Characterization of cardiac $I_{Ks}$ channel gating using voltage clamp fluorometry

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ABSTRACT

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Voltage-gated ion channels make up a superfamily of membrane proteins involved in selectively or non-selectively conducting charged ions, which can carry current in and out of cells, in response to changes in membrane voltage. Currents carried by ion channels influence the voltage across the cell membrane, which can trigger changes in the conductance of neighboring voltage-gated channels. In this way, signals, measured as transient changes in voltage called action potentials, can be sent through and between cells in order to transmit information quickly and efficiently throughout excitable systems. My thesis work focuses on elucidating the mechanisms underlying the voltage-dependent gating of a member of the voltage gated potassium (Kv) channel family, KCNQ1 (Kv7.1). Like other members of the voltage gated potassium family, the KCNQ1 channel is made up of four subunits, each containing a voltage sensing domain and a pore-forming domain. Tetrameric channels form with a single central pore domain, and four structurally independent voltage sensing domains. KCNQ1 plays roles both in maintenance of the membrane potential (it forms a leak current in epithelial cells throughout the body) as well as a very important role in resting membrane potential reestablishment (it forms a slowly activating current important in action potential repolarization in cardiac cells). In order to serve these varied functions, KCNQ1 displays uniquely flexible gating properties among Kv channels. Evidence of this flexibility is found in the observation that the presence or absence of various beta subunits can cause the channel to be non-conducting, slowly activating with a large conductance, quickly activating with a small conductance, or constitutively active. My thesis
project has been to unravel the mechanisms underlying these very different phenotypes, focusing on the role of the voltage sensor and its coupling to the channel gate.

Most of this work focuses on the role of KCNQ1 in the heart, where it comprises the alpha subunit of the slowly activating delayed rectifier current, \( I_{Ks} \). This current plays a major role in repolarization of the cardiac action potential, evidenced in part by its major role in shortening the action potential in the face sympathetic stimulation, which leads to phosphorylation-induced increase in \( I_{Ks} \) current. Further evidence for the importance of \( I_{Ks} \) to proper cardiac function is found through the identification of many mutations to \( I_{Ks} \) that result in cardiac arrhythmia, most notably Long QT syndrome, which results from loss of \( I_{Ks} \) current and an associated prolongation of the cardiac action potential. In addition, gain-of-function \( I_{Ks} \) mutations have been implicated in Short QT Syndrome and an inherited form of atrial fibrillation. In order to understand mechanisms underlying the physiological and pathophysiological functions of \( I_{Ks} \), a more complete picture of its structure and function are needed. One major goal in the pursuit of a more complete characterization of \( I_{Ks} \) is to understand the interaction between the \( I_{Ks} \) alpha subunit, KCNQ1 and its modulatory subunit KCNE1, which has been shown to profoundly affect the gating of the KCNQ1 channel. Among the effects of KCNE1 co-expression are a slowing of channel activation, a slowing of deactivation, a depolarizing shift in the voltage dependence over which the channel activates and an increase in conductance through the KCNQ1 channel pore. To this point, a complete structural and functional basis for these myriad biophysical alterations has not been established.

In order to better understand the gating of KCNQ1, this work develops a voltage sensor assay, voltage clamp fluorometry, to measure movements of the voltage sensor and explore changes to the voltage sensor induced by KCNE1 and disease-causing mutations. Chapter 1
validates this technique using mutagenesis to ensure the assay reports on voltage sensor movement. A preliminary characterization of voltage-dependent gating in homomeric KCNQ1 channels reveals an unexpected relationship between voltage sensor movement and channel opening. Chapter 1 then looks at the effect of KCNE1 on voltage sensor movement and coupling to the channel gate, finding both to be significantly altered in the presence of this beta subunit. Returning to the homomeric KCNQ1 channel, Chapter 2 further probes its gating and develops a model based on the prediction that KCNQ1 voltage sensors act as allosteric regulators of the channel gate. This scheme can make predictions about what gating processes are affected by permutations such as KCNE1 co-expression and the presence of disease-associated mutations. Finally, Chapter 3 explores the effects of two atrial fibrillation associated mutations on KCNQ1 gating using electrophysiology, biochemistry, and VCF. Through these results, this work provides novel insight into structures and interactions that are important for gating in both physiological and pathophysiological states.
Table of Contents

LIST OF CHARTS, GRAPHS, ILLUSTRATIONS.................................................................III
LIST OF ABBREVIATIONS ...................................................................................................... V
ACKNOWLEDGEMENTS .......................................................................................................... VI

INTRODUCTION ...................................................................................................................... 1
  THE I_Ks POTASSIUM CHANNEL IN CARDIAC ACTION POTENTIAL PHYSIOLOGY .................. 1
  PATHOPHYSIOLOGY ASSOCIATED WITH I_Ks ...................................................................... 2
  STRUCTURE OF VOLTAGE-GATED POTASSIUM CHANNELS .............................................. 4
  MECHANISMS OF Kv CHANNEL GATING .............................................................................. 6
MOLECULAR ARCHITECTURE OF THE I_Ks CHANNEL ............................................................ 9
  KCNE1-KCNQ1 stoichiometry ............................................................................................... 12
  KCNE1-KCNQ1 S6 interaction .............................................................................................. 12
  KCNE1-KCNQ1 S1 interaction .............................................................................................. 13
  KCNE1-KCNQ1 S4-S5 linker interaction .............................................................................. 13
  KCNE1-KCNQ1 S4 voltage sensor interaction .................................................................... 14
VOLTAGE SENSOR ASSAYS .................................................................................................... 15

CHAPTER 1: KCNE1 ALTERS THE VOLTAGE SENSOR MOVEMENTS NECESSARY TO
OPEN THE KCNQ1 CHANNEL GATE ................................................................................... 17
  SUMMARY .......................................................................................................................... 18
  INTRODUCTION ................................................................................................................ 18
  METHODS .......................................................................................................................... 21
    Molecular Biology ........................................................................................................... 21
    VCF recordings ............................................................................................................... 21
    Kinetic Modeling ............................................................................................................ 22
  RESULTS ............................................................................................................................ 25
  DISCUSSION ...................................................................................................................... 40

CHAPTER 2: AN ALLOSTERIC GATING SCHEME UNDERLIES THE FLEXIBLE GATING
OF KCNQ1 CHANNELS ......................................................................................................... 45
  SUMMARY .......................................................................................................................... 46
  INTRODUCTION ................................................................................................................ 47
  METHODS .......................................................................................................................... 50
    Molecular Biology ........................................................................................................... 50
    VCF recordings ............................................................................................................... 50
    Data Analysis .................................................................................................................. 50
  RESULTS ............................................................................................................................ 51
  DISCUSSION ...................................................................................................................... 64

CHAPTER 3: CHARACTERIZATION OF KCNQ1 ATRIAL FIBRILLATION MUTATIONS
REVEALS DISTINCT DEPENDENCE ON KCNE1 .................................................................... 68
  SUMMARY .......................................................................................................................... 69
  INTRODUCTION ................................................................................................................ 69
  METHODS .......................................................................................................................... 72
    Molecular Biology and Cell Culture ............................................................................... 72
    Electrophysiology .......................................................................................................... 72
    Voltage Clamp Protocols .............................................................................................. 73
    Crosslinking .................................................................................................................... 74
MTSEA-Biotin Experiments ................................................................. 75
Data Analysis ....................................................................................... 75
RESULTS............................................................................................... 76
DISCUSSION ....................................................................................... 97

CONCLUSIONS .................................................................................. 102

BIBLIOGRAPHY .................................................................................. 106
List of Charts, Graphs, Illustrations

Figure I1: The Cardiac Action Potential 1

Figure I1: Structure of voltage gated potassium channels. 5

Figure I2: Inactivation schemes in Kv channels 7

Figure I3: Physical and functional interaction between IKs proteins KCNQ1 and KCNE1 10

Figure 1.1: Voltage dependent fluorescence signals from KCNQ1 C214A/C331A G219C suggest 1:1 coupling between voltage sensor movement and channel activation. 25

Figure 1.2: Control data for psKCNQ1 27

Figure 1.3: The R243A mutation shifts the voltage dependence of both channel activation and fluorescence signal. 28

Figure 1.4: The F351A mutant fluorescence signal suggests movements of multiple independent voltage sensors precede channel opening 29

Figure 1.5: Fluorescence signals from psKCNQ1/KCNE1 suggest complex effects of KCNE1 on the Q1 voltage sensor and gating mechanism. 30

Figure 1.6: Cole-Moore shifts seen in psKCNQ1/KCNE1 but not psKCNQ1 alone. 32

Figure 1.7: Simulated behavior of KCNQ1 and KCNQ1-KCNE1 channels 34

Figure 1.8: Model structures for KCNQ1 and KCNQ1/KCNE1 channels 36

Figure 2.1: KCNQ1 voltage sensors move independently 48

Figure 2.2: KCNQ1 channels can open with only two activated voltage sensors 51

Figure 2.3: The I268A mutation increases the voltage-independent constitutive current in KCNQ1. 52

Gating Scheme 2.1 54

Figure 2.4: KCNQ1 mutant L251A introduces a brief delay in channel activation and fluorescence indicates that two voltage sensors are needed to cause the channel to open 55

Figure 2.5: Labeled KCNQ1 K218C is a more representative reporter of WT KCNQ1 voltage sensor movement 56

Figure 2.6: The I268A mutation introduces constitutive current and shifts
the voltage dependence of fluorescence relative to current in the K218C background

Figure 3.1: S140G, but not V141M, slows deactivation of homomeric channels.

Table 3.1: t of deactivation at different voltages for KCNQ1 S1 mutants

Figure 3.2: Function of wild-type KCNQ1 and wild-type KCNE1 (120mM K+ vs. 5mM K+). (A) G-V relationship for KCNQ1

Figure 3.3: S140G and V141M minimally affect KCNQ1/KCNE1 activation kinetics

Figure 3.4: KCNE1 slows deactivation of V141M heteromeric channels to a greater extent than S140G channels

Figure 3.5: Crosslinking of substituted cysteines in KCNQ1 and KCNE1 reveals orientation of S140 and V141 relative to KCNE1

Figure 3.6: Full Length Blot of Crosslinking Bands

Figure 3.7: Functional Consequences of Crosslinking Pair V141C / A44C is minor

Figure 3.8: Functional Consequences of Single Cysteines

Figure 3.9: Complete Proteolysis of HRV-3C Cleavage Site in the EQ Dimer

Figure 3.10: KCNE1 preferentially assembles next to proximal KCNQ1 subunit in tandem EQQ construct

Figure 3.11: Intersubunit location of KCNE1 impacts functional consequences of KCNQ1 mutations.

Figure 3.12: The S140G mutation slows equilibration of the voltage sensor during deactivation

Figure 3.13: In the presence of KCNE1, S140G and V141M mutations drastically slow equilibration of the VCF signal at hyperpolarized voltages

Figure 3.14: Predicted Orientation of S1 KCNQ1 relative to KCNE1
List of Abbreviations

Kv – Voltage-gated potassium channel
LQTS – Long-QT syndrome
VCF – Voltage clamp fluorometry
SA node – Sinoatrial node
AV node – Atrioventricular node
AP – Action potential
APD – Action potential duration
TM – Transmembrane
I_{Ks} – Slowly-rectifying cardiac potassium current
psKCNQ1 – KCNQ1 containing labeling construct mutations C214A C331A and G219C
KCNQ1L – alternate nomenclature to denote the presence of C214A C331A and G219C after labeling
fAF – Familial Atrial Fibrillation
SQTS – Short QT Syndrome
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Introduction

The I_{Ks} potassium channel in cardiac action potential physiology

The cardiac action potential (AP) is the electrical signal that propagates throughout the heart stimulating coordinated contraction of the heart muscle. Initiated in the sinoatrial (SA) node, it moves through the atria, AV node, ventricle and back up to the atria. The AP is shaped by the opening and closing of ion channel proteins found in the cell membrane of cardiac cells. In atrial, ventricular and purkinje cells, the action potential is initiated by activation of the voltage gated sodium channel, providing the upstroke (Phase 0 – Figure 1). Following the upstroke, voltage gated sodium channels rapidly and completely inactivate, which, along with the activation of rapid outward potassium current (I_{to}), creates the notch (Phase 1). The next phase of the action potential, termed the plateau phase, is defined by a balance of inward calcium current (mainly from Ca_{V}1.2) and outward potassium current. Finally, the repolarization phase (Phase 3) results from the inactivation of calcium channels and an increase in potassium current, mainly through the delayed rectifier currents I_{Ks} and I_{Kr}. Accompanying muscle contraction takes place due to excitation-contraction coupling:

Calcium, which enters cell during the plateau phase, changes roles from an electrical signal carrier to a chemical second messenger by triggering additional calcium release from intracellular stores and binding to Troponin C, promoting actin-myosin interaction and muscle contraction.  

Figure 11 The cardiac action potential. Representation of a ventricular action potential with phases noted by numbers 0-4.
Given the importance of calcium influx during the plateau phase to excitation-contraction coupling, it follows that the timing of the action potential is a critical determinant of proper cardiac function. As a result, the repolarization currents $I_{Ks}$ and $I_{Kr}$ are highly specialized ion channel proteins with unique biophysical profiles which allow them to respond to depolarization slowly, earning them the name ‘delayed rectifiers’ $^{3,4}$. Their slow response to depolarization allows for a prolonged plateau phase and adequate calcium entry. Too much potassium current too early in the action potential causes premature action potential repolarization that precludes vital calcium signaling as well as disrupting rhythmicity. Since the $I_{Ks}$ channel represents the predominant potassium current responsible for terminal repolarization in humans, its precise timing is particularly important in shaping the cardiac action potential duration.

Specific insight into the important role of $I_{Ks}$ in determining action potential duration was gained through the discovery that $I_{Ks}$ represents a major target for sympathetic stimulation through PKA-mediated phosphorylation of the channel following beta receptor activation and subsequent cellular increase in cAMP $^{5-7}$. Phosphorylation of $I_{Ks}$ results in gain-of-function effects including increased current, faster activation kinetics, and a hyperpolarizing shift in voltage-dependence. These effects result in increased outward current through $I_{Ks}$ earlier in the action potential, which shortens the action potential to accommodate elevated heart rate $^{8}$.

Pathophysiology associated with $I_{Ks}$

In contrast to the shortening effect of phosphorylation-induced increase in $I_{Ks}$ on APD, mutations to genetic components of $I_{Ks}$ that lead to loss of current can dangerously prolong APD $^{9}$. The discovery that mutations leading to loss-of-function of $I_{Ks}$ and $I_{Kr}$ underlie congenital Long-QT syndrome variant 1 (LQT1) and 2 (LQT2), respectively, further implicates these two
potassium currents as critical determinants of APD and identifies them as putative therapeutic targets in treating arrhythmia.

The first Long QT pedigree, identified by Jervell and Lange-Neilsen, was characterized by deafness, syncopy and sudden death. In ECG recordings from affected family members, a pronounced prolongation of the QT interval was observed. Later, Romano and Ward reported separate cases of families with prolonged QT interval and predisposition to syncopy and sudden death. The genetic basis for Long QT gradually came to light, with some mutations causing additional symptoms, such as deafness, due to additional roles for some Long-QT genes in other tissues, such as the inner ear. To date, 12 genes have been identified that can harbor mutations that associate with congenital LQTS. The vast majority of these genes code for ion channels or related genes, and mutations to the I\textsubscript{Ks} alpha subunit (called KvLQT-1 or KCNQ1) are the predominant form, representing approximately 50% of cases. The molecular mechanisms underlying this I\textsubscript{Ks} channelopathy range from non-functional and trafficking mutants to mutants causing subtle biophysical alterations affecting the voltage dependence and/or kinetics of channel currents.

Additionally, recent reports have implicated mutations to the KCNQ1 gene in congenital short-QT syndrome (SQTS) and familial atrial fibrillation (fAF). These pathological states result from gain-of-function mutations that increase repolarization current and shorten the action potential, though it is not presently understood why some of these mutations preferentially cause atrial arrhythmias. Several KCNQ1 linked to SQTS and fAF have a common biophysical characteristic: slowed deactivation kinetics which lead to use-dependent accumulation of repolarization current. The prominent role of repolarizing current from I\textsubscript{Ks} in determining action potential duration and the associated physiological and pathophysiological importance of
this current leads us to consider the detailed biophysical mechanisms that underlie the function of this current and in particular the processes underlying its highly specialized and important kinetics.

**Structure of voltage-gated potassium channels**

The Kv super-family of voltage-gated potassium-selective channels, mostly involved in the maintenance or reestablishment of resting membrane potential, open and close in response to changes in membrane voltage, with their voltage dependence uniquely tuned to their specific physiological role. These membrane-imbedded proteins contain four homologous or identical subunits, each containing six transmembrane helices, termed S1-S6. The first four helices (S1-S4) comprise the voltage sensing domain and the S5-S6 helices make up the pore (Figure I2A). A large body of work has established many biophysical mechanisms by which these proteins select for potassium and undergo voltage dependent gating processes. In particular, a high resolution crystal structure of a mammalian voltage-gated potassium channel has shed light on the molecular architecture of these important channels and has complemented functional studies to establish mechanisms and functional domains and residues important in the selectivity and gating of the channel. In Kv channels, each of the four channel-forming subunits contributes a structurally independent voltage-sensing domain, while the single pore domain is made up of S5-S6 from all four subunits so that the voltage-sensor-to-pore stoichiometry 4:1 in each Kv channel (Figure I2B).

Potassium selectivity is conferred to these channels by the selectivity filter, located toward the extracellular end of the pore, and contained within the “pore loop” between S5 and S6 helices. Potassium ions are dehydrated as they move into the selectivity filter, and the precise geometry of backbone carbonyl oxygen atoms in the filter mimics that of oxygen atoms in
hydrated potassium ion complexes (Figure I2C). As a result of the precise geometry of the selectivity filter required to efficiently dehydrate the potassium ion, the smaller sodium ion with its distinct hydration geometry is excluded.\(^{23}\)

Below the selectivity filter is the central cavity of the potassium channel and on the intracellular side of the membrane, a crossing of the four S6 helices comprises the channel gate, termed the bundle crossing (Figure I2C). The channel gate is thought to open and close by virtue of a rotation of S6 helices.\(^{24}\) This gating is coupled to the movement of the peripheral voltage sensors, likely mediated through a small helix in the linker between the S4 and S5 helices (S4-S5 linker), which contacts the pore helices.\(^{25-27}\)

The voltage sensing domain of Kv channels, comprised of helices S1-S4, is sensitive to membrane voltage by virtue of several positively charged residues residing

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![Figure I2 Structure of voltage gated potassium channels](image)
on S4. These charges sense changes in voltage and transfer the potential energy from this membrane voltage into kinetic energy to move the S4 helix in or out of the plane of the membrane. S1-S3, which surround the charge-containing S4, serve to shape the energy landscape of the S4 helix and stabilize S4 charges when they are not solvent-exposed\textsuperscript{28}.

**Mechanisms of Kv channel gating**

In order to serve their complex and varied functions in biological systems, Kv channels evolved several mechanisms for specialized voltage-dependent gating. Classic work by Hodgkin and Huxley in 1952 suggested that voltage-dependent transitions of four independent “voltage sensing particles” are required for the activation of the squid giant axon potassium current, long before ion channel proteins had been identified\textsuperscript{29}. Though this remarkable prediction has been largely confirmed, especially by the discovery of 4:1 stoichiometry between voltage sensor and channel gate\textsuperscript{22}, exceptions began to emerge with the observation that some voltage dependent channels open following the movement of fewer voltage sensors\textsuperscript{30,31}. Different relationships between voltage sensor movement and channel opening can underlie subtype-specific function in Kv channels. Indeed, this coupling seems to be a target for evolutionary modifications to channel gating that allow, for example, the channels underlying the hyperpolarization activated channels (so-called “funny current” in the heart) to open in response to hyperpolarization instead of depolarization even though it contains a canonical Kv channel S4 voltage sensor, which responds “normally” to voltage\textsuperscript{32}.

The nature and extent of the movement of S4 between resting and activated states of the voltage sensor has been a matter of extensive debate over recent years\textsuperscript{28,33-38}, but recent consensus estimates for this translocation are around 10-14 angstroms, though depending on the nature of the movement, the distance moved perpendicular to the membrane may be less\textsuperscript{39}. In
addition to the activated and deactivated positions of the voltage sensor, some studies have resolved pre-activated states, implying multiple voltage sensor movements before reaching the fully activated state \(^{40}\). The number of pre-open states of voltage sensors, their voltage dependence, kinetics, and coupling with the channel gate all can affect the kinetics of channel gating in response to voltage changes. In addition, other “relaxed” states of \(K_v\) channel voltage sensors have been resolved \(^{41-43}\). The various states and transitions of voltage sensors add complexity to the voltage and time-dependent transitions of channels between various conducting and non-conducting states of the channel. These states are further explored below.

Many \(K_v\) channels undergo inactivation, which is defined as entry into a non-conducting state of the channel that is structurally and functionally distinct from a deactivated state \(^{44}\). Inactivated states can be voltage- and/or time-dependent and can involve different structures in the channel. Potassium channel inactivation can be divided into two broad mechanisms, termed N-type and P/C-type inactivation \(^{45}\) (Figure I3). N-type inactivation refers to a so-called “ball and chain” mechanism whereby an intracellular N-terminal domain of the channel moves into and blocks the pore \(^{46}\). This inactivation process can be very rapid, and therefore most channels requiring fast inactivation employ the N-type variety.

In contrast, P/C-type inactivation involves alteration to the pore of the channel that renders it non-conducting.

**Figure I3 Inactivation schemes in \(K_v\) channels.** A) N-type inactivation involves movement of an N-terminal structure into the pore, which blocks conduction. B) P/C-type inactivation involves collapse of the pore, most likely localized to the selectivity filter.
Within P/C-type inactivation, multiple mechanisms and features have been proposed. Briefly, collapse of the selectivity filter seems to be a common mechanism of P/C-type inactivation\textsuperscript{47}, though conformational changes in other locations along the permeation pathway have also been proposed\textsuperscript{48,49}. Additionally, it seems that at least some P/C-type inactivation may be controlled by mode-shift of voltage sensors\textsuperscript{50-52}. In general, P/C-type inactivation is a slower process involving more extensive conformational changes than N-type inactivation. Despite the fact that prolonged depolarization is not a feature of healthy biological membranes, many physiological roles for P/C-type inactivation have been proposed based on their sensitivity to environmental conditions such as acidic pH\textsuperscript{53}. Inactivation is an additional voltage- and time-dependent gating process that evolved to increase gating complexity to accommodate specialized physiological roles of Kv channels.

Even more gating permutations arise from multiple Kv channel pore conformations that can result in multiple conductance states of the channel. These states likely correspond to intermediate states of the channel gate between fully open and fully closed\textsuperscript{54}. These “subconductance” states are clearly seen in single channel measurements, and while in some cases these are transient states, in other cases they are stable and sensitive to factors such as pH and signaling molecules\textsuperscript{55}. Subconductance states represent an additional functional moving part for use by evolutionary adaptation in designing highly specialized ion channel gating.

Activated, deactivated, inactivated and subconductance states are the parlance of kinetic models, which seek to define the presence of and rates of movement between these states for a given ion channel. These computationally demanding models allow for precise reproduction of the biophysical characteristics of these ion channels and, importantly, provide insight into mechanisms underlying physiology, pathophysiology and pharmacology of specific ion channel
subtypes and mutants. Models provide a means to test a gating scheme against experimental data and, in turn, refine and constrain these gating schemes based on data.

The view of potassium channels as rigid structures with discrete mobile domains results in models wherein the conformational states of these domains define the state of the channel. Simple models allow for direct entrance to the open state from only one closed state. The original Hodgkin-Huxley formulation proposes four independent “gating particle” movements resulting in gate opening with the final gating particle transition. Subsequent models incorporate an additional opening step between the closed state with four activated voltage sensors and the open channel. More recent models include intermediate voltage sensor states, multiple open states, and inactivated states that can be accessed from various open and/or closed conformational states. Together, structural determination, functional characterization and kinetic modeling of channels provide a detailed framework for understanding these important and complex molecular machines.

**Molecular architecture of the I\(_{\text{Ks}}\) channel**

The I\(_{\text{Ks}}\) channel is comprised of a Kv channel alpha subunit, KCNQ1 (Kv7.1), a modulatory beta subunit, KCNE1, and a host of interacting proteins, mostly involved in coordination of the phosphorylation state of the channel. KCNQ1 channels are functional voltage gated potassium channels which rapidly activate and partially inactivate over a relatively hyperpolarized set of voltages (Figure I4A). However, the biophysical characteristics of this current are distinct from those of the assembled I\(_{\text{Ks}}\) channel. Co-assembly with KCNE1 is required in order to reproduce the functional properties of I\(_{\text{Ks}}\): very slow activation over a depolarized range of voltages, slowed deactivation and the absence of inactivation. In addition, KCNE1 co-expression increases the surface expression and single channel conductance of
KCNQ1 and sensitizes channels to phosphorylation. Given the exhaustive range of effects of KCNE1 on KCNQ1 channel function, and the physiological and pathophysiological importance of this channel complex, much study in recent years has focused on unraveling the nature of KCNQ1-KCNE1 interaction.\textsuperscript{59-61}

KCNE1 is a single-pass transmembrane protein that belongs to a family of modulatory ion channel beta subunits, many of which also associate with KCNQ1 channels.\textsuperscript{62,63} Channels formed with KCNQ1 and other KCNE family members mediate currents with very different biophysical characteristics. KCNQ1-KCNE1 channels display slow, rectified currents upon heterologous expression; KCNQ1-KCNE2 channels display a small, constitutive current; KCNQ1-KCNE3 channels display large, constitutive current, KCNQ1-KCNE4 channels do not conduct; and KCNQ1-KCNE5 channels display rectified, slowly activating currents with voltage dependence even more depolarized than that of KCNQ1-KCNE1.\textsuperscript{64,65} It is also possible for KCNQ1 channels to co-assemble with multiple KCNE subunits within the same channel, in some cases creating biophysically distinct currents.

**Figure I4 Physical and functional interaction between 1Ks proteins KCNQ1 and KCNE1.** A) Schematic representation of single-pass transmembrane protein KCNE1 (green) and 6-TM Kv channel KCNQ1. B) Functional characteristics of KCNQ1 and KCNQ1/KCNE1 channels. KCNE1 induces increased conductance, slowed kinetics, and depolarized voltage dependence on KCNQ1 channels. C) Putative location of KCNE1 in a modeled KCNQ1 homotetrameric channel. KCNE1 assembles between the S1 and S6 helices of different KCNQ1 subunits.
The flexibility of KCNQ1 gating underlies its varied function in different tissues. In the gut, KCNQ1-KCNE3 leak currents provide potassium recycling that allows for vital chloride transport in epithelial cells. In heart and inner ear, KCNQ1-KCNE1 channels are important for repolarization and potassium secretion, respectively, explaining the linkage between Long-QT and deafness in Jarvell and Lange-Neilsen patients. How KCNE proteins are able to so profoundly alter the gating of KCNQ1 when their impact on related KCNQ channels is much more modest has prompted research into unique properties of KCNQ1. One major difference between KCNQ1 and most other members of the Kv family, including other KCNQs, is the low valence of the S4 voltage sensor. While the canonical shaker Kv channel contains 7 positively charged residues in its S4 helix, KCNQ1 contains only four, and it also contains an negative charge, bringing the net valence to +3. The charge paucity of the KCNQ1 voltage sensor has indeed been shown to impact the ability of beta subunits to modulate its gating, though prior to our work, no direct measurement of voltage sensor movement in the presence and absence of beta subunits has been possible, and therefore the impact of these subunits on voltage sensor movement was unclear.

Recent evidence from our laboratory and others has placed the KCNE1 subunit within the predicted structure of the KCNQ1 channel (based on homology with Kv1.2) between a peripheral voltage sensing domain and the central pore domain (Figure I4B). From this location, it is easy to imagine how this small subunit might make functionally important contacts with the voltage-sensing domain, coupling regions between the voltage sensor and channel gate, and/or the channel gate itself. Indeed, evidence has been presented to support each of these functional interactions. However, the details surrounding the nature and functional consequences of these contact points between KCNQ1 and KCNE1 are not fully understood.
Proposed structural and functional relationships for points of interaction between KCNQ1 and KCNE1 are detailed below.

**KCNE1-KCNQ1 stoichiometry**

Given the putative location of KCNE1 in the four-fold symmetrical KCNQ1 channel, four sites for KCNE1 assembly are present in each KCNQ1 channel. However, several lines of evidence suggest that in biological contexts, fewer than four KCNE1 subunits assemble with the KCNQ1 channel. First, it has been shown using tandem KCNQ1-KCNQ1-KCNE1 channels, that a stoichiometry of 4:2 KCNQ1:KCNE1 per channel is sufficient to recapitulate the major properties of KCNE1 modulation. Indeed, another study found that a predominant percentage of KCNQ1-KCNE1 channels contain only two KCNE1 subunits. However, other lines of evidence suggest that this is not a fixed stoichiometry, and the number of KCNE1 subunits may be variable. Importantly, different KCNQ1:KCNE1 stoichiometries seem to underlie different function of channel complexes, suggesting variable stoichiometry as a regulatory mechanism in I_Ks function.

**KCNE1-KCNQ1 S6 interaction**

Initial work on the location of KCNE1 within the KCNQ1 channel proposed that KCNE1 lines the pore of KCNQ1 channels, presumably in close contact with the S6 helix. Subsequent work confirmed an interaction between transmembrane KCNE1 and KCNQ1 S6, identifying three successive residues in KCNE1 and three successive residues in KCNQ1 that were thought to comprise a functional interaction point between the two proteins. Later work has revised the location of KCNE1 to a more peripheral location adjacent to the pore domain (Figure I3C) though the interaction with S6 remains a prominent feature. To date, interaction
points between KCNQ1 S6 and KCNE1 have been identified between the extracellular, transmembrane, and intracellular ends of the respective helices, exerting putative influence on KCNQ1-KCNE1 assembly as well as functional properties of KCNQ1 gating, in particular the S6 activation gate.61,68,81,82.

**KCNE1-KCNQ1 S1 interaction**

Studies that identify cysteine-cystein crosslinking between substituted residues atop KCNE1 and KCNQ1-S1 helices uncover an unexpected interaction point between KCNE1 and KCNQ1. Since there was previously limited evidence for functional significance of the S1 helix, uncovering the importance of this interaction in affecting channel gating would potentially lend insight into its role in Kv channels in general. The discovery that two naturally occurring mutations located toward the top of the S1 helix are associated with familial atrial fibrillation gave some clues as to the significance of S1. These mutations, S140G and V141M, cause a gain-of-function phenotype by altering the deactivation kinetics of IKs, such that repeated depolarization causes accumulation of non-deactivating current.15,16,20 Interestingly, preliminary reports postulated that these effects are dependent on the presence of KCNE1, further suggesting a functionally important KCNE1-KCNQ1 S1 interaction. These studies suggest that a functionally important interaction between KCNE1 and KCNQ1-S1 may preferentially affect channel deactivation.

**KCNE1-KCNQ1 S4-S5 linker interaction**

The S4-S5 linker is the putative physical entity that mediates coupling between the voltage sensor and channel gate. In the shaker potassium channel, mutations in KCNQ1, mutations to this linker and to the S6 gate have been shown to dramatically alter the biophysics
of channel gating in a similar fashion as KCNE1 co-expression, suggesting this interaction may be targeted by KCNE1 \(^{83-85}\). In agreement with this hypothesis, biochemical crosslinking data suggests a physical interaction between the KCNQ1 S4-S5 linker and KCNE1 \(^{81}\). Taken together, these previous studies suggest that KCNE1 may target the S4-S5 Linker-S6 interaction in KCNQ1, likely altering the coupling between voltage sensor and channel gate, in order to alter channel biophysics.

**KCNE1-KCNQ1 S4 voltage sensor interaction**

Finally, several recent studies focus on the ability of KCNE1 to directly or indirectly affect the KCNQ1 S4 voltage sensor. Biochemical crosslinking studies identify a putative direct physical interaction between KCNE1 and KCNQ1 S4, though this interaction may not be as close as KCNE1-KCNQ1 S1 or KCNE1-KCNQ1 S6 \(^{68}\). In addition, other studies propose an allosteric influence of KCNE1 on KCNQ1 S4 movement through interactions with other KCNQ1 structures such as S1 and the S4-S5 linker \(^{20,81}\). Still other studies suggest that assembly of KCNE1 alters the environment of KCNQ1 S4 and intra-subunit interactions between KCNQ1 S1 and S2 helices, changing S4 movement and channel gating \(^{86,87}\).

In order to quantify the extent of functional alteration to the KCNQ1 S4 voltage sensor due to the presence of KCNE1, some groups have measured movement of the KCNQ1 S4 voltage sensor in the presence and absence of KCNE1 using state-dependent modification of an introduced cysteine residue by a cysteine-modifying MTS reagent during activation to test the effect of the beta subunit on S4 equilibration kinetics \(^{70,71}\), though this method does not give detailed information on the voltage dependence and kinetics of voltage sensor movements, and assumes voltage-sensor-to-gate coupling is not affected by KCNE1. These methods therefore may not give a full picture of KCNE1-induced changes in S4 movement.
Voltage sensor assays

Given the uniqueness of the KCNQ1 voltage sensor, the location KCNE1 and its putative interaction with KCNQ1 S4, direct measurement of KCNQ1 voltage sensor movement might provide significant insight into the molecular mechanisms underlying this interaction. The traditional and definitive voltage sensor assay involves measurement of gating current, which arises from the movement of the charged S4 in the membrane voltage field. Resolution of these transient currents provides a direct measurement of the kinetics and voltage dependence of gating charge movement. However, two important limitations complicate measurement of gating currents from KCNQ1: 1) The low valence of KCNQ1 S4 implies fewer gating charges move through the membrane voltage field, thus lowering the magnitude of gating currents and 2) The slow kinetics of KCNQ1, especially in the presence of KCNE1, implies the equilibration of its voltage sensors in response to voltage changes may also be slow, thus making the resolution of transient gating currents more difficult.

An alternative approach is to employ an optical assay of the KCNQ1 voltage sensor using a technique called voltage clamp fluorometry. This assay requires attaching a fluorescent probe to the top of S4. As this helix moves relative to the membrane during voltage sensing, the environment of the fluorophore changes, thus altering its emission. By using highly environmentally sensitive dyes, the optical signal corresponding to voltage sensor movement can be easily resolved and used to infer voltage sensor movement. This technique has been widely used in voltage gated ion channels to resolve voltage sensor movement while at the same time measuring channel currents.

This work utilizes voltage clamp fluorometry along with other biophysical and molecular biology techniques to explore voltage dependent gating of the KCNQ1 channel to establish
mechanisms by which it achieves its unique and flexible gating properties. This work seeks to establish a general gating scheme for KCNQ1 channels and to define the impact on KCNQ1 from KCNE1 co-expression by analyzing alterations to voltage sensors and their relationship with channel gating. Finally, it seeks to understand how naturally occurring mutations associated with human disease alter channel gating by exploring the physical and functional interaction between specific mutations, beta subunits, and voltage dependent gating.
Chapter 1: KCNE1 alters the voltage sensor movements necessary to open the KCNQ1 channel gate.
**Summary**

The delayed rectifier $I_{Ks}$ potassium channel, formed by co-assembly of alpha (KCNQ1) and beta (KCNE1) subunits, is essential for cardiac function. Though KCNE1 is necessary to reproduce the functional properties of the native $I_{Ks}$ channel, the mechanism(s) through which KCNE1 modulates KCNQ1 is unknown. Here we report measurements of voltage sensor movements in KCNQ1 and KCNQ1/KCNE1 channels using voltage clamp fluorometry. KCNQ1 channels exhibit indistinguishable voltage dependence of fluorescence and current signals, suggesting a one-to-one relationship between voltage sensor movement and channel opening. KCNE1 co-expression dramatically separates the voltage dependence of KCNQ1/KCNE1 current and fluorescence, suggesting an imposed requirement for movements of multiple voltage sensors prior to KCNQ1/KCNE1 channel opening. This work provides the first insight into the mechanism by which KCNE1 modulates the $I_{Ks}$ channel and presents a novel mechanism for beta subunit regulation of ion channel proteins.

**Introduction**

Members of the super family of voltage-gated cation channels open and close in response to changes in the voltage across the cell membrane in order to generate electrical currents that underlie neuronal signaling and skeletal and cardiac muscle contraction. The voltage-gated openings of these channels are controlled by intrinsic voltage sensors, including several positively charged residues in the fourth transmembrane (S4) segment of the channel, which move in response to changes in the membrane voltage. The voltage sensor movements trigger opening of the channel gate through a coupling mechanism that is not well understood, and which may vary between specific channels and channel complexes. A fundamental
understanding of how voltage sensors move and how this movement couples to channel opening is important to understand how these channels function during physiological and pathophysiological conditions and how mutation of these channels and their associated accessory subunits cause disease. One channel whose gating properties are of particular interest is the IKs potassium channel; a slowly activating channel that is essential to normal cardiac function. That the unique biophysical properties of the IKs channel are important for normal cardiac physiology is evidenced by multiple pathophysiological clinical phenotypes associated with mutations that change the biophysical and regulatory properties of the IKs channel, such as Long QT Syndrome, Short QT Syndrome, and Familial Atrial Fibrillation.

The IKs channel consists of an alpha subunit (KCNQ1, also known as Kv7.1) and an accessory beta subunit (KCNE1). KCNQ1 belongs to the canonical voltage gated potassium channel family, forming a homotetrameric channel with a central pore domain and four peripheral voltage sensing domains. KCNE1 is a small (129 amino acid) single-pass transmembrane protein. Though four KCNQ1 subunits assemble to form functional tetrameric voltage-gated channels, the biophysical and regulatory properties of the KCNQ1 channel when expressed alone are completely distinct from IKs currents: KCNQ1 homomeric channels activate and deactivate rapidly and begin to open at voltages more negative than those that activate IKs channels (Figure 1.1C). Co-assembly of KCNE1 with KCNQ1 drastically slows the activation kinetics, shifts the voltage dependence of activation, slows deactivation, and increases single channel conductance, thereby reproducing the critical properties of the native IKs channel (Figure 1.4A).

Despite numerous studies of the mechanisms by which co-assembly of KCNE1 and KCNQ1 underlie properties of the assembled IKs channel, to date, the critical question of how
this beta subunit alters channel gating has remained unanswered\textsuperscript{92,93}. Two broad possibilities seem likely: (1) KCNE1 restricts the KCNQ1 voltage sensor, delaying its movement in response to changes in membrane voltage, and/or (2) KCNE1 restricts the movement of the KCNQ1 gate or changes the coupling between movement of the voltage sensor and opening of the channel gate so that the KCNQ1/KCNE1 channel opens more slowly in response to voltage sensor movement. Recent disulfide crosslinking experiments have established that KCNE1 is likely located between the peripheral voltage sensing domain and the pore domain of KCNQ1, in a unique position to affect voltage sensor movements, coupling between the voltage sensor and pore, or both\textsuperscript{68,69}.

In order to distinguish between the above two possible mechanisms, we assayed voltage sensor movements in the KCNQ1 channel in the presence or absence of KCNE1. To date, no gating current measurements have been reported for KCNQ1 channels, likely due to the low valence and slow movement of the KCNQ1 voltage sensor relative to other voltage gated potassium channels\textsuperscript{67,94}. Previous efforts to characterize the KNCQ1 voltage sensor in the presence and absence of KCNE1 relied on cysteine accessibility studies, and while they were able to gain insights into the movements of the voltage sensor, they provide only a limited view of its behavior\textsuperscript{70,71}. As an alternative technique to study IKs voltage sensing, we chose to pursue a fluorescence assay, voltage clamp fluorometry \textsuperscript{89}, which tracks changes in emission from a fluorophore attached to the extracellular end of the voltage-sensing fourth transmembrane segment of KCNQ1 (S4) as its environment changes during voltage sensing. This method has been used previously to assay voltage sensor movements in other members of the superfamily of voltage gated cation channels\textsuperscript{42,89,95}. 
Here, we present the first measurements of KCNQ1 voltage sensor movements using voltage clamp fluorometry (VCF). Using VCF, we independently track S4 voltage sensor movement (fluorescence) and channel opening (current), in order to understand the coupling between the KCNQ1 voltage sensor and channel gate in the presence or absence of KCNE1. Based on our data, we propose that the presence of the KCNE1 subunit alters the movement of the KCNQ1 voltage sensor and changes the coupling between the voltage sensor and the KCNQ1 gate. These results not only provide the first specific insights into the molecular basis of KCNE1-dependent IKs physiology but also reveal a novel modulatory mechanism for a potassium channel β subunits in general.

**Methods**

*Molecular Biology.*

Human KCNQ1 and KCNE1 were subcloned into the pGEM-HE oocyte expression vector. Mutations were introduced using Quikchange site-directed mutagenesis kit (Qiagen) and fully sequenced to ensure incorporation of intended mutations and the absence of unwanted mutations (sequencing by Genewiz). *In vitro* transcription of cRNA was performed using mMessage mMachine T7 RNA Transcription Kit (Ambion).

*VCF recordings.*

50ng of KCNQ1 RNA with or without 25ng of KCNE1 RNA was injected in to defolliculated *Xenopus Laevis* oocytes. VCF experiments were performed 3-7 days after injection: Oocytes were labeled for 30 min with 100 μM Alexa-488 maleimide (Molecular Probes) in high K⁺ ND96 solution (98mM KCl, 1.8mM CaCl₂, 1mM MgCl₂ 5mM, HEPES, pH 7.6 with NaOH) at 4°C. Following labeling, they were kept at 6°C to prevent internalization of
labeled channels. Oocytes were placed into a recording chamber animal pole “up” in nominally Ca\(^{2+}\)-free solution (96mM NaCl, 2mM KCl, 2.8mM MgCl\(_2\), 5mM HEPES, pH 7.6 with NaOH) in the presence (KCNQ1-KCNE1 and all Cole-Moore experiments) or absence (KCNQ1 alone experiments) of 100mM LaCl\(_3\) to block currents endogenous to *Xenopus* oocytes that activate at hyperpolarized potentials \(^96\). 100mM LaCl\(_3\) does not affect current or fluorescence signals in KCNQ1 alone (Data not shown).

Electrical measurements were carried out in the two electrode voltage clamp configuration using an OC-725C Oocyte Clamp (Warner Instruments). Microelectrodes were pulled to resistances from 0.5-2 MW and filled with 3 M KCl. Voltage clamp data were digitized at 5 kHz (Axon Digidata 1440A), collected using pClamp 10 (Axon Instruments, Inc.). Fluorescence recordings were performed using an Olympus BX51WI upright microscope. Light was focused on the top of the oocyte through a 20x water immersion objective after being passed through an Oregon green filter cube (41026 - Chroma). Fluorescence signals were focused on a photodiode and amplified with an Axopatch 200A patch clamp amplifier (Axon Instruments). Fluorescence signals were low-pass Bessell filtered (Frequency Devices) at 100-200 Hz, digitized at 1 kHz and recorded using pClamp 10. See Figure 1.1A for a schematic representation of the VCF technique.

**Kinetic Modeling**

Markov models are formulated to represent the gating of KCNQ1 and KCNQ1-KCNE1 channels, based on several assumptions. Channels are assumed to be homotetrameric, and as such, symmetry of four subunits is reflected in the structure; KCNE1 association affects all subunits equally. Each subunit of the tetramer is assumed to undergo two conformational
changes: first a voltage sensor movement, followed by an opening step. An activation process with two sequential steps was chosen since it is the simplest mechanism by which fluorescence can precede conductance while separating their voltage dependent behavior, and thus represents a minimal model for channel behavior.

The resulting model structures are presented schematically in Figure 1.8. Rightward transitions depicted in the schematic represent sequential activation of the voltage sensors in additional subunits, and downward transitions represent subunit opening. Scaling of forward and reverse rate constants reflects the assumption that these processes are identical and independent in each subunit. The two models considered differ in that for Model 1, all four subunits must undergo both movements for the channel to conduct current, and in Model 2, each subunit’s two-step activation produces $\frac{1}{4}$ of the total conductance, thus generating subconductance states (shaded in the schematic). Since the subconductance levels are assumed to be equally spaced, Model 2 can alternatively be represented as a 3-state model, where each state represents the kinetically identical behavior of four subunits in aggregate.

Conductance is defined in Model 1 as the open state probability; in Model 2 by the weighted occupancy of the subconductance states (0.25 for states 6 through 9, 0.5 for states 10 through 12, 0.75 for states 13 and 14, and 1.0 for state 15). For both models, fluorescence signal is assumed to be proportional to movement of voltage sensors, and thus is defined as the sum of the intermediate states, weighted corresponding to the number of subunits activated according to the equation below (where $S_i$ corresponds to the i’th state in the schematic):

$$F(t) = 1 \cdot S_2 + 2 \cdot (S_3 + S_6) + 3 \cdot (S_4 + S_7) + 4 \cdot (S_5 + S_8 + S_{10}) + 5 \cdot (S_9 + S_{11})$$
$$+ 6 \cdot (S_{12} + S_{13}) + 7 \cdot S_{14} + 8 \cdot S_{15}$$
Rate constants are formulated as exponential functions of voltage according to Eyring rate theory:

\[
k = k_{1/2} \cdot \exp \left( \frac{z(V - V_{1/2})F}{RT} \right),
\]

where \( V \) is transmembrane potential (mV), \( V_{1/2} \) is the equilibrium potential for each forward-reverse transition pair (mV), \( F \) is Faraday’s constant (96485 C/mol), \( R \) is the universal gas constant (8.314 J/(mol*K)), \( T \) is temperature (298 K), and \( z \) and \( k_{1/2} \) (msec\(^{-1}\)) are parameters for each rate constant.

The resulting models consist of eight free parameters. These parameters were selected as a best fit to experimental data, initially using a simulated annealing algorithm, which is felt to be more apt to identify a global best solution, and a Nelder-Mead simplex search algorithm for further refinement. Optimal parameters for KCNQ1 with Model 2 and KCNQ1-KCNE1 with Model 1 are listed in the Appendix Table S1.
Figure 1.1: Voltage dependent fluorescence signals from KCNQ1 C214A/C331A G219C suggest 1:1 coupling between voltage sensor movement and channel activation. (A) Schematic of the VCF technique. (B) Topology of the KCNQ1 and KCNE1 proteins in the cell membrane. Residues in the S3-S4 linker that were sequentially mutated to cys are shown, with the residue G219 highlighted in green. (C-D) Representative current (C) and fluorescence (D) from Alexa488-labeled KCNQ1 C214A/C331A G219C (psKCNQ1). Cells are held at -80mV and stepped to potentials between -120mV and +60mV for 2 seconds followed by a step to -40mV (current) or -80 mV (fluorescence). (E) Representative experiments showing activation kinetics of current and fluorescence signals in psKCNQ1 at different voltages. (F) Averaged steady-state activation for channel opening G(V) (n=7, black squares) and fluorescence F(V) (n=8, red circles). Error bars represent SEM.
Results

In order to avoid fluorophore-labeling of endogenous cysteines on the IKs channel, we first mutated two endogenous cysteine residues that are predicted to be accessible to the extracellular milieu, C214 and C331, to alanine as in Chung et al.\(^68\). These two mutations have limited functional consequences on the channel expressed either alone or with KCNE1 and thus we used this KCNQ1 construct (with C214A; C331A) as a background for subsequent experiments. We next mutated residues predicted to be in the S3-S4 linker, one at a time, to cysteine, functionally characterized the mutants, and tested whether they could be labeled with Alexa488 maleimide, which attaches covalently to externally accessible cysteine moieties, to yield a voltage-dependent fluorescence signal.

One mutant, KCNQ1 C214A/C331A G219C (from here called pseudo-WT, psKCNQ1) displays a consistent voltage-dependent fluorescence F(V) signal (maximum fluorescence change, $\Delta F/F \sim 5\%$) that saturates at negative and positive voltages (Figure 1.1), as if it reports on a voltage-activated conformational change of S4\(^89,95\). Importantly, both the G219C mutation and labeling with Alexa488 maleimide are well tolerated by the channel: psKCNQ1 channels activate normally and labeled channels activate with a voltage dependence that is only slightly left-shifted (Figure 1.2). Oocytes expressing KCNQ1 C214A/C331A channels do not display a voltage dependent fluorescence signal following labeling (Figure 1.2).
Comparing the activation time course of current and fluorescence for psKCNQ1 in

**Figure 1.2: Control data for psKCNQ1.** A) No fluorescence signal is detected from labeled KCNQ1 (C214/331A) channels expressing large currents (~5mA during +20mV depolarization). B) Labeling of psKCNQ1 and psKCNQ1/KCNE1 induces a modest left-shift in voltage dependent activation. Error bars represent SEM. C) Time course of normalized current (black trace) and normalized fluorescence raised to different powers (grey traces) show that the time course of F precedes the time course of current while F², F³ and F⁴ trail current time course. D) Normalized conductance and fluorescence from psKCNQ1. F(V) raised to the 2nd, 3rd, and 4th powers all lie to the right of the G(V).
response to a series of voltage steps between -40 and +20 mV, the fluorescence time course narrowly precedes the current time course at all voltages. The psKCNQ1 fluorescence signal has an almost identical voltage dependence to that of channel activation, as measured from the voltage-dependent conductance G(V) based on tail currents at -40 mV. This is a surprising result.
since other voltage-gated potassium channels require independent movements of all four voltage sensors prior to opening of the channel gate\textsuperscript{100,101}. Indeed, the fluorescence raised to the 2nd, 3rd and 4th power, which would mirror channel opening if the movement of 2, 3 or 4 independent voltage sensors, respectively, were needed for channel opening \textsuperscript{100}, all trail current time course and lie to the right of the G(V) (Figure 1.2).
Figure 1.4: The F351A mutant fluorescence signal suggests movements of multiple independent voltage sensors precede channel opening. (A-B) Representative current (A) and fluorescence (B) traces from psKCNQ1 F351A. For current measurements, cells are held at -80mV and stepped to potentials between -80 mV and +60 mV for 2 seconds followed by a common tail current to -40mV. Fluorescence voltage protocol is as in figure 1. (C) Representative fluorescence (red) and current (black) time courses in response to shown protocol. Fluorescence raised to the fourth power is shown in green. (D) Steady-state activation curves for current (n=5, black squares) and fluorescence (n=4, red circles). WT current (broken black line) and fluorescence (broken red line) are shown for comparison.

In order to confirm that the voltage dependent fluorescence changes we observe correspond to movement of the voltage sensor, we introduced mutations in S4 that we predict would alter its voltage sensitivity. The S4 charge neutralization mutant R243A was previously shown to shift to more depolarized potentials and decrease the slope of the G(V) curve for
KCNQ1. We tested the hypothesis that the fluorescence signal, if it reports the movement of the voltage sensor, would change in a similar manner for this mutation. In the psKCNQ1 background (psKCNQ1), R243A decreases the slope of ($\Delta dx = +8.9 \text{ mV}$) and right-shifts ($\Delta V_{1/2} = +35 \text{ mV}$) the G(V) curve, similar to its effect in the WT background (Figure 1.3). Fluorescence signals from Alexa488 maleimide-labeled psKCNQ1 G219C/R243A also display right-shifted and less steep voltage dependence, mirroring the changes in the G(V). This result is consistent with the fluorescence signal acting as a valid reporter of voltage sensor movement assuming that the R243A mutation affects movement of the S4.

However, from these results we cannot rule out that the fluorescence signal simply reports on a channel conformational change directly associated with channel opening (gating), since the voltage dependence of fluorescence and channel opening are similar for both psKCNQ1 and psKCNQ1 R243A. To rule out this possibility, we mutated a residue in the pore forming S6 helix, F351, which lies near the S4-S5 linker and is thought to participate in coupling the voltage sensor and channel gate in KCNQ1 channels. Previous studies have shown that F351A channels display an extremely right-shifted G(V) and a sigmoidal time course of current activation, similar to KCNQ1/KCNE1 channels. We reason that because this mutation lies far from the voltage sensor it could minimally affect voltage sensor movement while largely affecting channel opening. Therefore, if our fluorescence assay is a reporter of voltage sensor movement, this mutation might separate the voltage-dependent fluorescence F(V) from voltage-dependent channel opening G(V).

Figure 1.4 shows that the mutation F351A is well-expressed in our psKCNQ1 background and displays a sigmoidal current time course and a right-shifted G(V) as previously reported. Fluorescence signals are indeed separated from channel opening in this mutant: the
Figure 1.5: Fluorescence signals from psKCNQ1/KCNE1 suggest complex effects of KCNE1 on the Q1 voltage sensor and gating mechanism. (A-B) Representative current (A) and fluorescence (B) traces from psKCNQ1 co-expressed with KCNE1. For current measurements, cells are held at -80mV and stepped to potentials between -80 mV and +60 mV for 5 seconds followed by a common tail current to -40mV for 5 seconds. For fluorescence protocols, cells are held at -80 mV and stepped to voltages between -180 mV and +80 mV for 5 seconds and then returned to -80mV. (C) Kinetics of current (black) and fluorescence (red) in response to indicated depolarizations for 5 seconds (average of several cells, n=6-8). The fluorescence signal raised to the fourth power is shown in green. (D) Isochronal activation for channel opening (n = 9, black squares) and fluorescence (n = 5, red circles). F(V) raised to the fourth power is shown

Voltage dependence of the fluorescence is clearly left-shifted compared to the G(V) and the time course of the fluorescence signal markedly precedes channel opening (Figure 1.4C-D). This shows that the fluorescence reported by a fluorophore attached to G219C does not directly report voltage-gated channel opening, but, instead, a voltage-gated conformational change preceding channel opening. Taken together with the results from the R243A mutation, this result provides
confidence that the fluorescence signal from the psKCNQ1 G219C channels is due to the movement of the voltage sensor. Additionally, we find that the F(V) and fluorescence time course raised to the 4th power both mirrors the G(V) and time course of channel opening, respectively, as if in the F351A mutant the movement of four independent voltage sensors are required for channel opening (Figure 1.4D).

Next we explore the movement of the KCNQ1 voltage sensor in the presence of the beta subunit KCNE1. We find robust co-expression of psKCNQ1 and KCNE1 (psKCNQ1/KCNE1) characterized by currents very similar to those from WT KCNQ1/KCNE1 channels (Figure 1.5A). Fluorophore-labeled G219C psKCNQ1/KCNE1 channels generate a voltage-dependent fluorescence signal that is very different from the fluorescence from psKCNQ1 expressed alone (Figure 1.1C and 1.5B). Surprisingly, the main component of the F(V) for the psKCNQ1/KCNE1 fluorescence is drastically left-shifted relative to the F(V) for psKCNQ1 expressed alone, despite a 40 mV right shift in the voltage dependence of channel opening (G(V)) compared to psKCNQ1 expressed alone (Figure 1.5). Additionally, there is a small component of the fluorescence signal that activates at depolarized potentials, seemingly directly tracking channel opening, though we are unable to see saturation of either this small fluorescence signal or current within our working voltage range. We hypothesize that this small fluorescence signal corresponds to a late conformational change of S4 directly associated with channel opening, as has been previously seen in the ILT mutant of Shaker K channel. The marked separation between fluorescence and current suggests that like the F351A mutation, co-expression of psKCNQ1 with KCNE1 causes voltage sensors to respond to voltage well before channels open, as if independent movement of multiple voltage sensors is required before the channel conducts current. The F(V) and the time course of the fluorescence signal raised to the
4th power more closely resemble the $G(V)$ and the time course of the currents for psKCNQ1/KCNE1 channels, but do not completely overlap with the $G(V)$ and the time course of the current.
The fluorescence signals from psKCNQ1 and psKCNQ1/KCNE1 predict that voltage sensor movement occurs at very hyperpolarized potentials only in the presence of KCNE1. In
Figure 1.6: Cole-Moore shifts seen in psKCNQ1/KCNE1 but not psKCNQ1 alone. (A-B) Fluorescence (red) and current (black) from psKCNQ1 (A) and psKCNQ1/KCNE1 (B) in response to a hyperpolarizing prepulse protocol: cells are held at -80 mV and pulsed to voltages between -160 and -100 mV for 2 seconds (A) or 5 seconds (B) followed by a pulse to +20 mV for 2 seconds (A) or 5 seconds (B). (C) Cole-Moore shifts are quantified as the time to reach half the current level at the end of the depolarizing pulse following the -160 mV prepulse. Data are presented as mean of n = 5 (psKCNQ1) or n = 7 (psKCNQ1/KCNE1) experiments +/- SEM. Very significant (p<0.002) Cole-Moore shifts are seen between each 20 mV increment in prepulse potential between -160 and -60 mV for psKCNQ1/KCNE1 (paired Student’s T-test).

In order to test this prediction we tested the effect of conditioning prepulse potentials on the time course of channel opening in psKCNQ1 and psKCNQ1/KCNE1. This method was first
employed by Cole and Moore to study potassium channels in Squid Giant Axons \(^{102}\). They detected kinetic changes that were dependent on prepulse potential, indicating voltage-dependent transitions between closed states at these voltages. Cole-Moore shifts have previously been observed in KCNQ1-KCNE1 channels\(^{103}\) but there are no reports of hyperpolarizing prepulse experiments in KCNQ1 alone.

Figure 1.6 shows that prepulses to hyperpolarized potentials affect the kinetics of channel opening differently in labeled psKCNQ1 than in psKCNQ1/KCNE1 channels as predicted. For homomeric psKCNQ1 channels, there is no fluorescence change over the range of hyperpolarizing potentials tested, and the time course of the fluorescence change upon depolarization to +20 mV is the same regardless of prepulse potential. In addition, the time course of channel opening upon depolarization is not affected by hyperpolarizing prepulse potentials.

In contrast, psKCNQ1/KCNE1 channels display a marked prepulse-dependent time delay in the kinetics of channel opening in response to a broad range of prepulse potentials, as far negative as -160 mV (Figure 1.6B). In order to quantify this effect, we measure t1/2 of current following each prepulse where t1/2 is defined as the time to reach half the current level at the end of the 2 second depolarization following a prepulse to -160 mV (Figure 1.6C). This quantification shows a very significant (p < 0.002) change in activation kinetics between each 20 mV increment in prepulse potential between -160 and -60 mV. It is notable that the range of voltages that cause Cole-Moore effects in the psKCNQ1/KCNE1 channel is similar to that in which we detect fluorescence changes, F(V), for the psKCNQ1/KCNE1, suggesting that they measure the same voltage sensor movement. The quantification from prepulse experiments in
psKCNQ1 alone show no change in activation kinetics following different prepulses, confirming that voltage sensor movements do not occur at these negative voltages.

These results support the notion that the fluorescence signal at very negative voltages in psKCNQ1/KCNE1 reports on conformational changes that are coupled to channel activation. These data also support our conclusion that several voltage sensors need to move before the IKs channel can open, but that multiple separate voltage sensor movements are not needed to activate KCNQ1 alone. Finally, the Cole-Moore data and the fluorescence data strongly argue that KCNE1-dependent voltage sensor movements that take place across a range of very negative voltages profoundly affect activation of the channel, and that these voltage sensor movements along with the requirement that they precede channel opening defines the major mechanism by which KCNE1 slows activation of IKs.
Figure 1.7: Simulated behavior of KCNQ1 and KCNQ1-KCNE1 channels. (A-B) Activation time course for fluorescence (red) and current (black) for step pulse from -80 mV (KCNQ1) or -120 mV (KCNQ1/KCNE1). (C-D) Isochronal curves for fluorescence and current following a 2 s (KCNQ1) or 5 s (KCNQ1/KCNE1) pulse. (E-H) Activation of KCNQ1 and KCNQ1/KCNE1 models at +40 mV following hyperpolarizing prepulses as in Figure 1.6 show a pronounced Cole-Moore effect only in the presence of KCNE1. See Table 1.1 and Fig. 1.3 for model best fit parameters and schematic representation of the KCNQ1 and KCNQ1/KCNE1 model structures.


Discussion

Here for the first time, using VCF, we report that the presence of the KCNE1 β subunit in the IKs channel changes voltage sensor movements necessary to open the KCNQ1 α subunit channel gate to coordinate the mandatory physiological properties of the assembled IKs channels. Fluorescence measurements suggest that for KCNQ1 there is a one-to-one relationship between voltage sensor movement and channel opening since both the time course and voltage dependence of the fluorescence signal are similar to the time course and voltage dependence of the current, respectively. KCNQ1 mutagenesis supports the view that the fluorescence signal we measure reports S4 voltage sensor movement: S4 charge neutralization mutant R243A decreases the voltage dependence of the fluorescence signal and the S6 coupling mutant F351A separates F(V) and fluorescence time course from G(V) and current time course. Fluorescence data from the F351A mutant suggest that this mutation changes the voltage sensor-to-pore coupling such that movement of four independent voltage sensors now precedes channel opening. Co-expression of KCNE1 also separates the G(V) and F(V) and the time course of current and fluorescence signals, suggesting that KCNE1 also induces a requirement for movement of multiple voltage sensors prior to channel opening. Using a Cole-Moore protocol we find independent evidence for gating motions at extreme negative voltages in psKCNQ1/KCNE1, consistent with our conclusions from the fluorescence data. In psKCNQ1 expressed alone, the absence of a Cole-Moore shift in response to different hyperpolarizing prepulses is also consistent with fluorescence data, suggesting that there are no voltage sensitive gating movements at these hyperpolarized potentials in KCNQ1 expressed alone.
Figure 1.8: Model structures for KCNQ1 and KCNQ1/KCNE1 channels. A) Model 1, used to simulated KCNQ1-KCNE1 channels. Starting at the resting state 1, channels can undergo a voltage-sensitive sensor movement, represented by the rightward transitions. B) Model 2 is identical to model 1, with one change: it assumes that once each voltage sensor has activated, that subunit can then open and conduct current in a subconductance state (states outlined in gray and labeled S1).

The stark differences between fluorescence signals elicited from psKCNQ1/KCNE1 and those seen in the absence of the β subunit provide evidence that the voltage sensor is fundamentally changed by co-assembly of KCNQ1 and KCNE1. In the presence of KCNE1, fluorescence changes are seen over a very broad range of voltages and the time course of the voltage sensor equilibration in response to changes in voltage is markedly slower than in the absence of KCNE1. Additionally, we find fundamental differences in gating mechanisms between psKCNQ1 and psKCNQ1/KCNE1 channels. Our fluorescence data suggest that in psKCNQ1 there is a one-to-one relationship between voltage sensor S4 movement and channel opening. The empirical method of raising fluorescence signals to higher powers suggests that both F351A mutation and co-expression of KCNE1 alter this coupling as if there is a requirement for four independent voltage sensor movements prior to channel opening in both psKCNQ1 F351A and psKCNQ1/KCNE1.
In order to refine our analysis of gating in KCNQ1 and KCNQ1-KCNE1 channels, we considered a Markov kinetic model which simplifies each subunit’s opening into two distinct processes: voltage sensor movement, followed by subunit opening (for full model methods, see SI). As demonstrated in Figure 1.7, KCNQ1 channels are well-characterized by this simple scheme, with fluorescence preceding current, and similar steady-state voltage-dependence of fluorescence and current (Figure 1.7A, C and Figure 1.8). For KCNQ1-KCNE1 channels, a different model scheme is required to adequately separate F(V) and G(V) curves (Figure 1.7B, D and Figure 1.8). The scheme we chose requires sequential activation of the four voltage sensors prior to channel opening. This model accounts for a delay in current activation and sigmoidal current time course, and a wide separation in the fluorescence-voltage F(V) and conductance-voltage G(V) curves. A significant Cole-Moore effect is seen only in the presence of KCNE1 (Figure 1.7E-H), with a clear mechanism for the effect: at the end of the prepulse, some voltage sensors have activated, but the channel has not opened, allowing for more rapid activation during the subsequent test pulse. The modeling results shed light on the relation between voltage sensor movement and channel opening for KCNQ1 and KCNQ1-KCNE1. For KCNQ1-KCNE1 channels, a model that requires independent activation of multiple voltage sensors is needed to fit fluorescence and current data. In contrast, KCNQ1 data are well fit by a structurally distinct model where a single voltage sensor movement leads directly to channel opening.

We therefore propose that two fundamentally different gating schemes underlie activation in KCNQ1 and KCNQ1/KCNE1 channels. For KCNQ1 alone, a single voltage sensor movement places the channel into a state where it can transition to an open state, while for KCNQ1/KCNE1 channels, transitions of multiple independent voltage sensors move the channel between closed states along the activation pathway, towards a state where four activated voltage sensors allow
for transition to the open state. The gating scheme for KCNQ1 alone allows two physical interpretations: either all four KCNQ1 voltage sensors move in concert to allow the transition to the fully open state, or the four KCNQ1 voltage sensors move independently but the KCNQ1 channel transitions to the fully open state through a sequence of three stable subconductance states (with 1/4th, 2/4th, and 3/4th of the conductance of the fully open state) where the movement of each voltage sensor allows a transition into the next subconductance state. Based on noise analysis of KCNQ1 and KCNQ1/KCNE1, single channel conductance was reported to be approximately four times larger for KCNQ1/KCNE1 channels than for KCNQ1 channels expressed alone\textsuperscript{104,105}. Since the single channel conductance estimated with noise analysis is dominated by the conductance change induced by the most frequent and largest conductance transition, one explanation of the difference in single channel conductance could be that KCNQ1 mainly opens through subconductance states while KCNQ1/KCNE1 rarely opens through subconductance states.

Based on our data, we cannot distinguish between two possibilities: (1) all four KCNQ1 voltage sensors move in concert to allow the transition to the fully open state or (2) four KCNQ1 voltage sensors move independently but the KCNQ1 channel transitions to the fully open state through a sequence of subconductance states. Either represents a novel gating scheme in a Kv channel and this work will therefore serve as a basis for future studies into the detailed mechanisms of KCNQ1 gating. Our experimental results suggest that the gating of KCNQ1 is distinct from that of the related KCNQ4 channel, in which charge movement is much faster than channel opening\textsuperscript{94}.

Future studies will also refine our understanding of the KCNQ1/KCNE1 gating scheme and probe the detailed nature of the KCNE1-mediated remodeling of the KCNQ1 voltage sensor.
Since raising the fluorescence signal to the fourth power is not able to fully account for the separation between fluorescence signal and current, there might be additional effects of KCNE1 on the channel gate that inhibits its opening even after all voltage sensor movements have taken place. Also, the nature of the small fluorescence component seen in psKCNQ1/KCNE1 at depolarized potentials remains an open question.

What is clear from this work is that voltage sensor movements at hyperpolarized potentials have a profound impact on gating kinetics of KCNQ1/KCNE1 channels and that these motions are dependent on the presence of KCNE1. Importantly, the range of pre-pulse voltages over which Cole-Moore shifts are produced in KCNQ1/KCNE1 extends to similar voltages over which we observe S4 fluorescence changes, providing independent evidence of voltage dependent processes at very negative potentials and demonstrating these motions are coupled to channel gating.

In summary, this chapter establishes a novel mechanism for β-subunit modulation of ion channel proteins: auxiliary potassium channel β-subunits are able to both significantly alter the movement of voltage sensors and the manner in which that movement is coupled to channel gating. Additionally, the success of this methodology to assay modulation of KCNQ1 voltage sensor movement lays the groundwork for investigations both into the fundamental mechanisms underlying KCNQ1-KCNE1 phosphorylation-dependent changes in channel gating in response to stimulation by the sympathetic nervous system and into the molecular mechanisms of heritable arrhythmia mutations in KCNQ1 and its accessory proteins.
Chapter 2: An allosteric gating scheme underlies the flexible gating of KCNQ1 channels
Summary

KCNQ1 (Kv7.1) is a unique member of the superfamily of voltage gated K+ channels in that it displays a remarkable range of gating behaviors tuned by co-assembly with different β subunits of the KCNE family of proteins. Homomeric KCNQ1 channels activate quickly over a negative range of voltages; KCNQ1/KCNE1 channels activate very slowly over a depolarized range of voltages; and KCNQ1/KCNE3 channels are constitutively open. To better understand the basis for the biophysical diversity of co-assembled channels, we here investigate the basis of KCNQ1 gating in the absence of β subunits using voltage clamp fluorometry (VCF). Based on our previous work shown in Chapter 1, the kinetics and voltage dependence of voltage sensor movements are very similar to those of the channel gate, suggesting a one-to-one relationship. Here, we have tested two different hypotheses to explain KCNQ1 gating: 1) KCNQ1 voltage sensors undergo a single concerted movement that leads to channel opening, or 2) independent voltage sensor movements lead to channel opening before all voltage sensors have moved. Here, we find that KCNQ1 voltage sensors move independently, but that the channel can conduct before all voltage sensors move. In some mutant KCNQ1 channels, transition to the open state occurs even in the absence of voltage sensor movement. In these mutants, voltage sensors display more depolarized voltage dependence than the channel gate, implying that voltage sensors move after the channel has opened. To interpret these results, we propose an allosteric gating scheme wherein KCNQ1 is able to transition to the open state after 0-4 voltage sensor movements. This model allows for widely varying gating behavior depending on the relative strength of the opening transition, which physiologically is controlled by co-assembly with different KCNE family members.
Introduction

KCNQ1 (Kv7.1) is a member of the super-family of voltage-gated potassium channels (Kv), which contain 6 transmembrane helices and form functional tetramers with four peripheral voltage sensing domains surrounding a single potassium-selective pore domain. Much study spanning recent decades has focused on the detailed gating mechanisms of these molecular machines, establishing general principles of Kv channel gating as well as unique structural and functional properties that underlie the diverse physiological functions of different family members. Within the Kv family, KCNQ1 displays a unique flexibility in its gating properties depending on the tissue where it is expressed and the corresponding beta subunit with which it co-assembles: in the intestine KCNQ1/KCNE3 channels display voltage-independent current that supports chloride secretion, while in the heart KCNQ1/KCNE1 channels display slowly-activating voltage-dependent current that is critical to cardiac action potential repolarization. Remarkably, neither of these physiologically essential phenotypes resembles that of the KCNQ1 channel expressed alone, which activates rapidly over a hyperpolarized range of voltages. Still other KCNE proteins co-assemble with KCNQ1 to form heteromeric channels with distinct biophysical characteristics. This diverse array of gating schema allows this protein to play unique important roles in a vast number of systems in the body including the heart, brain, inner ear, kidney, lungs, and intestine.

In order to understand what underlies the flexibility of KCNQ1 gating, we sought to characterize the gating mechanisms of this channel, in particular the coupling between the peripheral voltage sensors and the channel gate. Classic work by Hodgkin and Huxley suggests that voltage-dependent transitions of four independent “voltage sensing particles” are required for the activation of the squid giant axon potassium current. This prediction has been largely
justified by subsequent research in K\textsubscript{v} channels, especially the discovery that there are four structurally independent voltage sensor domains for each channel pore\textsuperscript{22,25}. However, in some voltage sensitive potassium channels, movement of all four voltage sensors is not required in order for the channel to open. In the calcium- and voltage-gated BK channel, voltage-dependent gating follows an allosteric gating scheme (studied in the absence of Ca\textsuperscript{2+}) wherein voltage sensor movement acts as an allosteric promoter of gate opening\textsuperscript{30,31}.

In the previous chapter, we show using voltage clamp fluorometry that in the KCNQ1 potassium channel, the kinetics and steady-state voltage dependence of voltage sensor transitions appear to closely resemble channel currents, suggesting a 1:1 relationship between voltage sensor movement and channel opening. In addition, there is no Cole-Moore shift in KCNQ1 channels, in contrast to what would be expected from a Hodgkin-Huxley-type channel. In an attempt to explain this relationship in light of the predicted structure of the channel, we proposed two hypotheses: 1) All four voltage sensors in KCNQ1 move in concert, so that there is effectively one common voltage sensor transition that leads to channel opening, or 2) the voltage sensors move independently but that the KCNQ1 channel gate can open before all voltage sensors have moved, perhaps occupying stable subconductance states on the way to a fully open state.

Here, we show that KCNQ1 voltage sensors move independently, and that channels can indeed open before all voltage sensors have moved. Remarkably, we find that in some KCNQ1 mutant channels, transition to an open state can occur even in the absence of voltage sensor movement. In these mutants, we see that voltage sensors move with a more depolarized voltage dependence than the channel gate, implying that some voltage sensor movements can take place after the channel is open. These results lead us to adopt an allosteric gating scheme wherein
KCNQ1 is able to transition to the open state after 0-4 voltage sensor movements, with each successive voltage sensor movement strengthening the opening transition. This gating scheme allows for the flexible gating behaviors seen in channels formed with KCNQ1, controlled in physiological contexts by co-assembly with different accessory KCNE proteins.
Methods

Molecular Biology.

Human KCNQ1 and KCNE1 were subcloned into the pGEM-HE oocyte expression vector. Mutations were introduced using Quikchange site-directed mutagenesis kit (Qiagen) and fully sequenced to ensure incorporation of intended mutations and the absence of unwanted mutations (sequencing by Genewiz). *In vitro* transcription of cRNA was performed using mMessage mMachine T7 RNA Transcription Kit (Ambion).

VCF recordings.

50ng of KCNQ1 RNA was injected into defolliculated *Xenopus Laevis* oocytes. VCF experiments were performed 2-7 days after injection: Oocytes were labeled for 30 min with 100 µM Alexa-488 maleimide (Molecular Probes) in high K\(^+\) ND96 solution (98mM KCl, 1.8mM CaCl\(_2\), 1mM MgCl\(_2\) 5mM, HEPES, pH 7.6 with NaOH) at 4°C. Following labeling, they were kept on ice to prevent internalization of labeled channels. Oocytes were placed into a recording chamber animal pole “up” in nominally Ca\(^{2+}\)-free solution (96mM NaCl, 2mM KCl, 2.8mM MgCl\(_2\), 5mM HEPES, pH 7.6 with NaOH) or in high K\(^+\) ND96 solution. In experiments requiring hyperpolarized voltages, 100mM LaCl\(_3\) is used to block endogenous hyperpolarization activated currents. In chromanol-subtraction experiments, 100mM Chromanol 293B was introduced into the bath by pipetting.

VCF experiments were carried as previously reported (See Chapter 1).

Data Analysis

Steady state voltage dependence of current was calculated from exponential fits of tail currents following different test potentials. The fit of the tails were extrapolated to the beginning
of the tail pulse in order to avoid contamination by the “hook”, which results from inactivation that is removed within hundreds of milliseconds at the -40mV tail potential. Each $G(V)$ experiment was fit with a Boltzmann equation:

$$G(V) = A2 + (A1-A2)/(1 + \exp((V-V_{1/2})/K))$$

where $A1$ and $A2$ are the minimum and maximum, respectively, $V_{1/2}$ the voltage at which there is half-maximal activation and $K$ is the slope. Data were normalized between the $A1$ and $A2$ values of the fit. Fluorescence signals were bleach-subtracted and data points were averaged over tens of milliseconds at the end of the test pulse to reduce errors from signal noise. This data is fit with a Boltzmann and normalized between $A1$ and $A2$ parameters for each experiment.

For experiments in high K+ solution, tail currents are measured at -120 mV following 2 second test pulses to voltages between -140 mV and 60 mV. Experiments are performed before and after application of 100mM Chromanol 293B. $G(V)$ curves are measured by fitting the tail currents with single exponential functions and extrapolated to the beginning of the tail pulse, again to eliminate contamination from inactivation, which is rapidly relieved at -120 mV. $G(V)$ experiments are fit with a Boltzmann and normalized only to the $A2$ parameter and averaged. Constitutive current is calculated as $A2/A1$ for each experiment.

**Results**

In the previous chapter, we monitored movement of the KCNQ1 voltage sensor using a pseudo-WT KCNQ1 construct containing two cysteine neutralization mutations and a cysteine introduction at the top of the S4 helix at position 219 which we label with Alexa488 maleimide (KCNQ1 C214A/C331A/G219C). In this chapter, we will refer to KCNQ1 C214A/C331A
simply as KCNQ1 and denote the presence of G219C with attached fluorophore with a subscript ‘L’. From Chapter 1, we know that currents from KCNQ1\textsubscript{L} are similar to the WT KCNQ1 currents with two exceptions: the half-point of voltage dependent activation shifts by about -10mV and channels display relatively less inactivation. Fluorescence signals from this channel exhibit similar kinetics and steady-state voltage dependence as do channel currents, suggesting a one-to-one relationship between voltage sensor movement and channel opening. Based on this result, we initially hypothesized that all four KCNQ1 voltage sensors might move in concert to open the channel. If these voltage sensors move together, we reason that their movement must be cooperative.

In order to test for cooperativity between KCNQ1 voltage sensors, we design experiments wherein we look at channels containing heterogenous voltage sensors to determine their effect on each other and the gating of the channel. In order to identify mutations that alter voltage sensor movements, we turned to a recent study by Wu et al. that finds currents from two voltage sensor charge mutations, R228Q and R243Q, display very hyperpolarized and very depolarized voltage dependence, respectively, compared to the WT KCNQ1 channel\textsuperscript{86}. In agreement with previous findings, two-electrode voltage clamp recordings of KCNQ1 R228Q\textsubscript{L} and KCNQ1 R243Q\textsubscript{L} show dramatic hyperpolarizing and depolarizing shifts, respectively, in voltage dependence (Figure 2.1A). Using VCF, we find fluorescence signals are similarly shifted, as if the voltage sensor movement, but not the relationship between voltage sensors and channel gate, is altered by these mutations (Figure 2.1A).
In order to test for cooperativity between KCNQ1 voltage sensors, we next co-express KCNQ1 R228Q_L and KCNQ1 R243Q in order to assay currents from co-assembled channels, but only record fluorescence signals from R228Q-containing subunits. We reason that if voltage sensors are cooperative, than co-assembly of R243Q-containing subunits will shift the voltage
dependent movement of R228Q-containing voltage sensors. As shown in Figure 2.1A, currents from co-expression of R228QL and R243Q are significantly right-shifted relative to R228QL currents, but the fluorescence signal from R228QL is unchanged. Assuming equal expression and stochastic assembly of expressed subunits, the binomial distribution predicts that 87.5% of channels will assemble with mixed R228QL and R243Q subunits. Therefore, the presence of such a significant shift in currents without any change in R228QL fluorescence strongly suggests a lack of cooperativity between voltage sensors.

We next co-express KCNQ1 R228Q and KCNQ1 R243QL and record current and fluorescence. In this experiment, we again find that the voltage dependence of current is distinct from that of fluorescence, which is unchanged from R243QL monomer. This shows that while currents from co-assembled R228Q and R243QL channels are significantly shifted, fluorescence signals from R243Q are not affected by the presence of R228Q, again strongly supporting independent KCNQ1 voltage sensors. Currents from the two co-expression experiments display similar voltage dependence and both lie between the two fluorescence signals (Figure 2.1B), as if in channels containing mixed subunits, currents activate after some but not all voltage sensors activate. This observation is in agreement with our alternative hypothesis that current in KCNQ1 can activate before all voltage sensors have moved.

In order to more rigorously address the possibility that KCNQ1 channels can open before all four voltage sensors move, we explore another voltage sensor mutation, R231C, which leads to a constitutively active channel, by locking the voltage sensor in the activated position. Again using co-expression, we explore whether the presence of constitutively active voltage sensors within a co-assembled channel can open some percentage of these channels. We first co-inject KCNQ1 and KCNQ1 R231C subunits and look for voltage-independent (constitutive)
current. In order to distinguish constitutive current from KCNQ1 channels from background currents or leak, we perform activation protocols before and after application of 100µM chromanol, a KCNQ1-specific blocker to isolate chromanaol-sensitive current. We perform these experiments in high potassium external solution (see methods) to better resolve K\(^+\) current at hyperpolarized potentials. First, we assay constitutive current through homomeric KCNQ1 and KCNQ1 R231C channels. Figure 2.2A shows that KCNQ1 channels display 2.4% constitutive current, while R231C channels are completely constitutive. Next, we co-express KCNQ1 and KCNQ1 R231C subunits and assay constitutive current. According to the binomial distribution, 6.25% of channels will assemble with 4 R231C subunits, which will result in 6.25% constitutive current. Figure 2.2A shows that in KCNQ1 and KCNQ1 R231C co-expression experiments, we find 24% of current to be constitutive, according to the average A1 parameter from Boltzmann fits to each experiment (see Methods). Experiments extend to -140mV, where WT KCNQ1 voltage sensors are all in the resting state (see Chapter 1) and we see complete flattening of the G(V) curve at these voltages.
Figure 2.2: KCNQ1 channels can open with only two activated voltage sensors. (A) Chromanol-subtracted data from experiments expressing the constructs noted in the legend. Experiments are performed in high extracellular potassium and cells are held at -80mV, pulsed to voltages between -140mV and 60mV for 2 seconds, followed by 2 second pulses to -120mV to collect tail currents. Experiments are normalized to the maximum from Botzmann fits to the data (see Methods). (B) Chromanol-subtracted data from dimers containing tandem dimer constructs as noted in legend. Experiments are performed as in (A).

This result suggests that channels containing some voltage dependent and some constitutive voltage sensors do conduct current. However, since we cannot rule out higher expression or preferential assembly of channels with four R231C-containing subunits, it is difficult to be quantitative. We therefore construct a tandem KCNQ1 dimer containing KCNQ1 R231C and KCNQ1 within a single reading frame. Expression of this tandem construct will result in equal expression of these two subunits, and may promote assembly of channels containing two KCNQ1 R231C subunits and two KCNQ1 subunits as dimers of dimers. Expression of the tandem dimer KCNQ1(R231C)-KCNQ1 yields currents that display 29% constitutive current, while control tandem dimers containing KCNQ1-KCNQ1 subunits display only 4.4% constitutive current (Figure 2.2B). Therefore, if channels assemble as dimers of dimers containing two R231C and two WT voltage sensors, this result suggests that with half its voltage sensors activated, KCNQ1 channels are 29% open.
Figure 2.3: The I268A mutation increases the voltage-independent constitutive current in KCNQ1. (A-B) Activation traces taken before and after application of 100 µM chromanol. (C) Chromanol-subtracted data from experiments expressing the constructs noted in the legend. Experiments are performed in high extracellular potassium and cells are held at -80mV, pulsed to voltages between -140mV and 60mV for 2 seconds, followed by 2 second pulses to -120mV to collect tail currents. Experiments are normalized according to maxima from Botzmann fits to the data (see Methods). Error bars represent SEM (C) Comparison of normalized G(V) (black symbols) and F(V) (red symbols) curves from I268A<sub>L</sub>. Error bars represent SEM.

While the experiments in Figures 2.1 and 2.2 demonstrate that KCNQ1 channels are able to transition to the open state before all voltage sensors have activated, two possible mechanisms can explain this behavior. One possibility is that each independent voltage sensor movement partially opens a KCNQ1 channel to a stable subconductance state. Alternatively, each voltage
sensor movement might increase the open probability of a channel so that some percentage of channels with, for example, one activated voltage sensor would be in the open state. Based on our experiments to this point, it is not possible to distinguish between these possibilities. We gained some insight into this question from the recent study by Ma et al., which identifies a number of mutations in KCNQ1, including I268A, that cause an increase in voltage-independent constitutive current, leading them to propose an allosteric gating scheme for KCNQ1 channels wherein the channel can open in the absence of voltage sensor movement\textsuperscript{108}. In order to assay the movement of voltage sensors in one of these mutants, we introduce I268A into the KCNQ1\textsubscript{L} construct and monitor its fluorescence signal.

Again using high K\textsuperscript{+} external solution and examining chromanol-sensitive currents, we confirm that I268A introduces a significant amount of constitutive current into KCNQ1 channels. As shown in Figure 2.3, we find 37\% voltage-independent current in KCNQ1 I268A\textsubscript{L} vs 9.2\% in KCNQ1\textsubscript{L}. Measurement of the fluorescence signal from I268A\textsubscript{L} yields an F(V) curve that saturates at positive and negative voltages and is well-fit by a Boltzmann equation. This result shows that voltage sensor movement is relatively unperturbed by this mutation, and suggests that the in the presence of this mutation, a significant number of channels can open in the absence of voltage sensor movement. Intriguingly, when we plot the voltage dependence of the fluorescence signal from I268A\textsubscript{L} along with the normalized voltage-dependent portion of current, we find that the fluorescence signal activates with more depolarized voltage dependence than voltage-dependent gate opening (Figure 2.3B). This result suggests that voltage sensors can move even after the channel is fully open.

In order to interpret our results, we adopt an allosteric model of KCNQ1 channel gating wherein lateral movement between states represents movement of a voltage sensor between
resting and activated and vertical movement between states represents opening and closing of the channel gate (Gating Scheme 1 – parameters controlling the opening rate are shown). In this model, successive movement of voltage sensors strengthens the opening transition by a cooperativity factor (L), so that as more voltage sensors move, the probability of transitioning to the open state increases. This simple model is able to explain the 1:1 relationship between voltage sensor movement and channel opening in KCNQ1 as a balance of rightward and downward movements through states in the model during activation. It also explains how channels can open before all voltage sensors have moved since channel opening is possible with 0-4 voltage sensor movements. Therefore, the effect of I268A is simply to strengthen the downward opening rate (L₀) such that more channels open without any activated voltage sensors. In addition, the observation that the voltage-dependent fluorescence signal saturates after all channels are fully open in I268A is interpretable in the allosteric model as voltage dependent movement of voltage sensors between open states of the channel.

\[
\begin{align*}
C₀ & \leftrightarrow C₁ & C₂ & \leftrightarrow C₃ & \leftrightarrow C₄ \\
O₀ & \leftrightarrow O₁ & O₂ & \leftrightarrow O₃ & \leftrightarrow O₄ \\
\end{align*}
\]

\[L₀ \leftrightarrow L₀L \leftrightarrow L₀L² \leftrightarrow L₀L⁴ \leftrightarrow L₀L⁶ \]

_Gating Scheme 2.1_

If the I268A mutation has the effect of strengthening the opening transition in our proposed allosteric model, can we find other mutations that weaken the opening transition such that more voltage sensor movements are needed in order to observe channel opening? Indeed, we found a mutation like this, F351A, in Chapter 1. F351A homomeric channels activate well after voltage sensor movement is seen, suggesting that multiple voltage sensor movements are
required before the channel transitions to the open state (see Figure 1.4). This phenotype can be qualitatively explained as a decrease in the strength of the opening rate ($L_0$) in the allosteric model. In order to further explore mutations of this type, we express the S4-S5 linker mutation L251A, which was also previously shown to slow KCNQ1 channel activation, in our labeled KCNQ1 background. As shown in Figure 2.4, L251A, which was also previously shown to slow KCNQ1 channel activation, in our labeled KCNQ1 background. As shown in Figure 2.4, L251A,$L_0$ separates the kinetics of the fluorescence and current, though not to the extent induced by F351A. In agreement with this, steady-state $F(V)$ precedes steady-state $G(V)$ from L251A, such that fluorescence raised to the $2^{nd}$ power mirrors current, whereas in F351A, we must raise fluorescence to the $4^{th}$ power in order to reproduce the voltage dependence of current. We can interpret this result using the allosteric model by assuming that L251A weakens the opening transition, but not to the same extent as F351A. Taken together, results from I268A, L251A and F351A mutants support an allosteric
model of KCNQ1 gating wherein the opening transition is highly sensitive to mutation and can be strengthened or weakened to varying degrees by point mutations.
Given the high sensitivity of KCNQ1 channels to mutation, we next ask how closely our labeled construct KCNQ1_L resembles the true WT KCNQ1 channel. We compare constitutive current from KCNQ1 and KCNQ1_L. Figure 2.5A shows that while KCNQ1_L displays 9.2%
constitutive current, KCNQ1 displays only 2.4%. This difference in constitutive current is significant (p < 0.05) and we conclude that the G219C mutation increases constitutive current into the KCNQ1 channel. We therefore tested other reporter cysteines in order to find a construct

![Diagram](image)

Figure 2.6 The I268A mutation introduces constitutive current and shifts the voltage dependence of fluorescence relative to current in the K218C background. (A-B) Activation traces taken before and after application of 100 mM chromanol. (C) Chromanol-subtracted data from experiments expressing the constructs noted in the legend. Experiments are performed in high extracellular potassium and cells are held at -80mV, pulsed to voltages between -140mV and 60mV for 2 seconds, followed by 2 second pulses to -120mV to collect tail currents. Experiments are normalized according to maxima from Botzmann fits to the data (see Methods). Error bars represent SEM (C) Comparison of normalized G(V) (black symbols) and F(V) (red symbols) curves from I268A, Error bars represent SEM.
that would yield a fluorescence signal without increasing constitutive current. Figure 2.5 shows that K218C does not significantly increase constitutive current through KCNQ1 and that current and fluorescence signals from labeled KCNQ1 K218C are similar to labeled KCNQ1 G219C (Figure 1.1). The kinetics and steady-state voltage dependence of labeled KCNQ1 K218C show that voltage sensor movement precedes gate opening in this mutant to a larger extent than in the G219C background, suggesting that in K218C the opening transition is slightly weaker than in labeled KCNQ1 G219C. Given the similarity between constitutive current seen in WT KCNQ1 and KCNQ1 K218C, we take the fluorescence and current seen in this labeling background as the baseline for WT KCNQ1 channels. Finally, in order to ensure that the observed affects of KCNQ1 mutations on the opening transition are not dependent on G219C, we next retested the effect of I268A in the labeled KCNQ1 K218C background. As shown in Figure 2.6, both the dramatic increase in constitutive current (35%) and the depolarized voltage dependence of fluorescence relative to current are present in the K218C background.

Discussion

Here we provide evidence for allosteric voltage dependent gating in the KCNQ1 channel. Based on our previous observation that the KCNQ1 voltage sensor and gate have approximately the same voltage dependence, we first rule out concerted movement of KCNQ1 voltage sensors by showing that co-assembly of subunits containing heterogenous voltage sensors does not affect the behavior of each. We noted that in these channels, channels activate as if movement of all four voltage sensors is not required for channels to open. We next directly test this possibility using a voltage sensor mutation R231C, which constitutively activates the KCNQ1 voltage sensor. In channels co-assembled from KCNQ1 and KCNQ1 R231C channels, we see a large constitutive current both in co-expression and heterozygous tandem dimer experiments, as if
channels containing two activated voltage sensors have a significant probability of opening. We next identify a mutation, I268A, which displays a large constitutive current but normal voltage sensor movement, leading us to propose an allosteric gating scheme for KCNQ1. We use this allosteric gating scheme to interpret mutations that alter the relationship between voltage sensor movement and channel opening by surmising they change the strength of the opening transition. Finally, we propose that the fluorescence signal from K218C, which displays identical voltage-independent constitutive current as WT KCNQ1, to be the most representative of WT voltage sensor movement

KCNQ1 channels are expressed throughout the body and display widely varying biophysical properties, mostly based on co-assembly with various beta subunits of the KCNE family. The allosteric model presented here allows for interpretation of the affects these beta subunits have on channel gating properties. For example, KCNE1 co-assembly drastically slows current activation, which may be mostly due to a relative weakening of the opening transition in the allosteric model (though the movement of the voltage sensor itself is also affected-- see Chapter 1). In contrast, the constitutive phenotype of KCNQ1-KCNE3 and KCNQ1-KCNE2 channels might be due to a stabilization of the activated state of the voltage sensor or simply a large increase in the strength of the opening transition such that all channels are open before voltage sensors move. The flexibility in gating behaviors afforded by an allosteric framework therefore uniquely suits the widely variable KCNQ1 channel.

Our data not only suggests that KCNQ1 channels gate according to an allosteric scheme, but also that this channel is highly sensitive to mutation-induced changes to the strength of the opening transition. That KCNQ1 constitutes a highly allosteric protein is underlined by the observation that the labeling mutation G219C, located in the S3-S4 extracellular loop and far
from the channel gate, itself alters the opening transition. This alteration and the associated change in the relative positions of G(V) and F(V) curves is readily comprehendible within the allosteric model, since both are expected from a slight increase in the opening transition rate. How a mutation at this location within the channel might change the strength of the opening transition is a matter for further study.

Among the questions arising from this chapter is that of how generalizable this allosteric model is to voltage-gated potassium channels in general. Though this is the first member of the KV channel family to be show to gate in this way, the BK channel does employ its voltage sensors as allosteric regulators of the opening transition, along with binding of Ca$^{2+}$. For other Kv channels, current kinetic models dictate that opening is possible only after movement of all voltage sensors, and while these models faithfully reproduce biophysical gating properties of these channels, it may be possible to alter these channels in a way that would unmask allosteric properties.

With respect to the KCNQ1 channel itself, this work provides a baseline from which to look for structures and interaction points important in physiological and pathophysiological regulation of channel gating. Mutations identified in the S4-S5 linker alter both the strength of the opening transition as well as the cooperativity between voltage sensors. It is therefore not surprising that studies have found an intimate relationship between KCNE1 and the S4-S5 linker, since KCNE1 likely drastically alters these same gating parameters. However, mutations that alter the opening transition can be found throughout the channel, even in the voltage sensing domain (G219C). Therefore, what structures are most directly implicated in altering the strength of the opening transition, and how they do so are open questions.
This allosteric model also provides a framework with which to explore channel
deactivation, which plays an important role in both physiological and pathophysiologicacontexts. In particular, the preferential effects of familial atrial fibrillation-associated
mutations to slow deactivation, while sparing activation, imply separable pathways for these two
processes. Within the allosteric framework we can seek to identify rates, and therefore physical
processes, which may impact the kinetics of one process over another. Therefore, this work
provides a valuable tool for understanding not only how KCNQ1 behaves in physiological
contexts, but also for understanding the molecular mechanisms of disease-causing mutations that
alter gating.
Chapter 3: Characterization of KCNQ1 atrial fibrillation mutations reveals distinct dependence on KCNE1
Summary

The $I_{Ks}$ potassium channel, critical to control of heart electrical activity, requires assembly of alpha (KCNQ1) and beta (KCNE1) subunits. Inherited mutations in either $I_{Ks}$ channel subunit are associated with cardiac arrhythmia syndromes. Two mutations (S140G and V141M) that cause familial atrial fibrillation (AF) are located on adjacent residues in the first membrane-spanning domain of KCNQ1, S1. These mutations impair the deactivation process, causing channels to appear constitutively open. Previous studies suggest that both mutant phenotypes require the presence of KCNE1. Here, in collaboration with Priscilla Chan, I report that despite the proximity of these two mutations in the primary protein structure, they display different functional dependence on the presence of KCNE1. In the absence of KCNE1, the S140G mutation, but not V141M, confers a pronounced slowing of channel deactivation and a hyperpolarizing shift in voltage dependent activation. When co-expressed with KCNE1, both mutants deactivate significantly slower than wild-type KCNQ1/KCNE1 channels. The differential dependence on KCNE1 can be correlated with the physical proximity between these positions and KCNE1 as shown by disulfide crosslinking studies: V141C forms disulfide bonds with cysteine substituted-KCNE1 residues, while S140C does not. These results further our understanding of the structural relationship between KCNE1 and KCNQ1 subunits in the $I_{Ks}$ channel, and provide mechanisms for understanding the effects on channel deactivation underlying these two atrial fibrillation mutations.

Introduction

$I_{Ks}$ is the slowly activating component of delayed rectifier $K^+$ current in the heart and is a major contributor to the timing of repolarization of the cardiomyocyte membrane potential $^4$. 
The $I_{Ks}$ channel is composed of a tetramer of pore-forming alpha subunits, KCNQ1 (Q1), and accessory beta subunits, KCNE1 (E1) $^{57,58}$. Mutations in either KCNQ1 or KCNE1 have been linked to cardiac arrhythmia syndromes, including long QT syndrome (LQTS) $^9$, short QT syndrome (SQTS) $^{109}$ and familial atrial fibrillation (FAF) $^{15-17,110}$.

The biophysical properties of $I_{Ks}$ channel current are dramatically altered when KCNE1 associates with the KCNQ1 channel. Functional tetrameric channels can be formed by KCNQ1 alone, but co-assembly with KCNE1 is required for the unique kinetics necessary to regulate human cardiac electrical activity as well as for the channel’s functional response to the sympathetic nervous system. Specifically, KCNE1 co-assembly results in a depolarizing shift in the voltage dependence of activation, an increase in the single channel conductance, and an increase in current density $^{57,58,105}$. $I_{Ks}$ channel current is also characterized by slow activation and deactivation kinetics, with little or no inactivation, in contrast to the KCNQ1 homomeric channel, which is characterized by fast activation and deactivation kinetics and clear inactivation $^{111}$.

Recent studies reporting spontaneous crosslinking between substituted cysteine residues on KCNE1 and KCNQ1 have positioned KCNE1 between the first and sixth transmembrane helices (S1 and S6, respectively) of opposing KCNQ1 subunits, which is consistent with the current KCNQ1 structural model $^{68,69,80}$. In this region of S1, two gain-of-function disease mutations associated with atrial fibrillation (AF), S140G and V141M, are located in adjacent residues. When KCNQ1 containing either AF-related mutation in S1 (S140G or V141M) is co-expressed heterologously with KCNE1, the resultant channels activate immediately in response to depolarizing pulses applied from holding potentials similar to typical myocyte resting potentials $^{15,16}$. Subsequent analysis has revealed that the instantaneous current is due to
accumulation of open channels caused by incomplete deactivation between pulses at these holding potentials\textsuperscript{20}. To date, this channel property is believed to be manifested only in the presence of KCNE1 for both mutations\textsuperscript{20}.

Here we have explored the role(s) of KCNE1 in translating the effects of the KCNQ1 AF mutations S140G and V141M into pathological channel function by characterizing the mutations in the absence and presence of KCNE1. We have explored the structural proximity of KCNE1 relative to the two AF mutations located in S1 KCNQ1, using a biochemical assay to look for disulfide bridge formation between introduced cysteines. Our results demonstrate that even though both mutations exhibit extremely slow deactivation kinetics in the presence of KCNE1, they have distinct dependencies on this accessory subunit. V141M KCNQ1 channels resemble WT KCNQ1 channels in the absence of KCNE1, thus co-assembly with KCNE1 is required to alter channel deactivation kinetics. However, the S140G mutation in KCNQ1 itself is sufficient to dramatically slow the deactivation process and co-assembly with KCNE1 further slows channel closing. Biochemical evidence correlates function with structure, revealing orientation of the two proteins such that KCNQ1 V141, but not S140, is positioned close to KCNE1, providing a structural basis for distinct subunit dependence of channel function conferred by these two AF mutations. In line with the prediction of a direct interaction between V141M and KCNE1, we demonstrate that the location of KCNE1 within a heterozygous channel complex impacts the severity of the mutant phenotype. Our results support the notion of a physiologically important interaction between KCNE1 and KCNQ1 S1, sensitive to mutation, that dramatically affects the rate of channel deactivation. In this chapter, experiments were performed in collaboration with Priscilla Chan.
Methods

Molecular Biology and Cell Culture

Human KCNE1 was subcloned into the p3XFLAG-CMV-14 Expression Vector (Sigma-Aldrich E4901, St. Louis, MO) to generate a C-Terminal FLAG-tagged KCNE1. Mutations were engineered into human KCNQ1 and KCNE1 cDNA with the QuikChange Site-Directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). All biochemical experiments were conducted with these constructs. Human KCNE1 with an N-terminal HA tag was used for functional studies. A tandem construct, EQQ, was generated using a previously tested fusion construct, E1-Q1. E1-Q1 construct was engineered by linking the C-terminus of KCNE1 to the N-terminus of KCNQ1. Next, the EQQ construct was engineered by inserting an additional KCNQ1 subunit into the E1-Q1 construct. The E1-Q1 and Q1 dimer constructs were digested with Xhol and the 2-kb insert from the Q1 dimer was gel-purified and ligated into the Xhol site of E1-Q1. The nomenclature used is to reflect the individual subunits engineered into the construct, EQQ indicates one KCNE1 subunit linked to two KCNQ1 subunits. The human rhinovirus (HRV-3C) protease (Novagen) consensus cleavage site, LEVLFQGP, which is cleaved between the Q and G, was inserted into the C-terminus of KCNE1 to create a cleavage site between KCNE1 and KCNQ1. Chinese hamster ovary (CHO) cells (American Type Culture Collection, Manassas, VA) were cultured in Ham’s F-12 culture media with 10% FBS in a 37°C incubator with 5% CO₂.

Electrophysiology
CHO cells were transfected with wild-type or mutant KCNQ1 alone or co-transfected with wild-type or mutant KCNE1, and with eGFP (0.4 µg of each cDNA) and plated on 35 x 10 mm tissue culture dishes as previously described. Electrophysiological measurements were carried out 48h after transfection. Currents were recorded at 25°C using the whole-cell patch clamp configuration with an Axopatch 200A amplifier (Molecular Devices, Union City, CA) as previously described. Series resistance was 2-3 MΩ. The physiological K⁺ external solution contained (in mM): 132 NaCl, 4.8 KCl, 1.2 MgCl₂, 1 CaCl₂, 5 glucose, 10 HEPES, pH was adjusted to 7.4 with NaOH. Internal solution contained (in mM): 110 K⁺ aspartate, 1 MgCl₂, 1 CaCl₂, 11 EGTA, 5 K₂ATP, 10 HEPES. Cells were chosen based on eGFP fluorescence. For experiments in elevated K⁺, external solution contained (in mM): 17.8 NaCl, 120 KCl, 1.2 MgCl₂, 1 CaCl₂, 5 glucose, 10 HEPES, pH was adjusted to 7.4 with NaOH.

Voltage Clamp Protocols

To record the conductance–voltage (G–V) relationship for $I_{Ks}$, cells were voltage clamped at a negative holding potential (−80, −100 mV, or −120 mV). Test potentials were applied to a series of isochronal (2 sec) activation voltages with a fixed incremental increase between successive pulses (e.g. −100 to +40 mV in 20 mV steps). Activation was determined from the amplitude of deactivating tail currents measured at voltages indicated in the text for specific experiments as a function of the preceding test pulse voltage. The interval between test pulses was 15 or 20s, as described in figure legends.

To compare activation kinetics for channels with differing voltage dependences, voltages were chosen where open probabilities were similar. Time to half activation ($t_{1/2}$) was measured during 2 s pulses, taken at the voltage closest to the $V_{1/2}$ acquired from the G–V
relationship for each subunit combination. Time to half activation $t_{1/2}$, is measured with relation to the maximal current in the corresponding 2 s test pulse.

Unless otherwise indicated, kinetics of deactivating tail currents were analyzed using 2 s depolarizing steps to $+20$ mV applied every 15 seconds from a -100 mV holding potential followed by 2 s repolarizing steps ranging from -80 to -120 mV (20 mV increments). The time constant of $I_{Ks}$ deactivation ($\tau_{\text{deact}}$) measured at -80 mV, -100 mV, and -120 mV was obtained by fitting tail currents with unconstrained single exponential functions.

Crosslinking

Crosslinking procedures and the calculation of the crosslinking percentage have been described previously. CHO cells were co-transfected with mutant cDNAs of KCNQ1 C136V-C214A-C331A (pWTKCNQ1) and FLAG-KCNE1 (1.0 µg each) using PLUS reagent and Lipofectamine (Invitrogen, Carlsbad, CA). Cells were incubated for 48 h at 37°C in the presence of 5% CO$_2$.

Crosslinking occurred in the presence of ambient oxygen, no external oxidizing agents were added as described. In order to study only surface membrane proteins, the adherent cells were surface-biotinylated with EZ-Link Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific, Waltham, MA), the reaction was stopped with glycine methyl ester, the cells were lysed, and, after sedimentation of insoluble material, the supernatant was mixed with Ultralink Immobilized NeutrAvidin Protein Plus beads (Thermo Fisher Scientific) to bind the biotin-labeled membrane proteins, which were eluted from the beads by exposure for 3 min at 90°C to 8 M Urea, 4% SDS, 200 mM Tris pH 8.0, and 2 mM EDTA, all as previously described. Dithiothreitol (DTT) to a final concentration of 20 mM was added to half of each eluate. Samples were incubated at 50°C for 20 min, bromophenol blue was added, and the samples in SDS sample buffer were
electrophoresed on 4–20% acrylamide gels. The gels were transferred to nitrocellulose, and the blot was blocked with Blocking Buffer for Near Infrared Fluorescent Western Blotting (Rockland Immunochemicals Inc, Gilbertsville, PA) and incubated with goat anti-KCNQ1 C-20 antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) and mouse anti-FLAG M2 antibody (1:2000, Sigma-Aldrich, St. Louis, MO). Membranes were washed and incubated with donkey anti-goat AlexaFluor 680-labeled antibody (1:5000, Invitrogen, Carlsbad, CA) and donkey anti-mouse IRDye 800-labeled antibody (1:5000, LI-COR Biosciences, Lincoln, NE). Fluorescent signals were detected using the Odyssey Infrared Imager (LI-COR).

The extent of crosslinking was calculated as follows: \((\text{KCNQ1}/\text{KCNE1} + X/2)/\) \((\text{KCNQ1}/\text{KCNE1} + \text{KCNQ1} + \text{KCNQ1}_2 + X)\). Calculations were based on the intensities of four bands: the monomeric KCNQ1 band (\(~74\, \text{kD}\)), the crosslinked KCNQ1/KCNE1 band (\(~110\, \text{kD}\)), the KCNQ1\(_2\) band (KCNQ1 dimer \(~150\, \text{kD}\)), and a band labeled X (\(~250\, \text{kD}\)) (Figure 3.6)\(^68\).

**MTSEA-Biotin Experiments**

To verify that the S140C was accessible for crosslinking to Cys-KCNE1 residues, MTSEA-Biotin (Biotium, Hayward, CA) was used to assay the reactivity of this residue. MTSEA-Biotin was dissolved in DMSO at a concentration of 2 mg/100 μl and then diluted with PBS at a ratio of 1 to 100. A final concentration of 0.5 mM MTSEA-Biotin solution was added to the external bath solution. In these experiments, peak currents were measured at the end of a 2 s depolarizing pulse to +60 mV and normalized to capacitance.

**Data Analysis**

Data were collected using Clampex 8.0 (Molecular Devices, Union City, CA) and analyzed as described previously \(^{114}\) with Clampfit 8.0 (Molecular Devices) and Origin 7.0.
Statistical data analysis was assessed with Student's $t$ test; differences at $p < 0.05$ were considered to be significant.

**Results**
In order to probe the impact of KCNE1 on S140G and V141M mutant phenotypes, we first characterized the functional effects of these mutations in cells expressing channels encoded by the KCNQ1 subunit alone (Figure 3.1A-C). Because we were interested in determining the influence of each of these mutations on channel deactivation, particularly over a negative range
of voltages, experiments were carried out in external solutions containing elevated K⁺ concentration (120 mM, see Methods). Control experiments (Figure 3.2) verified that elevated K⁺ did not affect the voltage-dependence and deactivation of KCNQ1 channel compared with experiments in physiological K⁺ solution (5 mM). As had been shown for V141M channels in Xenopus oocytes 16, we find that channels encoded by both S140G and V141M close if voltages are sufficiently negative. Once closed, channels were then opened by application of positive test pulses. There was no significant difference in the V₁/₂ of activation obtained for wild-type (WT) KCNQ1 (V₁/₂ = -29 ± 2.1 mV, n=4) and V141M channels (V₁/₂ = -29 ± 0.9 mV, n=5, p > 0.05 vs. WT KCNQ1); however, the V₁/₂ of activation for S140G channels is significantly more negative than WT KCNQ1 channels (V₁/₂ = -57 ± 1.3 mV; n=5, p < 0.05 vs. WT KCNQ1) (Figure 3.1D). S140G channels also exhibited significantly slower deactivation over a range of voltages from -80 to -120 mV compared with WT KCNQ1 and V141M channels (Figure 3.1E and Table 3.1).

<table>
<thead>
<tr>
<th>Construct</th>
<th>-80 mV</th>
<th>-100 mV</th>
<th>-120 mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT KCNQ1</td>
<td>131 ± 31</td>
<td>104 ± 3</td>
<td>82 ± 14</td>
</tr>
<tr>
<td>KCNQ1 S140G</td>
<td>2240 ± 160*</td>
<td>1090 ± 71*</td>
<td>666 ± 110*</td>
</tr>
<tr>
<td>KCNQ1 V141M</td>
<td>153 ± 18</td>
<td>101 ± 32</td>
<td>80 ± 28</td>
</tr>
</tbody>
</table>

Table 3.1: τ of deactivation at different voltages for KCNQ1 S1 mutants
Data presented as mean tau of deactivation from exponential fit ± SEM (n = 4-6). * indicates the tau of deactivation is significantly different from WT at the indicated voltage.
We next investigated the impact of KCNE1 on channel properties when co-expressed with KCNQ1 carrying either of these two disease-associated mutations. A pulse protocol consisting of 2s isochronal activating pulses to +20 mV was applied at a very slow rate (once every 20 seconds) to ensure that the majority of channels were closed between pulses. We next measured the impact of KCNE1 co-expression on activation gating. Representative current traces

![Figure 3.2: Function of wild-type KCNQ1 and wild-type KCNE1 (120mM K⁺ vs. 5mM K⁺). (A) G-V relationship for KCNQ1/KCNE1 measured in 120mM K⁺ vs. 5mM K⁺ external solution. KCNQ1/KCNE1(120 mM K⁺): Holding potential = -120 mV. Cells were depolarized from -120 mV to +80 mV in 20 mV increments. Tail currents were measured at -120 mV for 5 s (left inset). KCNQ1/KCNE1 (5 mM K⁺): Holding potential = -80 mV. Test pulses were applied once every 11 seconds from -60 mV to +80 mV. Tail currents were measured at -40 mV for 2 s (right inset). (B) Deactivation time constant (tau) obtained at -100 and -120 mV from single exponential fits to tail currents following conditioning pulses (+20 mV, 2 sec).]
for S140G and V141M, assembled with KCNE1, reveal channels that exhibit the slow onset of activation seen in WT $I_{Ks}$ channels (Figure 3.3A-C). The voltages for half maximal (isochronal) activation for channels consisting of KCNE1 co-expressed with three KCNQ1 variants were measured to compare voltage dependence of activation gating (KCNQ1/KCNE1: $V_{1/2} = 17.5 \pm$
2.6 mV, \( n=6 \); S140G/KCNE1: \( V_{1/2} = 1.5 \pm 2.7 \) mV, \( n=4 \); V141M/KCNE1: \( V_{1/2} = 2.4 \pm 1.0 \) mV, \( n=3 \) (Figure 3.3D). \( I_{Ks} \) activation kinetics were then compared by measuring the time (\( t_{1/2} \)) at which \( I_{Ks} \) current is half maximally activated at the voltage closest to the \( V_{1/2} \) for each subunit combination. KCNE1 co-expression caused no significant change in activation kinetics of the S140G (\( t_{1/2} = 1.5 \pm 0.04 \) s, \( n=4 \)) and V141M (\( t_{1/2} = 1.45 \pm 0.06 \) s, \( n=4 \)) assembled channels (\( p>0.05 \) for S140G/KCNE1 vs. V141M/KCNE1). Although both were significantly different from WT KCNQ1/KCNE1 channels (\( t_{1/2} = 1.15 \pm 0.31 \) s, \( n=6 \)), this was a small difference that did not take away from our main focus, which was to look for changes in deactivation kinetics (Figure 3.3E).
We next focused on deactivation kinetics of the S140G and V141M mutant channels in the presence of KCNE1. To compare deactivation for the different KCNQ1/KCNE1 pairs, we determined the deactivation time constants by measuring tail currents at -120 mV following 2 s depolarizing pulses (Figure 3.4A-B). The deactivation time constant for S140G/KCNE1 channels ($t_{\text{deact}} = 4.09 \pm 0.23$ s, $n=5$) is significantly greater than that for WT KCNQ1/KCNE1 channels ($t_{\text{deact}} = 0.24 \pm 0.004$ s, $n=6$, $p<0.05$ for S140G/KCNE1 vs. WT KCNQ1/KCNE1). For V141M/KCNE1 channels, deactivation is significantly slower than both WT KCNQ1 and S140G

![Figure 3.4: KCNE1 slows deactivation of V141M heteromeric channels to a greater extent than S140G channels](image)
channels assembled with KCNE1 ($t_{\text{deact}} = 6.71 \pm 0.64 \text{ s}, n=4$, $p< 0.05$ vs. WT KCNQ1/KCNE1 and $p< 0.05$ vs. S140G/KCNE1) (Figure 3.4C).

Another way to analyze the KCNE1 functional interaction with S140G and V141M in terms of slowing deactivation is by examining the ratio of deactivation tau between the KCNQ1 subunit alone and co-expression with KCNE1. For the WT KCNQ1 channel, deactivation at -120mV is slowed about 3-fold in the presence of KCNE1 (Figure 3.4C). For KCNQ1 S140G, a slightly larger change in deactivation tau is seen from the addition of KCNE1 of about 7-fold. In contrast, KCNE1 co-expression with V141M subunits reveals a much more dramatic effect, slowing deactivation by more than fifty fold (KCNQ1: 2.74 ± 0.14, S140G: 7.75 ± 0.99, V141M: 51.39 ± 5.57) (Figure 3.4C).
The functional data suggest possible physical differences in the location of KCNQ1
residues S140 and V141 relative to KCNE1 in assembled channels. We thus sought to determine

Figure 3.6: Full Length Blot of Crosslinking Bands. KCNQ1 is represented as a red signal and KCNE1 as a green signal. The merged red and green in the crosslinked KCNQ1/KCNE1 band is yellow. The samples in the right lanes were reduced with DTT in sample buffer. X indicates a band of molecular weight greater than 250 kDa. The molecular weights of the markers are in kDa.
the proximity of S140 and V141 to KCNE1 by individually substituting cysteines in KCNQ1 and KCNE1 and monitoring spontaneous disulfide bridge formation (Figure 3.5A). To compare to the functional data we assayed crosslinking specifically of membrane proteins, as described in Methods. Two KCNQ1 Cys-substituted mutants (S140C and V141C) and twelve KCNE1 Cys-substituted mutants (G40C to L51C) were generated. These KCNE1 residues were chosen for cysteine substitution due to their location in the region of KCNE1 predicted to be aligned with
the region where Ser 140 and Val 141 are located. Crosslinking was determined by the percentage of crosslinked protein versus total protein (Figure 3.6), and then plotted for the twenty-four cysteine pairs tested (Figures 3.5B-C). Sample immunoblots are shown for crosslinking results obtained from S140C and V141C, in the absence and presence of a reducing agent, dithiothreitol (DTT). For S140C and each of twelve KCNE1 Cys mutants tested, disulfide bond formation was <10% (Figure 3.5B). This result suggests that S140 is not close enough to form contacts with KCNE1. In contrast, there is a high degree of disulfide bond formation between V141C and two Cys mutants on KCNE1: E43C and A44C. V141C/ E43C exhibited 35± 4.3% crosslinking, while V141C/ A44C exhibited 78± 2.4% crosslinking (Figure 3.5C), which are comparable to the high degree of crosslinking that was found for the extracellular flanks between KCNE1 and the S1 and S6 domains two KCNQ1 subunits.
Examination of the functional consequences of the crosslinking constructs revealed that substitution of a Cys at position 141 did not alter activation but slowed deactivation, in agreement with previous oocyte recordings. The single Cysteine substituted KCNE1 (Cys-KCNE1) mutations at positions 43 and 44 did not alter function when co-expressed with WT KCNQ1 (Figure 3.8). When V141C was co-expressed with A44C, the impact of crosslinking was minor; a further slight slowing of deactivation, in comparison to V141C alone, and no change in activation (Figure 3.7). Reduction of the crosslink with DTT shifted the voltage dependence of activation (V141C/A44C(-DTT) $V_{1/2} = 34.9 \pm 2.7$ mV, $n=5$, vs. V141C/A44C(+DTT): $V_{1/2} = 46.3 \pm 2.9$ mV, $n=5, p<0.05$) and caused a slight, statistically insignificant, speeding of deactivation (V141C/A44C(-DTT): $t_{\text{deact}} = 1.08 \pm 0.06$ s, $n=5$, vs. V141C/A44C(+DTT): $t_{\text{deact}} = 0.82 \pm 0.21$ s, $n=5, p>0.05$). Both of these effects, however, are consistent...
with the effect of DTT alone in the WT KCNQ1 channel or in single Cys-KCNQ1 or single Cys-
KCNE1 constructs, and therefore likely are independent of the reduction of the crosslink (Figure
3.8)\(^68\). This result suggests that crosslinking of KCNQ1 V141C to KCNE1 likely does not
perturb the native conformation of the channel, suggesting V141, but not S140, is oriented
towards KCNE1 in native channels.

Having shown evidence for the differential dependence of S140G and V141M mutations
on the presence of KCNE1, we next turned to the question of whether or not the location of
assembled KCNE1 subunits relative to mutation-containing S1 helices impacts the disease
phenotype. Our previous results suggest a direct physical interaction between KCNQ1 V141M
and KCNE1 is required for the disease-associated phenotype. In order to further test this
hypothesis, we constructed a tandem channel containing KCNE1 tethered to two KCNQ1
subunits (EQQ) (Figure 3.10), which has been used in previous KCNQ1 studies\(^74,76\).

We first wanted to test if in channels formed with this construct KCNE1 preferentially
assembles next to the S1 helix of a specific KCNQ1 subunit. We therefore engineered K41C into
KCNE1 and I145C into either the proximal or distal KCNQ1 subunit of the linked EQQ

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**Figure 3.9: Complete Proteolysis of HRV-3C Cleavage Site in the EQ Dimer.** Sample
immunoblots are shown for the EQ fusion construct, with an HRV-3C cleavage site inserted between the C-
terminus of KCNE1 and N-terminus of KCNQ1. The cell-surface expressed EQ protein is ~95 kD before (-)
protease treatment and the KCNQ1 monomer is ~75 kD after (+) protease treatment. The immunoblots
were developed with an antibody against a C-terminal epitope of KCNQ1.
construct where two endogenous cysteines have been removed as in Chung et al. KCNE1 K41C has previously been shown to spontaneously crosslink to KCNQ1 I145C. In order to visualize crosslinking, we also engineered an HRV-3C protease site into the linker between KCNE1 and proximal KCNQ1 subunit (Figure 3.10A). Figure 3.9 shows that proteolysis of the introduced HRV-3C site is complete.
After isolating channels expressed at the cell surface, we applied protease to cleave KCNE1 from the KCNQ1-KCNQ1 dimer (QQ). If a crosslink occurs between KCNE1 and the
proximal or distal KCNQ1, the EQQ band will remain after protease treatment. Subsequent treatment with DTT will reduce this crosslink, leaving just the KCNQ1-KCNQ1 dimer. As shown in Figure 3.10, when I145C is present in the proximal KCNQ1 subunit, we find ~90\%
crosslinking (Figure 3.10B-C). In contrast, when I145C is introduced into the distal KCNQ1 subunit we find significantly less (~50%) crosslinking. These results strongly suggest that in our tandem EQQ construct, KCNE1 preferentially assembles next to the S1 helix of the proximal KCNQ1 subunit.

We next functionally characterized tandem constructs containing the V141M mutation in either the proximal or distal KCNQ1 subunit (Figure 3.11A). We find that when V141M is present in the proximal KCNQ1 subunit, deactivation is much slower than WT EQQ channels (Figure 3.11B). In contrast, when the V141M mutation is introduced into the distal KCNQ1 subunit, deactivation is not significantly different from WT EQQ channels (Figure 3.11C). These results indicate that for the V141M mutation, the position of KCNE1 relative to the mutation-containing S1 helix is critical to its disease phenotype and that a direct physical interaction between KCNE1 and V141M underlies the defect in channel deactivation.

Based on our data with KCNQ1 S140G monomers with and without KCNE1, it is unclear whether or not the more severe phenotype of KCNQ1 S140G channels in the presence of KCNE1 was also dependent upon a direct interaction between KCNE1 and an S140G-containing S1 helix. In order to test this, we placed the S140G mutation in the proximal and distal KCNQ1 subunit of the EQQ tandem construct to see the effect of KCNE1-S140G proximity on channel function. As shown in Figure 3.11D-E, while the deactivation of tandem channels containing S140G in the distal KCNQ1 subunit is significantly slowed, there is a significant additional effect on deactivation with S140G in the proximal KCNQ1 subunit, next to the assembled KCNE1. This suggests that a direct physical interaction between KCNE1 and the S140G-containing KCNQ1 S1 has a functional impact on the expression of a more severe deactivation phenotype. Taken together, our results with tandem EQQ constructs supports a hypothesis that
the relative expression and assembly of KCNE1 in heterozygous patients harboring V141M and S140G will affect the expression of the disease-causing channel phenotype.

Finally, in order to better understand the mechanism underlying the mutation-induced phenotype from S140G and V141M mutations, we express these mutations in the background of the voltage clamp fluorometry reporter construct, KCNQ1 C214A/C331A/G219C (KCNQ1L). Using this construct, we can assay voltage sensor movement in KCNQ1 channels (see Chapter 1). First, we test the kinetics of the fluorescence signal during deactivation of KCNQ1L, KCNQ1

Figure 3.12: The S140G mutation slows equilibration of the voltage sensor during deactivation. Fluorescence signals from KCNQ1L (A), V141ML (B), and S140GL (C). (D) Bar graph showing T1/2 of fluorescence signal during deactivation at -120mV and -140mV. In all cases, vertical scale bar represents 0.5 DF/F (%) and horizontal scale bar represents 1 sec. Error bars represent SEM.
V141M and KCNQ1 S140G. Figure 3.12 shows that while V141M does not affect the kinetics of fluorescence equilibration during deactivation, S140G induces a significant slowing of the kinetics of fluorescence relaxation. This result is consistent with the hypothesis that S140G points toward the voltage sensing helix and slows channel deactivation by affecting the voltage sensor.
We next test voltage sensor equilibration during deactivation of S140G<sub>L</sub> and V141M<sub>L</sub> in the presence of KCNE1. Figure 3.13 shows that both S140G<sub>L</sub> and V141M<sub>L</sub> fluorescence signals show extremely slow relaxation at hyperpolarized potentials, and that V141M<sub>L</sub> fluorescence relaxes more slowly than S140G<sub>L</sub> in the presence of KCNE1, in agreement with the more severe channel deactivation phenotype of V141M in the presence of KCNE1. Also consistent with the
properties of mutant channel currents, fluorescence signals equilibrate quickly during depolarizing pulses, demonstrating again a preferential affect of these mutations during deactivation. Taken together, the fluorescence data supports a dramatic effect of these mutations on the equilibration of the voltage sensor during deactivation. This is consistent with the previously proposed mechanism of these mutations: that they slow channel deactivation by selectively stabilizing the activated state of the voltage sensor.

Discussion

We find that KCNE1 plays a critical role in distinguishing two effects underlying the atrial fibrillation-associated KCNQ1 mutations S140G and V141M. While both mutations disrupt deactivation in the presence of KCNE1, our experiments establish that S140G is able to slow channel deactivation in the absence of this subunit. We find that the V141M mutation in the KCNQ1 subunit alone is indistinguishable from the WT KCNQ1 subunit, confirming previous reports that this mutant phenotype requires the presence of KCNE1\textsuperscript{16}. The marked impact of KCNE1 on deactivation of the V141M channel occurs despite that fact that there is no effect of the mutation on WT KCNQ1 homomeric channel function. This indicates an important role for KCNE1 in determining the disease phenotype of the V141M assembled channels.

Based on our functional experiments, biochemical crosslinking was performed in order to establish a structural basis for the differential KCNE1 dependence of these two mutant phenotypes. KCNE1 has previously been shown by spontaneous disulfide crosslinking to be positioned in the I\textsubscript{KS} channel in a manner that allows KCNE1 communication with S1 and S6 of different KCNQ1 subunits\textsuperscript{68,69}. Our crosslinking results build onto this placement of KCNE1 within the assembled channel. We demonstrate that V141C, but not S140C, spontaneously
crosslinks with cysteines introduced in KCNE1 at positions 43 and 44. These results reveal a specific orientation of S1 whereby V141 is in close proximity to KCNE1, consistent with the KCNQ1 structural model (Figure 3.14)\(^\text{80}\). With this positioning, it is clear why there is such a dramatic effect on channel function when KCNQ1 with the V141M mutation is assembled in the tetrameric channel in the presence of KCNE1. In the absence of KCNE1, it is likely that the V141 residue is not close to any region of the channel where it could affect channel function, which is in agreement with this mutation having no functional impact on KCNE1-free
homomeric KCNQ1 channels. On the other hand, S140 is the neighboring residue on the S1 alpha helix and its position can be inferred to be rotated back towards the S2-S4 helices within its own subunit. Thus, it is not in a favorable position to form crosslinks with KCNE1 residues, but points in a direction that would allow it to impact the environment where S2 and S4 are located (Figure 3.14B). This prediction is consistent with our functional results showing that mutation of the S140 residue alone is sufficient to slow deactivation of homomeric KCNQ1 channels in the absence of KCNE1.

The functional consequences of the crosslink at V141C / A44C and V141C / E43C were observed to be minor, indicating that crosslinking does not perturb the native conformation of the channel; consequently these residues are likely in a relatively fixed position in the native state of the channel. Taken together, our results suggest an important region of proximity between the amino terminal end of KCNE1, near the transmembrane region and the extracellular end of KCNQ1 S1, that is particularly important in controlling deactivation kinetics of the KCNQ1/KCNE1 assembled channel. Based on these results, we propose that this KCNE1/KCNQ1 S1 interaction may mediate the KCNE1-induced changes in WT KCNQ1 deactivation.

The role of KCNE1 subunit assembly in channel gating was demonstrated using fusion constructs in which a KCNE1 subunit was positioned close to one KCNQ1 subunit but not to another KCNQ1 subunit. Using the results from our functional data characterizing the AF-associated mutations, S140G and V141M, we were able to demonstrate that the intersubunit location in the heteromultimeric channel is critical in translating the α subunit mutation into altered channel function. This is of particular relevance for congenital human disease in which heterozygote mutation carriers also carry one copy of WT KCNQ1. The requirement of KCNE1
for the most severe phenotype observed with these inherited mutations raises the interesting and important question of whether the pathological phenotype of these mutations may depend on the relative expression of KCNE1 in the heart and the resulting stoichiometry of KCNE1 in these channels. The tandem construct experiments in Figures 3.10 and 3.11 further strengthen this possibility as the data show that the location of KCNE1 relative to a mutant KCNQ1 in the assembled channel is critical in determining the severity of the channel defect. Mutation carriers in the S140G family have varied clinical phenotypes from no effect to AF to mild Long QT. Whether the stoichiometric ratio of KCNE1 to KCNQ1 is fixed at 2/4 as suggested by the work of the Kobertz group or variable as suggested by recent single molecule imaging experiments, our work would strongly suggest a mandatory proximity of KCNQ1 to a nearby KCNE1, especially in the case of V141M, subunit if the assembled channel is to be characterized by the key pathological phenotype: markedly slowed deactivation. In heterozygote patients, the number of KCNQ1 subunits carrying the disease causing mutation will vary from 1 to 4 in assembled channels, and thus alter the potential contributions of KCNE1/KCNQ1 interactions underlying arrhythmia risk. Similarly, if, in fact KCNE1/KCNQ1 stoichiometry is variable and may even vary during disease, then the severity of the disease phenotype, either for S140G or V141M mutation carriers, will be highly variable. Further, by this mechanism, chamber specific variation in KCNE1 may well play a role in these mutations predominant effect occurring in the atria.

In this chapter we find that neighboring AF mutations in KCNQ1 arrive at the same channel defect by two different pathways. For the S140G mutation, there are dramatic effects on KCNQ1 channel deactivation in the absence of KCNE1. However, for the V141M mutation, no change in channel behavior is observed without KCNE1 present. Biochemical crosslinking data also demonstrate that residue V141 is positioned close to and is facing KCNE1, revealing a
specific orientation of the KCNQ1 and KCNE1 subunits. We also demonstrate that the location of KCNE1 within a heterozygous channel complex impacts the severity of the mutant phenotype. Taken together, our results implicate a physiologically important interaction between KCNE1 and KCNQ1 S1 affecting the rate of channel deactivation in a mutation specific manner, underlying heritable cardiac disease.

Finally, we begin to uncover the mechanism underlying the mutant phenotype: slow deactivation kinetics. Since S1 is part of the voltage sensing domain and interacts closely with helicities which undergo voltage sensitive movements, previous studies have hypothesized that S140G and V141M directly affect the energetics of the position of the voltage sensor, promoting the activated over the resting state. Here, using voltage clamp fluorometry, we have shown that indeed the transition from the activated to the resting state of the voltage sensor is slowed by the presence of these mutations and KCNE1, and for the S140G mutation in KCNQ1 alone. Though this data supports the hypothesis that the activated state of the voltage sensor is relatively stabilized by the presence of these mutations, further experiments are needed to prove this mechanism, since indirect slowing of voltage sensor relaxation is also possible. Despite this caveat, these experiments provide the first measurement of voltage sensor movement that is perturbed by disease-causing mutations in KCNQ1, and provides a basis for further studies to firmly establish the molecular basis of this voltage sensor slowing.
Conclusions

This thesis focuses on the biophysics of KCNQ1 channel in order to elucidate gating mechanisms underlying this critical molecular machine. In the process, we have uncovered a novel gating scheme among Kv channels and gained insight into how certain disease-associated mutations alter channel biophysics to cause disease. In chapter 1, we develop a new voltage sensor assay for the KCNQ1 channel based on voltage clamp fluorometry (VCF). We first validate the use of this assay for the study of the KCNQ1 voltage sensor and then perform an initial characterization of voltage sensor movement in KCNQ1 expressed alone. Surprisingly, we find that voltage sensor movement and channel opening seem to display a 1:1 relationship, as if only one voltage sensor controls opening of the gate. This can be clearly seen by comparing the voltage dependences of conductance and voltage sensor movement in these channels, which are identical. This is a surprising result in light of the known stoichiometry of voltage sensors to pore of 4:1. To explain this result, we hypothesize that either all four KCNQ1 voltage sensors move in unison, or the channel transitions to the open state before all voltage sensors have moved. We next turn to the effect of KCNE1 co-expression on the voltage sensor. Remarkably, we find that in the presence of KCNE1, the voltage dependent movement of the voltage sensor takes place over a very wide range of voltages extending down to very negative voltages around -200 mV despite the fact that KCNQ1-KCNE1 channels do not begin conducting current until much more depolarized potentials, around -40 mV. Employing a classic electrophysiological assay called the Cole-Moore experiment, we find that these very negative voltage sensor movements do in fact impact channel gating. Taken together, the results from chapter 1 show that both KCNQ1 and KCNQ1-KCNE1 channels employ unique voltage-dependent gating mechanisms. In order to model these mechanisms, we use a typical framework for potassium channel gating and constrain
this model using our data. While KCNQ1/KCNE1 is well fit by a model that requires each voltage sensor to undergo multiple voltage dependent movements on the way to an opening step, KCNQ1 alone requires that states along the way are conducting: in this case modeled as each voltage sensor transition opening a fractional channel conductance, or subconductance state.

In order to begin addressing the nature of the coupling between voltage sensors and the channel gate in KCNQ1, in Chapter 2, we focus on gating of KCNQ1 alone by addressing the nature of the 1:1 relationship between voltage sensor movement and channel opening. We first address the hypothesis that voltage sensors move together to activate the KCNQ1 channel. We first rule out concerted movement of a channel’s voltage sensors by establishing a lack of cooperativity between them. We next explore the possibility that KCNQ1 channel transition to the open state before all voltage sensors move and find that indeed channels with fewer than four activated voltage sensors can open. In order to explain this gating mechanism we explore other gating mutations and find that in one mutant, channels can open even in the absence of voltage sensor movement. These results lead us to propose an allosteric gating scheme for KCNQ1 channels, wherein channels can open with 0-4 activated voltage sensors, with successive voltage sensor movement promoting opening. This gating scheme provides a framework not only for understanding the gating of KCNQ1 channels, but also for interpreting the effect of beta subunit modulation of the channel. This model is necessarily distinct from the model frameworks explored in Chapter 1, since our data suggests that channels can open in the absence of voltage sensor movement, which is not allowed in these models. This allosteric gating scheme provides the flexibility necessary for KCNQ1 channels to gate in very different ways, based on the relative strength of transitions between various states of voltage sensors and the closed or
conducted state of the channel gate. These rates may be significantly altered by the presence of different beta subunits, but also by mutations implicated in human disease.

Chapter 3 is concerned with addressing the effects on the voltage sensor of two mutations associated with inherited atrial fibrillation which have the effect of drastically slowing deactivation. These two mutations lie within the voltage sensing domain of the KCNQ1 channel, and previous studies hypothesize they alter channel deactivation by affecting movement of the voltage sensor specifically during deactivation. First, using electrophysiology, we establish a differential dependence of the mutation-induced effect on the presence of KCNE1. Next, we correlate this differential dependence with the relative position of these mutant residues with respect to KCNE1: the mutation for which a functional defect requires KCNE1 co-assembly lies at a position that is shown to be in close proximity to KCNE1 while the mutation which affects channel function in the absence of KCNE1 does not appear to interact with KCNE1, and instead this position may interact more directly with the voltage sensor. We next perform voltage clamp fluorometry on KCNQ1 channels expressing mutations in the presence and absence of KCNE1. We find that in KCNQ1 alone, the mutation that does not affect function of the channel similarly has no affect on the voltage sensor, while the mutation that changes deactivation of KCNQ1 alone indeed alters the voltage sensor movement preferentially during deactivation. In the presence of KCNE1, we find that voltage sensor relaxation is dramatically slowed by both mutations, while voltage sensor activation is spared. These results are consistent with the hypothesis that these mutations exert their effects by slowing the equilibration of the voltage sensor during channel deactivation.

By developing an assay for the movement of the KCNQ1 voltage sensor, we have been able to address longstanding questions related to the unique gating of this channel, its regulation
by beta subunits, and the effects of disease-causing mutations on its gating. In the process, we have uncovered many more questions, and continue to find the KCNQ1 channel enigmatic, compelling and at times frustrating. Notwithstanding, this work provides insight into some of mysteries of this channel and will serve as a stepping-stone towards a more complete understanding of its critical function.
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