Probing the molecular and structural basis of voltage sensor gating in Kv11.1 ion channels

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Congenital mutations in the cardiac Kv11.1 channel can cause Long QT Syndrome type 2 (LQTS2), a heart rhythm disorder associated with sudden cardiac death. Mutations act either by reducing protein expression at the membrane, and/or by perturbing the intricate gating properties of Kv11.1 channels. A number of clinical LQTS2-associated mutations have been reported in the first transmembrane segment (S1) of Kv11.1 channels but the role of this region of the channel is largely unexplored. In part this is due to problems defining the extent of the S1 helix, as a consequence of its low sequence homology with other Kv family members. Here we used NMR spectroscopy and electrophysiological characterization to show that the S1 of Kv11.1 channels extends from Trp410 to Leu432, and is flanked by unstructured loops.

Functional analysis suggests that pre-S1 loop residues His402 and Tyr403 play an important role in regulating the kinetics and voltage dependence of channel activation and deactivation. Multiple residues within the S1 helix also play an important role in fine-tuning the voltage dependence of activation, regulating slow deactivation, and modulating C-type inactivation of Kv11.1 channels. We demonstrate that for Kv11.1, activation and deactivation processes are not simple reversal transitions, but rather deactivation and inactivation processes are likely to be coupled. Analyses of LQTS2-associated mutations in the pre-S1 loop or S1 helix of Kv11.1 channels demonstrate perturbations to both protein expression and most gating transitions. Thus S1 region mutations would reduce both the action potential repolarizing current passed by Kv11.1 channels in cardiac myocytes, as well as the current passed in response to premature depolarizations that normally helps protect against the formation of ectopic beats.
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Chapter 1

Introduction

1.1 Background

Ion channels are important but complex molecular machines in the human body which act as gated pores that regulate the movement of ions across cell membranes. These macromolecules span the membrane bilayer and are responsible for electrical signalling in living organisms from bacteria to human, regulating physiology and behavior. Specifically, voltage-gated ion channels are responsible for electrical nerve activity, and the excitability and contraction of skeletal, cardiac and smooth muscle. Hundreds of genetic defects in ion channels are known to lead to various debilitating disease and life-threatening disorders. In the context of cardiac conditions, the cardiac myocyte has a diverse range of ion channels on its surface, each of which produces current, which take part in complex interplay of interactions to give rise to the cardiac action potential. Mutations in any of these myocyte ion channels can give rise to abnormalities in ion flux regulation and facilitating abnormal electrical currents, including Long QT Syndrome (LQTS), Short QT syndrome, Brugada syndrome, and catecholaminergic polymorphic ventricular tachycardia. In particular, LQTS type II are caused by mutations in Kv11.1 potassium channel, which may increase the risk of potential fatal arrhythmias such as torsade de pointes or sudden cardiac death (SCD). Especially when it occurs in young and otherwise healthy people, SCD not only results in a loss of productive years of life but has a devastating impact on families and communities.
Figure 1.1. (A) Ion channels which contribute to the underlying cardiac activity of the heart. Red boxes highlight Long QT Syndromes (LQTS), including LQT1, LQT2 and LQT3. LQT2 is caused by mutations in the Kv11.1 ion channels. Adapted from Bhuyian et al. 7 (B) Various ion channels exist on cardiomyocytes, each producing an electrical signal which combine to form the cardiac action potential, shown in black. Adapted from Bhuyian et al. 7
1.2 History

The human-ether-a-go-go gene (hERG) encodes for the α subunit of the delayed-rectifier potassium channel ($k_r$). This subunit can assemble to form a tetramer ion channel known as hERG or Kv11.1. These channels are widely expressed in physiological systems but play a particularly critical role in the heart where they are responsible for maintaining a normal heart rhythm and suppression of ectopic beats. $k_r$ is most apparent during the repolarization plateau of the cardiac action potential (Phase 3 of black cardiac action potential, Figure 1.1B) and acts to terminate the action potential and return the membrane potential to its resting level of $\approx -80$ mV. Mutations to Kv11.1 are of particular clinical interest, as a reduction in $k_r$ by drug block or inherited mutations will prolong the repolarization phase of the action potential, giving rise to Long QT Syndrome 2 (LQTS2). A prolonged repolarization can result in subsequent activation of early after-depolarizations and triggered activity, which can accelerate into rapid ventricular rhythms and predispose patients to potentially lethal arrhythmias such as torsade de pointes and sudden cardiac death (SCD).

Prior to our current knowledge of the variety and complexity of potassium ion channels in the heart, electrophysiology studies tended to treat outwardly directed delayed rectifier potassium current in the heart as $k_r$. It was Tsien and Noble who first showed that delayed rectifier currents in cardiac cells may exist as two separate entities, a rapid delayed rectifier and a slow delayed rectifier. Although not well received at the time, this work was later confirmed by Sanguinetti and Jurkiewicz and others when they showed that outward potassium current in isolated guinea pig heart cells could be isolated into two components: a fast component ($I_{Kr}$) and a slow component ($I_{ks}$). Aside from differences in their relative kinetics of gating, the two components could be differentiated pharmacologically, i.e. only the rapid component, $I_{Kr}$, was blocked by most anti-arrhythmic drugs, e.g. E-4031. Shortly thereafter, screening of a human hippocampus cDNA library...
with the *Drosophila* ether-a-go-go clone led to the identification of the human family of channels\(^{19}\), which now constitutes the Kv10.x, Kv11.x and Kv12.x subfamilies of voltage-gated K\(^+\) channels. Kv11.1 encoded a potassium channel with extensive homology of typical outward rectifying channels of the Kv10.1 family (49%), but with unusual gating kinetics characterized by slow transitions between closed and open states but fast transitions between open and inactivated states (discussed in further detail below). Finally, a series of breakthrough studies paved the way to our understanding of the significance and physiological role of Kv11.1, which would later propel this ion channel to the forefront of pharmacological and clinical arrhythmias research efforts. Curran et al.\(^{10}\) used linkage and physical mapping in 1995 to demonstrate that LQT2 loci and the *hERG* gene mapped to a similar region on chromosome 7q35-36, and suggested that mutations underlying congenital LQTS were attributed to Kv11.1 mutations, and proposed a cellular mechanism for *torsade de pointes*. Electrophysiological studies in *Xenopus laevis* oocytes then showed that Kv11.1 had biophysical properties almost identical to *Ikr* detected in cardiac myocytes.\(^{18,22}\)
1.3. Structure and function

An intimate understanding of the role of Kv11.1 channels in the pathophysiology of cardiac arrhythmias requires an understanding of channel function and gating on a molecular scale. Kv11.1 is part of the Kv channel family, with structure typical of voltage-dependent potassium channels\textsuperscript{19}. Present available structural information for Kv11.1 is based on homology with other potassium channels, given the lack of X-ray crystallography data for Kv11.1, to date\textsuperscript{23-25}. The ion channel is composed of four identical α subunits which forms a tetramer in the plasma membrane (Figure 1.2.). Kv11.1 comprises six transmembrane domains (S1-S6), intracellular N and C terminals, and a voltage sensor within the fourth transmembrane domain (S4). Functionally, Kv11.1 can be described in two parts including the voltage sensor domain (S1-S4) which detects changes in the membrane potential, and the pore domain (S5-S6) responsible for controlling the flux of ions through the central pore of Kv11.1. Like other Kv channels, Kv11.1 exists in three conformation states: open (activated), closed (deactivated) and inactivated states.
Figure 1.2. Schematic representation of topology of Kv11.1 ion channels. (A) Topology of the Kv11.1 ion channel showing two of the four subunits. Each subunit consists of six \( \alpha \)-helical transmembrane domains, S1-S6. The transmembrane S1-S4 form the voltage sensor. The S4 domain contains multiple positive charges and is thought to be the principle voltage sensor. The transmembrane domains S5-S6 and pore helix from each of the four subunits form the ion conducting pore. This figure is color-coded to match Figure 1.2.B. (B) A snapshot of the Kv11.1 homology model, generated using Swiss PdbViewer based on the crystal structure of a Kv1.2/2.1 channel chimera.²⁶
Even prior to structural studies of ion channels, seminal papers published by Hodgkin and Huxley in 1952 had proposed that K$^+$ and Na$^+$ channel families had four charged particles in the membrane electric field which acted as voltage sensors, responding to changes in membrane potential changes by alternating between activated and deactivated positions. This model was later supported experimentally, with Armstrong and Benzanilla demonstrating the movement of gating charges across the electric field in tandem with channel activation. Sequence alignment analysis demonstrated the conservation of four charged residues in the fourth transmembrane domain, suggesting its potential role in voltage sensing. It is now established that in Kv channels and also specifically Kv11.1, the primary voltage sensing domain is the S4 transmembrane domain, which consists of four basic residues (Arg or Lys) spaced by pairs of hydrophobic residues. During depolarization of Kv11.1, the charged residues of S4 detect the change in membrane potential and move outwards, resulting in a sequence of electromechanically coupled motions to open to the central pore and allow efflux of K$^+$ ions.

In contrast to typical Kv channels, Kv11.1 is unique in that it is characterized by slow activation (closed to open transition) but rapid inactivation (closed to inactivated transition) and recovery from inactivation (Figure 1.3.). The physiological importance of the unique fast inactivation of Kv11.1 is that it acts like an inward rectifier at positive voltages, such that at depolarized potentials the outward K$^+$ current is reduced and hence maintains the plateau phase of the cardiac action potential. Inactivation in Kv11.1 channels also appear to be voltage-dependent, although it is not clear whether this property is intrinsic or due to coupling with activation.
Figure 1.3. A schematic diagram demonstrating the gating of Kv11.1 channels. Kv11.1 can exist generally in three states: closed, open and inactivated. Closed and inactivated states are non-conducting whilst open states are conducting. Transitions between closed to open state is defined as activation, open to inactivated states defined as inactivation, and open to closed states defined as deactivation. Activation, deactivation, and inactivation transitions in Kv11.1 are voltage-dependent transitions, with activation and deactivation processes being slower compared to inactivation and recovery from inactivation. A schema showing the corresponding Kv11.1 current (blue) and action potential (gray) is shown below. This figure is reproduced and adapted from Vandenberg et al. 6
Typical Kv channels possess a mechanism for either rapid N-type inactivation or ball-type inactivation, or they may undergo slow C-type inactivation, which involves constriction of the selectivity filter at the mouth of the central pore to inactivate the channel\textsuperscript{6}. Inactivation in Kv11.1 is similar to C-type inactivation, although it has several unique features, namely fast gating kinetics\textsuperscript{32} and being voltage dependent.\textsuperscript{32-34} In order to elucidate the unique Kv11.1 inactivation gating, a series of N-tail truncations was performed, which did not significant affect rapid inactivation.\textsuperscript{8,33} However, when mutations were introduced in the selectivity filter of Kv11.1, this substantially altered inactivation kinetics\textsuperscript{8,33}. As such, these studies suggested that the fast-inactivation phenotype of Kv11.1 was more likely due to the C-tail collapse of selectivity filter mechanism. This hypothesis was later confirmed when it was discovered that other mutations in the selectivity filter such as S620T\textsuperscript{35} and G627Y\textsuperscript{36} which substantially altered inactivation. Whilst the exact electromechanical steps involved in Kv11.1 inactivation remain yet to be elucidated, recent studies using phi-value or rate-equilibrium free energy relationship (REFER) analysis\textsuperscript{37} have demonstrated that a concerted sequence of conformation changes underlie selective filter gating, much like the movements of a Japanese puzzle box, rather than simple collapse of the filter. This study and others also demonstrated the value of phi-value or REFER analysis in the context of potassium channels, which allow deduction of temporal energetic changes of different domains of the channel.\textsuperscript{37-39} The use of REFER analysis for the S1 helix in Kv11.1 channels is demonstrated and discussed in Chapter 4 of this thesis.

Aside from differences in gating, there are also structural differences between Kv11.1 and homologs which may underlie its unique gating phenotype. Firstly, in contrast to homologous Kv channels, Kv11.1 possesses and N-terminus Per-Arnt-Sim (PAS) domain\textsuperscript{40}. The equivalence of this region in mammalian CLOCK and Per proteins appears to play a homeostatic role in responding to hypoxia\textsuperscript{41}. However, the role of the PAS domain is less clear in the context of Kv11.1. Initial deletion studies reported acceleration of deactivation
gating kinetics following removal of the PAS domain. Supporting this work, others have showed that when recombinant 1-135 PAS proteins was added to truncated Δ2–373 Kv11.1 channels, slow deactivation gating was restored. Secondly, another feature of the Kv11.1 channel is the cyclic nucleotide-binding domain (CNBD) in the intracellular C-terminus. In other channels such as hyperpolarization-activated cyclic nucleotide–gated (HCN) channels and cyclic nucleotide–gated ion (CNG) channels, the CNBD appears to play a role in activation. However, mutagenesis studies does not appear to support a significant role for CNBD in activation of Kv11.1. Rather, there is increasing experimental data to support the role of CNBD in Kv11.1 trafficking, which several identified missense mutations and partial deletions of the CNBD identified. Thirdly, the S5P segment of Kv11.1 channels is longer compared to other K⁺ channels, approximately 40 amino acid long compared to 10-15 amino acid long in typical Kv channels. It is not clear whether this difference may contribute to rapid inactivation of Kv11.1 or selectivity for K⁺ ions. Fourthly, the sequence for selectivity filter of Kv11.1 (SVGFG) is different compared to the corresponding sequence in other VGKs (TVGYG), which may contribute to the unique inactivation of Kv11.1. Fifthly, the distribution of charges in the Kv11.1 VSD may also underlie its unique gating kinetics. In Kv11.1 VSD, there is an unusually high number of charges. The S1-S3 helices has six negatively charged residues, three of which are conserved (D411 in S1, D460 in S2, D509 in S3). Studies by Tseng’s group demonstrated that D411 may play a role in stabilizing the closed state, whilst D460 and D509 may play roles in stabilizing the Kv11.1 activated/open state. The other three charged residues may play various roles; D456 shown to be important for folding whilst D466 and D501 is implicated in voltage sensing during the activation transition.

Recently, the MacKinnon group was recently able to produce a single-particle cryo-electron microscopy structure for the voltage-gated K⁺ channel rKv10.1, which was bound to the channel inhibitor calmodulin. This is a related channel protein family member with high
sequence similarity to Kv11.1. The authors propose an alternative mechanism of voltage-dependent gating, based on their observation that the S4-S5 linker was different to prior potassium channel structures\textsuperscript{26,50,51}. In their proposal, the authors suggest a gating mechanism where the S4 enters the cytoplasm in a “down” state, interacts with the cytoplasmic C-linker and S6 in such a way which closes the channels. In the “up state”, the S4 moves into the membrane and interacts with the C-linker and S6 in order to open the helical bundle and allow ion flow. Their crystal structure indicates the helical extent of the S1 helix and shows no pre-S1 helical region, which may be the case for the Kv11.1 channel but is yet to be shown experimentally.
1.4. Role of voltage sensor domain

Whilst it well agreed that the positive charges of the S4 helix is important for electromechanical coupling during activation and deactivation of Kv11.1, the precise molecular nuances are yet to be established\textsuperscript{29-31}. Whilst such crystal structures have dramatically improved our understanding of the native conformation of the K\textsuperscript{+} channel\textsuperscript{49,52,53}, the molecular conformations underlying the coupling between the VSD and central pore remains to be well elucidated. It is generally believed that during activation, the charged residues of the S4 segment detect the membrane potential change and subsequently move upwards to form stabilizing interactions, particularly with charged residues S1-S3 via salt-bridge interactions\textsuperscript{54}. However, the exact nature of S4 translocation remains a subject of controversy, with inconsistent and conflict evidence and models proposed.

Three potential models for S4 movement during K\textsuperscript{+} channel gating have been proposed. The first potential model proposed was the helical-screw/sliding-helix model. Guy & Seetharamulu (1986)\textsuperscript{55} and Catterall (1986)\textsuperscript{56} suggested that the positive charges of S4 were paired with fixed negative charges on neighboring helices in the resting state. When the membrane was depolarized, an increasing outward force on S4 charges would be produced, and thus allowing the S4 helix to rotate through a 60\degree spiral and 0.45 nm outwards\textsuperscript{57,58}. In doing so, the positive charges would have all moved but kept their relative positions similarly in order to interact with another different set of four fixed negative charges. During activation gating, this concerted screw-helix motion occurs three times in succession, allowing transfer of charge across the membrane to account for the transient “outward gating current” originally described\textsuperscript{59,60}. A second model proposed for S4 voltage sensing movement across the transmembrane is the transporter-like model\textsuperscript{61}. This model is based on the assumption that the S4 segment does not move significantly during activation. Instead, the S4 charged residue activation is controlled by accessibility to either the internal
environment or external environment. Upon membrane depolarization, the open conformation takes such that the S4 charges essentially “flips” from one side of the membrane to the other. A third model for the movement of S4 during potassium channel activation is the paddle model, derived from observations of the KvAP crystal structure. In this hypothesis by Jiang et al., the S4 segment is associated with the helix of S3 to form a "paddle" located at the periphery of the KvAP subunit. Known as the S3b-S4 paddle, this structure moves from its peripheral location into the lipid layer during membrane activation.

In a study investigating Shaker potassium channels, neutralizing mutations were introduced into the S4 positively charged residues (K5Q and R6Q). These mutations resulted in non-functional channels. However, their function could be rescued if these mutations were paired with neutralizing mutations in the lower end of the S2 helix (E293Q) or S3 helix (D315N). These results suggest that the interactions between the charges in S4 with the distal end of S2/S3 may be important in folding and maturation of the ion channels, or activation/deactivation of the channel. In another study investigating similar residues, charge-reversal mutation analysis demonstrated important interactions between D4 in the S4 segment with E293 in S2 and D316 in S3. This study also showed important electrostatic interactions between S2 charges with R3 and R4 of the voltage sensor. In another study, mutations to charges in the S2/S3 helices disrupted the voltage-dependent of the potassium channel, resulting in a depolarizing shift of the G-V graph. However, after taking all of this into consideration, there is limited evidence investigating the role of charges and residues in the S1 segment, and potential interactions with the S2-S4 helices in the VSD.

1.5. Electromechanical coupling of VSD and pore

The electromechanical coupling between voltage sensing and pore gates is still relatively unclear. Multiple studies have demonstrated using high resolution structures and
structure-function analysis the importance of the S4-S5 linker in transmitting force from the VSD to pore domain\textsuperscript{50,66}. The S4-S5 linker is an amphipathic alpha helix both in crystal and in solution. Systematic scanning mutagenesis of the S4-S5 linker disturbed the electromechanical coupling of VSD and pore in Shaker K\textsuperscript{+} channels, supporting the role of the S4-S5 in this process\textsuperscript{67,68}. In particular, mutations introduced to the S4-S5 alongside with distal S6 segment resulted in significant phenotypes\textsuperscript{66}.

The S4-S5 linker is thought to act as a physical lever, pushing against the S6 segment in order to close the channel\textsuperscript{50}. In an attempt to provide experimental support for this hypothesis, a chimera was constructed between Shaker and KscA. The chimera comprised of Shaker K\textsuperscript{+} channels where the S5-S6 section was replaced by corresponding segments of KscA.\textsuperscript{69} KscA by itself is voltage-dependent, but when coupled with Shaker’s VSD, become voltage-sensitive. This results supports the importance of the S4-S5 linker in mediating the interaction between VSD and pore domains. The nature of the interaction between S4-S5 and the S6 activation gate was probed by systematically mutating combination of residues in the C-terminal end of S6. Sanguinetti’s group\textsuperscript{66} demonstrated that a single residue (R665) in S6 interacted with K540 via an electrostatic repulsion to mediate voltage sensor – pore gate coupling to allow hyperpolarization –dependent activation of Kv11.1. It has also been suggested that the lower part of the S6 segment plays an important role for voltage-dependent gating, supporting the above observations.\textsuperscript{70-72}

The remainder of the voltage sensor domain has traditionally thought to be relatively stable and immobile. There are a number of negatively charged residues\textsuperscript{73} distributed throughout the S1-S3 helices, which may act to stabilize the positively charged residues in the voltage sensor S4.\textsuperscript{1,74} Furthermore, these negative charges may play a role in defining the trajectory of S4 voltage sensor movement within the membrane.\textsuperscript{75} However, in contrast
to the traditional static role of S1-S3, there is recent merging evidence that S4 may interact with S1-S3 segments to contribute the regulation of voltage-dependent S4 movement during activation. Several studies have suggested that S4 movement and gating may be regulated by intra-unit interactions with the VSD.

1.6. Evidence for role of S1 region in Kv11.1 gating and function

Various studies that examined Shaker potassium channels have provided evidence for the role of the S1 region. There is evidence that Ser240 in S1 Shaker creates a steric gap that enables an intracellular pathway for transport of the S4 arg residues that are responsible for stabilization of depolarized VSD conformation. Ile241 in Shaker S1 positively shifts the voltage-dependence of VSD movement, altering functional coupling between VSD/pore domains. I241W immobilized VSD movements during activation and deactivation also drastically shifts the voltage act. These effects were reproduced by F244W, a helical turn upwards. Histidine scanning mutagenesis in the Shaker K channel identified that the mutant I241H (S1 segment) and I287H (S2 segment) generates inward currents at hyperpolarized potentials, suggesting that these residues are part of a hydrophobic plug that separates the water-accessible crevices. A multitude of studies have also examined the Kv11.1 channel. MD simulations demonstrated that in a Kv11.1 channel with L532P, there were greatly enhanced deactivation rates. This was attributed to lessened interactions across the S4-S5 interface. Along with this T421M clinical mutations have been identified and postulated to cause losses of Kv11.1 currents via reduced protein trafficking and defective gating. The data from the other five functional mutants suggest that D411 can stabilize the Kv11.1 channel in the closed state, while D460 and D509 have the opposite effect. D466 and D501 both may also contribute to voltage-sensing during the activation.
Evidence of the S1 segment in channel gating and function has been found in additional studies involving other potassium channels. For the Big Potassium (Bk) channel, during activation S4 diverges from S1 and S2, whilst S2 moves closer to S1. This reveals the structural arrangement and spatial constraint info for the Bk channel. For the Kv7.1 channels, in the closed state, there are disulfide and metal bridges, which connect S1 (C136) and S4 (S225) within the same VSD. In the open state, two neighboring S4 (I227) are constrained at proximity whilst R228 (S4) is confined closed to C136 (S1) of adjacent VSD. These constrains suggest potential cooperative subunit interactions, and suggest a key role of S1 segment in steering S4 motion during Kv7.1 gating. For Kv1.1-Kv1.4 channels, I177N in the S1 segment mutation was shown to reduce subunit surface expression, as well as increased rate of N-type inactivation. The S1 segments were also found to play an important role in making KCNQ1 channels constitutively active via KCNE3 subunit, suggests that equivalent subunits in S1 are important for inter-subunit interactions. The interface of S1/S5 has also been shown to be important for KCNQ1 channel gating. There were also two novel mutations of KCNH2, L413P in the transmembrane domain S1 and L559H in S5, which were found to be associated with protein trafficking defects in LQTS. A study examining the KCNQ2 channel, showed that disulfide bones and metal bridges of S4 (S195C, R198C, R201C) constrained S1 (C106) in the resting state, within the same VSD. They concluded that S1 (C106) can be in very close proximity to N-terminal S4 residues for stabilizing KCNQ2 resting conformations. Various other studies have also suggested that S1 and S2 of VSD are static relative to the motion of the membrane, whilst voltage-sensor paddle S3b is more mobile.

1.7. Kv11.1 deactivation and mode-shift

The majority of the earlier literature on Kv11.1 have focused on activation and inactivation processes, however to date there is no definitive conclusion regarding whether these processes are coupled or not. Piper et al. using cut-open oocyte voltage clamp
techniques and inactivation-deficient Kv11.1 mutant channels, suggested that Kv11.1 inactivation is coupled to activation. However, other studies have reported discordances between residues that affect activation and inactivation such as in the pore domain outer helix. Deactivation was traditionally presumed to be a basic reversal process of activation (from open to closed states transition), however there is increasing evidence in the literature to suggest that deactivation is a more complex process than this. Even from a physiological perspective, deactivation of Kv11.1 is an important component of the final repolarization phase of the cardiac action potential. One peculiar property of voltage gating Kv11.1 that supports the proposal that activation and deactivation are distinct processes, is the notion of mode-shift. This refers to the observation that the voltage range for upward movement of the VSD is lower than that compared to the voltage range for the VSD to return to its resting state. This phenomena was first observed by Bezanilla et al. in 1982 in squid axon sodium channels, and has since been observed in Kv11.1, HCN and Shaker ion channels. From this observation, it is deduced that the gating charges must move differently during activation compared to deactivation. One proposed explanation for mode-shift is that the VSD exists in three states: one “down state” and two “up state” positions. Upon initial activation, the VSD transitions from closed state to the initial up state, but quickly adapts the second up state position which acts to stabilize this activated conformation. Given this context, this raises the question of whether the slow deactivation kinetics of Kv11.1 is due to intrinsic property of the voltage sensor, or is it related to the fact that the open state is structurally stabilized by the relaxed “up-state” because of the mode-shift phenomena. The latter would be supported by potential coupling between deactivation and inactivation processes, however this hypothesis is yet to be explored in detail in the VSD of Kv11.1.
1.8. Clinical Significance of Kv11.1

Understanding the general function and gating mechanisms of Kv11.1 is not only of academic interest, but has significant clinical implications especially in the heart. As explained above, the unique gating kinetics of $I_{Kr}$ comprises slow activation and deactivation, but fast voltage-dependent inactivation and recovery from inactivation$^{32-34}$. This translates into a prolonged action potential, which increases the interval of time to allow for release of calcium ions from the sarcoplasmic reticulum, thus allowing for cross-bridge cycling and cardiac contraction$^6$. A secondary effect of a prolonged $I_{Kr}$ current is the suppression of premature stimuli. The rapid and voltage-dependent recovery from inactivation coupled with slow deactivation during early diastole allows the channel to suppress any premature stimuli or ectopic beats during the late repolarization phase$^{101}$. Any premature stimuli will cause rapid recovery from inactivation, leading to a huge surge in $I_{Kr}$ current to oppose the premature beat (Figure 1.4.). As such, Kv11.1 $I_{Kr}$ has a protective effect against arrhythmias.$^{33,102,103}$
Figure 1.4. Response of hERG current to premature action potential stimuli. As hERG channels recover from the inactivated state into the open state, during repolarization an 'ectopic beat' will result in a large increase in outward current followed by rapid re-inactivation and hence the transient current spike. The envelope of the peak current spike reflects the recovery from inactivation followed by deactivation. This figure and caption is from Perry et al. 101, and adapted from Lu et al. 103
Given the importance of Kv11.1 in repolarization of the cardiac action potential, mutations in this potassium channel such as in the case of Long QT Syndrome can decrease $I_{Kr}$ current which leads to delayed repolarization. Consequently, this can give rise of arrhythmias of which some are potentially be lethal including torsade de pointes\textsuperscript{9,16,18,104}. Congenital long QT syndrome is an electrical disorder of the heart, which manifests as an extended QT interval on electrocardiogram. Clinical manifestations of long QT syndrome often begin in adolescence or early adulthood but are highly variable, with some affected individuals being asymptomatic throughout life\textsuperscript{10,104}. Affected individuals are at a much greater risk of developing ventricular arrhythmias and sudden cardiac death\textsuperscript{10,104}.

Long QT syndrome type 2 (LQTS2), which accounts for approximately one third of long QT cases, is caused by mutations in the KCNH2 gene which encodes the Kv11.1 potassium channel protein\textsuperscript{10}. There are several different types of mutations which occur in KCNH2, classified into the following categories\textsuperscript{105}: (1) missense, (2) splice site, (3) in-frame deletion, and (4) frame-shift mutations. Previously, genotyping studies of LQTS2 clinical cohorts demonstrated differential phenotypes according to location of mutations. From 44 different Kv11.1 mutations identified from 201 patients, 14 different mutations were identified in the pore region of the channel in 35 patients. This was compared with 30 other non-pore mutations found in 166 patients\textsuperscript{106}. Patients with pore mutations developed a significantly higher proportion of arrhythmic events at follow-up (74\% vs 34\%, $P<0.001$), and this was attributed to the pore-location of the mutation after multivariate adjustment.\textsuperscript{106} More recently\textsuperscript{107}, the same group updated their analysis by performing a systematic mutagenesis study of 167 LQTS2 missense mutations. From their extensive analysis, Anderson et al. demonstrated that 88\% of LQTS2 missense mutations found in the PAS region, CNBD, and pore regions were associated with a defective trafficking phenotype\textsuperscript{107}. Subgroup analysis also demonstrated that pore mutations were strictly dominant negative, compared to mutations in the PAS and CNBD region. Studies have demonstrated that congenital LQTS
mutations in the PAS domain manifests as defective Kv11.1 deactivation and defects in intracellular Kv11.1 trafficking. As a “hotspot” for LQTS2 congenital mutations, atomic level structural data has also reveals its role in domain-domain interactions\textsuperscript{108}. As such the unique PAS domain and NH2 tail appears to be important for Kv11.1 gating.

LQTS is associated with potential lethal ventricular arrhythmias and is one of the underlying causes of sudden cardiac death in the young\textsuperscript{109}. Sudden death attributed to congenital arrhythmias contributes to 1% of deaths in the general population\textsuperscript{110}, and therefore, understanding the molecular basis and function of Kv11.1 channels is important for understanding the genetic substrate of sudden death in the young.

Additionally, pharmacological agents, which perturb the nuances of ion channel gating either by either blocking the pore of the channel to prevent conduction or altering the kinetics or the voltage-dependence of gating, can alter the fine balance of ionic flow, both mechanisms leading to clinical arrhythmias. Many drugs that cause ventricular arrhythmias do so by blocking cardiac Kv11.1 channels, resulting in acquired LQTS. Examples of drugs which have this action include terfenadine\textsuperscript{111}, astemizole\textsuperscript{112}, and sertindole\textsuperscript{113}. Drug companies now have mandatory testing of new drugs for their potential blocking effect on Kv11.1.
**Figure 1.5.** Cartoon of Kv11.1 with circles representing amino acids and cylinders representing transmembrane domain segments 1–6 and the S4–S5 linker. Black filled circles demonstrate the location of reported LQT2 clinical mutations, which is distributed throughout the Kv11.1 channel. Figure from Anderson et al. 107
1.9. Hypotheses and Aims

In summary, the voltage sensor domain of Kv11.1 clearly plays a critical role in normal gating function and kinetics of the channel, and is physiologically relevant in the context of repolarization of the cardiac action potential and maintenance of a normal heart rhythm. It is well established at the VSD plays an important role in electromechanical coupling during opening and closing of the activation gate. However, the role of the remainder of the VSD has not been characterized systematically. In particular, the exact role of the S1 domain of the voltage sensor, particularly the specific residues and interactions involved in Kv11.1 activation, deactivation, and inactivation gating, remains to be elucidated. The nature of deactivation voltage-dependence and gating in Kv11.1, and whether this is coupled to inactivation or activation processes in Kv11.1, remains to be established.

Therefore, this project aims to further elucidate the role of the VSD of Kv11.1 in gating kinetics and its potential physiological significance in the context of Type 2 LQTS. We wish to test the hypothesis that the S1 domain, in contrast to traditional thought regarding its static role, is involved in critical interactions with the remainder of the VSD to mediate Kv11.1 gating kinetics and to maintain a normal cardiac potential.

**Aim 1:** To determine the structure and helical transmembrane extent of the S1 helix in Kv11.1, and the nature of the pre-S1 region and whether there is any potential secondary structure that could indicate its possible physiological function.

**Aim 2:** To determine the residues and interactions of the S1 domain critical for voltage-dependence of Kv11.1 activation as well as the kinetics or rates of activation.
**Aim 3:** To also similarly determine the residues important in Kv11.1 inactivation gating and establish the energetic and temporal relationship in comparison to other parts of the Kv11.1 channel.

**Aim 4:** To assess residues important for voltage-dependence and kinetics of Kv11.1 deactivation, and probe whether correlates or may be coupled with inactivation or activation processes.

**Aim 5:** To investigate the gating phenotypes of known clinical LQTS mutants located in the putative S1 segment or pre-S1 region.
1.10. References


69. Lu Z, Klem AM, Ramu Y. Coupling between voltage sensors and activation gate in voltage-gated K+ channels. J Gen Physiol 2002;120:663-76.


Chapter 2

General methods

2.1. Molecular biology

Kv11.1 cDNA was obtained as a kind gift from Dr Gail Robertson, University of Wisconsin. pBluescript vector was donated by Professor Robert Vandenberg, University of Sydney, which contained the 5’ untranslated region (UTR) and 3’ UTR of the *Xenopus laevis* β–globin gene. Kv11.1 cDNA was subcloned into the pBluescript vector containing the *Xenopus laevis* β–globin gene. Primer design and mutagenesis was performed according to the QuikChange mutagenesis method (Stratagene Inc). Residues 394-438 were individually mutated to alanine (or if alanine to valine). Residues Thr399 and Thr436 were not investigated as clones could not be obtained. The QuikChange kit facilitated mutant strand synthesis reaction (thermal cycling). The control reaction was prepared (2.5 U/µL DNA polymerase, 10X reaction buffer, 100 ng/µL oligonucleotide control primer #1 and #2, dNTP mix, 5 ng/µL pBluescript) with double-distilled water. Thermal cycler was used according to manufacturer's cycling parameters. 10 U/µL *Dpn* I restriction enzyme was added directly to each amplification reaction, mixed and incubated at 37°C for 1 hour digestion, followed by transformation into XL1-Blue supercompetent cells (heat shock at 42°C for 30 seconds, followed by placement in ice for 2 minutes). Subsequently, the mutagenesis product sequence was confirmed by DNA sequencing.
Plasmids were linearized using *Bam*HI-HF (NEB, US) and the cRNA was transcribed *in vitro* using a mMessage mMachine T7 kit (Ambion, US) according to the manufacturer protocols.

### 2.2. Oocyte preparation and channel expression

Surgery was carried out in accordance with the Australian code for the care and use of animals for scientific purposes and all experiments were approved by the St Vincent’s Precinct animal ethics Committee (approval identifier 14/30). Female *Xenopus Laevis* frogs were anaesthetised by immersion with 0.17% w/v tricaine (Sigma, Castle Hill, NSW, Australia). The level of anaesthesia was assessed by testing if the frog could right itself when tipped over in the anaesthetic. Their reflexes were also tested by pinching the toes. Once unresponsive, a small abdominal incision was made and ovarian lobes were removed. The incision was closed in two layers and the frog was allowed to recover in a small freshwater tank whilst being observed before being returned to the main holding tank. Upon removal, the oocytes were left to stabilise by immersion in dissociation solution (ND96 containing (mm): KCl 2.0, NaCl 96.0, MgCl2 1.0 and Hepes 5.0 (pH adjusted to 7.5 with NaOH). The tissue was then disaggregated mechanically using forceps into small clumps of approximately 10 oocytes each, which was subsequently mixed with dissociation solution to wash off excess connective tissue ad blood. The small oocyte clumps were then mixed with 1 mg/mL Collagenase A in Ca

<sup>2+</sup>-free ND96 solution (96 M NaCl, 2 mM KCl, 1 mM MgCl2, 5 mM Hepes, pH 7.5) for 1-2 hours at room temperature. Circular rotation was performed to mix clumps until the outer follicular layer is removed. A second pass wash with dissociation solution allowed removal of dead cells and debris. The oocytes were then sorted, to allow selection of Stage V/VI oocytes, which were then placed in storage solution (ND96 supplemented with 2.5
mM pyruvic acid sodium salt, 0.5 mM theophylline and 10 μg/mL amikacin) and stored at 17°C.

2.3. Two-electrode voltage clamp electrophysiology

Xenopus oocytes were injected with cRNA and incubated at 17°C for 24–72 h prior to electrophysiological recordings. All experiments were undertaken at room temperature (21–22°C). Currents were amplified using a GeneClamp500B amplier (Molecular Devices, Sunnyvale, CA, USA), interfaced to an IBM compatible PC using a Digidata 1440 analogue digital convertor (Molecular Devices) controlled with pClamp9 software (Molecular Devices). Signals were filtered at 2 kHz using the built in 4-pole low-pass Bessel filter in the GeneClamp500B amplifier and digitized at 10 kHz.

Glass microelectrodes were made by pulling borosilicate glass using a PC-10 puller (Narishige, Tokyo, Japan) and filled with 3M KCl solution. Glass microelectrodes had tip resistances of 0.3–1.0 MΩ. Oocytes were perfused with ND96 solution (see above). The active bath clamp used an agar bridge containing 3M KCl agar. A single oocyte was placed in the recording chamber. The bath was perfused with recording solution (96mM NaCl, 2mM KCl, 1mM MgCl₂, 1.8mM CaCl₂, 10mM HEPES, pH adjusted to 7.5 with NaOH) for alanine mutations. For LQTS2-associated mutations, cells were perfused with ND94 solution in which the KCl was raised to 4mM, to better mimic physiological concentrations whilst NaCl was reduced to 94 mM. The current electrode and voltage electrode were inserted into the bath solution and resistance levels were checked, with voltage offset calibrated to zero. The two electrodes were then inserted into the oocyte, and the cells allowed to
stabilise for 1-2 minutes. Cells giving a background current of greater than 200 nA at -90 mV were discarded, however, this was not applied to mutants which were constitutively open at -90 mV. After the recording, micropipettes were taken out of the cell and the potential difference between the electrode tips and bath solution once again measured. If the voltage reading of electrode 1 after the recording was greater than 4 mV the recording was discarded.

2.4. Statistics

Initial data analysis was performed using the Clampfit module of the pClamp 9.0 and 10.0 software. Data acquisition and analysis were performed using pCLAMP version 9 (Axon Instruments, CA, USA), Microsoft Excel version 2013 (Microsoft, Redmond, WA, USA) and Graphpad Prism version 6 software (GraphPad Software, Inc, CA, USA). All data are shown as mean ± standard error of mean (S.E.M) for n experiments, where n denotes the number of different oocytes studied for each construct.
Chapter 3

Determining the extent of the S1 voltage sensor domain helix and its contribution to activation in Kv11.1 channels

3.1. Introduction

Kv11.1 channels play a crucial role in repolarization of the cardiac action potential and are therefore key regulators of heart rhythm. Inherited mutations in KCNH2, the gene which encodes Kv11.1 channels, or direct pharmacological block of Kv11.1 channels, can result in long QT syndrome type 2 (LQTS2), an electrical disorder of the heart which dramatically increases the risk of cardiac arrhythmias and sudden cardiac death. At present, over 750 KCNH2 variants have been identified.

Like other voltage-gated K+ channels, Kv11.1 channels function as tetramers, with each subunit composed of six helical transmembrane segments (S1-S6). The first four transmembrane segments (S1-S4) make up the voltage sensor domain (VSD), whilst the S5 and S6 helices, as well as the intervening pore helix (PH) and selectivity filter (SF), constitute the pore domain. Pore domains from each of the four subunits surround the potassium ion conduction pathway. Two gates control the flow of K+ ions through the conduction pathway: an activation gate, which is formed by the cytoplasmic ends of the four S6 helices, and a C-type inactivation gate located at the selectivity filter. In Kv11.1 channels, opening of the activation gate by depolarization occurs with much slower kinetics compared with other voltage-gated K+ channels. Channel deactivation also exhibits slow kinetics, mediated, at
least in part, by cytoplasmic N- and C-terminal interactions of the protein\textsuperscript{8-11}. In addition to slow activation and deactivation, Kv11.1 channels also undergo a rapid and intrinsically voltage dependent (i.e. not linked to the voltage dependence of activation) C-type inactivation\textsuperscript{12}. These unique features of Kv11.1 channels make them ideally suited to facilitate the delayed action potential repolarization, and suppression of arrhythmia is their response to premature stimuli, as observed in ventricular myocytes\textsuperscript{6}.

Several regions of the Kv11.1 protein, such as the S4 ‘voltage sensor’ helix and the pore domain, have been extensively studied\textsuperscript{6,13,14} and their role in the unique gating of Kv11.1 channels is well understood \textsuperscript{6,13,14}. The remaining components of the voltage sensor domain, including the S1 helix of the voltage sensor domain, have been less well studied. A major limitation in our understanding of the role of the S1 helix in Kv11.1 channels is that this region exhibits poor sequence homology to other members of the voltage-gated K\textsuperscript{+} channel (VGK) superfamily. Pairwise and global sequence alignments demonstrate sequence identities ranging between 5.9\% to 19\%, and sequence similarities ranging between 28\% and 44\% \textsuperscript{15-20} for the S1 helix. Thus, there is currently no consensus on the transmembrane helical extent of the S1 in Kv11.1 channels. More than 38 clinical LQTS2 mutations have been localized within the voltage sensor domain (VSD) of Kv11.1 channels (data from the International Long QT Registry), of which over one-third lie within the vicinity of the putative S1 helix, suggesting that this region must be important for channel expression and/or function.

There is also a limited understanding of the role and function of the S1 segment in the context of voltage sensitivity of Kv11.1 channels. It is well established
that the positively charged S4 helix of the VSD plays an important role in voltage sensing. The remaining VSD segments, including the S1 transmembrane segment have traditionally been thought to be relatively immobile, acting as a stabilizing anchor to allow the S4 voltage sensor to undergo motion. Recent studies on other voltage-gated K+ channels have suggested that the S1 helix forms potentially important interactions with the S2 and/or S4 helices, which may help the S1 helix steer S4 motion during activation gating. Other experimental data suggests that anchoring interactions between the S1 and S5 helices, are important for both gating and protein maturation of Kv2.1 channels. Emerging evidence suggests that the S1 segment may be involved in the regulation of voltage-dependent S4 movement during activation, which necessitates further study of the role of S1 in Kv11.1 channel gating.

Recently, a resolved cryo-electron microscopy structure of the rat TRPV1 and TRPA1 channels demonstrated the presence of a pre-S1 helix, which is involved in critical interactions with the TRP and linker domains to promote correct assembly and trafficking to the plasma membrane. The crystal structure of a chimeric Kv1.2-Kv2.1 voltage-dependent potassium channel also revealed an S0 helix, or pre-S1 helix. However, the existence of such a “pre-S1” helix in Kv11.1 channels, and its potential role in gating, has not been examined. The availability of the Kv10.1 cryo-electron microscopy (cryo-EM) structure as well as cryo-EM of Kv11.1 provides the opportunity for comparison with the Kv11.1 sequence in predicting the possible S1 segment as well as interacting residues with the voltage sensor. From the cryo-EM structure, the S1 helix extends from V215 – F241. Using a pairwise
alignment between the Kv11.1 sequence and Kv10.1 sequence, this corresponds to P405 - F431 in Kv11.1 with 50.0% identity and 69.4% similarity (Figure 3.1.A).

Figure 3.1. Pairwise alignment of the pre-S1 and putative S1 regions of Kv11.1 (top line) with rKv10.1 sequence (bottom line). The helical extent of the S1 helix of rKv10.1 from the cryo-EM structure data is highlighted in yellow.
We hypothesized that the S1 helix fine tunes the activation and rates of activation gating kinetics of Kv11.1 channels. Accordingly, the aims of this study were to: (1) use alanine scanning mutagenesis and NMR spectroscopy to determine the transmembrane limits of the S1 helix in Kv11.1; (2) determine whether there is a potential pre-S1 helix in Kv11.1 and its potential function in channel gating, and (3) investigate to what extent mutations of the S1 segment affect the voltage-dependence and rates of activation.
3.2. Methods unique to this chapter

3.2.1. Peptide synthesis

The 45-residue Kv11.1 S1 peptide (RIHRWTILHYSPKAVWDWLILLLLVIYTAVFTPYSAAFLKETEE) was commercially synthesised by Mimotopes (Melbourne, Australia).

3.2.2. Nuclear magnetic resonance (NMR) spectroscopy

Samples for NMR spectroscopy consisted of 1 mM of the S1 peptide dissolved in 350 μL of 90/10, H₂O/D₂O, v/v containing 100 mM deuterated sodium dodecyl sulfate (SDS; Cambridge Isotope Laboratories, Andover, MA). The paramagnetic relaxation agent gadolinium-diethylenetriamine pentaacetic acid-bismethylamide (a gift from Dr. Klaus Zangger, University of Graz, Graz, Austria) was introduced to this sample at a concentration of 2 mM. All of the experiments were performed on a Bruker Avance III 800 MHz narrow-bore NMR spectrometer (Bruker, Karlsruhe, Germany) equipped with TCI cryoprobe with sample temperature of 35 °C. Raw data were processed using TopSpin™ (Bruker, Karlsruhe, Germany) and analysed with SPARKY (T. D. Goddard and D. G. Kneller, University of California at San Francisco). The two-dimensional (2D) experiments that were performed before and after the addition of the relaxation agent, included total correlation spectroscopy (TOCSY) with MLEV spin-lock periods of 90 ms, and nuclear Overhauser enhancement spectroscopy (NOESY) with mixing time of 150 ms. Residue assignments were made according to the standard protocol from 2D TOCSY and NOESY experiments. To analyse the effect of the relaxation agent, integration of cross-peaks were compared in the NOESY experiments recorded before and after the addition of the relaxation agent.
3.2.4. Electrophysiology

*Xenopus* oocytes were prepared and two-electrode voltage clamp experiments were performed with the setup described in Chapter 2.

The ‘steady-state’ voltage dependence of activation (closed-to-open transition) was measured using a voltage clamp protocol in which cells were depolarized, from a holding potential of -90 mV, to membrane potentials in the range of -60 mV to +50 mV (test potentials), in 10 mV increments, for 10 s. During this depolarization step channels open slowly and then inactivate. The membrane potential was then repolarized to -70 mV for 500 ms, to allow channels to recover from inactivation and transition back into the open state before closing slowly. Peak tail currents measured during this step were normalised to the maximum tail current value ($I_{\text{max}}$), plotted against the test potential, and then fitted with a Boltzmann function (see Equation 1):

$$I / I_{\text{max}} = \left[1 + \exp(V_{0.5} - V_t) / k\right]^{-1}$$

(Eq. 1)

where, $I / I_{\text{max}}$ is the tail current normalised to the maximum tail current. $V_{0.5}$ is the half–maximal activation voltage, $V_t$ is the test potential and $k$ is the slope factor. The same data were also fitted with the thermodynamic form of the Boltzmann expression (see Equation 2):

$$g / g_{\text{max}} = \left[1 + \exp(\Delta G^0 - z_gEF) / RT\right]^{-1}$$

(Eq. 2)

where, $\Delta G^0$ is the difference in Gibb’s free energy between the states at 0 mV, $z_g$ is the effective number of electric charges crossing the transmembrane electric field, $F$ is Faraday’s constant, $R$ is the universal gas constant, and $T$ is absolute temperature.
Perturbations to Gibb’s free energy difference between closed and open states, for mutants compared to WT, were calculated as:

$$\Delta \Delta G^0 = \Delta G^0_{MT} - \Delta G^0_{WT \text{ mean}}$$  \hspace{1cm} (Eq. 3)

where $\Delta G^0_{MT}$ was from individual experiments and $\Delta G^0_{WT \text{ mean}}$ was the mean $\Delta G^0$ value from all WT replicates.

The rate of activation was measured using an envelope of tails protocol\(^6\). From a holding potential of -90 mV, cells were depolarized to the test potential for increasing time periods, starting at 10 ms and ending at 3 s. Cells were then repolarized to -70 mV in order to measure the peak tail current. The longer the time step at the test potential, the more channels have chance to open, and hence the larger the peak tail current. The protocol was repeated at test potentials of 0 mV. At each test potential, peak tail currents were plotted against the increasing time steps and then data was fitted with a single exponential function to obtain the time constant for activation ($\tau_{\text{act}, 0 \text{ mV}}$).
3.3. Results

3.3.1 Extent of the S1 helix of Kv11.1 channels:

Within the region of the putative S1 helix, there is poor sequence homology between Kv11.1 and other voltage-gated K⁺ channels. Several previous studies have reported putative S1 helices in Kv11.1 channels, but the starting amino acid varies from Ser404 (ending Phe424) to Val409 (ending Leu433)\(^{19}\), which are almost two helical turns different. As a result, there is no consensus to the start and end point of the S1 helix in Kv11.1 channels. To help resolve this discrepancy, we used NMR spectroscopy to analyse a 45 residue synthetic peptide spanning from Arg394 to Glu438, a region extending well beyond the N-terminal and C-terminal limits of any of the published S1 predictions. To mimic the membrane environment, we studied the synthetic S1 peptide in SDS micelles (see Figure 3.2). As this peptide contains several amino acids of the same type (i.e. 7 leucine, 4 isoleucine, 4 alanine and 3 valines) there was considerable overlap of signals in the aliphatic region of the NMR spectrum (see Figure 3.2). As a consequence we were not able to obtain a high resolution structure. Nevertheless, the stretch of negative chemical shift index (CSI) values of the \(\text{Ca}^\alpha-{\text{H}}\) resonances suggested that two regions, from Trp410 to Phe424 and from Tyr427 to Leu432, adopted helical conformations (see Figure 3.3). The positive CSI values for Thr425 and Pro426 suggest that there may be a break or ‘kink’ between the two helical segments. For the residues prior to Trp410, or after Leu432, there are no consecutive residues with negative CSI values, suggesting that these regions are not helical.

To investigate whether the helical region from Trp410 to Leu432 was likely to represent the transmembrane helix of S1, we repeated the NMR experiments in the...
presence of Gd$^{3+}$, a paramagnetic agent which suppresses signals from extra-micellar environments. Gd$^{3+}$ suppressed signals in the region Arg394 to Ala408, whereas the signals for residues Trp410 to Glu435 were largely unaltered (Figure 3.3). Combined, our NMR data suggests that the transmembrane extent of S1 in Kv11.1 extends from Trp410 to Leu432, with a probable break or kink at Thr425/Pro426. Our NMR data does not support a pre-S1 helical segment in Kv11.1 channels.
Figure 3.2. NOESY spectrum showing the amide-H\(\alpha\) cross peaks in the absence (red) and presence (blue) of Gd\(^{3+}\). Note the considerable spectral overlap in central region (4.1-4.4 ppm on the x axis and 7.8-8.1 ppm on the y axis). Examples of peaks that are clearly visible in both the presence and absence of Gd\(^{3+}\) are labelled in blue, and examples of peaks that are suppressed by Gd\(^{3+}\) are labelled in red. The suppressed peaks correspond to residues at the N- and C-termi whereas non-suppressed peaks correspond to residues in the middle region of the peptide, i.e. they are protected by the SDS micelles that exclude the Gd\(^{3+}\).
Figure 3.3. (A) Plot of the chemical shift index for Cα^1H resonances of S1 peptide in SDS micelles. Stretches of resonances with values <0.1 not interrupted by residues with a chemical shift index >0.1 is indicative of alpha helical secondary structure; (B) Fraction of suppression of Cα^1H resonance peaks by Gd^{3+}. Most of the residues from W410 to E435 are >50% protected from suppression by Gd^{3+}, indicating this region lies within the lipid membrane.
3.3.2. S1 helix and Kv11.1 channel steady-state activation

To investigate the functional role of the S1 region in Kv11.1 channels, we compared the gating properties of WT Kv11.1 channels with mutant channels in which each residue, from Arg394 to Glu38, was individually mutated to alanine (or from alanine to valine), with the exception of Thr399 and Thr436. First, we examined whether individual mutations altered the voltage dependence of channel activation, as this is one of the principle functions of the voltage-sensing domain. The voltage dependence of activation was assessed using the voltage protocol shown in Figure 3.4. Exemplar currents recorded from oocytes expressing WT or T421A mutant Kv11.1 channels are shown in Figure 3.4A. Current shown from hyperpolarizing mutants including Y403A and D411A are shown in Figure 3.5. Mutation T421A produced one of the largest perturbations to activation, causing a mean $\Delta\Delta G^{0}_{\text{act}}$ of 2.53±0.03 kCal/mol$^{-1}$ (Figure 3.6). Several other mutants within the S1 helix produced a biologically significant shifts in steady-state activation ($\Delta\Delta G^{0}_{\text{act}}$) including D411A (-0.96±0.29), L413A (1.17±0.07), I414A (0.76±0.52), L415A (0.88±0.16), L417A (0.65±0.13), V418A (-2.15±0.43), F424A(-0.63±0.03), Y427A (-1.05±0.51), A429V (1.41±0.05), A430V(-0.54±0.10) and F431A (0.86±0.09). There were several mutations in the pre-S1 region which also caused biologically significant shifts in steady-state activation including I395A (0.69±0.03), H396A (-0.92±0.16), L401A (-1.38±0.03), H402A (-2.31±0.26), Y403A (-2.39±0.28), F406A (0.77±0.11), A408V (1.23±0.32) and V409A (0.68±0.18) (Figure 3.6., Supplementary Table 1). Thus mutations located throughout the putative S1 segment, as well as the pre-S1 regions, are important for maintaining the voltage dependency of channel activation.
Figure 3.4. (A) Typical families of current traces recorded from (i) WT and (ii) T421A Kv11.1 channels during 10s isochronal activation protocols. The voltage specific protocol is shown in the inset. (B) Examples of steady state activation curves for WT (grey) and T421A (black).
Figure 3.5. (A) Typical families of current traces for (i) Y403A and (ii) D411A Kv11.1 channels during 10s isochronal activation protocols. The voltage specific protocol is shown in the inset. (B) Examples of steady state activation curves for Y403A (open triangle), D411A (open circle) and WT (closed grey circle) are shown. $V_m =$ voltage (mV).
Figure 3.6. Summary plot of the change in the energetics of the voltage-dependent distribution between the closed and open states for each S1 alanine mutant (measured as $\Delta \Delta G^0_{\text{act}}$ relative to WT, see Methods for details) at 0 mV. T399 and T436 were not recorded as clones were not obtained. Data shown are mean ± SEM with n=2-8 [errors bars not shown for mutants with n=2, see supplemental table 1]. Grey shading indicates putative helical extent of S1. Dotted black lines indicate mutants which produced a biological significant perturbation, which was defined as greater than ± 0.5 kCalmol$^{-1}$. Mutants which had a biological significant perturbation were coloured white.
3.3.3. S1 helix and Kv11.1 channel rates of activation

In order to further understand the role of the S1 segment in mediating transitions between the closed and open states of Kv11.1 channels, we examined the effect of alanine mutations on the kinetics of activation, by measuring the rate of activation at 0 mV, using an envelope-of-tails protocol. Typical example traces for WT and T421A are shown in Figure 3.7. Two other examples, Y403A with faster activation and A408V with slower rates of activation are shown in Figure 3.8. T421A produced a notable slowing of the rate of channel activation compared to WT, with mean Δln τ_{act, 0 mV} of 3.53±0.07 s⁻¹ (Figure 3.7). Perturbations to rate of activation of Y403A and A408V were -1.13±0.09 and 1.7±0.10, respectively (Figure 3.8.). Overall, mutations that significantly perturbed the rate of activation spanned the whole transmembrane extent of S1 (Figure 3.9), faster mutants including D411A, W412A, V418A and F431A, whilst slower mutants including W410A, L413A, I414A, L415A, L417A, I419A, Y420A, T421A Y427A, S428A and A429V. Outside the S1 helix, slower mutants included R394A, I395A, W398A, I400A, P405A, F406A, K407A, A408V, L433A, K434A, E435A and E438A. Faster mutant channels outside the S1 helix included Y403A channels.

In order to determine whether shifts in voltage dependence of activation was sufficient to account for the majority of perturbations observed in the rate of activation, we performed a correlation analysis between perturbations to steady-state activation versus changes to rates of activation at 0 mV. There was a statistically significant correlation (Figure 3.10, R²=0.58), however several mutations that lay outside the 95% confidence interval, including H402A, P405A, V409A, D411A, W412A, T421A, A429V, and F431A (Figure 3.10). Most notably, D411A and W412A
caused much greater perturbations to the rate of activation compared to the voltage dependence of activation.
**Figure 3.7.** Effect of alanine substitutions on rates of activation of Kv11.1. (A) Typical example of raw current traces obtained from WT and T421A Kv11.1 during the envelop of tails protocol illustrated at the top of the panel, at 0 mV. (B) $\tau_{fast}$ for rate of activation over a series of voltages (-40 mV to +40 mV) for WT (black) and T421A (red).
Figure 3.8. Effect of alanine substitutions on rates of activation of Kv11.1. (A) Typical example of raw current traces obtained from Y403A and A408V Kv11.1 during the envelop of tails protocol illustrated at the top of the panel, at 0 mV. (B) $\tau_{\text{fast}, 0 \text{ mV}}$ for rate of activation over a series of voltages (-40 mV to +40 mV) for WT (black), Y403A (red open circles) and A408V (red open triangles).
Figure 3.9. Summary plot of the changes in the rates of activation at 0 mV measured as ln(tau_{mut}/tau_{WT}). The grey shading indicates the putative transmembrane region. T399A and T436A were not recorded. Data shown are mean ± SEM with n=3-7 [see supplementary table 2]. Grey shading indicates putative helical extent of S1. Dotted black lines indicate mutants which produced a biological significant perturbation, which was defined as greater than ± 0.5 kCal mol⁻¹. Mutants which had a biological significant perturbation were coloured white.
Figure 3.10. Plots of changes in rates of activation versus $\Delta \Delta G^{\circ}_{\text{act}}$ for steady-state activation. Line of best fit and 95% confidence intervals (dashed lines) are shown. Labelled data points highlight those mutants that lie well away from the line of best fit. Error bars indicate SEM. T399A and T436A were not recorded. Data shown are mean ± SEM with n=2–8 [error bars not shown for mutants with n=2]. The solid line represents the linear fit using Pearson’s correlation, with dotted lines as 95% confidence interval bounds.
We then generated a homology model based on the structure of rKv10.1, a related channel protein family member with high sequence similarity to Kv11.1. In the homology model, the S1 helix (Figure 3.11.) begins at Pro405 and extends seven helical turns to Phe431, with no apparent breaks in the helical structure. However, there appears to be a noticeable kink in the S1 around Phe424-Thr425-Pro426. Based on the homology, the vicinity of residues suggest a network of interactions between S2 (cyan), S3 (light green), S4 (orange), and S5 (plum) helices and pore helix (dark green) (Figure 3.11.). The pre-S1 region is a flexible intracellular loop starting from Asp383 to Ser404. The region after S1 from Leu432 is also a flexible extracellular loop up to Glu437, which then transitions into S2 helix. These findings were consistent with the cryo-EM structure of Kv11.1 recently published by Wang and MacKinnon (Fig 3.12.).
Figure 3.11. Homology model of Kv11.1 based on cryo-EM structure of rKv10.1. (A) Amino acid sequence alignment of the extended S1 region (corresponding to the Kv11.1 S1 peptide shown Figure 3.1) of rKv10.1 and Kv11.1 channels. * denotes fully conserved amino acids, while colon (: ) and period (.) denote amino acids with strongly or weakly conserved properties, respectively. The grey box indicates the extent of the S1 helix based on the cryo-EM structure of rKv10.1 channels (50). (B) Homology model of a Kv11.1 channel based on the cryo-EM structure of the rKv10.1 channel\textsuperscript{32}. S1 helix (shown in white), S2 (cyan), S3 (light green), S4 (orange), S5 (blue), and the pore helix (PH, dark green). The homology structure and figure was produced by Dr Matthew D. Perry.
Figure 3.12. Cryo-EM structure of Kv11.1, based on publication of Wang and Mackinnon\textsuperscript{33}, 2017. PDB 5VA3 available from: http://www.rcsb.org/pdb/explore/remediatedSequence.do?structureId=5VA3
3.4. Discussion

There is a lack of consensus on the transmembrane helical extent of the S1 segment, its role in Kv11.1 gating, as well as the structural nature and functional role of the pre-S1 region. In this study, we determine the extent of the transmembrane S1 helix and demonstrate that the S1 segment contributes to both activation and deactivation transitions of potassium channel gating.

3.4.1. Extent and structure of S1 segment and pre-S1 region

To date, there have been considerably variation in bioinformatics predictions of the extent of the S1 helix in Kv11.1 channels published in the literature, with low identification and similarity scores. The N-terminal end of the S1 helix has been reported anywhere from Ile395 to Asp411, a span equivalent to more than 4 helical turns. Similarly the C-terminal end of the S1 helix has been reported from Ile419 to Glu435. Prior attempts of pairwise and global sequence alignments demonstrated low sequence homology for the S1 segment of Kv11.1, with identity ranging from 5.9%-19% and similarity ranging from 28%-44% in published alignments. In contrast to the pore regions of the Kv11.1 channel, the S1 segment is poorly conserved among canonical voltage gated potassium channels and cyclic nucleotide gated families, with few distinct markers, which can be used to align the S1 segments. Indeed, there is not a single amino acid position that is common across rat Kv1.1, human HERG and mouse HCN2, with significant variation in published available sequent alignments. NMR spectroscopy and Gd$^{3+}$ suppression investigations of an isolated peptide suggested that the transmembrane helical segment of S1 spans from W410-L432. However, we cannot exclude the possibility that the boundaries of the transmembrane S1 segment may vary by one or two
residues. Our sequence is most similar (56%) when aligned with the rKv10.1 cryo-
EM structure, recently reported by the MacKinnon group\textsuperscript{32}. Using homology of
Kv11.1 to the rKv10.1 cryo-EM structure, the equivalent transmembrane span for the
S1 helix was P405-F431 in the homology model\textsuperscript{32}. The differences in the exact
transmembrane extent of the S1 from our NMR data and based on the rKv10.1
homology model stems from the innate limitations of modelling. The structure of the
rKv10.1 channel was captured with the activation gate closed by the voltage sensor
likely in the up or activated conformation\textsuperscript{32}. There are currently no structures of any
Kv channel, which have the voltage sensor in the down or relaxed conformation.
Thus the potential interactions we discussed above, based on our homology model,
reflect only a snapshot in time during the closed-open transition.

In our NMR analysis, a large positive chemical shift index at T425 and
suppression at P426 also suggested a potential kink or break in the S1 segment at
this location. This would not be an unreasonable suggestion, given that proline
residues are known to cause kinks in helices\textsuperscript{36}. The cryo-EM structure recently
reported by Whicher et al.\textsuperscript{32} in the region Leu233-Val234-Pro235, which
corresponds to Phe424-Thr425-Pro426 in the Kv11.1 channel, which is consistent
with our NMR spectroscopy data. There is also a pronounced kink observed in the
crystal structure of the Kv1.2-2.1 chimera\textsuperscript{31}, at positions T184-L185-P186, which is
consistent with the notion of a kink. Our functional data demonstrates that mutations
of residues at the kink region, in particular Thr425 and Pro426, had a perturbing
impact on the voltage dependence of Kv11.1 activation but not kinetics of activation,
suggesting that these residues may be functionally important in maintaining the open
and closed ends states but not for the rates of transitions between these end states.
A range of ion channels have been postulated to have a short helical segment just prior to the S1 helix that has been speculated to play roles in biogenesis, protein folding and gating of these channels\textsuperscript{29-31,37-41}. Based on recent cryo-EM structures of the TRPV\textsubscript{1}\textsuperscript{30} and TRYP\textsubscript{A}1\textsuperscript{29} channels, as well as prior observations that mutants in the S0 region had faster gating phenotypes, we hypothesized that the pre-S1 region of Kv\textsubscript{11.1} may be helical and play a role in Kv\textsubscript{11.1} gating. However, our NMR and Gd\textsuperscript{3+} suppression data demonstrated no obvious helical characteristic to the pre-S1 region, with small chemical shift indexes and signals that were well suppressed by Gd\textsuperscript{3+}. Our NMR study results are consistent with the cryo-EM structure of rKv\textsubscript{10.1}\textsuperscript{32}, which demonstrates that the pre-S1 region in the rKv\textsubscript{10.1} is an unstructured loop. However, our functional studies of the voltage dependence of the S1 region demonstrates that the pre-S1 segment clearly has functional significance for Kv\textsubscript{11.1}, in particular with mutations to residues Leu\textsubscript{401}, His\textsubscript{402} and Tyr\textsubscript{403} producing significant hyperpolarizing shifts in voltage dependence and accelerated rates of activation. Clearly, the pre-S1 has functional significance in the Kv\textsubscript{11.1} channels – whilst the precise nature of this is not well understood the residues His\textsubscript{402} and Tyr\textsubscript{403} are likely key mediators in this process. Recent studies in TRPA1 and TRPV channels support a network of interactions between the pre-S1 helix with the TRP domain, an intracellular helix region after the S6 helix\textsuperscript{29,30,42}. In KCa\textsubscript{1.1} channels, the amphipathic S0’ helix forms part of an intracellular complex with C-terminal RCK1 N-lobe, the S2-S3 linker and S4-S5 linker regions\textsuperscript{38}. Certainly in Kv\textsubscript{11.1} channels, there is evidence to support interactions between the Per-Arnt-Sim (PAS), C-terminal linker, S4-S5 linker and cyclic nucleotide binding homology domain (cNBHD)\textsuperscript{8,10,43,44}. Based on this literature, it is not unreasonable to suggest that in Kv\textsubscript{11.1}, the pre-S1
region too also plays a role in a complex interaction network, likely contributing to cytoplasmic domain complexes of Kv11.1 to regulate rate of activation and deactivation.

3.4.2. Effect of S1 mutations on steady-state activation

To probe the role of each S1 residue, we used alanine scanning mutagenesis. Alanine was chosen as it would remove the interactions of the native functional group in any larger side chain amino acids, whilst causing minimal structural perturbation. T421A and V418A had the greatest impact on steady-state activation when mutated to alanine, whilst mutants D411A, L413A, I414A, L416A, L417A, A422V, F424A, T425A, P426A, Y427A, A429V, A430V, F431A had biological significant but moderate magnitude perturbation to voltage-dependence of steady-state activation. Of particular note, mutation of T421 produced the greatest perturbing effect on steady-state activation, with significant depolarizing shifts in the voltage dependence of activation. This residue is of particular interest given that it is the location of a characterized clinical Long QT Syndrome mutant T421M. Characterization of T421M in adult rate ventricular myocytes demonstrated significant loss of Kv11.1 current via defective gating, with positive shift in voltage dependence of activation and slower rate of activation. This clinical phenotype is supported by the gating phenotype of the T421A mutation tested the present study (see Chapter 6 of thesis). Collectively, this demonstrates that the Thr421 residue may be involved in specific interactions that stabilize the activated state of the Kv11.1 channel.
Mutations which perturbed steady-state activation were distributed throughout the S1 helix, although when mapped onto the homology structure, had many side-chains that were in close proximity to the S2 or S4 helices of the voltage sensor of the same subunit – with residues Val418, Thr421 and Thr425 side chains directly pointing into the S4 voltage sensor surface. Furthermore, residues Thr421, Asp456 and Arg2 of the voltage sensor were also in close proximity, suggesting a network of interactions which mediate the role of S1 in Kv11.1 gating. As such, this supports the notion that the S1 helix plays an integral role in the complex network of intra-subunit interactions with the S2 and S4 helices of the voltage sensor. There is evidence to support interactions between the positive charges of the S4 voltage sensors with negatively charged residues in S1, S2 and S3\textsuperscript{19,20,24,27}. Specifically for the S1 helix, the negatively charged D411 may interact with K438 of the S4 voltage sensor to stabilize the closed state of Kv11.1\textsuperscript{24}. The current findings of our functional analysis demonstrates that the S1 helix may play even a greater role, with multiple residues involved in a network of intra-subunit interactions between S1 helix with S2 and S4 helices of the voltage sensor domain.

Furthermore, the significant perturbations to voltage dependence is seen in the kink region Pro426, as well as the pre-S1 region (Leu401, His402, Tyr403) indicate that these positions may act to stabilize the open state in native WT Kv11.1 channels, providing further support for the role of the pre-S1 region in Kv11.1 activation gating.
3.4.3. Effect of S1 mutations on rate of activation

To assess the role of the S1 segment and pre-S1 region on the rates of activation of Kv11.1, rates of activation were measured for alanine mutants using an envelope of tails protocol. Mutations with significant perturbations to rate of activation were localized throughout the S1 helix as well as the pre-S1 region. This pattern of perturbation is similar to that noted for effects on steady-state activation gating and was also consistent with the NMR results. Generally, mutations that caused large perturbations to the voltage dependence of activation, also caused concomitant perturbations to the rate of activation, as demonstrated by the correlation analysis in Figure 3.10. This suggests for the majority of mutants where there was a significant perturbation to the kinetics of end-state transition, this could be explained by the perturbation to the voltage dependence of activation of Kv11.1.

However, it is notable that mutants D411A and W412A caused much greater perturbations to the rate of activation compared to the voltage dependence of activation, suggesting that perturbations to voltage dependence alone can only partially be responsible for the shift in transition kinetics observed. This was also found to be the case for F431A and V409A (Figure 3.10). It is possible that these mutations primarily affect one or more transition states between the two stable end states (closed and open) as well as the stable end states themselves. In contrast, mutation P426A caused much greater perturbation to the voltage dependence than to the rate of channel activation. It is notable that D411A and W412A corresponds approximately to the N-terminal end of S1, while A430V is located near the C-terminal end of S1, and P426A is likely a point at which the S1 helix kinks. Our
results support the notion that these residues may also be involved in interactions integral to the transition kinetics from the closed to open states of Kv11.1.

3.5. Conclusions

We determined the transmembrane helical extent of S1 to be from W410 to L432, with no evidence of a pre-S1 helix, based on our NMR data. Mutations within the S1 helix in Kv11.1 caused perturbations to steady-state activation as well as rates of activation. The critical residues in the S1 helix appears to form a surface facing the S4 helix of the voltage sensor which may play a role in stabilizing the end-states of activation transitions (Figure 3.11, 3.12). The pre-S1 region appears to have functional and clinical significance – our current results suggest that it plays a role in stabilising the open state via possible interactions with cytoplasmic domains. Our NMR data also indicates a potential kink at P425 location, consistent with cryo-EM data from the rKv10.1 channel.
3.6. References

### Supplementary Table 1. Steady-state activation parameters

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Yellow highlight indicates mutants which were recorded complete or in part by Dr Matthew D Perry and were included in this analysis. Green highlight indicates mutant that was completely recorded by Dr Peter Tan and were included in this analysis.
### Supplementary Table 2. Rates of activation parameters

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<th>Mutant</th>
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<th>mean Δln Tau act</th>
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Yellow highlight indicates mutants which were recorded complete or in part by Dr Matthew D Perry and were included in this analysis. Green highlight indicates mutant that was completely recorded by Dr Peter Tan and were included in this analysis.
Supplementary Figure 1. Full length steady-state activation traces for WT, Y403A, D411A and T421A on the same time scale.
Chapter 4

The role of the S1 voltage sensor domain in Kv11.1 inactivation gating

4.1 Introduction

The structure of Kv11.1 channels closely resembles other voltage-gated K⁺ channels (VGK). However, Kv11.1 channels have unique behaviour and gating kinetics [1, 2]. Most VGK channels open rapidly following depolarisation of the cell membrane potential, then inactivate slowly. In contrast, Kv11.1 channels have slow activation but very rapid inactivation [3, 4]. Furthermore, in contrast to the N-type inactivation in typical VGK channels mediated by occlusion of the pore with an intracellular N-terminal domain, the mechanism of inactivation in Kv11.1 has been suggested to be more similar to C-type inactivation, involving a series of conformational rearrangements in the selectivity filter to close the pore [3, 5].

Inactivation of Kv11.1 channels is of particular interest because most drugs bind preferentially to these channels in the inactivated state [6-8]. In addition to their clinical relevance, the unique gating kinetics of Kv11.1 inactivation also means that it is readily possible to use electrophysiology protocols to study inactivation by isolating the slower open-close transition, thereby making these channels an excellent model system for studying the mechanisms of inactivation [9].

In order to investigate the unique inactivation gating kinetics of Kv11.1 ion channels, knowledge of the structures and energetics of the stable ends states as well as that of the transition-state ensembles are required [10]. While X-ray crystallography / cryo-EM has been able to define the structures of several stable
end-state structures, the short-lived, high-energy transition-state structures are not amenable to direct structural analysis\textsuperscript{11-13}. Currently, Rate Equilibrium Free Equilibrium Relationship (REFER) analysis is the only protein engineering method that allows direct probing of the energetics of the transition state of a reaction\textsuperscript{14}.

REFER analysis allows the determination of the relative timing of domain motions during a transition process. Information about the transition pathway linking the initial open and final inactivated states during inactivation is obtained by introduction of mutations, and then by comparing the changes in free energy of the transition state (\(\Delta \Delta G^\ddagger\)) relative to changes in free energy between the ground states (\(\Delta \Delta G^0\))\textsuperscript{14-16}. The ratio of these perturbations is known as the \(\Phi\)-value. If a mutation is introduced into a domain which moves early, then \(\Delta G^\ddagger\) will be perturbed to a similar extent compared to \(\Delta G^0\), thus providing a \(\Phi\)-value close to 1 (Fig 3A). Conversely, if a mutation is introduced into a domain, which moves late, then \(\Delta G^\ddagger\) will be minimally perturbed compared to \(\Delta G^0\), thus producing a \(\Phi\)-value close to 0 (Figure 4.1.). For \(\Phi\)-values in the range from 0 – 1, domains with higher values are involved in earlier transitions, compared to those with lower values\textsuperscript{4,17-19}.

Previously, it was believed that Kv11.1 inactivation simply involved the collapse of the selectivity filter, effectively shutting the “inactivation gate”\textsuperscript{20}. However, extensive REFER analysis in recent years has demonstrated that Kv11.1 inactivation is likely to be more complex than this, involving interlinked motions of multiple domains (Figure 4.1.) analogous to that of opening and closing of a Japanese Puzzle Box\textsuperscript{4,21}.
Figure 4.1. Schematic representation of Φ-value analysis of inactivation in Kv11.1 channels. For Φ-value analysis, a point mutation is introduced into the channel (coloured line) and the change in free energy of transition state (ΔΔG‡) is compared with that of ground states (ΔΔG0). Φ-value is calculated by: ΔΔG‡ / ΔΔG0. (A) If particular domain moves early, then both ΔΔG‡ and ΔΔG0 are perturbed similarly, giving Φ-value close to 1. (B) If particular domain moves late in reaction, then ΔΔG‡ is minimal, giving Φ-value close to 0. (C) Japanese Puzzle Box model of Kv11.1 inactivation. Colour scheme indicates sequence of domain motions from open (red) to inactivated (purple). Figure 4.1A and B adapted from Wang et al. 20104. Figure 4.1C is adapted from Perry et al. 21
However, there are still several crucial missing pieces to this puzzle. The S1, S2 and S3 domains of the voltage sensor, as well as linkers between the transmembrane domains, have not yet been characterised by REFER analysis. How conformation changes within the voltage sensor are transmitted to the pore during inactivation is not well established.

Inactivation and activation gating has been suggested to be coupled processes in K+ channels, demonstrated in multiple studies using ion interactions and cysteine accessibility experiments. Of particular note, KcsA potassium channels also display an apparent C-type inactivation mechanism. In recent studies, this inactivation in KcsA has been shown to be strictly coupled with activation. For example, when testing a range of pH values, Gao and colleagues demonstrated that inactivation always accompanied activation. The rates of inactivation increased steeply in the pH range where rates of activation also increased correspondingly. However, the issue of whether activation and inactivation are coupled processes in Kv11.1 remains a point of contention. Systematic mutagenesis studies in the Kv11.1 outer pore helix as well as the S4 voltage sensor have demonstrated poor correlations between perturbations to steady-state inactivation and activation gating. Whether mutations in the S1 domain have similar or differential effects on activation and inactivation gating processes remains to be tested.

We hypothesised that the interface constituted by the S1 and S4 segments acts as an anchor, which allows effective transmission of mechanical forces from the voltage sensor onto the pore, ultimately collapsing the selectivity filter and inactivating Kv11.1 channels. In order to address some of the inadequacies in the current understanding of the role of the S1 segment in Kv11.1 inactivation gating, the aims of the present study were: (1) to use REFER analysis with an extensive
scanning mutagenesis to probe the contribution of the S1 segment during inactivation of Kv11.1 channels, and (2) to determine whether there is any correlation between voltage-dependence and gating kinetics between inactivation and activation or deactivation processes.
4.2. Methods unique to this chapter

4.2.1 REFER analysis

Any process where there are two stable ground states separated by a single dominant transition state, and where the forward and reverse rates are well described by single exponential processes, can be assessed using REFER analysis\(^{19}\). This is the case for Kv11.1 inactivation, which can be considered a transition pathway between open and inactivated states, and has rapid gating kinetics. In contrast, this method of analysis is not suitable for activation of Kv11.1, as it involves multiple transitions going from the closed to open end-states.

Specifically for Kv11.1 inactivation, the forward (open to inactivated) and reverse (inactivated to open) transitions are easily separable from the slower activation and deactivation gating transitions\(^{3,4,21}\). This allows for extraction of forward and reverse rate constants.

Rates for onset of inactivation \(k_{\text{inact},V}\) was measured using the triple pulse protocol shown in Fig 4A. From holding potentials of -90 mV, cells were depolarised to +40 mV for 500 ms so that channels become inactivated. A subsequent voltage-step to -90 mV or -110 mV for 10 ms enabled channels to recover from inactivation into the open state. The potential was then stepped to voltages between -70 mV and + 100 mV and rates of inactivation at each voltage were obtained by fitting a single exponential to the corresponding decaying current trace (see Fig. 4A).

Rates of recovery from inactivation \(k_{\text{rec},V}\) were measured using the two-step protocol shown in Fig 4B. Channels were opened and inactivated by an initial 1 s depolarising step to +40 mV from a holding potential of -90 mV, before being allowed to recover from inactivation by stepping to a range of negative potentials. Rates for
recovery from inactivation were obtained from the fastest time constant of a double
exponential function fitted to the hooked tail currents.

At each particular voltage (V), the observed rate constant \( k_{\text{obs},V} \) is equal to the sum of the forward \( k_{\text{inact},V} \) and backward \( k_{\text{rec},V} \) rates.

\[
k_{\text{obs},V} = k_{\text{inact},V} + k_{\text{rec},V}
\]  

(1)

The equilibrium rate constant \( K_{\text{eq}} \) at 0 mV was calculated as:

\[
K_{\text{eq},0} = \frac{k_{\text{inact},0}}{k_{\text{rec},0}}
\]  

(2)

The perturbations to inactivation gating for each mutant were measured relative to WT, as follows:

For a two state reaction, the transition state energy, \( \Delta G^\dagger \), is given by:

\[
\Delta G^\dagger = -RT \ln(k_{\text{inact}})
\]  

(3)

and the equilibrium free energy is given by:

\[
\Delta G^0 = -RT \ln(K_{\text{eq}})
\]  

(4)

Then the effect of a mutant on the transition state energy is given by:

\[
\Delta \Delta G^\dagger = -RT (\ln(k_{\text{inact,WT}}) - \ln(k_{\text{inact,mu}}))
\]  

(5)

and the effect of a mutant on the equilibrium free energy is given by:

\[
\Delta \Delta G^0 = -RT (\ln(K_{\text{eq,WT}}) - \ln(K_{\text{eq,mu}}))
\]  

(6)

A \( \Phi \)-value can then be calculated from the ratio of the change in the logarithm of the unidirectional forward rate constant, \( \log(k_{\text{inact,0}}) \), relative to the change in the logarithm of the equilibrium constant, \( \log(K_{\text{eq,0}}) \):

\[
\Phi = \frac{\Delta \Delta G^\dagger}{\Delta \Delta G^0} = \frac{\Delta \log(k_{\text{inact,0}})}{\Delta \log(K_{\text{eq,0}})}
\]  

(7)
An alternative method used to estimate the $\Phi$–value is derived from families of mutations at that position or domain. A REFER plot can be produced from the linear regression between $\log (k_{\text{inact},0})$ versus $\log (K_{\text{eq},0})$ of all mutants in a particular domain, and the overall $\Phi$-value is measured from the slope, i.e., $\Delta \log (k_{\text{inact},0}) / \Delta \log (K_{\text{eq},0})$.

4.2.2. Correlation analysis

Two-tailed Pearson correlation analysis was performed to examine correlations between perturbation to steady-state inactivation with rates of inactivation, and perturbations to steady-state inactivation with steady-state activation and deactivation. GraphPad Prism version 6 was used for all analysis, with $P<0.05$ deemed as significant.
4.3 Results

4.3.1. Determination of rate constants

To investigate the role of the S1 region in Kv11.1 channel C-type inactivation, we measured the voltage dependence of inactivation for WT and mutant channels. Typical examples of families of currents recorded to measure rates of onset of inactivation (triple pulse protocol) and rates of recovery from inactivation (double pulse protocol) for WT and T421A mutants are shown in Figure 4.2.

The observed rates ($k_{obs} = k_i + k_r$) were then plotted against voltage to generate a chevron plot (Figure 4.2C). The linear arms at each end of the chevron plot represent the unidirectional rate constants for the onset of inactivation (circles), and recovery from inactivation (squares). Unidirectional rate constants were then extrapolated to 0 mV ($k_i,(0\,\text{mV})$ and $k_r,(0\,\text{mV})$) and the equilibrium constant ($K_{eq,(0\,\text{mV})}$) for inactivation, which represents the voltage-dependence of inactivation, was calculated as $k_i,(0\,\text{mV}) / k_r,(0\,\text{mV})$.

A summary of all perturbations to the putative S1 and pre-S1 regions is summarized in Figure 4.3. The majority of perturbations were biological significant and are highlighted white in Figure 4.3. Significant mutations (Δlog $K_{eq}$) included D411A (-1.13±0.11), W412A (-0.79±0.05), L415A (-0.71±0.07), L417A (-0.54±0.02), V418A (-0.64±0.04), and T421A (-0.77±0.06) within the S1 segment. Outside the S1 helix, significant mutations which perturbed steady-state activation included Y403A (-1.04±0.16), E435A (-1.02±0.04), E437A (-0.63±0.03) and E438A (-0.63±0.04). The perturbations to steady-state inactivation were largely distributed throughout the N-terminal half of the putative segment before the kink at P426 in the S1 helix.
The effect of all S1 alanine mutations on the rates of inactivation are summarized in Figure 4.4. There were no mutations that caused >1 kCal mol\(^{-1}\) perturbation to the rates of inactivation. Moderately perturbing mutations (>0.5 kCal mol\(^{-1}\)) included D411A (0.77±0.10) and T421A (-0.50±0.05) within the S1 segment, and Y403A (-0.67±0.11) in the pre-S1 region. Outside the intracellular region of the S1 segment, E435A (-0.64±0.02) had the greatest perturbation to rates of inactivation (Supplementary Table 1). In terms of rates of recovery from inactivation, there were no mutations which perturbed rates of recovery from inactivation and achieved biological significance (Figure 4.5.).

We noted that the mutations that produced greater perturbations to rate of inactivation were similar to those that produced greater perturbations to steady-state inactivation. Therefore, a correlation analysis was performed between the perturbations to steady-state inactivation against perturbations to rate of inactivation by alanine scanning mutations of the S1 and pre-S1 regions. Significant positive correlation was observed between the forward, \(k_f(0\,mV)\), and the equilibrium for inactivation (\(K_{eq}\)) (\(R^2 = 0.88, p<0.0001\); Figure 4.6), indicating that perturbations to both the forward and reverse transitions contribute equally to the shift in equilibrium for each mutant.
Figure 4.2. Effect of alanine substitutions on steady-state inactivation. (A) Typical families of current traces recorded from WT Kv11.1 during (i) three step protocol and (ii) two step protocol to measure rates of inactivation and recovery from inactivation respectively. (B) Example current traces recorded from WT (grey) and T421A (black) Kv11.1 channels during a 0 mV step (left panel) and a -120 mV step (right panel). (C) Example chevron plots showing the voltage dependence of rates of inactivation and recovery from inactivation for WT (grey) and T421A (black) Kv11.1 channels. Arrows indicate the interpolated values for the forward and reverse rate constants at 0mV that were used to calculate the equilibrium values at 0 mV.
Figure 4.3. Summary plot of the perturbations to steady-state inactivation for each S1 alanine mutant (measured as $\Delta \log K_{eq,0}$ relative to WT, see Methods for details), measured at 0 mV. T399A and T436A were not recorded. Data shown are mean ± SEM with n=2-8 [errors bars not shown for mutants with n=2, see supplemental table 1]. Grey shading indicates putative helical extent of S1. Dotted black lines indicate mutants which produced a biological significant perturbation, which was defined as greater than ± 0.5 kCal mol⁻¹. Mutants which had a biological significant perturbation were coloured white.
Figure 4.4. Effect of alanine substitutions on rates of inactivation of Kv11.1. Bar charts summarising perturbations to forward rates of inactivation at 0 mV ($\Delta \log k_f$). T399A and T436A were not recorded. Data shown are mean $\pm$ SEM with $n=2-8$ [errors bars not shown for mutants with $n=2$, see supplemental table 2]. Grey shading indicates putative helical extent of S1. Dotted black lines indicate mutants which produced a biological significant perturbation, which was defined as greater than $\pm 0.5 \text{kCal mol}^{-1}$. Mutants which had a biological significant perturbation were coloured white.
Figure 4.5. Effect of alanine substitutions on rates of recovery from inactivation of Kv11.1. Bar charts summarising perturbations to forward rates of inactivation at 0 mV (Δlog $k_b$). T399A and T436A were not recorded. Data shown are mean ± SEM with n=2-8 [errors bars not shown for mutants with n=2, see supplemental table 2]. Grey shading indicates putative helical extent of S1. Dotted black lines indicate mutants which produced a biological significant perturbation, which was defined as greater than ± 0.5 kCalmol$^{-1}$. Mutants which had a biological significant perturbation were coloured white.
Figure 4.6. Plots of changes in forward rates of inactivation at 0 mV versus $\Delta K_{\text{inact},0}$ for alanine scanning mutagenesis of the S1 region. T399A and T436A were not recorded. Line of best fit and 95% confidence intervals (dashed lines) are shown. Error bars shown are SEM (except where $n=2$; $R^2 = 0.88$, $P<0.0001$). Data shown for $n=2$-8. Correlation analysis was performed using Pearson’s correlation test.
4.3.2. Individual $\Phi$-value determination for highly perturbing mutations

The obtained unidirectional rate constants were used to calculate an equilibrium constant ($K_{eq,0}$) for WT and mutant inactivation, using equation (2) from methods. Following the introduction of point mutation, for example T421A, the plot of log($K_{obs}$) against voltage demonstrated a distinct depolarising shift in the voltage dependence of inactivation. The relative change in log($K_{obs}$) between mutant and WT, denoted as $\Delta \log(K_{obs})$, describes how perturbing a particular mutation is. Shifts in $\Delta \log K_{eq,0}$ of $\geq 0.5$ log units were considered significant perturbations to inactivation gating, as these values were the cut-off criterion for generating a reliable $\Phi$-value from REFER plots $^{14-16}$. Using equation (6) from methods, the ratio of $k_{inact,0}$ to $K_{eq,0}$ allowed calculation of a $\Phi$-value for a particular individual residue, summarized in the Appendix. Systematic alanine-scanning mutagenesis was used to introduce destabilise mutations into the investigated domains.

4.3.3. REFER analysis

An alternative and more accurate method to determine $\Phi$-values for particular residues or domains is using REFER plots. These are linear regression plots of log($k_{inact,0}$) versus log($K_{eq,0}$) for a family of mutations at a single residue or domain of a channel, where the $\Phi$-value is derived from the gradient. Family mutations at T421 included T421C (log $K_{eq,0}$ 0.72±0.04, log $k_{inact,0}$ 2.00±0.03), T421V (log $K_{eq,0}$ 0.83±0.03, log $k_{inact,0}$ 1.99±0.03), and T421S (log $K_{eq,0}$ 0.10±0.07, log $k_{inact,0}$ 1.60±0.05). From the family mutations, a linear regression analysis was performed to deduce the overall $\Phi$-value for T421 ($\Phi = 0.63 \pm 0.08$, Figure 4.7A). This was similarly performed for the alanine scanning mutations for S1 ($\Phi$-value = 0.49 ± 0.05,
Figure 4.7B), and S4 (Φ-value = 0.49 ± 0.03, Figure 4.7C) shown. There was no significant difference between the Φ-values for S1, S4 and T421 as determined using ANOVA statistical testing (P=0.93).

Figure 4.7. Mutation of S1 residues affect inactivation at a midway point during the transition pathway. REFER plots of the forward unidirectional rate constant, log(\(k_{\text{inact,0}}\)), against equilibrium constant, log(\(K_{\text{eq,0}}\)), for inactivation for families of mutations at (A) T421 and (B) all S1 mutations tested. Data are presented as mean ± S.E.M for 3-8 cells. The slope of the linear regression analysis in each case represents the Φ-value as indicated. (C) REFER plot for all S4 mutations (published by Perry et al. 27 2013), (D) overlay of REFER plots of S1 and S4 mutations. S1 and S4 linear regression slopes were not significantly different, as determined by analysis of covariance statistical test (ANOVA). T399A and T436A were not recorded.
4.3.4. No correlation between inactivation and activation in Kv11.1 channels

The introduction of a mutation to Kv11.1 can alter the steady-state inactivation in two separate ways. One way is to alter the change in the equilibrium set point of the end-states of inactivation relative to WT. This is measured as the change in \( \log(K_{eq,0}) \). The second way to perturb inactivation is changing the degree of voltage dependence, measured as a change in slope or rate of forward (inactivation) or reverse (recovery from inactivation) rate constants. To assess whether perturbations to the steady-state activation is related to perturbations to the steady-state end states of Kv11.1 activation (see Chapter 3), we performed a correlation analysis as demonstrated in Figure 4.8. As observed, S1 mutation-induced perturbations to voltage-dependence of inactivation showed no correlation with mutation-induced perturbation to voltage-dependence of activation \( (R^2 = 0.003, \ p = 0.73, \ \text{Fig. 4.8}) \), consistent with activation and inactivation processes not being coupled for the S1 segment.
Figure 4.8. Perturbations to inactivation gating and activation gating show poor correlation for S1 residue alanine mutations. Plot comparing perturbations to inactivation equilibrium of S1 region of Kv11.1 (represented by $\Delta \log (K_{eq,0})$) compared with perturbations to steady-state activation ($\Delta \Delta G^0_{act}$) for each S1 residue mutation. Solid black lines represent linear regression analysis with 95% confidence interval. Error bars represent standard error of mean (S.E.M.) for n=2-8. Errors bars are not presented for mutants with n=2.
4.4. Discussion

Understanding the dynamic structural changes during Kv11.1 inactivation requires intricate knowledge of the interactions among the different protein domains during this process\textsuperscript{2,10}. Firstly, scanning alanine mutagenesis and REFER analysis of the S1 segment demonstrated simultaneous conformational changes (overall Φ-value of 0.5) in these domains, similar to the previous published Φ-value for the S4. It is proposed that the interface S1 and S4 domains, formed midway during inactivation, acts as an anchor which allows effective transmission of force from the voltage sensor through to the pore during gating. Secondly, we demonstrated a lack of any correlation between mutation-induced perturbations to the voltage-dependence of activation and inactivation in the S1 segment, providing support to the notion that activation and inactivation processes are not coupled in the S1.

4.4.1 REFER analysis of the S1 region

The perturbing mutations identified in S1 were subject to REFER in order to characterise the temporal motions of these domains. The S1 helix had an overall Φ-value of ~0.50, which was not significantly different from the previously obtained Φ-value for the S4 helix (Fig 4.7). This suggests that the S1 undergoes conformational changes approximately midway during inactivation gating. The Φ-value for S1 is smaller than that of previously published data for S5P (Φ-value = 0.6) and greater than that of S4-S5 linker (Φ-value = 0.45)\textsuperscript{4}. Thus, conformation changes to S1 and S4 domains occur after changes to S5P but precede changes to the S4-S5 linker.

The cryo-EM structure data recently published for rKv10.1 channels provide support for the above observations. The equivalent residue of T421 in Kv11.1
appears to be T231 in rKv10.1. The structure of rKv10.1 demonstrates that the side chains of this residue directly faces the S4 helix of the same subunit. T231 contains a polar side chain that interacts with the positive charges of the S4 voltage sensor as well as the negative charges in the S2 helix. As such, the cryo-EM structure demonstrates a network of interactions between the S1 and S4 interfaces which could be involved in mediating inactivation gating in Kv11.1, which supports the notion that S1 and S4 helices are involved in linked processes which occur approximately midway through the Kv11.1 inactivation process. Indeed, multiple studies in other potassium channels, including Kv1.2, Kv2.1-Kv6.4 and Kv1.2-Kv2.1 paddle chimera, have demonstrated that their equivalent residues play important roles in transmitting mechanical forces from the voltage sensor to the pore domain. Lee et al. (2009) demonstrated using statistical coupling analysis that the S1 helix in Kv1.2 forms a conserved small contact with the pore helix near the extracellular surface. Based on the present obtained Φ-values, it is also likely that this anchor interface forms after conformation changes in the S5P linker and prior to motions in the S4S5 linker (Figure 4.9).

Additionally, the perturbed residues of the S1 domain appears to form a hydrophobic cluster on one face of the helix. This cluster faces the transmembrane S4 helix. Recent studies have demonstrated that the key residues in the S4 helix during Kv11.1 inactivation include hydrophobic residues L529, L530, L532 and V535. These residues form a 3_{10} helical structure, similar to that in Kv1.2 and MlotiK1 channels. Interestingly, these residues are located all on the same helical face, pointing towards the important hydrophobic cluster identified in the S1 helix. One possibility is that interactions exist between the hydrophobic S1 cluster and S4
face which allow stabilisation of the inactivated state of Kv11.1, once the sequential motions of the domains have completed and the selectivity filter has collapsed.
Figure 4.9. Model of the interface between S1, S4 and extracellular S5 helix as an anchor which allows transmission of force from the pore domain to the voltage sensor domain during Kv11.1 ion channel inactivation. The channel schematic shows the transmembrane segments of two opposing subunits (labelled in right hand subunit). In the left hand subunit, the helices are number and color-coded according to the temporal sequence of events that occur during inactivation. Red represents the first step and purple represents the final step during the inactivation transition. The pore helix couples an initial loss of potassium ions from the selectivity filter, followed by the motion of transmembrane S5 and S5P. This allows the S1 helix and S4 voltage sensor to form an interface which acts as an anchor. This allows for subsequently motion in S4-S5 linker and S6 domains of the channel, which transmits force back to pore helix to collapse the selectivity filter inactivate the Kv11.1 channel. Figure adapted from Perry et al. 21, 2013.
4.4.2. Inactivation of S1 segment in Kv11.1 is not coupled to activation

In many Kv channels, the voltage dependence of channel inactivation is linked to voltage dependence of activation, whereas the unique feature of Kv11.1 channels is its fast and intrinsically voltage sensitive C-type inactivation mechanism\textsuperscript{2,31}. It has been previously demonstrated is that the voltage-dependence of activation and inactivation of Kv11.1 channels are separable\textsuperscript{32-36}. There still continues to be controversy in the literature regarding the nature of activation and inactivation processes and whether these transitions are coupled or not\textsuperscript{2,31}.

In the present study, alanine scanning mutagenesis significantly perturbed few residues in terms of steady-state inactivation. There were no severely perturbing residues with $\Delta$Keq >1kCal throughout the whole S1 transmembrane helix, emphasizing that the S1 segment does not contribute significantly to Kv11.1 inactivation. Using a biologically significant cut-off of 0.5 kCal, is used, then residues D411A and T421A within the S1 helix were moderately perturbing to the equilibrium of inactivation as measured from the forward (onset), although had minimal effects on reverse (recovery) rates of inactivation. This picture starkly contrasts what was observed for steady-state activation, where perturbing positions were distributed throughout the S1 helix (Chapter 3), forming potential network of interactions with the adjacent S2 and S4 helices of the voltage sensor domain. Our data also demonstrates a lack of correlation between mutation-induced changes in the equilibrium of inactivation compared with activation gating. When we examine the alanine mutations of S1 segment on rates of forward inactivation, a similar picture is seen with no major perturbations observed. This suggests that activation and inactivation processes may not be coupled in Kv11.1, and that the origin of the voltage-dependence of inactivation lies beyond the S1 segment.
In a study probing the gating of Shaker using fluorescent unnatural amino acids, it was shown that mutation of hydrophobic residues at the N-terminus of the S1 helix led to partial uncoupling of the S4 movement from pore opening\textsuperscript{37}. Our results together with this supports the notion that the S1 may have more roles than just providing passive structural support to merely stabilize the voltage sensor. Whether Kv11.1 inactivation is coupled to activation processes remains controversial, however, data from S1 alanine scanning mutagenesis provides further evidence that these processes are not closely coupled. This discordance is evident with mutants A429V and P426A, which had significant shifts in steady-state activation but not for steady-state inactivation. Moreover, mutants D411A, W412A, V418A, V423A and A430V has significant perturbations to rates of activation, whilst relatively non-significant effects on rates of inactivation. Recent studies have also suggested a discordance between rates of activation with rates of deactivation, via the phenomena of “mode shift”, where once the voltage sensor has moved upwards, it requires a more hyperpolarized voltage range for return movement to the resting state\textsuperscript{38-40}. Whether inactivation may be more closely to deactivation or closing of Kv11.1 channels remains to be addressed.

\textbf{4.4.3. Limitations}

The application of REFER analysis to obtain individual Φ-values (Appendix) for each mutant may not necessarily reflect the role of the native amino acid residue. Firstly, mutants need to cause a sufficient perturbation to the free energy difference between end states. Ideally the value for $\Delta \log (K_{eq,0})$ greater than 0.5 log units has been shown to be a biologically relevant cut-off\textsuperscript{4,41}. Φ-values obtained from mutants which have $\Delta \log (K_{eq,0})$ less than 0.5 log units will likely to have high levels of error.
Secondly, $\Phi$-values which lie outside the range between 0 and 1 are caused by a mutation which perturbs the end-states or alters the transition pathway. Thus, that individual mutation will not provide information regarding the native transition pathway. Even for mutants which do have $\Delta \log (K_{eq,0})$ greater than 0.5 log units as well as a $\Phi$-value within 0 - 1, there is a possibility that the mutation may have affected two or more different processes that by coincidence, cancel each-other out to give a $\Phi$-value within the desired range.

The REFER plots used in this investigation are able to overcome these limitations. If multiple mutations give the same $\Phi$-value, defined by a linear relationship between $\log(k_{inact,0})$ versus $\log(K_{eq,0})$ on a REFER plot, then the most likely explanation is that they perturb the same step in the transition pathway. The slope of the REFER plot provides an overall $\Phi$-value for the residue or the domain that has been mutated. Another advantage of this methodology is that particular residues which moving out of time relative to the remainder of the domain can be easily determined by observing for clear outliers on the REFER plots.

4.4.4. Future studies

In order to elucidate exactly which residues are energetically coupled within the interface formed by the S1 and S4 helices, double-mutant cycle analysis can be performed$^{21,27}$. Using this technique, the physical and energetic coupling between T421 from S1 and charged residues within the adjacent S4 and S2 helices can be confirmed. Residues from S4 or pore helix which are energetically coupled to this interface can also be identified. REFER analysis can also be used to characterise S2, S3 helices, as well as S1S2, S2S3 and S3S4 linkers. These experimental data
can then be used to derive and optimise simulation systems for models of Kv11.1 inactivation using Molecular Dynamics. Ultimately, knowledge of the structural dynamics of Kv11.1 inactivation characterised on an atomic and temporal scale should offer important insights into the mechanism by which Kv11.1 inactivation facilitates drug binding. This is the first step in designing therapeutic agents which do not trigger lethal arrhythmias.

4.4.5. Conclusions

While the role of the S1 helix in Kv11.1 gating kinetics has not been clearly established, this study supports that this domain plays a role in the mechanics of inactivation. The S1 voltage sensor domain of Kv11.1 is involved in inactivation gating, but this process is unlikely to be closely coupled with activation gating. Furthermore, REFER analysis demonstrated that the S1 helix undergoes conformation changes midway during Kv11.1 inactivation, approximately simultaneously with the S4 helix. We propose that an interface between S1 and S4 in the activated state acts as an anchor during potassium channel inactivation, and may facilitate force transmission from the voltage sensor to the pore domain.
4.5. References

Supplementary Table 1. Inactivation equilibrium parameters

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Yellow highlight indicates mutants which were recorded complete or in part by Dr Matthew D Perry and were included in this analysis. Cyan highlight represents inactivation data that was recorded by Dr David Wang or Dr Matthew D. Perry.

**Supplementary Table 2. Rates of inactivation parameters**
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Yellow highlight indicates mutants which were recorded complete or in part by Dr. Matthew D. Perry and were included in this analysis. Cyan highlight represents inactivation data that was recorded by Dr. David Wang or Dr. Matthew D. Perry.
Supplementary Figure 1. Additional full-length traces of WT and W412A on the same time scale for (A) onset of inactivation and (B) recovery from inactivation.
Chapter 5

S1 and coupling between deactivation and inactivation in Kv11.1 channels

5.1. Introduction

In Chapter 4, the discordance in the perturbations to voltage-dependence of activation and inactivation in Kv11.1 channels suggests that these two processes are not closely coupled. This result is broadly consistent with correlation analyses performed in other parts of the Kv11.1 ion channel, including the S4 voltage sensor\(^1\) as well as the outer pore helix domain\(^2\). However, given recent evidence suggesting that activation and deactivation in Kv11.1 may not be closely coupled (see below) we wondered whether inactivation may be more closely coupled to deactivation than to activation in Kv11.1 channels.

Recent studies have demonstrated a possible coupling between inactivation and “mode-shifting” in other ion channels, including sodium channels\(^3\) and Shaker channels\(^4\). Mode-shift is the observed phenomena where the voltage-dependence of the return of the voltage sensor domain (VSD) on repolarization appears to occur at a more hyperpolarized range of voltages compared to activation or VSD upward movements\(^5,5,6,7\). Whilst the precise molecular mechanisms remain to be elicited, Villalba-Galea et al.\(^8\) proposed that the mode-shift phenomena can be explained when considering that the VSD can exist in three states: VSD at rest in a "down state" and activated in two possible "up states". First, an active position which is populated immediately following depolarisation and second, a relaxed position populated as the VSD forms additional interactions to stabilise the ‘up’ conformation.
The second up state has been suggested to be responsible for the mode shift observed. Mode shifting of the VSD has been suggested to be coupled to entry into the inactivated state, based on good correlation of the time courses of C-type inactivation and mode shifting in other channels (minutes in Na channels and seconds in Shaker).

In Kv11.1 channels, Piper et al. analyzed gating currents and showed that mode shifting of the VSD does indeed occur in these channels, occurring over a time course of hundreds of milliseconds. Tan et al. used voltage-clamp fluorometry to measure VSD motion and cytoplasmic gate closure or deactivation in a series of N-terminal truncated constructs of Kv11.1. The authors demonstrated that deletion of the first 25 amino acid residues from the N-terminus abolished mode-shifting of ionic current, with the gating current or VSD mode shift remaining intact, thus demonstrating an uncoupling between VSD mode-shift and gate closure. Because the N-terminus of Kv11.1 is involved in regulation of this slow deactivation, these results suggest that in Kv11.1 the VSD mode shift may be coupled to deactivation gating. Given that mode-shifting in Kv11.1 has been shown to be disrupted by mutation-induced perturbations to inactivation or deactivation gating, this leads to the hypothesis of potential coupling interaction between inactivation and deactivation. However, the effect of mutations to the S1 segment and its role in coupling inactivation and deactivation remains less clear.

To address these questions, this chapter aims to: (1) determine the role of the S1 segment in voltage-dependence and kinetics of deactivation of Kv11.1 channels, and (2) determine whether there is any significant correlation between deactivation and inactivation in Kv11.1 which would support the notion that these processes are coupled.
5.2. Methods unique to this chapter

5.2.1 Electrophysiology

To measure 'steady-state' voltage dependence of deactivation (open-to-close transition), cells were depolarized to +40 mV for 1 s, to ensure channels were fully activated, then stepped to voltages between 0 and −120 mV for 10 s followed by a step to −70 mV to measure tail currents amplitude. Tail current amplitudes were normalized to the maximum tail current value and fitted with a Boltzmann expression (equation (1) below) to derive the $V_{0.5}$ of deactivation:

$$I / I_{\text{max}} = \left[1 + \exp(V_{0.5} - V_t) / k\right]^{-1}$$

(Eq. 1)

where, $I / I_{\text{max}}$ is the current normalised to the maximum, $V_{0.5}$ is the half–maximal activation voltage, $V_t$ is the test potential and $k$ is the slope. The same data were also fitted with the thermodynamic form of the Boltzmann expression (see Equation 2):

$$g / g_{\text{max}} = \left[1 + \exp(\Delta G^0 - z_g EF) / RT\right]^{-1}$$

(Eq. 2)

where, $\Delta G^0$ is the difference in Gibb’s free energy between the states at 0 mV, $z_g$ is the effective number of electric charges crossing the transmembrane electric field, $F$ is Faradays constant, $R$ is the universal gas constant, and $T$ is absolute temperature.

Perturbations to Gibb’s free energy difference between closed and open states, for mutants compared to WT, were calculated as:

$$\Delta \Delta G^0 = \Delta G^0_{\text{MT}} - \Delta G^0_{\text{WT mean}}$$

(Eq. 3)

where $\Delta G^0_{\text{MT}}$ was from individual experiments and $\Delta G^0_{\text{WT mean}}$ was the mean $\Delta G^0$ value from all WT replicates.

The rate of deactivation (open-to-closed transition) was measured using a double-step protocol. From a holding potential of -90 mV, cells were depolarized to +40 mV (or +80 mV for some mutants) for 1 s, in order to open then inactivate all
channels. Cells were then repolarized to a range of tail potentials between −20 to −160 mV, in 10 mV increments. The decaying portion of the tail currents were then fitted with a double exponential function in order to obtain the fast ($\tau_{\text{fast, } -120 \text{ mV}}$) and slow ($\tau_{\text{slow, } -120 \text{ mV}}$) time constants for deactivation at each tail potential. For each mutant and WT, we show a weighted average time constant for deactivation measured as:

$$\tau_{\text{mut or WT}} = \frac{\lambda_1 \cdot \tau_1 + \lambda_2 \cdot \tau_2}{\lambda_1 + \lambda_2}$$  \hspace{1cm} \text{(Eq. 3)}$$

where, $\tau_1$ and $\tau_2$ are respectively the fast and slow time constants measured at -120 mV, and $\lambda_1$ and $\lambda_2$ are the relative proportions of the fast and slow components respectively. We then compare each mutant to WT, as $\ln(\tau_{\text{mut}} / \tau_{\text{WT}})$.

5.2.2. Correlation analysis

Two-tailed Pearson correlation analysis was performed to examine correlations between perturbations to steady-state inactivation with perturbations to steady-state deactivation. GraphPad Prism version 6 (Graphpad Inc, CA, USA) was used for all analysis, with $P<0.05$ deemed as significant.
5.3. Results

5.3.1. S1 helix and Kv11.1 channel steady-state deactivation

The voltage dependence of the transition from open to closed states (deactivation) was assessed using a three step protocol, as shown in Figure 5.1. Typical families of tail currents recorded from WT and T421A mutant Kv11.1 channels are shown in Figure 5.1A. T421A produced a 45 mV shift in the $V_{0.5}$ of steady-state deactivation. A summary of the shifts in steady-state deactivation ($\Delta \Delta G^0_{\text{deact}}$, kCal mol$^{-1}$) for all mutants are shown in Figure 5.2 and Supplementary Table 1. Within the putative S1 helix, the mutations which produced biologically significant perturbations to steady-state deactivation were distributed throughout the S1 helix (D411A, W412A, L413A, L415A, L417A, I419A, Y420A, T421A, A422V, V423A, F424A, T425A, P426A, Y427A, S428A, A429V, A430V). Outside the S1 helix (based on our NMR structure), significant perturbations included I395A, Y403A, P405A, F406A, K407A, A408V, and V409A. It is notable that these mutations are all located in the N-terminal half of the S1 segment. The majority of these mutants overlap with those that disturbed steady-state activation. In terms of the mutants which produced the greatest shift in steady-state deactivation ($\Delta \Delta G^0_{\text{deact}}$), this was D411A (6.72±0.13), W412A (3.58±0.15), L413A (4.47±0.18), L415A (4.64±0.08), L417A (3.84±0.16) and T421A (5.58±0.06). These are the same set of residues which had a significant perturbing effect to rates of inactivation (Chapter 4 of thesis).
Figure 5.1. (A) Typical families of current traces recorded from (i) WT and (ii) T421A Kv11.1 channels during 10-s isochronal deactivation protocols. The voltage specific protocol is shown in the inset. (B) Examples of steady state deactivation curves for WT (grey) and T421A (black).
Figure 5.2. Summary plot of the change in the energetics of the voltage-dependent distribution between the closed and open states for each S1 alanine mutant (measured as ΔΔG^0_deact relative to WT, see Methods for details), at -120 mV. T399A and T436A were not recorded. Data shown are mean ± SEM with n=2-8 [errors bars not shown for mutants with n=2, see supplemental table 1]. Grey shading indicates putative helical extent of S1. Dotted black lines indicate mutants which produced a biological significant perturbation, which was defined as greater than ± 0.5 kCal/mol. Mutants which had a biological significant perturbation were coloured white.
5.3.2. Perturbations to deactivation kinetics of Kv11.1 channel deactivation

Next, we examined the effect of S1 mutations on the rate of Kv11.1 channel deactivation. Most S1 helix mutations had a limited impact on the rate of deactivation, i.e. \( \log(\tau_{\text{mut}} / \tau_{\text{WT}}) < 1 \). Within the S1 helix, the only mutations which produced biologically significant shifts in rates of deactivation (\( \Delta \ln \tau_{\text{deact \ adjusted}} \)) was L432A (-0.59±0.06), A430V (-0.55±0.19) and P426A (0.68±0.22) at the kink. Within the pre-S1 region, mutations which produced significant perturbations included H402A (-1.23±0.09), Y403A (-1.27±0.08), S404A (-0.72±0.06) and K407A (2.73±0.05). H402A, Y403A and S404A had accelerating effects on rates of deactivation, whilst K407A caused a dramatic slowing of the rate of deactivation across all membrane potentials from -160 mV to -110 mV (Figure 5.3). It was not possible to measure the deactivation rate of K407A at less negative membrane potentials, due to the apparent absence of channel closure at these potentials. A summary of the perturbations to rate of deactivation is summarized in Figure 5.4 and Supplementary Table 2.
Figure 5.3. (A) Typical examples of current traces recorded at -120 mV, following an activation step to +40 mV, for (i) WT and (ii) K407A Kv11.1 channels. Current traces were normalized to the same peak value to highlight the differences in the rates of deactivation. (B) Tau\(_{\text{deact}}\), -120 mV for rate of deactivation over a series of voltages (-160 mV to -80 mV) for WT (black) and K407A (red).
Figure 5.4. Summary plot of the changes in the rates of deactivation measured as $\Delta \ln k_{\text{deact, adjusted}}$ where $\tau_{\text{mut}}$ was measured as $(\lambda_1 \lambda_1 + \lambda_2 \lambda_2)/(\lambda_1 + \lambda_2)$ where $\lambda_1$ is the proportion of the fast component $\lambda_1$ and $\lambda_2$ is the proportion of slow component $\lambda_2$, from data measured at -120 mV. T399A and T436A were not recorded. Data shown are mean ± SEM with $n=2$-10 [errors bars not shown for mutants with $n=2$, see supplemental table 2]. Grey shading indicates putative helical extent of S1. Dotted black lines indicate mutants which produced a biological significant perturbation, which was defined as greater than ± 0.5 kCal/mol$^{-1}$. Mutants which had a biological significant perturbation were coloured white.
5.3.3. No correlation between voltage-dependence or rates of deactivation with activation in S1 segment

A mutation introduced into Kv11.1 can perturb deactivation via two different mechanisms. First, it can perturb deactivation relative to WT, by altering the chemical free energy change associated with deactivation\(^1\). A series of residues produced large perturbations to steady-state deactivation compared to WT, including residues D411, W412, L413, L415, L417 and T421. These were all negative shifts in voltage-dependence of deactivation. Second, a mutation can perturb deactivation by altering the rate constants for deactivation. Alanine scanning of the S1 segment showed little perturbation to the rates of deactivation, and a correlation analysis of perturbation to steady-state versus rates of deactivation yielded an \(R^2=0.006\) (Figure 5.5A).

Given that VSD upward motion has distinct voltage-dependence compared to VSD downward motion, it is not surprising that this may translate into differences in the voltage-dependence and kinetics of ionic current activation and deactivation. To test this, we performed correlation analyses between mutation-induced perturbations to steady-state and rates of activation with that of deactivation gating in Kv11.1. We found no significant correlation between perturbation to steady-state deactivation with steady-state activation (Figure 5.5.B, \(R^2=0.21\), \(P=0.07\)). No significant correlation was found between steady-state deactivation with rates of activation (Figure 5.5.C, \(R^2=0.03\), \(P=0.28\)). Furthermore, shifts in the rate of channel deactivation showed no significant correlation (\(R^2=0.01\), \(P=0.45\)) with the voltage dependence of channel activation (measured as \(\Delta\Delta G_{\text{act}}^0\)), as shown in Figure 5.5D.
Figure 5.5. Correlation analysis of deactivation and activation parameters in Kv11.1 channels following alanine scanning mutagenesis. (A) Plot of steady-state deactivation vs rate of deactivation; (B) steady-state deactivation vs steady-state activation; (C) steady-state deactivation vs rate of activation; and (D) steady-state activation vs rate of deactivation. Line of best fit and 95% confidence intervals (dashed lines) are shown, fitted according to Pearson’s correlation. Error bars indicate SEM for n=2-10.
5.3.4. Correlation between voltage dependence and rates of deactivation with inactivation in S1 segment and S4 voltage sensor

One of the objectives of this study was to probe whether inactivation was possibly linked to activation or deactivation processes in Kv11.1 channels. This was done by performing a correlation analysis of perturbations to activation with inactivation and deactivation with inactivation based on an alanine scan of the putative S1 segment (Figure 5.6). There was no significant correlation between perturbations to steady-state activation with steady-state inactivation (Figure 5.6.A, \(R^2=0.01, \ P=0.45\)). However, there was a significant, albeit modest, correlation between perturbations to voltage-dependence of deactivation with inactivation (Figure 5.6.B, \(R^2=0.26, \ P=0.0005\)).

To determine whether this property was unique to the S1 segment or potentially a global feature within the voltage sensor domain, similar analyses were conducted based on a serine scan of the S4 voltage sensor (Figure 5.7). Similar trends were observed, with significant correlation between deactivation with inactivation (Figure 5.7.B, \(R^2=0.57, \ P=0.003\)) producing the best fit. The correlations between steady-state activation and inactivation for the S4 sensor (Figure 5.7.A, \(R^2=0.22, \ P=0.10\)) were not significantly correlated. As such, this data suggests that deactivation and inactivation processes may be coupled, and this feature may be common to voltage sensor domain helices and not specific to the S1 segment.
Figure 5.6. Correlation analysis of perturbations to voltage-dependence of (A) activation vs inactivation, and (B) deactivation vs inactivation. Line of best fit and 95% confidence intervals (dashed lines) are shown, fitted according to Pearson’s correlation. Error bars indicate SEM for n=2-10.
Figure 5.7 Correlation analysis of the voltage sensor (S4) in terms of perturbations to voltage-dependence of (A) activation vs inactivation, and (B) deactivation vs inactivation. Line of best fit and 95% confidence intervals (dashed lines) are shown, fitted according to Pearson’s correlation. Error bars indicate SEM for n=3-6.
5.4. Discussion

In this study, we firstly demonstrate that the mutations to the S1 helix affect deactivation in a different manner to activation, with the critical residues being localized to the N-terminal half of the helix. No significant correlation was found between perturbations to the voltage-dependence of deactivation and activation, suggesting that deactivation is not just a simple reversal of activation and may involve more complex steps or interactions. Consistent with our hypothesis that deactivation may be coupled with inactivation processes, we found a significant correlation in perturbation to deactivation and inactivation processes. Furthermore, this observation was also observed from a serine scan analysis of the S4 voltage sensor. This suggests that deactivation and inactivation processes may be coupled during Kv11.1 gating, and that this feature is not unique to the S1 segment but may be a possible shared or mediated by interaction with the S4 voltage sensor.

5.4.1. Effect of S1 mutations on steady-state deactivation and rates of deactivation

Residues of positions D411, W412, L413, L415, L417 and T421 had the greatest shifts in steady-state deactivation. Interestingly, these residues are localized to the N-terminal half of the S1 helix before the kink at Pro426, and not distributed through the segment unlike those critical in S1 steady-state activation (see Chapter 3 of thesis). It is also interesting to note that in contrast to activation, the mutations to the pre-S1 region did not have an equally marked effect on the voltage-dependence of deactivation. It can be deduced that deactivation is not a simple reversal of activation in Kv11.1 channels. Rather, the transition from closed to open state likely involves interactions of the whole S1 segment in adjacent helices and modulated by
the pre-S1 region, in contrast to transition between open to closed state where only
the N-terminal half of the S1 segment appears to play a critical role.

Systematic alanine scan demonstrated limited impact on steady state
deactivation in the S1 transmembrane helix. However, the pre-S1 region appeared to
be important, with K407A significantly slowing rate of deactivation whilst Y403 and
H402 displayed faster rates of deactivation. This contrasts with the perturbations to
rates of activation (see Chapter 3), where the most significant perturbations were
localized throughout the S1 helix, including T421, V418, W412, D411, as well as in
the pre-S1 region including Y403, H402 and L401. There were no significant
correlation between perturbations to rates of deactivation with steady-state
activation.

There are several implications from this data for Kv11.1. Firstly, The major
implication from this is that Kv11.1 deactivation (open to closed state transition) is
not the simple reversal of activation (closed to open state transition). Rather, our
observations suggest that deactivation involves transition through a distinct set of
short-lived intermediate states. Previous work where N-terminal truncation of Kv11.1
causd perturbations to channel deactivation but not activation support this notion\textsuperscript{11}.
The S1 segment may act as an allosteric modulator, involved in important
interactions which help stabilize the intermediate states of the closed-to-open
transition and thus accelerating rate of activation. In contrast, the open-to-closed
transition is less reliant on the allosteric modulation of the S1 segment, and thus
alanine mutations do not have a significant perturbation.
One notable exception to the above trend is with K407A, a pre-S1 mutation that dramatically slowed down the rate of deactivation. This suggests that the positively charged lysine residue at this location in its native position serves to stabilize the closed state rather than an intermediate state. Based on the rKv10.1 cryo-EM structure, there is a ring of charged residues in the vicinity of K217, the equivalent position in rKv10.1\textsuperscript{12}. This ring of charged residues correspond to R472 in the S2 helix, R541 in the S4 helix, and R4 and R5 from the N-cap tail of the N-terminus. As such one possible mechanism is that these interact with the negatively-charged phosphate heads of the lipid bilayer, a charged-charged interaction which may serve to stabilise the closed state of Kv11.1 channels. Another potential mechanism is that these negative charges in the ring and K407 may interact with other negatively charged regions in Kv11.1, which may include the intracellular S2-S3 linker, C-linker, or cyclic nucleotide binding homology domain.

Our results also suggest that the pre-S1 region, despite not having helical secondary structure, appears to nevertheless contribute to S1 gating by accelerating both rates of activation and deactivation. In particular, H402A and Y403A caused some of the largest perturbations to rate of deactivation. In TRPA1 and TRPV channels, the pre-S1 helix forms a part of a network of interactions with the N-terminal Ankyrin domains, linker domains and C-terminal domains\textsuperscript{13,14}. In KCa1.1. channels, the pre-S1 region forms an intracellular complex with the C-terminus, the S2-S3 linker and S4-S5 linker\textsuperscript{15}. Based on these findings in other voltage-gated ion channels, we speculate that the pre-S1 region of Kv11.1 also interacts with the cytoplasmic domains of Kv11.1 to regulate deactivating gating.
5.4.3. Correlation between S1 deactivation and inactivation and implications

A significant correlation between these two processes found in both the S1 segment and S4 voltage sensor in this study supports coupling between Kv11.1 channel deactivation and inactivation within the entire VSD. However, whether this association is consistent in other parts of the channel, e.g. the S5P linker or pore helices, remains to be established. The notion of coupling of inactivation and deactivation processes is reminiscent of gating in canonical voltage-gated channels. For example, Barghaan et al.\textsuperscript{16} examined the relationship between deactivation and inactivation in Kv4.2 channels by investigating the role of the N-terminal domain and accessory subunits in controlling macroscopic inactivation and deactivation. Kv4.2 WT channels and N-terminal deletion mutants in the absence or presence of Kv channel interacting proteins were expressed. Although Kv4.2 undergoes N-type inactivation rather than C-type inactivation, the authors found a significant correlation between the modulatory effects of deactivation with effects on inactivation in terms of onset and recovery kinetics\textsuperscript{16}. Investigating of series of N-terminal deletions also lead the authors to identify a specific deletion in which both initial rapid phase of macroscopic inactivation and tail current deactivation were slowed, suggest that this particular portion of the N-terminal domain may control both early phase inactivation and deactivation\textsuperscript{16}.

In some ways, this mechanism may also be relevant for Kv11.1 but is yet to be verified experimentally. For example, the S631 residue in Kv11.1 demonstrated altered inactivation but not VSD mode shift\textsuperscript{4}, and mutations to S631 affect a late step in inactivation, according to REFER analysis\textsuperscript{17}. As such, the deactivation gating-dependent mode shift in Kv11.1 may be more related to an earlier part of the
inactivation process. If this is the case, it is possible that there is an equivalent region within the N-terminal domain of Kv11.1 which may be responsible for concurrent modulation of both deactivation and inactivation processes.

5.5. Conclusion

The S1 helix appears to play a role in Kv11.1 channel deactivation, with critical residues involved in voltage-dependence localised to the N-terminal half of the helix. Further, we demonstrate that perturbations to voltage-dependence of deactivation are significantly correlated with perturbations to inactivation. Lastly, we showed that this correlation between perturbations to deactivation and inactivation is also seen for mutations in the S4 voltage sensor, suggesting that inactivation and deactivation may be coupled throughout the VSD of Kv11.1.
5.6. References


## Supplementary Table 1. Steady-State deactivation parameters

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Yellow highlight indicates mutants which were recorded complete or in part by Dr Matthew D Perry and were included in this analysis. Green highlight indicates mutant that was completely recorded by Dr Peter Tan and were included in this analysis.
Supplementary Figure 1. Full traces of rates of deactivation (-120 mV) of WT, Y403A, K407A, W412A and T421A on time scale for comparison.
Chapter 6

*In vitro* voltage-dependence and gating perturbations of Long QT Syndrome Type 2 mutations found in the S1 segment of Kv11.1

6.1. Introduction

In an era of increasing emphasis on genetic screening and “personalised” medicine, understanding the precise phenotypes and molecular mechanisms of genetic variants has been brought to the forefront. The goal of precision medicine is to diagnose and treat patients according to their individual clinical phenotype, a notion that is dependent on an intimate comprehension of how aberrancies on a molecular level correlates with disease presentations\(^1\text{-}^3\). Understanding the link between genetic variation and clinical phenotypes and the ability to apply this knowledge on a wider scale and to novel mutations remains an elusive challenge, particularly pertinent to cardiac channelopathies\(^4\text{-}^5\).

In the context of Long QT Syndrome Type 2 (LQTS2), inherited mutations in the *KCNH2* gene, which encodes the Kv11.1 channel, is associated with increased risk of life-threatening lethal arrhythmias\(^6\text{-}^7\). The clinical presentation of Long QT Syndrome Type II is highly variable, and can range from being asymptomatic, mildly symptomatic in adulthood, to sudden death. This heterogeneity in clinical presentation has been attributed to various risk factors including the large number of genetic mutations, with over 500 known clinical mutations in the *KCNH2* gene\(^8\text{-}^11\). In
contrast to many other inherited diseases such as cystic fibrosis, in which one deletion mutant (Phe508del) in the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel accounts for the vast majority of cases\textsuperscript{12,13}, most LQTS2-affected families possess a novel Kv11.1 mutation\textsuperscript{14}. LQTS2-associated mutations in Kv11.1 can cause loss of channel function via several mechanisms, and has been classified broadly into 4 classes: class 1: abnormal transcription/translation, class 2: deficient protein trafficking, class 3: abnormal channel gating/kinetics, and class 4: altered channel permeability\textsuperscript{15-17}. It is also possible for mutations to have multiple modes of action, for example partially reduced expression as well as abnormal gating\textsuperscript{15-17}. Systematic studies of LQTS2 mutations suggest that the majority impair protein expression at the membrane, although it is not clear what proportion of mutations concomitantly perturb channel gating and/or permeation.\textsuperscript{15-17}

It has been previously hypothesised that the highly variable clinical presentation can be partly attributed to the specific mutation, which has its own specific “molecular pattern” or \textit{in vitro} phenotype of gating and kinetic phenotype\textsuperscript{10,11,18}. Correlation of \textit{in vitro} phenotype for Long QT Syndrome Type II mutations, both functional gating and expression characteristic, with clinical phenotype may provide a better risk stratification model. In a large scale mutation analysis of 167 missense mutations in Kv11.1, Anderson and colleagues\textsuperscript{18} demonstrated that deficient protein trafficking is the dominant mechanism for all domains except for the distal C-terminus. However, the S1 segment of Kv11.1 was not extensively investigated, as well as their functional gating phenotype. Given that a considerable proportion of voltage sensor domain clinical mutations is located
within the S1 segment or pre-S1 region, as well as the integral role of the S1 segment in Kv11.1 gating (Chapter 3, 4), understanding the *in vitro* phenotype of S1 clinical mutants is of great clinical significance\(^{19}\).

Therefore the aim of this study was two-fold: (1) to assess the *in vitro* phenotype of Long QT Syndrome clinical mutations in the S1 segment by determining steady-state and kinetic parameters, and (2) to assess the expression phenotype of the same mutants.
6.2. Methods unique to this chapter

6.2.1. Mutation database and mutagenesis

LQTS2 missense mutations were identified from the International Long QT Registry (Rochester) database. All missense mutations were made using the QuikChange II XL kit from Agilent (Santa Clara, CA) using primers designed with their primer design program. As previously described, Kv11.1 DNA was subcloned into a pBluescript vector containing the *Xenopus laevis* β-globin gene. Mutations were confirmed by DNA sequencing. Plasmids were linearised using *Bam*HI-HF and cRNA was transcribed in vitro using mMessage mMachine kit (Ambion P/L) according to the manufacturer’s protocols.

6.2.2. Western blot analysis of protein expression

Western blot methods have been previously described\(^\text{19}\). In brief, HA-tagged WT or mutant Kv11.1 constructs were transfected into HEK293 cells using the Lipofectamine 2000 transfection reagent (Life technologies, Mulgrave, VIC, Australia) with 200 ng of DNA. Cells were maintained in Dulbecco modified Eagles medium (DMEM, Life technologies) supplemented with 10% fetal bovine serum (Life technologies) and stored at 37 °C with 5% CO2. Cells were harvested 48 hours after transfection solubilized in Tris buffered saline (TBS, in mM: Tris 50, NaCl 137, pH 7.5) supplemented with 1% NP-40 and a protease inhibitor cocktail (Roche Diagnostics, Castle Hill, NSW, Australia) for 1 hour on a rotating wheel at 4°C. Cell lysates were cleared by centrifugation at 16,000 g at 4°C for 30 minutes. Lysates were then mixed with SDS-PAGE sample buffer containing 5% β-mercaptoethanol, then loaded and run on a 7.5% SDS-PAGE gel before transfer to Nitrocellulose
membrane (Bio-Rad, Gladesville, NSW, Australia). For quantitative Western blot analysis, membranes were probed simultaneously with mouse monoclonal anti-HA antibody (HA.11, Covance, Princeton, NJ, USA) and a rabbit or mouse anti-\( \alpha \)-actinin antibody (Santa Cruz Biotechnology, TX, USA), followed by anti-rabbit IRDye680 or anti-mouse IRDye800 to enable quantification with the Li-Cor Odyssey infrared imaging system (Li-Cor Biotechnology, Lincoln, NE, USA).

Kv11.1 channel protein was represented by two bands on the Western blot: a fully glycosylated band (FG, \( \sim 155 \text{kD} \)) which predominantly represents channel protein at the cell membrane, and a core glycosylated band (CG, \( \sim 135 \text{kD} \)) which represents immature protein that is intracellular and thus non-functional. To account for changes in expression levels from experiment to experiment, membrane expression was calculated from the ratio of the FG/CG bands (i.e. the CG band acts as an internal loading control). For each experiment, the mutant FG/CG ratio was compared to corresponding measures from a WT construct expressed on the same day (i.e., mutants were normalized to WT). Since FG/CG values were always normalized to WT protein (including for WT itself), then WT was expressed as 1 in every experiment and so no error bars are shown for WT (WT data from \( n=17 \) experiments). An FG/CG value of 0 indicates the absence of membrane protein on Western blots, 1 indicates an expression level comparable to WT (no defect) and >1 indicates improved expression compared to WT. An ANOVA test, with Dunnets post-hoc comparison, was used to determine statistical significance compared to WT (\( P<0.05 \)).
6.2.3. Electrophysiology assay

Functional assays were performed on LQTS2 mutant Kv11.1 channels expressed in *Xenopus* oocytes. The assay was performed via low temperatures (17°C) to incubate oocytes after injection with cRNA allows for the rescue of expression of the vast majority of mutations\(^{20,21}\). Low temperature rescue of expression in oocytes is used only as a tool to determine whether mutations would remain pro-arrhythmic (due to functional defects) if normal expression could be restored.

For this assay, a ventricular action potential was mimicked to examine the extent to which LQTS2 mutations perturb the amount of \(I_{Kr}\) contributing to cardiac repolarization. This was performed using a step-ramp voltage clamp protocol. Cells were clamped at a membrane potential of −80 mV prior to a depolarizing pulse to +30 mV for 500 ms (to mimic the plateau phase). This was followed by a ramp repolarization back to −80 mV over 220 ms (to mimic the repolarizing phase). WT or mutant currents elicited during the ramp repolarization phase were corrected for capacitance artefacts by subtracting the instantaneous drop in current observed at the onset of the ramp repolarization phase from the repolarizing current. Current amplitudes were normalized to peak \(I_{Kv11.1}\) conductance to compensate for the variable expression levels observed between cells. All functional measures of \(I_{Kv11.1}\) were normalized to the whole cell conductance (peak conductance) measured in the same cell from the peak negative currents elicited at voltages -140 to -160 mV following a 1s depolarization at +40 mV, as described in Perry et al.\(^{19}\), to exclude variations in channel expression and measure only the changes in current caused by functional gating defects caused by the mutation.
To examine the extent to which LQTS2 mutations perturb the amount of protective $I_{Kr}$ elicited in response to premature beats, we delivered premature depolarizations at 20 ms intervals during or after the ramp phase of the step-ramp protocol. Premature stimuli were mimicked by depolarizing the membrane potential to 0 mV for 40 ms, starting 120 ms after the start of the repolarizing ramp and continuing at 20 ms intervals in 23 successive sweeps, finishing 560 ms after the start of the repolarizing ramp. The resultant traces were compared with WT current to determine the extent of protective current produced by mutant channels.
6.3. Results

6.3.1. Expression of clinical LQTS2 causing mutations in the S1 region

Data from Chapter 3 and 4 suggests that residues within the S1 helix, in addition to residues within the pre-S1 regions, play a significant role in Kv11.1 channel activation. We next examined whether altered function is a characteristic of LQTS2 causing mutations that are located in the pre-S1 or S1 regions. According to the International LQT registry, six LQTS2 causing mutations have been identified within the pre-S1/S1 region (i.e. between R394 to E438), namely: I400N, H402R, W410S, Y420C, T421M, and S428L. There are two principle ways in which LQTS2 mutations perturb Kv11.1 channel function: 1) by reducing the expression of channel protein at the membrane, where it is functional, and/or 2) by perturbing the function of Kv11.1 channels in the membrane. First, we examined whether LQTS2 mutations in the S1 region alter protein expression at the membrane. Our western blot data performed on HEK293 cells transiently transfected with mutant or WT cDNA, demonstrates a reduced expression associated with all pre-S1/S1 mutations with the exception of T421M (see Figure 6.1), indicating that the S1 region may also be important for proper folding of channel protein. The specific Thr421 residue may have a greater role in gating mechanisms of Kv11.1 compared to trafficking or expression of the channel.
Figure 6.1. Phenotype of LQTS2 associated mutations in the pre-S1 region. Example western blot for WT, I400N, H402R, Y420C, T421M, S428L Kv11.1 channels and negative control. The FG and CG labels indicate the fully glycosylated and core-glycosylated forms. Data represent mean ± SEM for n=3-5.
6.3.2. Gating transitions of clinical LQTS2 causing mutations in the S1 and pre-S1 regions

We also analysed the perturbations of activation, deactivation and inactivation gating of the mutant channels was expressed in an oocyte system. Example traces for steady-state activation (Figure 6.2.), rates of activation (Figure 6.3.) and rates of deactivation (Figure 6.3.) for T421M, the least trafficking defective mutant, is shown. Analyses of the six clinical mutant channels demonstrated that I400N and H402R caused significant hyperpolarizing shifts in the voltage dependence of channel activation, with a change in Gibbs free energy of activation (ΔΔG$_{\text{act}}^0$) between 1-3 kCal mol$^{-1}$. Although the hyperpolarizing shifts in channel activation would be expected to enhance the magnitude of functional Kv11.1 current, the reduced protein expression of I400N and H402R mutant channel proteins likely makes the impact of these functional perturbations negligible. In contrast, S1 mutant T421M caused a dramatic depolarizing shift in the voltage dependence of activation, with a ΔΔG$_{\text{act}}^0$ ~ +4 kCal mol$^{-1}$, whilst showing slow activation, fast deactivation, and dramatically reduced inactivation.

A similar trend was seen for rates of activation, with T421M, H402R and I400N producing the greatest perturbations compared to WT, and T421M producing the greatest perturbation to rates of deactivation (Figure 6.4). W410S was demonstrated to be one of the least perturbing mutants, which had minimal effects on activation, inactivation and deactivation parameters. Consistent with the data presented in Chapter 3 and 4, alanine scanning mutagenesis had a lesser effect on rates of deactivation. Perturbations to the voltage dependence and kinetics of inactivation gating were also very minor in comparison to the mutant’s effect on activation processes in Kv11.1 channels.
Figure 6.2. Typical families of current traces recorded from WT and T421M Kv11.1 channels during 10-s isochronal activation protocols. The voltage specific protocol is shown in the inset (A). Examples of activation traces for WT (B) and T421M (C). Examples of steady-state activation curve for WT (grey) and T421M (black). Data represent mean ± SEM for n=3-5.
Figure 6.3. Effect of alanine substitutions on rates of activation and deactivation of Kv11.1. (A) Envelop of tails protocol used to obtain rates of activation. Example raw current traces obtained from WT (B) and T412M (C) Kv11.1 channels during envelop of tails protocol. (D) Protocol used to obtain rates of deactivation of Kv11.1 channels. Typical examples of current traces recorded at -120 mV, following an activation step to +40 mV, for (E) WT and (F) T421M Kv11.1 channels.
Figure 6.4. Summary of perturbations of clinical S1 and pre-S1 mutations of Kv11.1 channels on (a) ΔΔG$_{act}^0$ (b) rate of activation at 0 mV, (c) rate of deactivation at -120 mV, (d) ΔlogKeq, (E) rate inactivation forward. Data represent mean ± SEM for n=3-5.
6.3.3. Physiological phenotype of S1 LQTS2 mutations

In order to assess the impact of the mutations on the physiological phenotype of the Kv11.1 channels, the overall function of WT and mutant Kv11.1 channels was assessed using a step-ramp protocol to mimic a ventricular action potential (Figure 6.5.) and also a premature stimulation protocol to mimic early and delayed afterdepolarizations (Figure 6.6.). For the ventricular action potential protocol, this is used as a quick way of determining whether mutant channels have normal or abnormal function. We specifically used these protocols to assess T421M, which had minimal trafficking defect but the greatest perturbations to voltage dependence and transition kinetics of Kv11.1 channels, suggesting its main mechanism of mutation is affect Kv11.1 channel function rather than channel folding or assembly. We can see from Figure 6.5 that T421M had an $I_{\text{Kv11.1}}$ profile (shaded grey, area under curve) that was not significantly different to WT. This is consistent with a large-scale analysis by Perry et al.\textsuperscript{19} who demonstrated that clinical mutants which had significantly perturbed repolarizing $I_{\text{Kv11.1}}$ were only in the voltage sensor (S4) or pore domain of Kv11.1\textsuperscript{19}.
Figure 6.5. Response of WT and T421M Kv11.1 mutant channels to a ventricular action potential voltage protocol. A. Typical current traces for WT and T421M mutant Kv11.1 channels using the voltage protocol shown in panel (A). See methods for details regarding the ventricular action potential step-up voltage protocol designed to examine $I_{\text{Kv11.1}}$. 
The premature stimuli protocol (Figure 6.6.A) was designed to assess the protective ability of Kv11.1 mutant channels against a succession of pulses mimicking ectopic after depolarizations, in comparison to the WT channels’ natural protective ability. The protective current of WT, normalized against peak current, is shown as the black curve in Figure 6.6.B in comparison to T421M shown in red. We can clearly see that the magnitude of the spikes in T421M decayed more rapidly compared to WT. Thus, it appears that T421M has a relative normal phenotype and function when repolarizing $I_{Kv11.1}$ during the ramp phase of a step-ramp protocol or stimulated single ventricular action potential, but passed significantly less current when exposed to premature stimuli. These results confirm that the T421M mutant is of clinical relevance and affects Kv11.1 channel gating and reducing the ability of the channel to protect against ectopic after depolarizations.
Figure 6.6. Current in response to premature stimuli during and after an initial potential, for WT and T421M mutant Kv11.1 channels (n=3). Voltage protocol is shown in part (A). See methods for protocol used for premature depolarizations.
Similar protocols for ventricular action potential as well as premature ectopic beats were applied to mutants which did have a more considerable trafficking defect (compared to T421M). Despite their defective trafficking and expression, we hypothesized that the considerable perturbations to gating transitions caused by these LQTS2 mutations would likely alter the function of these channels under physiological influences i.e. ventricular action potential and premature beats in our assay. From Figure 6.7., it can be seen that that the functional phenotype of H402R and T421M were the most altered out of all the clinical mutants tested.

H402R had a large spike in current in the early plateau phase. The amount of current passed during the repolarization phase was normal (shaded grey), however, the amount of protective current passed as a response to premature depolarizations was greatly reduced compared to WT. In contrast, W410S had a similar amount of current passed in response to premature depolarizations compared with WT. The phenotype for T421M in response to ventricular action potential assay and premature depolarizations has already been described above.
Figure 6.7. Representative response curves of WT and S1 clinical mutant channels to a ventricular action potential voltage protocol as well as response to premature stimuli during and after an initial potential. The voltage protocol shown at the top of the figure. See methods for protocol used for ventricular action potential and premature depolarizations.
6.4. Discussion

Over 500 LQTS2-associated mutations have been associated with LQTS2. The majority of these are missense mutations, which result in reduced Kv11.1 channel protein expression at the membrane of cardiac myocytes. While the functional phenotype has been described for some expression defective mutant channels, most of the clinical mutations within the S1 segment and pre-S1 region have not been characterized. In Chapter 3, systematic alanine scanning of the pre-S1 region demonstrated significant gain of function perturbations to both steady-state and rates of activation in H402A and Y403A. Furthermore, there are several known clinical mutations in this region, namely I400N and H402R, as well as several more in the S1 segment. This suggests that the pre-S1 region and S1 segment play some important role in the normal regulation of Kv11.1 and when disturbed, corresponds to a clinically significant phenotype. Thus, we aimed to characterise the expression and functional gating profile of LQTS2 mutations found in the S1 and pre-S1 regions. The present study demonstrated that LQTS2 mutations in these regions broadly resulted in trafficking defects for all mutants with the exception of T421M. There were also perturbations to at least one of the gating transitions for all the clinical mutations studied in the S1 segment and pre-S1 region. The study also suggests that the gain of function effects in pre-S1 region clinical mutations is ameliorated by its trafficking defect. There are also severe perturbations to gating of T421M and loss of ability to protect against premature after depolarizations, despite minimal effects on trafficking of T421M. Therefore, S1 mutations likely reduce both action potential repolarizing current passed by Kv11.1 channels in cardiac myocytes and/or current passed in response to premature depolarizations, and thus increases risk of cardiac arrhythmias.
6.4.1. Trafficking and expression of pre-S1 and S1 mutants

The trafficking assay results suggest that all pre-S1 clinical mutations tested appeared to have a trafficking defective phenotype. The S1 clinical mutants were also broadly trafficking defect, with the exception of T421M. These results are consistent with prior literature suggesting that the majority of Long QT Syndrome Type II mutations mediate their clinical phenotype via loss of channel expression and appropriate trafficking\textsuperscript{10,18}. As such, the S1 helix is also important for the proper folding Kv11.1 channel proteins. Given that T421M had virtually no trafficking deficit, this suggests that the clinical phenotype associated with this channel must be mediated by its effects on Kv11.1 gating and channel kinetics.

6.4.2. Functional phenotype of pre-S1 and S1 clinical mutants

To assess the functional phenotype of pre-S1 and S1 clinical mutants, steady-state activation, deactivation, inactivation as well as perturbations to the transition rate constants were measured. It can be observed that both I400N and H402R in the pre-S1 region both residues had significant gain-of-function phenotypes with rightward shift in voltage dependence of activation, as well as accelerated rates of activation compared to wildtype, but limited perturbations to rate of deactivation and inactivation. As such, these results suggest that there is significant reduced trafficking of both I400N and H402R in the context of gain-of-function effects, and that LQTS2 mutations in the pre-S1 have their main mechanism of action likely to be loss of trafficking in contrast to defective gating kinetics.

In contrast to the pre-S1 clinical mutants, T421M had severely altered right-shifted voltage dependence, as well as slower rate of activation but accelerated rates
of deactivation. There were minimal effects observed on voltage-dependence of inactivation, as well as rates of inactivation and recovery from inactivation. This is consistent with the results presented in Chapters 3-5, where the T421 residue despite being an uncharged residue on the S1 transmembrane domain, appears to exert significant influence and regulation of Kv11.1 channel gating. Whilst the molecular and clinical significance of the T421M clinical mutation remains to be elucidated, it is suffice to say that this particular residue in the 421 location has a profound impact on normal Kv11.1 gating when mutated. As such, T421 would be a non-classical example of a mutation which has WT-like expression phenotype but a defective in-vitro functional gating which gives rise of the clinical phenotype of LQTS Type 2.

To further elucidate the effect of the T421M mutation on the physiological function of Kv11.1 channels, we tested the response of this mutation to (1) a protocol mimicking a single action potential, and (2) a protocol with a series of after depolarization pulses, to determine the protective effect of the mutant against ectopic beats relative to WT. We found that T421M did not significant effect the I\textsubscript{Kv11.1} current in response to a single action potential, but did result in a significant reduction in protective current in response to a series of after depolarization pulses. These spikes in response to premature stimuli contribute to the refractoriness of cardiomyocytes and play an important physiological role in protection from formation of ectopic beats. In the case of T421M, the reduced protective current is likely due to the significant accelerations in rates of deactivation concurrently. One possible mechanism since the protective I\textsubscript{Kv11.1} spike is proportion of the number of open channels when the premature stimuli is achieved, any mutation that enhances
deactivation will reduce the proportion of open channels, and thus reduce the ability of the mutated Kv11.1 channel to produce protective spikes and thus protective current. For other mutants with even more severe accelerations in rates of deactivation, it is likely that reduced $I_{Kv11.1}$ current would be observed even in response to a single ventricular action potential protocol, but this does not appear to be the case for T421M. Regardless, the apparently reduced protective current of T421M in response to premature depolarizations would underlie its proarrhythmic potential in patients with such mutations.

What are the possible interactions T421 could be involved with, given its importance in Kv11.1 channel gating? From a homology model published Perry et al.\textsuperscript{23}, the T421 residue is located in close proximity to charged residues in the S4 voltage sensor (Figure 6.8.). This is also very similar to the published Cryo-EM structure for Kv11.1 by Wang and MacKinnon\textsuperscript{30}, shown in Figure 6.9. The homology model and Kv11.1 structure both suggests that there may be plausible interactions between T421 and the S4 voltage sensor during gating, such that when the threonine residue is lost at the 421 position, such important interactions are disturbed resulting in sever perturbations to the voltage-dependence and rates of activation and deactivation of Kv11.1. One such candidate residue is D456, a charged residue which is in close proximity to T421 and may form important salt-bridge interactions to mediate ion channel gating.
**Figure 6.8.** Homology model based on Perry et al.\textsuperscript{24}, demonstrating the close vicinity of Thr421 of S1 helix to Asp456 of S2 helix and R2 of S4 helix. This model supports the notion that Thr421 may be involved in a network of interactions with charged residues in adjacent S2 and S4 helices, to mediate Kv11.1 gating.

**Figure 6.9.** Cryo-EM structure of Kv11.1 published by Wang and MacKinnon\textsuperscript{30} in 2017, which demonstrates close vicinity of Thr421 of S1 helix to Asp456 of S2 helix and R2 of S4 helix. This supports the notion that Thr421 may be involved in a network of interactions with charged residues in adjacent S2 and S4 helices, to mediate Kv11.1 gating.
Similarly, from Chapter 4 the REFER analysis data showed that the S1 segment had a phi-value of approximately 0.5, which is similar to that of the S4 helix\textsuperscript{23}. One hypothesis may be that T421 also plays an important role in inactivation Kv11.1, forming an anchor between S4 approximately midway through the inactivation process. From the homology model\textsuperscript{23,25}, Thr421 and Asn573 from the S5 helix are located in close proximity. Multiple studies in other potassium channels, including Kv1.2, Kv2.1-Kv6.4, and Kv1.2-2.1 paddle chimera, have demonstrated that their equivalent residues play important roles in transmitting mechanical forces from the voltage sensor to the pore domain\textsuperscript{26-28}. The formed anchor may provide the biomechanical leverage required to transmit subsequent conformational changes to the S5P linker and S4-S5 linker during inactivation of Kv11.1.

With regards to the other clinical mutants tested, all other pre-S1 (I400N, H402R) and S1 clinical mutants (W410S, Y420C, S428L) had reduced action potential repolarizing current and/or current passed in response to premature depolarizations, which together with their defective trafficking and perturbed effect on gating transition would result in increased risk of cardiac arrhythmias. However, these clinical mutants were all able to pass more current during premature depolarizations assay compared to T421M, suggesting that their clinical phenotype may not be as severe as T421M.

In this study, mutant channel protein expression Western Blot assays were performed using transfected mammalian HEK293 cells incubated at 37°C. This is preferable to the \textit{Xenopus} oocyte system for expression analysis because low temperature (17°C) incubation of oocytes allow for rescue of expression of some
mutants\textsuperscript{21}. In contrast, to test gating function we measured gating properties on the oocyte system. This performed at room temperature rather than physiological (37°C), which is known to slow gating kinetics in the oocyte system\textsuperscript{29}. It is important to note that interpretation of the presented results is limited by the fact that neither mammalian HEK293 cells nor \textit{Xenopus} oocytes contain the same exact combination of proteins found in cardiac myocytes. There are also other biochemical processes such as phosphorylation pathways and signal pathways that are not accounted for when using HEK293 cells and \textit{Xenopus} in our experiments. As a result, this limitation would likely underestimate the functional defects associated with the clinical S1 mutants studied here.

\textbf{6.4.3. Future directions and conclusions}

Clearly, further work is required to further characterize clinical mutations in the S1 segment and remainder of the voltage sensor domain and elucidate precisely their mechanism of action, effects on gating, trafficking and folding of Kv11.1. In the greater context of risk stratification for Long QT Syndrome, a comprehensive and systematic analysis of all known clinical mutations with detailed clinical data is required. An \textit{in vitro} phenotype model for all such mutations should be determined based on a combination of their basic trafficking phenotype, kinetics of assembly and degradation, domain-domain and subunit-subunit interactions. Simultaneously gating data in terms of voltage-dependence and gating kinetics data combined with expression profiles can create an \textit{in vitro} model of disease severity which can be correlated with robust clinical data. A large database analysis will be a first step towards improvement of risk stratification for Long QT Syndrome based on both clinical severity as well as specific mutation molecular mechanisms.
Additionally, further family mutations and double-mutant cycle analysis of critical residues in the S1 segment as well as voltage sensor and pore domains will assist in determining which residues are energetically coupled. Experimental data can be used to derive and optimize molecular model simulations of Kv11.1 inactivation. Ultimately, the knowledge of the structural dynamics of Kv11.1 gating should offer important insights into the mechanisms by which KCNH2 mutations give specific phenotypes seen in LQTS2.

In conclusion, clinical LQTS2 mutants that perturb the pre-S1 region or S1 helix can lead to disruption of the finely tuned properties of Kv11.1 channels, reduce the action of potential repolarizing currents passed by Kv11.1 channels in cardiac myocytes, as well as current passed in response to premature depolarizations. These mechanisms are likely predisposing patients to increased risk of cardiac arrhythmias and sudden cardiac death.
6.5 References

**Supplementary Table 1.** Trafficking phenotype of pre-S1 and S1 clinical LQTS2 mutants.

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Kevin Phan was involved in n=3 for all mutants listed in this table. Yellow highlights indicates mutants with additional data collected by Dr Chai Ann Ng and included in this analysis.

**Supplementary Table 2.** Gating parameters of pre-S1 and S1 clinical LQTS2 mutants.

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<td>0.53</td>
<td>0.04</td>
<td>0.08</td>
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<tr>
<td>S428L</td>
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<td>0.11</td>
<td>-0.49</td>
<td>0.10</td>
<td>-0.22</td>
<td>0.04</td>
</tr>
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Kevin Phan was involved in n=3 for all mutants listed in this table. Cyan highlight indicates additional data collected by Ms Errika David that was included in this analysis.
Chapter 7

Discussion

7.1. Concluding remarks

Elucidating the underlying mechanisms and developing more effective therapeutic strategies to reduce mortality from cardiac arrhythmias is a critical challenge for cardiology in the 21st century. The rhythmic contractions of the heart are initiated by cardiac action potentials, a complex interplay of ionic currents mediated by ion channel proteins expressed on cardiomyocytes. Inherited mutations may perturb the nuances of ion channel gating, either by altering the kinetics of gating or voltage-dependence of gating, giving rise to arrhythmias. In the case of the Kv11.1 channel, loss of function mutations result in congenital LQTS type 2, one of the commonest cause of sudden cardiac death in the young\(^1\). Kv11.1 is also the molecular target for the vast majority of cases of drug-induced arrhythmias and sudden cardiac death\(^2\). Therefore, understanding the basic normal structure and function of Kv11.1 in exquisite molecular detail will provide a foundation and platform to understand the complex clinical arrhythmias which arise when normal function is disrupted\(^3,4\).

Despite advances in our understanding of the voltage sensitivity and molecular detail of Kv11.1 gating in recent years, grasping a complete and comprehensive picture of the precise dynamic interactions remains elusive and challenging. Whilst some success has been heralded in the determination of atomic structures of several bacterial and mammalian K\(^+\) channels\(^5-7\), determination of the crystal structure for Kv11.1 has not been achieved, for reasons including that the expression and purification of membrane proteins remains a technical challenge. As
such, understanding the gating kinetics of Kv11.1 and other membrane potassium channels have relied on other means to probe function, including electrophysiology studies and functional assays including that mimicking ventricular action potentials and premature depolarizations used in Chapter 6.

Previous studies have demonstrated a crucial role for the voltage sensor domain (VSD) in activation and deactivation gating transitions in Kv11.1, with the S4 helix in particular acting as the principle voltage sensor\textsuperscript{6,8,9}. However, the functional role of the remainder of the VSD remain relatively unexplored. In the context of the S1 segment, there is a current lack of consensus in the literature regarding the extent of the helical transmembrane region\textsuperscript{10-15}, as well as a lack of understanding of its role in Kv11.1 gating. Although traditionally thought to be a static structure which other VSD helices can anchor against during gating\textsuperscript{10,16-18}, there is increasing evidence that the S1 segment may serve a greater dynamic purpose during the gating movements of Kv11.1\textsuperscript{19,20}.

7.2. Transmembrane extent of S1 helix and pre-S1 region

Firstly, we used NMR structural methodologies to gain insight into the helical transmembrane extent of the S1 segment of Kv11.1. Previously, the transmembrane regions for the S1 segment had been estimated using bioinformatics tools and prediction models, with conflicting alignments varying by up to 9 residues reported in the literature. The N-terminal end of the S1 helix has been reported from I395\textsuperscript{13} up to D411\textsuperscript{11}, spanning over 4 helical turns. The C-terminal end has been reported from I419\textsuperscript{13} up to E435\textsuperscript{11}. The challenge in defining accurately the extent of the S1 segment lies in the fact that in contrast to the pore regions of the Kv11.1 ion channel,
the S1 segment is poorly conserved among canonical voltage gated potassium channels, hyperpolarization activated channels and cyclic nucleotide gated families, with few distinct markers which can be used for alignment\textsuperscript{10-13}. Indeed, there is not a single amino acid position which is common across rat Kv1.1, human HERG and mouse HCN2. To address this issue, we tested and confirmed bioinformatics predictions using NMR spectroscopy experiments, where chemical shift index data and gadolinium suppression demonstrated that the approximate transmembrane helical extent spans from W410 to L432. Our chemical shift index data also supports a kink in the S1 segment in the vicinity of P425. Homology modelling based on the cryo-EM structure data for rKv10.1 potassium channel\textsuperscript{23} demonstrates that the S1 transmembrane extent ranges from P405 – F431. The differences in the exact transmembrane extent of the S1 from our NMR data and based on the rKv10.1 homology model stems from the innate limitations of modelling. The structure of the rKv10.1 channel was captured with the activation gate closed by the voltage sensor likely in the up or activated conformation\textsuperscript{23}. There are currently no structures of any Kv channel, which have the voltage sensor in the down or relaxed conformation. This limitation may explain the differences in the transmembrane extent of the S1 helix determined by the NMR data in Chapter 3 compared with homology model based on the rKv10.1 structure\textsuperscript{23}. Despite this, our data provides the first structurally-based attempt at determining the transmembrane helical extent of the S1 segment in specifically Kv11.1 channels.
7.3. Functional role of S1 helix in activation

Prior work has been performed to extensively study the pore-helix, S6, S5, S5P, S4, and S4-S5 linker of the Kv11.1 channel, using alanine scanning mutagenesis. In the present thesis, this systematic study of Kv11.1 has been extended to the S1 segment, a relatively understudied region within this channel. It is demonstrated that the S1 segment residues that were important for steady-state and rate of activation were distributed throughout the S1 region as well as localized in a cluster of residues in the pre-S1 region. Based in homology images shown in Chapter 3 and 4, it is likely that the S1 helix is involved in a network of interactions with charges in the adjacent S2 and S4 helices, to mediate Kv11.1 gating. It is likely that this network of interactions stabilizes the S4 voltage sensor and fine tunes the voltage sensitivity of Kv11.1 activation.

7.4. Functional role of pre-S1 region

Interestingly, a cluster of residues in the pre-S1 region was found to have significantly perturbed rates of activation and deactivation. Specifically His204 and Tyr403 play particularly important roles in regulating the voltage dependence of channel opening as well as regulating the slow closure which is an important feature of Kv11.1 channels. These perturbations resulted in much faster deactivation, similar to mutations seen in the N-terminal Per-Arnt-Sim (PAS) domain and C-terminal C-linker and cyclic nucleotide binding homology domain (cNBHD). Based on the similarities in phenotype seen with mutants in the pre S1 loop and mutants in the PAS and cNBH domains we suggest that the unstructured pre-S1 loop may interact with these intracellular domains to form a complex that modulates slow deactivation
phenotype in Kv11.1. This experimental data suggests that although the pre-S1 region lacks secondary structure, it may still play a functional role in Kv11.1 or a role in assembly and biogenesis via intracellular complexes and interactions. As such, our study provides the first systematic investigation of the role of the S1 segment in Kv11.1 gating.

7.5. Role of S1 helix in Kv11.1 inactivation

The defining feature of Kv11.1 channels is its unique inactivation gating kinetics, which has fast rate of inactivation, fast recovery from inactivation and is voltage dependent. Given that the unique gating kinetics plays an important physiological role in maintaining cardiac rhythm, understanding the underlying molecular basis of Kv11.1 inactivation is of great clinical interest. To elucidate the structural changes during Kv11.1 channel inactivation requires intricate knowledge of the interactions among the different protein domains during this process. Previously, REFER analysis has shown that Kv11.1 channel inactivation involves a very specific temporal sequence of interlinked domain motions, which allows interconversion between open and inactivated states. However, absent from this scheme are the temporal roles of the S1, S2, S3 domains and linkers of the voltage sensor. It is also not well established how the conformation changes within the voltage sensor are transmitted to the pore during gating, not only in Kv11.1 channels but across most potassium channels. In Chapter 4, changes in kinetics of inactivation gating following a point mutation was successfully used to derive temporal information about the role of the mutated residue during gating transitions of the S1 helix. In this study, scanning mutagenesis of the S1 helix alongside with
REFER analysis demonstrated an overall Φ-value of 0.5, which is very similar to that previously determined for the S4 segment (Perry et al., JGP 2013). It is proposed that the interface S1 and S4 domains, formed midway during inactivation, acts as an anchor which allows effective transmission of force from the voltage sensor through to the pore during gating. The importance of the T421 residue in the S1 segment has also been demonstrated, including its potential interactions with the surrounding S2 and S4 helices of the VSD.

7.6. Deactivation and inactivation processes in Kv11.1 are coupled

From the literature, it is unclear as to whether inactivation is coupled to either activation or deactivation in Kv11.1, and if so which residues are likely to be critical for this interaction\textsuperscript{27,36}. To probe this question, we performed a correlation analysis of perturbations to Kv11.1 voltage dependence of inactivation with perturbations to activation and deactivation. We found a reasonable correlation between steady-state inactivation and deactivation following alanine scanning mutation, whilst no correlation was found between inactivation and activation steady-state perturbations. There was no correlation found between steady-state deactivation with activation or rate of activation. This data suggests that in Kv11.1, the open-to-close transition (deactivation) is not a simple reversal of the closed-to-open transition (activation) and that rather inactivation and deactivation in Kv11.1 may be coupled processes. Similar patterns have been seen in N-terminal truncation studies of Kv11.1, which perturbed channel deactivation but not activation\textsuperscript{37}. Since there were more S1 mutants that accelerated the rate of deactivation, at least at physiological voltages, compared to those that perturbed the rate of activation, it may be that the S1 helical
segment acts as an allosteric modulator, involved in important interactions which stabilize intermediate states of the open-to-closed transition and thus contribute to the slow deactivation of WT Kv11.1 channels. To determine whether this relationship is isolated to only the S1 segment, or is also shared by the dominant voltage sensor S4, we performed a similar series of correlation analyses in S4 serine scan mutants. A similar relationship was also obtained, thus suggesting that inactivation and deactivation coupling in kv11.1 may be mediated by interactions between S1 and S4 voltage sensor domains, however, the precise nature and nuances of such interactions is yet to be defined.

7.7. Mechanistic insight into clinical mutants of the S1 region

Future investigation would also involve studying the phenotypes of the clinical LQTS mutants located in the S1 segment and pre-S1 region. Current literature suggests that the majority of clinical LQTS mutants have defective trafficking as the underlying mechanism for LQTS\textsuperscript{38,39}. Mutations of the channel can result in loss-of-function of Kv11.1 by either reduced mRNA synthesis, reduced assembly and trafficking of proteins from the endoplasmic reticular to the plasma membrane, defective gating or altered ion permeation through Kv11.1. The majority of clinical mutations investigated so far are missense mutations which results in defective protein trafficking and expression, decreasing the number of functional channels in the plasma membrane\textsuperscript{39}. Preliminary trafficking studies presented in this thesis have suggested that the pre-S1 clinical mutants have defective trafficking yet gain-of-function gating phenotype, supporting the notion that the pre-S1 region may be integral for Kv11.1 biogenesis and assembly. These mutations also had reduced
protective current passed in response to premature depolarizations, suggesting that their overall phenotype type is not only governed by their trafficking phenotype but also inherent deficits in gating transitions. Our results also demonstrate that the mechanism of S1 mutations in LQTS2 affect both the trafficking and gating kinetics of Kv11.1, which in turn reduces either the action potential repolarizing current passed by Kv11.1 channels in cardiac myocytes, and/or current passed in response to premature depolarizations. This increases the likelihood of cardiac arrhythmias in individuals with these mutations.

7.8 Future directions

Although a wealth of information regarding channel gating can be obtained by systematic mutagenesis, these studies are ultimately limited in that they are unable to elucidate the complex dynamic changes in structure and transitions between different gating states. The epitome of understanding ion channel gating and function would be an atom-by-atom visualisation of the changes in protein conformations and domain movements as a function of time during activation, deactivation and inactivation gating. To work towards this goal, future directions of study would involve: (1) identifying pairs or networks of interacting residues within the S1 helix and between the S1 region and the remainder of the VSD; (2) thermodynamic mutant cycle analysis to identify which stage of transition pathway does a particular interaction occur, and (3) the use of the above data to constrain homology models of the open, closed and inactivated states of Kv11.1, achieved using molecular dynamic simulations.
In the broader context, a detailed understanding of Kv11.1 gating and kinetics on a molecular level and how specific clinical mutants can affect these parameters has great potential for improving diagnosis and risk stratification of LQTS\textsuperscript{40}. Despite a common underlying genetic cause, the presentation of LQTS2 is highly heterogeneous. Whilst this may seemingly be explained by a vast number of mutations in Kv11.1, the variable penetrance phenotype and reports of asymptomatic siblings of sudden cardiac death patients demonstrates the complexity and multifactorial nature of LQTS phenotypic presentation.

Ultimately, a detailed understanding of the normal structure and function of the VSD of Kv11.1 will be the platform for the development of strategies to modify its function in disease states such as LQTS. Improved understanding of Kv11.1 ion gating on a molecular scale will also have broad implications for the understanding of basic mechanisms of voltage sensing which underlie electrical signalling in human physiology.
7.2. References

Appendix

Publications arising from this thesis


Publications related to this thesis


Conference Presentations

Matthew D Perry, Chai Ann Ng, Ying Ke, Kevin Phan, Jamie I Vandenberg (2014). “Assessing gating phenotypes of long QT syndrome type 2 causing mutations”. Oral presentation at the International Union for Pure and Applied Biophysics, Brisbane Australia
Kevin Phan, Jamie I Vandenberg and Matthew D Perry (2014). “Pore and voltage sensor interface may serve as an anchor during hERG K+ channel inactivation gating”. Poster presentation at the St Vincent’s and Mater Health Sydney Research Symposium, Sydney, Australia.

Kevin Phan, Jamie I Vandenberg and Matthew D Perry (2014). “The role the S1 segment in hERG K+ ion channel gating kinetics”. Poster presentation at the Annual Conference of the Australian Society for Biophysics, Melbourne, Australia.

*Awarded ASB Student Travel Award