhibit different kinetic and voltage-sensing properties (Wei et al., 1990). Subsequent cloning of Kv channels demonstrated that they are present in a vast number of organisms. In mammals, potassium channels homologous to the Shaker, Shab,
Shaw, and Shal channels of the fruit fly have been identified and have been separated into the Kv1, Kv2, Kv3 and Kv4 subfamilies, respectively, but are collectively classified as delayed rectifier-type (Kv1, 2 and 3) or A-type (Kv4) channels (Hille, 2001). Within each subfamily, multiple related channels have been reported, e.g., Kv1.5, 2.1, 4.2, etc. Together, these channels are characterized by their voltage-dependence, K⁺-selectivity and transmembrane topology. Other K-channels, such as the human ether a-go-go related gene (HERG) channel, the inward rectifiers (Kir), the tandem-pore leak channels, and calcium-activated K⁺ channels have also been identified, although they differ from the delayed rectifiers in terms of their transmembrane topology, voltage-sensitivity, or biophysical properties.

**Figure 1.1. Early potassium channel description.** (A) The Hodgkin-Huxley (HH) state diagram based on $I_K$ from the SGA (Hodgkin & Huxley, 1952a). At very hyperpolarized potentials, the channel resides in $C_0$, with the pore in a non-conducting conformation. During depolarization, the channel progresses through three additional closed states before entering the open state, through which K⁺ ions permeate. This depolarization-induced movement into the open state is called activation, while the opposite, the movement from the open state back into the closed states in response to a hyperpolarization, is called deactivation. (B) Structural representation of the HH model based on the presence of four subunits, each of which must be in a permissive state (red) to open the conduction pathway.
IV: General features of ion channel structure

Kv channels are composed of four $\alpha$-subunits that can assemble as homo- or heterotetramers in order to form the ion-conducting unit (Isacoff et al., 1990). Each $\alpha$-subunit contains six transmembrane (TM) domains (S1-S6) (Figure 1.2). The first four TM domains, especially the S4 helix that contains multiple charged arginine and lysine residues, are thought to comprise the voltage sensing domain of the channel (Aggarwal & MacKinnon, 1996). The S5-S6 region is often referred to as the pore domain, contains the permeation pathway, and is responsible for conferring potassium selectivity (Heginbotham et al., 1994). This latter region is also postulated to contain the activation gate (Armstrong, 1971), the slow inactivation gate (Hoshi et al., 1991; Liu et al., 1996; Cordero-Morales et al., 2006a), and the binding site for the blocking ball which mediates N-type inactivation in some channels (Zhou et al., 2001a). The N- and C-termini, which are both located intracellularly, are the targets for intracellular signalling pathways, and the large T1 domain, which comprises part of the N-terminus, has been shown to facilitate tetramerization during channel biogenesis (Deutsch, 2003). The N-terminal domain also confers N-type inactivation in some channels (Zagotta et al., 1990; Hoshi et al., 1990).

The recent crystal structures of the KcsA and Kv1.2 channels have contributed greatly to our overall understanding of Kv channel topology and have demonstrated that each $\alpha$-subunit of the channel contributes its pore domain, in a four-fold symmetrical arrangement, to form the ion conducting pathway (Doyle et al., 1998; Zhou et al., 2001b; Long et al., 2005a). Interestingly, the crystal structure of Kv1.2 has indicated that the voltage-sensing domains are not located in close proximity to the pore-forming domains from each $\alpha$-subunit. Instead, each voltage-sensing domain is located adjacent to the pore-forming domain of a
Figure 1.2. Overview of Kv channel structure. (A). Schematic of one Kv channel α-subunit. Transmembrane domains are shown as S1-S6, together with the re-entrant pore loop, P, the tetramerization domain, T1, and the N- and C-termini. (B) The X-ray crystal structure of the Kv1.2 channel illustrates the relative positions of the four colour-coded α-subunits. The transmembrane domains (TM; containing the voltage sensor and the conducting pore), the T1 domain and four bound β-subunits are seen from the plane of the membrane (Long et al., 2005a). (C) Kv1.2 structure seen from extracellular point of view (Long et al., 2005a).
neighbouring α-subunit (Long et al., 2005a). In the electromechanical coupling model proposed by MacKinnon and co-workers, the intracellular S4-S5 helices link the movements of the voltage sensing domain with the opening and closing of the pore domain (Long et al., 2005b).

V: Activation and permeation

The pore region and selectivity filter

Despite a divergence in sequence between many potassium channel families and subtypes, the pore domain, and especially the P (pore)-loop, is the defining region of the potassium channel and is highly conserved. In the Shaker Kv channel, the role of the S5-S6 region in forming the pore region was first elucidated by examining the effect of a mutation at position 422, in the region now known as the turret, on the affinity of charybdotoxin (CTX) binding (MacKinnon & Miller, 1989; MacKinnon et al., 1990). Similar studies in both Kv and voltage-gated sodium (Nav) channels concluded that the S5-S6 region contained residues that influenced the block by reagents including tetrodotoxin (TTX) and the classical open channel pore-blocker, tetraethylammonium (TEA⁺; see below), and therefore contained elements that constituted the pore (Noda et al., 1989; MacKinnon & Yellen, 1990; Yellen et al., 1991). An analysis of the pore regions from a range of potassium channels identified a conserved TXXTXGYG region in the P-loop that appeared to be essential for proper ion conduction (Heginbotham et al., 1994). The structure of the P-loop, the linker that connects S5 and S6, was identified in Nav channels as a re-entrant loop by molecular modeling studies (Guy & Seetharamulu, 1986). In Shaker, certain mutations at the first (T), fifth (V), sixth (G) and eighth (G) positions in this sequence led to channels that failed to selectively conduct K⁺
in comparison to other monovalent cations including Na\(^+\), Li\(^+\), Rb\(^+\), Cs\(^+\) and NH\(_4\)\(^+\) (Heginbotham et al., 1994). In addition, several mutations at positions 1, 3, 5, 6 and 7 either led to a loss of K\(^+\)-selectivity or ionic current. That study concluded by hypothesizing that this region of the channel may indeed be responsible for conferring ion selectivity and implied that the backbone carbonyls from these residues coordinated K\(^+\) ions.

In a landmark achievement in 1998, MacKinnon and co-workers crystallized a prokaryotic potassium channel (Doyle et al., 1998), providing the first direct view of the potassium channel pore (Figure 1.3). The \(\alpha\)-subunit of the KcsA channel, isolated from the bacterium Streptomyces lividans, contains only 2 transmembrane domains. As such, it does not possess a voltage-sensing domain, but does contain a pore domain homologous to the S5-S6 region of Kv channels, including the conserved TXXTXGYG signature sequence in the P-loop. Analysis of the KcsA structure indicated that the four signature sequences from each \(\alpha\)-subunit came together to form the selectivity filter of the channel, as shown by the locations of bound Cs\(^+\) or Rb\(^+\) ions (used in lieu of K\(^+\) due to their higher electron density) within the pore of the channel (Figure 1.3). The structure confirmed that the backbone carbonyls from positions 4-7 in the signature sequence, Thr75-Tyr78 in KcsA, were orientated towards the conduction pathway and created a series of oxygen rings throughout the 12 Å long filter in order to selectively coordinate permeant ions. This elegant arrangement of the backbone carbonyls underlies the high throughput of K\(^+\) ion conduction by effectively replacing the water oxygen atoms that normally interact with the ions in solution. As a result, the K\(^+\) ions are completely dehydrated during permeation with little energetic cost. This data corroborated theories of K\(^+\)-dehydration that had been proposed almost 30 years earlier to explain K\(^+\)-selectivity (Armstrong, 1971). Furthermore, the side chains of the filter residue
Y87 (TXXTXGFG) from each subunit interact with surrounding tryptophan residues from the pore-helix, located behind the filter, which are thought to support the filter’s structure by acting as an “aromatic cuff”.

Figure 1.3. The selectivity filter. (A) In KcsA, four $K^+$ ions are observed in the selectivity filter, and one in the inner vestibule (green spheres) (Morais-Cabral et al., 2001). (B) Alternate $K^+$ ion occupation of the filter in the 1,3 (left) and 2,4 (right) conformations. Green spheres represent $K^+$ ions while red spheres represent water. Note the presence of a $K^+$ ion near the extracellular terminus of the pore ($S_{\text{ext}}$) in the 1,3 configuration and a partially dehydrated ion ($S_0$) in the 2,4 configuration (Zhou et al., 2001b). (C) In KcsA, residue W68 hydrogen bonds with residue Y78 to form an “aromatic cuff” that supports the selectivity filter (Doyle et al., 1998).
Interestingly, the crystal structure showed the presence of a large water-filled vestibule within the permeation pathway, bounded on top by the selectivity filter and below by the S6 helix bundle crossing, an area where the intracellular S6 helices come into close proximity and appear to block the conduction pathway. Therefore, the authors proposed that the S6 helices moved apart during channel activation, thus allowing the vestibule to come in contact with the intracellular medium. This finding was important because it implied that in the open configuration, the intra- and extracellular bulk solutions were only separated by the short distance across the selectivity filter (~12 Å). Therefore, during permeation, the majority of the transmembrane voltage field would fall across the length of the filter, and not the entire membrane bilayer. As a result, ions need only cross a small distance, and the complex structural arrangements required to conduct K$^+$ ions are limited to a small region (Doyle et al., 1998).

The re-crystallization of the KcsA filter at a 2.0 Å resolution, using antibodies to stabilize the crystal structure in the presence of K$^+$ ions instead of Cs$^+$ or Rb$^+$ (Morais-Cabral et al., 2001; Zhou et al., 2001b), further clarified aspects of ion coordination that remained unclear from the initial data. Specifically, the new structure clearly demonstrated the presence of four K$^+$ binding sites within the filter (sites 1-4, or S$_1$ to S$_4$, extracellular to intracellular), as well as a partially coordinated ion at the extracellular entryway of the filter (S$_0$), a fully hydrated ion located extracellular to the pore (S$_{ext}$), and a fully hydrated ion within the inner cavity as seen in the original structure. Since the structure represented an averaged view of ions permeating the pore, the authors proposed that the structure actually depicted two distinct conformations, with the filter occupied by 2 ions at any given time either in the 1,3 or 2,4 states, coordinated by the backbone carbonyls from positions Thr75-
Gly78, as well as the side chain from Thr75. The outer ion at $S_0$ was partially coordinated by the backbone carbonyls of Gly79, which were oriented towards the extracellular medium (Figure 1.3).

The presence of multiple ions permeating successively through the channel pore validated earlier observations based on flux assays (Hodgkin & Keynes, 1955; Begenisich & De Weer, 1980; Stampe & Begenisich, 1996) and the anomalous mole fraction effect (Eisenman et al., 1986; Wagoner & Oxford, 1987), which suggested that ion channels utilized multi-ion pores. The presence of such a pathway is not a trivial detail, since it imparts both high flux rates and ion selectivity as a result of the electrostatic interactions between the ions as they conduct through the pore (Almers & McCleskey, 1984; Hess & Tsien, 1984; Neyton & Miller, 1988; Friel & Tsien, 1989; Berneche & Roux, 2001).

Subsequently, MacKinnon and co-workers have produced additional X-ray structures from the MthK Ca$^{2+}$-activated K$^+$-channel (Jiang et al., 2002a), the KvAP voltage-gated bacterial channel (Jiang et al., 2003a; Lee et al., 2005) and the human Kv1.2 channel (Long et al., 2005a). In all of these, the pore domain has showed remarkable consistency, highlighting the conserved nature of the Kv channel pore.

**The activation gate**

Early studies of $I_K$ in the SGA demonstrated that application of intracellular quaternary ammonium (QA) derivatives, such as TEA$^+$, could block the current, but only once the current had activated (i.e., the channels had to be opened first to become blocked) (Armstrong, 1971). In addition, QA’s such as C9 appeared to slow deactivation during repolarization and led to a pronounced rising phase or “hook” in these deactivating currents.
(also called “tail” currents) (Armstrong, 1966; Armstrong, 1969; Armstrong, 1971). These effects were mitigated either by increasing the extracellular potassium concentration ([K⁺]₀) or by hyperpolarizing the membrane to more negative potentials. These findings led to the hypothesis that QA’s might bind to K⁺ channels within the permeation pathway in order to block current (note that at this time the molecular architecture of the channel was largely speculative and this finding was important for the carrier versus pore model debate). The results also suggested that the inward potassium current through the pore must physically drive the bound QA ion from the pore, further implicating a binding site within the pore. Finally, the rising phase of current during deactivation indicated that the intracellularly applied QA ion likely hindered deactivation by binding near the activation gate. Since the QA ion was membrane-impermeant, these findings implied that the activation gate was located intracellularly (Armstrong, 1971). Similar experiments demonstrated that intracellular TEA⁺ could also bind within the pore region of the Shaker Kv channel upon opening (Choi et al., 1991), implying that the activation gate of this channel also resided intracellularly.

The search for the location of the activation gate was greatly enhanced by the introduction of substituted-cysteine accessibility method (SCAM) (Akabas et al., 1992; Kuner et al., 1996; Karlin & Akabas, 1998). This technique was originally developed to determine the accessibility of residues in a region of interest. Specifically, cysteine residues were individually substituted into a particular region, and the intra- or extracellular accessibility was assessed by monitoring the modification of the cysteine residues by methanethiosulfonate (MTS) reagents. The MTS reagents may be charged, such as the positively charged MTSET or negatively charged MTSES reagents, or can be neutral. By
using this assay, it can be determined if the residue is accessible from either the extra- or intracellular media, or if the accessibility is state-dependent. However, since this is a functional assay, it is dependent on the alteration of some aspect of channel function to signify successful modification (Holmgren et al., 1996b).

Yellen and co-workers (Liu et al., 1997) used the SCAM technique on the S6 region of Shaker-Δ (see section on N-type inactivation for definitions of the -Δ, -Δ6-46, -IR suffixes) to determine if the rate of cysteine modification by MTSET was different when the channels were treated while held closed, as compared to when held open. These studies demonstrated that the reactivity of several residues at the cytosolic end of S6 between positions 470 and 477 was up to 50,000-fold slower when channels were in the closed state, relative to the reactivity of the open state. In contrast, the reactivity of residues located more intracellularly, between positions 482 and 486, showed little state-dependence. Additionally, the V474C mutant strongly coordinated a Cd\(^{2+}\) ion when channels were opened. This implied that the four cysteines in the V474C mutant were located at a narrowing of the permeation pathway, which might constitute the activation gate. Modeling studies of Kv channel structure (Durell & Guy, 1992) had previously shown that V474 was part of a PVP motif that likely broke or “kinked” the α-helical structure of S6, and blocker protection studies of Shaker-Δ (del Camino et al., 2000) also demonstrated that the PVP motif separated residues that were inaccessible in the closed state, from those whose accessibility was state-independent. In 2005, the crystal structure of the Kv1.2 channel (Long et al., 2005a; Long et al., 2005b) demonstrated that this motif (more generally referred to as the PXP motif since the valine is not conserved in all Kv channels) did indeed produce a “kink” in the S6 helix and that residues near the equivalent of Shaker-V474 marked the narrowest part of the pore.
This also implied that the region of S6 near this area likely represented the activation gate. It was further hypothesized by some groups that intersubunit interactions between residues in this narrow region might interact with each other to stabilize the closed state by forming a "hydrophobic seal" (Kitaguchi et al., 2004).

Interestingly, the crystal structure did not support the concept that the lower segment of S6 (i.e., the region below the PXP motif) moved in a hinge motion to occlude the pore during gating, as had been hypothesized based on the crystal structures of the KcsA (Doyle et al., 1998), MthK (Jiang et al., 2002a; Jiang et al., 2002b) and KvAP (Jiang et al., 2003a) channels, which do not contain the PXP sequence. Instead, the region of S6 below the PXP motif appeared to be directed away from the pore region and was hypothesized to interact with the S4-S5 linker. In light of this, MacKinnon and co-workers suggested that during activation, a torsion is applied to this narrow region of the pore by the voltage sensor via the S4-S5 linker, causing the S5 and S6 helices to be pulled apart and thereby allowing ion conduction.

The structural basis of the voltage sensor

In the original experiments with the SGA, it was clear that changes in the membrane voltage regulated the activation and deactivation of the potassium conductance (Hodgkin & Huxley, 1952a). It was unknown at that time what might lend voltage sensitivity to the molecules underlying the currents observed, but it was hypothesized that some type of gating "particles" must be under the influence of the membrane electric field. Once the first Nav (Noda et al., 1984) and Kv (Tempel et al., 1987) channels were cloned and analyzed, it became clear that the S4 helices of these channels contained several positively charged
arginine and lysine residues, positioned at intervals of three or four amino acids as if they were on one face of the \( \alpha \)-helix. Since the other TM domains contained few charged residues, S4 was hypothesized to mediate the voltage sensitivity. In *Shaker*, the S4 segment contains 7 lysine and arginine residues (Tempel *et al.*, 1987), while in the *Electrophorus electricus* sodium channel, the S4 domains of the four linked subunits contain between 5 and 7 positively charged residues (Noda *et al.*, 1989). A simple interpretation of the molecular data was that each positive charge in the S4 helix contributed equally to the total gating charge moved \((Q_{\text{max}})\) during activation and deactivation. However, studies assessing the charge contribution of each lysine and arginine residue proved that this simple interpretation could not account for the observed gating behaviour. For instance, upon neutralizing each charged S4 residue in *Shaker*, Papazian and co-workers (Papazian *et al.*, 1991) discovered that the five expressing mutations did not equally affect gating. Instead, the R362Q mutation near the extracellular terminus of S4 shifted the \( V_{1/2} \) in the depolarizing direction by \(-20\) mV while the R368Q mutation produced a \(-50\) mV shift. Similar examinations of the S4 charges in sodium and potassium channels were equally ambiguous (Stühmer *et al.*, 1989; Liman *et al.*, 1991; Lopez *et al.*, 1991; Logothetis *et al.*, 1992; Tytgat & Hess, 1992; Schoppa *et al.*, 1992; Logothetis *et al.*, 1993). However, later studies suggested that these discrepancies may have been due to changes in the co-operativity of channel opening as a result of these mutations (Smith-Maxwell *et al.*, 1998).

To determine the actual number of charges moved during activation gating, several techniques were employed, including the limiting slope analysis (Almers, 1978; Liman *et al.*, 1991; Zagotta *et al.*, 1994b; Noceti *et al.*, 1996), variance analysis (Schoppa *et al.*, 1992; Seoh *et al.*, 1996; Noceti *et al.*, 1996), and the use of radiolabeled toxins, such as agitoxin, 

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(AT₁) (Aggarwal & MacKinnon, 1996). In general, these studies have suggested that the equivalent of 12-14 elementary charges (e₀) move per channel during activation and deactivation. This is consistent with the finding that in Shaker, the four outermost charges from each S4 helix make the most significant contribution to charge movement (Aggarwal & MacKinnon, 1996).

**Gating charge analysis**

Since gating charges move through the membrane electric field upon activation, a corresponding transient gating current (Iₙ) (Armstrong & Bezanilla, 1973; Schneider & Chandler, 1973) should be detectable. The first studies of gating currents from sodium channels in the SGA (Armstrong & Bezanilla, 1973; Keynes & Rojas, 1973; Keynes & Rojas, 1974) used a combination of signal averaging and the removal of permeant ions to eliminate ionic conductance and resolve the remaining gating currents. Subsequent studies have relied upon several factors to enhance the resolution of gating currents. These include: very high expression levels that can be achieved by heterologous expression of cloned channels; an improved *Xenopus* oocyte voltage clamp known as the cut-open oocyte vaseline gap (COVG) (Bezanilla *et al*., 1991; Perozo *et al*., 1992; Stefani *et al*., 1994); and the elimination of ionic current, either by the substitution of non-permeant ions (Armstrong & Bezanilla, 1973), block by toxins (Armstrong & Bezanilla, 1974; Bezanilla & Armstrong, 1974) or by the incorporation of mutations, such as Shaker-IR-W434F, that eliminate ionic conductance (Perozo *et al*., 1993).

In the Shaker-IR-W434F channel, on-gating currents (Iₙon) (i.e., gating currents in response to depolarizations) typically result in transient outward currents that rise rapidly
before decaying (Stefani et al., 1994). Integration of the on-gating current ($I_{g_{on}}$) yielded the gating charge moved during activation ($Q_{on}$) and plotting this relative to the test voltage ($V$) revealed the voltage-dependence of charge movement. Analysis of the $Q(V)$ relationship indicated that this curve lay to the left of the $g(V)$ curve (Stefani et al., 1994), implying that the majority of gating charge is moved prior to channel opening. The high bandwidth of the cut-open oocyte allowed the detection of a prominent rising phase in the currents elicited by potentials $>-30$ mV and revealed the presence of two components whose relative amplitudes varied with voltage (Bezanilla et al., 1994). In addition, the $Q(V)$ curves of both Shaker-IR-W434F and Kv1.5-W472F (W472F in Kv1.5 is the equivalent of W434F in Shaker-IR) were best fit by a double Boltzmann function (Bezanilla et al., 1994; Hesketh & Fedida, 1999).

Together, these studies provided evidence that the classical HH model, in which the transitions between closed states were identical and carried an equal amount of charge, could not account for the kinetic features of the observed gating currents. Instead, the results suggested that the transitions during activation may not all be identical, and that additional transitions needed to be incorporated to adequately describe channel activity.

Measurement of off-gating charge ($Q_{off}$) in Shaker-IR-W434F during deactivation showed that the charge returned is equal to and opposite the on-gating charge. The decay of off-gating currents ($I_{g_{off}}$) has been the source of considerable interest. Stefani and co-workers (Stefani et al., 1994) initially observed that following depolarizations $<-30$ mV, $I_{g_{off}}$ decayed with simple mono-exponential kinetics. However, with greater depolarizations, the decay displayed a pronounced rising phase, followed by a slow return of charge. This "charge immobilization" was first characterized in sodium currents (Armstrong & Bezanilla, 1977), and, like the slow on-gating currents, was another feature that could not be reconciled with...
earlier HH-type models. Charge immobilization was initially attributed to a slow closing step during deactivation (Bezanilla et al., 1994; Zagotta et al., 1994a), although some later argued for, and against, the additional influence of N-type inactivation (see below) (Bezanilla et al., 1991; Stühmer et al., 1991). Recently, charge immobilization has been attributed to a stabilization of S4 during the later phase of slow inactivation (see below) (Fedida, 1997; Olcese et al., 1997).

Since gating currents are one of the few sources of data regarding closed state transitions, they have been essential in constructing Kv channel gating models (Zagotta et al., 1994a; Schoppa & Sigworth, 1998). Gating charge analyses have also been useful in understanding the mechanistic bases of various mutations and pharmacological agents by evaluating their effects on closed state transitions.

**S4 gating movements**

Although it is clear that a number of charges must move during gating in response to changes in the membrane electric field, the type of movements made, and the manner in which this movement is coupled to the opening of the activation gate is still a matter of considerable debate. Early predictions of gating envisioned a motion in which complementary pairs of negative and positively charged residues in the membrane moved relative to each other such that a net outward movement of positive charge was achieved (Armstrong, 1981). This model was attractive because the pairing of positive and negative charges could explain how charged residues could exist in the otherwise energetically unfavourable environment of the lipid bilayer. However, while the S4 helix in Shaker does contain multiple positive charges (Tempel et al., 1987), a complementary set of negative
charges does not exist in the remaining TM domains, and of the few negative charges present, only one was shown to affect gating (Seoh et al., 1996). Clearly, another model of charge movement was required.

The use of the SCAM technique described earlier provided significant insight into the S4 movements in both the sodium and potassium channels. In a study of Shaker-IR, S4 residues were mutated to cysteines and assayed for state-dependence of MTSET-modification (Larsson et al., 1996). These studies suggested that the charges in S4 moved outward during gating, and more importantly, that S4 residues previously thought to be buried within the membrane were actually accessible to the intracellular solution, suggesting that it penetrated deep into the membrane. This picture was reminiscent of the open pore structure predicted for KcsA, in which the intracellular solution was in contact with much of the intracellular region of the permeation pathway. As a result, the extra- and intracellular solutions were separated by the length of the selectivity filter (~12 Å), and not the entire membrane (Doyle et al., 1998). In a similar scenario, the S4 region may be surrounded by one or more aqueous crevices or “cannaliculi” (Goldstein, 1996), which allows the extra-and intracellular solutions to penetrate into the membrane and come into close proximity (Figure 1.4). The presence of these cannaliculi elegantly solves the conundrum caused by the lack of negative counter-charges present in the membrane, because at any time, only a limited number of charges are buried within a small region of the hydrophobic protein, across which the entire membrane voltage drops. Additionally, the charges need only cross through this small barrier-type region, and not across the span of the entire membrane in order to move across the electric field, thereby allowing small movements to move large amounts of charge.
Using MTS reagents of variable lengths, the distance across this barrier was assessed in the *Shaker* channel to be less than 4 Å (Ahern & Horn, 2005).

**Figure 1.4. Hypothetical models of S4 movement.** (A) Two proposed motions to account for the MTS-accessibility of S4 residues: S4 may move exclusively, while the surrounding protein remains stationary (upper panel), or the opposite may occur (lower panel). Both utilize the presence of “cannaliculi”, or water-filled crevices, such that only a small portion of S4 is buried in protein at any given time (Yellen, 1998). (B) The motion depicted in the lower panel of A may result from the tilting and/or rotation of helices in the voltage-sensing domain. As a result, charged residues may move across the membrane voltage field with little vertical displacement of the S4 helix (Yellen, 1998).
Although these experiments demonstrated how a large number of charges could be efficiently moved across the entire membrane electric field, it was not completely obvious whether the S4 helix moved outward during gating, or whether rearrangements of the surrounding protein altered the locations of the cannaliculi, such that the barrier (and the voltage field which dropped across it) moved intracellularly (Figure 1.4). In either case, the net effect would be an outward movement of charge. In all likelihood, the true movements lie within these two extremes, and several experiments have attempted to show that while S4 does move during gating, the degree of translocation is likely limited and may involve a subtle twisting.

Much of the interest in clearly defining the gating movements of S4 was triggered by studies by MacKinnon and co-workers. Based on functional (Jiang et al., 2003b; Ruta et al., 2005) and structural data (Jiang et al., 2003a; Lee et al., 2005) from the KvAP channel, it was hypothesized that during gating S4 translocated in a “paddle” motion over a large (15 – 20 Å) distance. Consequently, many groups have focused on determining if this large translocation was unique to KvAP and whether the voltage-sensors of eukaryotic Kv channels might move over a smaller distance during gating. To answer these questions, many studies have conducted experiments using fluorophores or MTS reagents as probes of Kv channel movement.

In fluorescent imaging, a fluorophore probe (typically tetramethylrhodamine-maleimide, TMRM) is attached to a region of interest via a substituted-cysteine residue. The fluorescence from the probe can then be monitored to determine if its environment changes during gating (Mannuzzu et al., 1996). Conformational changes of the channel (including electrically silent transitions) can also be measured in parallel with ionic current recordings,
in a technique known as voltage-clamp fluorimetry (VCF). Refinements of this technique, known as fluorescence resonance energy transfer (FRET) (Glauner et al., 1999) and lanthanide resonance energy transfer (LRET) (Cha et al., 1999) which use both a donor and an acceptor fluorophore, have also been developed in order to provide a quantitative description of fluorophore movement.

By introducing cysteines into S2, the S3-S4 linker, S4, and the pore and then using LRET, Bezanilla and co-workers suggested that voltage-sensing led to S4 movements of only ~3.2 Å (Cha et al., 1999). This group then modified this technique further by placing the lanthanide donors in specific channel regions near the voltage sensor and using a pore-binding scorpion toxin containing the acceptor dye. Their results further refined estimates of S4 movement to only 2 Å in the vertical plane (Posson et al., 2005). An additional FRET study using the reagent dipicrylamine (DPA), reached similar conclusions (Chanda et al., 2005), in clear contradiction to the large “paddle” movements suggested by MacKinnon and co-workers.

The second major technique used to investigate S4 movement exploited the SCAM technique and was used to examine the state-dependent changes in the location of S4 relative to the outer pore region during gating, since the MacKinnon model suggested that neither were in proximity to each other, especially at rest (Jiang et al., 2003b). An examination of the E418C mutation at the extracellular terminus of S5 in Shaker-IR illustrated that the modification rate of this mutant by the negatively charged MTSES reagent was highest when the channel was in the activated conformation, as if the positive charges of S4 moved close to E418C during gating to create an electrostatically favourable environment for MTSES-binding (Elinder et al., 2001). In another set of experiments, a cysteine-substituted mutant of
the outermost S4 arginine was shown to form a disulfide bond with F416C, near the top of S5 (Broomand et al., 2003). Interestingly, their studies also suggested that the S4 domain might be located near the pore region of a neighboring subunit, as was later confirmed by the Kv1.2 crystal structure (Long et al., 2005a). Isacoff and co-workers combined the SCAM and fluorescence techniques to further critique the “paddle” model by examining the voltage-dependent movements of fluorophores conjugated to residues in the S1 to S3 helices, and by monitoring disulfide bond formation between substituted-cysteine residues in S4 and the pore (Gandhi et al., 2003). Their results indicated that the conventional model better predicted the accessibility of residues in S1 and S2 and that S4 came into close proximity with the pore during gating.

In general, these studies suggested that S4, and possibly other neighboring helices, underwent small vertical, twisting or tilting, rearrangements during gating. The movement of a large amount of charge (12-14 $e_0$) was made possible by the formation of one or more cannaliculi around the S4 helix, which confined the membrane electric field to a small distance through which S4 charges moved. In addition, as later confirmed by the Kv1.2 crystal structure, S4 lay near the pore region of a neighboring subunit (Figure 1.2), close enough to interact structurally, or electrostatically, with certain pore resides, including some in the turret.

VI: N-type inactivation

In the presence of a prolonged depolarization, the current elicited from Kv channels decays, i.e., it inactivates. In some channels, such as Shaker, this inactivation is rapid, occurring within milliseconds of channel opening, but can be removed by deletion of the N-
terminal amino acids 6-46 (Hoshi et al., 1990), creating an inactivation removed, Shaker-IR, channel (the suffixes –IR, Δ and Δ6-46 all denote the removal of N-type inactivation by cleavage of the N-terminus). The addition of an exogenous peptide fragment containing residues 1-20 could restore the block to Shaker-IR (Zagotta et al., 1990), confirming that the N-terminal fragment contained a particle that was the basis for the fast inhibition. Thus, this type of inactivation was dubbed “N-type”. The removal of N-type inactivation did not affect the normal activation and deactivation processes, suggesting that the inactivation step lay outside the activation pathway (Hoshi et al., 1990). Interestingly, however, off-gating currents from the inactivation-removed channel did not show the characteristic slowing, or charge immobilization, that the wild type channel did, suggesting that the binding of the inactivation particle may be responsible for conferring charge-immobilization, as discussed above (Bezanilla et al., 1991). These experiments implied that although inactivation was separate from activation, the two processes were coupled, such that just as activation preceded inactivation, recovery from inactivation occurred before deactivation of S4 occurred. This coupling has important consequences for the interpretation of the voltage-sensitivity of inactivation. In the sodium channel of the squid giant axon, inactivation was described as being intrinsically voltage-dependent (Hodgkin & Huxley, 1952c; Hodgkin & Huxley, 1952d). However, the coupling of activation and inactivation implies that there need not be any intrinsic voltage-dependence for the inactivation step. Indeed, unitary current analysis of sodium channels from neuroblastoma cells demonstrated that the apparent voltage-sensitivity of inactivation could actually be attributed to the voltage-dependence of activation, since this process was necessary for inactivation to occur (Aldrich et al., 1983).
In Kv channels, a similar voltage-independence of N-type inactivation has also been reported (Zagotta et al., 1990).

What characteristics of the N-terminal ball allow it to block K\(^+\) currents? The region that mediates the block contains a roughly 20 amino acid “ball” domain, which is thought to be the blocking particle, followed by approximately 60 amino acids that constitute the “chain”. Thus, the N-terminal ball was hypothesized to act as a “tethered blocker”, such that upon activation, its binding site becomes exposed and available to bind the N-terminal “ball” (Figure 1.5). This mechanism is mechanistically similar to the fast inactivation of sodium channels, for which a “ball and chain” or “hinged lid” mechanism had also been invoked (Armstrong et al., 1973; Armstrong & Bezanilla, 1977; Armstrong, 1981; Catterall, 1992). Thus, the Δ6-46 mutation abolishes N-type inactivation by cutting the chain and a portion of the blocking ball. The length of the chain is important since a reduction in its length actually increases the rate of inactivation, likely by restricting the mobility of the blocking ball and increasing the effective concentration of the ball near its binding site (Hoshi et al., 1990).

Interestingly, the properties of the ball itself give insight into the identification of the region to which it binds. Partial cleavage, such that only the C-terminal half of the ball is present, abolishes inactivation. In Shaker, the first 11 amino acid residues of the blocking ball are largely hydrophobic, while much of the C-terminal region contains charged side chain residues. The importance of the hydrophobic residues was demonstrated by mutagenesis of this region, which indicated that mutations, especially those involving a leucine at position 7 (Hoshi et al., 1990), could alter the off-rate of ball binding. Conversely, mutations of the charged C-terminal ball residues indicated that these residues mainly affected the association rate of ball block (Hoshi et al., 1990; Murrell-Lagnado & Aldrich,
These findings, plus additional kinetic analyses, led to speculation that the hydrophobic residues within the N-terminal half of the blocking ball were important for stabilizing the bound blocker within a confined, hydrophobic region of the channel at the site of block, while the hydrophilic residues likely made electrostatic interactions with other channel residues to effectively increase the ball concentration near its site of block (Murrell-Lagnado & Aldrich, 1993a).

As described above, internal TEA$^+$ likely binds to Kv channels at a position within the activation pathway, near the activation gate. Thus, the observation that internal TEA$^+$ slows the onset of N-type inactivation, as if both the blocking ball and the TEA$^+$ molecule compete for access to a similar binding site, is consistent with the conclusion that the N-terminal ball likely binds within an intracellular portion of the permeation pathway (Choi et al., 1991). This is further supported by the antagonistic effects of inward K$^+$ currents, whose movement through the pore is thought to repel the blocking ball (Demo & Yellen, 1991). Of significant interest, however, is that some mutations of the S4-S5 linker could affect N-type inactivation, although the concomitant effects of the mutations on activation necessitated caution in interpreting those results (Isacoff et al., 1991). Nevertheless, subsequent experiments demonstrated that modification of a cysteine mutant in the S4-S5 linker by the negatively charged MTSES reagent could enhance inactivation significantly, without affecting activation (Holmgren et al., 1996a). This result was consistent with the hypothesis that electrostatic interactions of the ball with charged residues near the intracellular pore mouth likely contribute to increasing the local ball concentration, but do not form the actual blocking site. Subsequently, based on mutant cycle analyses of mutations in a Kv$\beta$1 subunit (see below) and Kv1.4-IR, it was confirmed that N-type inactivation was initiated by
electrostatic interactions of the hydrophilic region of the blocking particle with residues near the intracellular pore mouth, after which the ball entered the hydrophobic interior of the inner pore cavity as an extended peptide to physically occlude the permeation pathway (Zhou et al., 2001a).

Figure 1.5. **N-type inactivation.** (A) The N-terminal ball, denoted "N", can access its binding site in the pore (shown as black subunits) once the channel has entered the open state, thereby resulting in a block of the conducting pathway. (B) N-type inactivation can also be conferred by Kvβ1 subunits (Gulbis et al., 2000).
It should be noted that not all Kv channels contain a blocking ball in their N-terminus, and consequently, do not show N-type inactivation when expressed in heterologous systems. However, N-type inactivation can be conferred by the presence of an auxiliary beta subunit. For the case of Kv1.5, fast inactivation can be conferred by either a Kvβ1 (Rettig et al., 1994) or Kvβ3 (Leicher et al., 1998) but not a Kvβ2 subunit (Heinemann et al., 1996). These differences can be attributed to the presence of residues in the N-terminus of the β1 subunit that are homologous to the N-termini of fast-inactivating Kv channels. Thus, N-type inactivation can be mediated either by an intrinsic blocking peptide, or by an accessory subunit (Figure 1.5).

VII: Slow (P/C-type) inactivation

Classical description

Currents from fast inactivation-removed Shaker channels (i.e., Shaker-IR, Δ, and Δ6-46) still exhibit the persistence of a very slow form of inactivation (Hoshi et al., 1990). Differences in the kinetic properties of slow inactivation, first described for different splice variants of Shaker were originally attributed to variations in the C-terminal sequences of these channels (Timpe et al., 1988). Subsequent analysis demonstrated that in the Shaker A (ShA) and Shaker B (ShB) splice variants, the only residue that could be directly linked to differences in the rate of slow inactivation was at position 463 in the S6 helix, at the level of the central cavity (Hoshi et al., 1991). In the ShA-IR variant, a valine is present at position 463 and inactivation proceeds with a time constant of 10-20 ms. However, in ShB-IR, the presence of an alanine at this position results in an inactivation time constant of 1-2 s. Thus, based on the difference in amino acid composition at position 463, in the C-terminal region
of the channel, the authors referred to this process as C-type inactivation rather than “slow” inactivation since it could indeed be very fast, as observed for the ShA variant. The authors also showed that as for N-type inactivation, C-type inactivation was independent of voltage and modeled it as being strictly coupled to channel opening. However, as will be discussed below, inactivation may also occur in some situations from closed states.

**External TEA\(^+\) and K\(^+\) antagonize C-type inactivation**

It was subsequently found that N- and C-type inactivation could be differentiated based on the effects of externally- or internally-applied TEA\(^+\) (Grissmer & Cahalan, 1989; Choi et al., 1991). As has already been described, internal TEA\(^+\) slows the rate of N-type inactivation in *Shaker* channels, and was hypothesized to do this by competing for access to a binding site in the pore (Choi et al., 1991). In this same study, it was also found that external TEA\(^+\) had no effect on the rate of inactivation in the wild type channel, which was dominated by N-type inactivation. However, in the Δ6-46 mutant, external TEA\(^+\) slowed the rate of the remaining C-type inactivation. Similar results were found independently for *Shaker-IR* (Molina et al., 1997) and for the Kv1.3 K\(^+\) channel which normally does not undergo N-type inactivation (Grissmer & Cahalan, 1989a). Together, these results implied that, just as internal TEA\(^+\) competed with N-type inactivation, external TEA\(^+\) might bind to the channel near the site where C-type inactivation occurred, such that it prevented the closure of the C-type inactivation gate.

Aldrich and co-workers demonstrated that increasing \([K^+]_o\) from 2 mM to 140 mM increased the time constant of C-type inactivation from \(~1.3\) s to \(3.1\) s in *Shaker-IR* (López-Barneo et al., 1993). Unitary current analysis confirmed that increased K\(^+\)_o relieved
inactivation by prolonging the duration of channel bursts, although small changes in the single channel conductance were detected. Substitution of extracellular K\(^+\) with Rb\(^+\) also slowed inactivation, while other cations, such as Cs\(^+\), Na\(^+\), NH\(_4\)\(^+\) and NMG\(^+\) did not. Of particular interest, the slowing of inactivation by high K\(^+_o\) was paralleled by a potentiation of the current magnitude in the Shaker-IR fast-inactivating T449 mutants (these mutants are further discussed below). As a result, in high K\(^+_o\), the peak current upon depolarization was greater than that in low K\(^+_o\). The interpretation of these results, and similar results in other channels (see below), has led to much speculation, but the authors from this study suggested that the fast-inactivating Shaker-IR-T449 mutant channels entered the inactivated state from both the open and closed states, and that K\(^+\) ions antagonized both types of inactivation by binding within the permeation pathway to impede the closure of the inactivation gate, which they hypothesized was located near the external pore mouth (López-Barneo et al., 1993). This relief of slow inactivation by K\(^+_o\) has also been referred to as a “foot in the door” mechanism (López-Barneo et al., 1993). Although this study provided evidence that C-type inactivation might occur from one or more closed states along the activation pathway, it was unclear if closed state inactivation occurred during activation, such that occupation of one or more partially-activated closed states allowed for a transition to an inactivated state, or if channels became inactivated from rest, in the absence of depolarization.

The possibility of closed state inactivation was also raised in an examination of the Kv1.4 channel. Similar to the fast-inactivating Shaker-IR-T449 mutants, the magnitude of current in Kv1.4 was reduced by lowering [K\(^+\)]\(_o\), until current was eliminated in the absence of K\(^+_o\) (Pardo et al., 1992; Jäger et al., 1998). The \(K_d\) for this effect was 2.2 mM, and other ions such as Rb\(^-\) and Cs\(^+\) could mimic the effects of K\(^+\) to some degree (Pardo et al., 1992).
Importantly, it was shown that despite a reduction of current in low K\(^+\), gating charge movement was conserved, suggesting that activation transitions still occurred in the non-conducting channels. These results were also compatible with the presence of closed state inactivation, but as with the fast-inactivating *Shaker*-IR-T449 mutants, it was uncertain from which closed state(s) the channels inactivated.

Interestingly, elevating [K\(^+\)]\(_o\) from 5 to 150 mM was also found to increase the peak current magnitude in *Kv1.3* (Levy & Deutsch, 1996b). However, in that study, the effects of [K\(^+\)]\(_o\) were interpreted very differently and were attributed to an increase in the rate of recovery from C-type inactivation. Despite this difference in the effect of [K\(^+\)]\(_o\), compared to the effects in *Shaker*, raising [K\(^+\)]\(_o\) also slowed the rate of inactivation in *Kv1.3* (Grissmer & Cahalan, 1989b; Levy & Deutsch, 1996b), thereby demonstrating that [K\(^+\)]\(_o\) was clearly affecting aspects of C-type inactivation.

Although studies had indicated that the wt *Shaker*-IR channel was relatively insensitive to [K\(^+\)]\(_o\) (López-Barneo *et al.*, 1993), Baukrowitz and Yellen (1995) demonstrated that the true sensitivity to [K\(^+\)]\(_o\) was obscured by the outward K\(^+\) flux that increased the effective [K\(^+\)]\(_o\) at the outer pore mouth to approximately 15 mM. By preventing K\(^+\) accumulation (either by using fast perfusion changes or protocols that did not create outward current), the *K\(_d\)* for the K\(^+\)-dependence of C-type inactivation in *Shaker*-IR, and wt *Shaker* (N-type inactivation intact), was found to be 1.5-2 mM (Baukrowitz & Yellen, 1995; Baukrowitz & Yellen, 1996), similar to the *K\(_d\)* for the K\(^+\)-dependence of *Kv1.4* (Pardo *et al.*, 1992). Thus, C-type inactivation in *Shaker*-IR is highly [K\(^+\)]\(_o\)-sensitive.

Baukrowitz & Yellen (1996) also hypothesized that if K\(^+\) current was blocked in *Shaker*-IR (either as a result of N-type inactivation or TEA\(^+\) blockade), the rate limiting step
in the transition to the inactivated state was the off-rate of the last ion bound in the pore, whose presence prevented inactivation. These experiments implied that inactivation is faster in low \([K^+]_o\) because lower concentrations increased the probability that the pore could become devoid of ions. However, it is possible that permeant ions need not be completely absent from the pore for slow inactivation to occur, as studies have also shown that the slow inactivation gate can close even if a \(K^+\) (Ogielska & Aldrich, 1999) or \(Ba^{2+}\) (Harris et al., 1998) ion still occupies an internal binding site of the pore. As a result, it is still unclear if all ions must leave the pore before C-type inactivation can occur. Nevertheless, together with the alleviation of C-type inactivation by \(TEA^+\), the effects of \(K^+\) provided an early phenotypic description of C-type inactivation. These studies also demonstrated that the presence of N-type inactivation could itself speed the rate of C-type inactivation by promoting a loss of \(K^+\) ions from the pore (Baukrowitz & Yellen, 1995).

Alternate mechanisms of inactivation

Despite the similarities in the sensitivity of C-type inactivation to \(K^+\) and \(TEA^+\) among Shaker-IR, Kv1.4 and Kv1.3, not all channels were found to share these qualities. In particular, raising \([TEA^+]_o\) or \([K^+]_o\) sped the rate of inactivation in Kv2.1 and Kv3.1 (Klemic et al., 1998; Klemic et al., 2001). These channels also displayed an anomalous “U-shape” in the voltage-dependence of inactivation (often referred to as “U-type” inactivation) which indicated that these channels inactivated preferentially from closed states (Klemic et al., 1998; Klemic et al., 2001). These differences have led some to suggest that U-type inactivation may be a separate mechanism by which channels inactivate (Klemic et al., 2001; Kurata et al., 2005). This is supported, to a certain extent, by the finding that a N-terminally
truncated form of the Kv1.5 channel also displays U-type inactivation, whose sensitivity to 
$[K^+]_o$ and $[\text{TEA}^+]_o$ can be distinguished separately from the C-type inactivation normally 
present in the wild type channel (Kurata et al., 2001; Kurata et al., 2002; Kurata et al., 2005). 
This indicates that it is possible for both types of inactivation to occur simultaneously in the 
same channel. This finding might also imply that the closed state inactivation proposed to 
 occur in fast-inactivating Shaker-IR-T449 mutants, Kv1.4 and 1.3, may be due to an 
enhancement of U-type inactivation. However, this is unlikely since the unique voltage-
sensitivity of inactivation that defines U-type inactivation is not observed in these channels.

As with Kv2.1 and 3.1, the properties of inactivation in Kv4.1 are not analogous to 
those in Shaker-IR. For example, extracellular TEA$^+$ does not slow down inactivation, and 
high $[K^+]_o$ speeds the entry into, and slows the recovery from, inactivation (Jerng & 
Covarrubias, 1997; Jerng et al., 1999). As a result, slow inactivation in Kv4.1 has been 
referred to by some as V-type inactivation (Jerng et al., 1999), and as with U-type 
inactivation, may represent a distinct type of inactivation. However, in Shaker-related 
channels, C-type inactivation appears to be the dominant form of slow inactivation and so U-
and V-type inactivation will not be considered further.

**The outer pore mouth contributes to C-type inactivation**

Given the hypothesis that external TEA$^+$ (Choi et al., 1991) and K$^+$ (López-Barneo et 
al., 1993) modulate C-type inactivation, perhaps by interacting with the outer pore mouth, it 
was reasonable to expect that mutation of residues in this region might also affect 
inactivation. This was confirmed by the finding that mutation of residue T449 in Shaker-IR 
could drastically alter the rate of C-type inactivation (López-Barneo et al., 1993; Schlief et
al., 1996). The T449-E, -K, -A, -Q and -S mutations increased the rate of depolarization-induced inactivation, while T449-H, -C and particularly -V slowed it. Interestingly, in those T449 mutations where inactivation was fast, a dependence of the peak current amplitude on $[\text{K}^+]_o$ was also found, as described above. This suggested that these two effects were both linked to an enhancement of slow inactivation. Not surprisingly, mutation of residue T449, especially T449Y, could also affect TEA$^+$ binding (Heginbotham & MacKinnon, 1992), as did mutation of D447 (Molina et al., 1997), although the exact location of the TEA$^+$ binding site in the outer pore mouth is still uncertain.

The residue at the equivalent of position 449 in Shaker is not well conserved among Kv channels, but mutation of the equivalent residues in other channels typically affects the rate of inactivation, TEA$^+$-sensitivity and $[\text{K}^+]_o$-dependence, suggesting that this position is an important determinant in C-type inactivation among Kv channels. For example, in Kv1.4, the K533Y mutation eliminated the $[\text{K}^+]_o$-dependence of the current amplitude and introduced TEA$^+$-sensitivity (Pardo et al., 1992). Similarly, in Kv1.3, protonation or mutation of the homologous H533 residue had variable effects on the rate of inactivation and $[\text{K}^+]_o$-dependency (Levy & Deutsch, 1996a; Jäger et al., 1998; Somodi et al., 2004). Interestingly, in Kv1.5, the R487V mutation (homologous to T449V), had little effect on the rate of inactivation (Wang et al., 2000b). However, when the permeant ion was sodium (see below) the rate of inactivation was greatly reduced, suggesting that this mutation was affecting C-type inactivation, although the exact mechanism was unclear (Wang et al., 2000a). In general, these results imply that the outer pore mouth, and in particular the equivalent residue of T449 in Shaker, is an important component in the C-type inactivation process.
This importance was highlighted dramatically by experiments that employed cysteine substitutions near the outer pore mouth and examined the state-dependence of both inter-subunit disulfide bond formation and Cd\(^{2+}\)-coordination. The T449C mutation was shown to coordinate Cd\(^{2+}\) ions with a 45,000-fold greater affinity when in the inactivated state, compared to the open state (Yellen et al., 1994). In addition, the M448C mutation formed inter-subunit disulfide bonds 50-65-fold faster in the inactivated state than in the closed state (Liu et al., 1996). With this data, it became clear that C-type inactivation likely resulted in a constriction of the outer pore mouth, and suggested that this region may constitute part of the C-type inactivation gate (Figure 1.6). Thus, external K\(^{+}\) and TEA\(^{+}\) likely relieved inactivation because their presence either in the selectivity filter or at the outer pore mouth impeded the constriction of the pore (Yellen et al., 1994). This model of the outer pore collapsing as a unit was compatible with experiments that indicated that C-type inactivation involved a concerted movement of the channel, such that the highly-cooperative movements of all four subunits were required (Panyi et al., 1995; Ogielska et al., 1995).

**P-type inactivation**

Continued investigations led to the phenomenological classification of another form of slow inactivation, namely “P-type” inactivation. This was borne out of studies of a chimeric channel, in which the pore of the Kv2.1 channel was replaced with that of Kv3.1. The 9 residues that differed between the channels were subsequently mutated back to that of 2.1 in order to determine if any one of these residues was responsible for conferring the particular characteristics of TEA\(^{+}\) block of either channel (Kirsch et al., 1992). While the results were inconclusive regarding the residues mediating TEA\(^{+}\) sensitivity, the V369I
mutation near the C-terminal end of the pore helix produced a fast-inactivation phenotype that was uncharacteristic of either native channel. As reported for the fast-inactivating Shaker-IR-T449 mutants, the faster inactivation in the V369I chimeric mutant was $[K^+]_o$-sensitive, and the rate of recovery from inactivation was also sped by $[K^+]_o$. However, due to anomalous effects of TEA$^+$ on the mutant ($[TEA^+]_o < 10$ mM enhanced the current magnitude), and the fact that the mutation lay in the P-loop of the channel, compared to the classical A463 residue in S6 that first identified C-type inactivation in Shaker-IR (Hoshi et al., 1991), the authors argued that the inactivation observed in the chimeric channel represented a novel form of inactivation, which they termed “P-type” (De Biasi et al., 1993). However, since the P-type designation was based on phenotypic data, and not definitive structural information, it was unclear if P-type inactivation truly represented a distinct form of inactivation. Indeed, the T449 mutations, which were discovered at roughly the same time as P-type inactivation (López-Barneo et al., 1993), were also located in the vicinity of the outer pore, and studies have indicated that mutations of Shaker-IR-T 449 and A463 both affect a shared mechanism of slow inactivation (Ogielska et al., 1995). This suggested that P-type inactivation might be a phenotypic variation of C-type inactivation.

Since P-type and C-type inactivation appeared to describe a similar structural process, the definition of P-type inactivation was subsequently altered to represent not a distinct type of C-type inactivation, but a particular phase of it. This began with the discovery of the W434F mutation in the pore helix of Shaker-IR, which resulted in a loss of ion conduction, but left gating currents intact (Perozo et al., 1993). To probe the mechanism by which the W434F mutation eliminated current, Sigworth and co-workers concatenated mutant and wild type subunits and demonstrated that as the proportion of mutant W434F subunits relative to
wild type increased, so too did the rate of inactivation and the occurrence of null sweeps in single channel records. The authors hypothesized that with W434F homotetramers, inactivation might be enhanced so much that it becomes essentially permanent, even at rest, thereby resulting in the loss of ionic currents (Yang et al., 1997). The hypothesis that the W434F mutation enhanced inactivation was further supported by the finding that the T449V mutation (which reduces the rate of C-type inactivation in Shaker-IR) appeared to alleviate the inactivation enhanced in the W434F channel by making Shaker-IR-W434F/R487V channels conducting again (Yang et al., 2002; Kitaguchi et al., 2004). Complications arose, however, when several groups showed that there was a clear correlation between the onset of C-type inactivation, and the immobilization of off-gating charge (Fedida et al., 1996; Olcese et al., 1997). Recall that the slowing of off-gating charge recovery had initially been modeled as the slow transition from the open to the closed state upon repolarization (Bezanilla et al., 1994; Zagotta et al., 1994a). Instead, studies now suggested that charge immobilization might actually be a manifestation of C-type inactivation, such that as inactivation progressed, S4 underwent a stabilizing transition, resulting in a left-shift of the voltage-dependence of off-gating charge movement relative to on-gating charge (Fedida et al., 1996; Olcese et al., 1997). As a result, once gating charge had been moved during activation, and subsequently “immobilized”, much larger voltage changes were required to return the voltage sensor to its resting state. In Shaker, this resulted in an approximately 50 mV shift to the left of the $Q/V$ curve of off-gating charge movement, compared to the $Q/V$ curve of on-gating charge (Olcese et al., 1997). It was then shown that W434F still underwent charge immobilization. However, the paradox then became, how could a “permanently inactivated” W434F channel feature a transition associated with C-type
inactivation, if it was already inactivated? To address this issue, both Sigworth (Yang et al., 1997) and Bezanilla (Olcese et al., 1997) proposed that slow inactivation might be composed of two processes (Figure 1.6). In an early, pore (P-type) transition, the pore mouth underwent a constricting motion as suggested by Yellen (Liu et al., 1996), which eliminated current. Upon further depolarization, a second (C-type) transition occurred in which gating charge was immobilized as S4 entered a stabilizing interaction with the pore, likely involving S5. The term “slow inactivation” was subsequently re-introduced, and is used in this thesis, to represent a combined P/C-type inactivation process.

![Figure 1.6. Movements associated with slow inactivation.](image)

In the closed state (top row, left), the S4 helices are in a resting conformation, the outer pore is in an open conformation, and the activation gate is closed. In response to a depolarization, the S4 helices move (shown by an upward translocation) as the channel progresses through several “partially-activated” closed states (only one is represented for simplicity). Once all four S4 helices are activated, the activation gate opens and the channel is in the “open” state. Continued depolarization results in closure of the outer pore (“P-type” inactivated) after which S4 enters into a stabilized conformation (“C-type”). Both inactivation movements are detected by a turret fluorophore (asterisk). A hypothetical model of closed state inactivation is shown within the box in the lower row and represents the closure of the inactivation gate either without prior S4 movement (lower row, left) or with limited S4 movement from partially-activated closed states. “Permanently inactivated” channels, such as the Shaker-IR-W434F mutant have been proposed to occupy only the states shown in the lower row. As a result, despite S4 movement, ionic current is not observed. Adapted from (Loots & Isacoff, 1998).
**Fluorophore movement follows P-type inactivation**

The advent of voltage-clamp fluorimetry (Mannuzzu et al., 1996), not only helped elucidate the motion of S4 during activation, as discussed above, but also aided in our understanding of the structural rearrangements during inactivation. By adding the TMRM fluorophore to a substituted cysteine at position 424 in the turret region of Shaker-IR, it was shown that the majority of the fluorescence signal from this site slowly increased in parallel with the onset of slow inactivation in ionic currents (Loots & Isacoff, 1998) (Figure 1.6). The fact that a fluorophore in the turret could sense rearrangements in the outer pore associated with P-type inactivation suggested that these regions must be rigidly linked together to some degree. In contrast, the fluorescence signal from the Shaker-IR-W434F mutant showed a fast component reporting activation but no inactivation component, further suggesting that this mutant had, at rest, already undergone the outer pore rearrangements associated with P-type inactivation (Loots & Isacoff, 1998).

**S4 movement may couple activation to slow inactivation**

As described previously, S4 had been shown to interact with residues in the pore domain (S5-S6 region) during activation (Elinder & Arhem, 1999; Gandhi et al., 2000; Broomand et al., 2003; Laine et al., 2003). The use of the TMRM fluorophore at positions 418C and 419C at the S5/turret boundary of Shaker-IR, also showed that both of these positions reported changes in fluorescence related to activation, suggesting a close proximity of the pore domain to S4 (Loots & Isacoff, 2000). However, residues 416, 417 and 419 also reported movements associated with inactivation. These results, like those obtained at position 424 (Loots & Isacoff, 1998), suggested that much of the pore behaved in a rigid
manner, such that outer pore constriction could be detected by fluorophores not located directly adjacent to the pore mouth.

Interestingly, the glutamate at the equivalent position of Shaker-E418 is conserved in many Kv channels and this position appears to be important for slow inactivation. This was shown by the fact that the Shaker-IR-E418Q mutant showed rapid inactivation (τ_{inact} = 16.4 ms in 30 mM [K⁺]₀), was potentiated by increasing [K⁺]₀, and displayed enhanced current in response to the T449V mutation (Ortega-Sáenz et al., 2000). Experiments also suggested that residue E418 interacted electrostatically with position G452 in S6 to stabilize the open state, but upon activation, this interaction was lost in favour of an interaction with position V451, which stabilized the inactivated state (Larsson & Elinder, 2000). Furthermore, this switch from an interaction favoring the open state to one favoring the inactivated state was hypothesized to occur as a result of interactions with S4 during activation (Loots & Isacoff, 2000; Larsson & Elinder, 2000).

Together, these findings supported two general conclusions. First, that Shaker-IR residue E418 interacted in a state-dependent manner with two S6 residues, with one interaction favouring the open state, the other favouring the inactivated state. Secondly, that in the open state, the presence of S4 near the pore enhanced the affinity of E418 for the inactivation-favouring interaction, thereby coupling activation with inactivation via S4 movement. It should be noted, however, that the interpretation of these results is problematic since mutation of Shaker-IR-E418 frequently results in a loss of functional current (Ortega-Sáenz et al., 2000; Loots & Isacoff, 2000; Larsson & Elinder, 2000). As a result, the functional state of the Shaker-IR-E418 mutant channel may not represent wild type channel and thus may not provide relevant data.
Is the selectivity filter the slow inactivation gate?

The role of the selectivity filter in regulating slow inactivation is clearly apparent from the studies described above which demonstrated that inactivation was modulated by the type and concentration of the permeant ion, as well as by mutations in the outer pore mouth, and by the finding that the conformation of the outer mouth changes during inactivation. More recently, conformational rearrangements of the selectivity filter associated with slow inactivation have also been detected by changes in ion selectivity and have led to the hypothesis that the selectivity filter (and not just the outer pore mouth) forms the slow inactivation gate.

In several Kv channels, removal of extra- and intracellular K\textsuperscript{+} results in a small Na\textsuperscript{+} current, suggesting that in the absence of K\textsuperscript{+} ions, the open conformation of the pore can become permeable to Na\textsuperscript{+} ions (Korn & Ikeda, 1995; Starkus et al., 1997; Ogielska & Aldrich, 1998; Wang et al., 2000a). Interestingly, it was subsequently discovered that the inactivated state was also permeant to Na\textsuperscript{+} ions, albeit with even less efficiency than the open state (Korn & Ikeda, 1995; Starkus et al., 1997; Ogielska & Aldrich, 1998; Wang et al., 2000a). These findings suggested that in the inactivated state, K\textsuperscript{+}-conduction was not lost because the selectivity filter collapsed and became physically occluded, but perhaps because it adopted an alternate conformation in which the filter binding sites no longer coordinated K\textsuperscript{+} ions with high affinity. Distortions of the selectivity filter were subsequently observed in the crystal structure of the KcsA channel in the presence of low K\textsuperscript{+} (3 mM), compared to the structure in high K\textsuperscript{+} (200 mM) (Zhou et al., 2001b). In low [K\textsuperscript{+}]\textsubscript{o}, the selectivity filter adopted a structure in which only two ions were bound, and the backbone carbonyls of V76 and G77, which normally coordinate K\textsuperscript{+} ions, pointed away from the conducting pathway and
interacted either with water molecules or other side chains. The α-carbon of G77 appeared twisted inwards and apparently occluded the pore. While the authors refrained from implicating such a conformation in the inactivated state (and some have argued that it is not the inactivated state (Cordero-Morales et al., 2006a), or the state in which Na⁺ ions are conducted in the absence of K⁺ ions, it nevertheless indicated that the filter was a flexible structure, capable of assuming different conformations. Furthermore, molecular dynamics simulations of the KcsA pore have confirmed that the filter is not a rigid structure (Noskov et al., 2004). Interestingly, these simulations have also suggested that during ion conduction, a series of spontaneous rearrangements deep within the filter that alter the interactions of T75, V76 and G77 can effectively stop ion conduction (Berneche & Roux, 2005). This might provide a mechanism by which the pore can become destabilized while in the open state, leading to inactivation.

So is the selectivity filter the slow inactivation gate? Recent studies in KcsA have suggested that this is a possibility. Mutation of position E71 in the pore helix of KcsA (equivalent to V369 in Kv2.1 (De Biasi et al., 1993); see “P-type”-inactivation above) was found to substantially increase the open probability of KcsA in conditions when the activation gate was already thought to be fully open (i.e., at low pH) (Cordero-Morales et al., 2006b). These findings led the authors to suggest that the selectivity filter is the inactivation gate in KcsA (Cordero-Morales et al., 2006a), and by extension, provided further support for the argument that the selectivity filter is the inactivation gate in Kv channels. The selectivity filter has also been implicated in the gating of cyclic-nucleotide gated (CNG) channels (Liu & Siegelbaum, 2000) and inward rectifier (Kᵢᵣ) channels (Lu et al., 2001; So et al., 2001), suggesting that it has a widespread role in the gating of different types of ion channels.
Summary of slow inactivation

Together, the available data suggest that in Kv channels, slow inactivation likely involves two distinct phases. In the initial P-type phase, the conformation of the selectivity filter is altered, such that $K^+$ ion conduction is lost. Subsequently, the voltage sensor enters a stabilized conformation (C-type inactivated), perhaps by interacting with the pore domain. In addition, while slow inactivation is typically coupled to channel opening, several studies have provided evidence that entry into the inactivated state may also occur from one or more closed states.

Since Kv channels play an important role in the repolarization of the membrane voltage, and inactivation influences this capability, a clear understanding of this process is an important step in determining the roles of Kv channels in cellular physiology.

VIII: Scope of thesis

This thesis deals primarily with an examination of slow inactivation in the Kv1.5 channel. As a member of the Kv1.x family, Kv1.5 is a mammalian homologue of Shaker, and the gene encoding it was originally cloned from fetal and adult human hearts (Tamkun et al., 1991; Fedida et al., 1993). This channel has also been shown to be present in the heart of the mouse (Attali et al., 1993), rabbit (Sasaki et al., 1995), guinea pig and ferret (Dobrzynski et al., 2000). The high level of Kv1.5 expression in the atria (including the sinoatrial (SA) node (Dobrzynski et al., 2000)) of the heart implies an important role in pacemaking, and as a result, Kv1.5 has become a pharmaceutical target for the treatment of atrial fibrillation (Roy et al., 2004). Differences in the expression of Kv1.5 have been associated with gender-based differences in the QT interval (Trepanier-Boulay et al., 2001), and changes in expression
have been detected in response to cardiac remodeling following atrial fibrillation (Van Wagoner et al., 1997; Brandt et al., 2000) or cardiac failure (Petkova-Kirova et al., 2005). Kv1.5 is also expressed in the smooth muscle of the airways (Adda et al., 1996), vasculature (Clement-Chomienne et al., 1999), and intestines (Overturf et al., 1994). Kv1.5 has also been localized to the brain (Takimoto et al., 1993) and the taste receptors of the tongue (Liu et al., 2005).

Unlike Shaker, Kv1.5 does not exhibit N-type inactivation but instead displays only slow, P/C-type inactivation. Although Kv1.5 channels can be modulated by the presence of β1-subunits (Sewing et al., 1996) or can form heteromultimers with other Kv1.x family members (Smirnov et al., 2002; Plane et al., 2005), we have studied the cloned channel as a homotetramer in isolation by expressing it in human embryonic kidney (HEK-293) or mouse lymphocyte (ltk−) cell lines. This affords us the opportunity to study Kv1.5 currents with minimal contamination from other current types.

The experiments presented here focus particularly on the role of the S5-P linker (turret (Doyle et al., 1998)) in the regulation of slow inactivation. In a previous report, a mutation in the Shaker-IR turret was found to have only a modest effect on inactivation (Perez-Cornejo, 1999). However, in Kv1.5 we show that the turret makes a significant contribution to the regulation of slow inactivation in response to the binding of protons, divalent cations, and MTS reagents. We have used these ligands as tools to better understand the mechanism by which the turret regulates inactivation. In the first study (Chapter 2) we demonstrate that the turret contains a histidine residue (H463) that mediates a profound inhibition of Kv1.5 current by protons (H+) and zinc (Zn2+) ions, and propose that this inhibition is likely related to an enhancement of slow inactivation (Kehl et al., 2002).
In Chapter 3, we extend the first study to include the effects of other divalent cations on Kv1.5, such as Ni$^{2+}$, Co$^{2+}$, and Mg$^{2+}$. In addition, by using unitary current analysis, we clearly demonstrate that the mechanism of current inhibition is unrelated to changes in the single channel conductance, but instead involves a loss of channel availability which we hypothesize is related to an enhancement of inactivation from the resting state (Kwan et al., 2004).

In the concluding study, we examine the role of the physico-chemical properties of the side chain at position 463 in modulating channel activity and show that, as with T449 mutations in Shaker-IR, substitutions of various amino acids by mutagenesis can have significant effects on the characteristics of inactivation. We then employ the SCAM technique to determine if H463 was unique in its ability to modulate inactivation by sequentially mutating individual turret residues to cysteines. By analyzing the functional consequences of MTS-modification on the substituted cysteine residues, we show that the turret of Kv1.5 contains a distinct locus of residues that are capable of modulating slow inactivation. These findings are novel since they identify a large cluster of residues near the outer pore mouth that can modulate inactivation and because they provide constraints on the possible interactions between the turret and pore.
REFERENCES


CHAPTER 2: MOLECULAR DETERMINANTS OF THE INHIBITION OF HUMAN Kv1.5 POTASSIUM CURRENTS BY EXTERNAL PROTONS AND Zn²⁺
INTRODUCTION

We have previously shown in human Kv1.5 (hKv1.5) channels that external Zn$^{2+}$ ions caused a depolarizing shift of the activation curve, an effect referred to as the gating shift, as well as a reduction of the current amplitude, which we termed block, that was relieved by external permeant ions such as K$^+$ and Cs$^+$ (Zhang et al., 2001a). Although a gating shift is very often associated with a change of the external concentration of divalent cations, as first detailed by Frankenhaeuser & Hodgkin (1957), the block of voltage-gated K$^+$ channels by divalent cations, in particular Zn$^{2+}$, is not a general finding and its mechanistic basis is therefore of some interest. In a follow-up study of the effects of Zn$^{2+}$ on-gating currents (Zhang et al., 2001b), we found that Zn$^{2+}$ ions caused a rightward shift of the voltage dependence of on-gating charge movement or the $Q_{on}(V)$ relationship. This Zn$^{2+}$-induced shift of the $Q_{on}(V)$ was approximately 2-fold greater than that observed for the conductance-voltage ($g(V)$) relationship and implied that Zn$^{2+}$ binding at a site in the outer channel pore could inhibit the ionic current as well as the movement of the voltage sensor. The latter effect is consistent with recent evidence for a close proximity of the outer pore mouth and the S4 segment which comprises a major part of the voltage sensor (Blaustein et al., 2000; Li-Smerin et al., 2000; Cha & Bezanilla, 1998; Loots & Isacoff, 1998) and with the view that the voltage sensing domain wraps around the outer rim, i.e., the turret of the pore (Loots & Isacoff, 2000).

Identifying the potential site(s) of action of Zn$^{2+}$ is facilitated by the fact that Zn$^{2+}$ shows high affinity binding to the imidazole ring of histidine (H) or to the sulphur atom of cysteine (C) residues. Lower affinity binding of Zn$^{2+}$ can also occur at the side chain carboxyl group of the acidic amino acid residues glutamate (E) and aspartate (D) (Vallee &
In the six transmembrane segment (6TM) α-subunit of hKv1.5 there is in the pore-forming (P-) region and the extracellular segments linking it to S5 (S5-P) and S6 (P-S6) only one high affinity Zn$^{2+}$ ligand, H463, which, based on the crystal structure of KcsA channels (Doyle et al., 1998), is found in the turret. Other potential Zn$^{2+}$ binding sites include, at the NH$_2$-terminal end of S5-P, a glutamate residue, E456, that is strictly conserved in voltage-gated channels (Ortega-Saenz et al., 2000) and which appears to interact with S4 (Loots & Isacoff, 2000). E456 is, however, an unlikely Zn$^{2+}$ binding site at least in part because its side chain carboxyl group is believed to form hydrogen bonds with residues in the P-S6 region (Larsson & Elinder, 2000). Two aspartate residues are also found in the outer pore mouth of hKv1.5, D469 in the outer pore helix and D485 that forms part of the "GYGD" motif in the pore signature sequence.

Interestingly, the inhibition of rat Kv1.5 (rKv1.5) currents by extracellular protons (H$^+_{o}$) (Steidl & Yool, 1999) has features similar to that of the Zn$^{2+}$ block of hKv1.5. Although not evident with Zn$^{2+}$ during 300 ms pulses (Zhang et al., 2001a), an acceleration by H$^+_{o}$ of current inactivation during long-lasting (> 1 s) depolarizations raised the possibility that the current inhibition was due to an accumulation of channels in the C-type inactivated state (Steidl & Yool, 1999). The term “C-type inactivation” originated with the observation that Shaker splice variants with different carboxy-terminal regions (including S6) showed different rates of slow inactivation (Hoshi et al., 1991). Subsequently it was reported that mutations in the pore (P) region of Kv2.1 channels increased the inactivation rate by a process having different characteristics than C-type inactivation and the term “P-type inactivation” was coined (De Biasi et al., 1993). A growing body of evidence now suggests that slow inactivation in Shaker-related channels such as Kv1.5 is in fact a complex process
involving either multiple and independent inactivation mechanisms or a single inactivation process that involves multiple steps (Olcese et al., 1997; Yang et al., 1997; Loots & Isacoff, 2000; Wang & Fedida, 2001; Kiss et al., 1999). P-type inactivation appears to involve a partial constriction of the outer pore mouth that eliminates K⁺ currents but has no effect on gating currents including their ability to undergo charge immobilization following the "closed to open" transition (Yang et al., 1997). C-type inactivation might be coupled to P-type inactivation and has been proposed to involve a further conformational change of the outer pore that stabilizes the S4 segments in the activated or outward position. This "stabilization" contributes to a voltage-dependent slowing of return or off-gating charge movement, an effect that is also known as charge immobilization (Olcese et al., 1997; Wang & Fedida, 2001). The possibility that a common mechanism of action, possibly involving an inactivation process, might account for the inhibition of Kv1.5 current by Zn²⁺ and H⁺ also pointed to the potential for a common site of action. In this connection it is known that Zn²⁺ and H⁺ bind to histidine residues and an rKv1.5 mutant in which histidine residues in the pore turret are replaced by glutamine (Q) (rKv1.5-H452Q) has a substantially reduced acid sensitivity (Steidl & Yool, 1999).

Against this background the experiments described here had two major goals. The first goal was to determine the concentration dependence of the inhibition of hKv1.5 channels by protons and to discover if, as with Zn²⁺, the inhibition by H⁺ was affected by changes of K⁺o. After confirming a K⁺o-sensitive inhibition of hKv1.5 currents by H⁺o, the second goal was to gain at least a preliminary insight into the molecular basis for that inhibitory action by assessing the effects of point mutations. We provide evidence that the binding of H⁺ or Zn²⁺ to histidine residues (H463) in the channel turret is a necessary but not
a sufficient condition for the inhibitory effect. Instead, H463s appear to function as sensors, such that H\(^+\) or Zn\(^{2+}\) binding permits a conformational change that involves an arginine (R) residue near the pore mouth (R487). An examination of gating currents revealed that H\(^+\) (pH 5.4) has no effect on the total gating charge movement (\(Q_{\text{max}}\)) and that charge immobilization persists following strong depolarizations. Based on these observations we propose that protons and Zn\(^{2+}\) ions inhibit hKv1.5 currents by affecting channel availability. The possibility that the H\(^+\) inhibition of hKv1.5 currents arises by the facilitation of a transition to a non-conducting state, possibly the P-type inactivated state, is discussed.

**METHODS**

**Cell preparation**

Wild type (wt) hKv1.5 channels were studied in a human embryonic kidney cell line (HEK293) as reported previously (Wang et al., 2000). Cells were dissociated for passage by using trypsin-EDTA and were maintained in minimum essential medium (MEM), 10 % fetal bovine serum, penicillin-streptomycin and 0.5 mg ml\(^{-1}\) gentamicin in an atmosphere of 5 % CO\(_2\) in air. All tissue culture supplies were obtained from Invitrogen (Burlington, ON, Canada).

Point mutations of the wt hKv1.5 \(\alpha\) subunit in the plasmid expression vector pcDNA3 were made using the Quikchange Kit (Stratagene, La Jolla, CA, USA) to convert the histidine (H) residue at position 463 to glutamine (Q) (H463Q) or glycine (G) (H463G). The double mutant H463Q,R487V was created by subcloning a cassette of hKv1.5-H463Q into hKv1.5 R487V (Wang et al., 2000) using BstEII and ClaI restriction enzymes (New England BioLabs, Beverly, MA, USA). Stable transfections of HEK293 cells were made using 0.8\(\mu\)g
of hKv1.5-H463Q, hKv1.5-H463Q, R487V or hKv1.5-H463G cDNA and 2 μL of Lipofectamine 2000 (Invitrogen). Geneticin (0.5mg/mL) was added 48 hrs after transfection.

**Recording solutions**

The standard bath solution contained, in mM, 140 NaCl, 3.5 KCl, 10 Hepes, 2 CaCl$_2$, 1 MgCl$_2$, 5 glucose and its pH was adjusted to 7.4 with NaOH. Where the effect of the external concentration of potassium (K$_{o}^+$) on the proton block was examined, zero K$_{o}^+$ solution was made by substituting NaCl for KCl and, for K$_{o}^+$ greater than 3.5 mM, NaCl was replaced by KCl. Cs$^+$-containing solutions were prepared by substitution of CsCl for KCl (3.5 mM Cs$_{o}^+$) or for both KCl and NaCl (20 mM and 140 mM Cs$_{o}^+$). In experiments addressing the effect of Na$_{o}^+$ on the current inhibition, N-methyl-D-glucamine (NMG$_{o}^+$) replaced Na$_{o}^+$ and the pH was adjusted with HCl. The external concentration of H$_{o}^+$ (H$_{o}^{+}$) was adjusted with 10 mM of buffer where the buffer was Hepes for the pH range 6.8 to 7.4, Mes for pH 5.4 to 6.7 or Taps for pH 8.4. Zn$^{2+}$-containing test solutions were made by the addition of ZnCl$_2$ from 0.1 or 1 M stock solutions. The low solubility of Zn(OH)$_2$ limits the maximum concentration of Zn$^{2+}$ that can be used at pH 7.4 to less than 5 mM. Our standard patch pipette solution for recording K$_{o}^+$ currents contained, in mM, 130 KCl, 4.75 CaCl$_2$ (pCa$_{2}^+$ = 7.3), 1.38 MgCl$_2$, 10 EGTA, 10 Hepes and was adjusted to pH 7.4 with KOH.

For gating current recordings the bath solution contained, in mM, 140 NMGCl, 1 MgCl$_2$, 10 Hepes (pH 7.4) or Mes (pH 5.4), 2 CaCl$_2$, 10 glucose and the pH was adjusted with HCl; the patch pipette solution consisted of 140 NMGCl, 1 MgCl$_2$, 10 Hepes, 10 EGTA and was adjusted to pH 7.2 with HCl. Chemicals were from the Sigma Aldrich Chemical Co. (Mississauga, ON, Canada).
In an experiment, a section of glass coverslip with cells attached to it was placed in the recording chamber (0.5 ml volume) and perfused with 5-10 ml of control solution. After recording control currents the chamber was flushed with 5-6 ml of test solution to ensure complete replacement of the bath solution before treated responses were recorded. If after perfusing 5-6 ml of control solution the post-treatment currents did not recover to within ± 10% of the pre-treatment amplitudes the entire data set was discarded. In most cells, however, virtually complete recovery was observed. We found no difference between experiments done with discontinuous perfusion, as described above, and experiments where the cells were continuously perfused (not shown).

Signal recording and data analysis

Macroscopic currents were recorded at room temperature (20-25 °C) using the patch clamp technique primarily in the whole cell configuration. In some of the cell lines expressing mutant hKv1.5 channels at a high level, i.e., the H463Q and some of the R487V mutants, the large amplitude of the whole cell currents necessitated recording macroscopic currents from outside-out patches. Voltage clamp experiments were done with an EPC-7 patch clamp amplifier and Pulse+PulseFit software (HEKA Electronik, Germany). Patch electrodes were made from thin-walled borosilicate glass (World Precision Instruments, FL, USA) and had a resistance of 1.0 to 2.5 MΩ measured in the bath with standard internal and external saline. Capacitance and series resistance compensation, typically 80%, were used. An on-line P/N method, for which the holding potential was -100 mV and the scaling factor was 0.25, was used to subtract leak and any uncompensated capacitive currents. Current signals filtered at 3
kHz (-3dB, 8-pole Bessel filter) were digitized (16 bit) at a sampling interval of 100 μs (10 kHz). Voltages have been corrected for the liquid junction potentials.

To quantify the inhibition of currents, tail currents were recorded at -50 mV following a depolarizing pre-pulse. Peak tail current amplitudes were then obtained by extrapolation of a single exponential function fitted to the tail current decay to the start of the step to -50 mV. After normalization of tail currents either to the maximum current of the control or the treated response, data points were fitted to a single Boltzmann function:

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

where, when \(y\) is the current normalized with respect to the control response, \(A\) is the proportion of the control \(g_{max}\). When \(y\) is the current normalized with respect to the maximal treated current, \(A\) is the best fit value for the normalized maximal response and ideally has a value of unity. \(V_{1/2}\) is the half-activation potential or mid-point of the activation curve, \(V\) is the voltage during the pre-pulse and \(s\) is the slope factor, in mV, reflecting the steepness of the voltage dependence of gating.

To quantify gating charge movement during activation or deactivation, charge-voltage \((Q(V))\) curves were generated by time integration of on- or off-gating currents as described previously (Chen et al., 1997). Activation gating in hKv1.5 is best fit by the sum of two Boltzmann functions where the larger component, known as \(Q_2\), represents ~80% of the total charge movement (Hesketh & Fedida, 1999). However, for simplicity, \(Q(V)\) data obtained at pH 7.4 and 5.4 were fitted to equation (1) where \(y\) is the charge moved, \(A\) is the maximal charge \((Q_{max})\) and \(V\) is the voltage at which the on-gating charge \((Q_{on})\) or off-gating charge \((Q_{off})\) is evoked. \(V_{1/2}\) and \(s\) remain as described above.
Concentration-response data for Zn$^{2+}$ were fitted to the Hill equation:

$$
y = \frac{1}{1 + \left(\frac{[Zn^{2+}]}{K_{zn}}\right)^{n_{Hill}}} \quad (2)
$$

where $y$ is the proportion of the control $g_{max}$, $K_{zn}$ is the equilibrium dissociation constant for Zn$^{2+}$ binding and $n_{Hill}$ is the Hill coefficient reflecting the number of Zn$^{2+}$ ions binding per channel. For protons, equation (2) was modified to account for the fact that the data points were normalized to the response at pH 7.4:

$$
y = \frac{K_{H}^{n_{Hill}} + (10^{-7.4})^{n_{Hill}}}{K_{H}^{n_{Hill}} + [H^{+}]^{n_{Hill}}} \quad (3)
$$

where $K_{H}$ is the equilibrium dissociation constant for proton binding.

The equation used to model the binding interaction between K$^{+}$, H$^{+}$, and the hKv1.5 channel has been described previously (Zhang et al., 2001a) and is also known as the ternary-complex model of interaction that is used to describe the binding of two ligands to the same receptor.
\[ y = \frac{1}{1 + \left[ \frac{[H^+]}{K_H} + \frac{[K^+]_o}{K_K} \right] \left( 1 + \frac{[K^+]_o}{K_K} \right)} \tag{4} \]

where \( K_H \) is the equilibrium dissociation constant for proton binding in zero \( K^+ \), \( K_K \) is the equilibrium dissociation constant for \( K^+ \) binding at pH 7.4 and the parameter \( \alpha \) is known as the cooperativity factor or the coupling constant/factor. A value for \( \alpha \) greater than unity indicates negative cooperativity. To reduce the number of parameters in the model the Hill coefficient for \( K^+ \) binding was assumed to be unity.

Data are expressed as the mean ± the standard error of the mean (SEM) except for the values obtained by non-linear least-squares fitting routines (Igor, Wavemetrics, OR, USA) which are expressed as the mean ± the standard deviation (SD). The paired-sample \( t \) test (control versus treated) was used to assess the actions of protons and \( \text{Zn}^{2+} \) on the inactivation rate. A \( P \)-value of 0.05 or less was considered significant.

RESULTS

**Increasing \([H^+]_o\) causes a gating shift and reduces the maximum conductance (\( g_{\text{max}} \))**

Representative traces in Figure 2.1 show the effect of changing \( \text{pH}_o \) from 7.4 to pH 6.4 in nominally \( K^+ \)-free medium (zero \( K^+ \)) in which \( \text{Na}^+ \) was the major extracellular cation. For the control currents the voltage protocol consisted of a 300 ms pulse command between -40
and 40 mV in 5 mV increments followed immediately by a 300 ms command to -50 mV to record the tail current. The robust pulse and ensuing tail currents, shown at a higher gain in the inset, obtained during or following strong depolarizations in pH 7.4 medium (Figure 2.1A) are consistent with a failure of hKv1.5 currents to disappear or “collapse” in zero K\textsuperscript{+} (Jäger & Grissmer, 2001). After switching to pH 6.4 medium the range of the pulse voltages was changed from -30 to 60 mV to compensate for a small rightward shift of the voltage dependence of gating, the so-called gating shift. As noted by Steidl & Yool (1999), there appeared also to be a slight slowing of the activation kinetics with extracellular acidification but this was not systematically studied and was certainly not as pronounced as the slowing caused by Zn\textsuperscript{2+} (Zhang et al., 2001a). A more profound effect of the increased extracellular acidity, and the main focus of this report, was a large reduction of pulse and tail current amplitudes. Figure 2.1C, which was derived in part from the traces shown in Figure 2.1A,B, plots the tail current amplitudes at -50 mV measured as described in Methods and fitted to a Boltzmann function. In this cell, increasing H\textsuperscript{+} caused V\textsubscript{h} to shift from -6.2 mV to 4.3 mV and the maximal tail current amplitude at pH 6.4 was 14 % of that measured at pH 7.4. Both the gating shift and the current reduction reversed completely and rapidly (e.g., Figure 2.9) after returning to pH 7.4.
Figure 2.1. Extracellular acidification decreases the maximum conductance ($g_{\text{max}}$) and causes a rightward shift of the conductance-voltage ($g(V)$) relationship for Kv1.5 currents. Panels A and B show, respectively, representative control (pH 7.4) and treated (pH 6.4) currents evoked by the voltage protocol shown above each family of traces. Successive pulse command voltages were incremented by 5 mV but for clarity only alternate traces are shown. The change of the range of pulse voltages at pH 6.4 was necessary to compensate for the gating shift. The holding potential in this and other figures was -80 mV, except where noted. Inset traces show the tail currents at a higher gain. Tail current amplitudes, obtained by extrapolating the fit of a single exponential function to the start of the step to -50 mV, are plotted in C and fitted to a Boltzmann function to obtain the equivalent of the $g(V)$ relationship. Acidification shifted the $V_\theta$ from -6.2 mV to 4.3 mV and the maximum current decreased from 1.7 nA to 0.24 nA which corresponds to a $g_{\text{max}}$ relative to that at pH 7.4 (relative $g_{\text{max}}$) of 0.14.
External protons have been reported to reduce the amplitude of rKv1.5 currents (Steidl & Yool, 1999) however the reduction (~40 %) was substantially less than shown in Figure 2.1 (~85 %) and in the left column of Figure 2.2 which summarizes the results obtained in 12 such experiments. Since our previous work showed that the reduction of hKv1.5 currents by Zn$^{2+}$ was affected by K$^+$, we next addressed the possibility that this apparently greater potency of the inhibition by protons of hKv1.5 currents shown in Figure 2.1 was due to the use of a zero K$^+$ bathing solution.

**Increasing [K$^+$]$_o$ inhibits the reduction of $g_{max}$ by extracellular acidification**

Current traces in the left, centre and right columns of Figure 2.2 were recorded from cells in which K$^+o$ was zero, 3.5 and 140 mM, respectively, and the pH$_o$ was changed from 7.4 (upper row of traces) to 6.4 (lower row of traces). The voltage protocol was similar to that described for Figure 2.1. Graphs at the foot of each column show the tail currents from a number of similar experiments for control (empty circles) and treated responses normalized either with respect to the maximum control tail current (filled circles) or to the maximum treated tail current (filled squares). That K$^+o$ inhibits the proton-induced current reduction is shown by the increase of the relative $g_{max}$ from $0.19 \pm 0.02$ ($n = 12$) in zero K$^+o$, to $0.56 \pm 0.01$ ($n = 6$) in 3.5 mM K$^+o$ and finally to $0.81 \pm 0.12$ ($n = 6$) in 140 mM K$^+o$. As with the Zn$^{2+}$ block, the gating shift at pH 6.4 was not significantly affected by changes of K$^+o$ (see Figure 2.2 legend for $V_1/s$ and $s$ values) suggesting that the proton-induced gating shift and current inhibition are independent effects.
Figure 2.2. Increasing K\textsuperscript{+}\textsubscript{o} reduces the inhibition of hKv1.5 current by protons. Traces obtained from three different cells showing the current at pH 7.4 (control, top row) and pH 6.4 (treated, lower row) in, from left to right, zero, 3.5 and 140 mM K\textsuperscript{+}\textsubscript{o}. In zero K\textsuperscript{+}\textsubscript{o}, control and treated pulse currents were evoked by 300-ms pulses from -50 to 45 mV in 5 mV steps; in 3.5 mM K\textsuperscript{+}\textsubscript{o}, the pulse command range was -50 to 45 mV at pH 7.4 and -30 to 65 mV at pH 6.4; in 140 mM K\textsuperscript{+}\textsubscript{o}, the range for pulse voltages was -40 to 55 mV. For clarity, only alternate current traces are shown. The corresponding control (○) and treated g(V) relationships, obtained from a number of similar experiments, are shown in the graph at the bottom of each column. Treated data were normalized with respect both to the g\textsubscript{max} at pH 6.4 (■) and to the control g\textsubscript{max} (○). The relative g\textsubscript{max} at pH 6.4 in zero, 3.5 and 140 mM K\textsuperscript{+}\textsubscript{o} was 0.19 ± 0.02 (n = 12), 0.56 ± 0.01 (n = 6), and 0.81 ± 0.12 (n = 6), respectively. In zero K\textsuperscript{+}\textsubscript{o}, V\textsubscript{1/2} and s changed from -21.4 ± 4.3 and 4.7 ± 0.5 mV at pH 7.4 to -8.2 ± 4.0 and 7.1 ± 0.3 mV at pH 6.4, respectively. In 3.5 mM K\textsuperscript{+}\textsubscript{o}, the corresponding values were -18.3 ± 1.9 mV and 3.9 ± 0.4 mV at pH 7.4 and -10.5 ± 1.2 mV and 3.9 ± 0.4 mV at pH 6.4; and, in 140 mM K\textsuperscript{+}\textsubscript{o}, -26.2 ± 1.1 mV and 3.8 ± 0.3 mV at pH 7.4 and -12.3 ± 1.1 mV and 3.8 ± 0.4 mV at pH 6.4.
Figure 2.3. The concentration dependence of the inhibition of Kv1.5 currents by protons in zero (○), 5 (■) and 140 mM (▲) K⁺. Data for zero K⁺ were obtained with either 143.5 mM Na⁺ (●) or 143.5 NMG⁺ (○) as the major extracellular cation. The lines represent the best fit to equation (3). The fitted values for the equilibrium dissociation constant for protons (K_H), the pH and n_Hill were, in zero K⁺ and 143.5 NMG⁺: 128 ± 53 nM (mean ± SD), 6.9 and 1.2 ± 0.5; in zero K⁺ and 143.5 mM Na⁺: 153 ± 13 nM, 6.8 and 1.5 ± 0.2; in 5 mM K⁺: 590 ± 85 nM, 6.2 and 1.6 ± 0.4; and in 140 mM K⁺: 1.1 ± 0.11 μM, 6.0, and 1.8 ± 0.3.

Although the pH estimates with either Na⁺ or NMG⁺ as the extracellular cation are similar, the increase of the relative g_max with NMG⁺ at pH 8.4 was significantly greater. Consistent with a non-competitive versus competitive interaction between H⁺ and K⁺ (see Figure 2.4) the increase of K_H going from zero to 5 mM K⁺ was greater than that going from 5 mM to 140 mM.
Data obtained by repeating experiments of the type shown in Figure 2.2 over a range of pHs were fitted to the Hill equation to generate the concentration-response curves shown in Figure 2.3. In zero $K^+_o$ medium in which $Na^+$ was the predominant metal cation (filled circles) the best fit to the data gave a $K_H$ of $153 \pm 13 \text{nM (pK}_H \sim 6.8)$ and a Hill coefficient, $n_{Hill}$, of $1.5 \pm 0.2$ which suggests that inhibition requires protonation of at least two sites. To determine if $Na^+$ ions affect the current inhibition by protons, the zero $K^+_o$ experiments were also done with NMG$^+$ as the major extracellular cation. With NMG$^+_o$ the $K_H$ was $128 \pm 53 \text{nM (pK}_H \sim 6.9)$ and $n_{Hill}$ was $1.2 \pm 0.5$ (open circles and dashed line of Figure 2.3). This suggests that external $Na^+$ ions do not affect the current inhibition by protons. With 5 mM $K^+_o$ the $K_H$ increased to $590 \pm 85 \text{nM (pK}_H \sim 6.2)$ but the value for $n_{Hill}$ of $1.6 \pm 0.4$ was not significantly different from that with zero $K^+_o$. In comparison to the substantial rightward shift caused by increasing $K^+_o$ from zero to 5 mM, a much smaller increase of the $K_H$ to $1.1 \pm 0.11 \text{\mu M (pK}_H \sim 6)$ was obtained when $K^+_o$ was increased from 5 to 140 mM. The $n_{Hill}$ in 140 mM $K^+_o$ was $1.8 \pm 0.3$.

$K^+_o$ relief of the effect of protons is fitted by a model of non-competitive inhibition

As noted with Zn$^{2+}$ block of hKv1.5 channels (Zhang et al., 2001a), the greater relief of the proton-induced current inhibition when $K^+_o$ was changed from zero to 5 mM $K^+_o$ compared to when it was changed from 5 to 140 mM $K^+_o$, suggested that $K^+$ ions and protons were not competing for a common site. For that reason we modelled the interaction between $K^+_o$ and $H^+_o$ as an allosteric inhibition (equation (4)), by which we mean that the interaction is mediated via separate binding sites and is therefore non-competitive.

For this analysis (Figure 2.4) we focussed in particular on the current inhibition at pH 6.4 with $K^+_o$ concentrations of zero, 1, 3.5, 5, 10, 20, 80 and 140 mM. The fit of these data to
equation (4) gave mean values (± SD) of 150 ± 1900 nM for $K_H$, 1.33 ± 17 for $n_{Hill}$, 0.68 ± 9 for $K_K$ and 6.2 ± 14.9 for $\alpha$, the factor by which bound $H^+/K^+$ inhibits the binding of $K^+/H^+$.

To reduce the SD of the estimates for $K_K$ and $\alpha$, we fixed the values for $K_H$ and $n_{Hill}$ in the fitting routine at 153 nM and 1.5, respectively, based on the data of Figure 2.3 (zero $K_{o}^+$, 143.5 Na$^+$). This was justified on the basis of the similarity to the values for $K_H$ and the $n_{Hill}$ from Figure 2.3 and the preliminary fit (i.e., with the four parameters free) of the data at pH 6.4. With $K_H$ and $n_{Hill}$ fixed, the fit of the data at pH 6.4 gave estimates for $K_K$ and $\alpha$ of 0.65 ± 0.27 mM and 5.5 ± 0.7, respectively.

At pH 6.9 and pH 5.9 the relative $g_{\text{max}}$ was measured with zero, 5, 20 and 140 mM $K_{o}^+$. For the data at pH 5.9 the best fit values for $K_K$ and $\alpha$, with $K_H$ and $n_{Hill}$ constrained as above, were 0.66 ± 0.48 mM and 6.2 ± 1, respectively; at pH 6.9 the corresponding values were 0.93 ± 2.8 mM and 6.2 ± 9.1.

**External Cs$^+$ ions mimic the block-relieving effect of K$^+$**

In hKv1.5 channels the permeability of Cs$^+$ ions relative to K$^+$ ions is approximately 0.2 (Fedida et al., 1999) and the $K_D$ for the relief by Cs$^+_o$ ($K_{Cs}$) of the Zn$^{2+}$ block is some 5-6 fold higher than the $K_K$ (Zhang et al., 2001a). Surprisingly, with the same experimental protocol but using Cs$^+$ at concentrations of 3.5, 20 and 140 mM (open inverted triangles of Figure 2.4) the ability of Cs$^+_o$ to antagonise the current inhibition by protons was indistinguishable from that of K$^+_o$. 

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Figure 2.4. The concentration dependence of the antagonism by K⁺₀ and Cs⁺₀ of the inhibition of hKv1.5 currents by H⁺₀. The relative \( g_{\text{max}} \) at different K⁺₀ concentrations is plotted for pH 6.9 (■), pH 6.4 (▲) and pH 5.9 (●). The data for pH 6.9 and 5.4 were obtained with zero, 5, 20 and 140 mM K⁺₀. At pH 6.4, K⁺₀ was zero, 1, 3.5, 5, 10, 20, 80 and 140 mM. Assessment of the block-relieving effect of Cs⁺₀ (▲) was done with concentrations of 3.5, 20 and 140 mM. The lines represent the best fit of the data to equation 4 (see Methods). With the values for \( K_H \) and \( n_{Hill} \) fixed to those obtained directly from the data in Figure 2.3 (153 nM and 1.5, respectively) the best fit values for \( K_K \) and \( \alpha \) were 0.65 ± 0.27 mM and 5.5 ± 0.7. Cs⁺₀ appears to be equivalent to K⁺₀ in its antagonism of the proton block. The best fit of the data at pH 6.9 was obtained with 0.93 ± 2.8 mM for \( K_K \) and 6.2 ± 9.1 for \( \alpha \); at pH 5.9 the corresponding values were 0.66 ± 0.48 mM and 6.2 ± 1. These estimates for \( K_K \) are very near those estimated for the K⁺₀ relief of the Zn²⁺ block (~ 0.5 mM) (Zhang et al., 2001a).
Sensitivity to $H^+$ and $Zn^{2+}$ inhibition is reduced in hKv1.5-H463Q

The range of $pK_{H^+}$s for the inhibition of hKv1.5 is consistent with the titration of one or more histidine residues and, as noted above, in rKv1.5 channels in which glutamine (Q) is substituted for H452, the homologue of H463 in hKv1.5, there is a substantially reduced proton sensitivity (Steidl & Yool, 1999). Based on the crystal structure of KcsA (Figure 2.5) (Doyle et al., 1998) H463 is presumed to be located in the outer rim or “turret” of the pore mouth. Since $Zn^{2+}$ ions also bind avidly to histidine residues this raised the possibility that the current inhibition caused either by $Zn^{2+}$ or $H^+$ involves binding to one or more of the H463s in the turret of the homotetrameric hKv1.5 assembly. To test that hypothesis we examined the concentration dependence of the conductance decrease by $H^+$ and $Zn^{2+}$ in the mutant hKv1.5-H463Q. These experiments were done in zero $K^+$ so that the interpretation of the results would not be complicated by a change, if any, of the affinity of the site at which $K^+$ ions bind to produce an allosteric inhibition of the actions of $Zn^{2+}$ and $H^+$.

The $g(V)$ relationships (Figure 2.6A) and concentration-response curves (Figure 2.6B) for the proton sensitivity of hKv1.5-H463Q, confirmed the results reported for rKv1.5. Thus, the gating shift was apparently intact but the decline of $g_{max}$ was seen only with much higher proton concentrations. Fitting of the concentration-response data (Figure 2.6B) to the Hill equation gave an estimate for $K_H$ of $4.7 \pm 1.9 \mu M (pK_H = 5.3)$ and an $n_{Hill}$ of $1 \pm 0.4$ versus the corresponding values of $0.15 \mu M (pK_H = 6.8)$ and $1.5$ in wt hKv1.5. The acid sensitivity of hKv1.5-H463Q is therefore quite comparable to that of rKv1.5-H452Q where the $pK_H$ is $\sim 5.2$ (Steidl & Yool, 1999).

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Figure 2.5. The structure of the S5, S6 and the pore (P) loop of Kv1.5 inferred from the crystal structure of KcsA. A. The sequence alignment for Kv1.5, Shaker and KcsA between the turret and the outer pore mouth. B. A side view of the KcsA channel in which the foreground and background α subunits have been removed for clarity. The α-subunit of voltage-gated K⁺ channels has an additional 4 transmembrane segments (S1-S4) that are not illustrated. Sites at which mutations were made, namely H463 and R487, are shown at their homologous positions in the KcsA crystal structure. The orientation of the side chains of these two residues is tentative.
Figure 2.6. A point mutation in the turret (S5-P loop), H463Q, reduces the inhibition but not the gating shift caused by $H^+$ and $Zn^{2+}$. A. The $g(V)$ relationship in zero $K^+$ at pH 8.4 (●), pH 7.4 (○), pH 6.4 (■), pH 5.9 (▲), and pH 5.5 (▼) after normalization with respect to the $g_{\text{max}}$ at pH 7.4. Values for the relative $g_{\text{max}}$, $V_{1/2}$, and $s$ were: at pH 8.4, 1.1 ± 0.02, -23.9 ± 1.3 mV and 5.4 ± 0.5 mV ($n = 5$); at pH 7.4, 1, -20.1 ± 1.0 mV and 4.6 ± 0.2 mV ($n = 28$); at pH 6.4, 1.06 ± 0.03, -13.0 ± 0.5 mV, and 4.0 ± 0.5 mV ($n = 3$); at pH 5.9, 0.86 ± 0.08, 7.6 ± 0.7 mV, and 5.6 ± 0.4 mV ($n = 8$); and, at pH 5.5, 0.63 ± 0.04, 19.2 ± 1.7 mV and 5.7 ± 0.4 mV ($n = 7$). B. The concentration dependence of the reduction of $g_{\text{max}}$ by protons. Fitting of the data to the Hill equation gave a $K_D$ of 4.7 ± 1.9 μM ($pK_H$ of 5.3) and $n_{\text{Hill}}$ of 1.0 ± 0.4. The $g_{\text{max}}$-$H^+$ concentration relationship for wt hKv1.5 is represented by the dashed line. C. The $g(V)$ relationship as described for (A) but with zero (○), 50μM (▲), 200μM (▼), 1mM (■) and 2.5mM (▲) of $Zn^{2+}$. The relative $g_{\text{max}}$, $V_{1/2}$, and $s$ were 1, -14.9 ± 0.9 mV, and 4.8 ± 0.3 mV for the control ($n = 27$); 0.87 ± 0.08, -1.9 ± 0.9 mV, and 6.2 ± 0.9 mV for 50μM $Zn^{2+}$ ($n = 5$); 0.77 ± 0.09, 7.1 ± 1.1 mV, and 5.9 ± 0.2 mV for 200μM $Zn^{2+}$ ($n = 6$); 0.47 ± 0.04, 18.5 ± 2.0 mV, and 6.0 ± 0.7mV for 1mM $Zn^{2+}$ ($n = 10$); and 0.52 ± 0.009, 27.2 ± 2.3 mV, and 6.4 ± 0.8mV for 2.5mM $Zn^{2+}$ ($n = 5$). D. As described for (B) but with $Zn^{2+}$. The best fit values for $K_{Zn}$ and $n_{\text{Hill}}$ were 1.7 ± 1 mM and 0.5 ± 0.2. The dashed line indicates the concentration-response relationship for wt Kv1.5 in zero $K^+$ ($K_{Zn} = 69$ μM, $n_{\text{Hill}} = 0.9$) (Zhang et al., 2001a).
Tests of the effects of Zn\(^{2+}\) on the H463Q mutant showed that the outcome (Figure 2.6C,D) mirrored that seen with protons. Because of the limited solubility of Zn(OH)\(_2\) the highest concentration of Zn\(^{2+}\) we tested was 2.5 mM and consequently a full concentration-response curve could not be obtained. From the limited concentration range over which data were collected the extrapolated \(K_{zn}\) was 1.7 ± 1 mM or roughly 25-fold higher than for wt hKv1.5 (Zhang et al., 2001a). The \(n_{Hill}\) for the inhibition by Zn\(^{2+}\) of wt hKv1.5 and hKv1.5-H463Q currents was 0.9 (Zhang et al., 2001a) and 0.5 (Figure 2.6), respectively.

In the course of this series of experiments we became aware of a report that currents through hKv1.5-H463G channels were completely suppressed upon changing from 4.5 mM to zero K\(^{+}\) medium at pH 7.4 (Jäger & Grissmer, 2001). This result was surprising since no such effect is apparent with the hKv1.5-H463Q mutant under the same recording conditions (Figure 2.6, 2.7A). Our experiments with hKv1.5-H463G confirmed this conductance collapse in zero K\(^{+}\) at pH 7.4 (Figure 2.7C) and we also noted that there was a striking increase in the inactivation rate in 3.5 K\(^{+}\) (Figure 2.7B) that was not previously reported.

Thus, in contrast to wt hKv1.5 (Figure 2.1) and hKv1.5-H463Q (Figure 2.7A) where there is little or no current decay evident during 300 ms pulse commands, in hKv1.5-H463G the current decay at 40 mV is well-fitted by a single exponential function with a time constant of 73 ± 8 ms \((n = 4)\) (Figure 2.7B).
Figure 2.7. In hKv1.5-H463G slow inactivation is greatly accelerated and the conductance collapses in zero K\textsuperscript{+} at pH 7.4. A. Shown for comparison are the currents from hKv1.5-H463Q evoked in zero K\textsuperscript{+} by 300 ms pulses to between -40 and 40 mV in 10 mV increments. B. hKv1.5-H463G currents recorded using the same stimulus protocol but with 3.5 mM K\textsuperscript{+}. The solid line superimposed on the current at 40 mV represents the best fit of the current decay to a single exponential function. The mean time constant for inactivation at 40 mV was 73 ± 8 ms (n = 4). C. From the same cell as in B and using the same voltage command protocol after switching to zero K\textsuperscript{+} at pH 7.4. Unlike either wt Kv1.5 (Figure 2.1) or Kv1.5-H463Q, K\textsuperscript{+} is required for hKv1.5-H463G channels to function normally at pH 7.4. Complete recovery was obtained after returning to K\textsuperscript{+}-containing bath solution (not shown).
**H\textsuperscript{+}\textsubscript{o} and Zn\textsuperscript{2+} accelerate inactivation**

In rKv1.5 H\textsuperscript{+} has been shown to accelerate inactivation, an effect that was evident only with long depolarizing commands (Steidl & Yool, 1999). Similarly, in hKv1.5 there was no obvious change of inactivation kinetics during 300 ms depolarizations but an increased inactivation rate was evident with H\textsuperscript{+} as well as Zn\textsuperscript{2+} during depolarizations lasting for several seconds (not shown). Fitting a single exponential function to the current decay during a 7 to 10 s depolarization at 60 mV in external medium with 5 mM K\textsuperscript{+} at pH 7.4 gave a time constant for inactivation ($\tau_{\text{inact}}$) of $2.63 \pm 0.11$ s ($n = 4$). In the same cells, extracellular acidification to pH 6.4 caused a roughly 50% reduction of $\tau_{\text{inact}}$ to $1.19 \pm 0.04$ s ($P < 0.05$).

Using the identical stimulation protocol we found that the changeover from Zn\textsuperscript{2+}-free medium at pH 7.4 to medium at the same pH and containing 1 mM Zn\textsuperscript{2+} reduced $\tau_{\text{inact}}$ by approximately 30% from $3.0 \pm 0.18$ s to $2.14 \pm 0.16$ s ($n = 4$, $P < 0.05$). Although these results confirm that current inhibition by H\textsuperscript{+} and Zn\textsuperscript{2+} is associated with a moderately increased rate of inactivation we suggest below that this cannot account for the reduction of $g_{\text{max}}$.

**Current inhibition by protons and Zn\textsuperscript{2+} is reduced in hKv1.5-R487V**

To more directly address the possibility that the reduction of $g_{\text{max}}$ reflected an effect on one or more inactivation processes we next examined the actions of H\textsuperscript{+} and Zn\textsuperscript{2+} in a hKv1.5 mutant in which an arginine (R) residue in the P-S6 region was mutated to valine (V) (R487V, Figure 2.5). This was motivated by the fact that mutations at the homologous site (T449) in N-type (fast) inactivation-removed Shaker channels (Shaker-IR) either accelerates (T449E, T449K, T449A) or slows (T449Y, T449V) inactivation (Lopez-Barneo et al., 1993).
A previous study of hKv1.5-R487V showed that inactivation was indeed dramatically slowed when channel currents were carried by Na\(^+\) but, curiously, the time course of K\(^+\) currents was relatively unchanged (Wang et al., 2000). It has also been proposed that a charged residue at position 487 is critical for the current inhibition by H\(^+\)\(_o\) (Jäger & Grissmer, 2001).

Figure 8 summarizes the results of experiments assessing the inhibition of hKv1.5-R487V by protons and Zn\(^{2+}\) ions in zero K\(^+\)\(_o\) (143.5 mM Na\(^+\)) medium. The \(g(V)\) relationships derived from tail current measurements (Figure 2.8A) show that the gating shift was apparently intact in the R487V mutant. However, there was a dramatic change of the concentration dependence of the H\(^+\)\(_o\)-induced conductance decline. For example, whereas in wt hKv1.5 the relative \(g_{\text{max}}\) at pH 5.9 was 0.07 ± 0.01 (\(n = 9\)) (Figure 2.4), in hKv1.5-R487V the relative \(g_{\text{max}}\) at the same pH was 0.92 ± 0.03 (\(n = 5\))(Figure 2.8A,B). An extrapolated \(pK_H\) of 4.6 obtained from the best fit of the concentration-response data of Figure 2.8B suggests a shift of ~2 pH units from the \(pK_H\) of wt hKv1.5 channels.

Tests of the sensitivity of hKv1.5-R487V channels to Zn\(^{2+}\) (Figure 2.8C,D) showed that the gating shift was, again, substantially unaffected and, as with H\(^+\)\(_o\), there was a clear increase of the Zn\(^{2+}\) concentration required to cause 50 % inhibition. Thus, whereas wt hKv1.5 currents were half-inhibited by 0.07 mM Zn\(^{2+}\) (Zhang et al., 2001a), in the R487V mutant 41.2 ± 1.7 % (\(n = 5\)) of \(g_{\text{max}}\) persisted in 2.5 mM Zn\(^{2+}\). Closer inspection of the concentration-response data of Figure 2.8D suggested that two Zn\(^{2+}\) binding sites might be involved in the inhibition of hKv1.5-R487V currents. Subsequent experiments with the double mutant hKv1.5-H463Q, R487V (open triangles of Figure 2.8D) implied that the higher affinity site (\(K_{Zn} = 29 \, \mu\text{M}\)) that accounted for approximately 20 % of conductance
A mutation near the pore mouth, R487V, substantially reduces the sensitivity to inhibition by H\(^+\) and Zn\(^{2+}\). A. The \(g(V)\) relationship in zero K\(^+\), at pH 8.4 (●), pH 7.4 (○), pH 6.4 (■), pH 5.9 (▲), and pH 5.5 (▼) after normalization with respect to \(g_{\text{max}}\) at pH 7.4. The values for the relative \(g_{\text{max}}, V_{1/2}\), and \(s\) were, respectively, 1.04 ± 0.02, -28.5 ± 1.1 mV, 4.6 ± 1.0 mV at pH 8.4 (\(n = 3\)); 1.04 ± 0.06, -18.1 ± 0.9 mV, 4.5 ± 0.2 mV at pH 7.4 (\(n = 17\)); 0.92 ± 0.03, 6.4 ± 1.3, 4.9 ± 0.4 at pH 5.9; and, 0.87 ± 0.03, 15.5 ± 1.6 mV, 5.5 ± 0.2 mV at pH 5.5 (\(n = 5\)). B. The concentration-response relationship for the reduction of \(g_{\text{max}}\) by protons. The continuous line, representing the best fit of the data to the Hill equation, was obtained with \(K_H = 23\ \mu\text{M}\) (\(pK_H\) of 4.6) and \(n_{Hil} = 0.8\). C. The \(g(V)\) relationship in zero K\(^+\) and with Zn\(^{2+}\) concentrations of 10 µM (●), 25µM (■), 100 µM (○), 200 µM (▼), 1 mM (■), and 2.5 mM (▲) after normalization with respect to the control (○) \(g_{\text{max}}\). The relative \(g_{\text{max}}, V_{1/2}\), and \(s\) were, respectively, 1.0, -13.4 ± 1.5 mV, 4.5 ± 0.3 mV for the control (\(n = 15\)), 0.99 ± 0.01, -5.9 ± 1.5 mV, 5.4 ± 0.4 mV in 10µM Zn\(^{2+}\) (\(n = 4\)); 0.92 ± 0.05, -5.7 ± 0.1 mV, 4.7 ± 0.5 mV in 25µM Zn\(^{2+}\) (\(n = 3\)); 0.80 ± 0.02, 2.8 ± 1.8 mV, 4.8 ± 0.5 mV in 100µM Zn\(^{2+}\) (\(n = 4\)); 0.78 ± 0.02, 5.2 ± 1.6 mV, 5.3 ± 0.3 mV in 200µM Zn\(^{2+}\) (\(n = 5\)); 0.70 ± 0.05, 21.0 ± 1.2 mV, 5.9 ± 0.3 mV in 1mM Zn\(^{2+}\) (\(n = 3\)); and, 0.59 ± 0.02, 28.9 ± 1.1 mV, 5.9 ± 0.3 mV in 2.5mM Zn\(^{2+}\) (\(n = 5\)). D. As described for (B) but with Zn\(^{2+}\). The continuous line represents the best fit of the hKv1.5 R487V data to the sum of two Hill equations. Binding at the higher affinity site (\(K_{Zn} = 29 ± 0.2 \mu\text{M}\)) accounted for ~20 % of the inhibition. The apparent elimination of the higher affinity site in the double mutant Kv1.5-R487V, H463Q (▲ and dashed line) suggests that it may reflect Zn\(^{2+}\) binding to H463. The extrapolated \(K_{Zn}\) for the lower affinity site in the R487V mutant was 6.4 ± 0.07 mM. Again, the dotted lines in B and D represent the corresponding concentration-response curves for wt hKv1.5 (Zhang et al., 2001a).
decline in the R487V mutant, was apparently eliminated. The latter observation could be accounted for in many ways, perhaps the simplest being that the higher affinity site in the R487V mutant reflects the binding of Zn\(^{2+}\) to one or more H463 residues. The concentration dependence of the reduction of \(g_{\text{max}}\) in the double mutant was best fitted by a single Hill function with a \(K_{Zn}\) of 2.2 mM, representing an approximately 30-fold increase over that measured in wt hKv1.5 under the same recording conditions.

**Current inhibition by H\(^{+}\)\(_{o}\) and Zn\(^{2+}\) is apparently not use-dependent**

If, as has been proposed to account for the block of rKv1.5 currents by H\(^{+}\)\(_{o}\) (Steidl & Yool, 1999), the inhibition of hKv1.5 currents by Zn\(^{2+}\) or extracellular acidification were due to an accumulation of inactivation then the degree of inhibition would be expected to show use-dependence. Figure 2.9 shows the results of a representative experiment addressing this issue. Peak tail current amplitudes following 300 ms depolarizations from -80 mV to 60 mV at pH 5.9 are bracketed by control and recovery responses at pH 7.4. \([K^+]_o\) was 3.5 mM. Two features of the current behaviour at pH 5.9 are significant. First, inhibition of the current is apparent with the first pulse and is more-or-less constant for each of the subsequent pulses during a train of 10 pulses delivered at 5 s intervals. Second, a 2 min stimulus-free interval in which the membrane was held at either -80 mV or -100 mV had no block-relieving effect. Consequently, despite the fact that both Zn\(^{2+}\) and H\(^{+}\)\(_{o}\) slightly enhance the rate of inactivation of residual hKv1.5 currents, there is no support for the hypothesis that accumulation of inactivation accounts for the reduction of \(g_{\text{max}}\). Finally, Figure 2.9 also demonstrates the rapid reversal, i.e., within the time course of fluid exchange in the bath, of the current inhibition after beginning the perfusion with pH 7.4 solution. The latter observation argues against a mechanism involving a change of the internal pH concomitant to extracellular acidification.
Figure 2.9. The effect of the stimulus frequency and holding potential on the inhibition of wt hKv1.5 currents by $H^+$. This graph, which is representative of the results obtained from six such experiments, three at pH 5.9 and three with 1 mM Zn$^{2+}$, plots the amplitude of tail currents measured at -50 mV following a 300 ms step to 60 mV to maximally activate channels. After 10 consecutive control responses in standard external saline (pH 7.4, 3.5 mM $K^+$) and evoked at 5 s intervals from a holding potential of -80 mV, pulsing was stopped and 5 ml of test solution was perfused to change the extracellular pH to 5.9 for the duration indicated by arrows. Resumption of the step commands approximately 2 minutes after extracellular acidification showed an immediate ~75 % reduction of the tail current amplitude. The identical effect was obtained for each of two subsequent pulse trains confirming that the inhibition was not affected by a period without stimulation. Changing the holding potential to -100 mV also had no effect on the current amplitude. Returning to pH 7.4 medium while pulsing shows the effect rapidly (within 15 s) and completely reverses implying that a change of the internal pH is not involved.
Protons cause a depolarizing shift of the \( Q(V) \) relationships but do not affect \( Q_{\text{max}} \)

Because gating currents can provide useful evidence on the conformational states available to a channel, we recorded gating currents in a stable HEK293 cell line expressing hKv1.5-W472F mutant channels (Chen et al., 1997). This mutant is analogous to the Shaker-IR-W434F non-conducting mutant in that it has no measurable \( K^+ \) current, perhaps because of permanent or greatly accelerated P-type inactivation (Yang et al., 1997).

Representative examples of gating current traces from hKv1.5-W472F recorded at pH 7.4 and pH 5.4 in the same cell are shown in Figure 2.10A-D. To prevent contamination of gating currents by endogenous HEK293 ionic currents, these recordings were made in symmetrical 140 mM NMG\(^+\). At pH 7.4, on-gating currents were evoked between -60 and 100 mV from a holding potential of -100 mV and at pH 5.4 the voltage range was from -60 to 150 mV to compensate for the proton-induced gating shift. As reported previously (Chen et al., 1997), on-gating currents at pH 7.4 were first apparent at -60 mV and as the strength of the depolarization increased both the peak amplitude and decay rate of the on-gating current increased. Following depolarizations up to 0 mV the return- or off-gating currents decayed rapidly as channels deactivated at -100 mV. In contrast, following depolarizations to 0 mV or more the off-gating currents are superimposable and have a clear rising phase that is followed by a slow decay. This slowing of charge return is such that integration of the off-gating current over a 15 ms period produces a \( Q_{\text{off}} \) that is reduced relative to \( Q_{\text{on}} \). This decrease of \( Q_{\text{off}}/Q_{\text{on}} \) or charge immobilization has been attributed to the conformational change underlying C-type inactivation (Chen et al., 1997; Yellen, 1997) since it is affected by the presence of permeant metal cations, much as C-type inactivation of ionic currents is affected by extracellular cations (Lopez-Barneo et al., 1993; Baukrowitz & Yellen, 1995).
Figure 2.10. Extracellular acidification to pH 5.4 causes a depolarizing shift of the $Q_{on}(V)$ and $Q_{off}(V)$ relationships but does not reduce $Q_{max}$. Panels A and B show at pH 7.4 and 5.4, respectively, the on- and off-gating currents recorded when the membrane was depolarized for 12 ms from a holding potential of -100 mV to between -60 and 100 mV (A) or -60 and 150 mV (B) in 10 mV increments before stepping back to -100 mV. Outward charge movement ($Q_{on}$) induced by the depolarization was determined by integrating the on-gating currents at pH 7.4 (○) and 5.4 (●), and is plotted in panel E. For the $Q_{on}(V)$ relationship in E, the fitted values for $V_{1/2}$ and $s$ were, respectively, -2.2 mV and 6.5 mV at pH 7.4 and 50.2 mV and 11.8 mV at pH 5.4. $Q_{max}$ was not significantly affected by extracellular acidification. C,D. From the same cell as in A and B, these panels show the off-gating currents following a 12 ms step from -80 mV to 50 mV in pH 7.4 (C) or to 100 mV at pH 5.4 (D) to move $Q_{max}$. Off-gating current was recorded in 10 mV increments between -200 and -10 mV at pH 7.4 and between -200 and 40 mV at pH 5.4. Charge return at pH 7.4 (△) and pH 5.4 (▲) is plotted against the repolarization voltage in E to obtain the $Q_{off}(V)$ relationship. Extracellular acidification changed the $V_{1/2}$ of $Q_{off}(V)$ from -100.5 mV to -72.9 and $s$ increased from 9.4 mV to 13.1 mV. Both at pH 7.4 and pH 5.4 there is a leftward shift of the voltage dependence of $Q_{off}(V)$ relative to $Q_{on}(V)$. 

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C-type inactivation is greatly accelerated in the recording conditions used here because there are no permeant metal cations on either side of the membrane. The transition of the voltage sensor from its outward “immobilized” position to the inward position remains voltage dependent but stronger hyperpolarizations are required to overcome the stabilizing interaction between the sensor and the C-type inactivated state. This accounts for the leftward shift, relative to the $Q_{on}(V)$ relationship, of the voltage dependence of charge return (Olcese et al., 1997; Wang & Fedida, 2001) (and see Figure 2.10E).

Figure 2.10B shows that changing the external pH from 7.4 to 5.4 caused a rightward shift of the voltage dependence of the on-gating currents such that the on-gating current evoked at 150 mV at pH 5.4 was comparable to that at 100 mV at pH 7.4. At pH 5.4 there was also a substantial increase of the peak amplitude and an increase of the decay rate of off-gating currents following strong depolarizations. To quantify the effects of changes of pH$_0$ on activation gating, the on-gating currents in Figure 2.10A and B were integrated to obtain the voltage dependence of on-gating charge movement shown in Figure 2.10E. A fit of the $Q_{on}(V)$ relationship at pH 7.4 (open circles) to a single Boltzmann function gave a maximum charge movement $Q_{max}$ of +2.5 pC, $V_{1/2}$ = -2.2 mV and $s$ = 6.5 mV. At pH 5.4 (filled circles of Figure 2.10E) $Q_{max}$, $V_{1/2}$ and $s$ were +2.5 pC, 50.2 mV and 11.8 mV. In the six cells examined $V_{1/2}$ was 4.3 ± 2.2 mV at pH 7.4 and 48.9 ± 1.2 mV at pH 5.4; $s$ increased from 7.1 ± 0.5 mV at pH 7.4 to 10.5 ± 0.4 mV at pH 5.4; and, the relative $Q_{max}$ ($Q_{max, pH5.4}/Q_{max, pH7.4}$) was 1.0 ± 0.003. Thus, changing pH$_0$ from 7.4 to 5.4 caused a ~45 mV rightward shift of the $V_{1/2}$ of the $Q_{on}(V)$ relationship and a decrease of the voltage-sensitivity of activation. Both of these effects are replicated by Zn$^{2+}$ (Zhang et al., 2001b) and interestingly, as with Zn$^{2+}$, the
shift of $V_{1/2}$ of the $Q_{on}(V)$ relationship is roughly twice that measured for the $g(V)$ curve. For example, at pH 5.9 the $V_{1/2}$ of the $g(V)$ relationship was shifted by ~21 mV (not shown).

Panels C and D of Figure 2.10 illustrate the outcome of experiments to determine if the change of off-gating current in Figure 2.10B was due to a shift of the voltage-dependence of off-gating charge movement (Olcese et al., 1997). The voltage clamp protocol consisted of a 12 ms step from the holding potential of -80 mV to 50 mV at pH 7.4 (C) or 100 mV at pH 5.4 (D) to evoke maximal charge movement. This was followed immediately by a pulse to between -10 and -200 mV at pH 7.4 or to between 40 and -200 at pH 5.4. Integration of the off-gating currents yielded the $Q_{off}(V)$ curves shown in Figure 2.10E at pH 7.4 (open triangles) and pH 5.4 (closed triangles). Considering first the data at pH 7.4, it can be seen that, as in the Shaker non-conducting mutant (Olcese et al., 1997), the voltage dependence of return charge movement was shifted leftward ($V_{1/2} = -100.5$ mV) by ~100 mV relative to the $Q_{on}(V)$ curve. Of particular importance is that a similar effect is seen at pH 5.4 where the $V_{1/2}$ of the $Q_{off}(V)$ curve was -72.9 mV, representing a leftward shift of ~124 mV from the $V_{1/2}$ of the $Q_{on}(V)$ relationship.

The values, respectively, for $V_{1/2}$ and $s$ of the $Q_{off}(V)$ relationship in 3 such experiments were, at pH 7.4, -102.8 ± 1.4 mV and 11.4 ± 1.0 mV and, at pH 5.4, -75 ± 1.4 mV and 14.8 ± 1.1 mV. Thus, at pH 7.4, there was, following a depolarization that moved $Q_{max}$, a ~107 mV leftward shift of the voltage dependence of return gating charge movement. A comparable leftward shift of ~124 mV of the voltage dependence of gating charge movement was seen at pH 5.4.
DISCUSSION

The first series of experiments (Figures 2.1-2.4) described in this paper show that, as with Zn$^{2+}$ ions, external protons cause a concentration-dependent and reversible inhibition of hKv1.5 currents. Although this effect is associated with a depolarizing shift of the activation ($g(V)$) curve, the two actions appear to be mechanistically unrelated. Both effects have been reported for rKv1.5 channels (Steidl & Yool, 1999) but we have extended the previous work by showing that external ions such as K$^+$ and Cs$^+$, but not Na$^+$, are able to relieve the inhibition but not the gating shift caused by protons. In zero K$^{+}_o$ the apparent $pK_H$ of the protonation site is 6.8 and this decreases to 6.2 with 5 mM K$^{+}_o$. The latter $pK_H$ accords well with the $pK_H$ of 6.2 for rKv1.5 responses recorded in 2 mM K$^{+}_o$ (Steidl & Yool, 1999) and the $pK_H$ of 6.1 in N-terminal deleted ferret Kv1.4 with 3 mM K$^{+}_o$ (Claydon et al., 2000). The influence of K$^{+}_o$ on this inhibition of hKv1.5 currents was modelled as a non-competitive interaction between K$^+$ and protons (Figure 2.4) and the estimated $K_D$ for this antagonism by K$^{+}_o$ is very near that estimated for the Zn$^{2+}$ block (Zhang et al., 2001a), i.e., $K_K = 0.5 - 1.0$ mM. This implies that the same K$^+$ binding site is involved in both cases and is perhaps homologous to the site ($K_D \sim 0.75$ mM) at which K$^+$ binds to lock Ba$^{2+}$ ions within the pore of Shaker B channels (Harris et al., 1998). Binding sites with a similar affinity for K$^+$ have also been shown to influence the availability of Shaker-T449A channels ($K_D = 0.8$ mM) (Lopez-Barneo et al., 1993) and to competitively inhibit C-type inactivation in Shaker-IR channels ($K_D = 1 - 2$ mM) (Baukrowitz & Yellen, 1996). Interestingly, the external lock-in site of Shaker B channels and the site at which K$^+$ binds to antagonise the inhibitory actions of Zn$^{2+}$ or H$^{+}_o$ in hKv1.5 also share the property of having a low affinity for Na$^+$ ions.
One of two clear differences between the actions of \( H^+ \) and \( Zn^{2+} \) is that while the \( K_D \) for the relief by \( Cs^+ \) of the \( Zn^{2+} \) block is roughly 5-fold higher than that for \( K^+ \) (Zhang et al., 2001a), \( Cs^+ \) is as effective as \( K^+ \) in antagonizing the current inhibition by protons (Figure 2.4). In the case of \( Zn^{2+} \) the higher \( K_D \) for \( Cs^+ \) was assumed to reflect the lower permeability of the \( Cs^+ \) in the pore. With \( H^+ \) it is conceivable that protonation of a negatively-charged, cation-binding site decreases that site's negativity and alters the selectivity sequence to one favouring \( Cs^+ \) binding (Hille, 1992). If so, the selectivity sequence of a binding site in the outer pore mouth must be involved since we have no evidence of a change of the reversal potential with extracellular acidification. The \( n_{Hill} \) of \( \approx 1.5 \) for the proton block suggests that at least two sites, most likely H463 residues in the tetrameric channel assembly, must be protonated. Although the \( n_{Hill} \) for \( Zn^{2+} \) block is near unity (Zhang et al., 2001a) this might still involve coordinated binding of histidine residues of two or more subunits.

**Evidence against a pore blocking mechanism**

The block of cardiac voltage-gated Na\(^+\) (Nav) channels by \( Zn^{2+} \) occurs by occlusion and is eliminated by the mutation of a cysteine residue in the pore (Backx et al., 1992). Similarly, the block by external protons of Nav channels in nerve and skeletal muscle has a voltage-dependence suggesting a site of action within the pore (Woodhull, 1973). It seems unlikely, however, that the inhibition of hKv1.5 current by \( H^+ \) and \( Zn^{2+} \) is due to pore block. First, in the voltage range where the open probability is maximal there is no indication of a voltage-dependent decline of the inhibition by \( Zn^{2+} \) (Zhang et al., 2001a) or \( H^+ \), e.g. Figure 2.1C, as would be expected were these ions binding at a pore site within the electric field. The latter
observation is consistent, however, with an interaction with one or more H463 residues which, being in the channel’s turret, are outside the electric field. It is well-established that Zn$^{2+}$ and H$^+$ bind to histidine residues and we have shown directly that the H463Q substitution shifts the $pK_H$ measured in zero K$^{+0}$ from 6.8 to approximately 5.4 (Figure 2.6). The acid sensitivity that persists in this mutant and in hKv1.5-R487V (Figure 2.8) is similar to that reported for Shaker ($pK_H \sim 5.4$) (Perez-Cornejo et al., 1998) and Kv1.2 channels ($pK_H \sim 4.9$) (Ishii et al., 2001). Exactly where protons and Zn$^{2+}$ act in these mutants is not known, but given their typical $pK_H$ values of 4-5, likely candidates are the aspartate residues in the outer pore mouth (see Introduction).

Since each of the H463s is approximately 14-16 Å from the central axis of the pore (Aiyar et al., 1995; Doyle et al., 1998) it is very unlikely that binding of either ion to H463 residues would directly occlude the permeation pathway since Zn$^{2+}$ has an ionic radius of 0.74 Å and H$^+$ is orders of magnitude smaller. Assuming that the site at which external K$^+$ binds to antagonise the current inhibition by Zn$^{2+}$ and H$^{+0}$ is in the outer pore mouth, our observation that this interaction is best described by a non-competitive versus a competitive model of inhibition also argues against direct pore block as a mechanism of action of either cation. It appears therefore that protonation or “zincification” of H463 residues indirectly leads to current inhibition. From this view of H463 as sensor arises the next question: what is the nature of the effector?

**A connection between current inhibition and an inactivation process**

Though it is clear from this study that inactivation is faster at acidic pHs ($\tau_{\text{inact}}$ in 3.5 mM [K$^+$]$_o$ is 2.6 ± 0.1 s at pH 7.4 versus 149 ± 7 ms at pH 5.5), our simulation studies (not
shown) indicate that this increased rate of inactivation cannot itself account for the reduction of $g_{\text{max}}$. While models that permit inactivation only from the open state can not successfully simulate the reduction in macroscopic conductance, single channel experiments have supported a modal gating model in which channels can inactivate from one or more closed states (Kwan et al., 2006). Furthermore, although increasing $K^+_o$ can speed recovery from C-type inactivation of Kv1.3 currents (Levy & Deutsch, 1996), an explanation for the inhibition that involves a slowing of recovery from inactivation and an accumulation of inactivation can be rejected since a two minute period without voltage pulsing has no effect on the degree of inhibition (Figure 2.9). Nonetheless, a simple interpretation of the effect of $K^+_o$ (or $Cs^+_o$) on the reduction of $g_{\text{max}}$ caused by $H^+_o$ or $Zn^{2+}$ is that, by a “foot-in-the-door” mechanism, $K^+_o$ acts as a competitive antagonist of a conformational change at the pore mouth that is believed to underlie inactivation. In this connection we think it is significant that a point mutation at a site (position 487; T449 in Shaker) that has been implicated in the regulation of inactivation (Lopez-Barneo et al., 1993) dramatically affects the proton block (Figure 2.8). In the studies of mutant Shaker channels the terms “potentiation”/“conductance collapse” were used to describe the increase/decrease of $g_{\text{max}}$ when $K^+_o$ was increased/decreased. It is likely that potentiation/collapse in Shaker-IR is analogous to block relief/block in hKv1.5 but there are some differences. Foremost among these is that, in contrast to the Shaker mutants, in Kv1.5 the block (conductance collapse) is $K^+_o$ and pH sensitive. That is to say at pH 7.4 removing $K^+_o$ has little or no effect on wt hKv1.5 currents whereas at pH 6.4 decreasing $K^+_o$ causes a substantial conductance decline. Additionally, although the tendency for the conductance of Shaker mutants to collapse in zero $K^+_o$ is strongly correlated with an accelerated inactivation rate, this does not extend to hKv1.5 where wt hKv1.5 is much more prone to block at pH 6.4.
than is hKv1.5-R487V even though both inactivate at approximately the same rate at pH 7.4 (Fedida et al., 1999). Nonetheless, the fact that current inhibition by $\text{H}^+$ is substantially reduced by increasing $\text{K}^+$ or by the R487V mutation implies that an inactivation process is involved. Additional insight into the possible basis for the proton block and, in particular, the role of C-type inactivation was provided by gating current results (Figure 2.10).

**External acidification and on-gating charge movement**

Based on the data of Figure 2.10 we can immediately exclude a mechanism of action in which protonation of H463 residues impedes on-gating charge movement and consequently prevents the opening of the activation gate since at pH 5.4 there is no significant reduction of $Q_{\text{max}}$. This is a second major distinction between the actions of $\text{H}^+$ and Zn$^{2+}$. At a concentration that reduces $g_{\text{max}}$ by more than 90 %, Zn$^{2+}$ decreases $Q_{\text{max}}$ by 10-15 % as though it were preventing the late, weakly voltage-dependent transitions in the activation pathway (Zhang et al., 2001b). However, even though $Q_{\text{max}}$ is unchanged by extracellular acidification, we cannot rule out the possibility that opening of the activation gate becomes uncoupled from the outward movement of the voltage sensor. Interestingly, as with Zn$^{2+}$ (Zhang et al., 2001b), the proton-induced depolarizing shift of $V_{1/2}$ for the $Q_{\text{on}}(V)$ relationship is roughly 2-fold greater than that measured from the $g(V)$ relationship. We have attributed this differential effect on the $g(V)$ and $Q(V)$ curves to the presence of two distinct binding sites. In our view the protonation of an as yet unidentified site on the channel surface affects the movement of the voltage sensor and culminates in a rightward shift both of the $g(V)$ and the $Q(V)$ curves. The protonation of a second site, which is probably H463, has two direct or indirect effects: it decreases $g_{\text{max}}$ and it causes a rightward shift of the voltage dependence of
activation gating. The latter effect possibly reflects the close proximity of S4 and H463 in the S5-P loop (Loots & Isacoff, 2000). Since H463-protonated and therefore non-conducting channels can report the gating shift in gating current measurements, but not in ionic current measurements, the gating shift attributed to protonation of this second site is evident only in the Q(V) curve.

**External acidification and off-gating charge movement**

At pH 7.4 and pH 5.4 the mid-point of the $Q_{\text{off}}(V)$ relationship was shifted leftward, relative to the corresponding $Q_{\text{on}}(V)$ curve, by 107 mV and 124 mV, respectively (Figure 2.10). That this shift occurs at both pHs is significant because it has been attributed to a stabilization of S4s in the outward position by a conformational change linked to C-type inactivation (Olcese et al., 1997; Wang & Fedida, 2001). We take this to mean that at pH 5.4 channels are not C-type inactivated prior to a depolarizing pulse but are able to become so when sufficiently depolarized. In other words, at a low pH wt hKv1.5 apparently behaves like the non-conducting mutant hKv1.5-W472F. That is to say, the gating shift notwithstanding, on- and off-gating charge movement is relatively normal but the channels are never or, at best, only very briefly in a conducting state. We speculate, as proposed for homotetrameric Shaker-IR-W434F (Yang et al., 1997), that in wt hKv1.5 protonation of, or Zn$^{2+}$ binding to, H463s allows an inactivation process to occur either from one or more of the closed states or at a greatly accelerated rate following the outward movement of the voltage sensor and channel opening. Since our data indicate that the transition to the C-type inactivated state is intact even at low pHs, this leaves P-type inactivation as a possible basis for the H$^{+}_{o}$-induced current inhibition. Some support for this suggestion comes from a study of the Shaker-IR-
FWFW mutant (Yang et al., 1997) where, as described for P-type inactivation (De Biasi et al., 1993), peak FWFW current was increased by external TEA\(^+\) and where there was also an enhancement of FWFW current when K\(^+\)_\(o\) was increased. In a similar manner, in hKv1.5 the block-relief by K\(^+\)_\(o\) would be due to the occupancy of a site, presumably near the outer pore, that inhibits P-type inactivation. The cooperativity factor, \(a\), of equation (4) would then be interpreted to mean that protonation of H463s, by virtue of a conformational change, inhibits the binding of K\(^+\) at its site, and \textit{vice versa}.

This proposed scheme is at least functionally equivalent to closed state inactivation proposed to account for the loss of current in \textit{Shaker}-IR-T449 mutants (Lopez-Barneo et al., 1993), to the decrease of channel availability proposed for the current loss in zero K\(^+\)_\(o\) in Kv1.4 (Pardo et al., 1992), and to the non-conducting “open” state proposed for Kv1.3 (Jäger et al., 1998) and hKv1.5 (Wang et al., 2000) channels.

\textbf{What is the connection between H463 and R487?}

To reiterate, our view is that H463 acts as a sensor and R487 is a required component in the effector mechanism, e.g., inactivation. Concerning the nature of the coupling between H463 and R487, it has been proposed that the charge of H463 reduces the \(pK_H\) of R487 by an electrostatic effect (Jäger & Grissmer, 2001). However, a number of our observations argue against such an electrostatic interaction. First, a strong, \textit{mutual} electrostatic interaction between R487 and H463 would be expected to affect the \(pK_H\) of H463. In this connection, a histidine residue substituted at the same position in the turret of \textit{Shaker} channels (F425H) has a \(pK_H\) of 6.4 in 2 mM K\(^+\)_\(o\) (Perez-Cornejo et al., 1998) that is similar to that for wt hKv1.5 (\(pK_H\sim 6.2\) in 5 mM K\(^+\)_\(o\), Figure 2.3). This suggests that the \(pK_H\) of a histidine in the turret is
weakly influenced, if at all, by the nature of the residue apposed to it in the tertiary structure (Doyle et al., 1998), be it either charged as with R487 in hKv1.5, or polar and uncharged as with T449 in Shaker. This also implies that an effect of the R487V mutation on the binding equilibrium for H⁺ or Zn²⁺ at H463 does not account for the decreased sensitivity of hKv1.5-R487V currents to inhibition by either cation. Another argument against an electrostatic interaction between a protonated H463 and R487 is that the proposed shift of the pKₜ of R487 by ~6 units would require that these two residues be in much closer apposition (Elinder et al., 2001) than the 8 Å (Cα to Cα) suggested by the crystal structure of KcsA (Doyle et al., 1998). We have also found, contrary to the expectation of an electrostatic mechanism, that increasing the Debye length by decreasing the ionic strength of the external solution does not substantially affect the block of wt hKv1.5 by Zn²⁺ (Minshall & Kehl, unpublished data). On these grounds, an electrostatic interaction between protonated H463 and R487 seems unlikely but the pH sensitivity of hKv1.5-R487H (Jäger & Grissmer, 2001) does imply that a positive charge near the pore mouth is necessary for the virtually complete suppression of outward current seen in zero K⁺₀.

An alternative view of the coupling between H463 and R487 is that, perhaps because of the change of its charge and a consequent increase of its hydrophilicity, the protonation of H463 permits a conformational change requiring R487. Although we have no direct evidence for such a conformational change, it is intriguing that studies of Kv2.1 have shown that the distribution of channels between two outer vestibule conformations is regulated by K⁺₀ (Immke et al., 1999). Additionally, a lysine residue (K356) which is positively charged at neutral pH, and which is homologous in position and charge to a protonated H463 of hKv1.5,
is crucial in this $K^+$-dependent conformational change (Immke et al., 1999). The K356 residue is also involved in the enhancement of Kv2.1 currents by $K^+_o$ (Wood & Korn, 2001).

**Inactivation and the influence of the charge on and size of the residue at position 463**

Jäger & Grissmer (2001) recently reported, and we have confirmed here, that in the mutant hKv1.5-H463G the conductance collapses at pH 7.4 after switching to zero $K^+_o$ (Figure 2.7). We also found that this mutant inactivates much faster than wt hKv1.5 which underscores the association in *Shaker*, noted above, between an increased inactivation rate and a tendency for the current to collapse in zero $K^+_o$. The differences in the properties of wt hKv1.5 and the H463Q and H463G mutants also imply that both the charge on and the size of the residue at position 463 influence the structural rearrangement leading to a conductance collapse in zero $K^+_o$. The importance of charge is evident in wt hKv1.5 at pHs where, when H463 residues are protonated, the conductance collapses in zero $K^+_o$. An influence of the size of the residue at position 463 is suggested by different behaviours of the H463Q and H463G mutants. Thus, there is no conductance collapse in zero $K^+_o$ at pH 7.4 in hKv1.5-H463Q where the substituted glutamine is uncharged, but polar, and occupies only a slightly smaller volume than histidine ($\sim 150 \ \AA^3$). In contrast, in the H463G mutant the uncharged but much smaller glycine residue ($\sim 60 \ \AA^3$) does allow the conductance to collapse in zero $K^+_o$ at pH 7.4. Additional indirect support for the idea that the size of the residue at this position in the turret affects inactivation comes from the report that substitution of glutamine for glycine at the homologous position in Kv1.3 (G380Q) slows inactivation roughly 7-fold (Nguyen et al., 1996).
Finally, the results of voltage clamp fluorimetry in Shaker suggest that, rather than being restricted to a structural collapse at the selectivity filter, slow inactivation may involve a coordinated movement extending to the outer rim (turret) of the pore (Loots & Isacoff, 2000). This is consistent with our results that in hKv1.5 both the charge on and the volume of the residue at position 463 in the turret influences a coordinated movement to an inactivated state.

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CHAPTER 3: THE EXTERNAL K⁺ CONCENTRATION AND MUTATIONS IN THE OUTER PORE MOUTH AFFECT THE INHIBITION OF Kv1.5 CURRENT BY Ni²⁺

INTRODUCTION

Kv1.5 (KCNA5) channels, which are expressed in cardiac myocytes (Fedida et al., 1993; Tamkun et al., 1991) and in smooth muscle cells of airways, the intestine and the vasculature (Adda et al., 1996; Clement-Chomienne et al., 1999), are members of a major structural class of K\(^+\) channels in which the \(\alpha\) subunit consists of 6 transmembrane (TM) segments with a pore-forming or P-region positioned between transmembrane segment five (S5) and S6. A characteristic feature of the 6TM-1P subunit is the charge-bearing S4 domain whose movement upon membrane depolarization (Baker et al., 1998; Larsson et al., 1996) is linked to the opening of the activation gate which is believed to comprise the cytoplasmic ends of the four S6 regions in the tetrameric channel assembly. Macroscopic currents through Kv1.5 channels resemble delayed rectifier currents. Thus, with a strong sustained depolarization, channel activation is rapid and voltage-dependent whereas inactivation is voltage-independent and occurs on a timescale of seconds.

Kv1.5 channels exhibit only outer pore (P/C-type) inactivation (Fedida et al., 1999) and in this regard are different from Shaker channels which also show inner pore (N-type) inactivation (Hoshi et al., 1991). The term C-type inactivation was coined to describe the slow inactivation process in Shaker that was uncovered when ball-and-chain or N-type inactivation was removed (Shaker-IR) by deletion of the cytoplasmic N-terminal residues 6-46. C-type inactivation is coupled to channel activation and is believed to involve a conformational change in the outer pore mouth that extends to the selectivity filter delimited by the highly conserved GYG sequence. Because C-type inactivated Shaker-IR (Starkus et al., 1997) and Kv1.5 (Wang et al., 2000a) channels are able to conduct Na\(^+\) ions, the current view is that the conformational change at the outer pore mouth involves an incomplete
constriction rather than a complete collapse. An important consequence of C-type inactivation is a leftward shift of the gating charge versus voltage relationship, or $Q-V$ curve, and charge immobilization (Fedida et al., 1996, Olcese et al., 1997).

In Shaker-IR channels the residue at position 463 in the S6 segment was the first shown to influence the rate of C-type inactivation (Hoshi et al., 1991). Subsequently, point mutations of the threonine residue (T449) in the outer pore mouth were shown to dramatically accelerate (T449E, T449A, T449K) or slow (T449Y, T449V) C-type inactivation (Lopez-Barneo et al., 1993). In Kvl.5 channels the residue homologous to T449 is R487 and it has been shown that inactivation is substantially slowed in Kvl.5-R487V when Na$^+$ is the charge carrier but not when K$^+$ is the permeant ion (Fedida et al., 1999; Wang et al., 2000a).

The finding that Shaker-IR/Kvl.5 channels with the pore mutation W434F/W472F were Na$^+$- but not K$^+$-conductive and showed wild-type gating charge behaviour, including gating charge immobilization following channel inactivation (Chen et al., 1997; Olcese et al., 1997), was one of the first indications of the complexity of outer pore inactivation. To account for the properties of the Shaker-IR-W434F non-conducting mutant it was proposed that there was also a so-called P-type inactivation process that prevented K$^+$ conduction but which was different from C-type inactivation in that it did not affect gating charge movement (Olcese et al., 1997; Yang et al., 1997). Restoration of ionic current in the double mutant Shaker-W434F-T449Y supports the hypothesis that enhanced inactivation accounts for the Shaker-IR-W434F conductance loss (Yang et al., 2002).

An intriguing divergence in the structure-function relationships of Kvl.5 and Shaker-IR is seen in the response to extracellular acidification. In Kvl.5 external protons cause, in
addition to a rightward shift of the $g-V$ curve that is often referred to as the gating shift, a concentration-dependent decrease of the maximum macroscopic conductance ($g_{\text{max}}$) as well as an acceleration of the inactivation rate of residual currents (Steidl & Yool, 1999; Kehl et al., 2002). In contrast, in Shaker-IR channels increasing $[\text{H}^+]_o$ does not reduce $g_{\text{max}}$ but the gating shift and the speeding of inactivation are observed (Perez-Cornejo, 1999; Starkus et al., 2003). A number of lines of evidence now support the view that protonation of a histidine residue (H463), the equivalent of Shaker-F425, in the pore turret (S5-P linker) plays an important role in the proton-induced conductance loss/block in Kv1.5. Thus, in the Kv1.5 H463Q mutant there is a large rightward shift of the concentration dependence of the $\text{H}^+\text{o}$ block (Kehl et al., 2002). The finding that the $\text{H}^+\text{o}$ block is antagonized by $\text{K}^+\text{o}$ and is also reduced in the R487V mutant (Jäger & Grissmer, 2001; Kehl et al., 2002) has suggested that the protonation of H463 facilitates an inactivation process requiring R487. An alternative explanation involving direct pore block by protons has been ruled out on the basis of single channel recordings (Kwan et al., 2003) and the finding that the $\text{Na}^+$ current through inactivated Kv1.5 channels is maintained following extracellular acidification (Zhang et al., 2003).

Additional support for a crucial role of H463 in the $\text{H}^+\text{o}$-induced decrease of $g_{\text{max}}$ is provided by reports showing that divalent cations known to bind to histidine residues also affect Kv1.5 currents. Harrison et al. (1993) first reported that extracellular $\text{Zn}^{2+}$ blocks Kv1.5 currents and, as with the $\text{H}^+\text{o}$ block, this effect of $\text{Zn}^{2+}$ is inhibited either by increasing $\text{K}^+\text{o}$ or by mutating H463 and/or R487 (Kehl et al., 2002). $\text{Ni}^{2+}$ is also a histidine ligand and although it too has been reported to block Kv1.5 currents expressed in Chinese hamster ovary (CHO) cells (Perchenet & Clement-Chomienne, 2001), the mechanism of, and the molecular
determinants for the block have not been resolved. To test the hypothesis that the mechanistic basis for the Ni$^{2+}$ block is essentially the same as that outlined above for Zn$^{2+}$ and H$^+$, we set out in this study to address the following questions. Is the block of Kv1.5 by Ni$^{2+}$ antagonized by increasing [K$^+$]? Does Ni$^{2+}$ speed the inactivation rate of residual Kv1.5 currents? Is the effect of Ni$^{2+}$ affected either by mutating H463, a putative Ni$^{2+}$ coordination site, or by mutating R487, a site implicated in the regulation of outer pore inactivation? Are gating currents affected by Ni$^{2+}$? And finally, is the blocking effect of Ni$^{2+}$ replicated by other divalent cations such as Co$^{2+}$, Cd$^{2+}$ and Mn$^{2+}$?

**METHODS**

**Cell preparation**

As described previously (Wang et al., 2000a), wild type (wt) and mutant human Kv1.5 channels, henceforth referred to simply as Kv1.5 channels, were studied in a human embryonic kidney cell line (HEK293) (Wang et al., 2000b). Cells were dissociated for passage by using trypsin-EDTA and were maintained in minimum essential medium (MEM), 10% fetal bovine serum, penicillin-streptomycin and 0.5 mg ml$^{-1}$ gentamicin in an atmosphere of 5% CO$_2$ in air. All tissue culture supplies were obtained from Invitrogen (Burlington, ON, Canada).

Point mutations of the wt Kv1.5 α-subunit in the plasmid expression vector pcDNA3 were made using the Quickchange Kit (Stratagene, La Jolla, CA, USA) to convert the histidine (H) residue at position 463 to glutamine (Q) (H463Q) or the arginine (R) at position 487 to valine (R487V). Stable transfections of HEK293 cells were made using 0.8 μg of Kv1.5-H463Q or Kv1.5-R487V cDNA and 2 μL of Lipofectamine 2000 (Invitrogen). Geneticin (0.5
mg/mL) was added 48 hrs after transfection. Because Shaker-related channels such as Kv1.5 are homotetramers (MacKinnon, 1991) a given point mutation will exist in each of the four subunits of the channel assembly.

**Recording solutions**

The standard bathing solution contained, in mM, 140 NaCl, 3.5 KCl, 10 Hepes, 2 CaCl₂, 1 MgCl₂, 5 glucose and its pH was adjusted to 7.4 with NaOH. Hepes was replaced by Mes when the pH of the extracellular solution was less than 6.8 in the experiments directly comparing the proton block and the Ni²⁺ block. Where the effect of the external concentration of potassium ([K⁺]₀) on the divalent metal cation block was examined, a nominally K⁺-free solution was made by substituting NaCl for KCl and, for [K⁺]₀ greater than 3.5 mM, NaCl was replaced by KCl. The standard patch pipette solution for recording K⁺ currents contained, in mM, 130 KCl, 4.75 CaCl₂ ($p\text{Ca}^{2+} = 7.3$), 1.38 MgCl₂, 10 EGTA, 10 Hepes and was adjusted to pH 7.4 with KOH. Solutions of divalent metal ions were made by dilution of 0.1 to 1 M stock solutions of the chloride salt in distilled water. At pH 7.4 the concentration of Ni²⁺ that can be used was limited to 10 mM or less by virtue of the solubility product for Ni(OH)₂ ($\sim 2 \times 10^{16}$).

Mouse fibroblasts expressing Kv1.5 channels at a low density were used to record unitary currents from outside-out patches. The inside face of the patch was exposed to standard patch pipette solution and the outside face was exposed to standard bath solution either with or without added Ni²⁺.

For gating current recordings the bath solution contained, in mM, 140 NMGCl, 1 MgCl₂, 10 Hepes, 2 CaCl₂, 10 glucose and the pH was adjusted to 7.4 with HCl. The patch
pipette solution contained 140 NMGlC, 1 MgCl₂, 10 Hepes, 10 EGTA and was adjusted to pH 7.2 with HCl. Chemicals were purchased from the Sigma Aldrich Chemical Co. (Mississauga, ON, Canada).

**Signal recording and data analysis**

Macroscopic currents were recorded at room temperature (20-22 °C) using the patch clamp technique primarily in the whole cell configuration. In some of the cell lines expressing mutant Kv1.5 channels at a high level, i.e., the H463Q and some of the R487V mutants, the large amplitude of the whole cell currents necessitated recording macroscopic currents from outside-out patches. Voltage clamp experiments were done with an EPC-7 patch clamp amplifier and Pulse+PulseFit software (HEKA Electronik, Germany). Patch electrodes were made from thin-walled borosilicate glass (World Precision Instruments, FL, USA) and had a resistance of 1.0 to 2.5 MΩ measured in the bath with standard internal and external solutions. Typically, 80 % series resistance compensation was used and an on-line P/N method, for which the holding potential was -100 mV and the scaling factor was 0.25, was used to subtract the leak current as well as any uncompensated capacitive currents. Current signals filtered at 3 kHz (-3dB, 8-pole Bessel) were digitized (16 bit) at a sampling interval of 100 μs (10 kHz). Voltages have been corrected for the liquid junction potentials.

In an experiment, a section of glass coverslip with cells attached to it was placed in the recording chamber (0.5 ml volume) and was continuously perfused with bathing solution. After recording currents in the control solution the inflow was switched to the test solution and once 5-6 ml had been flushed through the bath the treated responses were recorded. Recovery currents were taken after flushing the bath with 5-6 ml of control solution. If the
recovery currents were not within ±15% of the pre-treatment amplitudes the data for that cell were discarded. By this criterion most cells showed recovery.

To quantify the effect of Ni\(^{2+}\) and other metal cations on Kv1.5, tail currents were recorded at -40 or -50 mV following depolarizing pre-pulses of differing magnitude. Peak tail current amplitudes were obtained by fitting a polynomial function and taking the fitted value for the maximum current. After normalization of tail currents either to the maximum current of the control or the treated response, data points were fitted to a single Boltzmann function:

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

where, when \(y\) is the current normalized with respect to the control response, \(A\) is the proportion of the control \(g_{max}\). When \(y\) is the current normalized with respect to the maximal treated current, \(A\) is the best fit value for the normalized maximal response and ideally has a value of unity. \(V_{1/2}\) is the half-activation potential or mid-point of the activation curve, \(V\) is the voltage during the pre-pulse and \(s\) is the slope factor, in mV, reflecting the steepness of the voltage dependence of gating.

To quantify gating charge movement during activation, charge-voltage (\(Q_{on-V}\)) curves were generated by time integration of on-gating currents as described previously (Chen et al., 1997). Activation gating in Kv1.5 is best fit by the sum of two Boltzmann functions where the larger component, known as \(Q_2\), represents ~80% of the total charge movement (Hesketh & Fedida, 1999). However, for simplicity, \(Q-V\) data obtained at pH 7.4 and 5.4 were fitted to eq. 1 where \(y\) is the charge moved, \(A\) is the maximal charge (\(Q_{max}\)) and \(V\) is the voltage at which the on-gating charge (\(Q_{on}\)) is evoked. \(V_{1/2}\) represents the mid-point of the \(Q-V\) curve and \(s\) reflects the steepness of the voltage dependence of charge movement.
Concentration-response data were fitted to the Hill equation:

\[
y = \frac{1}{1 + \left( \frac{X^{2^+}}{K_D} \right)^{n_H}}
\]

where \( y \) is the proportion of the control \( g_{max} \), \( K_D \) is the equilibrium dissociation constant for the test cation (\( X^{2^+} \)) and \( n_H \) is the Hill coefficient reflecting the number of test cations binding per channel.

Microscopic currents were low-pass filtered at 3 kHz (8-pole Bessel), sampled at 10 kHz and digitally-filtered at 1 kHz for the data analysis using TAC and TACFit (Bruxton, Seattle). Leak and uncompensated capacitive currents were subtracted using a template generated from blank sweeps. Half-amplitude threshold analysis was used to idealize single channel recordings for the generation of dwell time histograms.

Data are expressed as the mean ± SE except for the values obtained by non-linear least-squares fitting routines (Igor, Wavemetrics, OR, USA) which are expressed as the mean ± SD. The paired-sample \( t \) test was used to compare the inactivation rates of residual currents in \( Ni^{2^+} \) and \( H^+ \). A \( P \)-value of 0.05 or less was considered significant.

RESULTS

Shown in Figure 3.1A are traces confirming the block of Kv1.5 currents by external \( Ni^{2^+} \). From a holding potential of -80 mV and with 0 mM \( K^+ \), currents were evoked by a family of 300 ms depolarizations from -45 to +35 mV with a cycle length of 5 s. Tail currents were recorded at -40 mV. After obtaining the control responses, the perfusate was switched to a test bathing solution containing 0.1 mM \( Ni^{2^+} \) and then to one containing 1 mM \( Ni^{2^+} \). Complete recovery was obtained after returning to \( Ni^{2^+} \)-free solution. As noted previously
(Perchenet & Clement-Chomienne, 2001), and in contrast to the effects with Zn\(^{2+}\) (Zhang et al., 2001a), with Ni\(^{2+}\) there was neither a significant change of the activation kinetics nor an obvious effect on the decay of residual pulse currents. The effect of Ni\(^{2+}\) on the current behaviour during longer depolarizing pulses is examined below (Figure 3.4).

**Increasing [K\(^{+}\)]\(_0\) causes a rightward shift of the concentration dependence of the Ni\(^{2+}\) block**

In order to quantify the block by Ni\(^{2+}\), g-\(V\) curves were constructed from peak tail currents as described in the Methods section. Panel B of Figure 3.1 plots the peak tail current amplitude *versus* the pulse voltage for the same cell in 0 mM K\(^{+}\)\(_0\) without Ni\(^{2+}\) and with 0.25 or 0.5 mM Ni\(^{2+}\). In this cell 0.25 mM and 0.5 mM Ni\(^{2+}\) decreased the maximum tail current, and by extension the maximum conductance (\(g_{\text{max}}\)), by approximately 70 % and 90 %, respectively.

To more clearly illustrate the effect of Ni\(^{2+}\) on the midpoint (\(V_{\text{y}}\)) of the g-\(V\) curve, the currents in panel B were normalized with respect to the maximum current for the same treatment group and are presented in panel C. It is evident that Ni\(^{2+}\) caused a rightward shift of the g-\(V\) curve and this is assumed to reflect a change of surface charge due to screening and/or binding to the channel. With 0.25 mM Ni\(^{2+}\) the shift of \(V_{\text{y}}\) determined from the best fit of the g-\(V\) data to the Boltzmann function was 10.6 ± 0.9 mV (\(n = 4\)). The gating shift with 0.5 mM Ni\(^{2+}\) was not determined because the standard deviation in the fitted values for \(V_{\text{y}}\) was quite large.
Figure 3.1. Ni$^{2+}$ block of Kv1.5 currents in 0 mM K$^+$. A. Control currents evoked by a family of 300 ms depolarizations from -45 to +35 mV, here shown in 10 mV increments, from a holding potential of -80 mV. Tail currents were recorded at -40 mV. Perfusion of solution containing 0.1 mM Ni$^{2+}$ and then 1 mM Ni$^{2+}$ caused a concentration-dependent inhibition of the current. Recovery traces illustrate the complete reversal of the Ni$^{2+}$ block. B and C. Ni$^{2+}$ decreases $g_{max}$ and shifts the g-V curve slightly rightward. B. Peak tail current at -40 mV following a 300 ms depolarization to the voltage indicated on the x-axis. Note the absence of any voltage dependence of the inhibition between 0 and 50 mV. C. The g-V relationship derived by normalizing tail currents with respect to the maximum tail current shows that Ni$^{2+}$ caused a 10 mV shift of the half-activation voltage. Current tails in 0.5 mM Ni$^{2+}$ were too small to be unequivocally analyzed.
Figure 3.2 shows the concentration-response relationship for the block of Kv1.5 by Ni\textsuperscript{2+} and the influence of [K\textsuperscript{+}]\textsubscript{o} thereon. Panel A illustrates representative current traces from 3 different cells in 0 mM (left), 3.5 mM (middle) and 140 mM (right) K\textsuperscript{+}\textsubscript{o}. In the absence of Ni\textsuperscript{2+} (-Ni\textsuperscript{2+}) the current in each of the K\textsuperscript{+}\textsubscript{o} concentrations had a similarly slow rate of decay. The inward tail current recorded at -40 mV in 140 mM K\textsuperscript{+}\textsubscript{o} is due to the shift of E\textsubscript{K} to ~0 mV. To produce a similar degree of block in the three different experiments it was necessary to increase the Ni\textsuperscript{2+} concentration to offset the effect of increasing [K\textsuperscript{+}]\textsubscript{o}. Note that in each example the Ni\textsuperscript{2+} block was not associated with an acceleration of pulse current decay. The latter observation, together with the fact that the tail current decay was not slowed, as best seen with the traces in 140 mM K\textsuperscript{+}\textsubscript{o}, supports the conclusion that a block of the open channel occurring with intermediate-to-slow kinetics (vis à vis the activation rate) is not involved. For the graph in Figure 3.2B the g\textsubscript{max} relative to the control value has been plotted against the concentration of Ni\textsuperscript{2+} for experiments in which [K\textsuperscript{+}]\textsubscript{o} was 0 mM (open circles), 3.5 mM (open triangles) or 140 mM (open squares). The solid lines overlaying the three data sets represent the best fit to eq. 2. With 0 mM K\textsuperscript{+}\textsubscript{o} the K\textsubscript{D} for the Ni\textsuperscript{2+} block was 0.15 ± 0.01 mM and n\textsubscript{H} was 1.3 ± 0.1. Increasing [K\textsuperscript{+}]\textsubscript{o} to 3.5 mM increased the K\textsubscript{D} to 0.44 ± 0.02 mM and n\textsubscript{H} was 1.6 ± 0.2. With 140 mM K\textsuperscript{+}\textsubscript{o} the K\textsubscript{D} was 3.1 ± 0.3 mM and n\textsubscript{H} was 0.9 ± 0.1. These results clearly demonstrate that, as with the block by H\textsuperscript{+} and Zn\textsuperscript{2+}, the block of Kv1.5 by Ni\textsuperscript{2+} is antagonized by increasing [K\textsuperscript{+}]\textsubscript{o}. 
Figure 3.2. Increasing $[K^+]_o$ changes the concentration dependence of the block of Kv1.5 by Ni$^{2+}$. A. Representative traces obtained from three different cells showing, superimposed, the currents evoked in the $K^+_o$ concentrations indicated either without (-) or with (+) the Ni$^{2+}$ concentration indicated. The voltage protocol consisted of a 300 ms step from -80 mV to 50 mV followed by a step to -40 mV. Increasing $[K^+]_o$ necessitates a higher concentration of Ni$^{2+}$ to produce roughly the same degree of block. The time calibration is the same for the three sets of traces. B. The concentration-response relationship for Ni$^{2+}$ in 0, 3.5 and 140 mM $K^+_o$ shows that increasing $[K^+]_o$ from nominally $K^+_o$-free to 3.5 mM shifted the $K_D$ from 0.15 ± 0.01 mM to 0.44 ± 0.02 mM. Increasing $K^+_o$ to 140 mM shifted the $K_D$ for the Ni$^{2+}$ block to 3.1 ± 0.3 mM. Each point represents the mean ± SE of measurements from 3-7 cells.
The time courses of the onset and the offset of the Ni\(^{2+}\) block are similar

Using a fast solution application system, Perchenet & Clement-Chomienne (2001) noted that the offset of the Ni\(^{2+}\) block was rapid but that the onset was comparatively much slower. They found, with test pulses delivered at 15 s intervals and using 1 mM Ni\(^{2+}\) and 5 mM K\(^+\), that steady-state block was reached only after 5 to 7 minutes. Since a similar phenomenon is not seen with H\(^+\) or Zn\(^{2+}\), we felt it was important to characterize the time dependence of the Ni\(^{2+}\) block and did so by comparing the time course of the current inhibition by Ni\(^{2+}\) with that by H\(^+\). Graphs summarizing the outcome of this comparison are shown in Figure 3.3. For each graph, the peak tail current, measured at -40 mV following a 300 ms pulse to 50 mV applied at 10 s intervals, was plotted against the elapsed time. In Figure 3.3A, Ni\(^{2+}\) and H\(^+\) were applied for the duration indicated by the horizontal bar at concentrations of 150 μM and 0.16 μM (pH 6.8) (Kehl et al., 2002), respectively, and in 0 mM K\(^+\) to cause roughly 50 % block of the current at the steady state. In 4 such experiments we consistently found that the time courses for the onset and offset of the block by Ni\(^{2+}\) and H\(^+\) were similar. Since the failure to uncover any asymmetry in the on- and off- time courses might be attributed to the absence of K\(^+\), experiments were also done with 5 mM K\(^+\) which necessitated using higher concentrations of Ni\(^{2+}\) and H\(^+\) to compensate for the effect of K\(^+\) on the block. Figure 3.3B shows that the outcome was still the same: after switching from the control to the test perfusate the relaxation to the steady-state was complete in less than a minute, a time frame that appears to reflect primarily the dynamics of solution exchange in the bath and is much shorter than the onset noted by Perchenet & Clement-Chomienne (2001).
Figure 3.3. A comparison of the time course of the onset and offset of the inhibition of Kv1.5 by Ni$^{2+}$ and H$^{+}$. Concentrations of Ni$^{2+}$ or H$^{+}$ producing ~50% steady-state inhibition with 0 mM K$^{+}$ (A) and, in a different cell, with 5 mM K$^{+}$ (B) were used. Peak tail currents measured at −40 mV following a 300 ms depolarization to 50 mV applied at a 10 s interval are plotted against the elapsed time. The horizontal bar indicates the duration of each application. The results do not reveal any obvious asymmetry in the onset versus the offset of the block either with or without K$^{+}$. As with H$^{+}$, the time course for the development or reversal of the Ni$^{2+}$ block was similar and in both cases is presumed to reflect the time course of solution exchange in the bath.
**Ni\(^{2+}\) block is associated with a slight acceleration of inactivation of residual currents**

In addition to blocking Kv1.5 currents, extracellular acidification accelerates the rate of inactivation of residual currents (Kehl et al., 2002; Steidl & Yool, 1999) and this was the motivation for determining if there was a similar association between block and inactivation with Ni\(^{2+}\). Our approach to addressing this question was to use cells expressing Kv1.5 channels at a very high density so that despite the reduction of \(g_{\text{max}}\) by 80-95\% the residual currents were virtually unfettered by endogenous HEK currents and could therefore be unambiguously analysed. The voltage protocol consisted of a 5 s step from -80 mV to 50 mV followed by brief depolarizations to 50 mV to track recovery from inactivation (Fedida et al., 1999). An interval of 120 s between the 5 s pulses was used to permit complete recovery from inactivation in the experiments with Ni\(^{2+}\). Initially, these experiments were done with 0 mM K\(^+\) but the interpretation of the data was confounded by a very slowly rising phase of current with a time constant of 1-1.5 s in 1 mM Ni\(^{2+}\) and 200-300 ms at pH 5.9 which followed a normally activating component of current (not shown). This slow component was not observed with 3.5 mM K\(^+\), consequently this was the [K\(^+\)]\(_o\) used when comparing the effects on inactivation of concentrations of H\(^+\) and Ni\(^{2+}\) that reduce \(g_{\text{max}}\) by 80 to 95\% (Figure 3.2 and Kehl et al., 2002). Results representative of those obtained in five experiments with 2 mM Ni\(^{2+}\) and 5 experiments with pH 5.4 are shown in Figure 3.4A,B where the effects of H\(^+\) and Ni\(^{2+}\), respectively, were tested on the same cell. At pH 5.4 the inactivation of the residual current during the 5 s pulse was well-fitted by a single exponential with a time constant of 91 ms and the steady-state current was ~25\% of the peak amplitude. At pH 5.4 the mean inactivation time constant \((\tau_{\text{inact}})\) at 50 mV was 101 ± 3 ms \((n = 5 \text{ cells})\).
Figure 3.4. A comparison of the residual current behaviour, done in 3.5 mM K$^{+}$ with a concentration of H$^{+}$ or Ni$^{2+}$ estimated to block 80 – 95 % of the channels, reveals divergent effects on inactivation. From a holding potential of -80 mV, the voltage protocol consisted of a 5 s step to 50mV followed by 50 ms steps at variable intervals to 50 mV to monitor recovery from inactivation. Current during the 5 s pulse is shown expanded on the right side of the figure. A. At pH 5.4 the inactivation of current during the 5 s pulse is well-fitted by a single exponential with a time constant of 91 ms. Peak currents, marked by the filled circles, that were evoked by the 50 ms test pulses were fitted to a single exponential with a time constant of 4.3 s. B. In the same cell after switching to solution containing 2 mM Ni$^{2+}$ at pH 7.4, inactivation was ~20 times slower ($\tau_{inact} = 1.69$ s) and recovery from inactivation was ~5 times slower ($\tau_{recovery} = 24.8$ s) than at pH 5.4.
In Figure 3.4A recovery from inactivation, tested by 50 ms depolarizations delivered from 0.5 s up to 96 s after the 5 s depolarization, was fitted to a single exponential with a time constant of 4.3 s. The mean $\tau_{\text{recovery}}$ at pH 5.4 was $4.2 \pm 0.1$ s. In contrast, currents recorded after switching from pH 5.4 solution to perfusate containing 2 mM Ni$^{2+}$ at pH 7.4 (Figure 3.4B) showed much slower inactivation as well as slower recovery from inactivation: $\tau_{\text{inact}} = 1.69$ s and $\tau_{\text{recovery}} = 24.8$ s. In the five cells tested with 2 mM Ni$^{2+}$ the mean value for $\tau_{\text{inact}}$ and $\tau_{\text{recovery}}$ was $1.71 \pm 0.07$ s and $23.5 \pm 2.1$ s, respectively. Because of their very large amplitude, currents in Ni$^{2+}$-free medium at pH 7.4 could not be recorded from these cells, however the best fit to a single exponential of the current decay during 7-10 s depolarizations to 60 mV at pH 7.4 in Kv1.5 is typically of the order of 2-3 s (Kehl et al., 2002) and the $\tau_{\text{recovery}}$ measured at -80 mV in 5 mM K$^{+}$ and using a similar voltage protocol is 1.1 s (Fedida et al., 1999).

The $K_D$ for the Ni$^{2+}$ block is increased in the H463Q and R487V mutants

We next examined the effect of Ni$^{2+}$ in Kv1.5 channels in which either a putative Ni$^{2+}$ binding site in the S5-P linker (turret) was mutated to a glutamine residue (H463Q) or the residue analogous to Shaker-T449 was changed from arginine to valine (R487V). To circumvent the potential problem of changes of the K$^{+}$-dependence of the block relief, the analysis of the effect of Ni$^{2+}$ on currents from these mutated channels was done with 0 mM K$^{+}$. Concentration-response curves for the Ni$^{2+}$ block of currents from Kv1.5-H463Q (filled squares) and Kv1.5-R487V (filled circles) are shown superimposed in Figure 3.5. As with the block by H$^{+}$ and Zn$^{2+}$ (Kehl et al., 2002), the concentration dependence for the block by Ni$^{2+}$ was shifted substantially to the right by either mutation. In Kv1.5-R487V the $K_D$ was
Figure 3.5. Ni\(^{2+}\) sensitivity is reduced in Kv1.5-H463Q and Kv1.5-R487V. Experiments with the mutant channels were done with 0 mM K\(^+\)\(_o\) to preclude a change of the K\(^+\)\(_o\) binding as the basis for the change of the sensitivity to Ni\(^{2+}\). Because Ni\(^{2+}\) is known to bind to histidine (H) residues, a mutant was constructed in which glutamine (Q) was substituted for H463, a residue in the S5-P linker that forms part of the outer pore vestibule. Kv1.5-H463Q (■) was 100- to 200-fold less sensitive to Ni\(^{2+}\) (K\(_D\) = 24 ± 8 mM; n\(_H\) = 0.4 ± 0.04) compared to the wt Kv1.5 responses (dashed line taken from Figure 3.2) measured in the same recording condition, i.e., 0 mM K\(^+\)\(_o\). In another mutant construct, the arginine (R) residue near the entrance to the pore mouth that has been implicated, by alignment with Shaker-T449, in the outer pore inactivation mechanism, was mutated to valine. The sensitivity of Kv1.5-R487V currents (●) to Ni\(^{2+}\) was approximately 20-fold less (K\(_D\) = 2.8 ± 0.004 mM; n\(_H\) = 0.7 ± 0.001) than that measured under the same recording conditions in wt Kv1.5. The dotted line, which was taken from Figure 3.2, represents the line fitted to the block of wt Kv1.5 in 140 mM K\(^+\)\(_o\).
estimated to be $2.8 \pm 0.004$ mM or roughly 20-fold higher than in wt Kv1.5. With Kv1.5-H463Q the concentration dependence of the block was much more shallow ($n_H \sim 0.4$) than in wt Kv1.5 and the $K_D$ was estimated by extrapolation to be $24 \pm 8$ mM which is 100- to 200-fold higher than in wt Kv1.5.

**Ni$^{2+}$ decreases channel availability**

Macroscopic current amplitude ($I$) is, in general, the product of the number of channels available ($N$), the single channel current ($i$) and the channel open probability ($P_0$) ($I = NP_0i$). To gain a clearer insight into which of these variables was affected by Ni$^{2+}$ ions, recordings were made from outside-out patches containing a single channel. Figure 3.6A shows representative, consecutive control sweeps evoked by a 300 ms depolarization from -80 mV to 100 mV applied at a frequency of 0.1 Hz. As reported previously (Chen & Fedida, 1998), channel openings occurred in bursts of varying duration and within bursts channel closings were frequent but brief. With seconds-long pulses (not shown) we observed closed states with longer mean dwell times which are assumed to reflect a multistep inactivation pathway. Double-Gaussian fits to the control all-points amplitude histogram (e.g., Figure 3.6C) indicates an open channel current ($i$) of $1.7 \pm 0.1$ pA ($n = 8$ patches). After switching to medium with 0.5 mM Ni$^{2+}$, which is the $K_D$ for the block in 3.5 mM K$^{+}$ (Figure 3.2), there was no significant change of the single channel current (e.g., Figure 3.6D; $1.6 \pm 0.1$ pA, $n = 6$ patches), and the $P_0$ in sweeps containing channel activity was also not significantly affected ($P_{0,-Ni} = 0.64 \pm 0.06$ versus $P_{0,+Ni} = 0.61 \pm 0.06$). There were, however, many more blank sweeps in the presence of the Ni$^{2+}$ (Figure 3.6B). Channel availability ($N$), defined as the number of sweeps with channel activity divided by the total number of sweeps, decreased significantly from the control value of $0.90 \pm 0.06$ ($n = 6$ patches) to $0.43 \pm 0.14$ ($n = 6$ patches) in Ni$^{2+}$. 

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Figure 3.6. Ni\textsuperscript{2+} effects at the single channel level. A. Shown here are 10 representative and consecutive control sweeps in a one-channel, outside-out patch that were evoked by a 300 ms pulse from -80 mV to 100 mV applied at 0.1 Hz and with [K\textsuperscript{+}]\textsubscript{i} = 140 mM and [K\textsuperscript{+}]\textsubscript{o} = 3.5 mM. Data were digitally filtered at 1 kHz. B. From the same patch as in A, 10 consecutive sweeps evoked with the same voltage protocol but with 0.5 mM Ni\textsuperscript{2+} in the external perfusate. The main effect of Ni\textsuperscript{2+} is to reduce channel availability. Representative all-point amplitude histograms from a different one-channel patch in control and 0.5 mM Ni\textsuperscript{2+}-containing perfusate are shown in panels C and D, respectively. A double Gaussian fit to data gave a mean current in each case of 1.6 pA.
Ni\textsuperscript{2+} causes a rightward shift of the $Q_{\text{on}}-V$ curve but does not affect $Q_{\text{max}}$

A possible explanation for the current block by Ni\textsuperscript{2+} is that one or more transitions in the gating pathway is prevented. To address that possibility, gating currents were recorded in an HEK293 cell line expressing Kv1.5-W472F channels. The W472F mutation produces channels that are not K\textsuperscript{+} conductive, but which have normal gating currents. Figure 3.7A shows gating current traces in control solution and in 1 mM Ni\textsuperscript{2+}. On-gating currents were evoked by 12 ms pulses between -60 and 130 mV from a holding potential of -100 mV. In the control traces, charge movement was first evident at approximately -50 mV and the peak amplitude and decay rate increased as the intensity of the depolarization increased. Following depolarizations up to -10 mV the off-gating current at -100 mV was rapid (e.g., Figure 3.7B, upper traces) but following stronger depolarizations there was a clear rising phase to the off-gating current and the peak current was substantially smaller and occurred much later (e.g., Figure 3.7B, lower traces) than was the case following steps to -10 mV or less. This pronounced change of off-gating current following stronger depolarizations has been attributed at least in part to a weakly voltage-dependent transition in the return pathway between the open and closed states (Perozo et al., 1993). To construct the charge-voltage ($Q-V$) curves shown in Figure 3.7C, on-gating currents were integrated and $Q_{\text{on}}$ was normalized with respect to the control maximal charge movement ($Q_{\text{max}}$). Although charge movement is better fitted by a double Boltzmann function to account for a smaller component of charge movement with depolarizations up to -20 mV (Hesketh & Fedida, 1999), the data of Figure 3.7C were fitted to a single Boltzmann function.
Figure 3.7. To determine if the conductance loss caused by Ni\(^{2+}\) was due to an inhibition of transitions in the activation pathway, the effect of 1 mM Ni\(^{2+}\) on gating charge movement in Kv1.5-W472F, a non-conducting mutant, was examined. Internal and external permeant ions were replaced by NMG\(^+\) to prevent ionic currents through endogenous HEK293 channels. The family of traces in the top of panel A shows control on-gating currents evoked between –60 and 90 mV from a holding potential of -100 mV and off-gating currents at –100 mV; the lower traces of panel A show the gating currents in 1 mM Ni\(^{2+}\). Control and treated traces, taken at the voltages indicated to account for the gating shift, have been superimposed in B to show that the kinetics of the on- and off-gating currents are not substantially affected by Ni\(^{2+}\). C. The $Q_{on}$-$V$ curve constructed from 6 cells by integrating the on-gating currents and normalizing with respect to the control $Q_{max}$ confirms that, although Ni\(^{2+}\) caused a ~10 mV rightward shift of the $V_{1/2}$, the $Q_{max}$ did not decrease. Fitting to a Boltzmann function gave control and treated $V_{1/2}$ values of -6.8 ± 1.2 mV and 2.2 ± 0.8 mV, respectively, and values of 7.0 ± 1.4 mV and 9.2 ± 1.2 mV for $s$. 

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In 6 experiments of the type illustrated in Figure 3.7, the control $V_y$ and $s$ were $-6.8 \pm 1.2$ mV and $7.0 \pm 1.4$ mV. After switching to 1 mM Ni$^{2+}$ $V_y$ was $2.2 \pm 0.8$ mV and $s$ was $9.2 \pm 1.2$ mV. The difference in $V_y$ between 1 mM Ni$^{2+}$ and control medium was $9.0 \pm 3.0$ mV. Aside from this gating shift, the gating current was essentially unaffected by 1 mM Ni$^{2+}$. In contrast to the situation with 1 mM Zn$^{2+}$ where $Q_{\text{max}}$ decreased by approximately 15% (Zhang et al., 2001b), $Q_{\text{max}}$ was unchanged by 1 mM Ni$^{2+}$.

**Co$^{2+}$ and Cd$^{2+}$, but not Mn$^{2+}$, block Kv1.5**

Other divalent transition metals that can bind to histidine include Cu$^{2+}$, Fe$^{2+}$, Co$^{2+}$, Cd$^{2+}$ and Mn$^{2+}$. Because a precipitate formed with Cu$^{2+}$ and Fe$^{2+}$, only the effects of Co$^{2+}$, Cd$^{2+}$ and Mn$^{2+}$ could be compared to those of Ni$^{2+}$. The experimental protocol was the same as that described for Figure 3.1 and was confined to tests with a 0 mM K$^+$ solution. Figure 3.8A shows a representative example of the effect of Co$^{2+}$ on currents evoked by the voltage protocol illustrated above the control responses. Switching from the control solution to one containing 0.1 mM Co$^{2+}$ had no significant effect on the current but 10 mM Co$^{2+}$ decreased the peak tail current following a +60 mV pulse by more than 90%. Virtually complete recovery occurred after returning to the control solution. Fitting of $g$-$V$ curves (not shown) to eq. 1 revealed that $V_y$ shifted by $11.4 \pm 0.9$ mV with 1 mM Co$^{2+}$ and by $25.3 \pm 1.3$ mV with 10 mM Co$^{2+}$. Neither concentration of Co$^{2+}$ significantly affected the slope factor of the $g$-$V$ curve (not shown). A fit of the Hill equation to the concentration-response data for Co$^{2+}$ (Figure 3.8B) gave an estimate for $n_H$ of $1.3 \pm 0.1$ and a $K_D$ ($1.4 \pm 0.1$ mM) that was roughly 10-times larger than that for Ni$^{2+}$ under the same recording conditions.
Figure 3.8. Co$^{2+}$ also causes a concentration-dependent block of Kv1.5 currents but is an order of magnitude less potent than Ni$^{2+}$. Shown in panel A are control and treated current traces evoked in 0 mM K$^{+}$o with the voltage protocol indicated above the control responses. In contrast to the ~35 % block of the current with 0.1 mM Ni$^{2+}$ (see Figure 3.2), 0.1 mM Co$^{2+}$ had no effect. However, with 10 mM Co$^{2+}$ the maximum peak tail current amplitude decreased by ~90 %. As with Ni$^{2+}$, the block by Co$^{2+}$ was completely reversible.

B. Concentration-response data obtained in 0 mM K$^{+}$o, with each point representing the mean ± SE of measurements in 3-8 cells, were fitted to eq. 2 which gave a $K_D$ of 1.4 ± 0.1 mM and an $n_H$ of 1.3 ± 0.1.
The effects of Cd\(^{2+}\) are not illustrated but closely resembled those of Co\(^{2+}\). The \(K_D\) was 1.5 ± 0.4 mM and the \(n_H\) was 1.3 ± 0.3. In 1 mM Cd\(^{2+}\) the \(V_H\) for the \(g-V\) relationship was shifted rightward by 19.5 ± 1.2 mV.

Of the divalent cations we tested for an ability to block Kv1.5, Mn\(^{2+}\) proved to be the least effective. At 10 mM, the highest concentration used, \(g_{\text{max}}\) was 73 ± 3 % of the control value. The mid-point of the \(g-V\) relationship was shifted rightward by 21.5 ± 0.7 mV (\(n = 3\)).

**Co\(^{2+}\) and Zn\(^{2+}\) mimic the effect of Ni\(^{2+}\) on Kv1.5 inactivation**

Figure 3.9A illustrates representative results of the effect of 10 mM Co\(^{2+}\) on inactivation and recovery from inactivation using a voltage protocol identical to that described for Figure 3.4. Again, a slowly rising phase of current seen in 10 mM Co\(^{2+}\), K\(^{+}\)-free medium (not shown) necessitated recording with 3.5 mM K\(^{+}\). In 10 mM Co\(^{2+}\) both the onset of and recovery from inactivation was comparable to that seen with 2 mM Ni\(^{2+}\) (Figure 3.4). In the four cells studied with 10 mM Co\(^{2+}\), \(\tau_{\text{inact}}\) and \(\tau_{\text{recovery}}\) were 1.3 ± 0.1 s and 24.6 ± 1.7 s, respectively. As noted above, Zn\(^{2+}\) also causes a concentration and K\(^{+}\)-dependent inhibition of Kv1.5 currents and for that reason its effects on inactivation were also examined (Figure 3.9B). Using a Zn\(^{2+}\) concentration of 2 mM, which is estimated to reduce \(g_{\text{max}}\) by 80-90 % in 3.5 K\(^{+}\), \(\tau_{\text{inact}}\) was 1.64 ± 0.3 s and \(\tau_{\text{recovery}}\) was 27.7 ± 2.1 s (\(n = 5\) cells). Thus, a feature which is shared by Ni\(^{2+}\), Co\(^{2+}\) and Zn\(^{2+}\) is an ability to substantially slow recovery from inactivation and to modestly accelerate inactivation. In this regard at least these divalent cations are clearly distinct from extracellular protons which, by comparison, accelerate inactivation to a far greater extent (\(\tau_{\text{inact}} \sim 100\) ms at pH 5.4) and slow recovery from inactivation much less (\(\tau_{\text{recovery}} \sim 4\) s at pH 5.4).
Figure 3.9. Co$^{2+}$ and Zn$^{2+}$ mimic the effect of Ni$^{2+}$ on macroscopic inactivation. A. Using a voltage protocol identical to that described for Figure 3.4, the $\tau_{\text{inact}}$ with 10 mM Co$^{2+}$ in 3.5 mM K$^{+}$ was well-fitted by a single exponential with a time constant of 1.6 s. The fit of an exponential function to the peak currents evoked by 50 ms test pulses following the 5 s pulse to 50 mV gave a $\tau_{\text{recovery}}$ of 21.8 s. B. With 2 mM Zn$^{2+}$ in 3.5 mM K$^{+}$, $\tau_{\text{inact}}$ was 1.86 s and the $\tau_{\text{recovery}}$ was 29.3 s. Because Zn$^{2+}$ slowed the activation rate the duration of test pulses used to monitor recovery was increased to 200 ms. These data indicate a clear difference in the effect on Kv1.5 inactivation of divalent cations versus external protons (Figure 3.4A).
DISCUSSION

As reported previously (Perchenet & Clement-Chomienne, 2001), external Ni\(^{2+}\) ions were shown to reversibly block human Kv1.5 currents (Figure 3.1). We have also shown here that Ni\(^{2+}\) block is affected by [K\(^+\)]\(_o\) (Figure 3.2). Thus, with 0 mM K\(^+\)\(_o\) the \(K_D\) for the Ni\(^{2+}\) block is approximately 150 \(\mu\)M whereas with 3.5 mM K\(^+\)\(_o\) the \(K_D\) increases to 400 \(\mu\)M. The latter value is consistent with the \(K_D\) of 570 \(\mu\)M obtained with 5 mM K\(^+\)\(_o\) in CHO cells (Perchenet & Clement-Chomienne, 2001). Increasing K\(^+\)\(_o\) to 140 mM increased the \(K_D\) to \(~3\) mM. The \(n_H\) of 1.2 to 1.6 derived from concentration-response data in 0 mM to 5 mM K\(^+\)\(_o\) (see also Perchenet & Clement-Chomienne, 2001)) suggests that the block requires the binding of at least two Ni\(^{2+}\) ions. In the study with Kv1.5 expressed in CHO cells the Ni\(^{2+}\) block was shown, regardless of the pulse frequency, to develop slowly over a 2 to 5 minute period (Perchenet & Clement-Chomienne, 2001) despite the use of a fast drug application system. These data were interpreted to reflect a large disparity in the association and dissociation rate constants for Ni\(^{2+}\) binding to the closed state of the channel. Although we agree that the Ni\(^{2+}\) block can occur from the closed state, we found no evidence for a slow development of that block (Figure 3.3).

One possible interpretation of the inhibition of the Ni\(^{2+}\) block by K\(^+\)\(_o\) is that it reflects an interaction in the channel pore either by competition for the same binding site or by an electrostatic effect between separate Ni\(^{2+}\) and K\(^+\) binding sites. However, as noted with the block by Zn\(^{2+}\) and H\(^+\)\(_o\) (Kehl et al., 2002), the block by Ni\(^{2+}\) shows no voltage dependence over a range of voltages where the open probability is maximal (Perchenet & Clement-Chomienne, 2001). This observation supports the conclusion that the Ni\(^{2+}\) binding site is at least not in a region of the pore that is within the electric field and, by extension, that Ni\(^{2+}\) is
not blocking by occlusion of the pore. The fact that Kv1.5 currents are blocked by $H^{+}$, $Zn^{2+}$ and $Cd^{2+}$, whereas Shaker channels are not, also suggests a binding site external to the pore (e.g., in the turret) since in Kv1.5 and Shaker there is complete homology from the N-terminal end of the pore helix to the GYG pore signature sequence.

As is the case with the block of Kv1.5 by $H^{+}$ and $Zn^{2+}$, the sensitivity of Kv1.5 channels to $Ni^{2+}$ is greatly affected (Figure 3.5) either by mutating H463 in the pore turret or by mutating R487, a residue in the outer pore mouth that has been shown in Shaker channels to play a pivotal role in P/C-type inactivation. These results with the 463Q and 487V mutant channels, as well as the sensitivity of the $Ni^{2+}$ block to $K^{+}$ and the outcome of other substitutions at position 463 (see below), are consistent with a model in which the binding of $Ni^{2+}$ to one or more H463 residues in the pore turret facilitates an inactivation process that involves the outer pore mouth. Although this model is the same as that proposed for the $H^{+}$ and $Zn^{2+}$ block of Kv1.5, there is not complete overlap of the effects of these three metal cations. For example, the inactivation rate of the residual currents is markedly different with the divalent cations ($Ni^{2+}, Co^{2+}, Zn^{2+}$) compared to $H^{+}$ (Figure 3.4 and Figure 3.9). Thus, for example, using concentrations that produce a similar degree of block in 3.5 mM $K^{+}$, the residual currents inactivated roughly twenty times faster with $H^{+}$ (pH 5.4) than with $Ni^{2+}$ (Figure 3.4). Additionally, the shift of the midpoint of the $g-V$ curve and the $Q_{on}-V$ curve by $Ni^{2+}$ was also much less than with either $H^{+}$ or $Zn^{2+}$. Finally, the dramatic slowing of the activation rate observed with $Zn^{2+}$ (Zhang et al., 2001a) is not seen with either $Ni^{2+}$ or $H^{+}$. It seems unlikely, though we cannot disprove, that these differences are due solely to the nature of ligand co-ordination by the histidine residues in the turret. Particularly in the case of $H^{+}$, the involvement of additional binding sites seems likely. This is suggested by the fact that
although Shaker-IR channels are largely resistant to the conductance collapse in low pH, acidification does accelerate current inactivation (Perez-Cornejo, 1999; Stárkus et al., 2003). Furthermore, we and others have shown that manipulations that reduce the block of Kv1.5 by metal cations do not affect the gating shift (Kehl et al., 2002; Trapani & Korn, 2003).

From the data in Figure 3.5 it is also apparent that neither of the outer pore mutations completely prevents current inhibition by Ni$^{2+}$. Currents through the Kv1.5-H463Q construct decreased by ~30% in 5 mM Ni$^{2+}$ and, as with the Zn$^{2+}$ block of this mutant channel (Kehl et al., 2002), the $n_H$ fitted to the concentration dependence of this block was quite small (~0.5) suggesting the involvement of a binding site and mechanism of action that is different. In the case of the R487V mutant, the $K_D$ and the $n_H$ for the Ni$^{2+}$ block with 0 mM K$^{+}$ are similar to that estimated for wt Kv1.5 in 140 mM K$^{+}$. Paradoxically, neither of these manipulations, increasing [K$^{+}$]$_o$ or mutating R487, substantially affects the inactivation rate of macroscopic currents carried by K$^+$ during sustained depolarizations (Fedida et al., 1999). Although the latter observations might be construed as evidence against an involvement of outer pore inactivation in the Ni$^{2+}$ block, that is to say neither manipulation can be shown directly to affect the current decay rate, an alternative explanation is that these manipulations inhibit an outer pore inactivation process occurring from a closed state but are much less effective against inactivation from the open state. In this connection, a K$^{+}$-sensitive ($K_D$ ~ 0.8 to 10 mM) inactivation process occurring from a closed state has been suggested to account for the decline of the macroscopic conductance seen in fast-inactivating Shaker-IR-T449 mutants when the [K$^{+}$]$_o$ is decreased (Lopez-Barneo et al., 1993) and there is evidence in Shaker-IR supporting, not exclusively, “multiple, independent pathways of which C-type is only one” (Yang et al., 1997).
As with some of the T449 mutations in Shaker, there are mutations of Kv1.5-H463 that can dramatically affect outer pore inactivation. For example, mutants in which glycine (G) (Kehl et al., 2002) or arginine (R) (Eduljee et al., 2003) is substituted for H463 display rapidly inactivating currents ($\tau_{\text{inact}} = 35$ to $75$ ms) and, again as in the Shaker-T449X mutants, these rapidly inactivating mutants show a collapse of the macroscopic conductance in 0 mM $K^+$. Furthermore, in the H463G mutant the conductance collapse in 0 mM $K^+$ is prevented by the R487V mutation (Trapani & Korn, 2003). The outcome of these H463G and H463R mutations is significant because it shows directly that the physico-chemical properties of the residue at this position can dramatically affect the time course of open- (and closed?) state inactivation and thus offers additional support for the proposition that non-covalent chemical modification of H463 by the binding of Ni$^{2+}$, in addition to other metal cations, can affect inactivation.

Another significant property of the H463G mutant is that $K^+$ affects the $g_{\text{max}}$ with a $K_D$ of approximately 1 mM (Eduljee et al., 2003). This low millimolar $K_D$ is comparable not only to that estimated for the fast-inactivating ShakerIR mutants (Lopez-Barneo et al., 1993) but to that obtained for the relief by $K^+$ of the $H^+$ and Zn$^{2+}$ block (Kehl et al., 2002; Zhang et al., 2001a). A detailed study of the $K^+$-dependence of the Ni$^{2+}$ block was not undertaken here. However, using the $K_D$ of the Ni$^{2+}$ block in zero and 3.5 mM $K^+$, and assuming, for simplicity, a competitive interaction, the $K_D$ for the relief of the block by $K^+$ is calculated to be $\sim 1.5$ mM. A consistent pattern that emerges from these studies, whether it is the spontaneously-occurring conductance collapse in Shaker-IR and Kv1.5 mutant channels or the metal ion-induced block/conductance collapse in wt Kv1.5, is that inhibition of the conductance loss occurs with low millimolar $K^+$ concentrations and that this inhibition
occurs in the absence of a change of the inactivation rate measured during depolarizing pulses. This implies that there is an outer pore inactivation process, perhaps that occurring from the closed state, that is much more sensitive to $K^+_o$ and, we suggest, given that its inactivation rate is not distinguishable from wt Kv1.5 channels, that in Kv1.5 the R487V mutation selectively affects this same inactivation process. With the fast-inactivating Shaker-IR mutants, Lopez-Barneo et al. (1993) remarked that the tendency for the conductance to collapse (inactivate from the closed state?) in 0 mM $K^+_o$ is associated with fast current inactivation. This correlation also applies to Kv1.5-H463G where the inactivation rate is some 20-fold faster than in wt Kv1.5 channels but it is much less evident with the Ni$^{2+}$, Co$^{2+}$ and Zn$^{2+}$ block where the inactivation rate of residual currents is only ~2-fold faster than in controls (Figures 3.4 and 3.9).

Particularly in view of the low concentrations of $K^+_o$ needed to relieve the metal cation block, a question that inevitably arises is whether the external $K^+$ binding site can also be populated by outward $K^+$ flux through the open channel. Though it has not been studied for Ni$^{2+}$ block, our recent finding (Zhang et al., 2003) of virtually identical $K_D$s for the block by $H^+_o$ of outward $K^+$ or Na$^+$ currents argues against a contribution of outward $K^+$ currents in the block relief. One explanation for this apparent absence of an effect of $K^+$ efflux through the open pore is that $K^+$ ions at the outer pore mouth rapidly equilibrate with the external solution. Alternatively, if Ni$^{2+}$-bound channels are inactivating from a closed state, or if the open time is very brief (Zhang et al., 2003), there would be no opportunity for block relief by outward $K^+$ currents.

A comparison of currents from one channel outside-out patches (Figure 3.6) prior to and following the application of 0.5 mM Ni$^{2+}$ showed: 1) that open channel current ($i$) at 100
mV did not change; 2) that the open probability ($P_o$) during 300 ms sweeps containing channel activity was not changed; and, 3) channel availability ($N$), decreased from a value of ≈0.9 in the control to ≈0.4 during treatment. Although a detailed analysis and comparison of open and closed time behaviours have not yet been done, these preliminary data are consistent with a model in which Ni$^{2+}$ binding facilitates a reversible transition from an available to an unavailable (closed state inactivated?) state.

Gating current analyses (Figure 3.7) showed that, as with H$^+$ (Kehl et al., 2002), Ni$^{2+}$ did not affect $Q_{max}$. This finding rules out the possibility that the prevention of one or more of the transitions in the activation pathway accounts for the Ni$^{2+}$-induced decrease of $g_{max}$. Ni$^{2+}$ treatment also caused a ≈-10 mV shift of the $Q_{on}$-$V$ curve but this was much less than the 50-60 mV shift seen with H$^+$ or Zn$^{2+}$ (Kehl et al., 2002; Zhang et al., 2001b). As noted above, it is not clear if this disparity in the gating shift reflects differences in ligand coordination with H463 residues or if the larger shift with H$^+$ and Zn$^{2+}$ reflects interactions with additional binding sites.

Transition metal ions that have now been shown to block Kv1.5 currents are Zn$^{2+}$, Cd$^{2+}$, Ni$^{2+}$ and Co$^{2+}$ (Figure 3.8). For the first-row transition metals the rank order for the inhibition of Kv1.5 in 0 mM K$^+$ is Zn$^{2+}$ ($K_D$ ~0.07 mM) ≥ Ni$^{2+}$ ($K_D$ ~0.15 mM) > Co$^{2+}$ ($K_D$ ~1.4 mM) > Mn$^{2+}$ ($K_D$ >10 mM) and, as such, is in accord with the Irving-Williams order (Glusker, 1991). Zn$^{2+}$, Ni$^{2+}$ and Co$^{2+}$, which are intermediate Lewis acids, are known to bind to the thiolate side group of cysteine and the imidazole nitrogen of the histidine. Zn$^{2+}$ is also able to bind to carboxylate and carbonyl oxygen atoms. Cd$^{2+}$, a second row transition metal, is a soft Lewis acid and typically has a higher affinity for a soft base such as the thiolate ion.
Preliminary work with the H463C mutant shows a sensitivity to block by Cd\(^{2+}\) that is greater than for wt Kv1.5.

In Cav2.3 (\(\alpha 1E\)) channels, external Ni\(^{2+}\) causes, in addition to a rightward shift of the \(g-V\) curve, a reduction of the slope conductance with an estimated \(K_1\) of 300 \(\mu M\) (Zamponi et al., 1996). The blocking reaction appears to be bimolecular and is also affected by the type of permeant ion (e.g., Ca\(^{2+}\) versus Ba\(^{2+}\)). It was suggested that the Ni\(^{2+}\) block of Cav2.3 reflected changes of permeation due to direct occlusion of the pore in addition to a possible change of the permeant ion concentration at the pore mouth. In voltage-gated \(K^+\) channels, divalent cations have proved to be useful probes of gating and permeation. However, whereas Zn\(^{2+}\) and Cd\(^{2+}\) have been studied in some detail (e.g., Gilly & Armstrong, 1982; Spires & Begenisich, 1994), Ni\(^{2+}\) has been used somewhat sparingly. In HERG \(K^+\) channels external Ni\(^{2+}\), as well as Cd\(^{2+}\), Co\(^{2+}\) and Mn\(^{2+}\), increased the maximum current amplitude, an effect that was imputed to an alteration of inactivation gating (Paquette et al., 1998). Interestingly, in HERG channels mutations at a number of sites in the S5-P linker can dramatically alter inactivation (Liu et al., 2002), a finding that underscores the findings with Kv1.5 that, either by substitution through point mutation, or by chemical modification through ligand binding, residues in this region can profoundly influence the rate and extent of one or more inactivation processes occurring at the outer pore mouth.

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REFERENCES


CHAPTER 4: SCAM ANALYSIS REVEALS A DISCRETE REGION OF THE PORE TURRET THAT MODULATES SLOW INACTIVATION IN Kv1.5

A version of this chapter has been submitted for publication. Eduljee, C., Clayton, T.W., Viswanathan, V., Fedida, D., and Kehl, S.J. Scam analysis reveals a discrete region of the pore turret that modulates slow inactivation in Kv1.5 (Am.J.Physiol. Cell Physiol.)
INTRODUCTION

Kv1.5 is a voltage-gated potassium channel whose macroscopic currents activate rapidly upon depolarization before inactivating slowly over several seconds (Fedida et al., 1999). This slow, P/C-type inactivation, is distinct from the fast, N-type inactivation seen with other potassium channels such as Shaker, which is due to occlusion of the inner pore mouth by the N-terminus or by a co-expressed beta subunit (Zagotta et al., 1990; Hoshi et al., 1990; Zhou et al., 2001). Our current understanding of P/C-type inactivation is largely the result of studies of the S5-S6 region of the Shaker-IR (fast inactivation removed) potassium channel, which have shown that mutation of residue A463 in S6 (Hoshi et al., 1991), E418 at the distal end of S5 (Ortega-Sáenz et al., 2000; Larsson & Elinder, 2000), F425 in the S5-P linker (Perez-Cornejo, 1999), W434 in the pore helix (Perozo et al., 1993), and T449 in the outer pore mouth (López-Barneo et al., 1993), can alter the rate of inactivation. Increasing the external potassium concentration ([K+]o), has been shown to inhibit inactivation in Shaker-IR (López-Barneo et al., 1993; Schlief et al., 1996), and together with studies involving the T449C mutant (Yellen et al., 1994), which indicate that the outer pore may constrict during inactivation, has led to the hypothesis that binding of external K+ ions within the conducting pathway inhibits inactivation via a foot-in-the-door mechanism that prevents constriction of the outer pore mouth (López-Barneo et al., 1993). Inactivation has also been demonstrated to lead to an immobilization of gating charge (Fedida et al., 1996; Olcese et al., 1997). However, the finding that the Shaker-IR-W434F mutant appears to be “permanently inactivated”, and yet still displays charge immobilization, has led to the hypothesis that slow inactivation is comprised of two phases (Olcese et al., 1997; Yang et al., 1997; Loots & Isacoff, 1998). In the initial P (pore)-type phase, the outer pore constricts, eliminating
potassium conductance, while with continued depolarization, S4 enters a stable, immobilized (C-type) conformation by interacting with the pore. We refer to these processes collectively as P/C-type inactivation.

Our previous work has shown that in Kv1.5, modification of residue 463 in the turret region (S5-P linker; Figure 4.1A and B) can also have profound effects on P/C-type inactivation. In particular, binding of extracellular H\(^+\) or Zn\(^{2+}\) ions to H463, increases the rate of depolarization-induced inactivation, and reduces the macroscopic current amplitude (Kehl et al., 2002; Zhang et al., 2005). The effects of H\(^+\) and Zn\(^{2+}\) binding could be antagonized by raising [K\(^+\)]\(_0\) (\(K_D \approx 1\) mM, a value similar to the \([K^+]_0\)-dependence of P/C-type inactivation in Shaker-IR (Baukrowitz & Yellen, 1996)) or by the R487V mutation (homologous to T449V in Shaker; Figure 4.1B) (Kehl et al., 2002). Therefore, we proposed that modification of turret residue H463 results indirectly in an enhancement of P/C-type inactivation.

In this study, we examined the effects of various substitutions at position 463 in order to understand the properties of this residue that are important in regulating Kv1.5 behaviour. To determine if residue 463 was unique in its ability to affect channel behaviour, we then used the substituted cysteine accessibility method (SCAM) (Holmgren et al., 1996) to introduce cysteine mutations into each turret position (Figure 4.1C) and tested for reactivity to the sulfhydryl-reactive MTSET and MTSES reagents. This was used to determine if modification by either MTS reagent could affect current, and whether any MTS-induced inhibition of current might be linked to the P/C-type inactivation process. The results indicate that modification of position 463 does not disrupt inactivation via a purely
Figure 4.1. The turret forms a rim at the perimeter of the outer pore mouth in Kv channels.  
A, A single subunit of the Kv1.5 channel highlighting the S5-P linker that forms the turret.  
B, Two opposing subunits of the S5-S6 region of the Kv1.2 crystal structure (Long et al., 2005a) generated using Accelrys DS ViewerPro 5.0 are shown to indicate the location of the turret (black carbonyl backbone) and the approximate positions of residues homologous to H463 and R487 are shown based on the locations of the equivalent residues in the Kv1.2 channel and energy minimization. The orientations of these side chains are not known.  
C, A sequence alignment of the S5-P linker, P-loop and P-S6 linker of Kv1.5 (NCB Accession # NM 002234), Kv1.2 (NCB Accession # NM 004974), Kv1.4 (NCB Accession # NM 002233), Shaker (NCB Accession # P08510), and KcsA (NCB Accession # 1K4C). Sequence comparison was made by aligning the GYG signature sequences of each channel. Conserved residues are indicated by a dash.
electrostatic interaction with residues in the pore, as proposed by others (Jäger & Grissmer, 2001), and suggest that position 463 is part of a discrete group of residues in the distal region of the turret (T462-P468) that are able to regulate P/C-type inactivation. These data are discussed in the light of reports on other channel types (e.g., TRPV5 and Kv2.1) that suggest a widespread role of the turret in the modulation of ion channel behaviour.

METHODS

Site-directed mutagenesis and cell preparation

Mutations were introduced into Kv1.5 in the pcDNA3 expression vector using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) and confirmed using fluorescent terminator-dye sequencing (Applied Biosystems, Foster City, CA, USA). Double mutations incorporating the R487V mutation were made by subcloning a cassette of DNA containing the mutation of interest into the R487V mutant vector using the BstEII and ClaI restriction enzymes. The wt Kv1.5 channel has one extracellular cysteine in the S1-S2 linker at position 268, and was present in all mutants, except for the C268V/T462C double mutant, which was made by subcloning the T462C mutant into the C268V vector using the BstEII and ClaI restriction enzymes. Wild type (wt) human Kv1.5 channels were studied in stably transfected HEK 293 and ltk cells lines and maintained as previously described (Kehl et al., 2002). Current properties were found to be similar in both lines. HEK 293 cells were used for the H463 mutants, while ltk cells were used for the cysteine mutants so that contamination of expressed currents by the endogenous current was minimal. Cells were stably transfected with mutant Kv1.5 cDNA as described previously (Kehl et al., 2002), or transiently transfected with 1 µg of plasmid cDNA using 2-4 µl of Lipofectamine 2000.
(Invitrogen) one day after passaging, and used for experiments 1-3 days after transfection. Cells were also co-transfected with 1 μg of CD8 cDNA. Before experiments, cells were incubated with OPTI-MEM (Invitrogen) containing CD8 antibody-conjugated beads (Dynal Biotech, Oslo, Norway) for 30 min. Cells were then washed with OPTI-MEM and those with bound beads were selected for recording (Jurman et al., 1994).

**Recording solutions**

The standard bath solution was nominally K⁺-free and contained, in mM: 143.5 NaCl, 10 Hepes, 2 CaCl₂, 1 MgCl₂, 5 glucose and its pH was adjusted to 7.4 with NaOH. The [K⁺]₀ was kept at 0 mM to eliminate the possibility of a K⁺₀-dependent relief of the MTS-mediated inhibition, except for the T462C mutation, where 3.5 mM K⁺₀ was required to sustain an appropriate level of resting availability. Where the effect of [K⁺]₀ on channel function was examined, KCl was substituted for NaCl. The standard patch pipette solution contained, in mM: 130 KCl, 4.75 CaCl₂ (pCa²⁺ = 7.3), 1.38 MgCl₂, 10 EGTA, 10 Hepes and was adjusted to pH 7.4 with KOH. Stock solutions of 2 M [2-(trimethylammonium)ethyl] methanethiosulfonate bromide (MTSET; Toronto Research Chemicals, Toronto, ON, Canada) and sodium (2-sulfonatoethyl) methanethiosulfonate (MTSES) were made weekly in distilled H₂O and kept at -20 °C. Before experiments, a 2 mM working concentration was made from the stock by dilution with the standard bath solution and kept for no more than 2 h at 4 °C before being used.

**Signal recording and data analysis**

Macroscopic currents were recorded in the whole cell or the outside-out patch configuration
as previously described (Kehl et al., 2002). To construct a $g$-$V$ curve, peak tail current was measured at -40 mV after 300 ms pre-pulses from -45 to +50 mV and fit to a single exponential function. After normalization, the data were fit to a Boltzmann function: $y = A / \{1 + \exp[(V_{1/2} - V)/s]\}$ where $y$ is the normalized current, reflecting conductance ($g$), $A$ is the best-fit of the maximal response, $V_{1/2}$ is the half-activation voltage, $V$ is the pre-pulse voltage, and $s$ is the slope factor.

MTS-induced inhibition was quantified by perfusing 5 ml of the MTS reagent, after which the cell was washed with 5 ml of the standard bath solution to reverse any non-specific effect of the MTS reagent.

Under normal cell culture conditions, currents from $\text{Kv1.5-T462C}$ were small and inactivation was fast ($\tau_{\text{inact}} = 121 \pm 24$ ms, $n = 5$, data not shown). Pretreatment with 0.5 mM of the reducing agent DTT (Fisher Scientific, Fairlawn, NJ, USA) in OPTI-MEM for a minimum of 30 min was found to maximally increase current amplitude and slow the rate of inactivation (see Figure 4.4) and was used prior to all experiments with this mutant. After pretreatment, currents from the T462C mutant were recorded in the absence of DTT and the current phenotype was not observed to change during the control current recordings. The C268V/T462C double mutant was also DTT sensitive, suggesting that disulfide bond formation between T462C and C268 could not account for the effects of DTT observed in the T462C mutant.

Data are expressed as the mean ± the standard error of the mean (S.E.) except for the values obtained by non-linear least-squares fitting routines, which are expressed as the mean ± the standard deviation (S.D.) (Igor, Wavemetrics, OR, USA). A one-way ANOVA and
Dunnett's test were used to test for significance between the control and test groups. A $P$-value of 0.05 or less was considered significant.

**Expression of cysteine mutants and Proteinase-K experiments**

While the majority of cysteine mutants displayed robust currents, less than 5% of P468C-transfected cells expressed currents. These currents were routinely $<1$ nA at $+50$ mV, except in the presence of the R487V mutation, which increased current amplitude and the proportion of expressing cells. We do not know the basis for the rescue of T462C current by the R487V mutation. No current was detected in cells transfected with either E456C, F464C or I467C cDNA, so a proteinase-K assay (Ebeling *et al.*, 1974) and immunoblot (Choi *et al.*, 2005) were conducted to examine the surface expression of these mutants. The results indicated that the E456C, F464C and I467C mutants were not fully glycosylated and did not express on the cell surface (not shown).

**RESULTS**

*The addition of a positive or negative charge or a decrease of volume of the side chain at position 463 increases the rate of slow inactivation and induces $[K^+]_o$-dependence*

Our previous work has shown that the H463G mutation increases the rate of Kv1.5 inactivation and almost completely eliminates current when potassium is removed from the extracellular solution (Kehl *et al.*, 2002; Zhang *et al.*, 2005). This motivated us to examine the effect of other substitutions at position 463 to determine whether a change of side chain volume or charge was an important determinant of the functional changes observed. Figure 4.2 illustrates typical currents in channels where H463 was mutated to glutamine (Q), tyrosine (Y), cysteine (C), arginine (R), lysine (K), glycine (G), alanine (A) or glutamate (E).
Compared to wt Kv1.5, the H463Q, H463Y and H463C mutations did not alter the rate of inactivation (see Figure 4.4 for quantification of inactivation in H463C) and the current amplitude was not decreased by removing extracellular K⁺. However, as with the H463G mutation, the H463R and H463K mutations increased the rate of inactivation and caused the current to collapse in 0 mM K⁺₀. The H463A and H463E mutants inactivated with a time course similar to that of H463K, but the sensitivity of current to removal of [K⁺]₀ was comparatively smaller. These results indicate that substitutions of large (cysteine = 109 Å³; tyrosine = 194 Å³; glutamine = 144 Å³; histidine = 153 Å³), neutral (at pH 7.4) sidegroups at position 463 have little effect on channel function, whereas small (<100 Å³), neutral side groups as well as large (>100 Å³), charged side groups increase the rate of inactivation and induce a [K⁺]₀-dependence of current amplitude.

Since several mutations at position 463 altered the rate of inactivation, we asked if other residues in the turret region could also affect inactivation. An alignment of the S5-S6 linkers from hKv1.2, hKv1.4, hKv1.5, Shaker and KcsA channels, reveals a large degree of homology at each end of the turret, while the intervening region shows considerable variation (Fig 1C). To investigate which turret residues might be important for regulating inactivation, a SCAM analysis was performed by substituting cysteines at each turret position, from E456 to P468. The effect of cysteine substitution alone was characterized prior to assessing the functional consequences of the covalent modification by either the MTSES or MTSET reagent.
Figure 4.2. Some mutations of turret residue 463 alter inactivation and confer sensitivity of the current magnitude to $[K^+]_o$. Wild type and 463 mutant currents were evoked by a depolarization to +50 mV from a holding potential of −80 mV. $[K^+]_o$ ranged from 0 to 140 mM. In this and subsequent figures, the dashed line denotes the zero current level. Currents from the wild type, H463Q, H463Y and H463C mutants showed little inactivation over a 300 ms period in the absence of $[K^+]_o$ (0 mM). Inactivation of H463R, H463K, and H463G mutant currents was rapid and well fit by a single exponential: $\tau_{\text{inact}} = 67 \pm 3$ ms, $n = 3$, in 140 mM $K^+$; $323 \pm 33$ ms, $n = 6$, in 140 mM $K^+$ and $73 \pm 8$ ms, $n = 4$, in 3.5 mM $K^+$ (taken from Figure 2.7), respectively. H463G, H463K and H463R did not conduct in 0 mM $K^+$, but the current level could be titrated by increasing $[K^+]_o$. H463A and H463E currents inactivated with a $\tau_{\text{inact}}$ of $390 \pm 33$ ms, $n = 6$, in 3.5 mM $K^+$ and $227 \pm 18$ ms, $n = 3$, in 3.5 mM $K^+$, respectively, and although $[K^+]_o$-sensitive, did not collapse completely in 0 mM $K^+$.
Cysteine substitutions have modest effects on the macroscopic properties of Kv1.5

Activation kinetics were largely unaffected by turret mutations. However, for D458C and P468C the $r_{act}$ was significantly slowed compared to the wt channel (Figure 4.3A-D). This slowing of activation was correlated with a depolarizing shift of the half-activation voltage ($V_{1/2}$) (Figure 4.3E). The $V_{1/2}$ of the S466C mutant also displayed a significant depolarizing shift, but the half-activation voltages of the other mutants were not significantly affected.

The slope factor obtained from the fit to a Boltzmann equation was significantly larger in the D458C, S466C and P468C mutants, compared to the wt channel, but unchanged in the remainder of mutants. As with activation, the deactivation kinetics were unaffected in the majority of the cysteine mutants. However, $r_{deact}$ was significantly slowed in S465C and sped in P468C, compared to the wild type channel (Figure 4.3D). Therefore, our results indicate that in Kv1.5, turret residues D458, S466 and P468 can affect activation kinetics and voltage sensitivity. This is consistent with studies that have suggested that the Shaker turret may interact with S4 to affect gating (Loots & Isacoff, 1998; Elinder & Arhem, 1999; Yifrach & MacKinnon, 2002; Laine et al., 2003) and structural data from the Kv1.2 channel indicating that S4 is in close proximity to the pore domain (Long et al., 2005b).

Representative traces of inactivation in wt Kv1.5 and the cysteine mutants are shown in Fig 4A. Analysis of the fast and slow components of inactivation of most mutants indicated that the majority were best fit by the sum of two exponentials and were not significantly different from wt Kv1.5 (Figure 4.4C). Only the Kv1.5-N459C mutation demonstrated a significant slowing of the fast component of inactivation compared to that of wt Kv1.5 (Figure 4.4C). No fast component of inactivation was observed in the D458C and P468C mutations, perhaps due to their slower activation (see above). The residual current at
Figure 4.3. Cysteine substitution in the turret has modest effects on activation and deactivation. A-C, Activation time constants (τ_{act}) were measured by fitting the activating phase (50 - 100 % of maximal) of currents at +50 mV to a single exponential function (Zagotta et al., 1994; Kanevsky & Aldrich, 1999), represented by the superimposed dashed line. Compared to wt Kv1.5 (A) (τ_{act} 3.0 ± 0.7 ms, n = 5), the τ_{act} of D458C (B) and P468C (C) were significantly slower at 14 ± 2 ms (n = 15) and 10 ± 2 ms (n = 3), respectively (activating wt current was scaled and shown as a dotted line in B and C). D, Average activation and deactivation (τ_{deact}) time constants are shown for the wt and cysteine-substituted channels. The τ_{deact} was measured at −80 mV by fitting a single exponential. The S465C mutation significantly slowed deactivation, τ_{deact} = 17 ± 4 ms (n = 5), compared to 8.7 ± 0.7 ms (n = 7) for wt Kv1.5, while the P468C mutation increased the rate of deactivation to 3.4 ± 0.3 ms (n = 5). In this and subsequent figures, a significant difference with respect to wt Kv1.5 (P < 0.05) behaviour is indicated by an asterisk, a dotted line indicates the wt Kv1.5 value and n.s.e. indicates there was no surface expression. E, The D458C, S466C and P468C mutations were also associated with a significant depolarizing shift of the g/V curve and an increase in the slope factor. The V_{1/2} and slope factor of wt Kv1.5 were −19.3 ± 0.8 mV and 4.7 ± 0.4 mV (n = 7), compared to −5 ± 2 mV and 7.9 ± 0.4 mV (n = 5) for D458C, −10 ± 3 mV and 9.2 ± 0.6 mV (n = 5) for S466C and −7 ± 3 mV and 11 ± 1 mV (n = 3) for P468C.
Figure 4.4. Slow inactivation was largely unaffected by cysteine substitution. A, Representative wild type and mutant currents in response to a depolarization to +50 mV for 5 or 6 s. Current decay was best fit to a double exponential to quantify the extent and rate of slow inactivation, except for D458C and P468C, where no fast phase of inactivation was detectable and a single exponential was used. B, The residual component from the double exponential fit is shown as a percentage of the peak current, and in S465C, was significantly smaller (18 ± 6%, n = 10), compared to wt Kv1.5 (47 ± 2%, n = 11). C, Average $\tau_{\text{slow}}$ and $\tau_{\text{fast}}$ of inactivation are shown for each mutant. The fast phase of inactivation in N459C was significantly slowed to 852 ± 162 ms ($n = 4, P < 0.05$) compared to 281 ± 46 ms ($n = 11$) for the wild type. $\tau_{\text{slow}}$ was 2.4 ± 0.3 s ($n = 11$) for the wt channel and was not different from the cysteine mutants.
the end of a 5 s pulse to +50 mV was also not significantly altered in most mutants, except for Kv1.5-S465C, where it was significantly reduced relative to the wt channel (Figure 4.4B). Analysis of the recovery from inactivation indicated that compared to the wild type channel (Figure 4.5A), H463C was significantly slowed (Figure 4.5B). P468C was not tested due to a typically small current amplitude, and the remainder of cysteine mutants were not significantly affected (Figure 4.5C).

**MTSET and MTSES irreversibly inhibit currents of cysteine-substituted mutants in the distal turret**

Having established that the majority of cysteine substitutions had only a modest effect on macroscopic current behaviour, we investigated the consequences of covalent modification of the cysteine mutants by either the positively charged MTSET or negatively charged MTSES reagent. Based on the crystal structures of KcsA (Doyle et al., 1998) and Kv1.2 (Long et al., 2005a) as well as accessibility studies of KvAP (Ruta et al., 2003), we have assumed that all introduced cysteines are accessible from the extracellular medium. In both wild type and mutant channels, treatment with either MTS reagent caused a non-specific inhibition that reversed after wash-out with reagent-free medium. A second component of inhibition observed in some mutants could not be washed off, even with prolonged perfusion of control solution (not shown). This inhibition was attributed to a non-reversible, covalent modification of one or more of the four introduced sulfhydryl groups per channel, and was quantified for each mutant as shown in Figure 4.6. The magnitude of this inhibition was the same, regardless of whether or not depolarizing steps were applied during the MTS
Figure 4.5. The H463C mutation slows recovery from the inactivated state. A and B, Recovery from inactivation was determined by inactivating channels with a 5 s prepulse to +60 mV and then repolarizing to −80 mV for 40 s. Throughout the recovery period, current amplitude was assessed without producing additional inactivation by applying 15 ms pulses to +60 mV, starting 25 ms after repolarization. Only the first 12 recovery pulses are shown for clarity (upper panel). Peak currents elicited during the recovery period were then normalized to the peak of the pre-pulse, plotted against the time elapsed since the end of the prepulse and fit to an exponential to obtain the time constant of recovery ($\tau_{\text{recov}}$) (lower panel). $\tau_{\text{recov}}$ was $3.9 \pm 0.4$ s ($n = 6$) for wt Kv1.5 (A) and slowed to $7.7 \pm 0.8$ s ($n = 5$) for H463C ($P < 0.05$, panel B). C, The $\tau_{\text{recov}}$ is shown for wt Kv1.5 and the cysteine-substituted mutants. Due to low expression, the $\tau_{\text{recov}}$ was not determined (n.d.) for P468C.
application (not shown), indicating that both the open and closed states were accessible to the MTS reagents and that an accumulation of inactivation did not contribute to the inhibition. Due to the incomplete inactivation of the cysteine mutants (Figure 4.4A), however, we were not able to accurately determine if the MTS-modification rate was different for the inactivated state, relative to the open or closed states. A possible role for cumulative inactivation was prevented by adjusting the cycle length of the depolarizations used to assess the peak current amplitude based on the recovery from inactivation time data from Figure 4.5. Furthermore, the degree of current inhibition following covalent modification was not affected by long periods of hyperpolarization (~5 min; not shown).

Peak wt Kv1.5 currents were irreversibly inhibited by 15 ± 2% (n = 13) after application and washout of 2 mM MTSET (Figure 4.6E). This degree of inhibition was not significantly different from that seen in the D458C, N459C and G461C mutants. Surprisingly, A457C was not inhibited to any degree by MTSET. In contrast, most cysteine substituted mutants at positions 462 through 468 were inhibited by 80 - 96% by MTSET (Figure 4.6E). Representative traces of the effect of MTSET on T462C are shown in panels A and B of Figure 4.6. Letters beside each trace indicate the current in control solution (a), at the end of MTS application (b), and after wash-out with 5 ml of control solution (c). A notable exception to the high MTSET sensitivity, however, was S465C which was not significantly inhibited.
Figure 4.6. MTSET and MTSES inhibited currents in mutants where the cysteine substitution was in the distal turret. The effect of 2 mM MTSET or MTSES was determined by pulsing channels to +50 mV for 300 ms every 10 s (20 s for H463C, to allow complete recovery from inactivation). A, Sample traces are shown for the control (a), MTSET application (b) and wash-out (c) for the T462C mutant. The exponential fit of the current inactivation after MTSET modification (c) is also shown scaled up (d) to allow comparison with the control trace. The $\tau_{\text{inact}}$ was 124 ± 12 ms, $n = 6$, after MTSET modification. B, Peak currents from the same cell as in panel A were plotted against time, and the steady-state level after wash-out was used to quantify the extent of current inhibition. Letters correspond to the traces in panel A. Inhibition was complete within 1-2 min of the application of MTSET, but a small component of the inhibition was reversed during the washout with control solution. C and D, As for A and B, but with MTSES. After MTSES modification, $\tau_{\text{inact}}$ was 66 ± 5 ms, $n = 7$. E, Average inhibition due to MTSET and MTSES modification in the wild type and cysteine mutant channels is shown. For wt Kv1.5, the mean percent inhibition by MTSET and MTSES was 15 ± 2 ($n = 13$) and 0 ± 3 ($n = 6$); 1 ± 4 ($n = 4$) and 20 ± 4 ($n = 5$) for A457C; 15 ± 4 ($n = 3$) and 30 ± 4 ($n = 5$) for D458C; 9 ± 5 ($n = 4$) and 1 ± 2 ($n = 6$) for N459C; 47 ± 3 ($n = 6$) and 18 ± 3 ($n = 8$) for Q460C; 19 ± 2 ($n = 8$) and -6 ± 4 ($n = 5$) for G461C; 81 ± 3 ($n = 6$) and 87 ± 3 ($n = 7$) for T462C; 82 ± 3 ($n = 5$) and 80 ± 4 ($n = 4$) for H463C; 24 ± 2 ($n = 3$) and 31 ± 4 ($n = 4$) for S465C; 94 ± 4 ($n = 4$) and 68 ± 4 ($n = 5$) for S466C; and 96 ± 4 ($n = 3$) and 99 ± 5 ($n = 3$) for P468C. $[K^+]_0$ was 3.5 mM for T462C and 0 mM for all other mutants.
To determine if the inhibition by MTSET was due to its positive charge, and as such mimicked the effect of $H^+$ or $Zn^{2+}$ binding to H463 in wt Kv1.5, the effect of MTSES, which carries a negative charge, was examined. Inhibition by MTSES followed roughly the same pattern as that of MTSET (Figure 4.6E). Wild type, N459C and G461C currents were not inhibited by MTSES, while A457C, D458C, and Q460C mutants were significantly inhibited, but to a maximum of only $\approx 30\%$ (Figure 4.6E). As with MTSET treatment, Kv1.5-T462C current was dramatically reduced by MTSES (Figure 4.6C and D). In the seven cells tested there was an $87 \pm 3\%$ ($P < 0.05$) reduction in peak current (Figure 4.6E). H463C, S466C and P468C currents were also significantly inhibited by 80 - 99 % (Figure 4.6E). Again, as with MTSET, the sensitivity of S465C to MTSES was much less than for cysteine-substitutions of neighbouring residues. T462C was unique amongst those mutants inhibited by MTS reagents in that currents recorded after treatment with either MTSET or MTSES exhibited a faster rate of inactivation as shown in Figure 4.6A and C. These effects were likely not due to changes in the oxidation state of the substituted cysteine (see Methods), since control currents were recorded in the absence of DTT and the current phenotype was not observed to change during this period. Consistent with an effect due to covalent modification, application of 0.5 mM of the reducing agent DTT reversed the inhibition due to either MTSET or MTSES. However, this reversal occurred slowly over a long period (>10 min.), such that full recovery was only observed in a small proportion of cells before the recording was lost (not shown).

These data indicate that substituted cysteine residues from positions T462-P468 of the turret, which we will refer to as the distal turret, are susceptible to covalent modification by MTS reagents, and that this modification results in a loss of current. The goal of the
following series of experiments was to determine the mechanistic basis for the MTS-mediated inhibition of current.

**The R487V mutation or elevation of [K+]o antagonizes the MTS-mediated inhibition of current**

Inhibition of wt Kv1.5 by H+ or Zn2+ is antagonized either by raising [K+]o or by the R487V mutation (Zhang et al., 2001; Kehl et al., 2002). Similarly, the [K+]o-dependence of Kv1.5-H463G currents is mitigated by the R487V mutation as implied in Figure 5 of Trapani & Korn (2003). Given that both of these manipulations, i.e., changing [K+]o or mutating R487, have been linked to effects on P/C-type inactivation (see Introduction), we examined the effect of MTSET and MTSES either in the presence of 140 mM K+o or with the addition of the R487V mutation, in the four mutants (T462C, H463C, S466C and P468C) that showed the greatest sensitivity to MTS treatment (>65 % inhibition, Figure 4.6E).

Representative traces for each of these four mutants before (-) and after MTSET exposure and wash off (+) in the presence of 140 mM K+o are shown in Figure 4.7A. As indicated in Figure 4.7B, the inhibition of each of the four mutants by MTSET in the presence of 140 mM K+o was significantly lower than that measured in 0 or 3.5 mM K+o. A similar effect of 140 mM K+o on the MTSES-mediated inhibition was observed in all four mutants (Figure 4.7B).

Figure 4.7A also shows representative traces of double mutants containing T462C, H463C, S466C or P468C together with the R487V mutation, before (-) or after (+) MTSET treatment. Compared to the degree of inhibition observed in the single cysteine mutants at the same [K+]o, the sensitivity of the double mutants to MTSET was significantly reduced.
Figure 4.7. Inhibition by MTSET and MTSES can be antagonized by raising \([K^+]_o\) or by the addition of the R487V mutation in the P-S6 linker. A, MTSET inhibition was examined, as described in Figure 4.6, in the T462C, H463C, S466C and P468C mutants but in the presence of 140 mM \(K^+_o\) or in double mutants including the R487V mutation (0 mM \(K^+_o\), except for T462C, where 3.5 mM was used; c.f. Figure 4.6). Sample traces on the left indicate current in 140 mM \(K^+_o\) before (-) and after incubation with and subsequent wash-out of MTSET (+). Representative traces in the right column of panel A indicate the current before (-) and after MTSET incubation and washout (+) in R487V double mutants. B, Average percent inhibition of current by MTSET and MTSES reagents in 140 mM \(K^+_o\) or with the R487V mutation (in 0 mM \(K^+_o\), except for T462C, where 3.5 mM was used) are shown versus the inhibition from Figure 4.6. The percent inhibition by MTSET and MTSES was 32 ± 9 (n = 5) and 18 ± 3 (n = 5) in 140 mM \(K^+_o\) and \(-4 \pm 2\) (n = 4) and \(-24 \pm 4\) (n = 5) with R487V for T462C; 4 ± 1 (n = 5) and \(-3 \pm 2\) (n = 4) in 140 mM \(K^+_o\) and 4 ± 2 (n = 4) and 0 ± 2 (n = 4) with R487V for H463C; 17 ± 9 (n = 4) and 6 ± 5 (n = 5) in 140 mM \(K^+_o\) and 4 ± 5 (n = 4) and 4 ± 3 (n = 6) with R487V for S466C; 52 ± 9 (n = 4) and 35 ± 8 (n = 4) in 140 mM \(K^+_o\) and 25 ± 1 (n = 5) and 9 ± 3 (n = 5) with R487V for P468C.
(Figure 4.7B). A similar result was observed with MTSES in the double mutants and, in the case of T462C-R487V, even produced a modest 24 ± 4 % (n = 5) potentiation (Figure 4.7B). Together, these results imply that modification by MTS reagents of cysteine residues substituted in this region of the turret inhibits current by facilitating a process akin to P/C-type inactivation.

**Elevating external potassium does not alter cysteine accessibility**

A possible explanation for the relief of the MTS-mediated inhibition in the presence of elevated [K⁺]₀ is that there was a change in the accessibility of the MTS reagents to the substituted cysteine residues. To address this possibility, T462C, H463C, S466C and P468C mutants were first exposed either to MTSET or MTSES in the presence of 140 mM K⁺₀ using the same protocol outlined in Figure 4.7, and following MTS washout, were then perfused with low K⁺₀ (0 or 3.5 mM). Representative current traces from Kv1.5-T462C in response to MTSES treatment are shown in Figure 4.8. Letters beside each trace in panel A indicate the current before (a) and after (b) MTSES application in 140 mM K⁺₀, after switching [K⁺]₀ to 3.5 mM (c) and then returning to 140 mM K⁺₀ (d). A plot of the peak current amplitude over time is shown in panel B. Currents recorded in 140 mM K⁺₀ were inhibited during MTSES exposure but this inhibition reversed upon wash-out. Subsequent lowering of K⁺₀ to 3.5 mM caused the current amplitude to decrease, despite an increase of driving force, and was consistent with the conclusion that the covalent modification had indeed occurred during the MTSES treatment in 140 mM K⁺₀. A similar pattern of [K⁺]₀-dependence was seen for both MTSET and MTSES in all four mutants (not shown). As with the MTS-mediated inhibition in low K⁺₀, the prevention of the inhibition by 140 mM K⁺₀ was independent of whether or not channels were pulsed during the MTS treatment. These results suggest that high K⁺₀
Figure 4.8. Increasing $[K^+]_o$ does not relieve MTS inhibition by preventing covalent modification of sulphydryl residues in cysteine-substituted mutants. The T462C mutant was exposed to MTSES in the presence of 140 mM $K^+_o$, after which $[K^+]_o$ was reduced to 3.5 mM to determine if the MTS-mediated inhibition could be restored. Sample traces of T462C in response to depolarizations to +50 mV every 10 s are shown on the left and peak currents are plotted against time on the right. Letters in the diary plot correspond to the sample traces shown. Control currents recorded in 140 mM $K^+_o$ (a), are compared to those after MTSES incubation (in 140 mM $K^+_o$) and washout with 140 mM $K^+_o$ (b) to establish that minimal inhibition occurred as a result of MTS-modification in the presence of 140 mM $K^+_o$. The $K^+_o$ concentration was then lowered to 3.5 mM (c) to determine if currents were modified during the preceding MTS wash-in, after which $[K^+]_o$ was changed back to 140 mM (d; trace shown as dashed line for clarity) to demonstrate the reversibility of the effect.
antagonizes the inhibition promoted by modification with the MTS reagents, but does not affect cysteine accessibility.

DISCUSSION

We have previously shown that turret residue H463 plays a key role in the H⁺ inhibition of Kv1.5. In this study we further explored the role of the Kv1.5 turret by examining which properties of position 463 are important in modulating channel behaviour and by demonstrating that residue H463 is part of a discrete set of residues in the distal half of the turret that can affect slow, P/C-type, inactivation.

Mutation of H463 speeds the rate of inactivation and induces [K⁺]₀-sensitivity

Figure 4.2 demonstrates that several substitutions at position 463 increased the rate of depolarization-induced inactivation and were associated with a decrease in macroscopic current in the absence of K⁺₀. This latter phenomenon has also been observed at pH 7.4 in wt Kv1.4 (Pardo et al., 1992) and fast-inactivating T449 Shaker-IR mutants (López-Barneo et al., 1993; Schlief et al., 1996) and has been suggested to represent a decrease in channel availability due to closed state inactivation (López-Barneo et al., 1993). However, as with T449 mutations in Shaker-IR (López-Barneo et al., 1993; Schlief et al., 1996), there was no clear correlation between the properties of substituted side chains at position 463 in Kv1.5 and the effects on the rate of inactivation or the [K⁺]₀-dependence of current amplitude. For example, both charged (H463K and H463E) and neutral substitutions (H463G) had similar effects on channel function. These results are inconsistent with the hypothesis that residue H463 interacts with position R487 by a purely electrostatic mechanism, as suggested by others (Jäger & Grissmer, 2001). Instead, modification of residue 463, either by mutation or
by protonation, appears to disrupt other interactions within the outer pore region that regulate inactivation gating. In support of this view, recent experiments have suggested that inactivation in KcsA is partially dependent on the ability of the side chain at position D80 of the GYG\textit{D} signature sequence (equivalent to Kv1.5-D485) in the outer pore mouth to become re-orientated into an outward, or “flipped”, conformation (Cordero-Morales \textit{et al.}, 2006a; Cordero-Morales \textit{et al.}, 2006b). Analysis of the KcsA crystal structure indicates that the side chain of residue D80 in the “flipped” conformation is directed towards the side chain of the equivalent turret residue of Kv1.5-H463 (Q58), and the closest atoms from both side chains are within 3 Å of each other. This raises the possibility that in Kv1.5, the effects of the turret on inactivation may be mediated in part by an interaction between H463 in the turret and D485 in the outer pore mouth.

Similar to the \([K^+]_o\)-dependent relief of slow inactivation in \textit{Shaker-IR} (López-Barneo \textit{et al.}, 1993; Baukrowitz & Yellen, 1995), raising external \(K^+\) also slowed the rate of depolarization-induced inactivation and increased the macroscopic conductance in several of the Kv1.5-H463 mutants. Interestingly, the estimated equilibrium dissociation constant \((K_K)\) for these two effects of \([K^+]_o\) varied between mutants (i.e., \(K_K = \sim 1\) mM and \(\sim 103\) mM for the \([K^+]_o\)-dependence of macroscopic conductance and the rate of inactivation in H463G, versus \(\sim 14\) M and \(\sim 190\) mM in H463R (Eduljee \textit{et al.}, 2003; Zhang \textit{et al.}, 2005)). These differences suggest that these are inactivation-related, but not equivalent, processes. However, whether there are two \(K^+\) binding sites that individually regulate these processes, or if a single “foot-in-the-door” site can account for both effects, is still unknown.
MTS reagents inhibit current by enhancing slow P/C-type inactivation

Using the SCAM technique, we found that MTS modification of cysteine-substituted residues in the distal region of the turret inhibited current to a greater degree than those in the remainder of the turret (Figure 4.6). As with the substitutions at position 463, the sign of the charge on the MTS reagent did not affect the current inhibition. Thus, in addition to position 463, modification of a discrete set of turret residues appears capable of inhibiting channel activity.

To date, studies employing the SCAM technique to probe the outer pore mouth of ion channels have not directly addressed the basis for the MTS-mediated inhibition, although some have provisionally attributed it to an occlusion of the outer pore mouth (Kürz et al., 1995). However, the crystal structure of Kv1.2 (Long et al., 2005a) predicts that the α-carbons of turret residues equivalent to positions T462 to P468 in Kv1.5, are ~16-21 Å from the central axis of the pore. Similar turret dimensions were also estimated for Kv1.3 using a functional (toxin-binding) assay (Aiyar et al., 1995). This suggests that when bound to the substituted cysteines, the 6-10 Å long MTS reagents, (Akabas et al., 1992; Andalib et al., 2004), are too far away to occlude the outer pore mouth. Without unitary current analysis of MTS-modified channels, however, we cannot exclude the possibility that MTS reagents cause a fast channel block as expected for a blocker tethered near the pore (Blaustein et al., 2000). Nevertheless, based on the following observations, we propose that at least in Kv1.5, the MTS inhibition is related to an enhancement of P/C-type inactivation, similar to the effects of select mutations and protonation of position 463.

The addition of the R487V mutation reduced the extent of MTS-mediated inhibition in the cysteine-mutants tested (Figure 4.7). Similarly, in Shaker-IR, the homologous T449V
mutation alleviated the enhanced inactivation observed in the W434F (Yang et al., 2002; Kitaguchi et al., 2004), E418C (Ortega-Sáenz et al., 2000) and A463V (Ogielska et al., 1995) mutations. Therefore, if the R487V mutation antagonizes inactivation similarly to T449V, as previously suggested (Fedida et al., 1999), then the relief of the MTS-mediated inhibition by R487V suggests the involvement of the P/C-type inactivation pathway in this process.

Figure 4.7 illustrates that raising $\left[K^+\right]_0$ also relieves the MTS-mediated inhibition. Because P/C-type inactivation in Shaker-IR is classically defined by its sensitivity to $\left[K^+\right]_0$ (López-Barneo et al., 1993; Baukrowitz & Yellen, 1995; Schlief et al., 1996), the $K^+_0$-sensitivity observed here also suggests that the MTS-mediated inhibition may represent an enhancement of inactivation.

Together, these results suggest that the inhibition due to MTS-modification of residues in the distal turret represents an enhancement of closed state P/C-type inactivation. This may occur either because each of these residues interacts with the inactivation gate, and MTS-modification affects this interaction, or because modification of each residue disrupts a common interaction between the turret and outer pore, such as the suggested interaction between H463 and D485. The existence of one or more interactions between the turret and pore is compatible with previous suggestions that the turret and pore may behave as a rigid structure in Shaker-IR (Loots & Isacoff, 1998). For example, conformational changes within the pore during slow inactivation are detected by a fluorophore bound at turret position Shaker-S424C (homologous to Kv1.5-T462C). Therefore, it is plausible that in Kv1.5 a reciprocal effect may occur, such that modification of turret residues results in a conformation change within the pore leading to an enhancement of inactivation.

Furthermore, the data suggest that the rigid connection between the turret and pore is
strongest in the distal turret (positions 462-468) such that modulation of these positions has the greatest effect on the pore. Interestingly, modification of S424C in the Shaker-IR turret by tetramethylrhodamine maleimide (TMRM) has little effect on currents (Loots & Isacoff, 1998), while modification of the homologous Kv1.5-T462C mutation by either TMRM or propyl-MTS does (unpublished observations). This lack of sensitivity to turret modification in Shaker-IR is also reflected by the comparatively smaller effect of pH on channel function when a histidine is substituted at the positional equivalent of H463 in Kv1.5 (Perez-Cornejo, 1999). These differences likely result from variations in the primary structures of these channels (Figure 4.1C) and suggest that a complex network of interactions is responsible for the phenotype of slow inactivation in Kv channels.

Functional role of the turret in other channels

The presence of a titratable side group in the turret appears to mediate pH-sensitivity in other channels, including H508 in fKv1.4 (Claydon et al., 2000), the F425H mutation in Shaker (Perez-Cornejo, 1999), H117 in Kir2.3 (Coulter et al., 1995) and E522 in the TRPV5 Ca\textsuperscript{2+} channel (Yeh et al., 2003). A compelling feature of this pH sensitivity is that in every case the titratable side group is at the position homologous to H463 in Kv1.5, suggesting that this position plays an important regulatory role in several diverse channel types.

A similar functional role of the turret is especially evident with regard to the TRPV5 channel, whose pore is hypothesized to exist in a conformation similar to that of KcsA (Dodier et al., 2004). Single channel analysis of TRPV5 (Yeh et al., 2003) showed that, as for Kv1.5 (Kwan et al., 2006), there is a decrease in the open probability ($P_o$), but not the single channel conductance ($\bar{g}$) in low pH. Although other mechanisms have not yet been
conclusively excluded, these data suggest that as with Kv1.5, an enhancement of closed state
inactivation underlies the H+ inhibition of this channel, and that the turret is functionally
coupled to the pore. Additionally, SCAM analysis of the TRPV5 (Dodier et al., 2004) turret
revealed that cysteine substitution results in robust current inhibition following MTS-
modification at positions similar to those that inhibit Kv1.5. These findings raise the
possibility of a conserved structure-function relationship of the turret across a wide range of
ion channels.

In conclusion, these data indicate that a discrete group of residues in the distal turret
are involved in regulating channel activity in Kv1.5, and perhaps other channel types, and
suggest that this occurs by a modulation of slow, P/C-type, inactivation via interactions with
the pore mouth.

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CHAPTER 5: GENERAL CONCLUSIONS AND DISCUSSION
The studies presented here have focused on the importance of the turret region of the Kv1.5 channel in the regulation of slow inactivation. Chapters 2 and 3 demonstrated that protonation of, or divalent cation binding to, turret residue H463 resulted in an enhancement of slow inactivation. In Chapter 4, these findings were extended to demonstrate that residue 463 was contained within a discrete set of residues in the distal turret (positions 462-468) that regulates slow inactivation in Kv1.5.

**Does the turret-mediated inhibition result from an enhancement of slow inactivation?**

In response to the binding of protons or divalent cations to residue H463, or the MTS-modification of distal turret residues, an inhibition of Kv1.5 current was observed as a $[\text{K}^+]_0$-dependent decrease in the magnitude of the macroscopic current, which was shown by a reduction in the maximal conductance ($g_{\text{max}}$), and an acceleration of the rate of depolarization-induced inactivation. We have used the term “depolarization-induced inactivation” instead of “open state inactivation” to account for the possibility that channels may be inactivating not just from the open state, but from transient occupations of a non-conducting state, accessed from the open state, which lies outside the activation pathway. The presence of this state accounts for the “flickery” nature of Shaker and Kv1.5 unitary currents (Zagotta et al., 1994; Schoppa & Sigworth, 1998; Kwan et al., 2006). A qualitatively similar increase in the rate of depolarization-induced inactivation and $[\text{K}^+]_0$-sensitivity of the current amplitude was also observed in the H463G, H463R and H463K mutants. To determine the mechanism by which turret modification (i.e., protonation, divalent-cation binding or mutation) reduced $g_{\text{max}}$ and accelerated depolarization-induced inactivation, we examined whether several treatments known to alleviate slow, P/C-type
inactivation in Shaker-IR, could also relieve the effects of turret modification. As described in Chapter 1, slow inactivation in the Shaker-IR channel is slowed by raising \([K^+]_o\) (López-Barneo et al., 1993; Baukrowitz & Yellen, 1995), raising \([\text{TEA}^+]_o\) (Choi et al., 1991; Molina et al., 1997), or mutating position Shaker-IR-T449 in the outer pore mouth to a valine, cysteine or histidine (López-Barneo et al., 1993). Since wt Kv1.5 is relatively insensitive to external \(\text{TEA}^+\) (Fedida et al., 1993; Snyders et al., 1993; Trapani & Korn, 2003), we assessed whether either raising \([K^+]_o\) or incorporating the Kv1.5-R487V mutation (equivalent to Shaker-IR-T449V) affected the inhibition induced by protonation, divalent cation binding, or MTS-modification.

Baukrowitz and Yellen (1995), proposed that elevated \(K^+\) inhibits slow inactivation by increasing the \(K^+\)-occupancy of an external binding site within the selectivity filter (see Figure 1.3, (Zhou et al., 2001)). There, acting as a "foot-in-the-door" (López-Barneo et al., 1993), the bound \(K^+\) ion limits the ability of the outer pore mouth to undergo the constriction believed to occur during slow inactivation (Yellen et al., 1994). Additionally, in some channels such as wt Kv1.4 and fast-inactivating Shaker-IR-T449 mutants, the removal of extracellular \(K^+\) results in a complete loss, or collapse, of current (Pardo et al., 1992; López-Barneo et al., 1993). Thus, changing \([K^+]_o\) can affect the rate of depolarization-induced inactivation and/or the magnitude of macroscopic currents. However, in Shaker-IR, the dependence of slow inactivation on \([K^+]_o\) is normally obscured by an apparent accumulation of \(K^+\) ions near the outer pore mouth, and so the effect of \([K^+]_o\) is best observed when this accumulation is minimized (Baukrowitz & Yellen, 1995) or when inactivation appears to be enhanced, as is the case for fast-inactivating T449 mutants in Shaker-IR (López-Barneo et al., 1993). Therefore, even though slow inactivation in Kv1.5 does not normally exhibit
much $[K^+]_o$-dependence (Fedida et al., 1999), it is still possible that the inactivation process in this channel is $[K^+]_o$-sensitive, but that this sensitivity is only observed under certain circumstances, such as when external potassium accumulation is minimized (as would certainly be the case for inactivation from a closed state) or when slow inactivation is enhanced. In support of this argument, our experiments have indicated that raising $[K^+]_o$ alleviated the reduction in $g_{\max}$ induced by the binding of protons (Figures 2.2-2.4) or divalent cations (Figure 3.2) to position H463, as well as the inhibition induced by MTS-modification of cysteine mutants in the distal Kv1.5 turret (Figure 4.7). In addition, raising $[K^+]_o$ increased the $g_{\max}$ and slowed the rate of depolarization-induced inactivation in the fast-inactivating Kv1.5-H463 mutants (Figure 4.2). Therefore, this $[K^+]_o$-sensitivity suggests that the inhibition arising from the modification of the Kv1.5 turret is related to an enhancement of slow inactivation. This association also implies that the turret can influence the state of the inactivation gate.

Similarly, the Kv1.5-R487V mutation (equivalent to Shaker-IR-T449V) also antagonized the inhibition induced by protonation and (Figure 2.8) divalent cation binding (Figures 2.8 and 3.5) to position 463 and MTS-modification of distal turret residues (Figure 4.7). Furthermore, Korn and co-workers implied that the R487V mutation abolished the $[K^+]_o$-dependency of the Kv1.5-H463G mutation (Trapani & Korn, 2003). As described in Chapter 1, mutation of the positional equivalent of Shaker-IR-T449 in several Kv channels affects slow inactivation, and the finding that mutation of this position also alleviated the inhibition due to turret modification in Kv1.5 provides further evidence that this inhibition shows similarities to slow inactivation in Shaker-IR.
The role of position R487 in regulating the effects of turret modification was especially evident with regard to the MTS-mediated inhibition of the T462C mutation. In the presence of the T462C substitution alone, either MTSET- or MTSES-modification substantially reduced the maximal conductance. In contrast, the addition of the R487V mutation to the T462C construct reversed the effects of the MTS reagents, especially for MTSES, such that currents were enhanced (Figure 4.7). Thus, it appears that while MTS reagents bind to cysteine-substituted residues in the turret, the effector residue, R487, determines the resulting effects on inactivation. Alternatively, the reduction in MTSES inhibition could have arisen from the loss of a favourable electrostatic interaction with the positively charged arginine side chain at position 487. However, such an electrostatic interaction is unlikely since the positively charged MTSET inhibited channel current despite the presence of the positive charge at position 487 (Figure 4.6). Furthermore, such an electrostatic interaction predicts that the R487V mutation would enhance MTSET inhibition, not attenuate it, as is shown in Figure 4.7.

**What is the functional basis for the importance of the residue at position 487 in Kv1.5?**

Although the R487V mutation attenuated the turret-mediated enhancement of slow inactivation, it is interesting that this mutation has little effect on the rate of slow inactivation of wt Kv1.5 K⁺ currents at pH 7.4 (Fedida et al., 1999). This is a clear departure from the effects of the equivalent T449V mutation in Shaker-IR (López-Barneo et al., 1993). On the other hand, the R487V mutation does prevent the inactivation of sodium currents recorded in wt Kv1.5 in the absence of K⁺₀ and K⁺ᵢ (Wang et al., 2000). Although it is unclear why the R487V mutation does not affect the inactivation of K⁺ currents, this may be a consequence of
the expression system used. This has been alluded to by experiments with Shaker-IR that showed that the T449V mutation substantially reduced the rate of slow inactivation when this mutant was expressed in Xenopus oocytes (López-Barneo et al., 1993; Ogielska et al., 1995; Schlief et al., 1996) but did not affect the rate of slow inactivation when expressed in the mammalian HEK-293 cell line (Holmgren et al., 1996), the same cell that was used in these studies. This suggests that slow inactivation might be modulated in part by cell-specific proteins, and that the effect of Kv1.5-R487V on the rate of inactivation may be only evident in the Xenopus oocyte expression system. Regardless, our studies have indicated that the residue at position 487 can be an important determinant of the rate, and possibly the state-dependence, of slow inactivation in Kv1.5.

Despite the functional impact of the residue at position 487 in Kv1.5 (and the equivalent position in Shaker and other Kv1 channels) on slow inactivation, the mechanistic basis of this regulation has yet to be fully addressed, which complicates an interpretation of how the turret might affect slow inactivation. Indeed, mutational analysis of the homologous T449 position in Shaker-IR has provided little insight into the physico-chemical properties of this residue that are important for the regulation of inactivation. For example, both neutral (T449A) and oppositely charged (T449E and T449K) substitutions accelerated inactivation (López-Barneo et al., 1993). This implies that the electrostatic properties of this residue are not of great importance to its ability to regulate inactivation, but few other mechanistic details are known. In addition, neither the KcsA (Zhou et al., 2001) nor Kv1.2 (Long et al., 2005) crystal structures provide any clues as to what interactions the equivalent positions might make with other outer pore residues. The lack of any side chain interactions is supported by functional experiments that found that the side chains of the T449C Shaker-IR
mutant were available to co-ordinate extracellular Cd$^{2+}$ ions (Yellen et al., 1994), while those of the T449Y mutant could co-ordinate TEA$^+$ ions (Heginbotham & MacKinnon, 1992; Molina et al., 1997). This might suggest that this position's largest influence on channel structure is on the packing of the channel protein in the outer pore mouth. In support of this, Korn and co-workers showed that substitution of a tyrosine at the equivalent position in Kv1.5 (R487Y) caused only minor changes in the voltage-dependence of activation and that the kinetics of activation and deactivation were unaltered (Trapani & Korn, 2003). Based on this, the authors suggested that this mutation resulted in limited changes in the structure of the pore domain that primarily led to a reorientation of undetermined charged residues relative to S4. This implies that while the equivalent position of Shaker-IR-T449 in Kv channels may be an important determinant of slow inactivation, mutations that affect inactivation likely do not cause large disruptions in the conformation of the outer pore mouth. Clearly, however, further experiments are required to clarify the exact role of this residue, and mutations at this position, in regulating slow inactivation.

The turret-mediated inhibition suggests that slow inactivation in Kv1.5 can occur from closed states

In general, slow inactivation has been described as being strongly coupled to activation, such that inactivation occurs primarily during depolarizations, either from the open state or possibly from a fast-inactivated state near the open state (Zagotta & Aldrich, 1990; Hoshi et al., 1991; Zagotta et al., 1994; Pathak et al., 2004). Discussions of a possible closed state mechanism of slow inactivation are typically avoided because the electrically silent transitions associated with closed state inactivation can not be observed in macroscopic
current recordings and can only be examined indirectly using gating current and fluorescence experiments. As a result, it can be difficult to conclusively determine from which closed state(s) the channels become inactivated. However, as described in Chapter 1, the concept of closed state inactivation has been invoked to explain the $[\text{K}^+]_0$-dependence of the peak current amplitude in fast inactivating Shaker-IR-T449 mutants since it was found that changes in the single channel conductance could not account for the effects on $g_{\text{max}}$ (López-Barneo et al., 1993). This closed state inactivation was hypothesized to be mechanistically related to depolarization-induced inactivation since both processes were similarly $[\text{K}^+]_0$-sensitive and since T449 mutations that enhanced depolarization-induced inactivation were typically associated with a sensitivity of the current to manipulations of $[\text{K}^+]_0$ (López-Barneo et al., 1993). In Kv1.3, it has been proposed that inactivation occurs preferentially from a closed state near the open state, again implying that inactivation need not be strictly coupled to channel opening (Marom & Levitan, 1994). The Shaker-IR-W434F mutation is also of particular interest to the topic of closed state inactivation since increasing the proportion of subunits carrying this mutation accelerated the rate of macroscopic slow inactivation and resulted in a greater frequency of null sweeps in single channel recordings (Yang et al., 1997). In the non-conducting W434F-homomultimer, it was proposed that the lack of ionic current was due to the channel becoming “permanently P-type inactivated” because the channels could enter the inactivated state directly from one or more closed states (Yang et al., 1997). Thus, despite the difficulties in precisely assigning the transitions associated with closed state inactivation in these channels, such a process provides a reasonable mechanistic explanation for the basis of this form of current inhibition.
As with the fast-inactivating Shaker-IR-T449 mutants, Kv1.5-H463 mutants that had a faster rate of depolarization-induced inactivation also exhibited a \([K^+]_o\)-dependence of the peak current amplitude. For example, as shown in Figure 4.2, this \([K^+]_o\)-sensitivity resulted in a near-complete abolition of current in the absence of external \(K^+\) in several mutants, thus mimicking the \([K^+]_o\)-sensitivity of Kv1.4 (Pardo et al., 1992). The same arguments that implied that the Shaker-IR-T449 mutants might exhibit closed state inactivation also suggest that the fast-inactivating Kv1.5-H463 mutants may undergo closed state inactivation. The subsequent loss of channel availability, resulting from closed-state inactivation, then accounts for the reduction of the maximal conductance. In support of a closed state inactivation mechanism, unitary current analyses of the proton- (Kwan et al., 2006) and nickel- (Figure 3.6) mediated inhibition have indicated that these treatments resulted in a reduction in channel availability and not the single channel conductance. Since this reduction in availability appeared to occur at rest, we have hypothesized that the reduction in \(g_{\text{max}}\) induced by proton and divalent cation binding to the turret of Kv1.5 resulted from an enhancement of closed state inactivation. This implies that, at least in response to modification of the turret in Kv1.5, slow inactivation can become uncoupled from activation.

If closed state inactivation does account for the \([K^+]_o\)-dependent loss of current following turret modification, our results indicate that depolarization-induced inactivation is not always affected equally. For example, while \(Ni^{2+}\) modification reduced the peak current amplitude, the rate of depolarization-induced inactivation was largely unaffected. In comparison, low pH affected both processes (see Figure 3.4). A similar disparity was observed with fast-inactivating H463 mutants. As noted in Chapter 4, the equilibrium constants \((K_K)\) for the \([K^+]_o\)-dependent enhancement of \(g_{\text{max}}\) and the slowing of the
depolarization-induced inactivation were not equivalent in the mutants studied. A similar variability in the $[K^+]_o$-dependence of the peak current amplitude was also reported in Shaker-IR-T449 mutants (Schlief et al., 1996). Finally, MTS-modification of the substituted-cysteine turret mutants (with the exception of T462C) affected only the current magnitude. In the Shaker-IR channel, a similar selective enhancement of the rate of depolarization-induced inactivation in the absence of effects on the current magnitude was observed in low pH (Starkus et al., 2003).

Since closed state and depolarization-induced inactivation have been hypothesized to result from a related P/C-type inactivation mechanism (López-Barneo et al., 1993), it is not readily apparent why different turret modifications should affect either process preferentially or why the concentration-dependent relief of these processes by $[K^+]_o$ should be different. As noted in Chapter 4, it is uncertain if these differences in the $[K^+]_o$-dependence imply that there are two $K^+$-binding sites, or whether one site can account for the relief of both depolarization-induced inactivation and the potentiation of the current magnitude. Although speculative, these results might imply that the conformation of the inactivation gate (i.e., the selectivity filter (Baukrowitz & Yellen, 1996; Cordero-Morales et al., 2006)) is different in the closed and open states, such that it is modulated differently by the turret or $K^+$ ions depending on whether the channel is in the open or closed conformation. These differences in the state of the selectivity filter may be the result of the high $K^+$ occupancy of the pore in the open state (Baukrowitz & Yellen, 1996), the concerted movements of the pore region during the later stages of activation gating (del Camino et al., 2005), or due to the influence of S4 on the pore when in its activated state (Loots & Isacoff, 2000; Larsson & Elinder, 2000).
Conserved role of the turret in channel regulation

Is the ability of the turret to modulate slow inactivation unique to Kv1.5? As briefly described in Chapter 4, studies from several channels, including Kv1.4 (Claydon et al., 2000; Claydon et al., 2002), the inward rectifier Kir2.3 (Coulter et al., 1995), and the epithelial Ca^{2+} channel TRPV5 have indicated that these channels also possess a titratable side group at the positional equivalent of Kv1.5-H463, and that protonation of this site also leads to an inhibition of current. However, this position may not be important exclusively for pH-sensitivity, as the equivalent position in Kv2.1 (K356) has been shown to influence ion permeation and may contribute to the external potassium ion binding site ($S_{ext}$, Figure 1.3 (Zhou et al., 2001)) (Consiglio et al., 2003). Protonation of the equivalent histidine in Kv1.1 (H355) altered TEA^{+}_o-sensitivity, leading to the conclusion that modification of this position could influence the conformation of the pore, although changes in TEA^{+} accessibility could also account for these effects. In HCN channels, MTS-modification of the equivalent C318 residue in the turret also resulted in a reduction of macroscopic current and alterations in the kinetics of activation and deactivation (Xue & Li, 2002). Thus, in a diverse range of channels, the turret residue at the positional equivalent to Kv1.5-463 may be important in modulating channel behaviour.

It must be noted, however, that modification of the equivalent turret residues in different channels does not necessarily produce identical effects on channel behaviour. This is especially true of the N-type inactivation removed Kv1.4 channel where, despite a similar primary structure of the turret (Figure 4.1), protonation of H508 (equivalent to Kv1.5-H463) affected the rate of, and the recovery from, depolarization-induced inactivation with no evidence for a concomitant reduction of $g_{max}$ (Claydon et al., 2000; Claydon et al., 2002). In
addition, MTS-modification of the Kv1.4-H508C mutant indicated that MTSET, but not MTSES, inhibited current (Claydon et al., 2002). In Shaker-IR, histidine-substitution at the equivalent position of Kv1.5-H463 (F425H), produced a channel whose slow inactivation, measured in Xenopus oocytes, was only mildly pH-sensitive compared to Kv1.5 (Perez-Cornejo, 1999). Furthermore, modification of the Shaker-IR-S424C channel with the TMRM fluorophore did not inhibit current (Loots & Isacoff, 1998), while currents from the homologous Kv1.5-T462C mutant were almost completely eliminated by similar modifications with TMRM (unpublished results), charged MTS-reagents MTSET or MTSES (Figure 4.6), or neutral MTS-reagents (unpublished results). In the absence of definitive structural information regarding these channels, we can only conclude that these differences are likely due to dissimilarities in the primary sequence and secondary structure of the turret/pore regions of these channels (Figure 4.1), or perhaps, other regions of the pore, that may alter the coupling between the effector residues in the turret and the conformational rearrangements in the pore.

Just as we have shown that slow inactivation can be regulated by a distinct group of residues in Kv1.5, large regions of the turret may modulate slow inactivation in other channels, including HERG. The turret of HERG is much larger compared to Kv channels (43 a.a., compared to 13 in Shaker and Kv1.5, measured from the extracellular terminus of S5 to the start of the pore helix (Liu et al., 2002)) and likely contains an α-helix (Liu et al., 2002; Torres et al., 2003). The substitution of several charged residues within this α-helix had dramatic effects on both activation and inactivation (Dun et al., 1999; Clarke et al., 2006). However, similar to our findings in Kv1.5, that the effects of turret modification are dependent on the type of side chain at position 487 (i.e., they are attenuated or reversed if the
wild type arginine is mutated to a valine), experiments in HERG and the related human ether-à-go-go (hEAG) and rat ether-à-go-go-like (rELK2) channels have also led to the hypothesis that the ability of turret mutations to affect inactivation in these channels is dependent on the presence of a serine at the positional equivalent of Kv1.5-R487 (Clarke et al., 2006). This raises the possibility that an interplay between the turret and the outer pore mouth may not be limited to Kv1.5, and instead represents a shared mechanism among channels by which the turret regulates inactivation. In a separate study, the incorporation of substituted-cysteines into the HERG turret demonstrated that many of the cysteines could cross-link via intersubunit disulfide bonds, and the authors proposed that the turrets may interact with each other to affect inactivation, perhaps by forming a structure that directly occludes the permeation pathway (Jiang et al., 2005). These results imply that the turrets of several channels may interact with the pore to regulate channel current and, as proposed for the HERG channel, may do so via intersubunit turret interactions.

How does the Kv1.5 turret interact with the slow inactivation gate?

Despite over 15 years of research, the molecular details of slow inactivation remain elusive. This is true in part because slow inactivation appears to involve a complex re-orientation of a large part of the pore domain and because many interacting residues are likely to be involved. The possibility that multiple inactivated states may contribute to slow inactivation further complicates our understanding of this process. These multiple pathways may account for the U-type inactivation described as in Chapter 1, the two inactivated states, I₁ and I₂, required to model inactivation in KcsA (Cordero-Morales et al., 2006), and the fact that the rates of depolarization-induced inactivation, as well as the recovery from
inactivation, are often fit best by a double exponential function. Nevertheless, several studies have concluded that the ultimate result of these processes is a re-organization of the selectivity filter (see Chapter 1), and therefore, the work presented here implies that the turret must be functionally coupled to this region of the pore in order to influence slow inactivation.

Do turret residues interact directly with the outer pore? The finding that a fluorophore bound to the Shaker-IR turret could detect slow inactivation rearrangements associated with the pore implies that the turret and outer pore mouth may indeed form interactions that rigidly link the two regions (Loots & Isacoff, 1998). However, data from the SCAM analysis of the Kv1.5 turret (Figure 4.6), suggests that this rigid interaction may be confined to the distal turret since MTS-modification of residues in this region had the greatest effect on inactivation. As noted earlier in Chapter 4, this may be because these residues can all interact independently with the pore, or because any single mutation disrupts a common interaction between the turret and pore. Jäger and Grissmer (2001) hypothesized that the interplay between H463 and R487 might provide one such interaction, and that protonation of H463 in low pH altered the electrostatic interaction between these residues, thereby enhancing inactivation. However, our data clearly exclude a purely electrostatic interaction between the turret and residue R487. Thus, if H463 and R487 do interact directly, this may be the result of other types of associations between these positions, such as hydrogen bonding. A coupling between these positions, however, is conceivable since the KcsA crystal structure (Zhou et al., 2001) indicates that the closest side chain atoms from the residues equivalent to Kv1.5-H463 (Q58) and R487 (Y82) come to within ~5 Å of each other. Furthermore, in KcsA, the central region of the turret containing the positional
equivalents of Kv1.5-N459 to S464 lies roughly parallel to the P-S6 linker, which contains the equivalent of Kv1.5-R487, such that the two regions are only separated by ~6-10 Å, measured Cα-Cα. Of particular interest, a recent study in KcsA proposed that an outer pore aspartate (D80, equivalent to D485 in Kv1.5) in the GYGD signature sequence may become re-orientated into an upward, or “flipped”, conformation (Cordero-Morales et al., 2006). The crystal structure also indicated that the atoms of the “flipped” D80 side chain were only 3.5 Å away from the closest side chain atoms of the equivalent position of Kv1.5-H463 (Q58). This suggests that in KcsA, turret residue Q58 may be capable of interacting directly with D80 in the outer pore mouth. The authors of this study hypothesized that movement of the D80 side chain may be an important factor in KcsA inactivation, although the functional state of the “flipped” structure was uncertain. Nevertheless, if the equivalent pore aspartate in Kv1.5 (D485) can undergo a similar rotation, then an interaction with H463 could account for the coupling between the turret and pore. Modelling studies have also indicated that the turrets of Shaker, and especially KcsA, can be mobile structures (Shrivastava & Bahar, 2006), and as a result, may move significantly during channel gating, and perhaps in doing so, facilitate interactions with residues in the pore. As stated earlier, however, the data presented here suggest that these interactions are influenced by more than just the presence or absence of charge at these positions.

Further questions

Together, these data suggest that in Kv1.5, a discrete set of turret residues can regulate slow inactivation by increasing the rate of depolarization-induced inactivation or by reducing the current magnitude, perhaps by enhancing closed state inactivation. This latter finding implies that in this channel, inactivation can become uncoupled from activation. As a
result, the ability of Kv1.5 channels to contribute to membrane repolarization is attenuated. Since extracellular pH can fall during periods of cardiac ischemia (Poole-Wilson, 1982) or in response to neuronal activity (Chesler & Kaila, 1992), the resulting modulation of this current by protons, or other extracellular factors including changes in \([K^+]_o\), may have a significant impact on the physiology of tissues expressing Kv1.5. Although not addressed in our experiments, the \(V_{1/2}\) of activation in Kv1.5 is sensitive to changes in pH, and some have suggested that physiological and pathological shifts in the extracellular pH can have significant effects on Kv1.5 current, based on these changes in voltage-sensitivity alone (Trapani & Korn, 2003).

Several questions regarding the role of the turret in Kv1.5 remain. Foremost among these is the nature of the structural changes that occur within the turret in response to turret modification (e.g., following extracellular acidification) and how these changes are coupled to the outer pore. With respect to the possible re-orientations of the turret upon modification, voltage-clamp fluorimetry may provide useful information. In particular, a clear demonstration of turret movement in response to turret modification in the absence of prior channel opening would greatly support the functional evidence for closed state inactivation in Kv1.5. Interestingly, in the aforementioned TRPV5 channel, it was shown that the \(H^+\)-mediated inhibition was accompanied by a movement of the pore helix (Yeh et al., 2005). Therefore, a detailed examination of pore helix movement in Kv1.5, in response to turret modification, might demonstrate a similar coupling of the turret to the pore via the pore helix. Of particular interest too, is whether closed state and depolarization-induced inactivation both lead to similar reorganizations of the pore. As alluded to above, an understanding of how apparently similar turrets (e.g., Shaker-IR versus Kv1.5; Kv1.5 versus Kv1.4) produce
significantly different inactivation phenotypes would also contribute greatly to our understanding of slow inactivation and may be advanced by the use of turret chimeras. Finally, an examination of the roles of R487, and in light of recent experiments, D485, in mediating the effects of turret modification is greatly required. It is possible that many of these questions may be aided by the growing number of X-ray crystal structures, especially with regard to demonstrating the range of conformational changes and interactions that the outer pore can undergo.

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