MOLECULAR MECHANISMS UNDERLYING GATING AND pH MODULATION OF THE hERG CARDIAC K⁺ CHANNEL

by

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In the Department of Biomedical Physiology and Kinesiology

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ABSTRACT

hERG encodes a $K^+$ channel that underlies the repolarizing cardiac current $I_{Kr}$. Inherited mutations and/or acidosis, as with myocardial ischemia, disrupt hERG gating and lead to life-threatening arrhythmias. Therefore, understanding the mechanisms that underscore hERG gating and proton modification is crucial. Here, we identify a unique glycine (G546) as being critically involved in the unusually slow gating of hERG channels. We show that G546 provides flexibility to the S4-S5 linker, stabilizing the closed state of WT hERG channels. Also, we demonstrate that low pH$_0$ inhibits hERG channel function by two independent mechanisms that are not mediated by native histidines: 1) an acceleration of channel closure due to acceleration of voltage sensor return; 2) a reduction of maximal conductance due to direct block of the pore. These data provide novel insight into the mechanisms underlying hERG’s slow gating and reveal the molecular basis underlying proton modulation as seen with myocardial ischemia.

Keywords: hERG; voltage-gated potassium channels; protons; pH modulation; fluorescence
For Mom
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GLOSSARY

A Amplitude of fit
Ca, Voltage-gated calcium channel
δ Fractional electrical distance
ΔG Change in free energy
F Faraday’s Constant = 96,500 C*mol⁻¹
fA Fractional Amplitude
F-V Fluorescence-voltage relationship
G-V Conductance-voltage relationship
Ikr Rapidly delayed rectifier current
k Slope factor
Kir Inward rectifying potassium channel
[K⁺]o Extracellular potassium concentration
Kv Voltage-gated potassium channel
LQTS Long QT syndrome
MgOR2 Ca²⁺ free Ringer’s solution
Nav Voltage-gated sodium channel
ND96 Extracellular recording solution
pHi Intracellular pH
pH₀ Extracellular pH
<table>
<thead>
<tr>
<th>Symbol</th>
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<tr>
<td>pK_a</td>
<td>Negative log of the acid dissociation constant</td>
</tr>
<tr>
<td>Q-V</td>
<td>Gating charge-voltage relationship</td>
</tr>
<tr>
<td>τ</td>
<td>Time constant</td>
</tr>
<tr>
<td>TEVC</td>
<td>Two-electrode voltage clamp</td>
</tr>
<tr>
<td>TMRM</td>
<td>Tetramethylrhodamine-5-maleimide</td>
</tr>
<tr>
<td>V_{1/2}</td>
<td>Voltage at half maximal activation</td>
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<tr>
<td>VCF</td>
<td>Voltage clamp fluorimetry</td>
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<tr>
<td>WT</td>
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<td>z</td>
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1: CHAPTER 1: INTRODUCTION

1.1 Thesis overview

Ion channels are expressed in every cell in the body. Their function underlies electrical events such as the cardiac action potential. There are many types of ion channels, which can be categorized according to the ion that they permeate, \( K^+ \), \( Na^+ \), \( Ca^{2+} \), \( Cl^- \). The potassium channel family are a diverse group of proteins that can be further classified by their response to membrane voltage, ligands and other molecules. Voltage-gated potassium (Kv) channels are a large family of ion channels that selectively allow potassium to move across the cell membrane in response to a change in voltage. Since these proteins play such a key role in many physiological processes, it comes as no surprise that genetic mutations or unexpected drug interactions can lead to life threatening illnesses. For example, dysfunction of the cardiac Kv channel studied in this thesis, encoded by the human ether-a-go-go-related gene (hERG) (Sanguinetti et al., 1995; Trudeau et al., 1995), can lead to life-threatening arrhythmias such as Long QT syndrome (Curran et al., 1995). It is therefore important that we not only understand the structure of these proteins, but the mechanisms underlying their function and its regulation.

The following introduction is a synopsis of the structure and gating of the hERG potassium channel, ending with a brief summary on what is known about pH modulation of hERG channels. This background will supplement the focus of
this thesis, which investigates the molecular mechanisms underlying hERG’s unique gating kinetics and modulation by extracellular protons.

1.2 hERG channel physiology

The ether a-go-go (eag) gene was first discovered in the *Drosophila melanogaster* fruit fly, when mutation at the eag locus resulted in a rapid leg-shaking phenotype in the presence of ether (Kaplan and Trout, 1969). Warmke and colleagues were the first to show the eag gene to encode a potassium channel with a similar consensus sequence to the Shaker channel superfamily (Warmke et al., 1991). The Shaker Kv channel from *Drosophila* was the first K⁺ channel to be cloned and is regarded as the archetypical K⁺ channel since it has been so well studied. Following the study of the eag channel, a human hippocampus cDNA library was screened using a mouse eag (meag) cDNA probe and the result was the isolation and sequencing of the human eag-related construct, the human ether a-go-go-related gene (hERG) (Warmke and Ganetzky, 1994). The hERG product shares 49% amino acid identity within the hydrophobic core with both eag and elk (ether-a-go-go like) genes, and thus was deemed erg (ether-a-go-go related gene). hERG is present on human chromosome 7 (Warmke and Ganetzky, 1994) and the gene product has been shown to be present in cardiac, brain, liver, pancreas tissue, and microglia (Curran et al., 1995; Zhou et al., 1998).

Meanwhile, as efforts were being made to sequence hERG, another part of the story was being unveiled. It was discovered that Iᵦ, a potassium current responsible for terminating the action potential in guinea pig ventricular myocytes
(Matsuura et al., 1987), had two components that could be separated by the application of a benzenesulfonamide Class III antiarrhythmic agent, E-4031. This E-4031 sensitive current was termed the cardiac rapid delayed rectifier current, $I_{Kr}$ (Sanguinetti and Jurkiewicz, 1990). $I_{Kr}$, named after its rapid activation (in comparison to other delayed rectifiers) and delayed rectification properties, is fully activated at potentials greater than or equal to 0 mV (with a $V_{1/2}$ of activation of -21.5 mV) but does not make a significant contribution to the cardiac action potential during depolarized potentials due to inward rectification (Sanguinetti and Jurkiewicz, 1990). Subsequent work led to the discovery that the molecular correlate of $I_{Kr}$ is the hERG channel (Sanguinetti et al., 1995; Trudeau et al., 1995). Similar to $I_{Kr}$, hERG channel conductance is maximal at potentials equal to or greater than +10 mV, at which hERG currents then begin to show rectification resulting in a progressive decrease in currents at more positive potentials. hERG channels also have a sensitivity to E-4031 application that is similar to $I_{Kr}$ (Sanguinetti et al., 1995; Trudeau et al., 1995). Currents recorded from hERG, like $I_{Kr}$, are characterized by slow activation and rapid inactivation at depolarizing potentials, and rapid recovery from inactivation and slow deactivation at hyperpolarized potentials (Sanguinetti et al., 1995; Trudeau et al., 1995). These unique gating kinetics result in a surge of repolarizing current during phase three of the cardiac action potential (Fig. 1A and B). Inherited mutations in the hERG channel that disrupt this gating process lead to life-threatening arrhythmogenic disorders such as Long QT syndrome (Curran et al., 1995; Perrin et al., 2008).
Figure 1. The unique gating kinetics of hERG channels result in a bolus of current, $I_{kr}$, during phase three of the cardiac action potential. 

A, The cardiac action potential and the respective currents contributing to each phase. $I_{kr}$ makes a significant contribution to phase three. B, hERG channel gating scheme. hERG channel activation and deactivation are slow, while inactivation and recovery from inactivation are fast. From Sanguinetti and Tristani-Firouzi (2006). hERG potassium channels and cardiac arrhythmia, *Nature*, 440, 463-469. Reprinted with permission from NPG.
Recently, it has been proposed that $I_{Kr}$ is conducted by a heteromeric $\alpha$-subunit composed of hERG 1a and 1b isomers; mutation of the novel 1b isomer has also been linked to LQTS and sudden cardiac death (Jones et al., 2004; Sale et al., 2008).

### 1.3 hERG channel structure

Since we do not have a crystal structure of the hERG channel, most of our knowledge of hERG channel structure comes from the crystal structures of other potassium channels, mainly bacterial potassium channels (KcsA (Doyle et al., 1998; Zhou et al., 2001), MthK (Jiang et al., 2002a; Jiang et al., 2002b), and KvAP (Jiang et al., 2003)) and a mammalian Kv channel (Kv1.2) (Long et al., 2005b; Long et al., 2005c). hERG channels have a predicted topology of a typical Kv channel (Fig. 2A) with four identical subunits showing four-fold symmetry (Fig. 2B), each composed of six transmembrane domains (S1-S6) and one pore helix domain (Sanguinetti and Tristani-Firouzi, 2006; Warmke and Ganetzky, 1994). These six transmembrane domains make up two separate functional regions that have their own distinct role in Kv channel function. The voltage-sensing unit is thought to comprise segments S1-S4, with S4 serving as the primary voltage sensor (Bezanilla, 2000); whereas the pore unit is thought to comprise S5 and S6, connected by a pore helix. This section will give a brief outline of what is known about the structure and functional role of these three regions.
Figure 2. Structure of the hERG Kv channel. A, Cartoon structure of the predicted hERG topology, highlighting the voltage sensing unit (S1-S4), S4-S5 linker, and pore region (S5-S6). From Sanguinetti and Tristani-Firouzi (2006). hERG potassium channels and cardiac arrhythmia. *Nature* 440, 463-469. Modified with permission from NPG. B, Extracellular view of the Kv1.2 crystal structure showing four-fold symmetry of the Kv tetramer. From Long et al. (2005). Crystal structure of a mammalian voltage-dependent Shaker family K⁺ channel. *Science* 309, 897-903. Reprinted with permission from AAAS. C,D, Kv1.2 crystal structure of the closed and open pore conformations, respectively. The S5-P linker, labeled in D, is 2-3 times longer in hERG than that shown. C, From Doyle et al. (1998). The structure of the potassium channel: molecular basis of K⁺ conduction and selectivity. *Science* 280, 69-77. Modified with permission from AAAS. D, From Jiang et al. (2002). The open pore conformation of potassium channels. *Nature* 417, 523-526. Modified with permission from NPG.
1.3.1 The pore

Fig. 2C and D shows the crystal structure of the pore region of the bacterial channels KcsA (closed state) and MthK (open state), respectively. The pore region compromises a selectivity filter at the extracellular end and an inner water-filled cavity that is formed by the four S6 α-helices (Doyle et al., 1998). The selectivity filter has a conserved TVGYG amino acid sequence that is responsible for the channel’s high selectivity for potassium; however, in hERG, the channel’s selectivity for potassium still remains despite a selectivity sequence of SVGFG (Doyle et al., 1998; Sanguinetti et al., 1995). As K⁺ ions enter the pore they dehydrate and are coordinated by the carbonyl oxygens of the selectivity filter. Ions are conducted through the pore in a single file manner by electrostatic repulsions of the neighbouring K⁺ ions (Zhou et al., 2001). The high-resolution images of the KcsA crystal structure reveal four K⁺ ion coordination sites (S1-S4), of which two are occupied at any one time (Zhou et al., 2001). As shown in Fig. 2C and D, the S6 α-helices form a bundle-crossing when the channel is in the closed state and then splay apart upon membrane depolarization into the open state to allow the passage of K⁺ ions (Jiang et al., 2002b). In most Kv channels, the hinge point where these S6 helices rotate around is formed by a Proline-Valine-Proline (PVP) motif; however, hERG channels lack this PVP motif and the position of the activation gate is therefore of great interest (Sanguinetti and Tristani-Firouzi, 2006). Mutagenesis within the S6 domain suggests that the gate may be in the lower portion of the S6 helical bundles (Wynia-Smith et al., 2008), but the definitive location of a gate is still
unknown.

1.3.2 The voltage sensing unit

Fig. 2B shows the crystal structure of the mammalian Kv1.2 channel with the S1-S4 transmembrane α-helices highlighted. In Shaker channels, the S4 segment is displaced in the outward direction upon membrane depolarization (Bezanilla et al., 1994; Perozo et al., 1992; Stefani et al., 1994; Stuhmer et al., 1989), and it has been shown that the extent of S4 movement in hERG channels is similar to that seen in Shaker channels (Elliott et al., 2009). In hERG channels, there are five positive charges in S4 uniformly spaced three amino acids apart (Liu et al., 2003; Subbiah et al., 2004) that are thought to form salt bridges with negative charges in S2 and S3 (Liu et al., 2003; Subbiah et al., 2004; Zhang et al., 2005). Three of these negative charges, aspartates D411, D460, and D509, are only present in members of the eag family and not in other Kv channels, and it is thought that these extra negative charges form additional salt bridges with the S4 domain – leading to a stabilization of the closed state in hERG channels (Liu et al., 2003). Gating currents and voltage clamp fluorimetry experiments in hERG channels have shown that voltage sensor movement is slow and rate limiting for channel opening (Piper et al., 2003; Smith and Yellen, 2002), supporting the notion that restricted voltage sensor movement may underlie hERG’s unusually slow activation kinetics, possibly by increasing constraints on the voltage sensor through extensive salt bridge networks.
1.3.3 The S4-S5 linker helix

The crystal structure of Kv1.2 suggests the S4-S5 linker is an $\alpha$-helix that connects the voltage-sensing unit to the pore domain of the channel (Long et al., 2005b). In hERG and other Kv channels, the S4-S5 linker has been proposed to serve as a communicating link between voltage-induced reconfigurations of the S4 segment and pore opening and closing (Ferrer et al., 2006; Long et al., 2005a; Sanguinetti and Xu, 1999; Shieh et al., 1997; Tristani-Firouzi et al., 2002). This process is commonly referred to as electromechanical coupling. It is thought that electromechanical coupling is achieved as movements of the voltage sensing domains perform work on the S4-S5 linker helix, which is then translated into a constriction or dilation of the S6 bundle crossing, resulting in closing or opening of the channel, respectively (Long et al., 2005a). Indeed, a recent study suggests that an interaction occurs between the S4-S5 linker and the S6 helix to stabilize the Shaker channel in the open state (Batulan et al., 2010). Interestingly, a similar interaction has been described for the hERG channel between D540 in the S4-S5 linker and residues in the bottom of the S6 helix, whose interaction seems to stabilize hERG channels in the closed state (Ferrer et al., 2006; Tristani-Firouzi et al., 2002).

1.4 hERG channel gating

Understanding the complexities of hERG channel gating has been a hot topic since it was discovered that disruption of this gating process leads to life-threatening arrhythmias (Curran et al., 1995). hERG channel activation is slow and occurs at depolarized potentials; however, the inactivation process in hERG
channels is rapid and also occurs over a similar depolarizing range so that as soon as hERG channels start to open they transfer into the inactivated state, resulting in little current during phases zero, one, and two of the cardiac action potential (see Fig. 1A). Upon repolarization, hERG channels rapidly recover from inactivation, and then start to slowly close. As shown in Fig. 1A, the result is a bolus of current during phase three of the cardiac action potential. Therefore, since this gating process plays such an important role in cardiac physiology, it is imperative that we understand these processes. The following is a brief introduction to what is known about hERG channel activation, deactivation and inactivation.

1.4.1 hERG channel activation

The molecular mechanisms underlying the unusually slow activation of hERG channels are not fully known. What we do know has been the result of efforts using conventional patch and voltage clamp techniques for a macroscopic understanding, combined with fluorescence and gating current records that have provided insight into conformational changes of the voltage sensor. hERG channel activation is unusually slow compared to that observed in other Kv channels. hERG channels activate with a time constant in the hundreds of milliseconds compared to tens of milliseconds for the archetypical Shaker Kv channel (Sanguinetti et al., 1995; Sanguinetti and Tristani-Firouzi, 2006; Trudeau et al., 1995). Fluorescence records and gating currents suggest that this slow activation process is the result of slow voltage sensor movement (Piper et al., 2003; Smith and Yellen, 2002), which has been shown to be rate-limiting for
hERG channel opening (Smith and Yellen, 2002). This differs from that seen in *Shaker* channels, where voltage sensor movement precedes channel opening and the rate-limiting step lies further along the activation pathway (Bezanilla, 2000). This suggests that additional constraints may be placed on the voltage sensor that have to be overcome in order to open hERG channels. Salt bridge interactions between the extra negative charges in the S1-S3 transmembrane domains (three more negative charges than that seen in *Shaker* channels) and positively charged residues in the S4 segment may explain the differences in voltage sensor kinetics between hERG and other Kv channels (Liu et al., 2003; Subbiah et al., 2004; Zhang et al., 2005).

The molecular mechanisms underlying hERG activation may however be more complex. For example, the S4-S5 linker helix has also been suggested to play a role in hERG channel activation (see section 1.3.3) (Ferrer et al., 2006; Sanguinetti and Xu, 1999; Tristani-Firouzi et al., 2002). In addition, although hERG channel activation is voltage dependent, with the S4 segment known to play a key role in the activation process (Piper et al., 2003; Smith and Yellen, 2002; Zhang et al., 2005), kinetic modelling suggests that a voltage-independent step in the hERG channel activation pathway becomes rate limiting for channel opening (Wang et al., 1997). Clearly, much remains to be understood about activation gating of hERG channels.

### 1.4.2 hERG channel deactivation

hERG channel closing is an unusually slow process that is still poorly understood. It is thought that a Per-Arnt-Sim (PAS) domain in the N-terminus
(amino acids 1-135) is one of the main contributors towards this slow process (Alonso-Ron et al., 2008; Gomez-Varela et al., 2002; Gustina and Trudeau, 2009; Morais Cabral et al., 1998; Schonherr and Heinemann, 1996; Spector et al., 1996a; Wang et al., 1998; Wang et al., 2000). It has been suggested that the PAS domain interacts with the permeation pathway of the channel, and possibly the S4-S5 linker, resulting in a slowing of deactivation via a “foot in the door” mechanism (Aydar and Palmer, 2001; Gustina and Trudeau, 2009; Morais Cabral et al., 1998; Wang et al., 1998). There is additional evidence that the N-terminus also interacts with the C-terminal region of the channel (Al-Owais et al., 2009); specifically that the PAS domain of the N-terminus interacts with the cyclic-nucleotide-binding domain of the C-terminus (Gustina and Trudeau, 2011). The importance of the contribution of the N-terminus to the closure of hERG channels is unmistakable since mutations within the N-terminus have been linked to LQTS (Splawski et al., 2000).

Similar to the slow activation process, the unusually slow channel closure in hERG channels has been suggested to result from slow voltage sensor movement (Smith and Yellen, 2002). This may be due to the size and orientation of certain positively charged residues in S4 (Subbiah et al., 2004), and/or the formation of a salt bridge between aspartate D456 in the S2 transmembrane domain and lysine K525 in S4 (Subbiah et al., 2004; Zhang et al., 2005). An alanine residue (A653) in the S6 helix has also been shown to stabilize the closed state through forming intersubunit interactions within the S6 bundle crossing (Stepanovic et al., 2009).
1.4.3 hERG channel inactivation

Inactivation is a non-conducting state common in Kv channels that generally occurs at depolarized potentials and follows channel opening. Two main types of inactivation have been described for Kv channels: rapid N-type and slow C-type inactivation. In Shaker, N-type inactivation results from blockage of the conducting pathway by the N-terminus (Hoshi et al., 1990), and removal of the N-terminus eliminates N-type inactivation revealing a much slower inactivation process known as C-type inactivation (Hoshi et al., 1991). C-type inactivation occurs through a collapse of the outer mouth of the pore; hence, mutations in this region, such as with mutation of T449, influence the mechanics of C-type inactivation in Shaker channels (Lopez-Barneo et al., 1993).

In hERG channels, inactivation has been shown to be voltage dependent (Sanguinetti et al., 1995). The S4 helix acts as the primary voltage sensor for inactivation just as it does for the activation process, although different regions of the voltage sensor contribute to one or the other processes (Piper et al., 2005b). Apart from its voltage dependence, one of the other characteristic features of hERG channel inactivation is its resemblance to C-type rather than N-type inactivation (Sanguinetti and Tristani-Firouzi, 2006). As such, deletion of the N-terminus does not remove hERG inactivation, whereas outer pore mutations have significant effects (Schonherr and Heinemann, 1996; Smith et al., 1996; Spector et al., 1996a). Specifically, S620T induces a dramatic depolarizing shift in the voltage dependence of inactivation (Ficker et al., 1998) and the double mutation G628C:S631C removes inactivation (Smith et al., 1996).
Despite mounting evidence that inactivation in hERG channels involves the extracellular face of the pore, the exact location of the inactivation gate is unknown. Previous reports have suggested an interaction between the S5-P linker and the outer mouth of the pore may comprise this gate, since mutations within the S5-P linker have been shown to remove inactivation and alter selectivity (Dun et al., 1999; Jiang et al., 2005; Liu et al., 2002). Further, replacing the S5-P linker of the non-inactivating eag channel with that from the hERG channel induces hERG-like inactivation into the chimera channel (Clarke et al., 2006).

Like with the activation and deactivation processes of hERG channels, the importance of hERG channel inactivation is highlighted by disease-causing mutations that affect this process. A mutation, N588K, which increases hERG channel conductance by removing inactivation is linked to short QT syndrome (Brugada et al., 2004). Two other mutations, A614V and V630L, which enhance hERG channel conductance by shifting the voltage dependence of inactivation in the hyperpolarizing direction are associated with LQTS (Nakajima et al., 1998).

1.5 hERG channel function is modulated by protons

Acidosis has been observed in cardiac tissue during ischemia (Clarke et al., 1993; Couper et al., 1984; Elliott et al., 1992; Gabel et al., 1997; Hirche et al., 1980; Weiss et al., 1984; Yan and Kleber, 1992), leading to life-threatening arrhythmias (Carmeliet, 1999; Crampin et al., 2006; Orchard and Cingolani, 1994). Previous reports have shown that intracellular pH (pHi) is reduced with myocardial ischemia (Camacho et al., 1993; Clarke et al., 1993; Elliott et al.,
1992; Gabel et al., 1997; Marban et al., 1990; Mohabir et al., 1991; Tani and Neely, 1989; Wagner et al., 1997; Yan and Kleber, 1992), reaching levels as low as pH 5.9 (Gabel et al., 1997). This accumulation of intracellular protons has been shown to 1) inhibit the current produced by ion channels such as Na,v,1.5, Ca,v,1.2, as well as a variety of Kv channels in the heart, such as Kir2.1; 2) reduce gap junction conductance; 3) decrease Ca$^{2+}$ release from the sarcoplasmic reticulum by inhibiting the ryanodine receptor; 4) inhibit the Na$^+$/Ca$^{2+}$ exchanger; 5) enhance the function of the Na$^+$/H$^+$ exchanger; 6) decrease the force of contraction of the cardiac myocyte through decreased Ca$^{2+}$ sensitivity of the troponin complex (Carmeliet, 1999; Crampin et al., 2006; Orchard and Cingolani, 1994). In addition to this change in pH$_i$, extracellular acidosis has also been reported during myocardial ischemia (Couper et al., 1984; Gabel et al., 1997; Hirche et al., 1980; Weiss et al., 1984; Yan and Kleber, 1992), and has been observed to reach values as low as pH$_o$ 5.5 (Gabel et al., 1997; Hirche et al., 1980). An accumulation of extracellular protons has been shown to reduce the conductivity of many ion channels, inhibit SR calcium release, reduce conduction velocity, and inhibit the Na$^+$/H$^+$ exchanger (Carmeliet, 1999; Crampin et al., 2006; Orchard and Cingolani, 1994).

Extracellular acidosis has been shown to reduce the contribution of I$_{kr}$ to the cardiac action potential by accelerating the rate of deactivation, shifting the voltage dependence of activation to the right, and decreasing the maximal conductance in a voltage dependent manner (Vereecke and Carmeliet, 2000). In the same study, fluctuations in pH$_i$ had no affect on I$_{kr}$ in ventricular myocytes.
This is consistent with previous reports that show that the repolarizing current during phase 3 of the cardiac action potential is reduced when extracellular pH\(_o\) is decreased in frog atrial fibres (Chesnais et al., 1975) and is unchanged when pH\(_i\) is decreased in guinea pig ventricular myocytes (Kurachi, 1982). These data suggest that inhibition of hERG channel function by acidosis may contribute to arrhythmogenesis during myocardial ischemia. It is therefore important to understand the molecular mechanisms by which protons mediate hERG channel current.

The effects of extracellular protons on hERG channel function have been studied previously; however, results are conflicting. While the consensus of evidence strongly suggests that low pH\(_o\) accelerates the rate of deactivation, the effect of extracellular protons on hERG activation parameters are conflicting (Anumonwo et al., 1999; Berube et al., 1999; Bett and Rasmusson, 2003; Jiang et al., 1999; Jo et al., 1999; Terai et al., 2000). More recently, a study showed that some of this inconsistency is due to the fact that extracellular protons affect hERG channel function via two distinct binding sites; one site modulating the rate of deactivation and the other responsible for the reduction of maximal conductance (Bett and Rasmusson, 2003). Interestingly, the effect of extracellular protons on both deactivation and maximal conductance had a pH dependency similar to the pK\(_a\) of a histidine residue (Bett and Rasmusson, 2003).

Despite the functional data, the mechanistic basis for these effects of pH\(_o\) on hERG channels is still unknown. Most of the efforts to date have explored the
mechanism underlying the acceleration of deactivation at low pH. The acceleration of deactivation has been determined not to be a result of charge screening, a phenomenon where protons bind negatively charged phospholipids on the membrane, changing the environment sensed by the voltage sensor. This possibility was ruled out since the extent of acceleration of deactivation at low pH was much greater than the extent of the shift in the voltage dependence of activation (Jiang et al., 1999). The acceleration of deactivation has also been shown not to be due to a destabilization of the inactivated state, for deactivation of an inactivation-removed mutant (G628C S631C) is still accelerated at low pH (Jiang et al., 1999). In addition, the acceleration of deactivation is not due to altered N-terminal interactions, for deactivation of an N-terminal deletion mutant is still accelerated at low pH (Jiang et al., 1999), nor is it due to titration of histidines H578 and H587 in the extracellular S5P linker (Jiang et al., 1999). Clearly more work is required to understand the mechanism of the action of extracellular protons on deactivation. Likewise is the case for the mechanism underlying the reduction in maximal conductance. A number of studies have observed this effect (Anumonwo et al., 1999; Bett and Rasmusson, 2003; Jo et al., 1999; Terai et al., 2000), but none have addressed the molecular mechanism.
2: CHAPTER 2: MATERIALS AND METHODS

2.1 Molecular biology

A pBluescript SKII expression vector was used to express hERG channels (a kind gift from Dr. D. Fedida) in *Xenopus* oocytes. Mutant constructs were generated using conventional overlap extension PCR with primers synthesized by Sigma Genosys (Oakville, Ontario). “Up” and “down” primers were used to generate the mutation within a specific region of the hERG gene. The mutated PCR product was ligated into the hERG gene within a pBluescript SKII vector. The construct was then transformed into DH5α competent cells and a Qiagen miniprep kit was used to extract plasmid DNA. All constructs were sequenced using Macrogen (Seoul, Korea) or Eurofins MWG Operon (Huntsville, Alabama). Constructs were linearized using XbaI restriction endonuclease and cRNA was synthesized from linear cDNA using the mMessage mMachine T7 Ultra cRNA transcription kit (Ambion, Austin, TX).

2.2 Oocyte preparation and injection

Terminal surgical procedures were performed on *Xenopus laevis* in order to remove oocytes. Frogs were terminally anaesthetized in 0.8% ethyl 3-aminobenzoic acid methanesulfonate (tricaine) solution for 25 min. To confirm death, both the tail and leg appendages were pinched to ensure lack of a reflexive kicking response, and then the frog was placed on its back to ensure
lack of the righting reflex. Finally, a syringe needle was inserted into the base of the skull to pith the brain, followed by a dislocation of the cervical portion of the spinal cord. All lobes of oocytes were removed through an incision made in the lower abdomen. Stage V-VI oocytes were isolated and defolliculated in two consecutive steps: 1) lobes were broken-up and teased apart with fine-tip forceps and then placed in a collagenase solution containing 1 mg collagenase type 1A in 1 mL of MgOR\(_2\) solution (in mM: 96 NaCl, 2 KCl, 20 MgCl\(_2\), 5 HEPES, titrated to pH 7.4 with NaOH). Oocytes were rocked gently for approximately 1 hour or until the follicular layer was loose. Oocytes were then removed from collagenase solution and washed 7-10 times in MgOR\(_2\) solution; 2) healthy oocytes (determined by the presence of a lightly coloured vegetal pole and dark animal pole, separated by a clearly defined band) were sorted and placed in SOS+ media (in mM: 96 NaCl, 2 KCl, 1.8 CaCl\(_2\), 1 MgCl\(_2\), 5 HEPES, 5% horse serum, 2.5 sodium pyruvate, 100 mg/l gentamicin sulfate, titrated to pH 7.4 with NaOH) for 30 min to further loosen the follicular layer before manual defolliculation with fine-tip forceps. It was important to ensure that oocytes were defolliculated in full for two reasons: 1) the follicular layer presents a collagenous barrier to the injection pipette that can result in cell damage during injections; 2) the follicular layer contains ion channels and transporters that can contaminate electrophysiological recordings (Bossi et al., 2007). Isolated oocytes were incubated in SOS+ media for 5-24 hours before injection. All injection equipment and gloves were cleaned with ethanol and then RNaseZAP (SIGMA) to ensure RNases were removed before injections. Injection electrodes were made using a
P-87 Flaming/Brown Micropipette puller (Sutter Instrument Co.) and tips were broken to create a bevelled shape. Electrodes were then filled with mineral oil and mounted onto the barrel of a Drummond digital microdispenser. Approximately 1.5 μL of mineral oil was dispensed from the electrode onto a piece of parafilm. RNA was then pipetted onto the mineral oil and sucked-up into the electrode. Each oocyte was then injected with 50 nl (5-10 ng) cRNA in MgOR₂ solution. Generally, 20 oocytes were injected at a time and incubated in SOS+ media in a 60 mm petri dish at 19 °C after injection. Solutions were replaced with fresh SOS+ media two hours following injection to remove any deceased oocytes. Oocytes stored for 1-10 days before recording, with SOS+ media being changed once every day.

2.3 Data acquisition

2.3.1 Two-electrode voltage clamp

Membrane currents were recorded from WT and mutant hERG channels using the two-electrode voltage clamp (TEVC) technique. In this technique, membrane potential is measured by an intracellular electrode. A silver chloride bath electrode present in the bath chamber solution serves as a reference electrode. The measured membrane potential is relayed to an Axoclamp 900A (Axon Instruments) amplifier that determines the potential difference between this and the command output from computer driven voltage protocols. Computer driven protocols were performed using pClamp 10.2 software and Digidata 1440 interface to convert the digital signal to an analog signal that is recognizable by the Axoclamp 900A amplifier. A second intracellular electrode injects current that
is proportional to the difference between command and measured membrane potentials. This current represents the flux of ions through the ion channels expressed at the membrane and is acquired by pClamp 10.2 computer software (Axon Instruments). To translate the bioelectricity of the cellular membrane into an electrical signal that can be interpreted by the amplifier, silver chloride wires are inserted into borosilicate glass. Electrons are transferred bidirectionally between the recording media and the silver chloride electrode based on the following equation:

\[ e^- + AgCl \leftrightarrow Ag(s) + Cl^-(aq) \]

To ensure high conductivity between the silver chloride electrode and the recording media, electrodes were filled with 3M KCl. Since the chloride concentration within the borosilicate microelectrode (3M) greatly differs from that around the bath electrode (chloride concentration of the recording solution was approximately 100mM), a liquid junction potential was created and was electrically subtracted before electrodes were inserted into the cell. Electrodes had a resistance of 0.2-2.0 MΩ for a given tip size when filled with 3 M KCl.

Membrane currents were recorded while cells were bathed in ND96 solution (in mM: 96 NaCl, 3 KCl, 1 MgCl₂, 0.5 CaCl₂, 5 HEPES, titrated to pH 7.4 with NaOH). For experiments where perfusion of different pHₖ were performed, an additional buffer, 5 mM MES, was added to all recording solutions to ensure buffering capacity. All current signals were acquired at a sampling rate of 10 kHz with a 4 kHz low-pass filter. Experiments were performed at 20-22 °C.
2.3.2 Voltage protocols

A number of voltage protocols were used in these studies. These are outlined below and are also shown diagrammatically in Fig. 3. Any modifications to these voltage protocols are noted in relevant figure legends.

2.3.2.1 Activation

As shown in Fig. 3A, oocytes were held at -80 mV and then depolarized to +60 mV for 2 s in 10 mV increments to activate and inactivate channels. After each depolarizing pulse, a 2 s hyperpolarizing pulse to -60 mV was applied to recover channels from inactivation and allow for channel closure. Since hERG channel deactivation is slow and recovery from inactivation is fast, peak tail currents represent the relevant channel conductance during the previous depolarizing potential.

2.3.2.2 Deactivation

As shown in Fig. 3B, oocytes were held at -80 mV and then depolarized to +60 mV for 500 ms to activate and inactivate channels, followed by 4 s hyperpolarizing pulses from -110 mV to -40 mV to recover channels from inactivation and allow for channel deactivation. Since hERG channel deactivation is slow, a 4 s pulse was required to obtain accurate time constant values.

2.3.2.3 Time constant of activation

To study the rate of hERG activation, an envelope of tails protocol was used (Wang et al., 1997). As shown in Fig. 3C, oocytes were held at -80 mV and then depolarized to +60 mV (or other depolarized potentials depending on the
Figure 3. Voltage protocols used to study the different gating processes of the hERG channel. A, voltage protocol used to study hERG channel activation parameters. B, voltage protocol used to study hERG channel deactivation. C, voltage protocol used to study the time course of hERG channel activation. D, voltage protocol used to study hERG channel inactivation.
voltage of interest) for durations from 10 to 500 ms (in 10 ms increments) to recruit channels into the open and inactivated state. After each depolarizing pulse, a 2 s hyperpolarizing pulse to -110 mV was applied to recover channels from inactivation and allow for channel closure. As the protocol continued, the length of the depolarizing pulse increased, recruiting more channels into the open state and this is represented by an increase in the size of the peak tail current. The magnitude of the peak tail current represents the relevant channel conductance during the previous depolarizing potential since the hyperpolarizing pulse to -110 mV quickly recovers the channels from inactivation before the channels begin to slowly close (Sanguinetti et al., 1995; Wang et al., 1997). The peak tail current upon repolarization increased with longer depolarizing durations until saturation – leading to an envelope of tails as shown in Fig. 7. Calculation of the activation tau from the envelope of tails is described in section 2.4.3.

2.3.2.4 Inactivation

As shown in Fig. 3D, oocytes were held at -80 mV and then depolarized to +40 mV for 500 ms to activate and inactivate channels. 30 ms conditioning pulses from -120 mV to +40 mV (in 10 mV increments) were then applied followed by a test pulse to 0 mV to measure the proportion of channels that were inactivated during the conditioning pulse. The voltage dependence of inactivation was determined by plotting the amplitude of current at the beginning of the 0 mV test pulse against the conditioning pulse potential (see Fig. 5).
2.3.3 Voltage clamp fluorimetry

Voltage clamp fluorimetry (VCF) is a powerful tool that enables observation of conformational changes of specific regions of an ion channel (like the voltage sensor, for example) in real-time. VCF has been previously used to study voltage sensor movement in hERG and other Kv channels (Bannister et al., 2005; Cha and Bezanilla, 1997; Claydon et al., 2007; Mannuzzu et al., 1996; Savalli et al., 2006; Smith and Yellen, 2002; Zheng and Zagotta, 2000). In hERG channels, conformational changes in the environment of the voltage sensor is monitored by attaching a sulphydryl-reactive fluorescent tag, tetramethylrhodamine-5-maleimide (TMRM), to a cysteine mutation engineered at L520 near the extracellular portion of the S4 segment (shown in Fig. 7A) (Smith and Yellen, 2002). This mutation created a site for fluorescence labelling via a disulfide interaction with TMRM. TMRM fluorescence probes were used for the following reasons: 1) unlike iodoacetamides, maleimides do not react with histidines or methionines under physiological conditions, therefore TMRM labeling is specific to cysteine residues; 2) unlike fluorescein and oregon green derivatives, TMRM is not pH sensitive (an important feature seeing as how one of the aims of this study was to examine how pH changes effect the fluorescence report of voltage sensor movement, see chapter two); 3) aside from eosin derivatives, TMRM has the highest extinction coefficient of the markedly available derivatives (eosin derivatives were not used since they have been shown to label lysine residues); and 4) in comparison with other dyes, TMRM is photostable, meaning it can undergo multiple repeated excitations before being destroyed in its excitable state (also known as photobleaching). Although the
quantum yield of TMRM is relatively low (0.2), this is well within the range of 0.05 to 1.0 needed to provide measurable fluorescence intensities, and is compensated for by the large extinction coefficient, resulting in reasonably high fluorescence intensities.

Cells were labelled in depolarizing solution (in mM: 98 KCl, 1 MgCl$_2$, 2 CaCl$_2$ and 5 HEPES, titrated to pH 7.4 using KOH) for 30 min at 10 °C in the dark. Depolarizing solution was used to ensure voltage sensors were in the upright activated position, providing TMRM better access to the L520C site (Cha and Bezanilla, 1997). It is important to label cells at 10 °C mainly because the quantum yield of TMRM is temperature sensitive and decreases as it is exposed to warmer temperatures. A Nikon TE2000S inverted microscope with Epi-Fluorescence attachment and photomultiplier tube (PMT) detection module were used for VCF experiments. White light was focused onto the cell via a 20x objective with a NA of 0.75. Light was filtered through a 525 nm band pass (45 nm) filter to excite fluorophores (TMRM has an excitation peak at 541 nm). Fluorescence emission was collected through the same objective, filtered with a 565 nm long pass filter (TMRM has an emission peak at 567 nm) and detected using a bialkali photocathode PMT recording module (Cairn Research). Fluorescence, current and voltage signals were acquired simultaneously using the Axon Instruments hardware described above. In some cases where bleaching of the fluorescence signal was observed, we compensated by subtracting the fluorescence recorded during potentials at which there was no channel opening.
2.4 Data analysis

Data throughout the text and figures are shown as means ± S.E.M. n stands for the number of cells recorded from. Statistical significance was determined using a one-way ANOVA and a Bonferroni post-hoc test for comparison of the parameters across different constructs studied, unless otherwise mentioned. In the case where comparisons were made before and after treatment with different pH, a two-way ANOVA and Bonferroni test was used; significance was determined if the p-value < 0.05. In figures showing raw current and/or fluorescence data, arrows mark the zero current level and dotted lines represent baseline current levels to guide the eye.

2.4.1 Conductance-voltage relationships

To describe the conductance-voltage (G-V) relations, peak tail current amplitudes were plotted. Values were normalized to the maximum tail current amplitude and the data was fitted with a single Boltzmann function:

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

where \( y \) is the conductance normalized with respect to the maximal conductance, \( V_{1/2} \) is the half-activation potential, \( V \) is the test voltage and \( k \) is the slope factor, which represents movement of charge across the membrane as the channel transitions from the closed to the open state.

2.4.2 Time course of deactivation

To determine the time course of deactivation, deactivating tail currents were fitted with either a single or double standard exponential function as shown
below, respectively:

\[
f(t) = A_{\text{deact}} \exp\left(-t/\tau_{\text{deact}}\right) + C
\]

\[
f(t) = A_{\text{slow}} \exp\left(-t/\tau_{\text{slow}}\right) + A_{\text{fast}} \exp\left(-t/\tau_{\text{fast}}\right) + C
\]

where \( A \) is the amplitude of the fit, \( t \) is time, \( \tau \) is the time constant of deactivation, and \( C \) is the residual. Deactivation was fit with a double exponential function at all potentials tested for the case of all channel constructs except for G546L, G546I, G546R, and G546E, which were best fit with a single exponential function at all potentials tested.

2.4.3 Time course of activation

To determine the time course of activation, peak tail currents were plotted against the depolarizing pulse duration. Data points during the late phase of activation were fitted with a single standard exponential function as described in the equation below. The late phase of activation was used for ease of comparison since hERG channel activation is sigmoidal, suggesting the presence of multiple steps in the activation pathway (Wang et al., 1997), and would require a polyexponential fit. Therefore, the activation tau derived from the single exponential fit is not sufficient to describe the entire activation process, but rather gives a good approximation of the predominant step in the activation pathway.

\[
f(t) = A(1-e^{(t/\tau_{\text{act}})}) + C
\]

where \( A \) is the amplitude of the fit, \( t \) is time, \( \tau \) is the time constant of
activation, and C is the residual.

2.4.4 Activation energy

To compare activation rates between mutants that exhibited different voltage-dependencies of activation, we corrected our data for activation energy so that comparison of activation rates could be made at comparable driving forces. Rather than just correcting for the voltage dependence of activation when comparing the activation time course amongst mutants, we corrected for the total activation energy because it is a more complete assessment of the perturbations of the activation pathway, since it incorporates electrical and chemical energy changes. As reported previously (Subbiah et al., 2004), the total energy of activation ($E_A$) can be described as:

$$E_A = - (\Delta G - zVF)$$

where $\Delta G$ is the work done at 0 mV (calculated using the formula $\Delta G = zFV_{1/2}$, with $V_{1/2}$ determined from Boltzmann fits of the G-V relations), $z$ is the apparent valence, $V$ is the membrane potential, and $F$ is Faraday’s constant.

2.4.5 Voltage dependence of block

The voltage dependence of proton block was quantified by plotting the fractional amplitude of peak hERG tail current at pH$_o$ 5.5 against the membrane potential. The data points were then fitted with the Woodhull model (Woodhull, 1973):

$$f_A(V) = f_A(0 \text{ mV}) \times \exp(z\delta FV/RT)$$
where $f_A$ is the fractional amplitude at the test voltage, $f_A(0 \text{ mV})$ is the fractional amplitude at 0 mV, $z$ is the apparent valence, $\delta$ is the electrical distance between the proton binding site and the rate-limiting barrier for exit (i.e. the fractional electrical distance), $F$ is Faraday's constant, $V$ is the test voltage, $R$ is the universal gas constant, and $T$ is the temperature in Kelvin’s.
CHAPTER 3: MUTATIONS WITHIN THE S4-S5 LINKER ALTER VOLTAGE SENSOR CONSTRAINTS IN hERG K⁺ CHANNELS

3.1 Introduction

The human ether-a-go-go related gene (hERG) encodes the pore-forming subunit of the cardiac K⁺ channel that underlies the repolarizing current, Iᵦ. The molecular mechanisms underlying the unique gating properties of hERG channels are not fully known. hERG activation is unusually slow, and this is thought to be due in part to: 1) slow voltage sensor movement that is rate-limiting for channel opening (Piper et al., 2003; Smith and Yellen, 2002), and 2) extensive salt bridge networks between positively charged residues in the S4 segment and negatively charged residues in the S1-S3 transmembrane domains that may place constraints on voltage sensor movement (Liu et al., 2003; Subbiah et al., 2004; Zhang et al., 2005). This situation is different from that seen in Shaker channels where voltage sensor movement precedes pore opening (Cha and Bezanilla, 1997; Mannuzzu et al., 1996) and only three negatively charged residues in S1-S3 are available to form salt bridge networks (Liu et al., 2003). However, despite these differences, the extent of S4 movement in hERG channels upon depolarization was recently shown to be similar to that in Shaker and KvAP channels (Elliott et al., 2009).

In addition to slow voltage sensor movement, the region connecting the S4-S5 linker helix has also been suggested to play a role in hERG channel
activation and electromechanical coupling (Sanguinetti and Xu, 1999; Tristani-Firouzi et al., 2002; Ferrer et al., 2006). The same has been reported in other Kv channels, such as the Shaker channel (Holmgren et al., 1996; Shieh et al., 1997; Chen et al., 2001; Batulan et al., 2010). One important contributor to the activation and electromechanical coupling processes in Shaker channels is a leucine heptad repeat that spans the S4-S5 linker. Fig. 4A shows a sequence alignment of the S4-S5 linker region from Shaker and hERG channels and highlights residues that contribute to this leucine heptad repeat in Shaker channels (McCormack et al., 1991). Multiple labs have reported that disruption of this leucine heptad repeat at the second leucine position (Shaker L382) drastically affects gating by: 1) dramatically shifting the voltage dependence of activation to more depolarized potentials, and 2) decelerating the time course of activation. Since the Q-V relation was unaffected, these data suggest that the mutation uncouples voltage sensor movement from pore opening (McCormack et al., 1991; McCormack et al., 1994; Schoppa et al., 1992; Schoppa and Sigworth, 1998a; Schoppa and Sigworth, 1998b). Interestingly, as shown in Fig. 4A, a glycine at position 546 naturally disrupts the leucine heptad repeat in hERG channels. In this Chapter, we present data from experiments designed to test the role of this unique glycine in hERG channels. Our hypothesis is that disruption of the leucine heptad repeat contributes to the characteristically slow gating kinetics of hERG channels by altering one of two processes: either electromechanical coupling between the voltage sensor and the pore gate or voltage sensor movement itself.
3.2 Results

3.2.1 Mutation of a critical site in the S4-S5 linker helix alters activation properties

Fig. 4B shows representative families of current traces recorded from WT and G546L channels in response to changes in the membrane potential (see Fig. 5B insets). Fig. 4C shows the conductance-voltage relationships for the two channel types calculated from the peak tail current amplitudes as described in section 2.4.1 of the methods. The G546L mutation had a large effect on activation, shifting the voltage dependence by nearly -50 mV, from -25.7 ± 0.1 mV in WT to -70.9 ± 1.2 mV in G546L channels.

Fig. 4D shows a plot of the fractional current amplitude measured at the end of the depolarizing pulse. These data highlight the presence of rectification in both channels, where channel conductance decreases at more depolarized potentials due to inactivation. The data is Fig. 4D show that rectification in G546L channels was not significantly different from WT. Given that rectification in hERG WT channels has been shown to be due to the onset of inactivation that becomes more prominent at more depolarized potentials (Sanguinetti et al., 1995; Smith et al., 1996; Spector et al., 1996b), these data also suggest that the G546L mutation does not affect inactivation. To test this, we measured the voltage dependence of channel inactivation in these two channels. Typical current traces recorded from WT hERG channels in response to a triple-pulse protocol (Fig. 3D and Fig. 5A) are shown in Fig. 5B. As shown in Fig. 5C, the voltage dependence of inactivation in G546L channels was not significantly different from that observed in WT: the $V_{1/2}$ of inactivation was -55.6 ± 7.2 mV.
Figure 4. The G546L mutation restores the leucine/isoleucine heptad repeat in hERG channels, affecting activation. A, sequence alignment of the S4-S5 linker in Shaker and hERG channels. The leucine/isoleucine heptad repeat motif is outlined by the boxes. B, typical current traces recorded from hERG WT and G546L constructs during the protocols shown in the insets. C, G-V relationships constructed from peak tail currents shown in B. G-V relations were fitted with a Boltzmann function (see Methods). D, plots of WT and G546L current at the end of depolarizing steps normalized to the peak current.
and -55.4 ± 3.1 mV for G546L and WT (n=2-4), respectively. Therefore, the G546L mutation specifically disrupts activation without affecting inactivation properties, consistent with previous observations in the literature that suggest that the activation and inactivation processes in hERG channels are not coupled (Piper et al., 2005a).

Fig. 6 shows that the G546L mutation also caused channels to activate more rapidly than WT channels. Since hERG channel activation and inactivation occur simultaneously at depolarized potentials, it is necessary to use an envelope of tails protocol, as described in section 2.3.2.3, because it separates these two processes. This is more accurate than simply assessing the rate of activation by fitting the rise of current upon depolarization (like those during pulse one of Fig. 4B, for example), where inactivation contaminates the records. Fig. 6A and C show typical current traces recorded from WT and G546L channels in response to the envelope of tails voltage protocol. To accurately compare the rates of channel activation, we first calculated the activation energy from the voltage dependence of activation (see section 2.4.4).

It is clear from Table 1 that in order to accurately compare the activation rates of WT and G546L at comparable driving forces, the time course of activation of G546L at +60 mV, for example must be compared to that of WT at +10 mV. After taking the hyperpolarizing shift of activation into account, G546L activated more rapidly than WT channels: at +10 mV the tau of activation for G546L channels was 43.2 ± 7.7 ms at an activation energy of 29.6 KJ/mol, compared to 70.9 ± 6.9 ms at 29.2 KJ/mol in the WT channel at +60 mV (n=4-6;
Figure 5. The G546L mutant inactivates in a manner similar to WT. A, B, Typical WT hERG currents (B) recorded during the triple pulse protocol shown in A. The holding potential was -80 mV. C, Normalized WT and G546L channel peak tail currents (taken at the point indicated by the arrow in B) plotted against the membrane potential during the conditioning pulse. Data were fitted with a Boltzmann function (see Methods). \( V_{1/2} \) and \( k \) values are (n=2-4; \( p > 0.05 \), two-tailed independent t-test).
<table>
<thead>
<tr>
<th></th>
<th>Tau (ms)</th>
<th>Voltage (mV)</th>
<th>-((\Delta G - zVF)) (KJ mol(^{-1}))</th>
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<td>20</td>
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<td>119.9 ± 5.9</td>
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<td>79.0 ± 5.9</td>
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<td></td>
<td>70.9 ± 6.9</td>
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<td>29.2</td>
</tr>
<tr>
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<td>98.2 ± 25.3</td>
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<td>15.0</td>
</tr>
<tr>
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<tr>
<td></td>
<td>43.2 ± 7.7</td>
<td>10</td>
<td>29.6</td>
</tr>
</tbody>
</table>

**Table 1.** Total potential driving force for activation of WT and G546L mutant hERG channels. Values for WT and G546L activation tau compared at similar total potential driving forces. Total potential driving force is the total activation energy described by -\((\Delta G - zVF)\). \(\Delta G\) was calculated from \(zFV_{1/2}\) where \(V_{1/2}\) and \(z\) (calculated from RT/kF) were obtained from Boltzmann fits of the \(G-V\) relationships of each channel.
Figure 6. The G546L mutation accelerates hERG channel activation by altering voltage sensor movement. A, C, WT and G546L currents recorded during an envelope of tails protocol to measure the time course of channel opening. Peak tail current at -110 mV was measured after a step to +60 mV of varying duration (10-500 ms in 10 ms increments). The holding potential was -80 mV (WT) or -120 mV (G546L). To account for the shift in the G-V relationship, WT and G546L traces are shown at +20 and -30 mV, respectively. B, D, normalized WT and G546L peak tail current following depolarizing steps to a range of potentials. Inset, plot of the time constant of activation of WT and G546L channels at each potential. Data points plot the tau from single exponential fits of the late phase of activation from the data in B (from t=100 ms) and D (from t=50 ms). The voltage dependence of the tau of the late phase of activation was fitted with a single exponential function. E, F, typical fluorescence reports from TMRM-labelled L520C and G546L L520C during a voltage step to +60 or +10 mV from a holding potential of -80 or -120 mV, respectively. The insets show the recordings on a faster time-base. Single exponential fits are shown as gray lines. G, plots of mean G-V and F-V relationships from L520C and G546L L520C. Data were fitted with a Boltzmann function. \( V_{1/2} \) and \( k \) values for the G-V relationships were -17.4 ± 4.5 mV and 10.9 ± 1.1 mV for L520C (n=4), and -55.8 ± 1.7 mV and 9.1 ± 0.7 mV for G546L L520C (n=7), respectively. \( V_{1/2} \) and \( k \) values for the F-V relationships were -15.2 ± 2.0 mV and 15.4 ± 1.0 mV for L520C (n=4), and -62.4 ± 10.2 mV and 12.8 ± 1.2 mV for G546L L520C (n=4), respectively.
This is shown more clearly in the inset to Fig. 6D, which plots the tau of the late phase of activation of WT and G546L channels as performed previously (Wang et al., 1997). In both WT and G546L, the tau of activation reached an asymptote at more depolarized potentials: 37.3 and 31.5 ms ($p > 0.05$), respectively. According to the hERG activation pathway described by Wang and colleagues, this would suggest that the voltage-independent step is not altered by the G546L mutation since the tau of the voltage-independent step in WT and G546L channels is statistically the same; further, the voltage-independent step becomes rate-limiting at more depolarized potentials in both channels (Wang et al., 1997). Since the voltage-independent step was unaffected by the leucine substitution, we can infer that the observed acceleration of activation was due to alteration of the voltage-dependent steps in the activation pathway.

To determine whether the G546L phenotype was the result of altered electromechanical coupling or altered voltage sensor movement, we measured the fluorescence report of voltage sensor movement using VCF as described in section 2.3.3. Attachment of a TMRM fluorophore at L520C has previously provided novel insight into the mechanics of voltage sensor movement in hERG channels (Smith and Yellen, 2002). Fig. 7A shows a schematic illustrating the predicted location of the TMRM fluorophore attached at L520C. As shown, this site is located at the C-terminal end of the S3-S4 linker, near the extracellular surface of the S4 voltage sensor. We interpret the fluorescence report from this site to be a direct report of voltage sensor movement since: 1) fluorescence
Figure 7. TMRM attached at L520C provides a faithful report of voltage sensor movement. A, schematic of the hERG channel showing the predicted position of the fluorophore attached at L520C. B, plot of the time constant of the TMRM fluorescence report from L520C upon depolarization and the late phase of ionic current activation of L520C channels, either in the presence or absence of TMRM label. Ionic current data points plot the tau from single exponential fits of the late phase of activation (from \( t=100 \) ms) from currents recorded during the envelope of tails protocol as described in section 2.3.2.3. C, typical ionic current (upper) and fluorescence (lower) traces recorded during a 2 s depolarizing voltage step to 0 mV followed by a 2 s hyperpolarizing step to \(-110\) mV from TMRM-labelled L520C channels in control conditions (ND96) and in the presence of 10 mM 4-AP. Fluorescence recordings are an average of three recordings. D, plot of the mean peak ionic current and fluorescence change (\( \Delta F/F \)) at the end of the 0 mV pulse in the absence and presence of 10 mM 4-AP (n=9). *significantly different (P<0.05). E, typical fluorescence traces recorded from TMRM-labelled WT channels (i.e. channels lacking L520C) during a 2 s depolarizing step to +60 mV followed by 2 s hyperpolarizing step to \(-110\) mV (holding potential \(-80\) mV). This was observed in 9 oocytes.
reports from TMRM labelling the equivalent residue to L520C in Shaker, Kv1.2, Kv1.4, and Kv1.5 channels have been shown to provide a faithful report of voltage sensor movement (Cha and Bezanilla, 1997; Claydon et al., 2007; Mannuzzu et al., 1996); 2) labelling of the native cysteines, C445 and C449, in the S1-S2 linker did not produce voltage-dependent fluorescence deflections (Fig. 7E); 3) application of 10 mM 4-aminopyridine (which binds and stabilizes the intracellular pore of hERG channels in the closed state) is associated with a reduction of the ionic current, but no change in the fluorescence report (Fig. 7C and D); and 4) the time-course of activation of the macroscopic current overlies the time-course of voltage sensor movement during activation (Fig. 7B), which is consistent with the observation that voltage sensor movement is rate-limiting for channel opening (Smith and Yellen, 2002).

Typical fluorescence reports of voltage sensor dynamics reported by TMRM attached at L520C show that voltage sensor activation was accelerated in G546L L520C channels compared to L520C (Fig. 6E and F): the tau of the fluorescence deflection observed upon membrane depolarization was 21.4 ± 1.0 ms at +10 mV in G546L L520C channels, and 43.1 ± 3.4 ms at +60 mV for L520C channels. It should be noted that these values were faster than the tau of activation derived from our ionic data; Fig. 7B plots the tau of the late phase of activation against voltage for the ionic, with and without the TMRM label, and the fluorescence data. Comparison of the activation tau of the ionic current after TMRM labelling with the fluorescence records show that the time course of activation overlies that of voltage sensor movement during activation (Fig. 7B).
Therefore, the apparent acceleration of voltage sensor movement in WT and G546L channels during activation, in comparison to the time course of activation of the ionic data, was caused by an unexpected effect of TMRM labelling.

Fig. 6G shows the G-V and fluorescence-voltage (F-V) relationships for L520C and G546L L520C channels. As reported previously (Smith and Yellen, 2002), the F-V relation overlaid the G-V relation in hERG L520C channels, suggesting that voltage sensor movement is the rate-limiting step for channel opening. We found that this was unchanged in G546L channels, although the relationships existed at more hyperpolarized potentials. Taken together, the data in Figs. 4 and 6 suggest that the G546L mutation causes a left shift in the voltage dependence of activation and an acceleration of activation kinetics – stabilizing the open state of the channel – by reducing the energy required for voltage sensor movement.

3.2.2 Reducing the flexibility of the S4-S5 linker helix destabilizes the closed state of the channel

To further explore the mechanism by which mutation of G546 disrupted the activation process, we replaced the glycine with a number of amino acids possessing different physico-chemical properties. Specifically, in addition to G546L, we tested the following mutants: G546Q, G546I, G546R, G546E, G546Y, G546V, G546C, and G546A. Fig. 8A plots the G-V relationships of all nine mutations tested. All, except G546C, induced large hyperpolarizing shifts in the voltage dependence of activation (view Table 2) similar to that observed with G546L. Given that the S4-S5 linker has been reported to be α-helical (Long et
al., 2005a; Sanguinetti and Tristani-Firouzi, 2006), and that glycine residues tend to provide flexibility to an $\alpha$-helix, we hypothesized that the native G546 residue permits flexibility to the S4-S5 linker that constrains voltage sensor movement and thus pore opening. To test this, we used the predictive algorithm, AGADIR, to investigate the $\alpha$-helical propensity of the S4-S5 linker region in hERG channels. These predictions are based upon helix/coil transition theory that includes potential side-chain-side-chain interactions, electrostatic effects and a comparison with a large database of experimentally measured helix contents in different peptides (Lacroix et al., 1998).

Fig. 8B shows the $\alpha$-helical propensity of the S4 segment, S4-S5 linker, and S5 segment. Consistent with the known hERG consensus sequence and Kv1.2 crystal structure that show that the S4 and S5 transmembrane domains are $\alpha$-helices, the $\alpha$-helical content of the S4 and S5 segments was high. However, in WT channels, the $\alpha$-helical propensity of the S4-S5 linker was generally low in comparison, and specifically G546 appeared to significantly reduce helical formation. This is more clearly shown in Fig. 8C, which plots the $\alpha$-helical propensity of just the S4-S5 linker at each residue. As shown in Fig. 8B and C, those mutations that destabilized the closed state of the channel (i.e. left-shifted the G-V relationship), were predicted to dramatically increase the $\alpha$-helical propensity of the S4-S5 linker. In contrast, the G546C mutant, which produced only minor changes in the activation free energy (consistent with previous reports (Alonso-Ron et al., 2008; Wang et al., 1998)), did not alter the $\alpha$-helical propensity of the S4-S5 linker. Therefore, the native glycine in hERG
Figure 8. G546 stabilizes the closed state of WT hERG channels by affording flexibility to the S4-S5 $\alpha$-helical linker. A, G-V relations recorded from WT and G546 mutants fitted with a Boltzmann function as described in the methods (n=4-6). B, C, AGADIR algorithm predictions of the $\alpha$-helical content of the S4, S4-S5 linker, and S5 (B), or just S4-S5 linker (C) regions. D,E, homology models of the hERG S4-S5 linker helix with (D) or without (E) the G546L mutation. Models were made using SWISSMODEL and the Kv1.2/Kv2.1 crystal structure as a template. * highlights putative hydrogen bonds formed by the G546 mutants.
WT channels disrupts helical formation of the S4-S5 linker and this is expected to increase flexibility – similar to how a glycine hinge provides flexibility to the S6 helical bundles in Shaker channels (Ding et al., 2005).

Calculations of the change in the free energy of activation ($\Delta \Delta G$) (see section 2.4.4) induced by each mutant (Table 2) suggest that substitutions at G546 reduced the activation energy in the range of -1.6-4.3 kcal/mol. Interestingly, this range correlates quite well with the energy of 1-2 hydrogen bonds (assuming 1.9 kcal/mol per H-bond). Fig. 8D and E show possible locations of hydrogen bonds in the S4-S5 linker of WT and G546L channels as predicted using the SWISS-MODEL workspace program. SWISS-MODEL outputs were derived from the known crystal structure of the mammalian Kv1.2 channel and Kv2.1/1.2 chimera (Long et al., 2005b; Long et al., 2007). The SWISS-MODEL output suggests that unlike the glycine present in WT channels, the substituted leucine may be in an orientation that permits the formation of two additional hydrogen bonds, consistent with the change in activation energy caused by this mutation (Table 2). While the model output is based on amino acid properties in free space rather than at a lipid aqueous interface, it is interesting to observe that the data is consistent with our AGADIR predictions and our calculation of changes in free activation energy. Taken together, these data suggest that increased $\alpha$-helical flexibility of the S4-S5 linker afforded by the native glycine stabilizes the closed state of WT hERG channels and contributes to the slow gating kinetics.
### Activation properties of WT and mutant hERG channels

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<th>$V_{1/2}$ (mV)</th>
<th>$k$ (mV)</th>
<th>$\Delta G$ (Kcal mol$^{-1}$)</th>
<th>$\Delta\Delta G$</th>
<th>$n$</th>
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<td>-</td>
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<td>5</td>
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<td>-6.4 ± 0.4</td>
<td>-4.3</td>
<td>5</td>
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<tr>
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<td>-4.0 ± 0.3</td>
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<td>6</td>
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<tr>
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<td>-5.1 ± 0.5</td>
<td>-3.0</td>
<td>5</td>
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<tr>
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<td>8.1 ± 0.1</td>
<td>-4.9 ± 0.1</td>
<td>-2.8</td>
<td>6</td>
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<tr>
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<td>-4.8 ± 0.2</td>
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<td>-1.6</td>
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Table 2. Activation properties of WT and mutant hERG channels. Free energy changes ($\Delta G$) were calculated from $zFV_{1/2}$ where $V_{1/2}$ and $z$ (calculated from $RT/kF$) were obtained from Boltzmann fits of the G-V relationships of each channel. Perturbation energies ($\Delta\Delta G$) were calculated using $\Delta\Delta G = \Delta G_{\text{mutant}} - \Delta G_{\text{WT}}$. 
3.2.3  Mutations at position G546 differentially affect deactivation gating

Unlike the effects on activation arising from mutation at G546, mutations at G546 had differential effects on the kinetics of deactivation. Fig. 9A-C shows deactivation data recorded from WT hERG channels. As previously observed (Sanguinetti et al., 1995), deactivation was best described by a double exponential fit (Fig. 10A-C). The average time constants and relative amplitudes of the fast and slow phases of current decay are plotted in Fig. 9B and C, respectively. Both the fast and slow tau values are voltage dependent; at more hyperpolarized potentials the fast phase is predominant and the rate of both phases of deactivation is faster than at less hyperpolarized potentials where the slow phase is the principal component. Fig. 9D-F plots the deactivation properties of G546L channels. Due to the left shift in the voltage dependence of activation for G546L channels (Figs. 4 and 8), deactivation was studied over voltages ranging from -70 to -150 mV. Interestingly, we observed that deactivation in the G546L mutant was best described by a single exponential function at all potentials tested (Fig. 9D-F); we did not observe a slow phase in G546L channels. Conversely, in the G546V mutant, deactivation remained biphasic, but the slow phase was markedly pronounced and voltage-independent (Fig. 9G-I). Similar observations were seen in the other G546 mutants tested, with mutation at this site resulting in one of three deactivation phenotypes: 1) WT-like, with two voltage dependent components of deactivation; 2) G546L-like, with one voltage dependent component; 3) G546V-like, with one voltage dependent component and a prominent slow phase showing no dependence on
Figure 9. Mutations at G546 affect the kinetics of deactivation differently than activation gating. A, D, G, typical current traces recorded from WT, G546L, and G546V channels in response to the voltage protocols shown in the insets. B, E, H, plots of deactivation tau-voltage relationships on a log scale. Deactivation in WT channels was best described by a double exponential function, yielding values for $\tau_{\text{fast}}$ and $\tau_{\text{slow}}$. In the case of G546L, deactivation was well described by a single exponential function ($\tau_{\text{deact}}$) at all potentials tested. G546V deactivation displayed an unusual prominent and voltage-independent slow phase of current decay. C, F, I, plots of the relative amplitudes of the phases of deactivation in each of the channel types (n=5-8). The G546L and G546V mutations specifically affect voltage sensor return during deactivation.
<table>
<thead>
<tr>
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<th>( \tau_{\text{fast}} ) (ms)</th>
<th>( \tau_{\text{slow}} ) (ms)</th>
<th>( A_{\text{fast}} )</th>
<th>( A_{\text{slow}} )</th>
<th>( \tau_{\text{slow ratio}} )</th>
<th>n</th>
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<td>WT</td>
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<td>61 ± 9</td>
<td>-100 mV 156 ± 20</td>
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<td>0.88 ± 0.05</td>
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</tr>
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<td>99 ± 4</td>
<td>-100 mV -</td>
<td>-150 mV -</td>
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<td>334 ± 89</td>
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<td>-150 mV -</td>
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<td>-100 mV -</td>
<td>-150 mV -</td>
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<td>-</td>
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<td>G546Y</td>
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<td>71 ± 2</td>
<td>-100 mV 980 ± 28</td>
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<td>0.44 ± 0.02</td>
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<td>-100 mV 1871 ± 203</td>
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<td>-150 mV -</td>
<td>0.68 ± 0.04</td>
<td>0.32 ± 0.04</td>
</tr>
<tr>
<td>G546A</td>
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<td>31 ± 7</td>
<td>-100 mV 623 ± 65</td>
<td>-150 mV -</td>
<td>0.38 ± 0.03</td>
<td>0.15 ± 0.02</td>
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Table 3. Deactivation properties of WT and mutant hERG channels. Values for deactivation tau, $\tau_{\text{fast}}$ and $\tau_{\text{slow}}$, and relative amplitudes, $A_{\text{fast}}$ and $A_{\text{slow}}$, were obtained from double exponential fits of WT and mutant deactivating tail currents. Values are shown at $-110$ mV for WT and G546C and at $-150$ mV for all other mutants to account for the shift of activation. G546I, G546L, G546R and G546E deactivating tail currents were fitted with a single exponential function at all potentials; hence there is no value for $\tau_{\text{slow}}$. $\tau_{\text{slow}}$ ratio values represent the ratio of $\tau_{\text{slow}}(-60\,\text{mV})/\tau_{\text{slow}}(-110\,\text{mV})$ (WT and G546C) or $\tau_{\text{slow}}(-100\,\text{mV})/\tau_{\text{slow}}(-150\,\text{mV})$ (all other mutants) to highlight the voltage-independence of the slow phase of deactivation observed in some mutant channels.
voltage. Table 3 summarizes these results by reporting the ratio of the slow phase tau at moderately and strongly hyperpolarized potentials ($\tau_{\text{slow}(-50 \text{ mV})}/\tau_{\text{slow}(-100 \text{ mV})}$). For WT, the ratio of $\tau_{\text{slow}(-50 \text{ mV})}/\tau_{\text{slow}(-100 \text{ mV})}$ was $10.5 \pm 1.8$, suggesting a strong voltage dependence; whereas for G546V, the ratio was $1.2 \pm 0.1$, suggesting the rate of the slow phase had little or no voltage-dependence. Ratios for G546L were not calculated since the slow phase was absent. These observations suggest that the nature of the amino acid at position 546 plays an important role in regulating deactivation, but not in the same way that it regulates activation. To better understand how the G546 mutations affected deactivation gating, we used VCF to measure voltage sensor return during channel closure.

Two possible mechanisms by which the G546 mutations could alter channel closing are by affecting electromechanical coupling, or by modifying voltage sensor movement. Fig. 10A shows example fluorescence traces recorded from TMRM-labelled hERG L520C channels over a range of hyperpolarizing potentials. Similar to that observed with the ionic current decay, the fluorescence report of voltage sensor movement was best fit with a double exponential and was accelerated at more negative potentials. Fig. 10B plots the relative amplitudes of the fast and slow phases of the fluorescence change for L520C channels during deactivation. The relative contributions of the fast and slow phases of voltage sensor return correlate well with the fast and slow components of the ionic current decay, suggesting that WT hERG channel closing is the result of at least two different conformational changes of the voltage sensor that occur upon repolarization. Example fluorescence reports
Figure 10. Mutations at G546 affect deactivation by altering voltage sensor return. A, C, E, typical fluorescence traces recorded from L520C, G546L L520C, and G546V L520C channels in response to the voltage protocols shown in the insets. Fluorescence traces were fitted with a double exponential function for L520C and G546V L520C, and a single exponential function for G546L L520C (gray lines). B, D, F, plots of mean relative amplitudes of the fast and slow components of fluorescence change during deactivation (n=5-8). The relative amplitudes of fast and slow components of ionic current decay (Fig. 9) are shown by the dotted lines. Fig. 10C shows typical fluorescence traces from G546L L520C channels. As with the ionic current decay, fluorescence deflections recorded from these channels were fit best with a single exponential function at all potentials tested (compare Fig. 10D with Fig. 9D); suggesting that the monoexponential deactivation phenotype present in G546L channels is due a specific modification of voltage sensor return.
from G546V L520C channels are shown in Fig. 10E. Again, similar to that seen with the ionic current decay in G546V channels, the fluorescence report from G546V L520C channels contained a slow phase of voltage sensor return that remained prominent at all potentials tested; further, the relative amplitudes of the fast and slow components of the fluorescence decay were voltage-independent (Fig. 10F). These data suggest that the deactivation phenotypes observed from mutation of G546 is due to a specific modification of the return of the voltage sensor during repolarization of the membrane.

3.2.4 The G546V phenotype is not the result of altered N-terminal interactions

Multiple studies have reported that the slow deactivation gating process observed in hERG channels is mediated by an N-terminal interaction with the core of the channel (Alonso-Ron et al., 2008; Gomez-Varela et al., 2002; Gustina and Trudeau, 2009; Morais Cabral et al., 1998; Schonherr and Heinemann, 1996; Spector et al., 1996a; Wang et al., 1998; Wang et al., 2000). More specifically, it has been suggested that this site of interaction may include regions of the S4-S5 linker (Wang et al., 1998). Therefore, we have constructed channels lacking the majority of the N-terminus (Δ2-354) to determine whether the unusual G546V phenotype is a result of altered interactions with the N-terminus. Fig. 11A and D show example current traces recorded from N-terminal deletion mutants with or without the G546V mutation, respectively. Study of these mutants over a range of hyperpolarizing membrane potentials show that, as reported previously, deletion of the N-terminus accelerates channel closing
Figure 11. Altered N-terminal interactions cannot describe the altered slow phase of G546V. A, D, typical current traces recorded from Δ2-354 and Δ2-354 G546V channels in response to the voltage protocols shown in the insets. B, E, plots of deactivation tau-voltage relationships on a common log scale (n=6-10). Although deactivation was best described by a double exponential fit in each channel, τ_{slow} was too slow in Δ2-354 G546V channels to obtain accurate measurements from the 4 s test pulse. C, F, plots of the relative amplitudes of the phases of deactivation in the two channel types. Values for A_{slow + residual} are plotted for Δ2-354 G546V rather than A_{slow}, because of the slow decay of current. Typical fluorescence traces recorded from Δ2-354 L520C and Δ2-354 G546V L520C channels in response to the voltage protocols shown in the insets. Respective relative amplitudes of the fast and slow components of deactivation were 91.4 ± 4.8 % and 8.6 ± 4.8 % for Δ2-354 L520C channels, and 32.4 ± 3.4 % and 67.6 ± 3.4 % for Δ2-354 G546V L520C channels.
Fast and slow components of deactivation were accelerated in Δ2-354 compared to that observed in WT channels (compare Figs. 11B and 9B); further, the fast phase in Δ2-354 channels was dominant at all potentials tested (Fig. 11C), unlike in WT channels (Fig. 9C). Interestingly, although the fast component of deactivation was accelerated in the G546V Δ2-354 channels – suggesting the N-terminus is still able to interact with the G546V channel – the prominent voltage-independent slow phase of deactivation remained (Fig. 11D-F). That the effect of the G546V mutation was evident regardless of the presence or absence of the N-terminus, suggests that the slow voltage-independent component of deactivation associated with the G546V mutation is not the result of altered N-terminal interactions but rather a specific effect on voltage sensor movement. To directly test this, we collected fluorescence emissions from TMRM attached at L520C from the N-terminal deletion mutants, with or without G546V (Fig. 11G and H). While the fluorescence report of the fast phase of voltage sensor movement was accelerated in both Δ2-354 and G546V Δ2-354 channels, the prominent voltage-insensitive component of voltage sensor return was still present in the G546V N-terminal deletion construct. These data support the notion that the G546V phenotype is due to a specific modification of voltage sensor movement.

3.3 Discussion

Despite recent efforts, the mechanism by which voltage sensor rearrangements in hERG channels are translated into pore opening and closing is unclear. In this chapter, our data suggest that the presence of a glycine in the
S4-S5 linker at position 546 increases the flexibility of the S4-S5 helix, resulting in increased constraints on voltage sensor movement and thus pore opening. Further, our VCF data demonstrates that mutations within the S4-S5 linker also alter the kinetics of deactivation, and do so by specifically affecting the way the voltage sensor moves.

3.3.1 Effects on activation

In Shaker K⁺ channels, a leucine heptad repeat is present in the S4-S5 linker and disruption of this motif at the second leucine position severely disrupts activation gating and electromechanical coupling (McCormack et al., 1991; McCormack et al., 1994; Schoppa et al., 1992; Schoppa and Sigworth, 1998a; Schoppa and Sigworth, 1998b). Interestingly, this leucine heptad repeat in the S4-S5 linker of hERG channels is naturally disrupted by a glycine in the second leucine position (Fig. 4A). Here, our data show that substitution of this glycine with leucine, in an attempt to restore the leucine heptad repeat, stabilizes the open state of the channel (Figs. 4 and 6). However, this effect is not specific to the leucine substitution, but is also present in a number of G546 mutations where the substituted amino acid varies in its physico-chemical property (Fig. 8A). Our AGADIR algorithm structural predictions suggest that these mutations increase the α-helical content of the S4-S5 linker and that the glycine at G546 in WT hERG channels stabilizes the closed state by placing constraints on S4 movement through increased flexibility of the S4-S5 linker α-helix.

In most Kv channels, the S6 helices of the intracellular gate contain a glycine residue that acts as a hinge permitting the splaying of the S6 helical
bundle required for channel opening (Cordes et al., 2002; Ding et al., 2005; Jiang et al., 2002b; Magidovich and Yifrach, 2004; Zhao et al., 2004). Interestingly, our SWISS MODEL predictions of the S4-S5 linker, using the known crystal structure of Kv1.2 as a template, suggest that G546 is located at a bend in the S4-S5 helix (Fig. 8D-E). Since the S4-S5 linker has been shown to interact directly with the S6 helices to stabilize the closed state of hERG channels (Ferrer et al., 2006; Tristani-Firouzi et al., 2002), we predict that this interaction is achieved through conformational rotations of the S4-S5 helix that may occur around G546. This would suggest that G546 is a glycine hinge. Substitution of glycines throughout the S4-S5 linker in the background of G546L, in an attempt to restore flexibility to the S4-S5 linker in different positions, would be a direct test as to whether flexibility of the S4-S5 linker is specific to the G546 position. Further, if G546 is a hinge point, then substitution with a proline should uncouple voltage sensor movement from pore opening; the proline may lock the linker in a specific conformation preventing the S4-S5 linker from initiating movement of the S6 bundle crossing. Further rationale and preliminary results from experiments designed to test this are presented in the Conclusions and Future Directions of this thesis (Section 5.1.1).

3.3.2 Effects on deactivation

Since substitutions at G546 had separable effects on channel activation and deactivation (Tables 2 and 3), and since there was little correlation of the nature of the amino acid with the effect on deactivation (see discussion and Fig. 12 below), we can infer that the changes in the α-helical propensity of the S4-S5
linker caused by G546 mutations are not responsible for the observed changes in deactivation properties. Our WT and G546V fluorescence data suggest the presence of at least two reconfigurations of the voltage sensor during deactivation that occur with different time courses and that can be independently modified (Figs. 10 and 11). This observation is consistent with hERG gating current records, which display a biphasic return of charge upon repolarization (Piper et al., 2003), and suggests that the voltage sensor experiences a different environment during deactivation than during activation. We propose that the G546 mutations specifically alter these reconfigurations; for example, the G546L mutation removed the slow phase of deactivation whereas the amplitude of the slow component in the G546V mutant was enhanced and voltage-independent (Fig. 9). Further, we suggest that these mutations alter these reconfigurations through modifying the way the voltage sensor moves as shown in Fig. 10. An interesting observation is that the fluorescence signal overshot the baseline at hyperpolarized potentials in both the G546V and G546L mutants; we did not observe this overshoot with L520C channels (Fig. 10). We interpret the observed overshoot to be the consequence of increased movement of the S4 segment at hyperpolarized potentials. This is consistent with the data presented in Figs. 4, 6 and 8 that suggest that the G546 mutants stabilize the open state of the channel by reducing constraints on the voltage sensor, particularly at negative potentials.

We observed no clear correlation between deactivation properties and the nature of the amino acid side chain (size, charge, and hydrophobicity) at position 546 (Fig. 12). From this, we conclude that the effect of the different substitutions
Figure 12. Correlations between hydrophobicity, size and charge of the residues at position 546 and the effects on deactivation. A-C, correlations of the weighted average tau of deactivation with side chain hydrophobicity (A), volume (B), and charge (C). The weighted average tau of deactivation was calculated from the data shown in Table 2 using the equation: weighted average tau (ms) = \( \tau_{\text{fast}} \times A_{\text{fast}} + \tau_{\text{slow}} \times A_{\text{slow}} \). Lines show Pearson correlations for each data set, and r, \( r^2 \) and p values are given. D, E, correlations of deactivation with hydrophobicity (D) or side chain volume (E) considering only the uncharged amino acids examined in this study. F, G, correlations of deactivation with side chain volume considering either only hydrophobic (F) or hydrophilic (G) amino acids. H, I, correlations of deactivation with hydrophobicity considering either “small” (<150 Å\(^3\)) (H) or “large/bulky” (>150 Å\(^3\)) (I) amino acids.
is the result of specific interactions or steric hindrances induced by each side chain. That being said, as shown in (Fig. 12H), we did observe a positive correlation between the mutational effect on deactivation and hydrophobicity of the mutated amino acid when large amino acids were not considered. This may suggest that hydrophobic interactions between the amino acid at G546 and other regions of the channel may slow deactivation; furthermore, bulky amino acids may additionally disrupt the deactivation process by interfering with these and other local interactions. Since activation was similar amongst the mutant channels, the specific interactions induced by the side chain at G546 must form when the voltage sensor is in the activated configuration and dictate the ability of the voltage sensor to return to its resting configuration upon repolarization. These results suggest that the voltage sensor experiences a different environment whether in the activated or deactivated state.
4: CHAPTER 4: MECHANISM OF THE ACTION OF PROTONS ON hERG K⁺ CHANNELS

4.1 Introduction

Extracellular acidosis has been shown to occur as a consequence of myocardial ischemia (Carmeliet, 1999; Crampin et al., 2006; Orchard and Cingolani, 1994). Low pH₀ reduces Ikr in ventricular cardiac myocytes (Vereecke and Carmeliet, 2000). The effect of pH₀ changes on hERG channel function in heterologous expression systems has been given much attention over the past decade. To date, every report that has studied proton modulation of hERG channels has observed an acceleration of deactivation. This observation has been observed in hERG channels across different expression systems, such as *Xenopus* oocytes, CHO, and L929 cells (Anumonwo et al., 1999; Berube et al., 1999; Bett and Rasmusson, 2003; Jiang et al., 1999; Jo et al., 1999; Terai et al., 2000). The effect of external protons on hERG channel inactivation properties is also fairly consistent. In oocytes, external protons have been shown to slightly accelerate the rate of recovery from inactivation (Jiang et al., 1999; Terai et al., 2000) while having little effect on the voltage dependence and rate of onset of inactivation (Jiang et al., 1999; Terai et al., 2000). These results differ slightly from those in a mammalian cell line, L929 cells (Anumonwo et al., 1999) where the authors found no significant change in the rate of onset or the recovery from inactivation (Anumonwo et al., 1999).
The major discrepancies in regards to hERG channel modulation by extracellular protons pertain to the effect on activation. In oocytes, changing the pHo in the range of 8.5-6.5 has been reported to have little effect on the voltage dependence of activation (Jiang et al., 1999); while at the same time, other studies with oocyte expression systems have reported significant depolarizing shifts when the pHo is dropped from of 8.6-5.6 (Terai et al., 2000), 8.5-5.5 (Bett and Rasmusson, 2003), 7.4-5.8 (Anumonwo et al., 1999), 8.0-6.2 (Jo et al., 1999). In CHO cells, one report suggests little effect of external protons on the voltage dependence of activation in the pHo range of 8.0-6.0 (Berube et al., 1999); while in L929 cells, dropping the pHo from 7.4 to 6.4 resulted in a depolarizing shift in the midpoint of the voltage dependence of activation (Anumonwo et al., 1999).

A similar discrepancy has been observed for the effect on peak tail current (or maximal conductance). In oocytes, most studies have reported a reduction in peak tail current at low pHo (Anumonwo et al., 1999; Bett and Rasmusson, 2003; Jo et al., 1999; Terai et al., 2000), while another study claims low pHo augments tail current amplitude (Jiang et al., 1999). In CHO cells, extracellular protons have little effect on maximal conductance (Berube et al., 1999); whereas in another mammalian cell line, L929 cells, low pHo reduces the peak tail current (Anumonwo et al., 1999). Recently, it has been suggested that low pHo slows the rate of activation by altering the voltage-independent step in the hERG activation pathway (Zhou and Bett, 2010).

Overall, the effect of external protons on hERG channel closing is well
established, whereas the effect on activation is conflicting. One study has
provided a reasonable explanation for this discrepancy by looking at the pH₀
dependence for the effect of protons on deactivation and maximal conductance
(Bett and Rasmusson, 2003). This study reports two different pKₐ values for the
effect on deactivation and maximal conductance: pH₀ 6.76 and 5.5, respectively
(Bett and Rasmusson, 2003). Therefore, based on the titration curves, any
development from pH₀ 7.4 will result in an alteration in the rate of deactivation;
whereas larger deviations from pH₀ 7.4 are required to observe reductions in the
peak tail current. This finding also has implications for the mechanism behind the
action of protons. The fact that pH₀ changes on deactivation and maximal
conductance can be described with two different pKₐ values suggests that
protons affect hERG channel function via two independent mechanisms.

Much attention has been given towards understanding the mechanism
behind pH₀ modulation of hERG channel function, yet little is understood. The
suggestion of histidines as pH₀ sensors in hERG channels is an interesting
proposal for two reasons: 1) the two pKₐ values for the effect of protons on
deactivation and maximal conductance are near that of a histidine (pKₐ = 6.04);
2) in other voltage-gated potassium channels, titration of a histidine side chain
mediates the effects of acidosis (Claydon et al., 2000; Jager et al., 1998; Kehl et
al., 2002; Steidl and Yool, 1999). Fig. 16A shows that hERG channels have five
native histidines that may be accessible to the extracellular solution (H485,
H492, H578, H587, and H562). Only the two histidines in the turret (H578 and
H587) have been studied and were shown not to be responsible for the
acceleration of deactivation at low pH$_o$ (Jiang et al., 1999). However, the role of these histidines pertaining to the effect of protons on maximal conductance, and the role of the remaining three histidines in modulation of both the activation and deactivation properties by pH$_o$ is yet to be determined. In this chapter, we explore the molecular mechanisms of action of external protons on hERG channel deactivation and maximal conductance. Our hypothesis is that native histidines act as pH sensors for both effects of protons on channel function.

4.2 Results

4.2.1 Protons inhibit hERG channel function via two distinct mechanisms

Figs. 13-15 summarize the effects of pH$_o$ on WT hERG channel function. Fig. 13 shows typical current traces recorded from WT hERG channels in response to various hyperpolarizing potentials preceded by a depolarizing pulse to +60 mV for 500 ms. Low pH$_o$ results in an acceleration of channel closing. This is more clearly shown in Fig. 15A, which shows the current decay at -60 mV recorded at pH$_o$ 5.5 normalized to that recorded at pH$_o$ 7.4 to highlight the acceleration of deactivation observed at low pH$_o$. Deactivating currents were fitted with a double exponential function at all potentials tested, and the resulting time constant values for the slow and fast phases of deactivation are plotted against membrane potential in Fig. 15C and D, respectively, and listed in Table 4. Both phases of deactivation were accelerated significantly at pH$_o$ 6.5, 5.5, and 4.5 in comparison to 7.4.

A second effect of low pH$_o$ that we observed was a reduction in maximal
Figure 13. Low pH₀ accelerates hERG channel deactivation. A–H, typical current traces recorded from hERG WT channels at pH₀ 7.4 (A,C,E,G), 8.0 (B), 6.5 (D), 5.5 (F), and 4.5 (H) in response to various hyperpolarizing pulses for 4 s, preceded by a depolarizing pulse for 500 ms (see section 2.3.2.2).
conductance. Fig. 14 shows typical current traces recorded from WT hERG channels in response to various depolarizing potentials. Similar to that shown in Fig. 13, the decay of the tail current (recorded in response to a -60 mV pulse for 2 s) in each trace was accelerated at low pH; however, it is also clear from Fig. 14E-H that the peak tail current at pH$_o$ 5.5 and 4.5 were reduced in comparison to that at pH$_o$ 7.4. Since the relative amplitude of the peak tail current in hERG channels is a measure of the relative conductance during the previous depolarizing pulse (see section 2.3.2.3 and 2.4.1), comparison of the peak tail current at different pH$_o$ values indicates the effect of protons on maximal conductance. Of the pH$_o$ values tested, maximal conductance is reduced at pH$_o$ values of 5.5 and 4.5. This is shown more clearly in Fig. 15B where current traces recorded at -60 mV at pH$_o$ 7.4 and 5.5 are overlapped to highlight the reduction in peak tail current observed at low pH$_o$. G-V relations of WT at the range of pH$_o$ tested are plotted in Fig. 15E; G-V relations at each pH$_o$ are normalized to pH$_o$ 7.4 to highlight the change in maximal conductance at each pH$_o$. Values for $V_{1/2}$, $k$, and fractional amplitude after perfusion of each pH$_o$ are listed in Table 4. At pH$_o$ 5.5 and 4.5, the G-V relationship was shifted to the right by approximately 20 mV. This pH-dependent shift in the voltage dependence of activation has been described in many other ion channels as an effect of charge screening (Hille, 2001); whereby protons titrate negative charges on the phospholipid membrane, resulting in a shift in the electrical environment sensed by the voltage sensor. Interestingly, maximal conductance is reduced by low pH$_o$ independent of the shift in the G-V. The mechanism behind this reduction in
Figure 14. Low pH reduces hERG channel maximal conductance. A-H, typical current traces recorded from hERG WT channels at pH 7.4 (A,C,E,G), 8.0 (B), 6.5 (D), 5.5 (F), and 4.5 (H) in response to a range of depolarizing pulses from -80 mV to +60 mV in 10 mV increments. Each pulse was followed by a -60 mV hyperpolarizing pulse where peak tail current was measured (see section 2.3.2.1).
 maximal conductance at low pH\textsubscript{o} is unknown.

Our data suggest that low pH\textsubscript{o} has two main effects on hERG channel function: 1) both the fast and slow components of deactivation are accelerated (Figs. 13 and 15), and 2) the maximal conductance is reduced (Figs. 14 and 15). Fig. 15F shows the pH\textsubscript{o} dependence for the effects of protons on deactivation (plotted as the change in weighted tau for simplicity) and maximal conductance. Protons affect deactivation and maximal conductance with a pK\textsubscript{a} of pH\textsubscript{o} 7.1 and 5.7, respectively. These observations are similar to that reported by Bett and Rasmusson, suggesting the presence of two separate binding sites for each effect (Bett and Rasmusson, 2003). Since the pK\textsubscript{a} for a histidine (pH 6.04) is comparable to the pK\textsubscript{a} of either of these effects, and since histidines serve as pH sensors for the effects of protons in other Kv channels (Claydon et al., 2000; Jager et al., 1998; Kehl et al., 2002; Steidl and Yool, 1999), we hypothesized that histidines may serve as the pH\textsubscript{o} sensor governing these effects in hERG WT channels. In the following sections, we first consider the role of histidines in the pH\textsubscript{o}-induced modulation of deactivation and then consider their role in the loss of conductance observed at low pH\textsubscript{o}.

4.2.2 Native histidines are not the pH\textsubscript{o} sensor for the effects of protons on deactivation

Fig 16A shows a schematic of the hERG channel highlighting the location of the five native histidines investigated in this study. H578 and H587 are located in the extracellular S5P linker, H485 and H492 are located in the S3 transmembrane domain, and H562 is in the S5 transmembrane domain. We
Figure 15. Protons have two separable effects on hERG WT channels. A, typical WT current traces during a 4 s pulse to -60 mV after a 500 ms pulse to +60 mV. pH 5.5 trace is normalized to pH 7.4 trace to highlight the change in deactivation rate (pH 7.4, black; pH 5.5, red). B, typical WT current traces during a 2 s pulse to +60 mV followed by a 2 s pulse to -60 mV (pH 7.4, black; pH 5.5, red). C, D, plots of the slow and fast deactivation tau-voltage relationships, respectively. E, G-V relations at pH 8.0, 7.4, 6.5, 5.5, and 4.5 normalized to peak tail current at pH 7.4. F, pH dependence of hERG WT deactivation and maximal conductance; pKₐ values were 7.1 and 5.7, respectively, derived from fits of the data in F with a Hill Equation. Values for deactivation and activation parameters, along with statistics and sample size are presented in Table 4.
<table>
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<th>pH&lt;sub&gt;o&lt;/sub&gt;</th>
<th>V&lt;sub&gt;1/2&lt;/sub&gt; (mV)</th>
<th>k (mV)</th>
<th>Fractional Amplitude (%)</th>
<th>τ&lt;sub&gt;fast&lt;/sub&gt; (ms) at -60 mV</th>
<th>τ&lt;sub&gt;slow&lt;/sub&gt; (ms) at -60 mV</th>
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<td>8.0</td>
<td>-25.2 ± 0.4</td>
<td>7.4 ± 0.1</td>
<td>109.1 ± 0.8*</td>
<td>490.3 ± 10.5*</td>
<td>2972.4 ± 59.9*</td>
</tr>
<tr>
<td>7.4</td>
<td>-26.2 ± 0.8</td>
<td>7.9 ± 0.2</td>
<td>-</td>
<td>403.8 ± 8.8</td>
<td>2403.3 ± 58.5</td>
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<tr>
<td>6.5</td>
<td>-23.0 ± 0.7</td>
<td>7.7 ± 0.1</td>
<td>98.8 ± 3.8</td>
<td>223.1 ± 7.3*</td>
<td>914.5 ± 86.0*</td>
</tr>
<tr>
<td>5.5</td>
<td>5.0 ± 1.5*</td>
<td>10.3 ± 0.3*</td>
<td>59.6 ± 4.2*</td>
<td>84.5 ± 9.0*</td>
<td>328.7 ± 36.5*</td>
</tr>
<tr>
<td>4.5</td>
<td>7.8 ± 2.5*</td>
<td>9.9 ± 0.1*</td>
<td>33.6 ± 2.5*</td>
<td>70.7 ± 3.9*</td>
<td>336.7 ± 16.3*</td>
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</table>

Table 4. pH<sub>o</sub> effects on WT hERG channels deactivation and activation properties. Values for V<sub>1/2</sub> and k were obtained from Boltzmann fits of the G-V relationships at each pH<sub>o</sub>. Values for deactivation tau, τ<sub>fast</sub> and τ<sub>slow</sub>, were obtained from double exponential fits of WT and mutant deactivating tail currents. * = Statistically different from pH<sub>o</sub> 7.4 (p < 0.05; n = 5-8 for pH<sub>o</sub> 4.5, 5.5, 6.5, 8.5, and n = 25 for pH<sub>o</sub> 7.4).
explored these five histidines because all have the potential to be exposed to the extracellular solution, and thus may be accessible to external protons. Several lines of evidence support this: 1) the S5P linker resides in the extracellular milieu and studies have shown that H587C is modifiable by extracellular application of reducing agents (Dun et al., 1999; Liu et al., 2002); 2) cystiene mutants located in the proximity of H485 and H492, are modifiable by extracellular application of reducing agents (Liu et al., 2003); 3) a recent report suggests that H562 is the pH\textsubscript{o} sensor mediating the effects on deactivation, since deactivation of the H562Q mutant is only slightly accelerated at pH\textsubscript{o} 6.6 (Bett et al., 2011).

Fig. 16B-G shows typical current traces recorded at -60 mV preceded by a depolarizing pulse to +60 mV in WT, H485Q, H492Q, H578Q, H587Q, and H562Q at pH\textsubscript{o} 7.4 (black trace) and 5.5 (red trace). Study of these mutants over a range of hyperpolarizing membrane potentials show that low pH\textsubscript{o} is still able to accelerate the time course of deactivation in all mutant channels. Fig. 16H-I plots the slow and fast components of deactivation against voltage, respectively. As was the case in WT channels, both phases of deactivation were accelerated significantly at pH\textsubscript{o} 5.5 in comparison to 7.4 in all mutants (see Tables 5 and 6). These data suggest that native histidines in hERG channels are not the pH\textsubscript{o} sensor for the effect on deactivation.

4.2.3 Protons accelerate deactivation by altering voltage sensor movement

The data in sections 3.2.4 and 3.2.5 suggested that voltage sensor return during normal channel deactivation is complex, undergoing at least two
Figure 16. Native histidines are not the pH_o sensor for the acceleration of deactivation at low pH_o. A, Schematic showing the predicted location of native histidines; H485Q, H492Q, H562Q, H578Q, H587Q are symbolized as blue, red, green, purple, yellow, respectively. B-G, typical current traces recorded from hERG WT, H485Q, H492Q H578Q, H587Q, and H562Q channels during a 4 s step to -60 mV applied immediately following a 500 ms step to +60 mV. pH_o 5.5 trace is normalized to pH_o 7.4 trace to highlight the change in deactivation rate (pH_o 7.4, black; pH_o 5.5, red). H,I, plots of $\tau_{slow}$ and $\tau_{fast}$ deactivation-voltage relationships, respectively. Neither mutation attenuated the acceleration of deactivation at low pH_o. Values, statistics and sample sizes are presented in Tables 5 and 6.
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<th>Construct (pH&lt;sub&gt;o&lt;/sub&gt;)</th>
<th>V&lt;sub&gt;1/2&lt;/sub&gt; (mV)</th>
<th>k (mV)</th>
<th>Fractional Amplitude (%)</th>
<th>τ&lt;sub&gt;fast&lt;/sub&gt; (ms) at -60 mV</th>
<th>τ&lt;sub&gt;slow&lt;/sub&gt; (ms) at -60 mV</th>
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<tr>
<td>WT (7.4)</td>
<td>-25.4 ± 2.1</td>
<td>8.0 ± 0.1</td>
<td>-</td>
<td>405.4 ± 25.6</td>
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<td>WT (5.5)</td>
<td>5.0 ± 1.5*</td>
<td>10.3 ± 0.3*</td>
<td>59.6 ± 4.2*</td>
<td>84.5 ± 9.0*</td>
<td>328.7 ± 36.5*</td>
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<td>H485Q (7.4)</td>
<td>-36.3 ± 1.6</td>
<td>7.5 ± 0.1</td>
<td>-</td>
<td>504.1 ± 12.0</td>
<td>2875.9 ± 110.3</td>
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<td>H485Q (5.5)</td>
<td>-4.0 ± 0.5*</td>
<td>10.9 ± 0.4</td>
<td>47.5 ± 1.8*</td>
<td>71.5 ± 3.3*</td>
<td>289.9 ± 8.1*</td>
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<td>H492Q (7.4)</td>
<td>-32.8 ± 1.4</td>
<td>7.4 ± 0.2</td>
<td>-</td>
<td>468.2 ± 15.6</td>
<td>2653.2 ± 118.4</td>
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<td>H492Q (5.5)</td>
<td>-6.5 ± 2.2*</td>
<td>10.4 ± 0.4</td>
<td>51.4 ± 1.4*</td>
<td>70.0 ± 2.9*</td>
<td>259.3 ± 15.3*</td>
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<td>H578Q (7.4)</td>
<td>-28.3 ± 1.6</td>
<td>7.0 ± 0.1</td>
<td>-</td>
<td>356.0 ± 19.4</td>
<td>2184.9 ± 130.6</td>
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<td>H578Q (5.5)</td>
<td>3.1 ± 1.1*</td>
<td>8.7 ± 0.2</td>
<td>27.7 ± 3.4*</td>
<td>70.2 ± 3.4*</td>
<td>272.5 ± 18.8*</td>
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<td>H587Q (7.4)</td>
<td>-30.6 ± 1.0</td>
<td>8.0 ± 0.6</td>
<td>-</td>
<td>417.4 ± 45.0</td>
<td>2620.9 ± 257.4</td>
</tr>
<tr>
<td>H587Q (5.5)</td>
<td>-1.6 ± 1.4*</td>
<td>10.7 ± 0.2</td>
<td>66.3 ± 3.1*</td>
<td>66.7 ± 4.1*</td>
<td>218.4 ± 18.2*</td>
</tr>
</tbody>
</table>

Table 5. pH<sub>o</sub> 5.5 effects on WT and mutant hERG channel deactivation and activation properties. Values for V<sub>1/2</sub> and k were obtained from Boltzmann fits of the G-V relationships at each pH<sub>o</sub>. Values for deactivation tau, τ<sub>fast</sub> and τ<sub>slow</sub>, were obtained from double exponential fits of WT and mutant deactivating tail currents. * = Statistically different from pH<sub>o</sub> 7.4 for each respective channel (p < 0.05; n = 5-8; paired t-test).
<table>
<thead>
<tr>
<th>Construct (pH&lt;sub&gt;o&lt;/sub&gt;)</th>
<th>V&lt;sub&gt;1/2&lt;/sub&gt; (mV)</th>
<th>k (mV)</th>
<th>Fractional Amplitude (%)</th>
<th>τ&lt;sub&gt;fast&lt;/sub&gt; (ms) at -60 mV</th>
<th>τ&lt;sub&gt;slow&lt;/sub&gt; (ms) at -60 mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (7.4)</td>
<td>-35.6 ± 1.5</td>
<td>7.4 ± 0.0</td>
<td>-</td>
<td>510.4 ± 13.9</td>
<td>3085.0 ± 186.3</td>
</tr>
<tr>
<td>WT (5.5)</td>
<td>2.4 ± 1.7*</td>
<td>11.0 ± 0.2*</td>
<td>36.4 ± 1.5*</td>
<td>76.0 ± 2.7*</td>
<td>322.3 ± 8.4*</td>
</tr>
<tr>
<td>H562Q (7.4)</td>
<td>-46.4 ± 5.3</td>
<td>7.4 ± 0.7</td>
<td>-</td>
<td>336.9 ± 9.6</td>
<td>2482.8 ± 62.5</td>
</tr>
<tr>
<td>H562Q (5.5)</td>
<td>4.9 ± 6.3*</td>
<td>15.4 ± 0.6*</td>
<td>36.6 ± 1.8*</td>
<td>83.1 ± 5.4*</td>
<td>460.0 ± 30.9*</td>
</tr>
</tbody>
</table>

Table 6. pH<sub>o</sub> 5.5 effects on WT and mutant hERG channel deactivation and activation properties (recorded in 30 mM KCl). Values for V<sub>1/2</sub> and k were obtained from Boltzmann fits of the G-V relationships at each pH<sub>o</sub>. Values for deactivation tau, τ<sub>fast</sub> and τ<sub>slow</sub>, were obtained from double exponential fits of WT and mutant deactivating tail currents. * = Statistically different from pH<sub>o</sub> 7.4 for each respective channel (p < 0.05; n = 5; paired t-test).
reconfigurations that can be independently modified. Given that voltage sensor movement has been reported to be rate-limiting for hERG channel pore opening (Piper et al., 2003; Smith and Yellen, 2002) (see also Fig. 6E and G), we hypothesized that extracellular protons, which modify hERG gating, may do so by altering voltage sensor movement. To test this, we investigated the effects of acidic pH₀ on voltage sensor movement using VCF.

Fig. 17A and B show example fluorescence traces recorded from L520C channels at either pH₀ 7.4 or 5.5. Fig. 17C shows the fluorescence trace recorded at -70 mV at pH₀ 5.5 scaled to pH₀ 7.4 to highlight the effect of extracellular protons on voltage sensor return. Both the fast and slow phases of voltage sensor return are accelerated at low pH₀. This is shown more clearly in Fig. 17D, which plots the time constant of the fast and slow phases of the fluorescence return and the ionic current decay at both pH₀ 7.4 and 5.5. Similar to that observed with the ionic current decay after perfusion of pH₀ 5.5, the amplitude of the fast component was dominant at all potentials tested and both phases of deactivation were accelerated significantly (n=7-9; p < 0.05, paired t-test). These observations suggest that the mechanism of action of external protons is via a specific action on the return of the voltage sensor.

4.2.4 Native histidines are not pH₀ sensors for the reduction of maximal conductance at low pH₀

Fig. 18A-F show typical current traces from WT, H485Q, H492Q, H578Q, H587Q, and H562Q channels at pH₀ 7.4 (black trace) and 5.5 (red trace). Cells were held at -80 mV and depolarized to +60 mV in 10 mV increments to activate
Figure 17. Protons accelerate deactivation by altering voltage sensor movement. A,B, fluorescence signals from TMRM attached at L520C during 4 s hyperpolarizing steps to -70 or -110 mV applied after a depolarizing step to +60 mV at pH_o 7.4 (A) or pH_o 5.5 (B). C, scaled fluorescence signals with pH_o 7.4 and 5.5 at -70 mV to highlight the effect of acidic pH on the fluorescence report of voltage sensor movement. D, plots of the voltage dependence of fast and slow phases of voltage sensor return on a common log scale (n=7-9).
and inactivate channels, followed by a hyperpolarizing pulse to -60 mV where peak tail current was measured. Substitution of each native histidine with glutamine did not remove the effect of low pH\textsubscript{o} on maximal conductance. Fig. 18G shows the G-V relationships for H485Q, H492Q, H578Q, and H587Q at pH\textsubscript{o} 7.4 and 5.5, with WT data shown as solid lines (pH\textsubscript{o} 7.4, black; pH\textsubscript{o} 5.5, red). In each case, G-V relationships at pH\textsubscript{o} 5.5 are normalized to pH\textsubscript{o} 7.4 to compare the extent of reduction of maximal conductance across constructs. Values for the change in activation parameters at pH\textsubscript{o} 5.5 for all constructs are listed in Tables 5 and 6. After perfusion of pH\textsubscript{o} 5.5, the fractional amplitude of the peak tail current was decreased to $47.5 \pm 1.8$, $51.4 \pm 1.4$, $27.7 \pm 3.4$, $66.3 \pm 3.1$ and $59.6 \pm 4.2\%$ of the original amplitude at pH\textsubscript{o} 7.4 for H485Q, H492Q, H578Q, H587Q and WT channels, respectively. Due to low expression levels, H562Q was recorded in 30 mM KCl and the G-V relationships at pH\textsubscript{o} 7.4 and 5.5 are compared to that of WT in 30 mM KCl in Fig. 18H. Again, after perfusion of pH\textsubscript{o} 5.5, the fractional amplitude of the peak tail current was decreased to $36.6 \pm 1.8$ and $36.4 \pm 1.5$ of the original amplitude at pH\textsubscript{o} 7.4 for H562Q and WT channels, respectively. It is clear from Fig. 18 that native histidines are not the pH\textsubscript{o} sensor for the effects on activation. We next explored the possibility that low pH\textsubscript{o} reduces maximal conductance via a stabilization of the inactivated state.

4.2.5 The reduction in maximal conductance is not due to a stabilization of inactivated states

In other voltage gated potassium channels, such as Kv1.4 and Kv1.5, the reduction in maximal conductance at low pH\textsubscript{o} has been shown to result from a
Figure 18. Native histidines are not the pH₀ sensor for the acceleration of deactivation at low pH₀. A-F, typical current traces recorded from hERG WT, H485Q, H492Q H578Q, H587Q, and H562Q channels during 2 s pulse to +60 mV followed by a 2 s pulse to -60 mV (pH₀ 7.4, black; pH₀ 5.5, red). G,H, plots of G-V relations at 3 mM KCl (G) and 30 mM KCl (H), constructed as described in the methods. Neither mutation attenuated the reduction in maximal conductance at low pH₀. Values, statistics, and sample sizes are presented in Table 5 and 6.
stabilization of C-type inactivation (Cheng et al., 2008; Claydon et al., 2000; Kehl et al., 2002; Steidl and Yool, 1999). Further, introduction of a histidine residue at position T449 in a pH₀ insensitive Shaker K⁺ channel confers pH₀ sensitivity to the C-type inactivation process, resulting in a stabilization of C-type inactivation and thus a reduction in maximal conductance (Lopez-Barneo et al., 1993). In hERG channels, inactivation is rapid and resembles the C-type inactivation process that is present in Shaker channels (Sanguinetti and Tristani-Firouzi, 2006; Schonherr and Heinemann, 1996; Smith et al., 1996; Spector et al., 1996a). Therefore, we hypothesized that protons might reduce the maximal conductance of hERG channels by stabilizing the C-type inactivated state. To test this, we measured the extent of reduction of maximal conductance at pH₀ 5.5 in the inactivation-removed S620T mutant (Ficker et al., 1998), and compared this with WT. Fig. 19A and B show example current traces recorded from hERG S620T channels in response to the activation voltage protocol described in the figure legend at pH₀ 7.4 and 5.5, respectively. As shown previously (Ficker et al., 1998), currents recorded from the S620T mutant at depolarizing pulses did not show rectification, consistent with the absence of inactivation in hERG S620T channels (Ficker et al., 1998). Further, as shown previously by Ficker and colleagues and in Fig. 19C, the G-V relation for S620T at pH₀ 7.4 was shifted in the depolarizing direction compared to WT channels (Ficker et al., 1998). It is clear from Fig. 19A and B that peak tail currents recorded at pH₀ 5.5 were reduced to a similar extent as that observed in WT channels (compare Fig. 19A and B with Fig. 14E and F). Fig. 19C plots the G-V
Figure 19. The reduction in maximal conductance at low pH is independent of the inactivation process. A, B, typical current traces recorded from hERG S620T channels at pH 7.4 (A) and 5.5 (B) in response to a range of depolarizing pulses for 2 s followed by a hyperpolarizing pulse to -60 mV for 2 s. C, G-V relations for hERG WT and S620T channels at pH 5.5 normalized to pH 7.4. The S620T mutant did not attenuate the reduction in maximal conductance observed at low pH in WT channels (n=7; p > 0.05; unpaired t-test).
relationship at pH_o 5.5 normalized to pH_o 7.4. The maximal conductance in S620T channels was reduced to a fractional amplitude of 65.1 ± 1.7 % at pH_o 5.5, which was not significantly different from that in WT channels (n=7; p > 0.05; independent t-test). These data suggest that, unlike in other Kv channels, the reduction in maximal conductance observed at low pH_o is not due to a stabilization of C-type inactivation. This is consistent with previous studies that show that low pH_o does not alter steady-state inactivation or the rate of onset of inactivation in hERG channels (Jiang et al., 1999; Terai et al., 2000). Clearly, the effect of low pH_o on hERG channel maximal conductance is independent of the mechanism of inactivation.

4.2.6 The reduction in maximal conductance at low pH_o is voltage dependent

The data in sections 4.2.4 and 4.2.5 suggest that the reduction in maximal conductance observed at low pH_o is not due to titration of native histidines or an involvement of the inactivation process. In Shaker Kv channels, L-type calcium channels, and the cardiac sodium channel, Na_v1.5, protons reduce maximal conductance by directly blocking the pore (Chen et al., 1996; Khan et al., 2006; Starkus et al., 2003). We hypothesized that this might be the case in hERG channels. To test this, we measured the voltage dependence of the reduction in maximal conductance at pH_o 5.5.

First, in order to more accurately compare the extent of proton block across different voltages, we extrapolated the tail current amplitude back to the instantaneous voltage change where recovery from inactivation does not
obscure our point of measurement (shown in Fig. 20A). The reasoning behind this was, since hERG deactivation is voltage dependent and faster at more hyperpolarized potentials, faster deactivation rates may result in an underestimation of the peak tail current and therefore an overestimation of the pH₀ dependent reduction at more hyperpolarized potentials. Tail currents were fitted with a double exponential function since deactivation is biphasic in hERG channels (Sanguinetti et al., 1995; Fig. 9 from this Thesis).

Fig. 20B plots the fractional amplitude of peak tail current at pH₀ 5.5 normalized to pH₀ 7.4 against voltage for WT, H578E, H587Q, and H587E channels (the rationale and data for testing the mutant constructs will be discussed later in section 4.2.7). It is clear from Fig. 20B that the reduction in maximal conductance at pH₀ 5.5 in WT channels was voltage dependent, with more pronounced block of peak tail current observed at the more hyperpolarized potentials. The voltage dependent nature of the reduction in maximal conductance at low pH₀ strongly suggests that the mechanism of action of external protons is by a direct block within the conducting pore (Woodhull, 1973). We then fit the data with the model proposed by Woodhull (Woodhull, 1973) to estimate the location of the proton binding site. The Woodhull model has been used extensively to estimate the binding site of a number of modifying agents, including protons, in different ion channels (Hille, 2001). For WT channels, the fractional electrical distance, δ, was 0.18 (δ was determined as described in section 2.4.5). This suggests that protons bind near the extracellular surface of
Figure 20. The reduction in maximal conductance at low pH is voltage dependent in hERG WT channels. A, typical peak tail current traces recorded from WT hERG channels at -60 mV after a depolarizing pulse to +60 mV for 2 s. Peak tail currents were fitted with a double exponential function and extrapolated back to the point of instantaneous voltage change (see section 4.2.6). B, plots of the fractional amplitude-voltage relations at pH 5.5 normalized to pH 7.4 for WT, H587E, H578E, and H587Q. Data were fitted with the Woodhull equation as described in the methods. Fractional amplitude at 0 mV was predicted as 1.24, 0.38, 1.15, and 1.27 for WT, H587E, H578E, and H587Q, respectively (n = 5-8).
the selectivity filter, possibly around the first K\(^+\) ion coordination site, S1 (Woodhull, 1973; Zhou et al., 2001).

4.2.7 Fixation of a negative charge at H587 enhances the loss of maximal conductance at low pH\(_o\)

The data in section 4.2.6 suggest that extracellular protons reduce hERG channel maximal conductance by interacting with the pore. In other ion channels, protons exert their effect by titrating negatively charged glutamate residues near the extracellular face of the pore (Chen et al., 1996; Jordt et al., 2000; Martinez-Francois et al., 2010). In hERG channels there is a glutamate residue (E637) situated at the extracellular region of the pore that may serve as the proton binding site. To test this, we have tried to mutate this residue to a neutral glutamine but have encountered problems engineering the construct. Interestingly, E637K is a long QT mutation that reduces I\(_{Kr}\) by decreasing hERG channel trafficking (Hayashi et al., 2002). Given this, it may be that the E637Q mutant channel may not traffic appropriately to the membrane even if we were able to successfully make the construct. As a different approach, given that protons titrate glutamates in other channels (Chen et al., 1996; Jordt et al., 2000; Martinez-Francois et al., 2010), and that the turret residue H587 has been described to be near or form part of the conducting pathway of the hERG channel (Liu et al., 2002), we introduced the H587E mutation to test whether protons bind within the conducting pore. Our rationale was that if protons interact with a negative charge within the pore, we should observe altered binding of protons in the H587E mutant channel. As a negative control, we also tested the
effects of the mutation H578E, a site which is also in the turret region, but that has been shown to lie outside of the permeation pathway (Liu et al., 2002).

Fig. 21A shows typical current traces recorded from the H587E construct in response to a depolarizing pulse to +60 mV followed by a hyperpolarizing pulse to -60 mV (pH_o 7.4 in black; pH_o 5.5 in red). The extent of the loss of maximal conductance at pH_o 5.5 was greater in the H587E mutant compared with WT (compare Fig. 21A with Fig. 15B, see also Table 7). This is more clearly shown in Fig. 21C, which plots the G-V relationships at pH_o 7.4 and at pH_o 5.5 normalized to pH_o 7.4 of the H587E channel, with WT shown in black (pH_o 7.4) and red (pH_o 5.5) solid lines. In contrast, the extent of loss of maximal conductance at pH_o 5.5 in H578E channels was similar to that in WT channels (Fig. 21B and D). Fig. 21B shows typical current traces recorded from H578E in response to a depolarizing pulse to +60 mV followed by a hyperpolarizing pulse to -60 mV (pH_o 7.4 in black; pH_o 5.5 in red) and G-V relations are shown in Fig. 21D (see also Table 7). These data suggest that fixed negative charges near the conducting pathway of hERG channels enhance pore block by external protons.

Fig. 20B plots the fractional amplitude of peak tail current at pH_o 5.5 normalized to pH_o 7.4 against voltage for WT, H578E, H587Q, and H587E channels. It is clear from Fig. 20B that the reduction in maximal conductance at pH_o 5.5 in H587E channels was voltage independent; whereas the reduction in maximal conductance in H578E showed a similar voltage dependence compared to WT. Woodhull predictions estimated the fractional electrical distance, δ, as 0.18, 0.17, and 0.04 for WT, H578E, and H587E, respectively (δ was determined
Figure 21. A fixed negative charge at position 587, but not 578, enhances the loss of conductance at pH 5.5. A,B, typical current traces recorded from H578E (A) and H587E (B) channels at pHo 7.4 (black trace) and pHo 5.5 (red trace) during a 2 s pulse to +60 mV followed by a 2 s hyperpolarizing pulse to -60 mV. C,D, G-V relationships for H578E (C) and H587E (D) showing the reduction in maximal conductance at pHo 5.5. WT GV relationships are shown as black (pHo 7.4) and red (pHo 5.5) line plots for comparison. Low pHo reduced maximal conductance of H578E to a similar extent as observed in WT, but H587E enhanced the reduction. Values and statistics are listed in Table 7 (n=5-8).
### pH\textsubscript{o} effects on WT and mutant hERG channel deactivation/activation properties

<table>
<thead>
<tr>
<th>Construct (pH\textsubscript{o})</th>
<th>(V_{1/2}) (mV)</th>
<th>(k) (mV)</th>
<th>Fractional Amplitude (%)</th>
<th>(\tau_{\text{fast}}) (ms) at -60 mV</th>
<th>(\tau_{\text{slow}}) (ms) at -60 mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (7.4)</td>
<td>-25.4 ± 2.1</td>
<td>8.0 ± 0.1</td>
<td>-</td>
<td>405.4 ± 25.6</td>
<td>2383.0 ± 119.1</td>
</tr>
<tr>
<td>WT (5.5)</td>
<td>5.0 ± 1.5*</td>
<td>10.3 ± 0.3*</td>
<td>59.6 ± 4.2*</td>
<td>84.5 ± 9.0*</td>
<td>328.7 ± 36.5*</td>
</tr>
<tr>
<td>H578E (7.4)</td>
<td>-23.0 ± 0.6</td>
<td>8.7 ± 0.3</td>
<td>-</td>
<td>354.8 ± 15.5</td>
<td>2095.6 ± 108.8</td>
</tr>
<tr>
<td>H578E (5.5)</td>
<td>9.1 ± 0.2*</td>
<td>9.9 ± 0.2*</td>
<td>58.9 ± 2.5*</td>
<td>67.9 ± 3.3*</td>
<td>318.9 ± 9.7*</td>
</tr>
<tr>
<td>H587E (7.4)</td>
<td>-30.3 ± 1.3</td>
<td>6.9 ± 0.1</td>
<td>-</td>
<td>591.6 ± 77.2</td>
<td>3122.3 ± 545.4</td>
</tr>
<tr>
<td>H587E (5.5)</td>
<td>1.4 ± 1.1*</td>
<td>11.7 ± 0.3*</td>
<td>28.7 ± 1.4*</td>
<td>70.1 ± 6.6*</td>
<td>301.4 ± 20.7*</td>
</tr>
</tbody>
</table>

Table 7. pH\textsubscript{o} 5.5 effects on WT and mutant hERG channel deactivation and activation properties. Values for \(V_{1/2}\) and \(k\) were obtained from Boltzmann fits of the G-V relationships at each pH\textsubscript{o}. Values for deactivation tau, \(\tau_{\text{fast}}\) and \(\tau_{\text{slow}}\) were obtained from double exponential fits of WT and mutant deactivating tail currents. * = Statistically different from pH\textsubscript{o} 7.4 for each respective channel (\(p < 0.05\); \(n = 5-8\); paired t-test).
as described in section 2.4.5). We also measured the voltage dependence of fractional amplitude change at pH$_o$ 5.5 in the H587Q mutant (which exhibits a similar reduction of maximal conductance at low pH$_o$ compared with WT as shown in Fig. 18 and Table 5) as a negative control to see if the enhanced loss of maximal conductance at low pH$_o$ in the H587E mutant was specific to the fixed negative charge. The voltage dependence of proton block in the H587Q mutation was similar to WT, with a $\delta$ of 0.20. This suggests that protons still bind near the extracellular surface of the selectivity filter in the H578E and H587Q channels, but bind at a site outside of the electrical field in the H587E channel, likely at the introduced glutamate (Woodhull, 1973; Zhou et al., 2001). We explain the voltage-independent proton binding in the H587E channel as a result of engineering an additional proton binding site outside of the conducting pathway with a higher affinity for protons than the site located within the conducting pathway. However, there is also the possibility that the H587E mutation has caused allosteric changes within the pore that prevent protons from accessing the binding site within the conducting pathway. We do not believe this to be the case since an allosteric change within the pore would be expected to change the selectivity of the channel, and selectivity of the H587E channel is not altered in comparison to WT (Ding et al., 1999).

4.2.8 The reduction in maximal conductance at low pH$_o$ is dependent on [K$^+$]$_o$

The previous data suggest that protons reduce maximal conductance by directly blocking the pore near the selectivity filter. It has previously been shown...
Figure 22. High $[K^+]_o$ alleviates the reduction in maximal conductance at low $pH_o$ in hERG WT channels. A, B, typical current traces recorded from hERG WT channels during a 2 s pulse to +60 mV followed by a 2 s pulse to -60 mV, in the presence of 0 mM (A) and 3 mM KCl (B) ($pH_o$ 7.4, black; $pH_o$ 5.5, red). C, bar chart plotting the fractional amplitude of peak tail current at $pH_o$ 5.5 normalized to $pH_o$ 7.4 for WT hERG channels in the presence of 0 mM and 3 mM KCl. Increasing $[K^+]_o$ alleviates proton block. * = statistical difference (n=5-8; p < 0.05; two-tailed paired t-test).
in Shaker channels that certain positively charged toxins block the pore by directly competing with a K\(^+\) binding site near the selectivity filter (Goldstein and Miller 1993; Ranganathan et al., 1996). In this case, increasing [K\(^+\)]\(_o\) resulted in greater K\(^+\) occupancy within the pore, which led to an electrostatic repulsion between K\(^+\) within its binding site and the toxin (Goldstein and Miller, 1993; Ranganathan et al., 1996). If protons bind directly within the hERG channel pore, increasing [K\(^+\)]\(_o\) might therefore be expected to reduce block. Fig. 22A and B show typical current traces recorded from WT hERG channels in the presence of 0 mM and 3 mM [K\(^+\)]\(_o\) (black and red traces represent current at pH\(_o\) 7.4 and 5.5, respectively). After applying the tail current extrapolation method (Fig. 20A), the extent of peak tail current reduction at pH\(_o\) 5.5 was 47.4 ± 5.3 % and 82.2 ± 5.0 % at 0 mM [K\(^+\)]\(_o\) and 3 mM [K\(^+\)]\(_o\), respectively (n=5-8; \(p < 0.05\); two-tailed paired t-test). This is plotted in Fig. 22C. That the reduction in maximal conductance was greater at a lower [K\(^+\)]\(_o\) concentration suggests that increasing [K\(^+\)]\(_o\), and thus occupancy of K\(^+\) coordination sites within the selectivity filter, positions a greater amount of positive charge around the conducting pathway that could repel like-positively charged protons, leading to less block. Further, based on the data from Figs. 20 and 22, it is tempting to speculate that the location of the proton binding site is around S1, since the Woodhull model suggests a site near the superficial surface of the channel, and a decrease in [K\(^+\)]\(_o\) provides protons with less competition for their binding site leading to enhanced block at pH\(_o\) 5.5. These observations provide strong evidence that protons reduce maximal conductance by directly blocking the conducting pore.
4.3 Discussion

Despite much focus, the mechanism behind the action of protons on hERG channel function remains unknown. For many Kv channels, pH modulation occurs via titration of histidine side chains. In Kv1.4 channels, titration of a turret histidine residue at pH_0 6.5 reduces maximal conductance via a stabilization of C-type inactivation, resulting in a slowing of the recovery from N-type inactivation (Claydon et al., 2000). A similar mechanism has been proposed in Kv1.5 channels: titration of a histidine residue in the turret is responsible for inducing C-type inactivation from the closed state, resulting in a reduction of maximal conductance at low pH_0 (Cheng et al., 2008; Kehl et al., 2002; Steidl and Yool, 1999). Interestingly, in hERG channels, extracellular protons affect the rate of deactivation and the maximal conductance with two different pK_a values that are close to that of a histidine (Bett and Rasmusson, 2003). hERG channels have five native histidines that may be accessible to the extracellular solution (H485, H492, H578, H587, and H562). Here we have shown that removal of these histidines does not remove the pH_0 sensitivity of hERG channels; however, while we have shown that removal of each individual histidine did not remove the effect of low pH_0, it is possible that the presence of one or more of these histidines underlies the pH_0 sensitivity of the hERG channel. Future experiments would be needed to rule this out.

Instead, our data suggest that protons accelerate deactivation by affecting voltage sensor movement, and reduce maximal conductance by directly blocking the pore. Our data in Fig. 17 suggest that protons accelerate hERG channel
deactivation by speeding up the return of the voltage sensor. A previous report suggests that protons accelerate deactivation by titrating negatively charged residues in the S1-S3 transmembrane domains (Liu et al., 2003). Titration of these negative charges may interrupt salt bridges that stabilize the voltage sensor in the late-activated state, resulting in acceleration of the return of the voltage sensor during deactivation. Further experiments are needed to explore this mechanism and are described in section 5.2.1.

The data in Figs. 20 and 22 suggest that protons reduce maximal conductance by blocking the pore. The evidence for this is 1) the reduction in peak tail current at low pHo is voltage dependent; 2) elevated levels of [K+]o can partially rescue the reduction in maximal conductance at low pHo; 3) introduction of negative charges within the permeating pathway increases the extent of block.

In hERG channels, the extracellular S5-P linker is unusually long compared to that of other Kv channels and is thought to be able to interact with the extracellular surface of the pore (Dun et al., 1999; Jiang et al., 2005; Liu et al., 2002). Furthermore, it has been suggested that the specific regions of the extracellular turret form part of the conducting pathway, and H587 is predicted to lie within one of these regions. In Fig. 21, we show that fixation of a negative charge at position 587 enhances the loss of maximal conductance observed at low pHo. Furthermore, this loss of maximal conductance is voltage independent and our Woodhull predictions suggest that protons now bind outside of the electric field (Fig. 20B). These data are interesting for two reasons: 1) these data are further evidence for the mechanism of proton block of the pore in WT
channels rather than a reduction in maximal conductance due to altered gating kinetics; 2) these data may provide insight into the structure of the S5-P linker, suggesting that H587 is situated at the mouth of the turret extension of the pore. Future experiments exploring the mechanism of pore block by external protons are presented in section 5.2.2.

Our results for the acceleration of deactivation observed at low pH are consistent with the data reported in the literature (Anumonwo et al., 1999; Berube et al., 1999; Bett and Rasmusson, 2003; Jiang et al., 1999; Jo et al., 1999; Terai et al., 2000). In general, the same is the case for our observed reduction in the maximal conductance at low pH (Anumonwo et al., 1999; Bett and Rasmusson, 2003; Jo et al., 1999; Terai et al., 2000). Similar to a previous report, we also found that the effect of protons occur with two different pH dependencies, suggesting two independent mechanisms for the effects (Bett and Rasmusson, 2003). This result explains why Berube and colleagues did not observe a reduction in maximal conductance but did see an acceleration in deactivation at low pH (Berube et al., 1999). In addition, while characterizing the effect on maximal conductance, we observed an increase in the peak tail current as an artifact due to perfusion of the bath solution (data not shown). To control for this, we perfused pH 7.4 to remove the artifact before conducting our experiments. This observation may explain why Jiang and colleagues reported an augmentation of peak tail current upon perfusion of pH 6.5 while most reports have not observed changes in peak tail current at pH 6.5 (Anumonwo et al., 1999; Bett and Rasmusson, 2003; Jo et al., 1999; Terai et al., 2000, see also
Figs. 14 and 15 from this thesis).

Our results are also consistent with the findings from Jiang and colleagues who showed that a double mutation in the turret (H578E H587E) did not remove the effect of low pH\textsubscript{o} on deactivation (Jiang et al., 1999). On the other hand, our findings are in contrast to those of Bett and colleagues who suggest that H562 is the pH\textsubscript{o} sensor for the effect of protons on hERG channel closing (Bett et al., 2011). However, the lowest pH\textsubscript{o} that Bett and colleagues recorded was pH\textsubscript{o} 6.6, whereas our recordings were made as low as pH\textsubscript{o} 5.5. Therefore, while we did not measure the pK\textsubscript{a} value for the H562Q channel, one possible explanation for the discrepancy between our’s and Bett and colleague’s results is that instead of removing pH\textsubscript{o} sensitivity of the channel, the H562Q mutation might have shifted the pK\textsubscript{a} for the effect of pH\textsubscript{o} on deactivation.
5: CONCLUSIONS AND FUTURE DIRECTIONS

hERG channels are the molecular correlate underlying $I_{Kr}$, which makes a significant contribution to the cardiac action potential. Disruption of hERG channel gating, whether by inherited mutations or metabolic by-products from pathophysiological processes like myocardial ischemia, can have drastic and life-threatening consequences on the electrical events of the heart. It is therefore of great importance that we understand the molecular mechanisms of hERG channel gating. Further, since heart disease is the number one leading cause of death in North America, it is equally pertinent that we know how metabolic by-products, such as protons, affect hERG channel function.

5.1 Mutations within the S4-S5 linker alter voltage sensor constraints in hERG $K^+$ channels

In this thesis, we have unveiled a novel mechanism to describe the unusually slow and poorly understood activation and deactivation gating of the hERG channel. We have identified a unique glycine residue (G546) in the S4-S5 linker that stabilizes the closed state of hERG channels; mutation at this site shifts the activation energy in the hyperpolarizing direction and significantly accelerates channel opening. We show with structural modelling and VCF, that mutation of G546 achieves both of these feats by decreasing the flexibility of the S4-S5 linker, resulting in fewer constraints on voltage sensor movement. Further, for the first time, we show that hERG’s complex deactivation kinetics are the
result of at least two reconfigurations of the voltage sensor that can be independently modified.

5.1.1 G546 is a hinge point in the S4-S5 linker

In Chapter 3, we showed that flexibility of the S4-S5 linker, afforded by G546, stabilizes the closed state of hERG channels. However, it remains to be determined whether the closed state of hERG channels is stabilized by flexibility of the linker specifically at G546, or whether flexibility of the linker as a whole is responsible for stabilizing the closed state. To test this, we have substituted glycines throughout the S4-S5 linker in the background of G546L, in an attempt to restore flexibility to the S4-S5 linker in different positions. If stabilization of the closed state is specific to flexibility of the S4-S5 linker about G546, then neither of these mutations should rescue the large hyperpolarizing shift in the voltage dependence of activation brought about by the G546L mutation. Fig. 23 shows the G-V relations of Y545G G546L, A547G G546L, A548G G546L, F551G G546L, G546L, and WT channels. It is clear from the data in Fig. 23 that none of the mutations tested restored the stable closed state observed in hERG WT channels. Additional mutant channels (V449G G546L, L550G G546L, L552G G546L) are still to be tested however. If neither of these mutations rescues the G546L phenotype, this would suggest that flexibility of the S4-S5 linker is specific to the G546 position.

If flexibility of the S4-S5 linker is specific to G546, it is tempting to speculate that G546 serves as a glycine hinge point in the S4-S5 linker. This is an interesting proposition since our SWISS MODEL calculations (Fig. 8)
Figure 23. Glycine residues substituted at different positions along the S4-S5 linker do not restore the activation properties observed in hERG G546L channels. G-V relations of hERG WT, G546L, Y545G G546L, A547G G546L, A548G G546L, and F551G G546L channels constructed from peak tail currents as described in the methods. (n=4-7).
predicted that G546 is located at a bend in the S4-S5 linker. To determine whether G546 acts as a glycine hinge, we intend to substitute a proline in its place and observe hERG channel gating kinetics. Given that the S4-S5 linker has been proposed to couple voltage sensor movement to channel opening by directly doing work against the S6 helices (Long, et al., 2005a; Sanguinetti and Tristani-Firouzi, 2006), and that the S4-S5 linker has been shown to stabilize the closed conformation of the S6 helices in hERG channels (Tristani-Firouzi et al., 2002; Ferrer et al., 2006), if G546 is a glycine hinge, we predict that the G546P mutation would place a permanent bend in the S4-S5 linker, resulting in a stabilization of the closed state at depolarized potentials and thus an uncoupling of voltage sensor movement from pore opening. To test this, we will perform VCF experiments on G546P L520C channels and monitor voltage sensor movement. If G546P uncouples voltage sensor movement from pore opening, then the F-V relation should exhibit the hyperpolarizing shift seen in the G546L construct (due to a loss of S4-S5 linker flexibility, see Fig. 6), whereas the G-V relation should exhibit a depolarizing shift in relation to the F-V. This is assuming that the G546P mutation locks the S4-S5 linker in the closed conformation, which is reasonable given that the S4-S5 linker has been shown to interact with the S6 helices to stabilize the channel in the closed state (Tristani-Firouzi et al., 2002; Ferrer et al., 2006).

5.2 Mechanism of the action of protons on hERG K+ channels

In the second part of this thesis, we identify two independent mechanisms for the action of external protons on hERG channel function. First, we provide
VCF data that suggest protons accelerate deactivation by modifying the return of the voltage sensor; second, we show evidence that indicates that protons reduce maximal conductance by directly blocking the conducting pathway. Both these mechanisms are explored in more depth in the discussion below, along with the future experiments that would strengthen the arguments presented for the mechanism behind proton modulation of hERG channels.

5.2.1 Protons accelerate deactivation by interrupting salt bridge formation within the voltage sensing domains

The only manipulation that has successfully removed the effect of low pH₀ on hERG channel deactivation is neutralization of the negative charges in the S2 and S3 transmembrane domains. This resulted in an acceleration of deactivation that was not further accelerated at low pH₀ (Liu et al., 2003). The authors suggest that titration of negative charges in the voltage sensing domains underscores the acceleration of deactivation observed at low pH₀, possibly by interfering with a “Master Switch” that regulates the rate of channel deactivation (Liu et al., 2003). Our data in Fig. 17 suggest that protons accelerate hERG channel deactivation by speeding up the return of the voltage sensor. Taken together with the data from Liu and colleagues (Liu et al., 2003), it is reasonable to suggest that titration of negative charges within the transmembrane domains accelerates voltage sensor return, resulting in the faster deactivation phenotype observed at low pH₀. To test the proposed mechanism by Liu and colleagues (Liu et al., 2003), we made the D466E mutation (which maintains the negative charge) and examined the effects of low pH₀ on hERG channel deactivation. Liu
and colleagues showed that neutralization of the negative charge at this site via the D466C mutation abolished the effect of low pH<sub>o</sub> (Liu et al., 2003). As shown in Fig. 24, low pH<sub>o</sub> did not accelerate channel closure in the D466E construct despite the preservation of the negative charge. This suggests that, contrary to what has been proposed by Liu and colleagues (Liu et al., 2003), the mechanism of the action of protons on hERG channel closure is more complicated than just a titration of negative charges in the transmembrane domains and may involve complex structural rearrangements of the voltage sensing domains.

Given that negative charges in the transmembrane domains have been shown to form salt bridges with positive charges in the S4 voltage sensing helix (Liu et al., 2003; Subbiah et al., 2004; Zhang et al., 2005), and that salt bridges stabilize the voltage sensor in different states, one possibility that we suggest is that H<sup>+</sup> ions accelerate deactivation by interrupting salt bridge formation when the voltage sensor is in the activated state. This would decrease the constraints placed on the voltage sensor when in the activated conformation and therefore accelerate the return of the voltage sensor during deactivation. A good candidate to begin exploring this hypothesis is D456, which is thought to form salt bridges with R531 when the voltage sensor is in the late-activated state (Subbiah et al., 2004; Zhang et al., 2005). To test this theory, we will mutate D456 to a neutral asparagine (which should destabilize the late-activated state of the voltage sensor) and test the effect of low pH<sub>o</sub>. If our hypothesis were correct, then interruption of this salt bridge would result in a channel that is insensitive to
Figure 24. Low pH_o does not accelerate deactivation in the D466E mutant channel. 

A, typical current traces recorded from hERG D466E channels during a 4 s step to -60 mV applied immediately following a 500 ms step to +60 mV, pH_o 5.5 trace is normalized to pH_o 7.4 trace to highlight the change in deactivation rate (pH_o 7.4, black; pH_o 5.5, red). B,C, plots of $\tau_{\text{slow}}$ and $\tau_{\text{fast}}$ deactivation-voltage relationships, respectively. Deactivation in the D466E mutant is insensitive to low pH_o (n=5).
changes in \( \text{pH}_o \), since the D456N mutation should have already destabilized the late-activated state.

### 5.2.2 Protons reduce maximal conductance by directly blocking the pore

The data from Fig. 20 suggest that protons reduce hERG channel maximal conductance in a voltage-dependent manner. This is consistent with results from Vermeke and colleagues, who showed that the reduction in \( I_{Kr} \) at low \( \text{pH}_o \) is voltage dependent (Vereecke and Carmeliet, 2000). Another study also suggests that protons have a voltage-dependent effect on hERG channel function (Jo et al., 1999); however, the authors estimated that protons traverse an electrical distance of 0.76 (Jo et al., 1999), a distance that is about four times greater than our estimate (Fig. 20). The reason for the difference between our predictions and those by Jo and colleagues is due to the methods used to measure the voltage dependence. Jo and colleagues measured the voltage dependence of block from the rate of deactivation to estimate the location of the proton-binding site (Jo et al., 1999). As mentioned by the authors, this method assumes that the accelerated decay in tail currents represents protons binding to the channel, resulting in a reduction in conductance (Jo et al., 1999). This assumption is not consistent with the evidence provided by Bett and colleagues and the data shown in this thesis (Bett and Rasmusson, 2003); further, Jiang and colleagues have shown that the reduction in maximal conductance is not due to the acceleration of channel closure observed at low \( \text{pH}_o \) (Jiang et al., 1999). Therefore, we feel our Woodhull model predictions, as shown in Fig. 20, are a
more accurate and direct representation of the true proton-binding site in hERG channels.

It has previously been shown in Shaker channels that increasing \([K^+]_o\) alleviates pore block observed with positively charged scorpion toxins (Goldstein and Miller, 1993; Ranganathan et al., 1996). In addition, intracellular protons block the intracellular pore of Shaker channels in a voltage dependent manner that has been characterized using the Woodhull model (Starkus et al., 2003; Woodhull, 1973). Here, we have shown that hERG channel maximal conductance is reduced in a voltage-dependent manner (Fig. 20), and can be rescued by increasing \([K^+]_o\) (Fig. 22), suggesting that the mechanism of action of extracellular protons on hERG maximal conductance is via direct block of the conducting pore. To directly test this, we will perform single channel experiments. If extracellular protons reduce maximal conductance by blocking the pore, then we would expect to see a reduction in the single channel conductance (Hille, 2001). This has been shown to be the case for intracellular proton block in Shaker channels and extracellular proton block of L-type Ca\(^{2+}\) channels, where proton block results in a reduction of the single channel conductance that is thought to be on the “very fast” time scale (Chen et al., 1996; Starkus et al., 2003). Given that the onset of the effect of extracellular protons is rapid in hERG channels (Vereecke and Carmeliet, 2000), we would also expect that protons reduce maximal conductance by “rapid flicker” block of the pore, similar to that seen in Shaker and L-type Ca\(^{2+}\) channels (Chen et al., 1996; Starkus et al., 2003).
Our data suggest that external protons reduce maximal conductance by blocking the pore at a superficial site, possibly near the top of the selectivity filter. In other ion channels, glutamate residues near the pore serve as the proton-binding site for the mechanism of action of low pH (Chen et al., 1996; Jordt et al., 2000; Martinez-Francois et al., 2010). Taking this into consideration, a potential candidate for the proton-binding site in hERG channels is the glutamate residue located at the top of the pore, E637. To investigate this further, we will mutate this residue to a neutral glutamine. If E637 is the proton binding site, then this mutant should not only be insensitive to changes in pH, but also, as shown in the L-type calcium channel by Chen and colleagues, the glutamine mutation should mimic the loss of single channel conductance observed at low pH, since a glutamine residue is equivalent to a protonated glutamate amino acid (Chen et al., 1996).

5.3 Accessory subunits may alter hERG gating by affecting voltage sensor movement

Our findings show that low pH accelerates hERG channel deactivation by speeding up both the fast and slow reconfigurations of voltage sensor return. As mentioned in Chapter 4, this suggests that protons modify hERG channel gating by altering the way the voltage sensor moves. This raises the possibility that modification of voltage sensor movement, rather than an alteration of electromechanical coupling or pore gate dynamics, may represent a general mechanism by which hERG channels are modulated by interacting partners or accessory subunits. Moreover, it is interesting to speculate that intracellular
interacting partners may also modify hERG channel gating in the same manner. Such is seen between the interaction of the ancillary subunit KCNE1 and the KvLQT1 channel (Osteen et al., 2011). It has been shown that KCNE1 interacts with the KvLQT1 channel, resulting in an alteration in channel gating through a modification of voltage sensor movement (Osteen et al., 2011). We predict that the KCNE1-related gene product of KCNE2, MiRP1, alters hERG channel function by altering voltage sensor movement as well. Coassembly of MiRP1 with hERG channels results in an acceleration of hERG channel deactivation that creates a current more representative of $I_{Kr}$ than the current conducted by hERG channels alone (Abbott et al., 1999); however, the interaction between MiRP1 and hERG remains unknown. It is possible that this coassembly may partially disrupt the interaction between the N-terminus and the core of the channel, since such a disruption has been shown to accelerate deactivation (Alonso-Ron et al., 2008; Gomez-Varela et al., 2002; Gustina and Trudeau, 2009; Morais Cabral et al., 1998; Schonherr and Heinemann, 1996; Spector et al., 1996a; Wang et al., 1998; Wang et al., 2000). Given that KCNE1 modulates KvLQT1 gating by altering voltage sensor movement (Osteen et al., 2011), that KCNE2 accelerates hERG channel deactivation (Abbott et al., 1999), and that deletion of the N-terminus accelerates hERG channel closing by speeding up voltage sensor return (Fig. 11), we propose that MiRP1 alters hERG gating by interrupting an interaction with the N-terminus, and thus changing the way the voltage sensor moves. To explore the action of MiRP1 on hERG channels, we will measure the fluorescence report of voltage sensor movement in hERG WT channels using
VCF with and without the presence of MiRP1. Further, we will modify the side chain of G546C with a bulky or charged adjunct group to see if the effect on deactivation seen with MiRP1 coassembly is altered. This would be an interesting observation since modification of G546C with an adjunct group has been shown to disrupt the interaction between the N-terminus and the core of the channel (Wang et al., 1998). Therefore, if the effect of MiRP1 on hERG channel deactivation is altered during this experiment, this may be evidence that MiRP1 and the N-terminus compete for a similar site, supporting the notion that MiRP1 accelerates channel deactivation by disrupting an interaction between the N-terminus and the core of the hERG channel.
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