MODULATION OF Kv1.5 SLOW INACTIVATION BY EXTERNAL CATIONS

by

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B.Sc.(Hon.), The University of British Columbia, 2000

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Physiology)

THE UNIVERSITY OF BRITISH COLUMBIA

December 2006

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ABSTRACT

Slow inactivation is an intrinsic biophysical property of voltage-gated potassium (Kv) channels that results in a non-conducting state under physiological conditions. It limits the amount of current through Kv channels and affects cellular excitability. However, the molecular basis of slow inactivation is not well understood. In this thesis investigation, the modulation of slow inactivation in the human Kv1.5 channel by extracellular Zn$^{2+}$, protons (H$^{+}$), Ni$^{2+}$, and other divalent cations was studied using standard voltage clamp techniques. Zn$^{2+}$, H$^{+}$, and Ni$^{2+}$ accelerated slow inactivation and caused a current inhibition in Kv1.5 expressed in HEK-293 and mouse ltk$^{-}$ cells. The current inhibition was hypothesized to result from the binding of Zn$^{2+}$, H$^{+}$, and Ni$^{2+}$ to the turret histidine residue (H463) which in turn promoted a slow inactivation process involving the outer pore mouth arginine residue (R487). The current inhibition induced by Zn$^{2+}$, H$^{+}$, and Ni$^{2+}$ was attenuated either by increasing extracellular [K$^{+}$] or by mutating H463 to glutamine (H463Q) or R487 to valine (R487V). Unitary current analysis revealed H$^{+}$ and Ni$^{2+}$ did not change the single channel current at +100 mV or the single channel conductance between 0 and +100 mV, but the number of blank (null) sweeps recorded with depolarizing pulses lasting up to 1 s was increased. The proportion of null sweeps correlated well with the extent of inhibition of macroscopic Kv1.5 current by external H$^{+}$. A model incorporating two modes of gating was employed to describe the transitions between the active sweeps (mode $A$) and the null sweeps (mode $U$), and external H$^{+}$ was proposed to inhibit Kv1.5 current by promoting mode $U$ gating. Consistent with this model was the finding that external K$^{+}$ antagonized mode $U$ gating induced by external H$^{+}$. Channels were observed to switch from mode $U$ back to mode $A$ during prolonged depolarizations ($\geq$ 6 s), and the delay in opening (first latency) was correlated with the dwell time in a depolarization-induced slow inactivated state. Together, the results suggest that Zn$^{2+}$, H$^{+}$, and Ni$^{2+}$ inhibit Kv1.5 current by promoting a slow (P/C-
type) inactivation process proceeding from closed states.
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<th>Amino Acids</th>
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4-AP - 4-aminopyridine

γ - single channel conductance

τ - time constants

ANOVA - analysis of variance

BK - Ca\textsuperscript{2+}-activated potassium channel with large conductance

Ca\textsubscript{v} - voltage-gated calcium (channel)

CHO - Chinese hamster ovary (cell)

CNG - cyclic nucleotide-gated (channel)

EDTA - ethylenediaminetetraacetic acid

EGTA - ethylene-glycol-bis(2-aminoethyl ether)-N,N,N′,N′-tetraacetic acid

\(E_K\) - equilibrium potential of potassium

\(g\) - macroscopic conductance

\(g_{\text{max}}\) - maximum macroscopic conductance

\(i\) - microscopic current

\(I\) - macroscopic current

\(I_{\text{Kur}}\) - ultra-rapid delayed rectifier current

\(I_{\text{max}}\) - maximum current

HEK - human embryonic kidney (cell)

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HERG - human ether a-go-go-related gene (channel)

\(K_D\) - dissociation constant
Kir - inwardly-rectifying potassium (channel)
Kv - voltage-gated potassium (channel)
MEM - minimum essential medium
MES - 2-[N-Morpholino]ethanesulfonic acid
MTS - methanethiosulfonate
Na\textsubscript{v} - voltage-gated sodium (channel)
n\textsubscript{H} - Hill coefficient
NMG\textsuperscript{+} - N-methyl-D-glucamine
NMR - nuclear magnetic resonance
p - probability
P\textsubscript{O} - Open probability
PxP - proline-x-proline (motif)
QA - quaternary ammonium
Q\textsubscript{max} - maximum gating charge
Q\textsubscript{on} - ON gating charge
Q\textsubscript{off} - OFF gating charge
r - correlation coefficient
s - second
SCAM - substituted cysteine accessibility method
SD - standard deviation
SEM - standard error of the mean
Shaker\textsubscript{IR} - (fast) inactivation Removed Shaker (channel)
s, - standard error of r (correlation coefficient)
subscript ‘i’ - intracellular
subscript ‘LG’ - long gap
subscript ‘LL’ - long first latency
subscript ‘o’ - extracellular
subscript ‘SG’ - short gap
subscript ‘SL’ - short first latency
TAPS - N-tris[Hydroxymethyl]methyl-3-amine-propanesulfonic acid
TEA\textsuperscript{+} - tetraethylammonium
t\textsubscript{crit} - critical time (burst analysis)
TM - transmembrane
V\textsubscript{1/2} - half-activation voltage
wt - wild-type
ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to all the people who have contributed their time and effort to my graduate study experience at UBC. To my graduate supervisor, Dr. Steven J. Kehl, I thank you for your boundless patience, your support, and all the time you have spent in teaching me what good science is about. I thank my supervisory committee members, Dr. David Fedida, Dr. Eric Accili, and Dr. Edwin Moore, who have been very supportive and have provided helpful insights into my research projects and into this thesis. It has been a great experience working with other graduate and undergraduate students, and I appreciate all the time we have shared together. I would like to thank all my friends, especially G.Y., for all the support and understanding you have given me. Finally, to my wonderful and loving parents, I am grateful for your encouragement, guidance, and patience.
DEDICATION

To my parents
CO-AUTHORSHIP STATEMENT


Daniel Kwan was responsible for i) performing the experiments and analysis of the effects of H$^+$ on Kv1.5 current at pH 6.4 (Figure 2.1); ii) performing some of the experiments and analyses of the K$^+$ attenuation of the H$^+$-induced current inhibition (Figure 2.2); iii) performing the experiments and analysis on the R487V mutant; iv) performing the experiments and analysis of the frequency dependence of the H$^+$-induced current inhibition (Figure 2.9); v) preparing and constructing the figures involved (50% contribution). Cyrus Eduljee performed the experiments on the H463G, H463Q, and H463Q/R487V mutants. Shetuan Zhang examined the effects of external H$^+$ on gating current. Steven Kehl conducted the rest of the experiments and wrote the manuscript.


Daniel Kwan was responsible for i) performing the experiments and analysis of the effects of Ni$^{2+}$ on Kv1.5 current; ii) performing the single channel recording and analysis on the effects of Ni$^{2+}$ (20% contribution). Cyrus Eduljee performed the experiments on the H463Q mutants. Shetuan Zhang examined the effects of external Ni$^{2+}$ on gating current. Logan Lee assisted with some of the experiments. Steven Kehl conducted the rest of the experiments and wrote the manuscript.


Daniel Kwan was responsible for i) performing all the whole-cell experiments and analyses of the effects of H$^+$ on Kv1.5; ii) performing all the single channel recording and analyses; iii) prepare all the figures in the manuscript; iv) designing all the experiment procedures for both the single channel and whole cell recordings; v) writing the manuscript except the discussion (85% contribution). Steven Kehl wrote the discussion for the manuscript and assisted in editing the rest of the manuscript.
1. Introduction to slow inactivation and voltage-gated potassium channels

Ion channels are indispensable molecular components of all living cells, from unicellular prokaryotic bacteria to multicellular eukaryotic organisms. These integral membrane proteins facilitate and regulate the movement of ions across the plasma membrane, which is an impermeable barrier for all charged particles. In non-excitable cells, potassium (K\textsuperscript{+} or K)-selective channels are involved in basic physiological functions such as maintaining K\textsuperscript{+} and electrolyte balance (Hille, 2001). In excitable cells, the flow of ions across the plasma membrane gives rise to bioelectric phenomena (Hodgkin and Huxley, 1952), and K channels, in particular the voltage-gated K (Kv) channels, are involved in repolarizing the membrane voltage from an excited state back to the resting level and in reducing membrane excitability (Hille, 2001). Surprisingly, more than 65 different genes in the human genome have been identified to encode various Kv channels (Alexander et al., 2006). This large repertoire of Kv channels highlights their important role in fine-tuning the repolarization step in an action potential and in other functions. In addition, mutations in various Kv channels can lead to inherited neurologic and cardiac disorders, such as episodic ataxia, epilepsy, long QT syndrome, and sudden cardiac death (Ashcroft, 2000). Therefore, a detailed understanding of these proteins, both in terms of their biophysical properties and pharmacological profiles, is of critical importance.

In this thesis, the modulation of slow inactivation in Kv1.5 by external cations is examined, and the purpose of this chapter is to review some of the basic biophysical properties of K channels and to lay out the background material for the reader to understand the results given in the subsequent chapters. An overview of K channel biophysics will first be introduced, including the Hodgkin and Huxley formalism and some early seminal experiments leading to the
elucidation of channel function and structure. Second, the molecular determinants for various K channel properties based on a number of K channel crystal structures (permeation, activation, electro-mechanical coupling, \textit{etc.}) will be described. Next, the different types of inactivation will be described with the focus on the so-called slow (P/C-type) inactivation. Finally, the scope of this research project will be defined.

1.1 Early Kv channel biophysics

There are two main aspects of Kv channel function: gating and permeation. The term gating is used to describe how a channel responds to stimuli. For example, Kv channels can populate different functional states in response to the membrane voltage, and the transitions between these functional states are collectively called the gating of the channel. The two basic functional states are the closed (non-conducting) state and the open (conducting) state. For most of the cases, Kv channels are assumed to predominantly reside in a closed state at rest. The probability of being open increases with depolarization. The gating process by which a channel makes transitions from a closed state to the open state is called activation, and the reverse process is called deactivation. Gating of channels can be described using gating models (schemes) in which the transitions between states are governed by voltage-dependent rate constants. Gating models can also be used for simulating the kinetic behaviour of channels to test hypotheses.

Probably the best known gating model for K current was proposed by Hodgkin and Huxley (1952) for the delayed rectifier Kv channel of the squid giant axon. In their model, four closed states and one open state were linked in series as shown in Figure 1.1 A. Each transition was assigned a voltage-dependent rate constant. The forward rate constants (toward the open
state) became larger with depolarization, whereas the backward rate constants had the opposite voltage dependence. To account for the delay in current onset and the sigmoidal kinetics of activation, the Kv channel was proposed to start primarily at Cl at rest (i.e., at negative holding potentials) and, upon depolarization, progressed through the other three closed states (C2 to C4) before finally reaching the open state (O). To account for this behaviour, Hodgkin & Huxley (1952) envisioned that a Kv channel contained four “particles,” which could be in either a non-permissive or in a permissive state, and that once all four “particles” were in the permissive state the channel opened. Conversely, as soon as a single “particle” became non-permissive, the channel reverted to a closed, non-conducting state. This model was consistent with the decay of ionic current upon repolarization being well-fit by a single exponential function. In the wake of the Hodgkin-Huxley model, more sophisticated and complex models for other Kv channels have been developed (Hoshi et al., 1994; Schoppa and Sigworth, 1998a; Schoppa and Sigworth, 1998b; Schoppa and Sigworth, 1998c; Zagotta et al., 1994b; Zagotta et al., 1994a). One common modification is the inclusion of another closed state (C5 in Figure 1.1 B) to account for a concerted opening step (Ledwell and Aldrich, 1999; Pathak et al., 2005; Smith-Maxwell et al., 1998). In this state, all the gating particles are activated (in the permissive state), but the channel is not yet open.

In their seminal 1952 paper, Hodgkin & Huxley also proposed that the voltage-dependent steps resulted from charged elements moving within the membrane voltage field (Hodgkin and Huxley, 1952), an hypothesis that accurately predicted the existence of “gating currents” in voltage-gated channels. However, it was about twenty years before these hypothetical gating currents could be measured. The first recording of gating current was made by Armstrong & Bezanilla (1974) from voltage-gated sodium (Na\textsubscript{v}) channels in a preparation where all the
Figure 1.1 Gating schemes of Kv channels. A. A gating scheme for generic Kv channels based on the Hodgkin-Huxley model (Hodgkin and Huxley, 1952). A channel can either be in one of the closed, non-conducting states (C1 to C4) or in the open state (O). Transitions between states are all voltage-dependent. B. In more recent models of activation, another closed state (C5) is included before the open state to account for the concerted opening step observed. In C5, all the gating particles are assumed to have activated. No inactivated state is included.

In the absence of structural information in the early days, the activated voltage sensor was hypothesized to open a “gate” in the Kv channel that otherwise prevented the flow of K⁺. Interestingly, internally applied quaternary ammonium compounds, such as tetraethylammonium (TEA⁺), were found to block the Kv channel in squid giant axon and in the frog node of Ranvier only when the activation gate was open (Armstrong, 1966; Armstrong and Hille, 1972). In addition, bound TEA⁺ and other small quaternary ammonium compounds could become trapped in the Kv channel if the activation gate was closed by repolarization, which suggested that the
activation gate acted as a physical barrier and was located between the cytoplasmic face of the channel and the receptor site for quaternary amines (Armstrong, 1966; Holmgren et al., 1997). These data provided additional insights into Kv channel structure, and, as we shall see later, these structural interpretations were elegantly confirmed in the crystal structures of several K channels.

Once the activation gate is open, K$^+$ ions start to move down their electrochemical gradient, a process known as permeation. Hodgkin & Keynes (1955) were the first to propose that the Kv channel of the cuttlefish giant axon was a multi-ion pore, meaning that multiple ions occupied the conducting pathway during a single-file permeation process. However, at that time the structural basis for the high selectivity of Kv channels for K$^+$ over Na$^+$ was unknown. Under normal physiological conditions, the selectivity sequence for most Kv channels is Tl$^+ > K^+ > Rb^+ > NH_4^+ > Cs^+ >> Na^+ = Li^+$ (Blatz and Magleby, 1984; Heginbotham and MacKinnon, 1993; Hille, 1973; Latorre and Miller, 1983; Shapiro and DeCoursey, 1991), and selectivity in K channels is clearly not based solely on the ionic crystal radius since that of Na$^+$ is less than that of K$^+$. In a series of studies, Mullins (1959) proposed that the selectivity of Kv channels was based on the energy required for dehydration (reviewed in Hille, 2001). Roughly a decade later, Bezanilla & Armstrong (1972) proposed that the Kv channel pore contained “coordinating cages” that replaced the hydration shell for K$^+$, in effect acting as surrogate water molecules. It took another 25 years later before the chemical basis for these coordinating cages was revealed in the very first crystal structure of a K channel (Doyle et al., 1998).

With a sustained depolarizing pulse, most ion channels do not stay in the open state(s) indefinitely but instead undergo a process called inactivation that results in a non-conducting state that is distinct from the closed states described above. Hodgkin & Huxley (1952) proposed that the transient Na$^+$ conductance of the squid giant axon was due to the closing of a separate,
inactivation gate which functioned independently from the activation gate. This view of inactivation gating was adopted for the transient K currents (Connor and Stevens, 1971). A number of different types of inactivation, with distinct molecular mechanisms, have been discovered. Inactivation is discussed in greater detail later in this chapter.

To summarize, a number of ingenious experiments between the 1950’s and 1980’s provided functional data from which structural insights for ion channels were drawn, but the overall structure of Kv channels was just starting to emerge. It became clear that the different functional states of Kv channels arose from different conformations, and starting in the 1980’s, a growing number of studies began to focus mainly on the structural aspect of ion channels (Noda et al., 1984; Papazian et al., 1987; Steinbach, 1989; Tanabe et al., 1987) as the ion channel field entered the era of molecular cloning. Various ion channels were cloned in the last twenty years, and a movement towards studying cloned channels in heterogenous systems was evident. The cloning and structural studies on ion channels have revolutionized the field of channel biophysics by providing a physical framework for the functional data.

1.2 Structure of Kv channels

In 1987, the Jan group published the primary sequence of the first Kv channel, the Shaker channel, from the fruit fly Drosophila melanogaster (Papazian et al., 1987). At that time, several voltage-gated sodium (Na\textsubscript{v}) and calcium (Ca\textsubscript{v}) channels had already been cloned (Noda et al., 1984; Tanabe et al., 1987); therefore, comparisons were quickly drawn between the cloned voltage-gated channels. A major difference was that the size of Shaker was about a quarter of the cloned Na\textsubscript{v} and Ca\textsubscript{v} channels. The cloned Shaker gene was found to encode a channel-forming α-subunit of a Kv channel, which was proposed and later confirmed to contain six
transmembrane helices with intracellular N- and C-termini, as shown in Figure 1.2 A (Doyle et al., 1998; Jiang et al., 2003; Long et al., 2005a). It was shown that a Kv α-subunit is analogous to a single domain of a Na$_v$ or Ca$_v$ channel, and a Kv channel was proposed to be a homotetrameric construct with a four-fold rotational symmetry, as shown in Figure 1.2 B and C (Doyle et al., 1998; Jiang et al., 2003a; Long et al., 2005a; MacKinnon, 1991). Following the cloning of the Shaker channel, three other homologues (Shab, Shaw, and Shal) appeared in relatively rapid succession (Butler et al., 1989) and were also confirmed to encode functional Kv channels (Wei et al., 1990). The topology of all the cloned Kv channels was later found to be conserved; therefore, they were grouped into the so-called 6TM-1P channel family (Alexander et al., 2006; Hille, 2001).

As the list of cloned K channels grew, the need for a systematic nomenclature for all K channels became clear. Originally, channels were named based on the species and tissue of origin. However, without knowing the corresponding gene, a channel might, and often did, acquire more than one name. In 1991, a naming system based on homology and evolutionary relationship between the different K channels was proposed and implemented shortly afterward (Chandy, 1991). Each Kv channel protein is now identified as Kv$x$.y in which $x$ and $y$ are numbers representing a specific subfamily and the order of discovery in that particular subfamily, respectively (Hille, 2001). In addition, the Kv channel genes are labelled as KCNM$M_N$ in which $M$ is a letter assigned to a specific subgroup, and $N$ is a number assigned to the particular channel. The four Drosophila Kv channels (Shaker, Shab, Shaw, and Shal) are now known as the prototypes of the Kv1 to Kv4 subfamily members, and their gene families have been named as KCNA to KCND, respectively.

By comparing their primary sequences, Kv channels are seen to differ mainly in the
Figure 1.2 Structural representations of Kv channels. A. Schematic drawing of the pore-forming α-subunit of Kv channels. B. Side view of the ribbon representation of the Kv1.2 crystal structure showing the transmembrane domains (TM), the T1 domain (T1), and an associated β-subunit (β) (Long et al., 2005a). Each of the 4 subunits is represented by a different colour. C. Stereoview of the Kv1.2 crystal structure from the extracellular side directly above the pore (Long et al., 2005a). The transmembrane segments for the subunit in red are labelled from S1 to S6. The pore domain (S5 and S6) of one subunit is adjacent to voltage-sensing domain from another subunit.

Composition of the intracellularly-located N- and C-termini, whereas the transmembrane segments and their linkers are, in the main, very similar. Likewise, the different members within the same subfamily often have subtly different biophysical and pharmacological properties that arise from the slight differences in their primary sequences. Based on structural similarities, a number of functional domains have been identified in all Kv channels. For example, the T1 domain in the cytoplasmic N-terminus of each α-subunit is responsible for tetramerization, in
which only subunits from the same subfamily can form homo- or heterotetramers (Bixby et al., 1999; Pfaffinger and DeRubeis, 1995; Shen et al., 1993; Shen and Pfaffinger, 1995; Xu et al., 1995; Zerangue et al., 2000). The T1 domains are thought to associate with each other during protein translation before individual subunits are folded properly (Robinson and Deutsch, 2005; Strang et al., 2001). T1 domains also serve as the docking sites for the N-type inactivation (see below) particle and auxiliary β-subunits (Gulbis et al., 2000). The β-subunits, which are shown with the Kv1.2 crystal structure in Figure 1.2 B (Long et al., 2005a), can modulate channel function and also act as chaperone proteins to increase the surface expression of Kv channels (Wible et al., 1998). The N- and C-termini may contain additional motifs and/or domains which affect channel function (Jerng and Covarrubias, 1997; Ju et al., 2003; Nishida and MacKinnon, 2002; Wray, 2004) but lie outside the focus of this study.

In addition to the T1 domain, a Kv channel has two other functional domains: the pore-forming (pore) domain and the voltage-sensing domain. The pore domain constitutes S5, the P-loop, S6, and the associated linkers; the voltage-sensing domain is formed by S1, S2, S3, S4 and the associated linkers, with S4 being the main voltage sensor.

1.2.1 The pore-forming domain

The pore domain is a basic functional unit of K channels. Even though K channels from different families may have substantial differences in their primary sequences that give rise to their specific conduction and gating properties (De Biasi et al., 1993; Harris and Isacoff, 1996; Kirsch et al., 1992), the general pore architecture and basic permeation process are thought to be very similar among K channels (Doyle et al., 1998; Heginbotham et al., 1992; MacKinnon et al., 1998; MacKinnon and Doyle, 1997). Figure 1.3 A shows an alignment of the outer pore regions
(from the end of S5 to the start of S6) from a number of K channels including Shaker. From the alignment, it is clear that the two linkers (S5-P and P-S6) are the least conserved, whereas the P-loop is highly conserved within a subfamily and differs only slightly between subfamilies. The P-loop contains the signature sequence TxxTxyG/FGD which forms the selectivity filter (Doyle et al., 1998; Heginbotham et al., 1992; Heginbotham et al., 1994). Much of the theoretical work on permeation and selectivity has used the KcsA channel pore as a structural model.

The KcsA channel is an intracellular-proton activated K channel from the bacterium Streptomyces lividans, and it was the first K channel for which high resolution (2-3 Å) crystal structures were obtained (Doyle et al., 1998; Zhou et al., 2001b). The sequence of the pore for KcsA is included in Figure 1.3 A for comparison. KcsA is homologous to the pore domain of Kv channels or the inward rectifier K (Kir) channel in the 2TM-1P family (Caprini et al., 2005; Lu et al., 2001). Indeed, KcsA can be substituted into the pore domain of Shaker to form a functional Kv channel (Caprini et al., 2005; Lu et al., 2001; Lu et al., 2002). The α-subunit of KcsA consists of two transmembrane helices: the outer helix, which is homologous to S5 in Kv channels or M1 in Kir, and the inner helix, which is homologous to S6 in Kv and M2 in Kir channels. These helices line the hydrophilic pore and form the interface between the pore lumen and the hydrophobic lipid environment of the membrane (Aiyar et al., 1994; Lu and Miller, 1995; Shieh and Kirsch, 1994; Yellen et al., 1991). In addition, the KcsA structure reveals two constrictions along the central axis of the pore: the cytoplasmic bundle crossing formed by the convergence of the inner helices and the selectivity filter formed by the signature sequence (Figure 1.3 B grey-blue sticks). The bundle crossing is thought to be the activation gate for K channels, whereas the selectivity filter is a major regulatory site for permeation and selectivity as discussed above.
Figure 1.3 Structural representations of the pore domain.  

A. Sequence alignments of the outer pore region from the indicated K channel compared to that of Shaker. The letters represent the standard single letter code for each amino acid. The amino acid sequence of Shaker is listed in full, while the dash (-) in the sequence of other channels represents a residue identical to that in Shaker. The number in front of each sequence show the position of a highly conserved glutamate (E) residue.  

B. Side view of ribbon representation of the KcsA crystal structure with the front and back subunits removed for clarity (Roux, 2005). The S5 helices (green), pore helices (orange), and S6 helices (blue) are colour coded, whereas the turret residues are shown as a continuous grey line. The selectivity filter is shown as grey-blue sticks with the carbonyl oxygen atoms shown in red. The green spheres represent K⁺ at the coordination sites as labelled.  

C. Electron density map of the selectivity filter (Zhou et al., 2001b). The green spheres represent K⁺ ions, whereas the red dots represent water molecules. The selectivity filter orientation is similar to that shown in B. Seven coordination sites are shown, with S₁ and S₃ labelled. K⁺ ions are shown to occupy the two external binding sites (S₀ and S_ext) external to S₁, with the K⁺ ion occupying S_ext being surrounded by 4 water molecule. The K⁺ ion at the central cavity site is surrounded by 8 water molecules, i.e., fully hydrated.
1.2.1.1 The activation gate

As noted previously, access of $K^+$ to the conducting pathway is regulated by the activation gate. From the KcsA structure, the location of the bundle crossing on the cytoplasmic side of the channel, and the large central cavity behind it, agrees well with the description for the activation gate proposed previously (Armstrong, 1966; Holmgren et al., 1997). As shown in Figure 1.3B, this bundle crossing arises from the convergence of the inner helices (blue) to form an “inverted teepee.” The dimensions of the bundle crossing are such that the passage of $K^+$ is prevented; therefore, the KcsA structure is considered to represent the closed conformation of $K$ channels (Doyle et al., 1998). On the other hand, in the crystal structure from MthK, a prokaryotic $Ca^{2+}$-activated $K$ channel isolated from the bacterium *Methanobacterium thermoautotrophicum*, the inner helices were shown to be displaced laterally from the central pore thus creating an opening sufficient for $K^+$ to gain access to the central cavity and the selectivity filter (Jiang et al., 2002a; Jiang et al., 2002b). From a comparison of the two structures, a conserved glycine residue in the inner helix was proposed to act as a hinge point in opening of the activation gate (Ding et al., 2005; Jiang et al., 2002b; Magidovich and Yifrach, 2004). In eukaryotic $K$ channels, a proline-x-proline (PxP) motif is located below this conserved glycine residue, and this motif is seen in the Kv1.2 structure to form a kink in S6 and constitutes part of the activation gate (del Camino et al., 2000; del Camino et al., 2005; del Camino and Yellen, 2001; Long et al., 2005a; Long et al., 2005b; Webster et al., 2004). Between the activation gate and the selectivity filter, an internal cavity is clearly evident both in the KcsA and the Kv1.2 structure. The size of this central cavity permits the accommodation of quaternary amines such as TEA$^+$. In addition, the inner cavity also contains the binding sites for the N-terminal inactivation peptides and other compounds (Armstrong and Loboda, 2001; Chen and Fedida, 1998; Choi et al., 1993; Fedida, 1997; Zhou et
1.2.1.2 The selectivity filter

External to the bundle crossing, the selectivity filter forms a functional unit which, as its name implies, selects K⁺ over Na⁺ and other physiological cations during the permeation process. The selectivity filter is formed by the K channel signature sequence located in the C-terminal half of the P-loop (Heginbotham et al., 1994; Heginbotham and MacKinnon, 1993), whereas the N-terminal half of the P-loop forms the pore helix, which has its long axis pointing towards the central cavity (Doyle et al., 1998). The pore helix was originally proposed to have a dipole that could stabilize the ion in the central cavity (Doyle et al., 1998). However, this dipole of the pore helix has been shown not to contribute significantly to cation binding in the central cavity, at least in Kir channels (Chatelain et al., 2005). Residues in the pore helix form an extensive hydrophobic and hydrogen bond network with the side chains of the valine and tyrosine residues in the V-G-Y-G sequence of the selectivity filter, as shown in Figure 1.4 (Doyle et al., 1998). The V-G-Y-G sequence is folded such that the side chains are all facing the pore helix while the carbonyl oxygen atoms are pointing into the conduction pathway, an arrangement that agrees well with previous models for ion selection in K channels (Bezanilla and Armstrong, 1972; Hille, 1973; Mullins, 1959). The hydrophobic network surrounding the selectivity filter is thought to act like a spring that holds the selectivity filter open. In addition, the glutamate residue E71 at the internal end of the pore helix has been proposed to interact with the aspartate residue in the G-Y-G-D sequence (Cordero-Morales et al., 2006b), and these interactions may underlie a gating function of the selectivity filter (Berneche and Roux, 2005; Cordero-Morales et al., 2006a; Cordero-Morales et al., 2006b). The interaction between the selectivity filter and permeant ions,
and the role of the selectivity filter in inactivation gating, is discussed in more detail later in this chapter.

Figure 1.4 Stick model of the hydrophobic network surrounding the selectivity filter. The figure is oriented parallel to the plasma membrane with the "pore" at the centre. Residues are as labelled. Y78 is the tyrosine residue in the GYGD signature sequence. Hydrogen bonds are shown as blue sticks. [Doyle et al. (1998); reprint with permission.]

1.2.1.3 The outer pore region

The outer pore region, which is formed by the S5-P and P-S6 linkers, is the least conserved region of the pore domain in the Kv channel family (Figure 1.3 A). Within this region, however, a glutamate residue at the start of the S5-P linker (E418 in Shaker) is absolutely conserved among all Kv channels, but its functional role in normal gating is not well understood (Larsson and Elinder, 2000; Ortega-Saenz et al., 2000). Mutating this residue to an alanine (E418A) or a cysteine (E418C) accelerates slow inactivation (see below) (Larsson and Elinder, 2000; Yifrach and MacKinnon, 2002), whereas the E418D mutation slows the rate of inactivation (Ortega-Saenz et al., 2000). In both the KcsA and Kv1.2 crystal structures, the S5-P linker is folded as a random coil at the outer most part of the channel and forms the so-called turret region (Doyle et al., 1998; Long et al., 2005a). The turret region varies in length, with the human ether-a-go-go gene-related channel (HERG) having one of the longest (38 residues compared to 12 residues in Shaker). The turret regions of Shaker and HERG have been proposed
to form an ordered helical structure (Clarke et al., 2006; Elinder and Arhem, 1999; Jiang et al., 2005; Torres et al., 2003). The role of the turret in the regulation of channel function is less well known, but mutations in this region have been shown to affect the gating of Kv channels (Steidl and Yool, 1999).

On the other end of the P-loop, the P-S6 linker is relatively short but functionally important. The residue T449 in Shaker, or the homologous residue in other Kv channels, has been shown to affect the kinetics of slow inactivation (Lopez-Barneo et al., 1993; Ogielska et al., 1995; Schlief et al., 1996) as well as the binding of TEA+ in Kv channels (Heginbotham and MacKinnon, 1992; MacKinnon and Yellen, 1990). However, the molecular mechanism by which slow inactivation is modulated by this site is still largely unknown.

1.2.1.4 Permeation and selectivity in K channels

The main determinant of permeation and selectivity in K channels is the selectivity filter. Based on the high resolution KcsA structure, there are up to seven K+ binding sites along the conducting pathway as shown in Figure 1.3 C (Roux, 2005; Zhou et al., 2001b). At the most external site (S_ex), K+ is proposed to be fully hydrated, whereas at the adjacent binding site (S_0), the K+ ion is seen to be partially hydrated by three to four water molecules with the rest of the hydration shell filled by the carbonyl oxygen from each of the four glycine residues (G79) in the G-Y-G-D sequence (Berneche and Roux, 2001; Zhou et al., 2001b). Based on electrostatic calculations, occupancy of S_ex and S_0 is mutually exclusive. In the selectivity filter, a fully dehydrated K+ may bind to any of the four different sites (S 1 to S 4 ) coordinated by the backbone carbonyl oxygen of Y78-G77 (S 1 ), G77-V76 (S 2 ), V76-T75 (S 3 ) or T75-V74 (S 4 ) (Berneche and Roux, 2001), but at any given time, only 2 K+ ions may occupy the selectivity filter (Aqvist and
Luzhkov, 2000; Berneche and Roux, 2001; Morais-Cabral et al., 2001; Zhou et al., 2001b). The two K⁺ ions may either be in the S₁-S₃ or S₂-S₄ configuration, and the other two sites are occupied by water molecules. A fully hydrated K⁺ can bind to the central cavity site positioned between S₄ and the bundle crossing; however, other ions (Na⁺, Tl⁺, Rb⁺, and Cs⁺) may have to lose part of their hydration shell before binding to this site, which may contribute to some degree of selectivity (Berneche and Roux, 2001; Zhou et al., 2001b). In addition, S₂ shows differential affinity towards different ions, and this selectivity for permeant ions over non-permeant ions may underlie the different permeability ratio for the permeant ions (Aqvist and Luzhkov, 2000; Berneche and Roux, 2001; Noskov et al., 2004; Zhou and MacKinnon, 2004). These results are consistent with a model in which selectivity in a Kv channel is based on competitive binding between ions (Immke and Korn, 2000; Kiss et al., 1998; Korn and Ikeda, 1995). Furthermore, K⁺ and other permeant cations binding to these sites may modulate channel function and gating, especially in slow inactivation of Kv channels.

In summary, the pore domain is critical for all K channels as it forms a highly selective conduit for K⁺ movement across the plasma membrane. However, permeation of ions does not start until the activation gate is opened, a process that is coupled to the activation of the voltage sensor.

1.2.2 Voltage-sensing domain

In Kv channels, although S1, S2, and S3 contribute to voltage-sensing, the S4 segment is considered to be the main voltage sensor. Each S4 segment contains basic residues (arginine (R)) or lysine (K)) at every third or fourth position (reviewed in Hille, 2001; Wei et al., 1990). The total number of equivalent gating charges per channel has been estimated to be 13 e₀ for
Shaker (Noceti et al., 1996; Schoppa et al., 1992); that is, each S4 contributes between 3-4 gating charges during voltage-sensing. This is consistent with the finding that in Shaker, neutralizing each of the four outer most arginine residues (R362, R365, R368, R371) decreases the total number of gating charges in the mutant channel by 4 (Aggarwal and MacKinnon, 1996; Seoh et al., 1996). Using the substituted cysteine accessibility method (SCAM), the four outermost arginine residues were shown to be accessible to extracellular methanethiosulfonate (MTS) reagents at depolarization, but only the second to fourth arginine residues were accessible to intracellular MTS reagents at hyperpolarization (Larsson et al., 1996). These results strongly support the role of these four residues as the primary gating charges in Shaker that move across the voltage field during voltage-sensing. Besides the basic arginine residues, some acidic residues in S2 (Shaker E283, E293) and S3 (Shaker D316) are also involved in voltage sensing (Aggarwal and MacKinnon, 1996; Seoh et al., 1996), and they may form salt bridges with the gating charges in S4 and stabilize them (Papazian et al., 1995; Sato et al., 2003). In $\text{Na}_{\text{v}}$ and $\text{Ca}_{\text{v}}$ channels, the S4 in each of the homologous domains also contains positive charges (Noceti et al., 1996; Yang et al., 1996; Yang and Horn, 1995), however, the four S4 segments may contribute differently in voltage-sensing and other processes (Cha et al., 1999a; Chanda et al., 2004; Kuhn and Greeff, 2003; Lam et al., 2005).

Even though S4 has generally been accepted as the major carrier of the gating charges, the precise molecular motion of S4 is still not fully understood. A number of models have been proposed over the years (reviewed by Hille (2001) and Horn (2004)), and two crystal structures of Kv channels, the KvAP channel from the bacterium Aeropyrum pernix and the mammalian (r)Kv1.2 from rat brain, have been solved in the search for possible mechanisms for voltage sensing (Jiang et al., 2003; Long et al., 2005a). The two crystal structures show different
orientations of some of the transmembrane helices, and from the KvAP structure, an unconventional view described as the voltage-paddle model was proposed. However, the voltage-paddle model was found to be inconsistent with a number of functional studies that preceded and followed its publication (Gandhi et al., 2003; Gonzalez et al., 2005; see Laine et al., 2004). On the other hand, the Kv1.2 structure agreed with most of the predictions deduced from the various functional studies. Based on the Kv1.2 crystal structure, a possible model for voltage sensing is described below.

1.2.3 Possible structural model for Kv channels

The crystal structure of Kv1.2 may be the best representation of native eukaryotic Kv channels to date. The six transmembrane segments are oriented more-or-less perpendicular to the plane of the membrane, with the S1-S2 linker, S3-S4 linker, the S5-P linker, and P-S6 linker being extracellular. This is consistent with the S1-S2 linker being glycosylated (Gandhi et al., 2003; Shen et al., 1993). However, S4 is seen in the crystal structure to be partially exposed to the lipid environment without being completely protected by S1, S2, and S3 (Long et al., 2005a), which differs from the conventional view in which the hydrophilic S4 segment is thought to be shielded completely from the lipid environment by S1, S2, and S3 to minimize the thermodynamically unfavourable charge-lipid interaction. A low resolution KvAP model obtained from electron microscopy in a more native state also suggested that S4 was exposed to the lipid environment (Jiang et al., 2004). The counter-charges in S1, S2, and S3 may be sufficient to stabilize the gating charge within the lipid core.

Given the above structural model, how does S4 move during voltage sensing?

Unfortunately, the Kv1.2 crystal structure did not give much insight into the movement of S4
with respect to the membrane. However, a number of functional studies have suggested S4 is surrounded by a small canaliculus, and that the voltage field drops across a very small region around S4 (Baker et al., 1998; Goldstein, 1996; Larsson et al., 1996; Starace and Bezanilla, 2004). For example, when the outermost arginine residue in S4 of Shaker was mutated to histidine (R362H), a proton current could be recorded upon hyperpolarization in the presence of a proton gradient. It appeared that the imidazole ring of the histidine residue could bind a proton on the more acidic side and transfer it to the more basic side with very small movement (Starace and Bezanilla, 2004). The simplest explanation for this result is that the external and cytoplasmic solutions are separated by a very small space, and that the electric field is focussed onto a small region.

In most models, S4 moves as a rigid structure. Upon depolarization, the electrostatic force drives the gating charges outward. However, the movement of S4 is probably more a rotational than an outward (translational) movement (Cha et al., 1999b). S4 may also become tilted, and both the rotational and tilting motion of S4 may occur over a small region as the four outermost gating charges are shuttled to the external milieu (Ahern and Horn, 2005; Cha et al., 1999b; Glauner et al., 1999). This model is proposed on the basis of a fluorescence study which shows limited S4 movement upon depolarization (Chanda et al., 2005). In addition, the structural requirements for this model are consistent with the Kv1.2 crystal structure (Long et al., 2005a). However, the actual “canaliculi” around S4 have not yet been mapped onto the Kv1.2 crystal structure, and the relative movements of the different transmembrane segments has also not been measured. The above model can account for most functional data available and seems to be the most probable model thus far.
1.2.4 Electro-mechanical coupling in Kv channels

One of the most interesting questions in studying Kv channel activation is how the conformational changes in S4 lead to the opening of the activation gate formed by S6. As discussed above, the opening of the activation gate appeared to involve only the inner helices (S6) with the positions of both the selectivity filter and the outer helices (S5) remaining essentially unchanged. Opening of the activation gate involved a rotation of S6 at a conserved glycine (Ding et al., 2005; Lee, 2005; Magidovich and Yifrach, 2004; Seebohm et al., 2005; Wang et al., 2005; Zhao et al., 2004) or at the PxP motif (Bright et al., 2002; Labro et al., 2003; Rich et al., 2002; Webster et al., 2004) which may act as a hinge. But the question remains: how is the movement of S4 coupled to the conformational changes of S6?

This question has been partly answered by studies of a chimeric channel. As mentioned, the pore domains from different K channels are structurally very similar; therefore, it is possible to "swap" the pore domains between two different K channels. Moreover, the machinery necessary for the coupling between the voltage sensor and the pore domain may be revealed by progressively substituting the pore domain of a non-voltage-gated K channel into that of a Kv channel. Such an experiment has been performed, and when the KcsA pore domain was substituted into Shaker, two phenylalanine residues (F401 and F404) in the S4-S5 linker were required to confer voltage-dependent activation (Caprini et al., 2005; Lu et al., 2002). This intimated that the conformational changes in S4 were coupled to the movement of the S4-S5 linker. That is, S4 may be indirectly coupled to S6 via the S4-S5 linker.

The coupling between the S4-S5 linker and S6 is further suggested by the Kv1.2 crystal structure (Long et al., 2005a). In the crystal structure, the pore domain of each subunit has its S4-S5 linker associated with its own C-terminal half of S6 below the PxP motif, as shown in
Figure 1.2 C (Long et al., 2005b). It is proposed that an “outward” movement of S4 during depolarization would pull on S6 via the S4-S5 linker, and that this pulling motion results in a rotation, either at the conserved glycine residue or at the PxP motif, that opens the intracellular gate (Long et al., 2005b). This simple model seems to have merit for eukaryotic Kv channels; however, it is not clear whether a similar interaction exists in other voltage-gated channels. For example, the human Na$_{v}$1.5 does not have the PxP motif in any of the S6 segments, but it does contain a glycine residue in each of the S6 in domains I, II, and III (Wang et al., 2005).

In summary, the crystal structures from a number of K channels (KcsA, MthK, KvAP, Kv1.2, etc.) have contributed significantly in our understanding of the molecular mechanisms for activation, permeation, and selectivity. The crystal structures have provided a snapshot of the channels. However, channels are dynamic proteins, and the detailed conformational changes rely more on the functional studies.

### 1.3 Inactivation in Kv channels

Inactivation is an intrinsic property of Kv channels (Bezanilla, 2000). In the presence of a continuous depolarization, Kv channels enter a stable non-conducting inactivated state, and this process is manifested as a time-dependent current decay under voltage clamp conditions. The time course of inactivation can be described by exponential functions with one or more components, with each component described by an amplitude term ($A$) and a time constant ($\tau$). With the exception of the fast C-type inactivation in HERG, inactivation is generally voltage-independent, but it may be coupled to other voltage-dependent processes or states (Hoshi et al., 1991; reviewed in Yellen, 1998). In Kv channels, the three main types of inactivation, namely N-type, U-type, and P/C-type, are classified based on their interactions with intra- or extracellular
TEA⁺ and K⁺, and they may also differ in their molecular basis. Table 1.1 summarizes the differences among the three types of inactivation. In this section, a brief description of the biophysics of N- and U-type inactivation is followed by a somewhat more detailed description of the properties of P/C-type inactivation.

**Table 1.1 Comparison of properties of N-type, U-type, and P/C-type inactivation.**

<table>
<thead>
<tr>
<th>Inactivation</th>
<th>N-type (fast)</th>
<th>U-type</th>
<th>P/C-type (slow)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structural basis</td>
<td>N-terminal peptide</td>
<td>Similar to C-type inactivation (?)</td>
<td>S4, Selectivity filter, outer pore</td>
</tr>
<tr>
<td>Time course</td>
<td>Fast (tens of milliseconds)</td>
<td>Intermediate (hundreds of milliseconds)</td>
<td>Usually slow (seconds)</td>
</tr>
<tr>
<td>Gating</td>
<td>Directly coupled to open state</td>
<td>Coupled to closed states</td>
<td>Mainly proceed from open states</td>
</tr>
<tr>
<td>Extracellular K⁺</td>
<td>Accelerates recovery from inactivation</td>
<td>Accelerates inactivation</td>
<td>Slows rate of inactivation</td>
</tr>
<tr>
<td>Extracellular TEA⁺</td>
<td>No effect</td>
<td>Accelerates inactivation</td>
<td>Slows rate of inactivation</td>
</tr>
<tr>
<td>Intracellular K⁺</td>
<td>No effect</td>
<td>N/A</td>
<td>Slows inactivation kinetics</td>
</tr>
<tr>
<td>Intracellular TEA⁺</td>
<td>Slows inactivation competitively</td>
<td>N/A</td>
<td>No effect*</td>
</tr>
</tbody>
</table>

*Other quaternary amines with longer mean dwell times can accelerate inactivation (Baukrowitz and Yellen, 1996).

**1.3.1 N-Type inactivation**

Of the three types of inactivation, N-type inactivation, also known as fast inactivation, is by far the best characterized. In the early 1970's, a transient A-type K current was identified in neurons of gastropods (Connor and Stevens, 1971). The A-type current, a term now used to
describe all transient K current, was envisioned to arise from an inactivation process similar to that giving rise to the transient Na current in Na\textsubscript{v} channels. Just a few years later, Bezanilla and Armstrong (1977) used internally applied pronase, a proteolytic enzyme, to remove (fast) inactivation in the Na\textsubscript{v} channel of squid giant axon, and they proposed a “ball-and-chain” model as the mechanism for Na\textsubscript{v} channel (fast) inactivation. Based on this model, pronase was seen simply to remove the cytoplasmic “inactivation ball.”

The Shaker channel from the fruit fly Drosophila melanogaster also gives rise to a fast inactivating A-type current (Iverson et al., 1988; Timpe et al., 1988). Using an approach similar to that for studying fast inactivation in the Na\textsubscript{v} channel (Bezanilla and Armstrong, 1977), Hoshi et al. (1990) were able to remove (fast) inactivation from Shaker by applying trypsin, another proteolytic enzyme, to the cytoplasmic side of the channel. Moreover, deleting residues 2-46 from the N-terminus of Shaker using mutagenesis also resulted in a current that did not inactivate, at least with short depolarizing pulses (Hoshi et al., 1990), and this mutant became known as the inactivation-removed Shaker channel, or ShakerIR. Reapplying the N-terminal peptide back into the intracellular medium reconstituted fast inactivation in ShakerIR and conferred fast inactivation to other Kv channels (Stephens and Robertson, 1995; Zagotta et al., 1990). These results showed conclusively that fast inactivation is mediated by the N-terminus and thus is now commonly referred to as N-type inactivation.

N-type inactivation has been demonstrated in Shaker (Hoshi et al., 1990; Zhou et al., 2001a), Kv1.4 (Stuhmer et al., 1989; Tseng-Crank et al., 1993; Zhou et al., 2001a), Kv3.3 (Fernandez et al., 2003), Kv3.4 (Schroter et al., 1991; Stephens and Robertson, 1995), and Kv channels associated with Kvβ1.1 subunits (Heinemann et al., 1996) and Kvβ1.3 subunits (Decher et al., 2005), and its mechanism is analogous to a ball-and-chain model. The N-terminal
peptides from Shaker B, hKv3.4, and Kv1.4 have been shown to block the non-(fast) inactivating mouse Kv1.1 channel (Antz and Fakler, 1998; Stephens and Robertson, 1995), and Kv1.4 can be blocked by the N-terminal ball from Shaker in addition to its own N-terminal ball (Ruppersberg et al., 1991). However, despite this apparent non-specific blocking of Kv channels, the inactivation balls (around 30-40 amino acids in length) show very little homology in their primary sequence or tertiary structure, as revealed by their NMR and crystal structures (Antz et al., 1997; Antz and Fakler, 1998; Schott et al., 1998). The “non-specificity” arises from the fact that the N-terminal ball receptor site in the internal cavity is structurally very similar between Kv channels (del Camino et al., 2000; Gulbis et al., 2000; Zhou et al., 2001a). The length of the linker (i.e., the “chain”) between the N-terminal ball and the rest of the channel can affect the kinetics of N-type inactivation, such that the rate is slowed if this linker is lengthened, or accelerated if part of the linker is deleted (Zagotta et al., 1990).

The ball-and-chain model describes N-type inactivation very well; however, the manner in which the N-terminal ball binds to its receptor site in the central cavity is not as straightforward as the inactivation cartoons sometime imply. In Kv channels, the T1 domains are folded into a four-fold symmetric structure that is suspended from the transmembrane segments like a hanging gondola, as shown in Figure 1.2 B (Kobertz et al., 2000; Kreusch et al., 1998; Long et al., 2005a; Sokolova et al., 2001). Between the T1-S1 linkers are lateral openings that the N-terminal ball must pass through to gain access to the central pore (Gulbis et al., 2000; Long et al., 2005a; Zhou et al., 2001a). The N-terminal inactivation particle may be in a “pre-inactivated” state as it binds to the T1 domain and/or the S4-S5 cytoplasmic loop prior to activation (Isacoff et al., 1991; Zhou et al., 2001a). The transition between this pre-inactivated state and the inactivated state may be too fast to be recorded and thus giving rise to an apparent closed-state
inactivation. However, N-type inactivation is strictly coupled to activation (Demo and Yellen, 1991) in that the N-terminal ball can access its binding site only if the activation gate is open.

The binding of the N-terminal peptide to the internal cavity is mechanistically very similar to that for an open channel blocker such as intracellular TEA\(^+\). Internal TEA\(^+\) can slow N-type inactivation by competing with the N-terminal ball for an overlapping binding site (Choi et al., 1991; del Camino et al., 2000). However, unlike TEA\(^+\) and other quaternary ammonium (QA) compounds that can be trapped in the internal cavity upon deactivation, the N-terminal peptide with the tethered chain prevents the closing of the activation gate (Demo and Yellen, 1991; Ruppersberg et al., 1991). In addition, increasing the extracellular K\(^+\) concentration can speed up the rate of recovery from N-type inactivation as if the inward K\(^+\) can knock off the inactivation particle (Demo and Yellen, 1991). Interestingly, mutating the cationic (basic) lysine at position 7 in the N-terminal peptide in *Shaker* dramatically reduces fast inactivation (Hoshi et al., 1990). Furthermore, both the NMR structures of the N-terminal peptides from Kv3.4 and Kv1.4 show localized positively-charged surface residues. On the basis of the two latter observations, it is tempting to speculate TEA\(^+\) and the N-terminal peptide mimic K\(^+\) in binding to the receptor site in the central cavity.

### 1.3.2 U-type inactivation

U-type inactivation has been described in Kv3.1 (Klemic et al., 2001), Kv2.1 (Klemic et al., 1998), the *Shaker* channel (Klemic et al., 2001), the N-terminal truncated form of Kv1.5 (Kurata et al., 2001; Kurata et al., 2002; Kurata et al., 2005), as well as in N-type and R-type Ca\(_v\) channels (Patil et al., 1998). The term U-type inactivation was coined on the basis of the U-shaped inactivation-voltage curve, in which the degree of inactivation at intermediate voltages is
greater than at higher voltages (Klemic et al., 2001). It has been proposed that this U-shaped inactivation-voltage relationship arises from inactivation that proceeds preferentially from partially-activated states (Klemic et al., 1998). Since the mean dwell time in partially activated states is longer with intermediate depolarizations, the proportion of inactivated channels is larger than observed with stronger depolarizations. This property of U-type inactivation may underlie the strong frequency dependence of U-type inactivation, in which the extent of inactivation with short repetitive depolarization steps is larger than that with a single-pulse depolarization of the same duration (Klemic et al., 1998; Klemic et al., 2001; Kurata et al., 2001). This “excessive cumulative inactivation” has also been observed in other Kv channels (Aldrich, 1981; DeCoursey, 1990), but whether excessive cumulative inactivation is an exclusive property of U-type inactivation is still unknown.

A number of properties distinguish U-type inactivation from N- and P/C-type inactivation. U-type inactivation is accelerated by increasing the extracellular concentration of K⁺ or TEA⁺ (Klemic et al., 1998; Klemic et al., 2001; Kurata et al., 2005), which is directly opposite to its effects on slow inactivation (Choi et al., 1991). In addition, the rate of recovery from U-type inactivation at negative potentials is faster than the rate of U-type inactivation at depolarized potentials (Klemic et al., 2001). This is different from P/C-type inactivation in which the rate of recovery is usually slower than the rate of inactivation. Even though the molecular mechanism for U-type inactivation is not known, at least in the Shaker channel, U-type and P/C-type inactivation can be observed simultaneously at different voltages (Klemic et al., 2001). The coexistence of these two types of inactivation argues for separate underlying mechanisms for the two processes. It has been proposed that slight changes in the pore structure may be sufficient for the channel to switch from U-type inactivation to P/C-type inactivation and
vice versa (Klemic et al., 2001). However, it is still unclear why some channels preferentially inactivate from the closed-state. Kv4.1 has been proposed to inactivate preferentially from a closed-state (Gebauer et al., 2004; Jerng et al., 1999), but its inactivation properties are not completely identical to U-type inactivation, suggesting the existence of several forms of closed-state inactivation (Klemic et al., 2001). Yet, U-type inactivation may be more prevalent than previously thought (Klemic et al., 2001; Patil et al., 1998).

1.3.3 Slow (P/C-type) inactivation

When N-type inactivation is removed by N-terminal deletion, the resulting ShakerIR current shows a slow decay process termed slow inactivation (Hoshi et al., 1990). In early studies, the properties of slow inactivation were found to depend on the C-terminal splice variant; therefore, this type of inactivation was also called C-type inactivation (Hoshi et al., 1991), and the terms have been used synonymously for some time. By comparing the different C-terminal splice variants of Shaker, the residue at position 463 in S6 was found to determine the kinetics of slow inactivation: a valine residue (ShakerA) slowed the decay rate, whereas an alanine at residue 463 (ShakerB) resulted in a faster decay. This residue was the first of many residues in the pore domain that have been shown to affect slow inactivation. Slow inactivation manifested as a time-dependent current decay with a multi-exponential time course, which suggested more than one process was involved. Indeed, a number of experiments have shown that slow inactivation is at least a two-step process involving conformational changes in the pore region and the S4 segment (see below); therefore, slow inactivation is now referred to as P/C-type inactivation (Chen et al., 2000; Loots and Isacoff, 1998).

The terms P-type and C-type inactivation are applied somewhat differently than originally
proposed. Historically, P-type inactivation was proposed to describe an inactivation process in
the V369I mutant of a chimera made by substituting the pore of Kv2.1 with that from Kv3.1
(Kirsch et al., 1992). It was found that the inactivation properties in this mutant were different
from both C-type and N-type inactivation, and the term P-type inactivation was coined because
residue 369 is located deep in the pore (De Biasi et al., 1993). Nowadays, the term P-type
inactivation is used to describe the conformational changes in the selectivity filter and/or the
outer pore region (Loots and Isacoff, 1998). Similarly, C-type inactivation was originally used to
describe the slow inactivation process observed in Shaker1R; however, it now is taken to mean
the process resulting in gating charge immobilization (Cha and Bezanilla, 1997; Olcese et al.,
1997). We are only beginning to understand the role of the outer pore mouth, the selectivity
filter, and S4 in mediating this complicated process.

1.3.3.1 Role of the outer pore in slow inactivation

The outer pore has been implicated as one of the most important structural determinants
of slow inactivation. The classical features of slow inactivation (Kiss and Korn, 1998), namely
its modulation by the mutations at position 449 in Shaker (Lopez-Barneo et al., 1993) and its
inhibition by external TEA⁺ (Choi et al., 1991) and external K⁺ (Lopez-Barneo et al., 1993), are
all related to the outer pore. The important role of Shaker T449 in slow inactivation is
highlighted by the dramatic changes of current decay observed in the different T449 mutants. A
glutamate (E), lysine (K), alanine (A), glutamine (Q) or serine (S) at the position 449 can
dramatically accelerate slow inactivation, whereas a valine (V) or tyrosine (Y) residue at this
position results in a very slow or non-inactivating mutant (Lopez-Barneo et al., 1993; Schlief et
al., 1996). Moreover, the T449H mutant shows K⁺-dependent inhibition of slow inactivation
only at low pH, and in Kv1.3, the histidine residue H399 at the site homologous to Shaker T449 also slows the rate of slow inactivation only at low pH (Somodi et al., 2004). In addition, Shaker T449 is also involved in the binding of external TEA\(^+\) (Heginbotham and MacKinnon, 1992; MacKinnon and Yellen, 1990). Mutating this residue to tyrosine (T449Y) or phenylalanine (T449F) increases the TEA\(^+\) affinity by 25- to 50-fold (Heginbotham and MacKinnon, 1992; Kavanaugh et al., 1992; MacKinnon and Yellen, 1990).

The inhibition of slow inactivation by external TEA\(^+\) and \(K^+\) was explained by the “foot-in-door” process (Almers and Armstrong, 1980; Choi et al., 1991; Grissmer and Cahalan, 1989; Lopez-Barneo et al., 1993; Matteson and Swenson, Jr., 1986; Pardo et al., 1992; Swenson, Jr. and Armstrong, 1981). Support for this hypothesis was provided by Yellen et al. (1994) who showed that the outer pore constricted during slow inactivation. Mutating the outer pore residues M448, T449, or P450 to a cysteine (M448C, T449C, or P450C) resulted in a state-dependent block by external Cd\(^{2+}\) and Zn\(^{2+}\) (Yellen et al., 1994), and the T449C mutant was 45,000-fold more sensitive to Cd\(^{2+}\) in the inactivated state than the open state. The M448C and P450C mutants also show a similar state dependent reaction to MTS reagents, and the M448C mutant can also be cross-linked between two subunits by a thiol-reactive compound (Liu et al., 1996). During slow inactivation, the outer pore regions of the four subunits are thought to constrict in a highly cooperative manner (Ogielska et al., 1995; Panyi et al., 1995). This constriction may be inhibited physically by external \(K^+\) and TEA\(^+\) as they bind to the outer pore.

It is possible that the external coordination sites (\(S_0\) or \(S_{\text{ext}}\)) are the regulatory sites at which external \(K^+\) and TEA\(^+\) bind and consequently slow P/C-type inactivation. This hypothesis predicts that ions (\(K^+\), TEA\(^+\), or other permeant ions such as Rb\(^+\), NH\(_4^+\), Cs\(^+\), and Na\(^+\)) binding to the external coordination site could prevent the collapse of the outer pore. Indeed, the extent of
attenuation of C-type inactivation by permeant ions is similar to the permeability ratio with respect to K⁺ (Lopez-Barneo et al., 1993), which is consistent with the above hypothesis. Similarly, TEA⁺ was shown to be bound at S₀ in a molecular dynamic simulation (Crouzy et al., 2001). In addition, in a KcsA crystal structure, a TEA⁺ analog was shown to bind to the coordination site S₀ and to be coordinated by Y82 (homologous to Shaker T449) at the outer pore (Lenaeus et al., 2005). These results suggest that the external TEA⁺ binding site is similar in KcsA and in Shaker, and that TEA⁺ may act as a “partially hydrated K⁺” at S₀. However, TEA⁺ can block certain mutants (e.g., Shaker D447E T449Y) without affecting the rate of slow inactivation (Molina et al., 1997). Therefore, the mechanism by which external TEA⁺ inhibits slow inactivation may be more complicated than once thought.

1.3.3.2 Role of the selectivity filter in slow inactivation

Several lines of evidence have suggested that the selectivity filter undergoes conformational changes during slow inactivation, and that the slow inactivation gate may in fact be the selectivity filter in a collapsed, non-conducting state (Cordero-Morales et al., 2006a; Cordero-Morales et al., 2006b; Kiss et al., 1999; Kiss and Korn, 1998). The possible involvement of the selectivity filter in slow inactivation was first implied by the inhibition of slow inactivation by permeant ions (Lopez-Barneo et al., 1993). It was later suggested that when the selectivity filter was depleted of permeant ions, either following N-type inactivation (Baukrowitz and Yellen, 1995) or block by internal quaternary amines with long mean dwell times (Baukrowitz and Yellen, 1996), slow inactivation could be dramatically accelerated. It was also proposed that slow inactivation could not proceed before the last ion had evacuated the selectivity filter (Baukrowitz and Yellen, 1996; Yellen, 1998). According to this hypothesis,
external TEA⁺ and external permeant ions may attenuate slow inactivation by preventing the evacuation of K⁺ from the selectivity filter (Kiss and Korn, 1998).

The involvement of the selectivity filter in slow inactivation can also be appreciated from the various mutations in the P-loop that result in a change in the rate of slow inactivation. Mutating W434 in the pore helix of Shaker to phenylalanine (W434F) results in a non-conducting mutant (Perozo et al., 1993) that is thought to be permanently P-type inactivated (Olcese et al., 1997; Yang et al., 1997), and the homologous mutation in Kv1.5 (W472F) also results in a similar non-conducting mutant (Chen et al., 1997). When these residues are mapped onto the KcsA structure, the equivalent residue (W67) is in a network of hydrophobic residues that includes W68 and Y78 (in the G-Y-G-D signature sequence; Figure 1.4) and which is proposed to hold the selectivity filter in the open conformation (Doyle et al., 1998).

Interestingly, the W434F mutant becomes conducting with the T449V mutation (Kitaguchi et al., 2004), which suggests the two mutations affect the same process but in an opposite manner. The tryptophan-to-phenylalanine mutation is thought to disrupt the hydrophobic network resulting in the collapse of the selectivity filter, and the channel becomes permanently P-type inactivated. This idea is supported by the fact that normal gating charge movement is observed in this mutant, which also suggests that the gating charges are not immobilized and that the channel is not C-type inactivated.

An apparent “collapse” of the selectivity filter is also observed in the absence of external permeant ions, during which some K channels may enter a non-conducting state that is related to slow inactivation (Yellen, 1997) and distinct from the defunct state (Loboda et al., 2001; Melishchuk et al., 1998). Thus, in Kv1.4, removal of external permeant ions results in a conductance collapse due to a large decrease of channel availability. This external K⁺-dependent
conductance collapse is absent in the K533Y-I535M mutant (Pardo et al., 1992). The involvement of K533, which is homologous to Shaker T449, suggests that the collapse of Kv1.4 conductance is related to a slow inactivation process. In the absence of external K⁺ or other permeant ions, the BK channel and the plant inward rectifier K channel KAT1 and ZmK2.1 also fail to conduct current (Hertel et al., 2005; Su et al., 2005; Vergara et al., 1999). This "conductance collapse" is also observed in Kv1.3 at pH 6.0, the Shaker T449A, T449E, T449K mutants, and the Kv1.5 H463G mutant (Jäger et al., 1998; Jäger and Grissmer, 2001; Lopez-Barneo et al., 1993; Su et al., 2005). These channels are all proposed to enter a stable non-conducting state resulting in channels being unavailable for activation. In the inward rectifier Kir1.1, reducing external [K⁺] promotes an inactivated mode of gating which is similar to the conductance collapse described above (Sackin et al., 2001). Together, these results highlight the importance of permeant ions in channel function, in which K⁺ in the pore prevents a "conductance collapse" related to an inactivation process.

Another clue to the involvement of the selectivity filter in slow inactivation is the change of selectivity observed during slow inactivation (Yellen, 1998). In the absence of K⁺ on both sides of the membrane, slow inactivation was estimated to be complete in milliseconds (Baukrowitz and Yellen, 1996). It was expected that, under this K⁺-free condition, the outer pore of the channels would become constricted due to slow inactivation, and channels would be non-conducting. However, removing K⁺ from both sides of the channels resulted in measurable currents through Shaker, Kv1.5, and Kv2.1 that were carried by Na⁺ (Korn and Ikeda, 1995; Starkus et al., 1997; Wang et al., 2000). That is, inactivated channels became Na⁺ permeable (Kiss et al., 1999). This Na⁺ current was also observed in the permanently P-type inactivated "non-conducting" Shaker W434F mutant in the absence of K⁺ on both sides of the membrane.
(Starkus et al., 1998). It appeared that the selectivity filter became constricted during slow inactivation such that the coordination sites could accommodate Na⁺ better than in the "open" conformation.

Besides the more "defined" functional studies, a number of recent structural studies have proposed the molecular basis for the conformational changes during slow inactivation. From the KcsA crystal structure, a structural rearrangement at T75 and V76 during K⁺ conduction was predicted by a molecular dynamics study to occur spontaneously, and this rearrangement was suggested to be an initial step in slow inactivation (Berneche and Roux, 2005). In a recent study, the residues E71 and D80 in KcsA are suggested to undergo a conformational change during some gating processes (Cordero-Morales et al., 2006a). It is uncertain whether this conformational change is related to a slow inactivated state in Kv channels, but these studies have opened up new possibilities for further investigations.

1.3.3.3 Role of S4 in slow inactivation

In addition to participating in voltage sensing during activation, S4 is also involved in slow inactivation. Studies using fluorescent probes have shown that changes in fluorescence project from the pore out to S4 during slow inactivation (Gandhi et al., 2000; Loots and Isacoff, 1998; Loots and Isacoff, 2000). However, the precise interaction of the voltage-sensing domain and the pore domain during slow inactivation is still largely unknown. The involvement of S4 in slow inactivation is implicated most directly in the process called gating charge immobilization (Fedida et al., 1996; Olcese et al., 1997).

Gating charge immobilization was first described in Naᵥ channels (Armstrong and Bezanilla, 1977). With short depolarizing pulses, the ratio of off-gating charge to on-gating
charge ($Q_{\text{off}}:Q_{\text{on}}$ ratio) was close to one, suggesting that most of the gating charges activated (mobilized) during activation returned to the resting level upon repolarization. However, with longer depolarizing pulses, the $Q_{\text{off}}:Q_{\text{on}}$ ratio became progressively smaller, and the kinetics of some of the $\text{off}$-gating current was much slower. Together, these observations indicated that the gating charges moved back to the resting state but only very slowly, as if some of the gating charges were being "immobilized." This apparent loss of gating charges is described as gating charge immobilization.

In ShakerIR, a similar leftward shift of the $Q_{\text{off}}-V$ curve (i.e., charge immobilization) was observed after the channels were inactivated, but all the gating charge could be seen to return to the resting state if a strong hyperpolarizing pulse was given (Olcese et al., 1997). It was shown that the time course of gating charge immobilization followed that of slow inactivation, and the time course of recovery from gating charge immobilization also correlated well with that of recovery from slow inactivation (Olcese et al., 1997). In addition, gating charge immobilization in Kv1.5 was inhibited by 4-aminopyridine (4-AP), which inhibited slow inactivation by inhibiting channel opening (Fedida et al., 1996). It was proposed that gating charge immobilization is part of the C-type inactivation process (Loots and Isacoff, 1998).

The conformational changes occurring during charge immobilization and C-type inactivation are still unknown, but an interaction between the pore domain and S4 was postulated (Loots and Isacoff, 2000). A possible interacting pair is the activated S4 and a conserved glutamate residue (E418 in Shaker) at the base of the turret (Larsson and Elinder, 2000; Loots and Isacoff, 2000). In the Kv1.2 crystal structure the pore domain (in particular the S5) of one subunit is adjacent to the S4 of another subunit (Long et al., 2005a). In Shaker, E418 is suggested to be positioned adjacent to the top of S4 during activation (Loots and Isacoff, 2000),
and it is thought to interact with G452 in the P-S6 linker to stabilize the open state. This interaction is thought to be broken during slow inactivation, as shown by E418 coming close to V451 in an inactivated state (Loots and Isacoff, 2000). Yet, it is uncertain which residues actually move during slow inactivation and how S4 fits into this picture. Nevertheless, as suggested by the Shaker ILT mutant, the movement of S4 itself may not trigger slow inactivation. In this mutant, the movement of S4 is “energetically separated” from the concerted opening of the activation gate (Pathak et al., 2005); that is, the voltage range over which S4 movement occurs is not sufficient to open the activation gate, which requires a much stronger depolarization. Even at potentials in which S4 is fully activated, this mutant does not seem to undergo slow inactivation until the activation gate is open. Conversely, slow inactivation has been suggested to occur from one or more closed states (Olcese et al., 1997; Yang et al., 1997). It is uncertain whether slow inactivation occurring from open state is structurally equivalent to that occurring from closed states.

To summarize, slow (P/C-type) inactivation has been shown to result from a constriction of the outer pore and/or a constriction at the selectivity filter, both of which may underlie P-type inactivation. A slower process that involves an interaction between the pore domain and S4 results in gating charge immobilization that underlies C-type inactivation. Surprisingly, the turret region, which forms part of the outer vestibule, has, except for a few reports (Perchenet and Clement-Chomienne, 2001; Steidl and Yool, 1999; Zilberberg et al., 2001, been largely ignored in the study of slow inactivation. That the turret may play an important role in slow inactivation is suggested by results described in this thesis.
1.4 Kv1.5 and scope of thesis investigation

The human voltage-gated potassium channel Kv1.5, encoded by the gene KCNA5, is a delayed rectifier K channel expressed in atrial and, to a smaller extent, ventricular myocytes (Fedida et al., 1993; Mays et al., 1995), vascular and intestinal smooth muscles (Overturf et al., 1994), pulmonary artery smooth muscles (Archer et al., 1998), pancreatic islets (Philipson et al., 1991), and microglia in the central nervous system (Jou et al., 1998). In human atrial myocytes, Kv1.5 mediates the ultra-rapid delayed rectifier current (I_{kur}), which is partly responsible for repolarizing the atrial action potential and for the determination of the plateau duration (Fedida et al., 1998; Feng et al., 1997). Simulations have shown that a reduction in I_{kur} can lead to a prolongation of the atrial action potential (Gomez et al., 2005), and have suggested that a reduction in Kv1.5 current may have therapeutic benefit for treating atrial fibrillation (Brendel and Peukert, 2003; Matsuda et al., 2001). The role of Kv1.5 in the central nervous system is not well understood.

The homotetrameric Kv1.5 channel is a Shaker-related channel with some biophysical properties that differ from those of Shaker. In contrast to the Shaker channel, wild-type Kv1.5 does not have an N-terminal inactivation ball and thus does not undergo N-type inactivation unless associated with Kv β1.1 or β1.3 subunits (Heinemann et al., 1996). Furthermore, wild-type Kv1.5 is not sensitive to external TEA^+ block, presumably due to the positively charged arginine residue at position 487, homologous to Shaker T449, given that the R487V mutation confers (external) TEA^+ sensitivity to Kv1.5 (Fedida et al., 1999). Similar to the Shaker channel, Kv1.5 is sensitive to block both by 4-AP and quinidine (Bouchard and Fedida, 1995; Fedida, 1997; Wang et al., 1995). The blocking mechanism for 4-AP and quinidine is thought to be similar in Kv1.5 and Shaker, in which 4-AP stabilizes an activated closed-state (Armstrong and
Loboda, 2001) and quinidine acts as an open channel blocker (Fedida, 1997). Given that the single channel conductance is similar between Kv1.5 and Shaker, it is thought that the conducting pore of these two channels is similar.

As mentioned above, in the absence of intracellular and extracellular K⁺, both the Kv1.5 (Wang et al., 2000) and the Shaker channel (Starkus et al., 1997) are permeable to Na⁺, and both the Shaker W434F mutant and the equivalent Kv1.5 W472F mutant do not conduct K⁺ current (Chen et al., 1997; Hesketh and Fedida, 1999; Perozo et al., 1993). Interestingly, the Kv1.5 R487V mutation strongly attenuates slow inactivation in Kv1.5 when Na⁺ is the charge carrier, but no significant difference in the inactivation kinetics is observed with K⁺ as the charge carrier (Wang et al., 2000), a phenomenon that is not yet explained. This is in contrast to the finding in the Shaker channel where a valine at the equivalent position (T449V) strongly inhibits slow inactivation with K⁺ current (Lopez-Barneo et al., 1993; but see Holmgren et al., 1996). In addition, external K⁺ has been shown to inhibit slow inactivation in the Shaker channel (Lopez-Barneo et al., 1993), but external K⁺ seems to have a much more muted effect on the rate of slow inactivation in Kv1.5 (Fedida et al., 1999). The molecular basis for these differences has not yet been identified, but the molecular mechanisms underlying slow inactivation are assumed to be similar in Kv1.5 and in Shaker.

This dissertation examines the mechanistic basis for the modulation of hKv1.5 (Kv1.5) slow inactivation by external H⁺, Ni²⁺, K⁺, and some other divalent cations. The goal for this research project is to increase our understanding of slow inactivation gating of ion channels, which may in turn lead to a better understanding of the structure-function relationship of slow inactivation and the conformational changes associated with slow inactivation. The impetus for this work was the observation in our laboratory that external Zn²⁺ could inhibit Kv1.5 current in a
$K^+_o$-dependent manner (Zhang et al., 2001). However, the binding site for $Zn^{2+}$ and the mechanistic basis for its inhibitory effect were not identified in that study. Therefore, the first goal of this research project was to ascertain the molecular basis for the $Zn^{2+}$-induced current inhibition (Chapter 2). To do this, Kv1.5 macroscopic currents were studied in various conditions using conventional voltage clamp techniques in the whole-cell and outside-out configurations. The turret histidine residue at position 463 (H463) was proposed to be the binding site for $Zn^{2+}$, and this binding of cations resulted in a current inhibition mediated by a process involving the outer pore arginine residue (R487). In addition, the properties of the current inhibition were similar to the classical features of slow inactivation. It was also found that external $H^+$ also caused an inhibition of Kv1.5 with similar properties as that induced by $Zn^{2+}$. Therefore, the current inhibition induced by external $Zn^{2+}$ and $H^+$ in Kv1.5 was hypothesized to result from an enhancement of a slow inactivation process that led to the reduction in channel availability.

This hypothesis was tested further with external $Ni^{2+}$ and other divalent cations, and a similar current inhibition was observed, albeit with somewhat different effects on other biophysical properties (Chapter 3). From the unitary currents recorded with $Ni^{2+}$, the current inhibition was found to result from an increased probability that a channel failed to report current upon depolarization, which was again consistent with our hypothesis. The single channel analysis was continued in Chapter 4 but focussed on the effects of changes of external pH on gating behaviour. When the unitary current behaviour of Kv1.5 was systematically analysed at different pHs, a shift in the mode of gating was revealed, and the shift was proposed to result from an inactivation process proceeding from closed states. Further support for channels being in an inactivated state at low pH was obtained by analysing the first latency behaviour and the
gap lengths between bursts (Chapter 5). Using long ($\geq$ 6 s) depolarizing pulses, channels were sometimes shown to open with a long delay (first latency), which was proposed to result from the recovery from an unavailable mode of gating (channel failed to conduct; mode $U$) to a normal mode of gating (channel able to conduct; mode $A$). The mean (long) first latency was found to correlate with the mean (long) gap length. This result suggested that mode $U$ gating involved an inactivated state. Chapter 5 also presents evidence that $K^+$ antagonizes the $H^+$-induced current inhibition by promoting mode $A$ gating. Together, the results support the hypothesis that the external $H^+$-induced current inhibition resulted from promotion of an inactivation process from one or more closed states. A discussion of all the experimental results and the implication of these findings for our view of the mechanistic basis for slow inactivation in Kv1.5 and in other channels is provided in the concluding chapter.
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2. Molecular determinants of the inhibition of human Kv1.5 potassium currents by external protons and Zn$^{2+}$

2.1 Introduction

We have previously shown in human Kv1.5 ($h$Kv1.5) 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., 2001b). Although a gating shift is very often associated with a change of the external concentration of divalent cations, as first detailed by Frankenhaeuser and 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 if therefore of some interest. In a follow-up study of the effects of Zn$^{2+}$ on gating currents (Zhang et al., 2001a), 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; Cha and Bezanilla, 1998; Li-Smerin et al., 2000; Loots and Isacoff, 1998) and with the view that the voltage sensing domain wraps around the outer rim, i.e., the turret of the pore (Loots and 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 and Auld, 1990). In the six transmembrane segment (6TM) \(\alpha\)-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 and 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 and Elinder, 2000). Two aspartate residues are also found in the outer pore mouth of hKv1.5, D469 in the outer pore helix and D485 which 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\(^{+}\)) (Steidl and Yool, 1999) has features similar that of the Zn\(^{2+}\) block of hKv1.5. Although not evident with Zn\(^{2+}\) during 300 ms pulses (Zhang et al., 2001b), an acceleration by H\(^{+}\) 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 and 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 (Kiss et al., 1999; Loots and Isacoff, 2000; Olcese et al., 1997; Wang and Fedida, 2001; Yang et al., 1997). 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 and 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 a 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 and Yool, 1999).

Against this background the experiments described here had two major goals. First, 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⁺. After confirming a K⁺-sensitive inhibition of hKv1.5 currents by H⁺, 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 Zn^{2+} 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_{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.

2.2 Materials and Methods

2.2.1 Cell preparation

Wild type (wt) hKv1.5 channels were studied in a human embryonic kidney cell line (HEK-293) 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% CO₂ in air. All tissue culture supplies were obtained from Invitrogen (Burlington, ON, Canada).

Point mutations of the wt hKv1.5 α-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 Clal restriction enzymes (New England BioLabs, Beverly, MA, USA). Stable transfections of HEK-293 cells were made using 0.8 µg 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.

2.2.2 Recording solutions

The standard bath 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. Where the effect of the external concentration of potassium (K⁺) on the proton block was examined, zero K⁺ solution was made by substituting NaCl for KCl and, for K⁺ 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⁺) or for both KCl and NaCl (20 mM and 140 mM Cs⁺). In experiments addressing the effect of Na⁺ on the current inhibition, N-methyl-D-glucamine (NMG⁺) replaced Na⁺ and the pH was adjusted with HCl. The external concentration of H⁺ (H⁺₀) 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. Zn²⁺-containing test solutions were made by the addition of ZnCl₂ from 0.1 or 1 M stock solutions. The low solubility of Zn(OH)₂ limits the maximum concentration of Zn²⁺ that can be used at pH 7.4 to less than 5 mM. Our standard patch pipette solution for recording K⁺ currents contained 130 KCl, 4.75 CaCl₂ (pCa²⁺ = 7.3), 1.38 MgCl₂, 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 MgCl₂, 10 HEPES (pH 7.4) or MES (pH 5.4), 2 CaCl₂, 10 glucose and the pH was adjusted with HCl; the patch pipette solution consisted of 140 NMGCl, 1 MgCl₂, 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).

2.2.3 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:

\[ y = \frac{A}{1 + \exp\left(\frac{V_y - V}{s}\right)} \quad (2.1) \]

where, when \( y \) is the current normalized with respect to the control response, \( A \) is the proportion of the control \( g_{\text{max}} \). 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. \( V_y \) 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 and Fedida, 1999). However, for simplicity, \( Q(V) \) data obtained at pH 7.4 and 5.4 were fitted to Equation (2.1) where \( y \) is the charge moved, \( A \) is the maximal charge \( (Q_{\text{max}}) \) and \( V \) is the voltage at which the on-gating charge \( (Q_{\text{on}}) \) or off-gating charge \( (Q_{\text{off}}) \) is evoked. \( V_y \) and \( s \) remain as described above.

Concentration-response data for Zn\(^{2+}\) were fitted to the Hill equation:
where \( y \) is the proportion of the control \( g_{\text{med}} \), \( K_{Zn} \) is the equilibrium dissociation constant for Zn\(^{2+}\) binding and \( n_H \) is the Hill coefficient reflecting the number of Zn\(^{2+}\) ions binding per channel. For protons, Equation (2.2) was modified to account for the fact that the data points were normalized to the response at pH 7.4:

\[
y = \frac{K_H^{-n_H} + (10^{-7.4})^{n_H}}{K_H^{-n_H} + [H^+]_o^{n_H}}
\]  

(2.3)

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., 2001b) 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.

\[
y = \frac{1}{1 + \left( \frac{[H^+]}{K_H} \right) \left( \frac{1 + \frac{[K^+]_o}{K_K}}{1 + \frac{[K^+]_o}{K_K}} \right)^{n_{\text{H}K}}}
\]

(2.4)

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 $Zn^{2+}$ on the inactivation rate. A $p$-value of 0.05 or less was considered significant.

2.3 Results

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

Representative traces in Figure 2.1 show the effect of changing pH$_o$ from 7.4 to pH 6.4 in nominally $K^+$-free medium (zero K$_o^+$) in which Na$^+$ was the major extracellular cation. For the control currents the voltage protocol consisted of a 300 ms pulse command to 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 2.1 A) are consistent with a failure of hKv1.5 currents to disappear or “collapse” in zero K$_o^+$ (Jäger and 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\(^{2+}\) (Zhang et al., 2001b). 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 2.1 C, which was derived in part from the traces shown in Figure 2.1 A and B, plots the tail current amplitudes at -50 mV measured as described in Materials and Methods and fitted to a Boltzmann function. In this cell, increasing H\(^{+}\) caused \(V_h\) 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.

External protons have been reported to reduce the amplitude of rKv1.5 currents (Steidl and 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\(^{+}\)\(_o\), 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.

2.3.2 Increasing [K\(^{+}\)]\(_o\) inhibits the reduction of \(g_{\text{max}}\) 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 circles).
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 \( V_c \) 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.

That \( K^+_{o} \) inhibits the proton-induced current reduction is shown by the increase of the relative \( g_{\text{max}} \) from 0.19 ± 0.02 \((n = 12)\) in zero \( K^+_{o} \) to 0.56 ± 0.01 \((n = 6)\) in 3.5 mM \( K^+_{o} \) and finally to 0.81 ± 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 \( V_c \) and \( s \) values) suggesting that the proton-induced gating shift and current inhibition are independent effects.
Figure 2.2 Increasing $K^+_o$ reduces the inhibition of $h$Kv1.5 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 $K^+_o$. In zero $K^+_o$, control and treated pulse currents were evoked by 300-ms pulses from -50 to 45 mV in 5 mV steps; in 3.5 mM $K^+_o$, 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 $K^+_o$, 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 $g_{\text{max}}$ at pH 6.4 (■) and to the control $g_{\text{max}}$ (●). The relative $g_{\text{max}}$ at pH 6.4 in zero, 3.5 and 140 mM $K^+_o$ was $0.19 \pm 0.02 \ (n = 12)$, $0.56 \pm 0.01 \ (n = 6)$, and $0.81 \pm 0.12 \ (n = 6)$, respectively. In zero $K^+_o$, $V_{1/2}$ and $s$ changed from $-21.4 \pm 4.3$ and $4.7 \pm 0.5$ mV at pH 7.4 to $-8.2 \pm 4.0$ and $7.1 \pm 0.3$ mV at pH 6.4, respectively. In 3.5 mM $K^+_o$, the corresponding values were $-18.3 \pm 1.9$ mV and $3.9 \pm 0.4$ mV at pH 7.4 and $-10.5 \pm 1.2$ mV and $3.9 \pm 0.4$ mV at pH 6.4; and, in 140 mM $K^+_o$, $-26.2 \pm 1.1$ mV and $3.8 \pm 0.3$ mV at pH 7.4 and $-12.3 \pm 1.1$ mV and $3.8 \pm 0.4$ mV at pH 6.4.
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\text{\textsuperscript{o}} medium in which Na\text{\textsuperscript{+}} was the predominant metal cation (\textit{filled circles}) the best fit to the data gave a $K_H$ of 153 ± 13 nM ($pK_H \sim 6.8$) and a Hill coefficient, $n_H$, of 1.5 ± 0.2 which suggests that inhibition requires protonation of at least two sites. To determine if Na\text{\textsuperscript{+}} ions affect the current inhibition by protons, the zero K\text{\textsuperscript{o}} experiments were also done with NMG\text{\textsuperscript{+}} as the major extracellular cation. With NMG\text{\textsuperscript{+}}, the $K_H$ was 128 ± 53 nM ($pK_H \sim 6.9$) and $n_H$ was 1.2 ± 0.5 (\textit{open circles} and \textit{dashed line} of Figure 2.3). This suggests that external Na\text{\textsuperscript{+}} ions do not affect the current inhibition by protons. With 5 mM K\text{\textsuperscript{o}}, the $K_H$ increased to 590 ± 85 nM ($pK_H \sim 6.2$), but the value for $n_H$ of 1.6 ± 0.4 was not significantly different from that with zero K\text{\textsuperscript{o}}. In comparison to the substantial rightward shift caused by increasing K\text{\textsuperscript{o}} from zero to 5 mM, a much smaller increase of the $K_H$ to 1.1 ± 0.11 $\mu$m ($pK_H \sim 6$) was obtained when K\text{\textsuperscript{o}} was increased from 5 to 140 mM. The $n_H$ in 140 mM K\text{\textsuperscript{o}} was 1.8 ± 0.3.

### 2.3.3 K\text{\textsuperscript{o}} relief of the effect of protons is fitted by a model of non-competitive inhibition

As noted with Zn\textsuperscript{2+} block of hKv1.5 channels (Zhang \textit{et al.}, 2001b), the greater relief of the proton-induced current inhibition when K\text{\textsuperscript{o}} was changed from zero to 5 mM K\text{\textsuperscript{o}} compared to when it was changed from 5 to 140 mM K\text{\textsuperscript{o}}, suggested that K\text{\textsuperscript{o}} ions and protons were not competing for a common site. For that reason we modelled the interaction between K\text{\textsuperscript{o}} and H\text{\textsuperscript{o}} as an allosteric inhibition (Equation (2.4)), 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\text{\textsuperscript{o}} concentrations of zero, 1, 3.5, 5, 10, 20, 80 and 140 mM. The fit of these data to
Figure 2.3 The concentration dependence of the inhibition of Kv1.5 currents by protons in zero (●,○), 5 (■) and 140 mM (▲) $K^+_o$. Data for zero $K^+_o$ 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 (2.3). The fitted values for the equilibrium dissociation constant for protons ($K_H$), the $pK_H$ and $n_H$ were, in zero $K^+_o$ and 143.5 NMG$^+$: 128 ± 53 nM (mean ± SD), 6.9 and 1.2 ± 0.5; in zero $K^+_o$ and 143.5 mM Na$^+$: 153 ± 13 nM, 6.8 and 1.5 ± 0.2; in 5 mM $K^+_o$: 590 ± 85 nM, 6.2 and 1.6 ± 0.4; and in 140 mM $K^+_o$: 1.1 ± .11 µM, 6.0, and 1.8 ± 0.3. Although the $pK_H$ 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 $K^+_o$ (see Figure 2.4) the increase of $K_H$ going from zero to 5 mM $K^+_o$ was greater than that going from 5 mM to 140 mM.

Equation (2.4) gave mean values (± SD) of 150 ± 1900 nM for $K_H$, 1.33 ± 17 for $n_H$, 0.68 ± 9 for $K_x$ 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 $K_x$ and $\alpha$, we fixed the values for $K_H$ and $n_H$ 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 $n_H$ 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 $n_H$ fixed, the fit of the data at pH 6.4 gave estimates for $K_x$ and $\alpha$ of 0.65 ± 0.27 mM and 5.5 ± 0.7, respectively.
Figure 2.4 The concentration dependence of the antagonism by $K_{\text{in}}^+$ and $Cs_{\text{in}}^+$ of the inhibition of $h$Kv1.5 currents by $H_{\text{in}}^+$. The relative $g_{\text{max}}$ at different $K_{\text{in}}^+$ 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 $K_{\text{in}}^+$. At pH 6.4, $K_{\text{in}}^+$ was zero, 1, 3.5, 5, 10, 20, 80 and 140 mM. Assessment of the block-relieving effect of $Cs_{\text{in}}^+$ (▼) was done with concentrations of 3.5, 20 and 140 mM. The lines represent the best fit of the data to equation 2.4 (see Materials and Methods). With the values for $K_H$ and $n_H$ 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 $0.65 \pm 0.27$ mM and $5.5 \pm 0.7$. $Cs_{\text{in}}^+$ appears to be equivalent to $K_{\text{in}}^+$ in its antagonism of the proton block. The best fit of the data at pH 6.9 was obtained with $0.93 \pm 2.8$ mM for $K_K$ and $6.2 \pm 9.1$ for $\alpha$; at pH 5.9 the corresponding values were $0.66 \pm 0.48$ mM and $6.2 \pm 1$. These estimates for $K_K$ are very near those estimated for the $K_{\text{in}}^+$ relief of the Zn$^{2+}$ block (∼0.5 mM) (Zhang et al., 2001b).

At pH 6.9 and pH 5.9 the relative $g_{\text{max}}$ was measured with zero, 5, 20 and 140 mM $K_{\text{in}}^+$.

For the data at pH 5.9 the best fit values for $K_K$ and $\alpha$, with $K_H$ and $n_H$ constrained as above, were $0.66 \pm 0.48$ mM and $6.2 \pm 1$, respectively; at pH 6.9 the corresponding values were $0.93 \pm 2.8$ mM and $6.2 \pm 9.1$.

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

In $h$Kv1.5 channels the permeability of $Cs^+$ ions relative to $K^+$ ions is approximately 0.2
(Fedida et al., 1999) and the $K_f$ for the relief by $Cs^+$ ($K_{(Cs)}$) of the $Zn^{2+}$ block is some 5-6 fold higher than the $K_f$ (Zhang et al., 2001b). 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 $Cs^+$ to antagonise the current inhibition by protons was indistinguishable from that of $K^+$.  

### 2.3.5 Sensitivity to $H^+$ and $Zn^{2+}$ inhibition is reduced in $hKv1.5 H463Q$

The range of $pK_H$ 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 and 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 2.6 A) and concentration-response curves (Figure 2.6 B) 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 2.6 B) to the Hill equation
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.

gave an estimate for $K_H$ of $4.7 \pm 1.9$ μM ($pK_H = 5.3$) and an $n_H$ of $1 \pm 0.4$ versus the corresponding values of $0.15$ μM ($pK_H = 6.8$) 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 $-5.2$ (Steidl and Yool, 1999).

Tests of the effects of Zn$^{2+}$ on the H463Q mutant showed that the outcome (Figure 2.6 C and 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 $1.7 \pm 1$ mM or roughly 25-fold higher than for wt hKv1.5 (Zhang et al., 2001b). The $n_H$ for the inhibition by Zn$^{2+}$ of wt hKv1.5 and hKv1.5 H463Q currents was $0.9$ (Zhang et al., 2001b) and $0.5$ (Figure 2.6), respectively.

In the course of this series of experiments we became aware of a report that currents
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, 1.0 ± 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 $n_H$ 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 (□), 50 μM (■), 200 μM (▲), 1 mM (▲) and 2.5 mM (▲) of $Zn^{2+}$. The relative $g_{\text{max}}$, $V_{1/2}$, and $s$ were 1.0 ± 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 50 μM $Zn^{2+}$ ($n = 5$); 0.77 ± 0.09, 7.1 ± 1.1 mV, and 5.9 ± 0.2 mV for 200 μM $Zn^{2+}$ ($n = 6$); 0.47 ± 0.04, 18.5 ± 2.0 mV, and 6.0 ± 0.7 mV for 1 mM $Zn^{2+}$ ($n = 10$); and 0.52 ± 0.009, 27.2 ± 2.3 mV, and 6.4 ± 0.8 mV for 2.5 mM $Zn^{2+}$ ($n = 5$). D. As described for (B) but with $Zn^{2+}$. The best fit values for $K_{Zn}$ and $n_H$ 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, $n_H = 0.9$) (Zhang et al., 2001b).
through hKv1.5 H463G channels were completely suppressed upon changing from 4.5 mM to zero K\textsubscript{o} medium at pH 7.4 (Jäger and 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 and 2.7 A). Our experiments with hKv1.5 H463G confirmed this conductance collapse in zero K\textsubscript{o} at pH 7.4 (Figure 2.7 C), and we also noted that there was a striking increase in the inactivation rate in 3.5 K\textsuperscript{+} (Figure 2.7 B) that was not previously reported. Thus, in contrast to wt hKv1.5 (Figure 2.1) and hKv1.5 H463Q (Figure 2.7 A) 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 2.7 B).

![Figure 2.7 In hKv1.5 H463G slow inactivation is greatly accelerated and the conductance collapses in zero K\textsubscript{o} at pH 7.4. A. Shown for comparison are the currents from hKv1.5 H463Q evoked in zero K\textsubscript{o} 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\textsubscript{o} at pH 7.4. Unlike either wt Kv1.5 H463 (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).]
2.3.6 $H^+\mathrm{_{o}}$ and $Zn^{2+\mathrm{_{o}}}$ accelerate inactivation

In $rKv1.5$, $H^+\mathrm{_{o}}$ has been shown to accelerate inactivation, an effect that was evident only with long depolarizing commands (Steidl and 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 $H^+\mathrm{_{o}}$ as well as $Zn^{2+\mathrm{_{o}}}$ 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$^+$ 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 $Zn^{2+\mathrm{_{o}}}$-free medium at pH 7.4 to medium at the same pH and containing 1 mM $Zn^{2+\mathrm{_{o}}}$ 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 $H^+\mathrm{_{o}}$ and $Zn^{2+\mathrm{_{o}}}$ is associated with a moderately increased rate of inactivation we suggest below that this cannot account for the reduction of $g_{\text{max}}$.

2.3.7 Current inhibition by protons and $Zn^{2+\mathrm{_{o}}}$ 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 $H^+\mathrm{_{o}}$ and $Zn^{2+\mathrm{_{o}}}$ 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 (ShakerIR) 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 were 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⁺ (Jäger and Grissmer, 2001).

Figure 2.8 summarizes the results of experiments assessing the inhibition of hKv1.5 R487V by protons and Zn²⁺ ions in zero K⁺ (143.5 mM Na⁺) medium. The g(V) relationships derived from tail current measurements (Figure 2.8 A) 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⁺-induced conductance decline. For example, whereas in wt hKv1.5 the relative gₘₐₓ at pH 5.9 was 0.07 ± 0.01 (n = 9; Figure 2.4), in hKv1.5 R487V the relative gₘₐₓ at the same pH was 0.92 ± 0.03 (n = 5; Figure 2.8 A and B). An extrapolated pKₜₜ of 4.6 obtained from the best fit of the concentration-response data of Figure 2.8 B suggests a shift of ~2 pH units from the pKₜₜ of wt hKv1.5 channels.

Tests of the sensitivity of hKv1.5 R487V channels to Zn²⁺ (Figure 2.8 C and D) showed that the gating shift was, again, substantially unaffected and, as with H⁺, there was a clear increase of the Zn²⁺ concentration required to cause 50% inhibition. Thus, whereas wt hKv1.5 currents were half-inhibited by 0.07 mM Zn²⁺ (Zhang et al., 2001b), in the R487V mutant 41.2 ± 1.7% (n = 5) of gₘₐₓ persisted in 2.5 mM Zn²⁺. Closer inspection of the concentration-response data of Figure 2.8 D suggested that two Zn²⁺ 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 2.8 D) implied that the higher affinity site (K_Zn = 29 µM) which accounted for approximately 20% of conductance 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 Zn²⁺ to one or more
Figure 2.8 A mutation near the pore mouth, R487V, substantially reduces the sensitivity to inhibition by H⁺ and Zn²⁺. 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_max at pH 7.4. The values for the relative g_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, -18.1 ± 0.9 mV, 4.5 ± 0.2 mV at pH 7.4 (n = 17); 1.04 ± 0.06, -1.8 ± 1.3 mV, 5.6 ± 0.4 mV at pH 6.4 (n = 5); 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_max by protons. The continuous line, representing the best fit of the data to the Hill equation, was obtained with K_H = 23 μM (pK_H of 4.6) and n_H = 0.8. C. The g(V) relationship in zero K⁺ and with Zn²⁺ concentrations of 10 μM (●), 25 μM (●), 100 μM (○), 200 μM (▼), 1 mM (■), and 2.5 mM (▲) after normalization with respect to the control (○) g_max. The relative g_max, V_{1/2}, and s were, respectively, 1, -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 10 μM Zn²⁺ (n = 4); 0.92 ± 0.05, -5.7 ± 0.1 mV, 4.7 ± 0.5 mV in 25 μM Zn²⁺ (n = 3); 0.80 ± 0.02, 2.8 ± 1.8 mV, 4.8 ± 0.5 mV in 100 μM Zn²⁺ (n = 4); 0.78 ± 0.02, 5.2 ± 1.6 mV, 5.3 ± 0.3 mV in 200 μM Zn²⁺ (n = 5); 0.70 ± 0.05, 21.0 ± 1.2 mV, 5.9 ± 0.3 mV in 1 mM Zn²⁺ (n = 3); and, 0.59 ± 0.02, 28.9 ± 1.1 mV, 5.9 ± 0.3 mV in 2.5 mM Zn²⁺ (n = 5). D. As described for (B) but with Zn²⁺. 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_{2H} = 29 ± 0.2 μM) 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 Zn²⁺ binding to H463. The extrapolated K_{2H} 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., 2001b).
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.

2.3.8 Current inhibition by $H^+_o$ and $Zn^{2+}_o$ is apparently not use-dependent

If, as has been proposed to account for the block of $rKv1.5$ currents by $H^+_o$ (Steidl and 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 Zn²⁺, 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.

2.3.9 Protons cause a depolarizing shift of the Q(V) relationships but do not affect Q_max

Because gating currents can provide useful evidence on the conformational states available to a channel, we recorded gating currents in a stable HEK-293 cell line expressing hKv1.5 W472F mutant channels (Chen et al., 1997). This mutant is analogous to the Shaker 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 2.10 A-D. To prevent contamination of gating currents by endogenous HEK-293 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 $Q_{off}$ that is reduced relative to $Q_{on}$. This decrease of $Q_{off}/Q_{on}$ 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 (Baukrowitz and Yellen, 1995; Lopez-Barneo et al., 1993). 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 and Fedida, 2001; see Figure 2.10 E).

Figure 2.10 B 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
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 ($\bigcirc$) and 5.4 ($\bullet$), 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 ($\bigtriangleup$) and pH 5.4 ($\bigstar$) 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 $Q_{off}$ relative to $Q_{on}(V)$.
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 on activation gating, the on-gating currents in Figure 2.10 A and B were integrated to obtain the voltage dependence of on-gating charge movement shown in Figure 2.10 E. 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, $V_0 = -2.2$ mV and $s = 6.5$ mV. At pH 5.4 (filled circles of Figure 2.10 E), $Q_{max}$, $V_0$, and $s$ were +2.5 pC, 50.2 mV and 11.8 mV. In the six cells examined, $V_o$ was $4.3 \pm 2.2$ mV at pH 7.4 and $48.9 \pm 1.2$ mV at pH 5.4; $s$ increased from $7.1 \pm 0.5$ mV at pH 7.4 to $10.5 \pm 0.4$ mV at pH 5.4; and, the relative $Q_{max}$ ($Q_{max, pH5.4}/Q_{max, pH7.4}$) was $1.0 \pm 0.003$. Thus, changing pH from 7.4 to 5.4 caused a -45 mV rightward shift of the $V_o$ 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., 2001a) and interestingly, as with Zn$^{2+}$, the shift of $V_o$ of the $Q_{on}(V)$ relationship is roughly twice that measured for the $g(V)$ curve. For example, at pH 5.9 the $V_o$ 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 2.10 B 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 2.10 E 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 \((V_s = -100.5 \text{ mV})\) by \(-100 \text{ mV}\) relative to the \(Q_{on}(V)\) curve. Of particular importance is that a similar effect is seen at pH 5.4 where the \(V_s\) of the \(Q_{off}(V)\) curve was \(-72.9 \text{ mV}\), representing a leftward shift of \(-124 \text{ mV}\) from the \(V_s\) of the \(Q_{on}(V)\) relationship.

The values, respectively, for \(V_s\) and \(s\) of the \(Q_{off}(V)\) relationship in 3 such experiments were, at pH 7.4, \(-102.8 \pm 1.4 \text{ mV}\) and \(11.4 \pm 1.0 \text{ mV}\) and, at pH 5.4, \(-75.3 \pm 1.4 \text{ mV}\) and \(14.8 \pm 1.1 \text{ mV}\). Thus, at pH 7.4, there was, following a depolarization that moved \(Q_{max}\), a \(-107 \text{ mV}\) leftward shift of the voltage dependence of return gating charge movement. A comparable leftward shift of \(-124 \text{ mV}\) of the voltage dependence of gating charge movement was seen at pH 5.4.

2.4 Discussion

The first series of experiments (Figures 2.1 - 2.4) described in this paper show that, as with \(\text{Zn}^{2+}\) ions, external protons cause a concentration-dependent and reversible inhibition of \(h\text{Kv1.5}\) 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 \(r\text{Kv1.5}\) channels (Steidl and Yool, 1999) but we have extended the previous work by showing that external ions such as \(\text{K}^+\) and \(\text{Cs}^+\), but not \(\text{Na}^+\), are able to relieve the inhibition but not the gating shift caused by protons. In zero \(\text{K}^+\), the apparent \(pK_H\) of the protonation site is 6.8 and this decreases to 6.2 with 5 mM \(\text{K}^+\). The latter \(pK_H\) accords well with the \(pK_H\) of 6.2 for \(r\text{Kv1.5}\) responses recorded in 2 mM \(\text{K}^+\) (Steidl and Yool, 1999) and the \(pK_H\) of 6.1 in N-terminal deleted ferret \(\text{Kv1.4}\) with 3 mM \(\text{K}^+\) (Claydon et al., 2000). The influence of \(\text{K}^+\) on this inhibition of \(h\text{Kv1.5}\) currents was modelled as a non-competitive interaction between \(\text{K}^+\) and protons (Figure 2.4) and the estimated \(K_D\) for this antagonism by \(\text{K}^+\) is very near that estimated.
for the Zn\(^{2+}\) block (Zhang et al., 2001b), 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 (\(K_D \approx 0.75\) mM) at which K\(^+\) binds to lock Ba\(^{2+}\) ions within the pore of ShakerB 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 and Yellen, 1996). Interestingly, the external lock-in site of ShakerB channels and the site at which K\(^+\) binds to antagonise the inhibitory actions of Zn\(^{2+}\) or H\(^+\) 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., 2001b), 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, 2001). 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 \(n_H\) 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 \(n_H\) for Zn\(^{2+}\) block is near unity (Zhang et al., 2001b), this might still involve coordinated binding of histidine residues of two or more subunits.
2.4.1 Evidence against a pore blocking mechanism

The block of cardiac voltage-gated Na⁺ (Naᵥ) channels by Zn²⁺ 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 Zn²⁺ 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 Zn²⁺ (Zhang et al., 2001b) or H⁺, e.g. Figure 2.1 C, 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 Zn²⁺ and H⁺ bind to histidine residues, and we have shown directly that the H463Q substitution shifts the pK₉ measured in zero K⁺ 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 (pK₉ ~5.4) (Perez-Cornejo et al., 1998) and Kv1.2 channels (pK₉ ~4.9) (Ishii et al., 2001). Exactly where protons and Zn²⁺ act in these mutants is not known, but given their typical pK₉ 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 Zn²⁺ 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 Zn²⁺ and H⁺ 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?

2.4.2 A connection between current inhibition and an inactivation process

Though it is clear from this study and that of rKv1.5 currents (Steidl and Yool, 1999) that
inactivation is faster at acidic pHs, our simulation studies (not shown) indicate that this increased
rate of inactivation cannot itself account for the reduction of $g_{\text{max}}$. Furthermore, although
increasing $K^+_o$ can speed recovery from C-type inactivation of Kv1.3 currents (Levy and 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) which 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 ShakerIR 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

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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 $H^+_o$ is substantially reduced by increasing $K^+_o$ 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 about the role of C-type inactivation was provided by gating current results (Figure 2.10).

2.4.3 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 $Q_{max}$. This is a second major distinction between the actions of $H^+_o$ and $Zn^{2+}$. At a concentration that reduces $g_{max}$ by more than 90%, $Zn^{2+}$ decreases $Q_{max}$ by 10-15% as though it were preventing the late, weakly voltage-dependent transitions in the activation pathway (Zhang et al., 2001a). However, even though $Q_{max}$ 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., 2001a), the proton-induced depolarizing shift of $V_o$ for the $Q_{on}(V)$ 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 and 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.

2.4.4 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 and 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 ShakerIR 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 ShakerIR 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 $\text{TEA}^+$ and where there was also an enhancement of FWFW current when $K^+\_o$ increased. In a similar manner, in $h\text{Kv1.5}$ 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, $\alpha$, of equation (2.4) 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 vice versa.

This proposed scheme, which remains to be tested by single channel analysis, is at least functionally equivalent to closed-state inactivation proposed to account for the loss of current in ShakerIR T449 mutants (Lopez-Barneo et al., 1993), to the decrease of channel availability ($N$) 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 $h\text{Kv1.5}$ (Wang et al., 2000) channels.

2.4.5 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 and Grissmer, 2001). However, a number of our observations argue against such an electrostatic interaction. First, a strong, 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 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 Zn$^{2+}$ 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 $pK_H$ of R487 by $\sim 6$ 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 Zn$^{2+}$ (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 and 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$_{o}$.

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$_{o}$ (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 and Korn, 2000).

2.4.6 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 $h$Kv1.5 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 $wt$ $h$Kv1.5 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 $wt$ $h$Kv1.5 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 $wt$ $h$Kv1.5 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 $h$Kv1.5 H463Q where the substituted glutamine is uncharged, but polar, and occupies only a slightly smaller volume than histidine (~150 Å³). In contrast, in the H463G mutant the uncharged but much smaller glycine residue (~60 Å³) 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 and 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.

Acknowledgements

Supported by a grant to S.J.K. from the Natural Sciences and Engineering Research Council and by grants to D.F. from the Canadian Institutes for Health Research and the Heart and Stroke Foundation (HSF) of British Columbia and Yukon. S.Z. was in receipt of a Research Fellowship from the HSF of Canada and D.C.H.K. was supported by a Trainee Award from the Michael Smith Foundation for Health Research. We thank Qin Wang who prepared the cells and Dr. Simon Baudrexel who assisted with some of the experiments.
2.5 References


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3. The external \( K^+ \) concentration and mutations in the outer pore mouth affect the inhibition of Kv1.5 current by \( \text{Ni}^{2+} \)

3.1 Introduction

Kv1.5 (KCNA5) channels, which are expressed in cardiac myocytes (Fedida \textit{et al.}, 1993; Tamkun \textit{et al.}, 1991) and in smooth muscle cells of airways, the intestine and the vasculature (Adda \textit{et al.}, 1996; Clement-Chomienne \textit{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 \textit{et al.}, 1998; Larsson \textit{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 \textit{et al.}, 1999) and in this regard are different from \textit{Shaker} channels which also show inner pore (N-type) inactivation (Hoshi \textit{et al.}, 1991). The term C-type inactivation was coined to describe the slow inactivation process in \textit{Shaker} that was uncovered when ball-and-chain or N-type inactivation was removed (\textit{ShakerIR}) 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 ShakerIR (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 ShakerIR 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 Kv1.5 channels the residue homologous to T449 is R487 and it has been shown that inactivation is substantially slowed in Kv1.5 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 ShakerIR/Kv1.5 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 ShakerIR 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 ShakerIR W434F conductance loss (Yang et al., 2002).

An intriguing divergence in the structure-function relationships of Kv1.5 and ShakerIR is seen in the response to extracellular acidification. In Kv1.5 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 (Kehl et al., 2002; Steidl and Yool, 1999). In contrast, in ShakerIR channels increasing [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 H^+\_o block (Kehl et al., 2002). The finding that the H^+\_o block is antagonized by K^+_o and is also reduced in the R487V mutant (Jäger and 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 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 H^+\_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 Zn^{2+} blocks Kv1.5 currents and, as with the H^+\_o block, this effect of Zn^{2+} is inhibited either by increasing K^+_o or by mutating
H463 and/or R487 (Kehl et al., 2002). 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 and 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 $[K^{+}]_{o}$? 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$^{2+}$?

3.2 Materials and Methods

3.2.1 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 (HEK-293) (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 $\alpha$-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 the arginine (R)) at position 487 to valine (R487V). Stable transfections of HEK-293 cells were made using 0.8 $\mu$g 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.

3.2.2 Recording solutions

The standard bathing 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. 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 Ni$^{2+}$ block. Where the effect of the external concentration of potassium ([K$^+$]$_o$) on the divalent metal cation block was examined, a nominally K$^+$-free solution was made by substituting NaCl for KCl and, for [K$^+$]$_o$ greater than 3.5 mM, NaCl was replaced by KCl. The standard patch pipette solution for recording K$^+$ currents contained 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. 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 Ni$^{2+}$ that can be used was limited to 10 mM or less by virtue of the solubility product for Ni(OH)$_2$ ($\approx 2 \times 10^{-16}$).

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 Ni$^{2+}$.

For gating current recordings the bath solution contained, in mM, 140 NMGCl, 1 MgCl$_2$, 10 HEPES, 2 CaCl$_2$, 10 glucose and the pH was adjusted to 7.4 with HCl. The patch pipette
solution contained 140 NMGC1, 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).

3.2.3 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:

$$ y = \frac{A}{1 + \exp \left( \frac{V_{h} - V}{s} \right)} $$

(3.1)

where, when $y$ is the current normalized with respect to the control response, $A$ is the proportion
of the control $g_{\text{max}}$. 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. $V_{h}$ 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 ($Q_{\text{on}}-V$) 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 and
Fedida, 1999). However, for simplicity, $Q-V$ data obtained at pH 7.4 and 5.4 were fitted to
Equation (3.1) where $y$ is the charge moved, $A$ is the maximal charge ($Q_{\text{max}}$) and $V$ is the voltage
at which the on-gating charge ($Q_{\text{on}}$) is evoked. $V_{h}$ 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 ± SEM 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 \( \text{Ni}^{2+} \) and \( \text{H}^+ \). A \( p \)-value of 0.05 or less was considered significant.

3.3 Results

Shown in Figure 3.1 A are traces confirming the block of \( \text{Kv}1.5 \) currents by external \( \text{Ni}^{2+} \). From a holding potential of -80 mV and with 0 mM \( \text{K}^+ \), 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 and Clement-Chomienne, 2001), and in contrast to the effects with Zn$^{2+}$ (Zhang et al., 2001b), 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).

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 $g_{\text{max}}$ 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 analysed.
3.3.1 Increasing $[K^+]_o$ 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 $K^+_o$ 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 ($V_s$) 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 $V_s$ 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 $V_s$ was quite large.

Figure 3.2 shows the concentration-response relationship for the block of Kv1.5 by Ni$^{2+}$ and the influence of $[K^+]_o$ thereon. Panel A illustrates representative current traces from 3 different cells in 0 mM (left), 3.5 mM (middle) and 140 mM (right) $K^+_o$. In the absence of Ni$^{2+}$ (-Ni$^{2+}$) the current in each of the $K^+_o$ concentrations had a similarly slow rate of decay. The inward tail current recorded at -40 mV in 140 mM $K^+_o$ is due to the shift of $E_K$ to ~0 mV. To produce a similar degree of block in the three different experiments it was necessary to increase the Ni$^{2+}$ concentration to offset the effect of increasing $[K^+]_o$. Note that in each example the Ni$^{2+}$ 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 $K^+_{o}$, 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 3.2 B, the $g_{max}$ relative to the control value has been plotted against the concentration of Ni$^{2+}$ for experiments in which $[K^+]_o$ 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 Equation 3.2. With 0 mM $K^+_o$ the $K_D$ for the Ni$^{2+}$ block was $0.15 \pm 0.01$ mM and $n_H$ was $1.3 \pm 0.1$. Increasing $[K^+]_o$ to 3.5 mM increased the $K_D$ to $0.44 \pm 0.02$ mM and $n_H$ was $1.6 \pm 0.2$. With 140 mM $K^+_o$ the $K_D$ was $3.1 \pm 0.3$ mM and $n_H$ was $0.9 \pm 0.1$. These results clearly demonstrate that, as with the block by H$^+_o$ and Zn$^{2+}$, the block of Kv1.5 by Ni$^{2+}$ is antagonized by increasing $[K^+]_o$.

### 3.3.2 The time courses of the onset and the offset of the Ni$^{2+}$ block are similar

Using a fast solution application system, Perchenet and 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 $K^+_o$, that steady-state block was reached only after 5 to 7 minutes. Since a similar phenomenon is not seen with H$^+_o$ 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$^+_o$.

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 3.3 A, Ni$^{2+}$ and H$^+_o$ were applied for the duration indicated by the horizontal bar at concentrations of 150 $\mu$M and 0.16 $\mu$M (pH 6.8) (Kehl et al., 2002), respectively, and in 0 mM $K^+_o$ to cause roughly 50% block of the current at the
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 $K^+$ 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 $K^+$ shows that increasing $[K^+]_o$ from nominally $K^+$-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 ± SEM of measurements from 3-7 cells.

steady state. In four such experiments we consistently found that the time courses for the onset and offset of the block by Ni$^{2+}$ and $H^+_o$ were similar. Since the failure to uncover any asymmetry in the on- and off- time courses might be attributed to the absence of $K^+_o$, 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 3.3 B 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 and Clement-Chomienne (2001).

3.3.3 $Ni^{2+}$ 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 and 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

![Figure 3.3](image-url)
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 3.4 A and 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 ($n = 5$ cells). In Figure 3.4 A 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 ± 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 3.4 B) 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 ± 0.07 s and 23.5 ± 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).

Figure 3.4 Inactivation and recovery kinetics of Kv1.5 at pH 5.4 or with 2 mM Ni\(^{2+}\). A comparison of the residual current behaviour, done in 3.5 mM K\(^+\) with a concentration of H\(^+\)\(_o\) (A) or Ni\(^{2+}\) (B) 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 50 mV 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 \(\approx\) 20 times slower (\( \tau_{\text{inact}} = 1.69 \text{s} \)) and recovery from inactivation was \(\approx\) 5 times slower (\( \tau_{\text{recovery}} = 24.8 \text{s} \)) than at pH 5.4.
3.3.4 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 estimated to be 2.8 ± 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 ± 8 mM which is 100- to 200-fold higher than in wt Kv1.5.

3.3.5 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 ($P_o$). 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 3.6 A 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 and 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
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 (filled squares) was 100- to 200-fold less sensitive to Ni$^{2+}$ ($K_D = 24 \pm 8$ mM; $n_H = 0.4 \pm 0.04$) 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 (filled circles) to Ni$^{2+}$ was approximately 20-fold less ($K_D = 2.8 \pm 0.004$ mM; $n_H = 0.7 \pm 0.001$) 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$_o^+$.

which are assumed to reflect a multistep inactivation pathway. Double-Gaussian fits to the control all-points amplitude histogram (e.g., Figure 3.6 C) 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$_o^+$ (Figure 3.2), there was no significant change of the single channel current (e.g., Figure 3.6 D; $1.6 \pm 0.1$ pA, $n = 6$ patches), and the $P_o$ in sweeps containing channel activity was also not significantly affected ($P_{o-Ni} = 0.64 \pm 0.06$ versus $P_{o-Ni} = 0.61 \pm 0.06$). There were, however, many more blank sweeps in the presence of the Ni$^{2+}$ (Figure 3.6 B). 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 ± 0.06 (n = 6 patches) to 0.43 ± 0.14 (n = 6 patches) in Ni$^{2+}$.

Figure 3.6 Ni$^{2+}$ 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$^+$]$_o$ = 140 mM and [K$^+$]$_i$ = 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$^{2+}$ in the external perfusate. The main effect of Ni$^{2+}$ is to reduce channel availability. Representative all-point amplitude histograms from a different one-channel patch in control and 0.5 mM Ni$^{2+}$-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.

3.3.6 Ni$^{2+}$ causes a rightward shift of the $Q_{on}$-$V$ curve but does not affect $Q_{max}$

A possible explanation for the current block by Ni$^{2+}$ is that one or more transitions in the gating pathway is prevented. To address that possibility; gating currents were recorded in an HEK-293 cell line expressing Kv1.5 W472F channels. The W472F mutation produces channels
that are not K$^+$ conductive, but which have normal gating currents. Figure 3.7 A shows gating current traces in control solution and in 1 mM Ni$^{2+}$. 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 3.7 B, 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 3.7 B, 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 3.7 C, on-gating currents were integrated and $Q_{on}$ was normalized with respect to the control maximal charge movement ($Q_{max}$). 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 and Fedida, 1999), the data of Figure 3.7 C were fitted to a single Boltzmann function.

In 6 experiments of the type illustrated in Figure 3.7, the control $V_h$ and $s$ were -6.8 ± 1.2 mV and 7.0 ± 1.4 mV. After switching to 1 mM Ni$^{2+}$ $V_h$ was 2.2 ± 0.8 mV and $s$ was 9.2 ± 1.2 mV. The difference in $V_h$ between 1 mM Ni$^{2+}$ and control medium was 9.0 ± 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 $Q_{max}$ decreased by approximately 15% (Zhang et al., 2001a), $Q_{max}$ was unchanged by 1 mM Ni$^{2+}$.
Figure 3.7 Gating charge movement with 1 mM Ni$^{2+}$ in Kv1.5. 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 HEK-293 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 $V_{0.5}$, the $Q_{max}$ did not decrease. Fitting to a Boltzmann function gave control and treated $V_{0.5}$ 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 $s$.

3.3.7 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 3.8 A 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\textsuperscript{2+} had no significant effect on the current but 10 mM Co\textsuperscript{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 Equation (3.1) revealed that \( V_l \) shifted by 11.4 ± 0.9 mV with 1 mM Co\textsuperscript{2+} and by 25.3 ± 1.3 mV with 10 mM Co\textsuperscript{2+}. Neither concentration of Co\textsuperscript{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\textsuperscript{2+} (Figure 3.8 B) gave an estimate for \( n_H \) of 1.3 ± 0.1 and a \( K_D \) (1.4 ± 0.1 mM) that was roughly 10-times larger than that for Ni\textsuperscript{2+} under the same recording conditions.

**Figure 3.8** Co\textsuperscript{2+} also causes a concentration-dependent block of Kv1.5 currents but is an order of magnitude less potent than Ni\textsuperscript{2+}. Shown in panel A are control and treated current traces evoked in 0 mM K\textsuperscript{+} with the voltage protocol indicated above the control responses. In contrast to the -35% block of the current with 0.1 mM Ni\textsuperscript{2+} (see Figure 3.2), 0.1 mM Co\textsuperscript{2+} had no effect. However, with 10 mM Co\textsuperscript{2+} the maximum peak tail current amplitude decreased by ~90%. As with Ni\textsuperscript{2+}, the block by Co\textsuperscript{2+} was completely reversible. B. Concentration-response data obtained in 0 mM K\textsuperscript{+}, with each point representing the mean ± SEM of measurements in 3-8 cells, were fitted to Equation (3.2) which gave a \( K_D \) of 1.4 ± 0.1 mM and an \( n_H \) of 1.3 ± 0.1.

The effects of Cd\textsuperscript{2+} are not illustrated but closely resembled those of Co\textsuperscript{2+}. The \( K_D \) was 1.5 ± 0.4 mM and the \( n_H \) was 1.3 ± 0.3. In 1 mM Cd\textsuperscript{2+} the \( V_l \) 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 \((n = 3)\).

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

Figure 3.9 A 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\(^{+}\)\(_{o}\)-free medium (not shown) necessitated recording with 3.5 mM K\(^{+}\)\(_{o}\). 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\(^{+}\)\(_{o}\)-dependent inhibition of Kv1.5 currents and for that reason its effects on inactivation were also examined (Figure 3.9 B). Using a Zn\(^{2+}\) concentration of 2 mM, which is estimated to reduce \(g_{\text{max}}\) by 80-90% in 3.5 K\(^{+}\)\(_{o}\), \(\tau_{\text{inact}}\) 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 \((\tau_{\text{inact}} \approx 100\) ms at pH 5.4\) and slow recovery from inactivation much less \((\tau_{\text{recovery}} \approx 4\) s at pH 5.4\).
Figure 3.9 Co\textsuperscript{2+} and Zn\textsuperscript{2+} mimic the effect of Ni\textsuperscript{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\textsuperscript{2+} in 3.5 mM K\textsuperscript{+} 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\textsuperscript{2+} in 3.5 mM K\textsuperscript{+}, $\tau_{\text{inact}}$ was 1.86 s and the $\tau_{\text{recovery}}$ was 29.3 s. Because Zn\textsuperscript{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 3.4 A).

3.4 Discussion

As reported previously (Perchenet and Clement-Chomienne, 2001), external Ni\textsuperscript{2+} ions were shown to reversibly block human Kv1.5 currents (Figure 3.1). We have also shown here that Ni\textsuperscript{2+} block is affected by [K\textsuperscript{+}]\textsubscript{o} (Figure 3.2). Thus, with 0 mM K\textsuperscript{+}, the $K_D$ for the Ni\textsuperscript{2+} block is approximately 150 $\mu$M whereas with 3.5 mM K\textsuperscript{+}, 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\textsuperscript{+} in CHO cells (Perchenet and Clement-Chomienne, 2001). Increasing K\textsuperscript{+} 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\textsuperscript{+} (see also Perchenet and Clement-Chomienne, 2001) suggests that the block requires the binding of at least two Ni\textsuperscript{2+} ions. In the study with Kv1.5 expressed in CHO cells the Ni\textsuperscript{2+} block was shown, regardless of the pulse frequency, to develop slowly over a 2 to 5 minute period (Perchenet and 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\textsuperscript{2+} binding to the closed state of the channel. Although we agree that the Ni\textsuperscript{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\textsuperscript{2+} block by K\textsuperscript{+} 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\textsuperscript{2+} and K\textsuperscript{+} binding sites. However, as noted with the block by Zn\textsuperscript{2+} and H\textsuperscript{+} (Kehl et al., 2002), the block by Ni\textsuperscript{2+} shows no voltage dependence over a range of voltages where the open probability is maximal (Perchenet and Clement-Chomienne, 2001). This observation supports the conclusion that the Ni\textsuperscript{2+} binding site is at least not in a region of the pore that is within the electric field and, by extension, that Ni\textsuperscript{2+} is not blocking by occlusion of the pore. The fact that Kv1.5 currents are blocked by H\textsuperscript{+}, Zn\textsuperscript{2+} and Cd\textsuperscript{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\textsuperscript{+} and Zn\textsuperscript{2+}, the sensitivity of Kv1.5 channels to Ni\textsuperscript{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\textsuperscript{2+} block to K\textsuperscript{+} and the outcome of other substitutions at position 463 (see below), are consistent with a model in which the binding of Ni\textsuperscript{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\textsuperscript{+} and Zn\textsuperscript{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., 2001b) 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 ShakerIR channels are largely resistant to the conductance collapse in low pH, acidification does accelerate current inactivation (Perez-Cornejo, 1999; Starkus 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 and 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$^+$], 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 $K^+_o$-sensitive ($K_D \sim 0.8$ 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 ShakerIR T449 mutants when the $[K^+]_o$ is decreased (Lopez-Barneo et al., 1993) and there is evidence in ShakerIR 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 ($\tau_{macro} = 35$ 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 $K^+_o$. Furthermore, in the H463G mutant the conductance collapse in $0$ mM $K^+_o$ is prevented by the R487V mutation (Trapani and 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 $K^+_o$ affects the $g_{macro}$ 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 $K^+_o$ of the $H^+_o$ and Zn$^{2+}$ block (Kehl et al., 2002; Zhang et al., 2001b).
A detailed study of the $K^+_{o}$-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 $K^+_o$, and assuming, for simplicity, a competitive interaction, the $K_D$ for the relief of the block by $K^+_o$ is calculated to be $\sim1.5$ mM. A consistent pattern that emerges from these studies, whether it is the spontaneously-occurring conductance collapse in ShakerIR 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 $K^+_o$ 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 ShakerIR 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 $\sim2$-fold faster than in controls (Figure 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., 2001a). 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 $Zn^{2+} (K_D \sim 0.07 \text{ mM}) > Ni^{2+} (K_D \sim 0.15 \text{ mM}) > Co^{2+} (K_D \sim 1.4 \text{ mM}) > Mn^{2+}$
(\(K_D > 10\) mM) and, as such, is in accord with the Irving-Williams order (Glusker, 1991). \(\text{Zn}^{2+}\), \(\text{Ni}^{2+}\) and \(\text{Co}^{2+}\), which are intermediate Lewis acids, are known to bind to the thiolate side group of cysteine and the imidazole nitrogen of the histidine. \(\text{Zn}^{2+}\) is also able to bind to carboxylate and carbonyl oxygen atoms. \(\text{Cd}^{2+}\), 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 \(\text{Cd}^{2+}\) that is greater than for \(wt\) Kv1.5.

In \(\text{Ca}_\text{v}2.3\) (\(\alpha 1\text{E}\)) channels, external \(\text{Ni}^{2+}\) causes, in addition to a rightward shift of the \(g-V\) curve, a reduction of the slope conductance with an estimated \(K_c\) of 300 \(\mu\)M (French et al., 1996). The blocking reaction appears to be bimolecular and is also affected by the type of permeant ion (e.g., \(\text{Ca}^{2+}\) versus \(\text{Ba}^{2+}\)). It was suggested that the \(\text{Ni}^{2+}\) block of \(\text{Ca}_\text{v}2.3\) 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 \(\text{Zn}^{2+}\) and \(\text{Cd}^{2+}\) have been studied in some detail (Gilly and Armstrong, 1982; Spires and Begenisich, 1994), \(\text{Ni}^{2+}\) has been used somewhat sparingly. In HERG \(K^+\) channels external \(\text{Ni}^{2+}\), as well as \(\text{Cd}^{2+}\), \(\text{Co}^{2+}\) and \(\text{Mn}^{2+}\), 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.
3.5 References


4. Single channel analysis reveals different modes of Kv1.5 gating behaviour regulated by changes of external pH

4.1 Introduction

Kv1.5 is a Shaker-related, voltage-gated potassium channel encoded by the gene KCNA5. In the human heart it mediates the ultra-rapid delayed rectifier current ($I_{\text{Kr}}$) involved in repolarizing the atrial action potential (Fedida et al., 1993; Feng et al., 1997). In a previous study, we reported that low external pH inhibits macroscopic Kv1.5 currents and causes a depolarizing shift of the conductance-voltage ($g-V$) relationship without substantially affecting activation kinetics (Kehl et al., 2002). Based on structure-function analyses, a histidine residue (H463) in the pore turret (S5-P linker) has been suggested to form part of the site to which external protons bind to produce these effects. Current inhibition by external protons can be attenuated either by raising the external [K$^+$] or by mutating an arginine residue near the outer pore mouth to valine (Kv1.5 R487V) (Kehl et al., 2002), which is equivalent to the T449V mutation in Shaker. These latter manipulations also influence an inactivation process involving the selectivity filter which is best characterized in Shaker channels where it is known as slow, P/C-type inactivation (Lopez-Barneo et al., 1993) but is often referred to below as outer pore inactivation. Together with the observation that the inactivation rate of residual currents increases in a pH-dependent manner with a $K_p$ similar to that for the current inhibition (Kehl et al., 2004; Steidl and Yool, 1999), these findings suggested a possible link between the proton-induced inhibition of Kv1.5 and outer pore inactivation. Pore occlusion as the basis for the effect
has been considered but deemed unlikely given that Na⁺ currents, reflecting ion flux through the inactivated pore, are little affected by decreasing pH (Zhang et al., 2003).

To get a clearer picture of the mechanistic basis for the current inhibition by external protons in Kv1.5, currents through single Kv1.5 channels were studied. Our results show that single channel current (i) is not affected by changing the extracellular pH and indicate that a change of $P_o$ is the basis for the effect. Consistent with the acceleration of inactivation of residual macroscopic currents at low pH, there was, in sweeps showing channel activity, a decrease of the mean burst duration and an increase of the mean interburst duration, suggesting a stabilization of inactivation. Within bursts, channel gating between the open state and closed states outside of the activation pathway was only slightly affected, if at all. The main cause of the pH-induced current inhibition of Kv1.5 was an increased probability of a gating mode where the channel was unavailable for activation.

4.2 Materials and Methods

4.2.1 Cell preparation

A stable mouse cell line, ltk, expressing hKv1.5 channels at a low density was generated by transfection with the cDNA of the human potassium channel Kv1.5 (hKv1.5) subcloned in the gentamicin-resistant gene-containing pcDNA3 vector using methods described previously (Wang et al., 2000). Cells were maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% gentamicin and incubated at 37°C in an atmosphere of 5% CO₂ in air. Cells were dissociated and plated onto cover slips for experimental use within 1-3 days. All tissue culture supplies were obtained from Invitrogen (Burlington, Ontario, Canada).
4.2.2 Electrophysiology

Single-channel current ($i$) and whole-cell current ($I$) were recorded in either the cell-attached or, in experiments where responses at two different pHs were compared, the outside-out patch configuration. For cell-attached recordings, the bath solution was assumed to depolarize the cell to 0 mV; it contained (in millimolar) 140 KCl, 3.5 NaCl, 2 CaCl$_2$, 1 MgCl$_2$, 5 glucose, 10 HEPES and was adjusted to pH 7.4 with NaOH; the patch pipette (external) solution contained 140 NaCl, 3.5 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 5 glucose and was adjusted either to pH 7.4 with 10 HEPES/NaOH or to pH 6.4 with 10 MES/NaOH. For recordings from an outside-out patch or in the whole-cell configuration, the bath (external) solution contained 140 NaCl, 3.5 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 5 glucose and was adjusted to pH 7.4 (10 HEPES/NaOH), 6.4 (10 MES/NaOH), or 5.9 (10 MES/NaOH); the patch pipette (internal) solution contained 130 KCl, 10 EGTA, 10 HEPES, 4.75 CaCl$_2$, 1.38 MgCl$_2$ and was adjusted to pH 7.4 with KOH. The free [Ca$^{2+}$] and [Mg$^{2+}$] were calculated to be 50 nM and 1 mM, respectively, using the MaxChelator program (Stanford University, Stanford, CA, USA). In subsequent text the reference to pH means the extracellular pH. All chemicals were purchased from Sigma-Aldrich (Mississauga, ON, Canada).

Voltage commands and current measurements were made with an EPC-7 patch clamp amplifier connected to an ITC-18 digital interface (Instrutech, Port Washington, NY, USA) and controlled by Pulse software (HEKA Electronik, Germany). Patch electrodes pulled from thin-walled borosilicate glass (World Precision Instruments, FL, USA) had a resistance measured with recording solutions of 8-25 MΩ. Current signals were low-pass filtered at 3 kHz (-3dB, 8-pole Bessel, NPI Electronics, Tamm, Germany) and digitized at 10 kHz with a 16-bit A/D converter. Cells were held at -80 mV at rest. A junction potential of -4 mV was compensated in the voltage measurements in the outside-out patch configuration; no junction potential correction was made.
for data acquired in the cell-attached recording mode.

4.2.3 Data analysis

Outside-out recordings were made at room temperature (20-25°C) and involved exclusively one-channel patches identified as such by the absence of overlapping open events in pH 7.4 solution at a potential, usually 100 mV, where the open probability was high. In cell-attached recordings it was not possible to change the pH of the solution in the recording electrode and although patches containing one channel could be identified unequivocally at pH 7.4 this was not the case at pH 6.4 where the open probability was lower. Single-channel records were analysed with TAC and TACFit software (Bruxton, Seattle, WA, USA) after digital filtering at 1 kHz. Capacitive currents were removed by subtracting the average of sweeps obtained at the same voltage that showed no channel activity (i.e., blank or "null" sweeps). Given a combined analogue and digital filter frequency of 950 Hz, the dead time of the system was calculated to be 0.3 ms using the formula $0.253/f$, where $f$ represents the overall combined analogue and digital filter cut-off frequency in Hz (Hoshi et al., 1994). Half-amplitude threshold analysis, with a rise time of 0.3 ms, was used to detect events and generate idealized records from which dwell time histograms and ensemble time courses were constructed. Events with durations shorter than the dead time were censored. Although there was evidence for a conductance substate in some traces (e.g., Figure 4.1), this phenomenon was not analysed in detail due to its relatively infrequent occurrence. For the analysis of gating kinetics a burst was defined as a group of brief open and closed events followed by a closed interval of 20 ms or more. There was no correction for missed events in the analysis of dwell time histograms that were fitted to exponential functions. The choice of the number of components used for fitting was based on a maximum likelihood
technique, in which the least number of components with a significant improvement was used (Saftenku et al., 2001). A value of twice the difference in log-likelihood (i.e., $2|LL_1 - LL_2|$) being greater than the $\chi^2$ value with a given degrees of freedom ($v$) is considered significant. The number of degrees of freedom is equal to the difference in free parameters between the models (Saftenku et al., 2001). Averaged results are expressed as the mean ± SEM unless otherwise stated. Results for fitting histograms and the Hill equation are given as the mean ± SD.

Statistical tests (Student’s $t$-test, ANOVA) were performed with Jmp In Software (SAS Institute, Cary, NC, USA). A probability less than 0.05 was considered significant.

A “runs analysis” was performed (Gibbons, 1985) to check the randomness of the occurrence of sweeps in which the channel was available or unavailable (Horn and Vandenberg, 1984). Data were obtained from one-channel cell-attached patches stepped to 100 mV for 150 ms with a cycle length of 3, 5, 10, or 15 seconds. A run ($R$) is defined as a sequence of sweeps showing similar gating behaviour which, for the purposes of this paper, means either a sequence of null sweeps in which the channel was in the unavailable mode ($U$) or a sequence of active ($A$) sweeps in which at least one open event was detected. When the number of trials is > 40, a normalized statistical value ($Z$) can be calculated by the equation,

$$Z = \frac{R - 2np(1-p)}{2\sqrt{np(1-p)}} \quad (4.1)$$

where $R$ is the number of runs, $n$ is the number of trials or sweeps, and $p$ is the probability of the event. The expected number of runs is $2np(1-p)$. A smaller than expected number of runs implies clustering and generates values of $Z$ greater than 0. Conversely, a value of $Z$ less than 0 indicates a tendency to alternate between null and active sweeps (Horn et al., 1984). The value of $Z$ was compared to the normal distribution to determine the statistical significance; a value
greater than 1.64 indicated behaviour that was non-random and clustered (*p* < 0.05, one-tail test (Plummer and Hess, 1991; Nilius, 1988)).

Contingency tables (2x2) were constructed to determine whether, at pH 6.4, there was a correlation between a sweep ending in an inactivated or non-inactivated state and the following sweep being active or null. Chi-square (*χ²*) analyses were performed using the equation,

\[
χ² = \sum \sum \frac{(f_{ij} - \hat{f}_{ij})^2}{\hat{f}_{ij}}
\]

where *f*<sub>ij</sub> and *\hat{f}*_<sub>ij</sub> represent, respectively, the experimental and expected frequency for row *i* and column *j* (Zar, 1984). A *χ²* value greater than 3.841 is considered significant (*p* < 0.05, *v* = 1).

### 4.3 Results

Representative, consecutive unitary current traces recorded at pH 7.4 from an outside-out patch during a 1-s depolarizing pulses at 100 mV with 3.5 mM K<sup>+</sup> are shown in Figure 4.1 A. As noted above, Kv1.5 exhibits conductance substate behaviour (marked by arrows) but these were not systematically analysed. As with ShakerIR channels (Hoshi et al., 1994; Schoppa and Sigworth, 1998a), at this voltage and pH the latency to first opening was consistently very brief (< 1 ms) and the channel rapidly flickered between short-lived open and closed states. Save for a few traces in which the channel entered a long-lived closed state presumed to be due to outer pore inactivation, the open probability was high for the duration of the depolarization. At pH 7.4, null sweeps occurred rarely, e.g., Figure 4.4 A. Fitting the cumulated all-points amplitude histogram (Figure 4.1 A bottom panel) with a three-component Gaussian function gave a single channel current of 1.7 ± 0.2 pA for the main conductance state, which is comparable to previously published results for *h*Kv1.5 (Fedida *et al*., 1993). The intermediate component is
due to a conductance substate and incomplete opening/closing events.

Figure 4.1 B shows unitary currents from the same patch as in Figure 4.1 A using the identical voltage protocol but after switching to pH 6.4. As at pH 7.4, flickery channel behaviour and conductance substates (arrows) were observed. There was no obvious difference in the probability of substates at pH 7.4 and 6.4. However, more sweeps showed the channel entering a long-lived closed state before the end of the voltage step, and the proportion of null sweeps was higher. Fitting of the all-points amplitude histogram at pH 6.4 gave a single channel current of $1.7 \pm 0.2 \text{ pA}$.

Figure 4.1 C and D shows representative current traces recorded from another outside-out patch at pH 5.9 and during recovery at pH 7.4 using the same voltage protocol as in Figure 4.1 A. At pH 5.9, the number of null sweeps was even higher; there was no evidence of channel activity in eleven of the twelve sweeps shown. The absence of activity in the first sweep suggests that depolarization-induced inactivation is unlikely to account for the null sweeps. In addition, the series of 10 consecutive null sweeps observed over a period of ~150 s is not likely to be due to a failure to recover from depolarization-induced inactivation since the recovery of residual macroscopic currents from inactivation at pH 5.4 with 3.5 mM $K^+_o$ is $\approx 4$ s (Kwan et al., 2004). This long period of inactivity is not likely to be due to entry into a defunct state since $K^+$ was present in both the intracellular and extracellular solution and recovery did not require a long depolarization pulse (Loboda et al., 2001). Recovery responses were obtained after switching the bath solution back to pH 7.4, demonstrating that the dramatic decrease of channel availability at pH 5.9 was also not due to the spontaneous loss of the channel from the patch. Furthermore, the single channel current ($i$) did not change between pH 5.9 and pH 7.4 (Figure 4.1 C and D).
Figure 4.1 The single channel current amplitude of Kv1.5 does not change between pH 7.4, 6.4, and 5.9. A. Representative unitary current through a one-channel, outside-out patch at pH 7.4. Current traces were evoked by 1 s depolarizing pulses at 100 mV from a holding potential of -80 mV every 15 s. Kv1.5 unitary currents show flickering behaviour. The failure of the channel to open (e.g. last trace in pH 7.4 column) was an infrequent observation at this pH. The corresponding cumulative all-points histogram based on traces in A is shown at the bottom of the panel. Fitting the histogram to a three-component Gaussian function gave a value of 1.7 pA for the major conducting level. B. Unitary current from the same outside-out patch using the same voltage protocol after switching to external solution at pH 6.4. The open channel current does not change but there are more null sweeps. The all-points amplitude histogram shown at the bottom gave a single channel current of 1.7 pA for the major conducting level. The higher proportion of the non-conducting points reflects the higher proportion of null sweeps and the more frequent termination of active sweeps by a long-lived non-conducting state. C. Unitary current from a different outside-out patch using the same voltage protocol as in A but with an external solution at pH 5.9. Channel activity is observed in only one of the twelve sweeps. This dramatic decrease of channel activity was not due to the loss of the channel from the patch since recovery of activity was obtained after returning to pH 7.4 solution (D). The all-points histograms at the bottom of panel C and D give a value of 2.0 pA for the mean open channel current.
Similar results were observed in 2 other patches. Together, these results show that decreasing external pH decreases the availability but not the single channel current of Kv1.5.

Analysis of single channel currents over a range of voltages confirmed that the single channel conductance is not affected by changing pH. Representative unitary currents recorded at 0 to 100 mV in 20 mV increments at pH 7.4 or 6.4 from different outside-out patches are shown in Figure 4.2 A. The graph in panel B shows the i-V relationship based on data from 3 cells, derived from all-points histograms as described for Figure 4.1, at pH 7.4 (unfilled circles) and pH 6.4 (filled circles). A line fitted to the data gave a slope conductance (γ; mean ± SD) of 11.8 ± 0.6 pS at pH 7.4 and 11.3 ± 0.8 pS at pH 6.4, which were not significantly different. This finding, together with the data of Figure 4.1, allowed us to reject the hypothesis that the reduction in macroscopic current by acidification is due to the reduction of the single channel conductance arising either by a change of the permeation pathway or by occlusion of the pore. Instead, the results of Figure 4.1 and 4.2 indicate: 1) that the low pH-induced decrease of macroscopic current must arise solely by an effect on channel gating; and, 2) that depolarization-induced inactivation is unlikely to play a significant role in the mechanism responsible for the null sweeps.

Macroscopic currents and the ensemble current behaviour generated from idealized records, obtained in each case from channels expressed in Itk cells, are shown for comparison in Figure 4.3 A and B, respectively. Macroscopic currents evoked by a 1 s pulse to 100 mV at pH 7.4, 6.4 and 5.9 showed a pH-dependent decrease of the peak current along with an increased rate of inactivation (Figure 4.3 A; see inset of normalized currents and figure legend for numerical values) as reported previously (Kehl et al., 2002). The reductions in peak currents are comparable to the reduction in macroscopic conductance measured from Kv1.5 expressed in
**Figure 4.2** Open channel current-voltage relationship showing the single channel conductance does not change with pH. *A.* Representative unitary currents at voltages between 0 and 100 mV in 20 mV increments at pH 7.4 and 6.4. All traces shown were digitally filtered at 1 kHz. *B.* Fitting a line to the $i-V$ relationship at pH 7.4 (unfilled circles) and pH 6.4 (filled circles) gave a slope conductance (mean ± SD) of 11.8 ± 0.6 pS and 11.3 ± 0.8 pS, respectively. These values for the slope conductance were not significantly different.

HEK-293 cells with similar recording conditions (5 mM $K_0^+$; Figure 4.3 of (Kehl et al., 2002)) and that measured from the ensemble current (Figure 4.3 B; see figure legend for details).

Furthermore, the time courses of inactivation measured in the whole-cell configuration at different pH also agreed well with that measured from the ensemble currents. This correlation shows that a change of $P_0$ nicely accounts for the effect of pH on the amplitude and kinetics of macroscopic currents. Inspection of the single channel responses reveals that this low pH-induced decrease of $P_0$ arises for two reasons: 1) channel availability decreases; and, 2) the burst duration decreases. Of these two actions, the influence of pH on channel availability was much greater and is considered first.

### 4.3.1 pH affects channel availability

Figure 4.3 C shows a plot of channel availability, defined as the proportion of sweeps
Figure 4.3 Current behaviour at the macroscopic level and the ensemble average of unitary current of Kv1.5 at pH 7.4, 6.4, and 5.9 are qualitatively similar. A. Representative, superimposed macroscopic currents at pH 7.4 (top trace), pH 6.4 (middle trace), and pH 5.9 (bottom trace) evoked with a 1 s depolarizing pulse to 100 mV from a holding potential of -80 mV. Peak current was reduced by about 22% at pH 6.4 and 81% at pH 5.9. Fitting the currents at pH 7.4, 6.4, and 5.9 to a single exponential function gave mean inactivation time constants of 558 ± 75 ms, 556 ± 61 ms, and 346 ± 46 ms, respectively. Normalized currents are shown in the inset to better illustrate the acceleration of inactivation. B. Ensemble open probability generated from idealized single channel records at pH 7.4, 6.4, and 5.9. The ensemble behaviour reproduces the changes of the peak amplitude and kinetics observed in the macroscopic currents. Compare to that at pH 7.4, the peak ensemble current was reduced by 34% at pH 6.4 and 82% at pH 5.9. A single exponential fitted to the ensembles gave time constants of 548 ms, 401 ms, and 197 ms for pH 7.4, 6.4, and 5.9, respectively. Normalized ensembles are shown in the inset. C. A reduction in channel availability accounts for the reduction of peak macroscopic current by external H⁺. Channel availability (filled circles) agrees well with the normalized relative \( g_{\text{max}} \) recorded in HEK-293 cells (unfilled triangles) or the normalized peak macroscopic current in ltk (unfilled circles). Availability is defined as the proportion of sweeps with one or more open events. Data from HEK-293 cells were obtained from our previous study (Kehl et al., 2002). Briefly, whole-cell currents were recorded from a series of 300-ms depolarizing steps at -50 to +60 mV, and the instantaneous tail currents at -50 mV were analysed to give the relative \( g_{\text{max}} \) values at different pH. The composition of the bath and pipette solutions were identical to that listed in Materials and Methods except the bath solution contained 5 mM K⁺ and 138.5 Na⁺. Fitting the whole-cell data to the Hill equation gave a \( pK_H \) of 6.2 ± 0.2 with a Hill coefficient of 1.6 ± 0.4 (dashed line) for the reduction in relative \( g_{\text{max}} \). Fitting channel availability to the Hill equation (solid line) gave a \( pK_H \) of 6.4 ± 0.2 with a Hill coefficient of 1.9 ± 0.2.
with at least one open event, against pH (filled circles). Availability changed from 0.97 ± 0.02 at pH 7.4, to 0.64 ± 0.6 at pH 6.4 and to 0.17 ± 0.03 at pH 5.9 (n = 3 -12 patches). Included for comparison in Figure 4.3 C is the relationship between pH and either the normalized

Figure 4.4 Modal gating of Kv1.5 at different pHs. A. A diary plot of Kv1.5 constructed by plotting the open probability per sweep for 150 consecutive sweeps depolarized at 100 mV for 150 ms every 3 s at pH 7.4 with a cell-attached patch. In this example, only one sweep (#79) shows mode A gating. B. Diary plot from another cell-attached patch using an identical protocol but at pH 6.4. Mode A (available) and mode U (unavailable) gating appear in clusters, as suggested by runs analyses (see text). C. Frequency histogram of the number of consecutive sweeps showing mode U gating at pH 6.4. The length of runs with mode U gating was pooled from 7 patches, and the resulting histogram was fitted to a single exponential distribution to give a time constant of 2.8 sweeps (8.4 s). D. Frequency histogram of the number of consecutive sweeps with mode A gating at pH 6.4. Fitting the histogram to a single exponential distribution gave a time constant of 1.9 sweeps (5.7 s).
macroscopic peak current at 100 mV for Kv1.5 expressed in ltk cells (unfilled circles) or the relative macroscopic conductance data taken from our study using Kv1.5 in HEK-293 cells (unfilled triangles; Kehl et al., 2002). The similarity of these three relationships indicates that a change of availability is the primary cause of the inhibition of Kv1.5 currents by extracellular protons and is consistent with our previous conclusion (Kehl et al., 2002) that the pH-induced increase of the depolarization-induced inactivation rate alone is an insufficient explanation.

The decrease in availability may result from an increase in the number of random null sweeps or from a modal gating scheme in which the null (or active) sweeps are clustered together. To determine if gating was modal, unitary currents through cell-attached patches were recorded using a pipette solution buffered to pH 7.4 or 6.4, and the voltage protocol consisted of a 150 ms pulse at 100 mV applied at an interval of 3 s. The attached cell was depolarized by a high [K+] bath solution and its resting potential was assumed to be 0 mV. It was also assumed that the absence of more than one (main conductance) open level indicated that the patch contained just one channel. A representative diary plot of \( P_a \) per trace at pH 7.4 (panel A) and 6.4 (panel B) is shown in Figure 4.4. At pH 6.4, the diary plot indicates a clear tendency for alternating periods of null and active traces and for that reason a runs analysis was done (see Materials and Methods) to determine if sweeps showing similar channel behaviour were clustered, i.e., that gating was modal. Sweeps were labelled as either unavailable (\( U \)) for null traces or available (\( A \)) for traces showing one or more openings of the channel. Using Equation 4.1, the Z values were calculated to be 5.0 ± 1.3 (range 1.77-19.6; \( n = 6 \)) at pH 7.4 and 6.3 ± 1.0 (range 2.85-9.17; \( n = 7 \)) at pH 6.4. Both values indicate a significant clustering (\( p < 0.001 \)) of active and null sweeps.

To estimate the average lifetimes of epochs of \( U \) and \( A \) sweeps, histograms were
constructed from the numbers of consecutive sweeps with either type of gating. Data at pH 7.4 or 6.4 were combined from 6 and 7 one-channel, cell-attached patches, respectively (Plummer and Hess, 1991) and represent a total of 1502 sweeps (1308 $A$ sweeps, 194 $U$ sweeps) at pH 7.4 and 843 sweeps (385 $A$ sweeps, 458 $U$ sweeps) at pH 6.4. Despite the large number of sweeps recorded at pH 7.4, there were only 93 runs (49 in mode $A$, 44 in mode $U$) and the histogram for mode $A$ was inconclusive due to a large proportion of very long runs (not shown). Fitting the histogram for mode $U$ to a single exponential gave a time constant of 0.76 ± 0.06 sweeps (or 2.3 ± 0.2 s given a stimulus period of 3 s; not shown). On the other hand, 385 runs (194 in mode $A$, 191 in mode $U$) were recorded with pH 6.4, and histograms for mode $U$ and mode $A$ are shown in Figure 4.4 $C$ and $D$, respectively. A log-likelihood test revealed the distribution was better fitted by a single exponential function (difference in log-likelihood < 0.2, not significant, $\nu = 2$) with a time constant of 2.8 ± 0.4 sweeps (8.4 ± 1.2 s) for mode $U$ and 1.9 ± 0.3 sweeps (5.7 ± 0.9 s) for mode $A$. Together, these results suggest decreasing pH promotes mode $U$ gating while increasing pH favours mode $A$ gating.

If, for simplicity, we assume that the transition between the two modes is first order, i.e.,

$$U \leftrightarrow A$$

then $k_1$ and $k_{-1}$ are equal to the reciprocal to the mean lifetime of $U$ and $A$, respectively. At pH 6.4, the estimates for $k_1$ and $k_{-1}$ were 0.12 s$^{-1}$ and 0.18 s$^{-1}$, respectively. These numbers translate to a probability for $A$ of 0.4 at pH 6.4. This analysis was based on the assumptions that: 1) a channel could make at most one transition during a 3 s period; and, 2) the probability of switching between the two gating modes during a 150 ms pulse to 100 mV was negligible. The first assumption is probably valid since changing the interpulse interval to either 5 or 10 s did not
alter the outcome of the runs analysis; however, when the interpulse interval changed to 15 s the grouping of $U$ and $A$ sweeps showed no statistical tendency to cluster (not shown). The second assumption seems valid for the transition from $U$ to $A$ because very few traces (less than 1%) had a latency to first opening longer than a few ms. However, the long-lived closure sometimes observed before the end of a voltage pulse could be due to either a depolarization-induced inactivation or a transition to mode $U$.

An important question regarding mode $U$ is whether it simply reflects depolarization-induced inactivation. With a 1 s depolarization to 100 mV at pH 6.4 there was, compared to pH 7.4, a higher probability of entering a long-lived closed state (Figure 4.1 B) but this was not invariably linked to the channel being unavailable on the next sweep evoked 15 s later. In Figure 4.1 B, for example, five of eleven active sweeps terminate in a long-lived closed state, which is presumed to reflect depolarization-induced inactivation, and four of those five sweeps are followed by an active sweep. Statistical analysis of a 2x2 contingency table (sweep ending in inactivation versus sweep active at end) x (next sweep null versus next sweep active) showed there was no correlation between a pulse ending in an inactivated state and the next sweep being blank (27 pairs, $\chi^2$ value = 2.41, $p > 0.1$). With shorter depolarizations (150 ms), such as those used for Figure 4.4 B, the probability of inactivating before the end of the pulse was low, as indicated by the similarity of the $P_o$ per active trace at either pH, but clustering of null sweeps persisted. Similarly, runs analysis at pH 6.4 with 20 ms depolarizing pulses to 100 mV delivered at 0.33 Hz (not shown) showed clustering of null sweeps ($Z = 12.0 \pm 3.5$; $n = 6$; not shown). These results indicate that mode $U$ gating is not simply due to depolarization-induced inactivation. This is consistent with our previous finding that decreasing external pH has no effect on the maximum gating charge ($Q_{max}$) mobilized during activation (Kehl et al., 2002), in
contrast to what is expected were the channels in the depolarization-induced C-type inactivated state. The gating charge data also imply that mode $U$ gating includes voltage-sensitive transitions between several non-conducting states. However, the data do not allow us to say whether transitions between mode $U$ and the depolarization-induced inactivated state are possible.

**4.3.2 pH does not substantially affect intraburst behaviour**

As noted above, inspection of channel behaviour during active sweeps at either pH 7.4 or 6.4 revealed bursts comprised of brief closures and brief openings. Dwell time histograms of open (conducting) and closed (non-conducting) events within bursts at pH 7.4 and pH 6.4 are shown in Figure 4.6 and are based on depolarizations lasting for up to 180 s (Figure 4.5) to prevent the censoring of a long-lived, non-conducting state that usually occurred with 1 s depolarizations to 100 mV, particularly at pH 6.4 (e.g., Figure 4.1 B). The open duration histogram at pH 7.4 (Figure 4.6 A) was fitted by the sum of two exponentials. The slower component of the frequency distribution had a mean open time ($\tau_o$) of 1.5 ± 0.1 ms ($n = 8$ patches) and represented the more frequently observed open event ($a_o = 0.70 ± 0.04$). The faster component of the distribution represented 0.3 ± 0.04 of the open events and had a mean duration ($\tau_f$) of 0.34 ± 0.02 ms, which is probably an underestimate given the limited frequency response of the system. At pH 6.4 the open duration histogram was also biexponential (Figure 4.6 C). The slower component had a longer mean open time ($\tau_o$) of 1.4 ± 0.1 ms and represented approximately 0.80 ± 0.04 of the events. The less frequent ($a_f = 0.20 ± 0.04$), faster component had a mean dwell time ($\tau_f$) of 0.35 ± 0.05 ms ($n = 7$ patches). Neither the mean dwell times nor the proportion of time spent in these two open states changed significantly between pH 7.4 and
Figure 4.5 Decreasing external pH to 6.4 decreases the mean burst length and increases the apparent interburst duration.  

A. Representative unitary currents at pH 7.4 in a cell-attached patch. High $K^+$ bath solution was assumed to depolarize the cell to 0 mV; the pipette solution contained 3.5 mM $K^+$. Contiguous traces of the first 80 s of activity at 100 mV are shown. Bursts of flickering channel behaviour were bracketed by gaps longer than 20 ms (see Materials and Methods).  

B. Representative contiguous traces from another cell-attached patch but at pH 6.4. The within-burst behaviour was only slightly changed, the mean burst duration was decreased and the apparent interburst duration was increased. Both traces were digitally filtered at 1 kHz.

6.4 ($p > 0.05$; ANOVA).

Panels B and D of Figure 4.6 plot, at pH 7.4 and 6.4, respectively, the frequency distribution of the closed events during a long depolarization to 100 mV. At pH 7.4 the closed duration histogram was fitted to the sum of four exponentials but we focus initially on the three fastest components that largely reflect gating behaviour within a burst. A critical time of 20 ms was used as the minimum gap length that signified the end of a burst (defined below). The time constants (and amplitudes) for the fastest, intermediate-fast and slow-fast components were $0.22 \pm 0.1$ ms ($a_f = 0.63 \pm 0.02$), $0.42 \pm 0.03$ ms ($a_{i-f} = 0.35 \pm 0.02$), and $2.6 \pm 0.2$ ms ($a_s = 0.02 \pm 0.003$) ($n = 8$ patches). The corresponding values at pH 6.4 were $0.17 \pm 0.04$ ms ($a_f = 0.81 \pm 0.03$), $0.45 \pm 0.05$ ms ($a_{i-f} = 0.18 \pm 0.03$), and $2.4 \pm 0.3$ ms ($a_s = 0.01 \pm 0.002$) ($n = 7$ patches). The fastest closed component had a time constant shorter than the dead time of our system and consequently its mean lifetime should be taken as a rough estimate only. Although the mean dwell time of each of the three fastest components of the closed duration histograms did not
Figure 4.6 Extracellular acidification does not significantly affect gating transitions within bursts. Unitary current from cell-attached patches recorded at pH 7.4 and 6.4 with 2- or 3-minute depolarizing pulses to 100 mV were idealized using a half-amplitude method. A. The open duration histogram obtained from the current trace shown in Figure 4.5 A was fitted to a biexponential distribution to give time constants of 0.5 ms and 2.6 ms, and an area of 0.30 and 0.70, respectively. B. The closed duration histogram obtained at pH 7.4 was fitted to a four-component exponential distribution, and the fastest three time constants were 0.2, 0.5, and 4.8 ms, with a respective area of 0.63, 0.36, and 0.02. The slowest component (time constant 0.48 s) is thought to represent one or more inactivated states. It has a relative area less than 1% of the total closed events. C. The open duration histogram generated from the current trace shown in Figure 4.5 B at pH 6.4. The fast and slow time constants are 0.5 and 2.1 ms with an area of 0.2 and 0.8, respectively. D. The closed duration histogram at pH 6.4 was fitted to a 4-component exponential distribution. The three fastest components have time constants 0.2, 0.4, and 2.6 ms and an area 0.81, 0.18, and 0.01, respectively. The slowest component (time constant 5.5 s) represents less than 1% of the total non-conducting events.

change significantly between pH 7.4 and pH 6.4 ($p > 0.05$; ANOVA), their relative proportions did change. Compare to that at pH 7.4, the relative proportion of the fastest component increased
significantly ($p < 0.01$) at pH 6.4 while the intermediate-fast component decreased significantly
($p < 0.01$) at pH 6.4; however, the slow-fast component was unchanged ($p > 0.1$). These results
showed that intraburst gating behaviour was not dramatically affected by changing pH from 7.4
to 6.4.

4.3.3 pH affects the burst- and interburst duration

A burst of openings is conventionally defined as a series of openings separated by gaps
that are less than a critical length ($t_{cr}$) (Colquhoun and Sigworth, 1995). A value for $t_{cr}$ was
determined by numerically solving the equation, $\exp(-t_{cr}/\tau_j) = 1 - \exp(-t_{cr}/\tau_s)$ where $\tau_{sf}$ is the
time constant for the slow-fast component during a burst and $\tau_i$ is the time constant for the
slowest component in the closed duration histogram (Figure 4.6). Using conservative estimates
for $\tau_{sf}$ and $\tau_i$ of 3.5 and 500 ms respectively, $t_{cr}$ was set to 20 ms for either pH. Despite pooling
the data from patches at pH 7.4 ($n = 8$) or pH 6.4 ($n = 7$) the frequency of events remained low
and therefore it was not possible to obtain a reliable estimate of the number of interburst non-
conducting states. Additionally, at pH 6.4 the $P_o$ was low and, although overlapping channel
openings were never observed in the data that were analysed, it was not possible to be certain that
there was only one channel in the patch, thus making a meaningful analysis of the interburst data
problematic. An analysis of the mean burst duration was more straightforward because
overlapping openings were never observed during bursts at either pH. By dividing the
accumulated burst length by the number of bursts observed, the mean burst duration was
determined to be 1.7 s at pH 7.4 and 0.73 s at pH 6.4. The data of Figure 4.5 also show that the
slow, non-conducting state at either pH is non-absorbing. Compared to the pH 7.4 data, the
probability of the slow, non-conducting state was higher at pH 6.4 and it is more stable.
4.4 Discussion

Whether Kv1.5 channels are expressed in HEK-293 cells (Kehl et al., 2002) or in ltk cells, as in this study, extracellular acidification has two major effects on Kv1.5 macroscopic current: 1) the peak current amplitude decreases, and, 2) the inactivation rate of residual currents increases. Macroscopic current, $I$, is equal to $NP_0i$ or, in an expanded form, $NP_0\gamma(V-E_K)$ where $N$ is the channel density, $P_0$ is the open probability which has voltage and time dependence, $\gamma$ is the single channel conductance, and $E_K$ is the reversal potential. Analysis of one-channel patches showed that decreasing pH did not affect $i$ at pHs between 7.4 and 5.9 and over a range of voltages (Figure 4.1 and 4.2) indicating that $\gamma$ had not changed. This provided direct evidence that the decline of macroscopic currents is not due to occlusion of the open pore or a change of the permeation pathway. A similar conclusion has been reached for the effect of external protons on macroscopic Shaker1R current (Starkus et al., 2003).

Ensemble data constructed from idealized traces (Figure 4.3 B) replicated the main features of macroscopic currents indicating that the observed changes of single channel behaviour can account for most of the previously reported effects. One effect of low pH, a rightward shift of the $g-V$ relationship (Kehl et al., 2002) that is presumably due to screening and/or binding to surface charges (Trapani and Korn, 2003), was not apparent at the single channel level because of the strong depolarization that was typically used. The main effect of acidification was to decrease $P_0$: 1) by decreasing channel availability as shown by an increased proportion of blank or null sweeps; and, 2) by decreasing the average burst duration during active sweeps. Of these two changes, the decrease of channel availability was the primary cause of the decrease of the peak macroscopic conductance. This was illustrated by the overlap of the availability-pH curve derived from single channel analysis and the $g_{\text{max}}$-pH curves derived from...
the analysis of macroscopic currents (Figure 4.3 C). Diary plots and the outcome of runs analyses of the channel behaviour at pH 6.4 (Figure 4.4) showed that null and active sweeps were clustered and consequently that the behaviour likely represented different sets or modes of gating. The three criteria typically used to define modal gating were met (Nilius, 1988). First, the two distinct kinetic behaviours were consistently observed in one-channel patches, thus ruling out the possibility of two populations of channels. Second, the probability of a given gating mode could be experimentally manipulated, in this case by changing pH. And, third, the rate for the transition between the two modes was slow, as shown by clustering in diary plots and by the absence of clear evidence of a switch from mode $U$ to mode $A$ during 1 s depolarizations. Active sweeps at pH 6.4 and 5.9 frequently ended with a long, censored closed state (Figure 4.1 B) that we assume is due primarily to depolarization-induced inactivation, but we cannot preclude the possibility of transitions to mode $U$.

Three lines of evidence lead us to reject a model in which depolarization-induced inactivation accounted for the null sweeps. These are, first, that mode $U$ gating observed at low pH is not dependent on prior channel opening (first sweeps in Figure 4.1 B and C). The mean burst length, which is a reflection of the rate of depolarization-induced inactivation, is much longer than the dead time of our system so that a failure to detect very brief open events is an unlikely explanation for the null sweeps. Second, clustering of null sweeps was observed even with very brief (20 ms) depolarizations where the probability of depolarization-induced inactivation was very low. Third, in a previous study of macroscopic currents the decline of the peak current was evident on the first sweep after a 2 min period in which the potential was held continuously at -80 mV while the pH was changed (Kehl et al., 2002). These observations are inconsistent with a model in which null sweeps resulted from the occupancy of a long-lived
inactivated state entered during a depolarizing pulse.

The lumping together of two distinct gating behaviours into $U$ and $A$ modes, and the assignment of a first order reaction scheme for transitions between these two modes is very likely an oversimplification of a more complex gating behaviour. The details of states and the kinetics of transitions within a gating mode, as well as the exact connectivity between states in the two modes, will be required to develop a full understanding of the kinetic processes involved. A more detailed scheme of mode $A$ would include several closed states, closed-inactivated states, an inactivated state, an open state as well as closed states outside of the activation pathway (Olcese et al., 1997; Zagotta et al., 1994b; Kurata et al., 2004). Much less is known about the states traversed in mode $U$. However, save for a rightward shift of the $Q-V$ curve, gating currents are unchanged at low pH indicating that voltage sensor movement is more-or-less intact in channels gating in mode $U$.

Analysis of active sweeps at pH 7.4 or pH 6.4 suggests that in mode $A$ the opening and closing transitions within a burst were largely pH-insensitive (Figure 4.6). At either pH, Kv1.5 channel activity is characterized by rapid flickering that, especially at 100 mV, most likely reflects transitions between short-lived open and closed (i.e., non-inactivated) states outside of the normal activation pathway (Schoppa and Sigworth, 1998b; Zagotta et al., 1994a). There was evidence for two states in the open duration histogram but the faster of these two was near the dead time of the system and is therefore equivocal. Aside from this second open state, channel gating within a burst, including the existence of the three fastest components in the closed duration histogram, is very much as described for ShakerIR channels (Schoppa and Sigworth, 1998b).

The slowest component of the closed duration histogram represented a state that
terminated a burst and was attributed to inactivation. However, because of the relatively small number of these long non-conducting events, we cannot be certain of either the number of inactivated states or their mean dwell times. To address the simpler question of how a change of pH affected the equilibrium between bursting behaviour and gaps between bursts, channel activity was studied during minutes-long depolarizations to 100 mV (Figure 4.5). Decreasing pH from pH 7.4 to 6.4 decreased the burst duration and appeared to increase the interburst interval which can account for the faster inactivation rate of macroscopic and ensemble currents (Figure 4.3, A and B) as well as the slower rate of recovery from inactivation at low pH (Kwan et al., 2004).

Extracellular protons increase the depolarization-induced inactivation rate in ShakerIR (Starkus et al., 2003), N-terminal deleted Kv1.4 (Claydon et al., 2002), rat Kv1.5 (Steidl and Yool, 1999) and human Kv1.5 (Kehl et al., 2004). This enhancement of the inactivation rate is attenuated by the T449V mutation in ShakerIR and by the homologous mutation, R487V, in Kv1.5 and as such is consistent with a connection to outer pore inactivation. However, despite the fact that depolarization-induced inactivation both in ShakerIR and Kv1.5 is accelerated by decreasing pH, there are several important, unexplained differences in the response of these two structurally related channels to extracellular acidification. Protonation of H463 appears to be the primary event that triggers both a change of availability and the acceleration of depolarization-induced inactivation in Kv1.5 since both effects are substantially reduced in Kv1.5 H463Q (Kehl et al., 2002). In contrast, in ShakerIR the acceleration of depolarization-induced inactivation has been provisionally attributed to protonation of the aspartate residue in the GYGD sequence of the selectivity filter. The most striking disparity is that acidification decreases channel availability (increases mode U gating) in Kv1.5 but not in ShakerIR or Kv1.4. Interestingly, however,
mutations of the threonine residue at position 449 in Shaker (Lopez-Barneo et al., 1993) or its positional equivalent in Kv1.4 (Pardo et al., 1992) can produce currents that collapse in K⁺-free external medium, an effect that has been attributed to a change of availability due to closed-state inactivation (Pardo et al., 1992). We have not yet assessed the consequences of mutations of R487 on channel availability at pH 7.4 in Kv1.5 but have found that mutations of residues at or near the putative proton-binding site (H463) in the pore turret can produce currents at pH 7.4 that show fast inactivation and have a K⁺₀-sensitivity similar to that observed both for ShakerIR mutants and for wt Kv1.5 channels at low pH₀ (Eduljee et al., 2004; Kehl et al., 2002). Although a faster rate of depolarization-induced inactivation is associated with a K⁺₀-sensitive change of availability in ShakerIR T449 mutants, this is not a consistent correlation. For example, with wt ShakerIR decreasing the external pH can dramatically speed inactivation but does not appear to affect channel availability (Starkus et al., 2003), and, conversely, the inhibition of Kv1.5 current by divalent cations such as Ni²⁺, Co²⁺ and Zn²⁺ is correlated with a substantial decrease of channel availability that is associated with relatively small changes of depolarization-induced inactivation rate (Kwan et al., 2004). This indicates that “closed-state inactivation” and “open-state inactivation” do not always show parallel changes.

An important question that remains to be answered is whether the decreased availability or mode U gating we observe at low pH in Kv1.5 is due to a constriction of the outer pore mouth, as has been proposed for P/C-type inactivation. Typically, P/C-type inactivation is assumed to be strongly coupled to channel activation such that it occurs from the open state or perhaps from one or more closed states accessed outside of the activation pathway. If, as suggested by the effects of K⁺₀ and the R487V mutation on the inhibition of Kv1.5 current at low pH, the decreased channel availability does indeed reflect outer pore inactivation, this would mean that this process
becomes uncoupled from activation. Evidence obtained from the non-conducting mutant $ShakerIR\ W434F$ for “permanent” or resting P-type inactivation provides some support for this idea (Yang et al., 1997).

**Acknowledgement**

This work was supported by a grant from the Canadian Institutes of Health Research to S.J.K. and D.F. D.C.H.K. received trainee awards from the Michael Smith Foundation for Health Research and the Natural Sciences and Engineering Research Council of Canada.
4.5 References


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5. The microscopic changes in Kv1.5 slow inactivation gating caused by external K$^+$ and H$^+$

5.1 Introduction

Increasing external K$^+$ antagonizes the inhibition of macroscopic current caused by H$^+$ in Kv1.5 (Kehl et al., 2002). While the mechanism by which external H$^+$ inhibits Kv1.5 current was investigated at the single channel level in Chapter 4 (Kwan et al., 2006), the effects of external K$^+$ on this inhibition were not studied previously. With a fixed [K$^+$]$_o$ (3.5 mM), both the single channel conductance (between 0 and 100 mV) and the mean dwell times within bursts were unaltered by external H$^+$. Instead, H$^+_o$ increased the number of sweeps showing no channel activity (null sweeps). This behaviour was modelled as a shift in the mode of gating, in which mode A gating was proposed to result in normal gating behaviour (i.e., with open channel activity), and mode U gating resulted in channels that failed to open (i.e., null sweeps). Based on the idea that external H$^+$ inhibits Kv1.5 current by promoting mode U gating, external K$^+$ is hypothesized to antagonize the effect of H$^+$ by inhibiting the promotion to mode U gating.

In addition to the aforementioned effect, removing all external K$^+$, either at low extracellular pH or in the presence of external Ni$^{2+}$, Kv1.5 current evoked by long depolarizing pulses (≥ 1 s) showed an unexpected “slow rising phase” instead of a time-dependent decay (i.e., slow inactivation) (Kwan et al., 2004). The mechanism underlying this unusual behaviour remains uncertain, but this phenomenon appears to result from channels coming out of a non-conducting state that may be related to slow inactivation. Therefore, a detailed understanding of slow inactivation in Kv1.5 is needed to answer these questions.

To investigate the molecular mechanisms of external K$^+$ modulation of H$^+$-induced effects and the slow rising phase, two different experimental approaches were used. In Part I, the
mechanism by which external K$^+$ modulates Kv1.5 current at a fixed pH was examined. Unitary currents were recorded at pH 6.4 with 0, 1, 3.5, and 20 mM external K$^+$ in the cell-attached configuration. By using long depolarizing pulses ($\geq$ 6 s), some sweeps were seen to have long first latencies to opening, which was thought to represent channels switching from mode $U$ back to mode $A$. With increased external [K$^-$], the proportion of sweeps starting in mode $A$ was increased, which was consistent with an inhibition of mode $U$ gating. In Part II, the slow rising phase of current was studied using an external solution in which the external [K$^+$] was maintained at 0 mM while varying external pH from 7.4 to 5.9. When the sweeps showing long latency were summed together, a prominent slow rising phase was seen. The result suggested the slow rising phase resulted from a recovery from mode $U$ gating. In addition, the mean long first latency correlated with the mean dwell time of a depolarization-induced inactivated state. Together, these results suggest that a slow inactivation process underlies mode $U$ gating.

5.2 Materials and Methods

5.2.1 Cell preparation

A stable mouse cell line $ltk^-$ expressing Kv1.5 was made from the cDNA of Kv1.5 subcloned into the gentamicin-resistant gene-containing pcDNA3 vector as described previously (Wang et al., 2000). Cells were maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% gentamicin and incubated at 37°C in an atmosphere of 5% CO$_2$ in air. Cells were dissociated enzymatically and were plated onto coverslips for experimental use within 1-3 days. All tissue culture supplies were obtained from Invitrogen (Burlington, Ontario, Canada).
5.2.2 Electrophysiology

Whole-cell (macroscopic) and unitary (microscopic) currents were recorded using standard voltage clamp techniques in the whole-cell and cell-attached configurations, respectively. For whole-cell recording, cells were bathed in nominally $K^+$-free bath (external) solution containing (in millimolar) 143.5 NaCl, 2 CaCl$_2$, 1 MgCl$_2$, and 5 glucose adjusted to pH 7.4 (10 HEPES/NaOH), 6.9 (10 HEPES/NaOH), 6.4 (10 MES/NaOH), or 5.9 (10 MES/NaOH), or $K^+$-containing external solutions made by substituting equimolar amounts of KCl for NaCl at pH 6.4. The patch pipettes contained Ca$^{2+}$- and Mg$^{2+}$-free (internal) solution with 130 KCl, 10 EGTA, 10 HEPES adjusted to pH 7.4 with KOH. For cell-attached single channel recording, the bath solution contained 140 KCl, 3.5 NaCl, 2 CaCl$_2$, 1 MgCl$_2$, 5 glucose, and 10 HEPES buffered to pH 7.4 and was assumed to depolarize the cell to 0 mV. The pipettes for cell-attached recordings contained either nominally $K^+$-free (extracellular) solution with 143.5 NaCl, 2 CaCl$_2$, 1 MgCl$_2$, and 5 glucose adjusted to pH 7.4 (10 HEPES/NaOH), 6.9 (10 HEPES/NaOH), 6.4 (10 MES/NaOH), or 5.9 (10 MES/NaOH), or $K^+$-containing solution made by substituting an equimolar amount of KCl for NaCl at pH 6.4. In subsequent text, references to $K^+$ and pH refers to the extracellular $[K^+]$ and extracellular pH. All chemicals were purchased from Sigma-Aldrich (Mississauga, Ontario, Canada).

Voltage commands and current measurements were made at room temperature (20-25°C) with an EPC-7 patch clamp amplifier connected to an ITC-18 digital interface (Instrutech, Port Washington, New York) and controlled by Pulse software (HEKA Electronik, Lambrecht, Germany). Patch electrodes pulled from thin-walled borosilicate glass (World Precision Instruments, Sarasota, Florida) had a resistance of 1-3 MΩ for whole-cell experiments and 8-15 MΩ for cell-attached single channel recordings when measured in recording solutions.
Cells were held at -80 mV, and a liquid junction potential of -4 mV (or 4 mV) was compensated in all voltage measurements in the whole-cell (or cell-attached) recording modes. Current signals were low-pass filtered at 3 kHz (-3dB, 8-pole Bessel, NPI Electronics, Tamm, Germany) and digitized at either 5 or 10 kHz. For analysis, unitary currents were further filtered digitally at 1 kHz which gave an effective filtering frequency of 950 Hz.

5.2.3 Data analysis

Whole-cell macroscopic currents were analysed with Igor Pro 5.0.4 (WaveMetrics, Lake Oswego, Oregon). The (fast) activation time course was estimated by fitting currents within the first 20 ms between 50% and 100% of the peak current to a single exponential function. Inactivation kinetics were measured by fitting the current decay with a double exponential function. The time course of the slow rising phase was measured by fitting the current from 200 ms after the start of the pulse to the end of the pulse with double exponential functions to avoid an initial “hook” of current that was observed in some recordings (e.g., Figure 5.7 A, pH 6.4).

Single channel currents recorded by voltage steps at +100 mV from a holding potential of -80 mV were first corrected for capacitive currents using blank (null) sweeps recorded from the same patch and then idealized in TAC (Bruxton, Seattle, Washington) using a half-amplitude threshold criterion with a rise time of 0.3 ms for event detection (Kwan et al., 2006). Only patches with a single (major) level of event were used for all analyses except for the first latency histograms, in which all unequivocal latencies to first opening in multi-channel patches were also included (see below). Conductance substates were observed, but this phenomenon was not analysed in detail. Single channel current amplitude was determined from the difference in
amplitude between the two major peaks in the all-points histogram that was fitted to a Gaussian distribution function with three components (see Figure 4.7). Given a combined filtering frequency of 950 Hz, the dead time of the system was estimated to be 0.3 ms (Hoshi et al., 1994), and events shorter than the dead time were censored. Based on the analysis in Chapter 4, a 20-ms critical time ($t_{cri}$) was implemented for determining the termination of bursts (Kwan et al., 2006). No correction was made for missed events in the analysis of duration histograms.

Duration (dwell time) histograms were fitted to exponential distribution functions with multiple components in TACFit (Bruxton, Seattle, Washington) and in Igor Pro. The number of components was determined by a maximum likelihood technique, in which the least number of components with a significant improvement was used (Saftenku et al., 2001). A significant improvement was achieved if, given the log likelihood values of the two models ($LL_1$ and $LL_2$), a value of $(2|LL_1 - LL_2|)$ was greater than the $\chi^2$ value with a degree of freedom ($v$) equal to the difference in free parameters between the two models. Averaged results were expressed as mean ± SEM (standard error of the mean) unless otherwise stated, and the time constants for the histograms are given as mean ± SD (standard deviation). Statistical tests (Student’s $t$-test, ANOVA) were performed with JMP In Software (SAS Institute, Cary, North Carolina). The correlation coefficient ($r$) and its standard error ($s_r$) were calculated as described previously (Zar, 1984). A probability of less than 0.05 was considered significant.

5.3 Results

Part I Mechanism for the relief of $H^+$-induced inhibition by $K^+_{o}$

5.3.1 External $K^+$ antagonizes the $H^+$-induced Kv1.5 current inhibition in ltk$^-$ cells

Based on the analysis of tail current behaviour of Kv1.5 in HEK-293 cells, it was shown that
external K⁺ antagonizes the macroscopic current inhibition induced by external H⁺ (Chapter 2). Figure 5.1 A shows superimposed representative current traces evoked by 6 s depolarizing pulses at +60 mV with 0, 1, 3.5, and 20 mM external K⁺ at pH 6.4 from a mouse ltk cell expressing Kv1.5. As noted in Chapter 3, with 0 mM K⁺, Kv1.5 showed a slow rising phase at pH 6.4 instead of a time-dependent decay. This slow rising phase was not evident with 1 mM or higher concentrations of K⁺. The basis for this slow rising phase is considered in more detail in the second part of this chapter.

By increasing external [K⁺], current amplitude is increased, and this result can be seen more clearly by expanding the first 100 ms of the current traces (Figure 5.1 B). During the first 100 ms, Kv1.5 reached a peak within the first 20 ms (Ip20ms). Figure 5.1 C shows the relative Ip20ms with different [K⁺] normalized with respect to that with 0 mM K⁺. With 1, 3.5, and 20 mM K⁺, the peak current had an average fold-increase of 3.68 ± 0.14, 4.14 ± 0.07, and 5.64 ± 0.61, respectively (p < 0.01), compared to that with 0 mM K⁺. This enhancement of current caused by increasing [K⁺] was similar to that shown in Figure 2.4. These values did not take into account the changes in driving force or single channel conductance and thus may underestimate the relief of the pH-dependent inhibition by external K⁺.

Figure 5.1 D shows the current traces normalized to their respective Ip20ms, an approach that emphasizes the slow rising phase at 0 mM K⁺. The current traces with 0 mM K⁺ (at pH 6.4) were fitted to a double exponential function, which gave a mean time constant (τ slowdown) of 1.44 ± 0.12 s (n = 11; Figure 5.1 E) for the slow rising phase and a slower component (τ = 7.2 ± 0.6 s) for the decay phase. In contrast, the current traces with 1, 3.5, and 20 mM K⁺ show a multi-component time-dependent decay (i.e., slow inactivation) which can be fit to a double exponential function. The values for τfast and τslow were 0.35 ± 0.07 s and 1.87 ± 0.33 s with
Figure 5.1 Effects of external K\(^+\) on the macroscopic current through Kv1.5 expressed in ltk~ cells at pH 6.4. Kv1.5 currents were recorded using 6 s depolarizing pulses to +60 mV with 0, 1, 3.5, and 20 mM external K\(^+\) at pH 6.4 to study the changes in current amplitude and gating kinetics. A. Representative Kv1.5 currents from the same cell are superimposed to show the relief of current inhibition at pH 6.4 by external K\(^+\). Note that the current recorded at pH 6.4 with 0 mM K\(^+\) has a slow rising phase as opposed to the prominent current decay observed with higher [K\(^+\)]\(_o\). B. The traces in A are shown on an expanded time scale. The difference in current amplitude and the activation kinetics can be seen more clearly, but the slow rising phase with
0 mM K\(^+\) was not evident on this time scale. C. The relief of current inhibition was quantified by calculating the relative peak current amplitude during the first 20 ms \(I_{p,20ms}\) with respect to that during the first 20 ms at 0 mM K\(^+\). The relative current was 3.68, 4.14, and 5.64 with 1, 3.5, and 20 mM K\(^+\), respectively. D. Current traces were normalized to their respective \(I_{p,20ms}\) to allow a comparison of the current kinetics. This approach emphasizes the slow rising phase observed with 0 mM K\(^+\), which has a mean time constant of 1.44 ± 0.12 s \((n = 11)\). Inactivation kinetics were affected only slightly by changing external K\(^+\) between 1 and 20 mM. E. Current traces were fitted to double exponential functions, and the fast \((\tau_{fast}; \text{black bars})\) and slow \((\tau_{slow}; \text{grey bars})\) time constants were 0.35 ± 0.07 s and 1.87 ± 0.33 s with 1 mM K\(^+\) \((n = 5)\), 0.20 ± 0.03 s and 1.41 ± 0.27 s with 3.5 mM K\(^+\) \((n = 5)\), and 0.35 ± 0.03 s and 2.08 ± 0.45 s with 20 mM K\(^+\) \((n = 4)\), respectively. Only \(\tau_{fast}\) of 3.5 mM K\(^+\) was significantly different from that with 1 and 20 mM K\(^+\). Included in this panel is the mean time constant for the slow rising phase \(\tau_{slow,rise}\) \(\text{hatched bar}\) for comparison. F. The proportions of the steady-state \(\text{empty bars}\), fast \(\text{black bars}\), and slow \(\text{grey bars}\) components were unaltered by changing external K\(^+\). Their respective values were 0.30 ± 0.02, 0.19 ± 0.02, and 0.51 ± 0.03 with 1 mM K\(^+\) \((n = 5)\), 0.20 ± 0.01, 0.15 ± 0.01, and 0.65 ± 0.01 with 3.5 mM K\(^+\) \((n = 5)\), and 0.23 ± 0.02, 0.24 ± 0.05, and 0.53 ± 0.04 with 20 mM K\(^+\) \((n = 4)\). Notice that \(\tau_{slow,rise}\) with 3.5 mM K\(^+\) is very similar to \(\tau_{slow}\) with 3.5 mM K\(^+\). The average \(\tau_{fast}\) with 3.5 mM K\(^+\) was significantly different from that with 1 and 20 mM K\(^+\), while the values of \(\tau_{fast}\) with 1 and 20 mM K\(^+\) were not significantly different. The values of \(\tau_{slow}\) with 1, 3.5, and 20 mM K\(^+\) were also not significantly different from each other. The proportion of each of the components did not show a clear trend except that the proportion of the slow component was dominant, and the proportions of all three components with 1 and 20 mM K\(^+\) were not significantly different (Figure 5.1 F; see figure...
legend for values). Together, these results show that slow inactivation is somewhat enhanced with 3.5 mM $K^+_o$, or that a progression out of the slow rising phase contributed to the apparently slower inactivation with 1 mM $K^+_o$. Otherwise, external $K^+$ has a very limited effect on slow inactivation (Chen et al., 1997).

Figure 5.1 $G$ shows the current amplitudes at the end of 6 s depolarizing pulses to +60 mV at different $[K^+]_o$ with respect to their corresponding $I_{p,20 ms}$. With 0 mM $K^+$, the current was potentiated to $1.22 \pm 0.05$, reflecting the contribution of the slow rising phase. With 1, 3.5, and 20 mM $K^+$, the current amplitude was reduced to $0.32 \pm 0.2$, $0.21 \pm 0.01$, and $0.26 \pm 0.02$, respectively. Figure 5.1 $H$ shows the activation time constants ($\tau_{act}$) with different $[K^+]$. The activation time constant, as measured by fitting the last 50% of the rising phase within the first 20 ms of the current traces, was $1.5 \pm 0.1$, $1.8 \pm 0.2$, $1.9 \pm 0.3$, and $1.8 \pm 0.1$ ms with 0, 1, 3.5, and 20 mM $K^+$, respectively (not significantly different; ANOVA; $p = 0.28$). Together, these results are consistent with the findings presented in Chapters 2 and 3 and suggest that the relief by external $K^+$ of the $H^+$-induced macroscopic Kv1.5 current inhibition observed in $ltk$ cells and HEK-293 cells is similar. Since the current inhibition induced by $H^+$ is due to a promotion of mode $U$ gating, external $K^+$ may relieve this inhibition by antagonizing this promotion to mode $U$ (i.e., by promoting mode $A$ gating).

5.3.2 External $K^+$ increases the mean $P_o$ but not the unitary current amplitude

The relief of $H^+$-induced macroscopic current inhibition by $K^+$ may result from an increase in open probability ($P_o$) or from an increase in the single current amplitude ($I = N \cdot P_o \cdot i$). To determine the mechanistic basis for this relief of inhibition, unitary currents were recorded in the cell-attached configuration from mouse $ltk$ cells expressing Kv1.5 with depolarizing pulses.
lasting 6, 31, or 61 seconds. A 6 s pulse was used so that a comparison with the macroscopic current could be made. In contrast, the 31 s and 61 s pulses were used to increase the number of reopening events in each sweep and to observe the transition out of mode U. Figure 5.2 A-D shows representative unitary current traces evoked by 31 s depolarizing pulses to +100 mV (pulsed every 60 s) from different cell-attached patches with 0, 1, 3.5, and 20 mM K\textsuperscript{+} at pH 6.4. The traces show that increasing K\textsuperscript{+} causes an apparent increase in \( P_0 \) but no change of the unitary current amplitude (\( i \)).

To quantify \( P_0 \), unitary current traces were idealized using a half-amplitude technique, and the mean \( P_0 \) within the first 20 ms (hatched bars), 6 s pulses (black bars), and 31 s pulses (grey bars) with 0, 1, 3.5, and 20 mM K\textsuperscript{+} was calculated and shown in Figure 5.2 E. The mean \( P_0 \) within the first 20 ms gave an estimate of the proportion of channels starting in mode U. However, the capacitive currents of some of the sweeps had a complex waveform and thus could not be compensated completely. Consequently, the onset of current could not be determined unequivocally, and the \( P_0 \) within the first 2 ms was a rough estimate. Given this uncertainty, an additional 10% error was added to the mean \( P_0 \) within the first 20 ms. Using this criterion, increasing external K\textsuperscript{+} significantly increased \( P_0 \) within the first 20 ms from 0 to 20 mM K\textsuperscript{+} (but not between the \( P_0 \) with 1 and 3.5 mM K\textsuperscript{+}). This result is qualitatively similar to that shown in Figure 5.1 B. Interestingly, the \( P_0 \) during the first 20 ms was not significantly different from that with 6 s pulses with 0 mM K\textsuperscript{+}. This result reflects the relative \( I_{6s} \) for the slow rising phase as shown in Figure 5.1 G.

For the 6 s pulses (applied every 30 s), the mean \( P_0 \) was significantly different for each pair except for that between 0 and 3.5 mM K\textsuperscript{+}. The \( P_0 \) of 6 s pulses with 1 mM K\textsuperscript{+} was significantly higher than with 3.5 mM K\textsuperscript{+}, which was consistent with a slower slow inactivation with 1 mM.
Figure 5.2 Unitary current traces and the mean open probability with 0, 1, 3.5, and 20 mM external K\(^+\) at pH 6.4. Unitary current traces with 0, 1, 3.5, and 20 mM K\(^+\) were recorded from different cell-attached patches using 6 s, 31 s, or 61 s depolarizing pulses to 100 mV.

Representative traces (short latency only) lasting 31 s are shown in the upper panels in A-D. Selected 1 s segments of the current traces (horizontal bars) are expanded in the lower panel to better show the intraburst behaviour at different [K\(^+\)]\(_0\).

E. The mean open probability (\(P_o\)) during the first 20 ms of depolarizing pulses (hatched bars), the entire 6 s pulses (black bars), and the entire 31 s pulses (grey bars) was 0.10 ± 0.01, 0.13 ± 0.01, and 0.06 ± 0.01 with 0 mM K\(^+\), 0.34 ± 0.01, 0.16 ± 0.01, and 0.06 ± 0.01 with 1 mM K\(^+\), 0.29 ± 0.01, 0.11 ± 0.01, and 0.07 ± 0.01 with 3.5 mM K\(^+\), and 0.55 ± 0.01, 0.29 ± 0.02, and 0.17 ± 0.02 with 20 mM K\(^+\). These data show that each increment of [K\(^+\)]\(_0\) significantly increased the mean open probability.

F. Single channel current amplitude is unaltered by external K\(^+\). Mean single channel current amplitude was determined by fitting the all-points histograms derived from individual unitary current sweeps to a Gaussian distribution with 3 exponential components and calculating the difference between the two major components. The single current amplitudes were 1.5 ± 0.1 pA with 0 mM K\(^+\) and 1.6 ± 0.1 pA with 1, 3.5, and 20 mM K\(^+\). Error bars represent the standard error of the mean (SEM).
K⁺ as shown in Figure 5.1 A. On the other hand, the mean $P_0$ during 31 s pulses with 0, 1, and 3.5 mM K⁺ was not significantly different from each other; only that with 20 mM K⁺ was significantly higher than the rest. Together, these results show that increasing external K⁺ changes the mean $P_0$ mainly within the first 6 s, whereas the effect of K⁺ on $P_0$ is diminished with longer pulses.

The unitary current amplitude at +100 mV with 0, 1, 3.5, and 20 mM K⁺ at pH 6.4 was determined by plotting the all-points amplitude histograms (see Methods; data not shown). It was found that the mean current amplitude was not significantly different with varying [K⁺] between 0 and 20 mM (Figure 5.2 F). It is unclear why the single current amplitude did not change with the change of driving force. Nonetheless, the result shows the relief of current inhibition by K⁺ is due to an increase in $P_0$ rather than an increase in the single channel current amplitude.

5.3.3 External K⁺ does not change the intraburst behaviour of Kv1.5 dramatically

The increase in mean $P_0$ could result from changes in the intraburst behaviour (i.e., an increase in the dwell time of the open state(s) and/or a reduction in the dwell time of the closed states), in the bursting behaviour (increased burst length and/or reduction in gap (interburst) duration), and/or an increase of the probability of mode A gating. The lower trace from each panel in Figure 5.2 A-D shows a selected section of the upper trace with channel activities on an expanded time scale as indicated by the horizontal bars. The intraburst behaviour at each [K⁺] tested appears to be very similar, suggesting that a change in the intraburst behaviour is not responsible for the K⁺-induced current relief. Figure 5.3 A shows representative open duration histograms for 0, 1, 3.5, and 20 mM K⁺ from 4 different cell-attached patches. Each of the
histograms was fitted to a double exponential distribution function with time constants \( \tau_{of} \) and \( \tau_{os} \) for the fast and slow components, respectively. Figure 5.3 B shows the averaged \( \tau_{of} \) and \( \tau_{os} \) at each of the [K']_o tested. The values of \( \tau_{of} \) were very close to the dead time of the recording system and thus cannot be compared with any degree of confidence. On the other hand, \( \tau_{os} \) did not show a clear trend with external [K'], except that \( \tau_{os} \) with 0 mM K' was slightly larger than the others (significant; ANOVA; \( p < 0.05 \)).

As shown in Figure 5.3 C, both the relative proportion of \( \tau_{of} \) and \( \tau_{os} \) did not change significantly with [K']. These results show the open states are not dramatically affected by external K'.

Similarly, the closed states within bursts were not affected by external K'. Figure 5.3 D shows representative closed duration histograms for 0, 1, 3.5, and 20 mM K' at pH 6.4. Each histogram was fitted to a 5-component exponential distribution function. Among the 5 time constants, \( \tau_{c,1}, \tau_{c,2}, \) and \( \tau_{c,3} \) were smaller than the critical time (\( t_{crit} = 20 \) ms; red line) and thus taken to represent the mean durations of non-conducting states within a burst. On the other hand, \( \tau_{c,4} \) and \( \tau_{c,5} \) were larger than \( t_{crit} \) thus representing the mean durations of inactivated states. On this basis, \( \tau_{c,4} \) and \( \tau_{c,5} \) were treated separately from the other three components and are considered in greater detail below. The mean dwell times and the proportion for \( \tau_{c,1}, \tau_{c,2}, \) and \( \tau_{c,3} \) with 0, 1, 3.5, and 20 mM K' were summarized in Figure 5.3 E and F, respectively. Again, the values of \( \tau_{c,1} \) are too small to be compared unequivocally. Both the mean closed times and the proportion for each of these components did not change significantly with external [K'].

Together, the data of Figure 5.3 show that both the open times and closed times within bursts were at most modestly affected, if at all, by changing external [K'].

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Figure 5.3 External K\textsuperscript{+} does not alter gating within bursts at pH 6.4. A. Representative open duration histograms from four different cell-attached patches with 0, 1, 3.5, and 20 mM K\textsuperscript{+} are shown. Each open duration histogram was fitted to a distribution with the sum of 2 exponential components ($\tau_{o,f}$ and $\tau_{o,s}$). B. Both $\tau_{o,f}$ (black bars) and $\tau_{o,s}$ (grey bars) with 0, 1, 3.5, and 20 mM K\textsuperscript{+} are shown, and the respective values were 0.4 ± 0.1 ms and 1.9 ± 0.1 ms with 0 mM K\textsuperscript{+} ($n = 16$ patches), 0.3 ± 0.1 ms and 1.4 ± 0.1 ms with 1 mM K\textsuperscript{+} ($n = 7$ patches), 0.2 ± 0.1 ms and 1.4 ± 0.1 ms with 3.5 K\textsuperscript{+} ($n = 14$ patches), and 0.2 ± 0.1 ms and 1.5 ± 0.1 ms with 20 mM K\textsuperscript{+} ($n = 7$ patches). Only the value of $\tau_{o,s}$ with 0 mM K\textsuperscript{+} was significantly different from that with 1 and 3.5 mM K\textsuperscript{+}. C. Changing [K\textsuperscript{+}] did not affect the proportion of the fast (black bars) and slow (grey bars) components, which were 0.19 ± 0.02 and 0.81 ± 0.03 with 0 K\textsuperscript{+} ($n = 16$), 0.24 ± 0.04 and 0.76 ± 0.04 with 1 mM K\textsuperscript{+} ($n = 7$), 0.29 ± 0.03 and 0.71 ± 0.03 with 3.5 mM K\textsuperscript{+} ($n = 14$), and 0.26 ± 0.03 and 0.74 ± 0.03 with 20 mM K\textsuperscript{+} ($n = 7$). D. Representative closed duration histograms were generated from idealized unitary current traces recorded with 0, 1, 3.5, and 20 mM K\textsuperscript{+} from 4 different cell-attached patches. Each closed duration histogram was fitted to a 5-component exponential distribution function. The two slowest components had mean durations that were longer than the critical time (20 ms; red line) and thus are taken to represent gating activities occurring outside of bursts. E. Increasing [K\textsuperscript{+}] did not change significantly the three fastest components ($\tau_{c,f}$, black bars; $\tau_{c,2}$, grey bars; and $\tau_{c,3}$, hatched bars) of the closed duration histograms. The values of $\tau_{c,f}$, $\tau_{c,2}$, and $\tau_{c,3}$ were 0.3 ± 0.1 ms, 0.7 ± 0.1 ms, and 3.9 ± 0.4 ms with 0 mM K\textsuperscript{+} ($n = 16$), 0.3 ± 0.1 ms, 0.8 ± 0.1 ms, and 3.7 ± 0.4 ms with 1 mM K\textsuperscript{+} ($n = 7$), 0.2 ± 0.1 ms, 0.6 ± 0.1 ms, and 2.9 ± 0.3 ms with 3.5 mM K\textsuperscript{+} ($n = 14$), and 0.2 ± 0.1 ms, 0.8 ± 0.1 ms, and 3.5 ± 0.6 ms with 20 mM K\textsuperscript{+} ($n = 7$), respectively. F. Similarly, the proportions of these
three components were also not significantly different. The proportion of \( \tau_{C,1} \) (black bars), \( \tau_{C,2} \) (grey bars), and \( \tau_{C,3} \) (hatched bars) were 0.82 ± 0.02, 0.5 ± 0.02, and 0.03 ± 0.01 with 0 mM K\(^+\) (\( n = 16 \)), 0.85 ± 0.03, 0.13 ± 0.03, and 0.01 ± 0.01 with 1 mM K\(^+\) (\( n = 7 \)), 0.85 ± 0.02, 0.13 ± 0.02, and 0.02 ± 0.01 with 3.5 mM K\(^+\) (\( n = 14 \)), and 0.89 ± 0.02, 0.10 ± 0.02, and 0.01 ± 0.01 with 20 mM K\(^+\) (\( n = 7 \)). Error bars represent the standard error of the mean (SEM).

5.3.4 External K\(^+\) promotes mode A gating

Thus far, external K\(^+\) has been shown not to affect either the unitary current amplitude or the intraburst behaviour. This implies that external K\(^+\) antagonizes the H\(^+\)-induced current inhibition by changing the burst behaviour (burst length and/or interburst duration) and/or channel availability (the proportion of mode A gating). Mode U gating was defined in Chapter 4 as a failure to open during depolarizing pulses shorter or equal to 1 s. With longer depolarizing pulses, the probability of observing a transition from mode U to mode A was expected to increase. For this reason, depolarizing pulses lasting for 6, 31, and 61 seconds were used. As expected, the proportion of null sweeps in these long pulses was reduced, and with 0 mM K\(^+\) many sweeps showed delayed opening (long first latency; Figure 5.8). These delayed openings were assumed not to result from missed brief opening events. This was supported by the analysis of burst duration and the probability of observing bursts with duration shorter than the time resolution of the recording system (see below).

First latency data were obtained from idealized unitary current traces recorded at pH 6.4 with 0, 1, 3.5, and 20 mM K\(^+\) by measuring the time between the start of the depolarizing pulse and the first apparent opening event. With multi-channel patches, the first latency value for channel (level) \( x \) (where \( x > 1 \)) was included only if it occurred within the first burst of channel opening \((x - 1)\). The duration histograms for first latency with 0, 1, 3.5, and 20 mM K\(^+\) are plotted in Figure 5.4 A. All four duration histograms show a bimodal distribution, with a peak at around
1 ms and another peak in the range of seconds. However, as with measuring $P_0$, a maximum estimated error of 2 ms was added to the error of the latency values due to imperfect compensation of the capacitive currents. Given an error as large as 2 ms, the fast component is only an estimate. In addition, no correction of first latency for filter time delay was performed.

Normally, the shape of the probability density function for first latency is a skewed bell-shaped curve and deviates from an exponential function especially in the shorter duration domain. However, the probability density function can be well approximated by an exponential distribution with two components for all durations longer than the mean short first latency.

Therefore, the first latency histograms were fitted to a double exponential distribution to estimate the mean short latency ($\tau_{sl}$) and mean long latency ($\tau_{ul}$). The values for $\tau_{sl}$ and $\tau_{ul}$ are shown in Figure 5.4 C and E, respectively. Values for $\tau_{sl}$ were around 1 to 1.5 ms and did not appear to change significantly with external $K^+$. This is consistent with the lack of an effect of external $K^+$ on the macroscopic activation kinetics shown in Figure 5.1 H. The mean long first latency ($\tau_{ul}$) increased from $2.9 \pm 0.4$ s with 0 mM $K^+$ to $4.0 \pm 0.7$ s and $10.3 \pm 3.1$ s with 1 and 3.5 mM $K^+$. The value of $\tau_{ul}$ with 20 mM $K^+$ had a large standard deviation due to the small number of long latencies (22 events) with this $[K^+]$ and was therefore omitted. Since $\tau_{ul}$ was well over 1 s, the probability of seeing sweeps coming out from mode $U$ was very low with a 1 s pulse; hence, these sweeps would have appeared as “null” sweeps in Chapter 4. To be consistent with the analysis performed in Chapter 4 and the definition of mode $U$ (unavailable) gating, the channels with a long first latency were assumed to have shifted from mode $U$ to mode $A$ gating during the depolarizing pulses. Alternative models are considered (see Discussion), but the above interpretation was favoured for its simplicity.
Figure 5.4 External K\(^+\) causes parallel changes in both the mean long first latency and the mean long gap at pH 6.4. A. First latency histograms at pH 6.4 with 0, 1, 3.5, and 20 mM K\(^+\) were generated by measuring the first latency values from idealized unitary current traces recorded at 100 mV from different cell-attached patches. All four histograms show bimodal distributions and were fitted to a double exponential distribution function to obtain the mean short (\(t_{SL}\)) and long (\(t_{LG}\)) latencies, which are presented as bar graphs in panel C and E, respectively. B. Gap (interburst) duration histograms were generated from closed events longer than 20 ms. Each of the four gap duration histograms was fitted to a distribution representing the sum of three exponential components. The mean duration of the fastest component (\(t_{C,3}\)) for each of the four histograms was shorter than 20 ms and thus assumed to represent the “tail” of the slow closed state (\(C_3\); see Figure 5.3 D). The data in A and B are presented on the same time scale to emphasize the similarity between \(t_{SL}\) and \(t_{LG}\). Values for \(t_{SG}\) and \(t_{LG}\) at different pH are shown in panel D and E (grey bars), respectively. C. Values of \(t_{SL}\) were plotted against [K\(^+\)], and they were not significantly different (a maximum error of 2 ms was assumed). The fitted values of \(t_{SL}\) were 1.0 ms for 0, 1, and 20 mM K\(^+\) and 1.4 ms for 3.5 mM K\(^+\). D. Values of \(t_{C,3}\) (black bars) and \(t_{SG}\) (grey bars) are shown against pH. The dash line represents the critical time set for this study (20 ms). Values of \(t_{SG}\) changed from 36 ± 6 ms with 0 mM K\(^+\) to 59 ± 9 ms, 76 ± 9 ms, and 78 ± 7 ms with 1, 3.5, and 20 mM K\(^+\). E. Values of \(t_{LL}\) (black bars) and \(t_{LG}\) (grey bars) were plotted against [K\(^+\)]. No significant difference between the two was detected at any pH. The values for \(t_{LL}\) were 2.9 ± 0.4 s, 4.0 ± 0.7 s, and 10.3 ± 3.1 s with 0, 1, and 3.5 mM K\(^+\), respectively, and the values for \(t_{LG}\) were 2.5 ± 0.2 s, 3.0 ± 0.2 s, and 5.8 ± 0.3 s with 0, 1, and 3.5 mM K\(^+\), respectively. The error for \(t_{LL}\) with 20 mM K\(^+\) was too large and thus omitted. All error bars represent the standard deviation of the fit.
To test the hypothesis that sweeps with a long (first) latency represented channels shifting from mode $U$ to mode $A$ during the depolarizing step, the following analysis was done. In Chapter 4, the reduction in current by external $H^+$ was attributed to the reduction in availability; that is, a promotion of mode $U$ gating. For this study, sweeps obtained from one-channel, cell-attached recordings were divided into three groups based on their first latency. Sweeps in group $S$ had a first latency shorter than 20 ms; sweeps in group $L$ had a first latency $\geq 20$ ms; and, sweeps in group $N$ did not show any opening during the entire depolarizing pulse (up to 31 s). The use of a 20-ms criterion for group $S$ was based on the fact that the first peak of the first latency histogram occurred at approximately 2 ms, and a value 10 times longer should clearly separate the short latencies from the long latencies, given that the mean long latency was in seconds. Figure 5.5 A shows the proportion of group $S$, $L$, and $N$ at different $[K^+]_o$. From 0 mM to 20 mM $K^+$, the proportion of sweeps in group $S$ was increased and the proportion of sweeps in group $L$ and $N$ was decreased. If both group $S$ and $L$ were combined as “mode $A$” gating, the availability with 0 mM $K^+$ would be 0.55. Yet, from Chapter 2, the residual current is predicted to be around 0.2, a number much closer to the proportion of sweeps in group $S$ alone (0.26). In Chapter 4, the proportion of sweeps in mode $A$ at pH 6.4 with 3.5 mM $K^+$ was calculated to be approximately 0.60, which was comparable to the proportion of sweeps in group $S$ (0.63). The current inhibition observed under macroscopic current correlated with the proportion of sweeps in group $S$ alone much better than with the combined proportion of sweeps in group $S$ and $L$. These correlations suggest that sweeps belonging to either group $L$ or $N$ contribute to the current inhibition by $H^+$ and that sweeps belonging to group $L$ start in mode $U$.

In Figure 5.5 A, the proportion of sweeps in each group was calculated from all sweeps pooled from different patches to compensate for a very low number of sweeps obtained from
some patches. For that reason, no standard error is given. However, with such a large number of cumulated events (> 150 events in total for each treatment), the data were considered to be normally distributed and hence a 5% error was used to determine significance. Using this criterion, external K⁺ from 0 to 20 mM significantly increases the proportion of mode A (i.e., group S); there was no significant change of the probability of mode A between 1 and 3.5 mM K⁺. This result is consistent with the hypothesis that external K⁺ inhibits mode U gating.

5.3.5 External K⁺ slows the onset but not the recovery from slow inactivation

By increasing the number of long-lived non-conducting events, the use of long depolarizing
steps (6, 31, and 61 seconds) allowed a better characterization of the microscopic rate of the onset of, and recovery from slow inactivation. In Figure 5.3 D, each of the four closed duration histograms showed two components (τc,J and τc,S) that were longer than the critical time (τcrit) of 20 ms as determined in Chapter 4 (Kwan et al., 2006). Since these two components represent < 0.1% of all the closed events, the resolution was not very good with individual patches. To obtain a better estimate of the dwell times, all the closed events with duration longer than 20 ms were binned together from the 7-16 single-channel cell-attached patches recorded with 0, 1, 3.5, and 20 mM K⁺ to generate their respective gap (interburst) duration histograms (Figure 5.4 B). Each of the gap duration histograms was fitted to an exponential distribution function with three components.

The mean duration for the fastest component (τc,J) of each of the histograms was shorter than 20 ms and is assumed to represent the “tail” of the longest closed state (i.e., τc,J; see Figure 5.3 D), whereas the other two components (τSG and τLG) were assumed to represent the dwell times of two inactivated states. Both τc,J (black bars) and τSG (mean short gap; grey bars) are shown in Figure 5.4 D. Surprisingly, the value of τSG increased significantly from 0 to 3.5 and from 0 to 20 mM K⁺ (ANOVA; p < 0.05). This result suggests that external K⁺ increases the dwell time of a fast component of slow inactivation. Similarly, the value of τLG with 3.5 mM K⁺ was significantly different from the others, but the values of τLG with 0 and 1 mM K⁺ were not significantly different from each other. Interestingly, with each [K⁺]o tested the values of τLL and τLG were not significantly different. Moreover, τLL and τLG correlate significantly with each other (r = 0.93; p < 0.05). This can also be seen by comparing the histograms in Figure 5.4 A and B, in which the duration axes span the same time scale. This approach emphasizes the correlation between the mean (peak) long latency and the mean (peak) long gap. This result suggests that
the long first latency is related to a transition out of an inactivated state. The proportion of the short (\(\tau_{sc}\)) and long (\(\tau_{lc}\)) gap are shown in Figure 5.5 B to be around 0.5 for 0, 1, and 3.5 mM \(K^+\), but the proportion of short gaps appeared to dominate (0.63 ± 0.05) with 20 mM \(K^+\). Together, the above results suggest that recovery from inactivation may at best be slowed slightly by external \(K^+\).

Panels A-D of Figure 5.6 show the burst duration histogram with 0, 1, 3.5, and 20 mM external \(K^+\), respectively. Surprisingly, given that the macroscopic inactivation time course contains multiple components, each histogram was well fitted to a single exponential distribution function. Figure 5.6 E shows the mean burst duration with each of the \([K^+]_o\) tested. A burst was defined, as in Chapter 4, as a collection of openings terminated by a closing event longer than \(t_{crit}\) (20 ms). The mean burst length changed from 0.42 ± 0.01 s and 0.44 ± 0.01 s with 0 and 1 mM \(K^+\) to 0.37 ± 0.01 s and 0.55 ± 0.01 s with 3.5 and 20 mM \(K^+\). The significant reduction in mean burst length with 3.5 mM \(K^+\) is consistent with an acceleration of slow inactivation observed at the macroscopic level. The above result shows that external \(K^+\) causes a small but significant slowing of the macroscopic slow inactivation kinetics.

Using the shortest mean burst duration measured (0.37 s), the proportion of bursts shorter than 2 ms was ~0.5%. That is, with uncertainties in the first 2 ms of the pulses, at most 0.5% of the long latency sweeps may have grouped inappropriately (i.e., in group L instead of group S). On this basis, missed events are not likely to account for the long first latencies.

Part II Molecular mechanism for the slow rising phase at low pH with 0 mM \(K^+\)

In this section, the effects of changing external pH in 0 mM \(K^+\) are examined in relation to the slow rising phase. As discussed in connection with Figure 5.1 A, there is a prominent slow
Figure 5.6 At pH 6.4 increasing \([K^+]_o\) increases the burst duration. Burst duration histograms with 0, 1, 3.5, and 20 mM K\(^+\) are shown in A-D. A burst was defined as a series of openings terminated by a closing event longer than 20 ms. Each burst duration histogram was fitted to a single exponential distribution function. E. The time constants for the burst duration histograms were 0.42 ± 0.01 s, 0.44 ± 0.01 s, 0.37 ± 0.01 s, and 0.55 ± 0.01 s for 0, 1, 3.5, and 20 mM K\(^+\), respectively. The values were significantly different from each other but constituted only a modest effect.

rising phase of current during a 6 s pulse to +60 mV at pH 6.4 that is not evident with 1 mM and higher \([K^+]_o\). To investigate this slow rising phase of current, an approach similar to that employed in Part I was used. Macroscopic and single channel currents were analysed to deduce the microscopic changes that underlie the slow rising phase.

5.3.6 Kv1.5 shows a slow rising phase at low pH with K\(^+\)-free solution

To characterize the slow rising phase at different pHs, Kv1.5 currents were recorded with 6 s depolarizing pulses to +60 mV at pH 7.4, 6.9, 6.4, and 5.9 in nominally K\(^+\)-free solutions. Representative traces at pH 7.4, 6.4, and 5.9 are shown superimposed in Figure 5.7 A.

Consistent with previous findings (Chen et al., 1997), Kv1.5 current during the 6 s pulse at pH
7.4 inactivated with a time course that was well fitted by a double exponential function (see figure legend for time constants). After switching to bath solution at pH 6.4 or 5.9, currents through Kv1.5 were strongly reduced as reported in Chapters 2 and 4. Figure 5.7 B shows the relative peak current within the first 20 ms ($I_{p,20ms}$) with respect to that at pH 7.4, which was decreased to 64 ± 8% at pH 6.9, 13 ± 1% at pH 6.4, and 3.1 ± 0.4% at pH 5.9, and this reduction in peak current is comparable to the data obtained with HEK-293 cells (Figure 2.3; 0 mM K⁺).

As in HEK-293 cells (not shown), currents at both pH 6.4 and 5.9 showed a slow rising phase. The mean time constants for this slow rising phase ($\tau_{slow rise}$) at pH 6.4 and 5.9 were 1.44 ± 0.12 s ($n = 11$) and 0.47 ± 0.08 s ($n = 7$), respectively, and were significantly different (ANOVA; $p < 0.001$).

Compared to $I_{p,20ms}$, the maximum current amplitude ($I_{max}$) at pH 6.4 and 5.9 was potentiated by 1.32 ± 0.04 and 1.47 ± 0.17-fold due to the slow rising phase (Figure 5.7 C, black bars). In contrast, since no potentiation was observed at pH 7.4 and 6.9, the maximum current occurred within the first 20 ms. Figure 5.7 C also shows the relative current amplitude at the end of the 6 s pulse ($I_{6s}$, grey bars) relative to $I_{p,20ms}$. At pH 7.4 and 6.9, the current amplitudes were reduced to 0.52 ± 0.03 and 0.60 ± 0.03 of their respective $I_{p,20ms}$ due to slow inactivation. Conversely, the relative $I_{6s}$ at pH 6.4 and 5.9 was potentiated to 1.26 ± 0.05 and 1.31 ± 0.16 due to the slow rising phase.

As reported in Chapter 2, decreasing pH increased the time constant for activation ($\tau_{act}$). Values of $\tau_{act}$ were obtained by fitting current traces between 50% and 100% of $I_{p,20ms}$ within the first 20 ms. Figure 5.7 D shows the increase in $\tau_{act}$ with decreasing pH, and the values were significantly different (except between pH 7.4 and 6.9; see figure legend for details). This slowing of the activation kinetics may be due to a rightward gating shift related to screening of
Figure 5.7 Kv1.5 current shows a slow rising phase at low pH with 0 mM external K⁺.

Whole-cell Kv1.5 currents at pH 7.4, 6.4, and 5.9 with 0 external K⁺ were recorded with voltage commands to +60 mV for 6 s from a holding potential of -80 mV. A. Representative Kv1.5 current obtained from the same cell. A prominent current inhibition, along with a slow rising phase, can be seen in the current traces recorded at both pH 6.4 and 5.9. 

B. To quantify the current inhibition with decreasing external pH, the peak current within the first 20 ms of the depolarizing pulse ($I_{p,20ms}$) was normalized with respect to that at pH 7.4. The relative $I_{p,20ms}$ was reduced to 64 ± 8%, 13 ± 1%, and 3 ± 4% at pH 6.9, 6.4, and 5.9, respectively. 

C. The relative peak current within the 6 s pulse ($I_{max}/I_{p,20ms}$; black bars) and the relative current at the end of the 6 s pulse ($I_{ad}/I_{p,20ms}$; grey bars) at different pHs are compared. Values at different pHs were normalized with respect to individual $I_{p,20ms}$ within the group. The slow rising phase at pH 6.4 and 5.9 results in a maximum current that is larger than $I_{p,20ms}$ by 32 ± 4% and 47 ± 17%, respectively. In contrast, $I_{ad}/I_{p,20ms}$ at pH 7.4 and 6.9 were reduced to 0.52 ± 0.3 and 0.60 ± 0.3, whereas at pH 6.4 and 5.9, the relative $I_{ad}$ had increased to 1.26 ± 0.05 and 1.31 ± 0.16, respectively. 

D. External [H⁺] increases the activation time constant ($\tau_{act}$). By fitting the current traces between 50% and 100% of $I_{p,20ms}$ to single exponential function, $\tau_{ac}$ at different pHs was measured, and it increases from 1.0 ± 0.1 ms at pH 7.4 and 0.9 ± 0.1 ms at pH 6.9 to 1.5 ± 0.1 and 2.3 ± 0.4 ms at pH 6.4 and 5.9, respectively. Error bars represent the standard error of the mean (SEM).
surface charges by protons (Deutsch and Lee, 1989; Kehl et al., 2002; Trapani and Korn, 2003).

To obtain an insight into the mechanistic basis for the slow rising phase of current in 0 mM K\textsuperscript{+}, unitary currents from Kv1.5 were recorded at different pHs in the cell-attached mode, and the data were pooled to determine the mean first latencies, mean gap durations, and mean burst length.

5.3.7 External H\textsuperscript{+} promotes group L (long first latency) and N (null) behaviour

As for the study of the effect of different [K\textsuperscript{+}]\textsubscript{o} (Part I), unitary currents were recorded from cell-attached patches with depolarizing pulses lasting up to 61 s in order to study the microscopic inactivation kinetics. Representative current traces from different cell-attached patches evoked by 31 s depolarizing pulses to +100 mV from -80 mV at pH 7.4, 6.9, 6.4, and 5.9 are shown in Figure 5.8 A-D, respectively. In each panel, the top trace shows a sweep with a short latency (i.e., group S), and the bottom trace shows a sweep with a long latency (i.e., group L). Both the intraburst behaviour and single channel current amplitude were shown in Chapter 4 to be virtually unaffected by decreasing external pH with 3.5 mM K\textsuperscript{+}, and a similar effect was observed with 0 mM K\textsuperscript{+} (data not shown). From idealized unitary current traces, P\textsubscript{0} was determined at different pHs. Values for P\textsubscript{0} calculated from the 6 s and 31 s sweeps at different pHs are shown in Figure 5.8 E. Both the 6 s and 31 s pulses showed a reduction in mean P\textsubscript{0} with decreasing external pH, with the 6 s pulses showing a higher P\textsubscript{0}. The reduction in P\textsubscript{0} with 31 s pulses suggests the existence of a long-lived non-conducting state.

Similar to the results obtained when changing [K\textsuperscript{+}]\textsubscript{o} at pH 6.4, unitary current traces recorded at different external pHs with 0 [K\textsuperscript{+}]\textsubscript{o} showed three distinct gating behaviours and were divided into group S (first latency < 20 ms), L (first latency ≥ 20 ms), and N (no channel opening).
Figure 5.8 Unitary current, mean open probability, and the proportion of sweeps in groups $S$, $L$, and $N$ at different pHs. Representative unitary current traces recorded from different cell-attached patches with 0 mM K$^+$ at +100 mV for 31 s at pH 7.4 (A), 6.9 (B), 6.4 (C), and 5.9 (D) are shown. In each panel, the top trace shows a representative sweep with a short latency, whereas the bottom trace shows a sweep from another patch with long latency. Current amplitudes were unaltered with pH (~1.6 pA at 100 mV in each case). E. Decreasing external pH decreased the mean open probability. Mean open probability ($P_o$) during 6 s (black bars) and 31 s (grey bars) pulses at different pHs are compared in the bar graph. Error bars represent the standard error of the mean (SEM). F. Decreasing external pH also reduced the proportion of sweeps in group $S$. Unitary current traces lasting up to 31 s were divided into groups $S$, $L$, and $N$ (see Figure 5.5). Group $S$ contains traces with a short (< 20 ms) latency to first opening; group $L$ contains traces with long (≥ 20 ms) latency to first opening; and, group $N$ contains traces without opening during a depolarizing pulse lasting up to 31 s. The proportion of group $S$, $L$, and $N$ were 0.66, 0.13, and 0.21 at pH 7.4, 0.54, 0.28, and 0.18 at pH 6.9, 0.27, 0.30, and 0.43 at pH 6.4, and 0.04, 0.38, and 0.58 at pH 5.9.

Figure 5.8 $F$ shows the proportion of sweeps in the three groups at the 4 different pHs. The proportion of sweeps in group $S$ decreased significantly from 0.66 at pH 7.4 to 0.54, 0.27, and
0.04 at pH 6.9, 6.4, and 5.9, respectively. In other words, if sweeps with long first latency were assumed to be in mode $U$ at the start of the depolarizing pulse, then the proportion of sweeps starting with mode $U$ gating (i.e., group $L$ and $N$) was increased with external $H^+$. This reduction in the proportion of group $S$ was qualitatively similar to the reduction in availability reported in Chapter 4 (Kwan et al., 2006), which is consistent with the above interpretation of group $L$ behaviour.

### 5.3.8 The mean long first latency correlates significantly with the mean long gap

From the idealized traces, duration histograms for first latency, gaps between bursts, and burst lengths at different pHs were plotted to determine the changes the bursting behaviour. Figure 5.9 $A$ shows the first latency histograms at pH 7.4, 6.9, 6.4, and 5.9. As in Figure 5.4 $A$, the histograms fit well to a double exponential distribution function except at pH 7.4, for which very few long latencies (17 in total) were observed; therefore, the long latency ($\tau_{LL}$) at pH 7.4 is only an estimate. Again, the mean short latency ($\tau_{SL}$) corresponds to sweeps in group $S$, and the mean long latency ($\tau_{LL}$) represents the mean delay in opening of sweeps in group $L$. The histograms showed a shift in the relative distribution towards the longer component with decreasing pH as shown in Figure 5.8 $F$. The pH-dependent changes in $\tau_{SL}$ and $\tau_{LL}$ are shown in Figure 5.9 $C$ and $E$, respectively. The value of $\tau_{SL}$ increased from 0.4 ms at pH 7.4 to 1.0 ms at pH 6.9, 1.0 ms at pH 6.4, and 1.6 at pH 5.9. However, given an estimated maximum error of 2 ms (see Figure 5.4 $A$), the changes in $\tau_{SL}$ with pH cannot be resolved unambiguously.

The gap (interburst) duration histograms at different pHs are shown in Figure 5.9 $B$. As in Figure 5.4 $B$, the gap duration histograms were best fitted with an exponential distribution function with 3 components, except at pH 5.9 where the data were best fitted with a double
Figure 5.9 The mean long first latency correlates with the mean long gap between bursts.  
A. First latency histograms are generated from idealized unitary currents recorded at pH 7.4, 6.9, 6.4, and 5.9 with depolarizing pulses at 100 mV. All four histograms show bimodal distributions and were fitted to a double exponential distribution function giving the mean short (τ_{SL}) and long (τ_{LL}) first latencies. Values of τ_{SL} and τ_{LL} were summarized in panel C and E, respectively. B. Gaps (interbursts) were defined as closing events longer than 20 ms in the idealized traces and were binned to generate the gap duration histograms. Histograms for pH 7.4, 6.9, and 6.4 were best fitted to distributions representing the sum of 3 exponential components, and the histogram for pH 5.9 was fitted to a distribution comprising the sum of 2 exponential components. The fastest component (τ_{C3}) for all four histograms has a mean value smaller than 20 ms and was assumed to represent the “tail” of a long closed state (C3, see Figure 5.3 D). The data in A and B are presented on the same time scale to emphasize the similarity between τ_{LL} and τ_{LG}. Values for τ_{SG} and τ_{LG} at different pHs are shown in panel D and E, respectively. C. Values of τ_{SL} plotted against pH, increased from 0.4 ± 0.1 ms at pH 7.4 to 1.0 ± 0.1, 1.0 ± 0.1, and 1.6 ± 0.3 ms at pH 6.9, 6.4, and 5.9, respectively. D. Values of τ_{SG} (black bars) and τ_{LG} (grey bars) at different pHs were summarized. The dashed line represents the critical time (20 ms). Values of the mean short gap (τ_{SG}) changed from 70.7 ± 10 and 71.1 ± 6 ms at pH 7.4 and pH 6.9 to 36.2 ± 6 ms at pH 6.4. The histogram for pH 5.9 does not show a component corresponding to short gap. E. Comparison of the mean long first latency and the mean long gap. Values of τ_{LL} (black bars) and τ_{LG} (grey bars) are shown against pH; no significant difference was detected between the two values at each pH tested, and τ_{LL} and τ_{LG} correlated significantly (r = 0.99 ± 0.08; p < 0.01). The values for τ_{LL} were 2.35 ± 1.3 s, 2.78 ± 0.35 s, 2.87 ± 0.39 s, and 4.53 ± 0.36 s at pH 7.4, 6.9, 6.4,
and 5.9, respectively. The values for $\tau_{LG}$ were $1.76 \pm 0.19$ s, $1.77 \pm 0.12$ s, $2.54 \pm 0.18$ s, and $4.44 \pm 0.40$ s at pH 7.4, 6.9, 6.4, and 5.9, respectively. $F$. The proportion of short and long gaps at different pHs. The proportion of short gaps was reduced from 0.64 at pH 7.4 to 0.59 and 0.48 at pH 6.9 and 6.4, respectively. Short gaps were not detected at pH 5.9. All error bars represent the standard deviation from the fit to the histograms.

exponential distribution function. The fastest component ($\tau_{C3}$) in all four histograms had a mean dwell time shorter than 20 ms and was interpreted as the "tail" of the distribution of a long closed state. The other 2 components ($\tau_{SG}$ and $\tau_{LG}$) are shown in Figure 5.9 D and E, respectively. With decreasing pH, $\tau_{SG}$ decreased significantly from $71 \pm 9$ ms at pH 7.4 and 71 ± 6 ms at pH 6.9 to $36 \pm 6$ ms at pH 6.4, suggesting that decreasing external pH reduces the dwell time of a "fast" inactivated state. This result was unexpected as it appears to contradict the acceleration of slow inactivation observed in Kv1.5 as discussed in Chapter 4 (Kwan et al., 2006). On the other hand, the proportion of $\tau_{SG}$ was reduced with pH as well (Figure 5.9 F). The proportion of $\tau_{SG}$ decreased from 0.64 at pH 7.4 and 0.59 at pH 6.9 to 0.48 at pH 6.4 and 0 at pH 5.9. That is, the overall time spent in all inactivated states may still be increased, which is consistent with the reduction in mean $P_o$ with decreasing external pH (Figure 5.8 E). The absence of $\tau_{SG}$ at pH 5.9 is interesting since the macroscopic inactivation time course at pH 5.9 with 3.5 mM external K$^+$ is well fitted by a single exponential function (Kehl et al., 2004; Kwan et al., 2006).

Figure 5.9 E shows the comparison between $\tau_{LL}$ and $\tau_{LG}$ at different pHs. Both $\tau_{LL}$ and $\tau_{LG}$ increase with decreasing pH, from $2.3 \pm 1.3$ s and $1.8 \pm 0.2$ s at pH 7.4, to $2.8 \pm 0.4$ s and $1.8 \pm 0.1$ s at pH 6.9, $2.9 \pm 0.4$ and $2.5 \pm 0.2$ s at pH 6.4, and $4.5 \pm 0.4$ and $4.4 \pm 0.4$ at pH 5.9, respectively. The values of $\tau_{LL}$ and $\tau_{LG}$ were not significantly different at each of the pHs tested. Moreover, they correlated significantly ($r = 0.99 \pm 0.08; p < 0.01$). These results show an intriguing correlation between the dwell time of the non-conducting states prior to channel
opening and that of an inactivated state visited after channel opening.

To test this idea that the long first latency gives rise to the slow rising phase shown in the macroscopic current, ensemble traces were generated from all the 6 s sweeps with long latency (group L) at either pH 6.4 (*Top row; n = 135 sweeps*) or 5.9 (*Bottom row; n = 389 sweeps*) and are shown in Figure 5.10. Indeed, the ensemble traces show a slow rising phase which, when fitted to a single exponential function, has a time constant of 1.10 s at pH 6.4 and 0.252 s at pH 5.9. These values are comparable to that shown in Figure 5.7 (1.52 s at pH 6.4 and 0.36 at pH 5.9). However, when the sweeps from groups S and L were added, the resulting ensemble traces did not show the slow rising phase (data not shown). It is uncertain how this discrepancy occurs (but see Discussion).

**Figure 5.10** Ensemble of idealized unitary current traces with long first latency at pH 6.4 and 5.9. The ensemble traces from all the unitary current traces with delayed (> 20 ms) first opening (*i.e.*, group S traces) at pH 6.4 (*top*) and 5.9 (*bottom*) are shown. The ensemble traces show a slow rising phase, and the red lines represent the best fit single exponential function to the data. The slow rising phase at pH 6.4 has a time course slower than that at pH 5.9, and the time constant at each pH is comparable to that for the macroscopic current. The dotted lines represent zero current.
In addition to the dwell time in the inactivated states that are related to the microscopic rate of recovery from inactivation, the burst duration, which at 100 mV is related to the microscopic rate of inactivation, was also determined. Figure 5.11 A-D shows the burst duration histograms at pH 7.4, 6.9, 6.4, and 5.9, respectively. As in Figure 5.6, all four duration histograms show a single component. However, whereas changing $[K^+]_o$ at pH 6.4 showed an inconsistent trend (Part I), reducing pH$_o$ at a fixed $[K^+]_o$ (0 mM) decreased the mean burst duration. The mean burst duration decreased from $0.53 \pm 0.02$ s at pH 7.4 to $0.40 \pm 0.01$ s at pH 6.9, $0.42 \pm 0.01$ s at pH 6.4, and $0.22 \pm 0.01$ s at pH 5.9 (Figure 5.11 E). The mean burst length at pH 6.9 was not significantly different from that at pH 6.4. However, from pH 7.4 to pH 5.9, the mean burst

![Figure 5.11 Mean burst durations of Kv1.5 at different external pH with 0 external K⁺.](image)

Histograms plotting burst duration at pH 7.4 (A), 6.9 (B), 6.4 (C), and 5.9 (D) were fitted to a single exponential distribution function with the indicated time constant. A burst is defined as a series of openings terminated by a closing event longer than 20 ms. The plot of the time constants versus pH$_o$ in E show that the burst length decreased with decreasing pH. The values of the mean burst lengths were $0.53 \pm 0.02$ s, $0.40 \pm 0.01$ s, $0.42 \pm 0.01$ s, and $0.23 \pm 0.01$ s at pH 7.4, 6.9, 6.4, and 5.9, respectively. The mean burst durations at pH 6.9 and 6.4 were not significantly different; otherwise, all values are significantly different from each other. Error bars represent standard deviation.
duration was significantly decreased, which was consistent with an acceleration of slow (P/C-type) inactivation observed at the macroscopic level. A single component in the burst duration histograms is consistent with the hypothesis that slow inactivation proceeds in a sequential manner, which is similar to the model proposed by Loots and Isacoff (1998). Alternatively, Kv1.5 may inactivate through multiple pathways with similar transition rates such that the peaks cannot be resolved unambiguously.

5.4 Discussion

In this study, unitary currents through Kv1.5 were recorded using long depolarizing pulses either at a fixed pH (6.4) with varying $[K^+]_0$ (0, 1, 3.5, and 20 mM) or with a fixed $K^+_0$ (0 mM) at various pH (pH 7.4, 6.9, 6.4, and 5.9) to determine the changes in microscopic gating (Figure 5.2 to 5.6) and the mechanism leading to the slow rising phase at pH 6.4 and 5.9 (Figure 5.8 to 5.11). The mean dwell times for the open and closed states within bursts were virtually unaltered by external $K^+$. That is, the transitions between the open state(s) and the “closed” states outside the normal activation pathway were largely independent of external $K^+$ and $H^+$. Instead, external $K^+$ decreased the probability of mode $U$ gating (Figures 5.5 A and 5.8 F), which was evident as an increased proportion of sweeps with short latency (< 20 ms, group S) and a reduction in the proportion of sweeps either with long latency ($\geq$ 20 ms, group L) or without open channel activity (group N). The sweeps with long latency were interpreted as channels switching from mode $U$ to mode $A$, and the null sweeps represented channels that stayed in mode $U$ or entered a more stable inactivated state from mode $U$. The mean long first latency ($\tau_{UL}$) correlated significantly with the mean long gap ($\tau_{UL}$) (Figures 5.4 E and 5.9 E), which suggested that the delayed opening is related to the dwell time in an inactivated state. This result is consistent with
the idea that channels in mode $U$ gating have undergone a slow inactivation process either at rest or from states visited during activation. The ensemble traces generated from all 6 s sweeps in group $L$ at either pH 6.4 and 5.9 with 0 mM $K^+$ resembled the slow rising phase observed at the macroscopic level (Figure 5.10), which suggested that recovery from mode $U$ could be the basis for the slow rising phase of current observed at low pH. That is, the slow rising phase may reflect the time dependence for recovery from one or more inactivated states. Together, these data show that mode $A$ and mode $U$ are linked by at least a common inactivated state and by a common closed state at rest (see Scheme 5.1 below).

### 5.4.1 External $K^+$ and mode $U$ gating

Similar to the classical slow (P/C-type) inactivation in *Shaker* (Lopez-Barneo *et al.*, 1993), mode $U$ gating is inhibited by external $K^+$. The molecular basis for this inhibition is unknown; however, if mode $U$ gating results from an outer pore inactivation process as proposed, a “foot-in-door” mechanism may also be involved.

Compared to its effect on Kv1.5, external $K^+$ plays a more direct role in regulating current amplitude in some other channels. Removing external $K^+$ has been shown to result in a partial or complete “conductance collapse” in Kv1.4, the large conductance calcium-activated potassium channel (BK), and the plant inward rectifier K channel KAT1 (Hertel *et al.*, 2005; Pardo *et al.*, 1992; Vergara *et al.*, 1999). In some *ShakerIR* mutants and Kv1.3, reducing external $K^+$ was proposed to inhibit current by decreasing channel availability due to inactivation occurring in closed state(s) (Jäger *et al.*, 1998; Lopez-Barneo *et al.*, 1993). This reduction in channel availability has clear parallels with the external $H^+$-induced current inhibition in Kv1.5, as seen in this and previous chapters. However, it is uncertain why this regulation of channel availability
by external $K^+$ is pH-dependent in Kv1.5 but not, apparently, in Shaker (Lopez-Barneo et al., 1993; Starkus et al., 2003). This question may be answered by swapping the outer pore of Kv1.5 with Shaker with progressively larger regions to determine if the outer pore is involved.

An unresolved issue during the testing of external $K^+$ was the unaltered single channel amplitude (Figure 5.3), which was expected to decrease with increasing $[K^+]_o$ due to the reduction in driving force. We cannot provide a satisfactory explanation for this result. In Kv2.1, the residue K356 was proposed to regulate the single channel conductance by adopting different conformations (Trapani et al., 2006); however, whether a similar regulation of the single channel conductance exists in Kv1.5 is uncertain. Additional experiments are needed to see whether the rectification property of Kv1.5 changes with $K^+_o$, as our data suggest the outward current is $K^+_o$-independent. In any case, the results in Figure 5.2 shows the single channel current amplitude was not significantly increased by $K^+_o$.

5.4.2 Mode $U$ or not mode $U$?

In Chapter 4, mode $U$ gating was proposed to explain the current inhibition induced by increasing external $[H^+]$. The use of a model incorporating two different gating modes was justified since the properties of the $H^+$-induced current inhibition satisfied the criteria of modal gating (Nilius, 1988). First, the two modes had distinct gating kinetics that could consistently be observed in single-channel patches. Second, the proportion of the two modes of gating in Kv1.5 could clearly be manipulated by changing external $H^+$. Third, the two modes occurred in different clusters with 150-ms test pulses repeated up to every 10 s. In this study, the first two criteria were clearly met. In addition, a transition from mode $U$ to mode $A$ could be clearly seen from the sweeps with long first latency, which was consistent with the criterion of a distinct.
gating behaviour in each mode. Therefore, the use of a gating model incorporating two modes is still consistent with the data shown in this chapter. An alternative interpretation for the long latencies is that the sweeps in group L may represent channels in another mode of gating. However, this view of a modal gating is more complex (3 modes instead of 2) but provided no additional insights into the gating of Kv1.5; therefore, this 3-mode model is not considered further.

5.4.3 Mode U gating and slow inactivation

One of the key observations in this Chapter is the correlation between $\tau_{LC}$ and $\tau_{LL}$. The simplest structural interpretation of this result is that the inactivation gate is closed when a channel is in mode U, such that the channel remains non-conducting even after S4 is activated and the activation gate is presumed to be open. This view of mode U is engendered in the gating model shown in Scheme 5.1, which is offered as a preliminary model of Kv1.5 gating at low pH and is adopted from gating models for Shaker (Olcese et al., 1997) and Kv1.5 (Kurata et al., 2001). This model was proposed to explain closed-state inactivation in the truncated form of Kv1.5. In Scheme 5.1, the top row represents mode A gating, whereas the bottom row represents mode U gating, and an inactivation process is represented by the downward arrows. That is, during mode A gating (top row), a channel can reach the open state ($O'$) through the normal closed states (C0 to C4), resulting in a burst of openings. ($O'$ in this model represents the composite of states within a burst including the non-conducting states outside of the activation pathway and the open states (Hoshi et al., 1994)). A burst of openings terminates when the channel enters an inactivated state (I). In contrast, at low pH, a closed inactivation process puts the channel in the closed-inactivated state (I0) in the bottom row (mode U), and upon
depolarization, the channel progresses through the closed-inactivated states ($I0$ to $I4$) to the inactivated state ($I$) that is shared between mode $U$ and mode $A$. With a sufficiently long depolarizing pulse, a channel may recover from state $I$ back to $O'$, which results in a delayed opening at the single channel level or the slow rising phase of current at the macroscopic level. It is possible that a channel may switch to the other row during activation (i.e., $C1 = II$, etc.), but the probability for these transitions is assumed to be very low based on the transition rates between mode $A$ and mode $U$ from both resting state and activated states; however, the rates for the vertical transitions from the pre-open states have not been determined explicitly from this study.

![Scheme 5.1](image)

**Scheme 5.1** A gating scheme proposed for the modal gating observed in Kv1.5. The top and bottom row represents mode $A$ and mode $U$ gating, respectively. Increasing [$H^+$]$_o$ promotes the transition to the bottom row, whereas increasing [$K^+$]$_o$ promotes the upward transition. The open state ($O'$) represent the composite of all the open and closed states within a burst. The inactivated state ($I$) also represents multiple inactivation processes. This gating scheme is adopted from Olcese *et al.* (1997) and Kurata *et al.* (2001).

Scheme 5.1 is a slight departure from early models of modal gating where no specific reference was given in relating how the states in each mode were connected to those in other modes (Hess *et al.*, 1984). However, in models of Ca$^{2+}$-mediated inactivation of the L-type Ca$^{2+}$ channel, two modes, Mode Normal and Mode Ca, were connected explicitly through all of the states in the activation pathway (Shirokov *et al.*, 1998; Tanskanen *et al.*, 2005). In particular,
Mode Ca consisted of Ca\(^{2+}\)-inactivated states with a very low probability of visiting the open state in Mode Ca (O\(_{Ca}\)), whereas gating in Mode Normal resulted in bursts of openings. In this light, Scheme 5.1 is considered to be a reasonable first approach to describing the modal gating in Kv1.5 at low pH.

Although Scheme 5.1 can explain the closed-inactivation of Kv1.5, it does not replicate all of the details for the depolarization-induced inactivation. For example, state I in this model is an oversimplification of the inactivation process, which is shown to have at least two components, \(I_{SG}\) and \(I_{LG}\), with mean dwell times \(\tau_{SG}\) and \(\tau_{LG}\), respectively. Since the inactivated state \(I_{SG}\) does not show a corresponding component in the first latency histogram, it is likely not connected directly to mode \(U\) (i.e., the bottom row in Scheme 5.1). Moreover, the burst duration histogram contains only a single exponential component, which suggests there may only be a single inactivation process that terminates a burst. Alternatively, there may be multiple processes with similar kinetics that can terminate a burst. Unfortunately, the connection between the inactivated states and the states within bursts cannot be determined unequivocally from the limited data presented here. In addition, the assumption that \(\tau_{LG}\) represents the dwell time of one inactivated state is possibly an oversimplification; that is, \(\tau_{LG}\) may also represent the mean duration spent in more than one state.

5.4.4 Molecular basis for the slow rising phase

Based on our interpretation of mode \(U\) gating, the slow rising phase is assumed to reflect recovery from inactivation. The concept of recovery (from inactivation) during depolarization may at first seem unusual, but this is an innate ability of Kv1.5 channel even under normal physiological conditions. For example, at pH 7.4 the steady-state current is about 50% with a 6 s
pulse (Figure 5.7), which suggests the inactivated state is non-absorbing and inactivated channels can recover from an inactivated state during a depolarizing pulse. It is possible that, given a large number of inactivated channels at the start of a depolarizing pulse (e.g., low pH with 0 mM $K^+$), a significant number of them recover to the open state to give rise to an apparent slow rising phase. However, the data presented here do not exclude other possibilities, such as slow activation, as the basis for this slow rising phase.

A number of unresolved issues presented in this chapter warrant caution in our preliminary interpretation of the molecular basis for the slow rising phase. For example, although the ensemble of traces in group $L$ shows a slow rising phase, the ensemble average of group $S$ and $L$ sweeps did not show a slow rising phase (data not shown). It is unclear what underlies this discrepancy. The use of different voltages between the macroscopic data and the single channel data has been ruled out as a possibility, as unitary currents recorded from 60-mV or 100-mV did not show a substantial difference that would result in the absence of the slow rising phase (data not shown). One possibility is that in the cell-attached mode, fewer channels show group $L$ behaviour (or recovery from inactivation). In whole-cell recordings, increasing the apparent rate of slow inactivation by increasing intracellular $\text{Mg}^{2+}$ and/or $\text{Ca}^{2+}$ can inhibit the slow rising phase (data not shown). It is possible that the extent of channels recovering from either mode $U$ gating or from inactivated states was smaller than that going into an inactivated state with cell-attached patches. This is similar to the absence of slow rising phase at pH 7.4 with 0 mM $K^+$ even though both group $S$ and $N$ sweeps were present; that is, the proportion of group $S$ was too small to show the slow rising phase. To minimize the variability, both unitary current and macroscopic current should be recorded in the outside-out configuration so that a direct comparison can be made.

Another issue with the ensemble traces shown in Figure 5.10 was the apparent discrepancy
between the mean long first latency and the time course of the slow rising phase. The mean long latency ($\tau_{LL}$) became longer with decreasing pH, yet the time course of the slow rising phase was faster at pH 5.9 compared to that at pH 6.4. A preliminary explanation for the difference between $\tau_{LL}$ and the time constant of the slow rising phase is the possible involvement of the burst duration during the slow rising phase. The slow rising phase may be interpreted as an interplay between channels inactivating and channels coming out from a non-conducting state. At pH 5.9, the burst duration was short (Figure 5.11 D and E) and $\tau_{LL}$ was relatively long; therefore, only a small proportion of channels with relatively short latency contributed to the rising phase, while the channels with longer latencies made up the quasi-steady-state current. In contrast, in the case of pH 6.4, the mean burst duration was longer and the mean latency was shorter. Upon depolarization, a larger proportion of channels, even those with longer first latencies, contributed the rising phase before channels opening with a shorter first latency started to close. Therefore, the time course of the slow rising phase at pH 6.4 was slower than that at pH 5.9. In an extreme hypothetical situation, in which the burst length is much longer than the mean (long) first latency, the time constant for the slow rising phase would be the same as that of the first latency (i.e., the ensemble becomes the cumulative first latency graph). Together, it is believed that the correlation between first latency and recovery from inactivation is genuine, and that recovery from a slow inactivation process underlies the slow rising phase observed at low pH.

To summarize, the mechanism by which external K$^+$ antagonizes the H$^+$-induced current inhibition in Kv1.5 was shown to result from an inhibition of mode $U$ gating. With long depolarizing pulses (> 1 s), some sweeps (group L) showed delayed opening that was interpreted to reflect channels “recovering” from mode $U$ gating into mode $A$. Furthermore, this long latency
correlated with a depolarization-induced inactivated state (long gap), which suggested a common inactivated state visited by both mode $U$ and mode $A$. This delayed opening may also result in the slow rising phase at low pH with 0 external $K^+$, suggesting a link between recovery from slow inactivation and the slow rising phase.

Acknowledgement

This study was supported by a grant from the Canadian Institutes of Health Research to S.J.Kehl and D.Fedida. We thank Fifi Chiu for preparing the cells. D.C.H.K. was in receipt of a Doctoral Trainee Award from the Michael Smith Foundation for Health Research.
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6. Slow inactivation in Kv1.5

The molecular mechanism for the external H\(^{+}\), Zn\(^{2+}\), and Ni\(^{2+}\)-induced inhibition of human Kv1.5 (\(h\text{Kv1.5}\), or simply Kv1.5) current has been investigated. Some of the changes in the biophysical properties of Kv1.5 induced by these ions are listed in Table 6.1, and the major findings that relate to slow inactivation are summarized and discussed in the following section.

6.1 Mechanism for the external cation-induced current inhibition

In a previous study, Kv1.5 current was shown to be inhibited by external Zn\(^{2+}\), and this inhibition was antagonized allosterically by external K\(^{+}\) (Zhang et al., 2001b). In search of the binding site for Zn\(^{2+}\) that mediates this inhibition, a histidine residue (H463) in the turret came to our attention (Steidl and Yool, 1999). When Kv1.5 currents were recorded at low pH, a prominent inhibition was observed, and we showed that this inhibition was also antagonized by external K\(^{+}\) in an allosteric fashion (Figures 2.2-2.4). The similarities between the effects of H\(^{+}\) and Zn\(^{2+}\) suggested that the mechanism by which these ions inhibited Kv1.5 was similar and possibly involved the same binding site.

To test further whether H463 mediated the Zn\(^{2+}\)- and H\(^{+}\)-induced current inhibition in \(h\text{Kv1.5}\), H463 was mutated to glutamine (H463Q), as the equivalent mutation in \(r\text{Kv1.5}\) (H452Q) was found to reduce H\(^{+}\)-sensitivity (Steidl and Yool, 1999). As expected, this mutation attenuated current inhibition induced by these ions and shifted the concentration-response curves for both H\(^{+}\) and Zn\(^{2+}\) to the right (Figure 2.6). Other ligands for histidine, such as Ni\(^{2+}\), were also shown to inhibit Kv1.5 (Chapter 3) and this effect was similarly attenuated by the H463Q mutation. These results were consistent with H463 being the binding site for H\(^{+}\), Zn\(^{2+}\), and Ni\(^{2+}\) to inhibit Kv1.5.
Table 6.1 Comparison of the effects of external Zn\(^{2+}\), Ni\(^{2+}\), and H\(^+\) in Kv1.5. Listed below are some of the biophysical parameters measured either in control condition (pH 7.4, 3.5 mM K\(^+_o\)) or with Zn\(^{2+}\), H\(^+\), or Ni\(^{2+}\). Values for \(V_{1/2}\) and slope factor (\(k\)) were obtained by fitting the corresponding date to a Boltzmann function. The time constants for activation (\(\tau_{\text{activation}}\)), deactivation (\(\tau_{\text{deactivation}}\)), inactivation (\(\tau_{\text{inact}}\)), and recovery (\(\tau_{\text{recover}}\)) were measured by fitting the data with single exponential functions (see the Method sections in Chapter 2 and 3 for detail). Availability is defined as the proportion of sweeps (duration ≤ 1 s) showing channel activity.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Zn(^{2+})</th>
<th>H(^+)</th>
<th>Ni(^{2+})</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Macroscopic current properties</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Activation</td>
<td></td>
<td></td>
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<tr>
<td>(V_{1/2})</td>
<td>-10.2 ± 0.4 mV</td>
<td>+21.1 ± 0.7 mV (1 mM) (^1)</td>
<td>+3 ± 4 mV (pH 6.4)</td>
<td>0.4 ± 0.9 mV (0.25 mM)</td>
</tr>
<tr>
<td>slope factor ((k))</td>
<td>6.8 ± 0.4 mV</td>
<td>9.4 ± 0.7 mV (1 mM) (^2)</td>
<td>9.2 ± 0.3 (pH 6.4)</td>
<td>No change (0.25 mM)</td>
</tr>
<tr>
<td>(\tau_{\text{activation}})</td>
<td>1.76 ms (+50 mV)</td>
<td>16.9 ms (1 mM; +50 mV) (^2)</td>
<td>2.3 ± 0.4 ms (pH 5.9; +60 mV)</td>
<td>No change (0.25 mM)</td>
</tr>
<tr>
<td>Deactivation</td>
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<tr>
<td>(\tau_{\text{deactivation}})</td>
<td>15.1 ms (-40 mV)</td>
<td>9.1 ms (-40 mV; 1 mM) (^2)</td>
<td>11.0 ± 0.4 ms (-50 mV, pH 5.9)</td>
<td>No change (0.25 mM)</td>
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<td>Current inhibition</td>
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<tr>
<td>(K_D)</td>
<td>69 μM (0 mM K(^+_o)) (^2)</td>
<td>153 ± 13 nM (pK(_{1/2}) = 6.8, 0 mM K(^+_o))</td>
<td>150 ± 10 μM (0 mM K(^+_o))</td>
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<tr>
<td>Hill coefficient</td>
<td>0.892</td>
<td>1.5 ± 0.2</td>
<td>1.3 ± 0.1</td>
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</tr>
<tr>
<td>K(^+_o)-dependence ((K_D))</td>
<td>~500 μM (^2)</td>
<td>680 ± 90 μM</td>
<td>~1.0 mM (est.)</td>
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<tr>
<td>Inactivation</td>
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<tr>
<td>(\tau_{\text{inact}}) (+50 mV)</td>
<td>2.63 ± 0.11 s (5 mM K(^+))</td>
<td>2.14 ± 0.16 s (1 mM, 5 mM K(^+_o)) (^2)</td>
<td>1.19 ± 0.04 s (pH 6.4, 5 mM K(^+_o))</td>
<td>1.71 ± 0.07 s (1 mM, 3.5 mM K(^+_o))</td>
</tr>
<tr>
<td>(\tau_{\text{recover}}) (-80 mV)</td>
<td>4.3 s</td>
<td>27.7 ± 2.1 s (2 mM) (^2)</td>
<td>4.2 ± 0.1 s (pH 5.4)</td>
<td>23.5 ± 2.1 s (2 mM)</td>
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<tr>
<td>Gating current properties</td>
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<tr>
<td>(Q_{\text{max}})</td>
<td>1</td>
<td>0.85 (1 mM) (^1)</td>
<td>1 (pH 5.5)</td>
<td>1 (1 mM)</td>
</tr>
<tr>
<td>(V_{1/2}) (Q(_{1/2}))</td>
<td>-2.2 mV</td>
<td>60.2 ± 1.5 mV (1 mM) (^1)</td>
<td>48.9 ± 1.2 mV (pH 5.4)</td>
<td>2.2 ± 0.8 mV (1 mM)</td>
</tr>
<tr>
<td>Single channel properties</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single channel current / conductance</td>
<td>1.7 pA (+100 mV) / 11.8 pS</td>
<td>ND (^3)</td>
<td>1.7 pA (+100 mV) / 11.3 pS</td>
<td>1.6 pA (+100 mV) / ND (^3)</td>
</tr>
<tr>
<td>Availability</td>
<td>0.97</td>
<td>ND (^3)</td>
<td>0.18 (pH 5.9)</td>
<td>0.3 (0.5 mM)</td>
</tr>
</tbody>
</table>

\(^1\)From Zhang et al. (2001a)
\(^2\)From Zhang et al. (2001b)
\(^3\)Not Determined
After showing the binding site for these cations, the next step was to deduce the mechanism for the current inhibition. Based on the KcsA structure, the distance between H463 and the central axis of the pore was ~14-16 Å, which was probably too far for H⁺ to occlude the conducting pore directly (Aiyar et al., 1995; Doyle et al., 1998). Therefore, we surmised that H⁺, and possibly Zn²⁺, probably did not cause a fast block in Kv1.5. Conversely, the inhibition induced by Zn²⁺, H⁺, and Ni²⁺ showed a property that was similar to the classical slow (P/C-type) inactivation, namely, inhibition by external K⁺. We tested this idea by measuring the current inhibition in the mutant in which the outer pore residue R487 was changed to valine (R487V), as the homologous mutation in Shaker (T449V) was shown to inhibit slow inactivation (Lopez-Barneo et al., 1993). Indeed, the current inhibition was dramatically antagonized by this mutation (Figure 2.8 and 3.5), which suggested that a slow inactivation process was involved in the cation-induced current inhibition.

A number of findings support a conclusion that this slow inactivation process proceeded from resting closed states rather than from depolarized/activated states. First, the current inhibition induced by H⁺ or Ni²⁺ was “instantaneous;” that is, the extent of inhibition remained the same after a complete solution exchange to that at low pH or with Ni²⁺ (Figures 2.9 and 3.3). Second, at concentrations that produced roughly the same amount of inhibition, the kinetics of the onset of, and recovery from, slow inactivation were still much faster at low pH than with metal divalent cations (Figures 3.4 and 3.9; Table 6.1). This result was inconsistent with the hypothesis that the current inhibition resulted from channels that failed to recover from an inactivated state in the interval between pulses. Third, the maximum gating charge movement ($Q_{max}$) was unaltered by H⁺ (Figure 2.10) and Ni²⁺ (Figure 3.7), which suggested that the activation machinery was largely unaffected by these ions even under conditions where a large
proportion of channels were unable to conduct. Based on these results, it was hypothesized in Chapter 3 that the binding of cations to H463 promoted a closed-inactivation process which resulted in a reduction in the number of channels available for activation and hence a reduction in the macroscopic current amplitude. A similar hypothesis of reduction in channel availability was proposed to explain the reduction in current amplitude with lowering external K⁺ in fast-inactivating Shaker mutants (Lopez-Barneo et al., 1993) and in Kv1.4 (Pardo et al., 1992).

To gain some mechanistic insights into the cation-induced current inhibition, unitary currents were studied. The above hypothesis of a reduction of channel availability predicted that the number of blank (null) sweeps should be increased. This prediction was confirmed both with Ni²⁺ (Figure 3.6) and with H⁺ (Figures 4.1 and 4.2). In addition, the proportion of null sweeps correlated well with the extent of current inhibition (Figure 4.3). At the same time, the single channel current amplitude was unaltered by either Ni²⁺ or H⁺. The latter result directly refuted the hypothesis that H⁺ and the divalent cations inhibit macroscopic Kv1.5 current by a “fast block” mechanism. Furthermore, the unaltered gating behaviour within bursts (Figure 4.6) is inconsistent with the hypothesis that H⁺ acts as an “intermediate” block (increased flickering within a burst and prolonged burst length). Together, the single channel results show that H⁺, and possibly Zn²⁺ and Ni²⁺, act as “slow blockers” that modify channel gating.

The change in the gating behaviour induced by H⁺ was proposed to be a promotion of Kv1.5 from an available mode (mode A) of gating to an unavailable mode (mode U) of gating. During mode A gating, a channel can open and conduct current (giving rise to active sweeps), whereas during mode U gating, a channel remains non-conducting despite normal gating charge movement (resulting in null sweeps). In other words, mode U gating was suggested to result from the previously proposed closed-inactivation process which underlies the cation-induced
current inhibition. Based on this modal gating model, conditions that attenuate the H⁺-induced current inhibition, such as increasing external [K⁺], were predicted to inhibit mode U gating. This was confirmed by an increase in the proportion of sweeps in mode A with increasing [K⁺] (Figure 5.5). By using long depolarizing pulses (≥ 6 s), some channels were seen to switch from mode U back to mode A, which appeared as sweeps showing long first latencies. That is, the long first latencies represented the duration that a channel spent in one or more states of mode U during a depolarizing pulse. The mean long latency was found to correlate with the mean long gap duration between bursts, and the latter value was taken to represent the mean dwell time in some inactivated states during a depolarizing pulse. Together, these results suggest mode A and mode U are connected through some depolarization-induced inactivated states, and support the hypothesis that a closed-inactivation process with many of the properties of slow inactivation underlies mode U gating.

6.2 Implications of slow inactivation gating

For the remainder of this chapter, the term slow inactivation is used to describe the process by which the outer pore becomes constricted, without regard to the particular state from which this process occurs. Conversely, the term depolarization-induced inactivation is used to describe the inactivation process that proceeds from any states within in a normal burst of openings during a depolarizing pulse. These terms are so defined in hope of avoiding any confusion in the following discussion of the possible mechanism for the cation-induced current inhibition and mode U gating.
6.2.1 Mode $U$ gating versus U-type inactivation

Based on the proposed mechanism for mode $U$ gating, it is worthwhile to compare it with other known closed-inactivation processes. U-type inactivation is proposed to occur from closed states, and it is defined by a number of biophysical properties (Klemic et al., 2001). The most obvious one is the “U-shaped” inactivation curve, from which the term “U-type” is derived. However, U-type inactivation seems to have little in common with mode $U$ gating. For example, a U-shaped inactivation curve is not observed with the current inhibition induced either by $H^+$ (Figure 2.2), $Ni^{2+}$ (Figure 3.1), or $Zn^{2+}$ (Zhang et al., 2001b) in Kv1.5, suggesting that U-type inactivation was not involved. In addition, although it can occur in truncated Kv1.5 (Kurata et al., 2002), U-type inactivation has never been shown with full-length Kv1.5; conversely, mode $U$ is shown in wt Kv1.5. A second property of U-type inactivation is its sensitivity to the holding potential (Klemic et al., 2001); that is, by changing the holding potential from -80 to -30 mV, more channels dwell in the ‘pre-open” states from which the transition to U-type inactivated state is faster, and hence more channels are inactivated. However, most experiments performed with regard to the $H^+$-induced current inhibition were done at a holding potential of -80 mV, yet mode $U$ was consistently observed. In addition, at pH 5.9 with 0 mM $K^+$, raising the holding potential to -30 mV had no effect on the $H^+$-induced current inhibition (personal communication, Dr. S. J. Kehl). Both of these results are inconsistent with an involvement of U-type inactivation. A third property of U-type inactivation is the “reversed” sensitivity to external TEA$^+$ and $K^+$, both of which promote U-type inactivation (Klemic et al., 1998; Klemic et al., 2001; Kurata et al., 2005). However, mode $U$ gating was inhibited by $K^+_o$ (Figure 5.5). A fourth property is the “excessive cumulative inactivation,” which arises because repetitive pulsing results in a greater extent of inactivation (Aldrich et al., 1983; Klemic et al., 2001). Repetitive pulsing does not
increase the extent of the H\(^+\)-induced current inhibition (Figure 2.9). Together, the properties of the cation-induced current inhibition are inconsistent with that of U-type inactivation and point to a distinct form of closed-inactivation process in mode \( U \) gating. Is this closed-state inactivation related to slow (P/C-type) inactivation?

### 6.2.2 Slow inactivation from closed states

The concept of an inactivation process proceeding from the resting closed state is not new. It has been used to explain the reduction in channel availability in various Kv channels (Lopez-Barneo et al., 1993; Pardo et al., 1992; Steidl and Yool, 1999; Teisseyre and Mozrzymas, 2006; Yang et al., 1997), and a P-type inactivation process may be involved. P-type inactivation was originally proposed to be distinct from C-type inactivation based on the finding that external TEA\(^+\) increased the current amplitude in addition to causing a slowing of the time-dependent decay in the chimeric Kv2.1-3.1 V369S mutant (De Biasi et al., 1993). In that report, a stabilization of transitions from an inactivated state to both the resting closed state and the open state was proposed to account for the paradoxical potentiation of the peak current by low concentrations of TEA\(^+\). That is, some channels proceeded directly from the closed state to the inactivated state, and the recovery of these channels into the open state gave rise to the potentiation. This is similar to our hypothesis that the potentiation of current during the slow rising phase of Kv1.5 current at low pH with 0 mM K\(^+\) (Figure 5.7) is due to channels recovering from mode \( U \) gating and/or an inactivation process.

Another possible example of P-type inactivation proceeding from closed state is the Shaker W434F mutant (Yang et al., 1997). With more subunits expressing phenylalanine at position 434 in a concatameric construct, the rate of inactivation became faster, and when all the
W434s were mutated to phenylalanine, the channel was predominantly inactivated. However, the W434F homomeric mutant was shown to have normal gating charge movement (Perozo et al., 1993), which suggests the mutant either inactivates from closed states or the depolarization-induced inactivation rate exceeds the resolution of the recording system. In the Shaker1R 434WFWF concatamer, active sweeps and null sweeps were seen to be clustered. In addition, the intraburst behaviour was unaltered by the mutations. The above observations were surprisingly similar to that shown for the H⁺-induced inhibition presented in this dissertation (Figures 2.10, 4.3, 4.4 and 4.6). This parallelism argues for a similar mechanism underlying both the non-conducting W434F mutation and the cation-induced current inhibition; that is, a P-type inactivation process. However, as conceded by Yang et al. (1997), we cannot rule out the possibility of ultra-short bursts that are not resolved because of the limited response time of our recording system.

The model of a slow inactivation process involved in the cation-induced current inhibition is very attractive based on the comparison between our data and previous studies. However, this interpretation appears to be inconsistent with some of the properties ascribed to mode U gating and depolarization-induced slow inactivation. The most obvious difference between the two is their K⁺-dependence (Chen et al., 1997). In fact, compared to the depolarization-induced inactivation, mode U gating is more similar to the “typical” slow (P/C-type) inactivation; that is, mode U gating is inhibited by external K⁺ and (probably) by the R487V mutation, whereas the depolarization-induced inactivation is relatively insensitive to both K⁺,o and the R487V mutation. In addition, the K₉ for the K⁺ effect on slow inactivation in Shaker (1 mM) estimated by Baukrowitz and Yellen (1996) was very similar to the K₉ for the K⁺ effect on the current inhibition induced by Zn²⁺, H⁺, and Ni²⁺ (0.5-1 mM; Figures 2.3 and 3.2). We do not yet have a
satisfactory explanation for why external K\(^+\) and R487V do not inhibit the depolarization-induced inactivation in Kv1.5 under physiological conditions. However, external K\(^+\) did not attenuate slow inactivation in the *ShakerIR* 434WWWWW concatamer (the residue at position 434 was tryptophan in each of the four subunits) expressed in *Xenopus* oocytes (Yang *et al.*, 1997). This phenomenon was proposed to result from K\(^+\) accumulation at the pore mouth. Yet, the current traces recorded with 0 and 1 mM K\(^+\) consistently show significant differences (*e.g.*, Figure 5.1), suggested that the accumulation of K\(^+\) at the outer pore was minimal and hence could not be the reason for the K\(^+\)-insensitivity. Similarly, the *Shaker* T449V mutant expressed in HEK-293 cells, as opposed to *Xenopus* oocytes, was found to have an inactivation rate similar to that of *wt Shaker* (Holmgren *et al.*, 1996). These results suggest the regulation of slow inactivation may be more complicated than once thought, and these phenomena may warrant additional attention in the future.

6.2.3 Possible mechanism for closed state inactivation

A number of results shown in this dissertation and in other studies are inconsistent with the dogma that slow inactivation is coupled to the open state. That is, closed state inactivation may arise by an uncoupling of the inactivation machinery from the activation apparatus. This idea is consistent with the result that the kinetics of the *on*-gating charge and \(Q_{\text{max}}\) are unaltered by external H\(^+\) (Figures 2.10 and 3.7), which suggests S4, and possibly the activation gate, is functional even in channels exhibiting mode \(U\) gating. This view of a possible uncoupling between the activation and inactivation machineries is supported by the uncoupling of gating charges and recovery from slow inactivation in Kv1.5 reported by Wang & Fedida (2002), in which the kinetics of gating charge recovery is faster than the kinetics of recovery from slow
inactivation, suggesting, at least during recovery, that the movement of the gating charges (and the activation gate?) can be uncoupled from the movement of the slow inactivation gate. As mentioned, P-type inactivation has been suggested to proceed from closed states, at least in mutated channels (De Biasi et al., 1993; Yang et al., 1997). Furthermore, it should be noted that depolarization is not required for Kv1.5 to be in mode U (i.e., closed-inactivated; Figures 2.9 and 4.1), which suggested that this inactivated state did not result from channels failing to recover from slow (P/C-type) inactivation as in the case in Kv1.4 at low pH. However, the physical basis for the coupling between slow inactivation and activation is currently unknown, so a coherent model cannot be constructed. Given its role in activation, S4 is expected to play a role in the coupling, but the details remain unknown.

6.2.4 Possible interactions between H463 and the outer pore

Compared to the physical coupling between inactivation and activation, the interaction between the turret and slow inactivation is clearer. At the very least, the binding of H⁺ or other cations to H463 appears to result in a conformational change involving R487. From this relationship, the most obvious question is how these two residues might interact. A direct electrostatic interaction between H463 and R487 has been considered in Chapter 2, but based on the finding that reducing ionic strength has no effect on the extend of current inhibition (not shown), a direct electrostatic interaction between the two residues has been deemed unlikely. From the KcsA structure (Doyle et al., 1998), the distance between the α-carbons (Cα) of Q58 (homologous to H463) and Y78 (homologous to R487) is approximately 8 Å, but their side chains are much further apart (~12 Å) and point in different directions. Therefore, a direct interaction between H463 and R487 is not favoured.
Based on the KcsA structure, H463 may interact with two regions. The first one is the pore helix, which has been implicated in slow inactivation, with the best example being Shaker W434 (or Kv1.5 W472). As mentioned, the effect of the W434F mutation on Shaker is very similar to the effect of H⁺ on Kv1.5, which raises the possibility that a similar mechanism is involved. The side chain of W434 is proposed to form a “hydrophobic cuff” that interacts with the side chains of the tyrosine residue in the selectivity filter (Y76) thus holding it in the open conformation (Doyle et al., 1998). Changing this tryptophan residue to phenylalanine removed this interaction, and the channel became permanently P-type inactivated (Yang et al., 1997).

Similarly, any movement of the pore helix may also weaken the wt W67-Y78 interaction. Since the pore helix is likely to move as a rigid rod (as with other α-helical structures), any movement along the length of the pore helix may result in significant movement at W67. In Shaker, mutating the aspartate residue at position 431 (at the top of the pore helix) to asparagine (D431N) accelerates slow inactivation slightly (Loots and Isacoff, 2000). When mapped onto the KcsA structure, this residue is approximately 1 “turn” away from W434 and is proposed to interact with the turret residue at position 424 (Loots and Isacoff, 2000), which is homologous to Kv1.5 T462. In addition, the side chain of this residue (D469 in Kv1.5) is about 6 Å away from that of Kv1.5 H463. The charge on H463 may interact with that on D469, and such interaction may affect the tilting/orientation of the pore helix at the N-terminal end, resulting in the breaking of a hydrophobic interaction between W472 and Y483 and hence P-type inactivation.

Another region with which H463 may interact is a short stretch of polypeptide between the selectivity filter and the start of the P-S6 linker. Based on the KcsA structure, the side chain of H463 is only ~5 Å away from the side chain of a methionine (M486) between a conserved aspartate (D485) and R487. In Shaker, mutation of M448 (homologous to Kv1.5 M486) to
cysteine has been shown to accelerate slow inactivation in the presence of Cd\textsuperscript{2+} (Liu et al., 1996). M448, along with T449 and P450, has been suggested to move during slow inactivation (Liu et al., 1996), and it is possible that a conformational changes of any one of these residues could affect the orientation of the side chain of the other two residues. In addition, the side chains of these residues seem to be inaccessible to MTS reagents applied from the external milieu during slow inactivation, and Liu et al. (1996) envisioned the side chains of T449 may "flip" and become exposed to the external milieu. In a similar vein, the side chain of D80 in KcsA (homologous to Kv1.5 D485 or Shaker D447) has indeed been shown to "flip" or rotate about 180°, and it has been proposed that a large rotation about the polypeptide backbone of the residues in this outer pore region is possible. It is conceivable that a protonated Kv1.5 H463 may affect the conformational change of M486 or other neighbouring residues and thus accelerate slow inactivation.

6.2.5 Gating within bursts and depolarization-induced inactivation

Besides mode $U$ and closed state inactivation, the states within bursts and the depolarization induced inactivated states were also studied. The single channel analyses in both Chapters 4 and 5 have consistently shown that Kv1.5 has 2 open states and 3 non-conducting states within bursts. In Shaker, only 1 open state was observed, but 3 non-conducting states were found within bursts (Hoshi et al., 1994). It is uncertain if the presence of two functional open states in Kv1.5 is significant, as both of these states appear to have the same conductance. It is possible the open state with shorter dwell time may be an artifact arising from the flickery behaviour of Kv1.5. The mean dwell time for the three non-conducting states were similar between Kv1.5 and Shaker, suggesting their pore structures are very similar. Unfortunately, no
physical basis for the "closed" states outside the normal activation pathway is known, and the connections between these states and the open state(s) are unknown. It is also unclear whether these closed states are involved during depolarization-induced inactivation, and the study of these state may require additional structural information and/or some methods to separate these state from one another.

To my knowledge, no report has investigated depolarization-induced inactivation in detail at the single channel level; therefore, a direct comparison between the results found in this study and others is not possible. Two depolarization-induced inactivated states ($I_{SG}$ and $I_{LG}$) were identified from the gap duration histograms shown in Chapter 5 (Figures 5.4 and 5.9). In addition, given a mean dwell time of $\sim 4$ s for $I_{LG}$, another inactivated state ($I_N$) should be included to account for the null sweeps lasting for more than 60 seconds. If mode $U$ gating results from a process other than a failure of recovery from inactivation, $I_N$ is likely not connected directly to any closed-inactivated states (10-14). However, how $I_N$ is connected to the other inactivated states is unclear. These three inactivated states may represent three different steps in slow (P/C-type) inactivation. For example, since mode $U$ gating was only seen to connect to $I_{LG}$, this state may represent a P-type inactivated state. If this is the case, the inactivated state $I_N$ may represent a C-type inactivated state, since the time course of C-type inactivation, as judged by charge immobilization, is slower than P-type inactivation (Loots and Isacoff, 2000). However, it is uncertain what process underlies $I_{SG}$.

In summary, the binding of H$^+$ and other cations to H463 promotes a closed state inactivation resembling P-type inactivation. This effect may result from a conformational change of the pore helix and/or the outer pore that uncouples the inactivation machinery from the activation apparatus. However, several issues with regard to the coupling between activation and
slow inactivation have not yet been resolved. More direct structural information on the
conformational changes during closed-state and open state inactivation is needed to show how if
they are related.

6.3 Comparison of H\(^+\)-induced effects in Kv1.5 and in other channels

In addition to Kv1.5, a number of Kv channels are sensitive to changes of the extracellular
pH. The binding sites for H\(^+\)\(_o\) in these channels are mainly in the pore domain versus the turret
region. Comparing the effects of H\(^+\) between that in Kv1.5 and that in other channels may
provide additional insights into the modulation of slow inactivation and other biophysical
processes.

6.3.1 External H\(^+\) modulation of Shaker

One of the standards in the study of Kv channels, especially in the context of slow
inactivation, is Shaker, and in particular, the N-type inactivation removed ShakerIR channel. In
ShakerIR, the current amplitude was reduced by 25\% at pH 5.0 in 2.5 mM K\(^+\)\(_o\) \((pK_H = 4.7)\), an
effect that coincided with an acceleration of slow inactivation (the time constants were \(~1.64\ s\) at
pH 7.4 and \(~190\ ms\) at pH 5.5) (Starkus et al., 2003). The inhibition of current at low pH in
ShakerIR was roughly 1.5 orders of magnitude less sensitive to external H\(^+\) than wt Kv1.5 but
similar to that of the H463Q \((pK_H = 5.3)\) and R487V \((pK_H = 4.6)\) mutants. A comparison of the
turret of Kv1.5 and ShakerIR reveals that the site homologous to Kv1.5 H463 in Shaker is F425,
which does not constitute a H\(^+\) binding site. There are four acidic residues (E418 and E422 in the
turret, D431 in the pore helix, and D447 in the outer pore mouth) near the external surface that
might act as H\(^+\) binding sites, and Starkus et al. (2003) proposed the outer pore aspartate (D447)
residue as the H\(^+\) binding site as this residue was proposed to modulate slow inactivation (Olcese et al., 1997). In contrast to our result with Kv1.5, the authors proposed an acceleration of slow inactivation was primarily involved in the inhibition of peak current. This idea was supported by the finding that the effects of H\(^+\) in ShakerIR were antagonized but not eliminated by the outer pore mutation T449V (R487V in Kv1.5).

The results of the H\(^+\)-induced current inhibition in ShakerIR might be relevant to our findings in the Kv1.5 H463Q and R487V mutants. Neither of these mutations eliminated the H\(^+\)-induced current inhibition but instead shifted the concentration-response curve to the right (Figures 2.6 and 2.8). This latter result suggests a second, lower-affinity, binding site for H\(^+\) exists in Kv1.5. Given a similar sensitivity to external H\(^+\) in ShakerIR and in these Kv1.5 mutants, a common mechanism may be involved in both channels. This “second” mechanism may be studied in the Kv1.5 mutants at low pH by recording their unitary currents.

6.3.2 External H\(^+\) modulation of Kv1.4

The Shaker-related Kv1.4 channel, which exhibits intrinsic N-type inactivation, also shows an external H\(^+\)-dependent current inhibition (Claydon et al., 2000; Claydon et al., 2002; Ishii et al., 2001). Alignment of the Kv1.5 and the Kv1.4 sequence (Figure 1.3 A) shows the turret, the P-loop, and the P-S6 linker of the two channels are very similar. There is a histidine residue (H508) at the site homologous to Kv1.5 H463, and a positively-charged lysine residue (K532) is located at the site homologous to Kv1.5 R487. The close resemblance of these two channels, at least around the pore mouth, suggests that Kv1.4 might behave like Kv1.5. Indeed, as with Kv1.5, mutating H508 to glutamine (H508Q) or K532 to tyrosine (K532Y) antagonizes the H\(^+\)-induced current inhibition (Claydon et al., 2000). However, the effects of H\(^+\) in Kv1.4
differ from those in Kv1.5 in that the current inhibition was frequency dependent, arising in large part because of a slowing of recovery from inactivation (Claydon et al., 2000). These results suggested, in contrast to those in Kv1.5, that activation of the channel was required for the current inhibition by $H^+$. Furthermore, in the N-terminal deleted Kv1.4 mutant, lowering external $H^+$ accelerated slow inactivation without causing a significant current inhibition (Claydon et al., 2002), and when either the H508Q, H508C, H508E, or K532Q mutation was introduced into the N-terminal deleted mutants, the time course of slow inactivation became insensitive to external pH between pH 8.5 and 6.5. These results suggested that even though H508 could act as a pH sensor and K532 was probably a component of the effector mechanism, binding of $H^+$ to H508 did not result in an closed-inactivation dependent current inhibition, at least at a pH $\geq$ 6.5. It is uncertain whether reducing extracellular pH further (e.g. 5.5) will induce any changes in the inactivation kinetics as in Kv1.5 or ShakerIR. Together, the $H^+$-induced current inhibition in Kv1.4 was mechanistically very different from that in Kv1.5, and the molecular basis for these surprising differences remains unknown.

### 6.3.3 External $H^+$ modulation of KCNQ2/KCNQ3

The heteromeric KCNQ2/KCNQ3 channel is a voltage-gated channel that is proposed to be the molecular correlate of the neuronal M-current (Wang et al., 1998). It does not show slow inactivation, but external $H^+$ inhibits the channel with a $pK_H$ of 6.68 (Prole et al., 2003), and this inhibition showed some similarities to that of Kv1.5. First, increasing external $K^+$ antagonizes the $H^+$-induced current inhibition. Second, the single channel conductance is unaltered by changing external pH. These results are consistent with $H^+$ modulating a gating process rather than directly occluding the pore. However, the gating changes in KCNQ2/KCNQ3 were due to a
decrease of the mean open time and the presence of a long-lived closed state, in contrast to the unaltered intraburst behaviour observed in Kv1.5 (Figure 4.6). Interestingly, when the basic lysine residue at position 260 in the turret of KCNQ3 was mutated to glutamine (K260Q), the current inhibition was enhanced. A similar result was also observed with the KCNQ2 H260Q (homologous to Kv1.5 H463Q) mutation. Together, these results are consistent with H$^+$ modulating an outer pore process that makes the channel non-conducting; however, whether this process is similar to the slow (P/C-type) inactivation in other Kv channels is uncertain, if not unlikely, given that this channel does not normally show slow inactivation. Nevertheless, an involvement of the turret in the pH effect is clearly illustrated.

In summary, external H$^+$ can inhibit many Kv channels, and also affect the rate of slow inactivation through its interaction with the turret and/or the outer pore. While the mechanism for the current inhibition may be different in some of these channels compared to that proposed for Kv1.5, it nevertheless underscores a role for the turret in the regulation of channel function.

6.4 Physiological significance and future directions

External H$^+$, Zn$^{2+}$, Ni$^{2+}$, and some other divalent cations have been shown to inhibit macroscopic Kv1.5 current by binding to the histidine residue (H463) in the turret region. This current reduction, at least that caused by external H$^+$, has a number of implications for cardiac pathophysiology. During ischemic heart attack or acidosis, extracellular pH may drop to a value as low as 6.0 (Rehncrona, 1985). At this pH, Kv1.5, which mediates the ultra-rapid delayed rectifier current ($I_{Kur}$) in atrial myocytes (Feng et al., 1997; Feng et al., 1998) that is responsible for repolarizing the action potential (Snyders, 1999), is inhibited. If no other channels or transporters are being affected, a direct consequence of acidosis would be a reduction in the total
repolarizing current and a prolongation the action potential, which may decrease the risk of atrial fibrillation (Eun et al., 2005). However, during ischemic heart attack, K⁺ may accumulate in the extracellular fluid, and this elevated external K⁺ would be expected to antagonize the H⁺-induced current inhibition of Kv1.5, diminishing the anti-atrial fibrillation effect induced by H⁺o. As suggested by Trapani and Korn (2003), even with a small change in pH, Kv1.5 currents may also be reduced by H⁺o because of a rightward gating shift of the g-V curve. Of course, with the presence of other channels and transporters in the native tissue, the interplay between the membrane currents makes a prediction of the functional outcome more complicated. Given the important role of Kv1.5 in atrial repolarization, therapeutic agents targeting Kv1.5 for treating atrial arrhythmia are currently in development (Brendel and Peukert, 2003; Peukert et al., 2003). In light of this, the results presented here have provided one possible mechanism for modulating Kv1.5, in which H⁺ has been proposed to shift the equilibrium from a normal mode of gating towards an “unavailable” mode of gating that may relate to a slow inactivation process occurring from closed states. This mechanism for modulating Kv1.5 availability may be exploited pharmacologically in the rational design of drugs.

Several new questions arise from this report. The inactivation process is suggested to be similar to the depolarization-induced inactivation; however, whether they reflect the same conformational changes in the outer pore and/or selectivity filter is still unclear. If mode U gating involves conformational changes similar to those occurring in slow (P/C-type) inactivation, the outer pore would be expected to be constricted most of the time at low pH (P₀ < 0.05 at pH 5.9 with 0 mM K⁺o). This hypothesis could be tested either by voltage clamp fluorimetry with rapid perfusion to determine if a movement of the turret and/or the outer pore occurs when pH₀ is lowered. It can also be tested by “trapping” experiments at low pH using
Cd$^{2+}$ with cysteine mutants (Yellen et al., 1994) or with Ba$^{2+}$ (Harris et al., 1998). For example, external Cd$^{2+}$ has a higher affinity for the Shaker T449C mutant when the channel is in an inactivated state. If a similar observation can be made in Kv1.5 with the R487C mutant, perfusion with solution at pH 5.9 in 0 mM K$^+$ and suitable concentration of Cd$^{2+}$ should stabilize most channels in the inactivated state, such that even when the bath solution is switched back to the control solution (pH 7.4 with 0 mM K$^+$ and 0 mM Cd$^{2+}$), the current amplitude would still be very small. Conversely, if such a constriction of the outer pore is absent during mode $U$ gating, the current amplitude would remain unchanged before and after the Cd$^{2+}$ treatment.

In this chapter, the voltage sensor movement is proposed to be uncoupled from the inactivation machinery during closed-state inactivation, which predicts the activation gate may be open despite the closing of the inactivation gate. This hypothesis may be tested using electron pair resonance (EPR) similar to the shown in KcsA (Cordero-Morales et al., 2006), in which the relative distance between the labelled residues can be determined. Alternatively, channels may be reconstituted in lipid bilayer such that conditions on both sides of the membrane can be varied, and an open channel blocker (e.g. internal TEA$^+$) can be tested for trapping in the central cavity at low pH with short depolarizing pulses. Similarly, auxiliary $\beta$ subunit with N-terminal ball (e.g. Kv $\beta$1.1) can also be used to determine the accessibility of the N-terminal ball to the central cavity by comparing the recovery rate after switching from low pH to pH 7.4. However, this last experiment may be complicated by interactions between the N-terminal ball and slow inactivation.

Slow inactivation is an important biophysical property of Kv channels. By regulating the total repolarizing current through a reduction in the number of channels available for activation, slow inactivation can play a role not only in neuronal action potentials but also in cardiac action
potentials which last for several hundreds of milliseconds. This dissertation has provided a number of findings regarding the modulation of slow inactivation, and these results can lead to a better understanding of the molecular mechanisms underlying this intrinsic process of Kv and other voltage-gated ion channels.
6.5 References


