Mechanistic basis for LQT1 caused by S3 mutations in the KCNQ1 subunit of \( I_{\text{Ks}} \)

Jodene Eldstrom, Hongjian Xu, Daniel Werry, Congbao Kang, Matthew E. Loewen, Amanda Degenhardt, Shubhayan Sanatani, Glen F. Tibbits, Charles Sanders, and David Fedida

1Department of Anesthesiology, Pharmacology and Therapeutics, and 2Department of Pediatrics, Children and Women’s Hospital, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada
3Department of Biochemistry, Vanderbilt University, Nashville, TN 37235
4Department of Biomedical Physiology and Kinesiology, Simon Fraser University, Burnaby, British Columbia V5A 1S6, Canada

Long QT interval syndrome (LQTS) type 1 (LQT1) has been reported to arise from mutations in the S3 domain of \( KCNQ1 \), but none of the seven S3 mutations in the literature have been characterized with respect to trafficking or biophysical deficiencies. Surface channel expression was studied using a proteinase K assay for \( KCNQ1 \) D202H/N, I204F/M, V205M, S209F, and V215M coexpressed with \( KCNE1 \) in mammalian cells. In each case, the majority of synthesized channel was found at the surface, but mutant \( I_{\text{Ks}} \) current density at +100 mV was reduced significantly for S209F, which showed \( \sim 75\% \) reduction over wild type (WT). All mutants except S209F showed positively shifted \( V_{1/2} \)’s of activation and slowed channel activation compared with WT (\( V_{1/2} = +17.7 \pm 2.4 \text{ mV and } \tau_{\text{activation}} \) of 729 ms at +20 mV; \( n = 18 \)). Deactivation was also accelerated in all mutants versus WT (126 \( \pm \) 8 ms at \( -50 \text{ mV; } n = 27 \)), and these changes led to marked loss of repolarizing currents during action potential clamps at 2 and 4 Hz, except again S209F. \( KCNQ1 \) models localize these naturally occurring S3 mutants to the surface of the helices facing the other voltage sensor transmembrane domains and highlight inter-residue interactions involved in activation gating. V207M, currently classified as a polymorphism and facing lipid in the model, was indistinguishable from WT \( I_{\text{Ks}} \). We conclude that S3 mutants of \( KCNQ1 \) cause LQTS predominantly through biophysical effects on the gating of \( I_{\text{Ks}} \), but some mutants also show protein stability/trafficking defects, which explains why the kinetic gain-of-function mutation S209F causes LQT1.

INTRODUCTION

Long QT interval syndrome (LQTS) is a potentially fatal condition, characterized by a prolonged QT interval (usually defined as longer than 440 ms for males and 460 ms for females on the electrocardiogram; Rossenbacker and Priori, 2007) and the development of serious arrhythmias (ventricular tachycardia, torsade de pointes, which may degenerate into ventricular fibrillation). Hereditary LQTS is predominantly caused by mutations in \( KCNQ1 \), the \( \alpha \) subunit for the slow delayed rectifier potassium channel, \( I_{\text{Ks}} \) (Sanguinetti et al., 1996). This mutation is present with a high prevalence, perhaps as high as 1:200, in an isolated Northern Canadian population that suffers a high incidence of sudden arrhythmic death (Arbour et al., 2008). V205M is not the only mutation associated with LQT1 found in the S3 domain of \( KCNQ1 \) (Inherited Arrhythmias Database [IAD]: http://www.fsm.it/cardmoc/), which also includes D202H/N, I204F/M, S209F, and V215M (Yamaguchi et al., 2003; Napolitano et al., 2005; Tester et al., 2005), but to our knowledge, none of these have been functionally characterized. Here, we studied all reported S3 mutations in \( KCNQ1 \) in the presence of \( KCNE1 \) electrophysiologically to better understand their role in the generation of LQTS in affected individuals. We have recently identified a novel missense mutation, V207M, in the S3 transmembrane region of \( KCNQ1 \), the \( \alpha \) subunit for the slow delayed rectifier potassium channel, \( I_{\text{Ks}} \) (Sanguinetti et al., 1996). This mutation is present with a high prevalence, perhaps as high as 1:200, in an isolated Northern Canadian population that suffers a high incidence of sudden arrhythmic death (Arbour et al., 2008). V205M is not the only mutation associated with LQT1 found in the S3 domain of \( KCNQ1 \) (Inherited Arrhythmias Database [IAD]: http://www.fsm.it/cardmoc/), which also includes D202H/N, I204F/M, S209F, and V215M (Yamaguchi et al., 2003; Napolitano et al., 2005; Tester et al., 2005), but to our knowledge, none of these have been functionally characterized. Here, we studied all reported S3 mutations in \( KCNQ1 \) in the presence of \( KCNE1 \) electrophysiologically to better understand their role in the generation of LQTS in affected individuals. In addition, we sought to resolve the issue of whether V207M, also in the S3 domain, should remain as a polymorphism or be characterized as a mutation.
reclassified as pathogenic. Originally, this mutation was found in one individual as part of a screen of 744 apparently healthy subjects (Ackerman et al., 2003). Subsequently, the same mutation was found in an individual during a screen of sudden unexplained death (Nishio et al., 2009). In mice homozygous for this mutation, the QT interval was significantly longer than in the wild-type (WT) mice, but no mention was made of whether arrhythmias or sudden unexplained death were observed in these animals. In the present study, we distinguished between mutations giving rise to LQTS primarily due to deficiencies in surface expression from those where LQTS was most likely to be caused by effects on KCNQ1 channel gating and kinetics and functional $I_{Ks}$ currents.

The role and movement of the S3 helices within the voltage sensor are generally considered along with those of S4. From the recent crystal structure of Kv1.2 (Long et al., 2005), it is predicted that during activation, the voltage-sensing domain undergoes conformational changes with translation and rotation of S4 coupled with movement of the S1 to S3 helices around S4 (Yarov-Yarovoy et al., 2006). This is thought to allow for sequential interactions between basic residues in S4 and acidic residues in the S2 and S3 helices, which stabilize the charged residues in the hydrophobic environment of the membrane (Papazian et al., 1995; Tiwari-Woodruff et al., 2000). In addition to allowing for more sophisticated predictions for Kv channel activation, these crystal structures of Kv1.2 have enabled the generation of open- and closed-state models of KCNQ1, onto which many disease-causing mutations have been mapped (Smith et al., 2007) and KCNE1 interaction modeled (Kang et al., 2008). After we completed our electrophysiological analysis, the KCNQ1 models were used to examine the structural context of the residues in S3 that lead to LQTS and the other amino acid substitutions that we made at those sites. Modeling studies have informed the role of residue size, hydrophobicity, and charge on gating effects of S3 structures, as well as inter-residue van der Waals interactions and potential hydrogen bond formation. We suggest that such interactions between residues in S3 with those in S2 and S4 have significant effects on both closed- and open-state stability of the KCNQ1 channels and the resultant $I_{Ks}$ currents, and that these changes have an important role in the causation of LQTS in affected individuals.

### MATERIALS AND METHODS

#### Molecular biology

$KCNQ1$ and $KCNE1$ genes were purchased from OriGene Technologies, Inc. Mutations within the third transmembrane (S3) region of the $KCNQ1$ gene were constructed using two-step PCR reactions and sequenced to verify the presence of only the desired mutation.

#### Cell preparation and transfection

Electrophysiology was performed on transiently transfected mouse $ltk$- cells grown in MEM with 10% fetal bovine serum at 37°C in an air/5% CO$_2$ incubator. 1 d before transfection, cells were plated onto sterile glass coverslips in 35-mm Petri dishes with 20–30% confluence. On the day of transfection, cells were washed once with MEM with 10% fetal bovine serum. To identify the transfected cells efficiently, channel DNA was cotransfected with a vector encoding green fluorescent protein using the following DNA ratios: 1:2.5:1 in µg of $KCNQ1$|$KCNE1$|$GFP$ and LipofectAMINE 2000 (Invitrogen). In some experiments, a mouse $ltk$- $KCNE1$ stable line was used to express mutant and WT $KCNQ1$ channels. Cells were allowed to grow overnight before recording.

#### Electrophysiological procedures

Coverslips containing cells were removed from the incubator before experiments and placed in a superfusion chamber (volume of 250 µl) containing the control bath solution at 35°C and perfused continuously with bathing solution heated to 35°C by an inline heater (Warner Instruments). Whole cell current recording and data analysis were done using an Axopatch 200B clamp amplifier and pClamp 8 and 10 software (MDS Analytical Technologies). Patch electrodes were fabricated using thin-walled borosilicate glass (World Precision Instruments, Inc.). Electrodes had resistances of 1–2 MΩ when filled with control filling solution. Capacity compensation and 80% series resistance compensation were used in all whole cell recordings. No leak subtraction was used when recording currents, and 0 current levels are denoted by the dashed lines in the current panels. Data were sampled at 10 kHz and filtered at 2 kHz. Membrane potentials have not been corrected for small liquid junction potentials between bath and pipette solutions. Action potential (AP) protocol was generated using LabHEART version 4.9.5.

### Table 1

$LQT$-associated mutants in the IAD

| Nucleotide | Amino acid | Mutation type | Region | Phenotype | Reference |
|------------|------------|---------------|--------|-----------|-----------|
| G604C      | D202H      | Missense     | S3     | RWS       | Napolitano et al., 2005 |
| G604A      | D202N      | Missense     | S3     | RWS       | Yamaguchi et al., 2003 |
| A610T      | I204F      | Missense     | S3     | RWS       | Tester et al., 2005    |
| G612G      | I204M      | Missense     | S3     |           | Napolitano et al., 2005 |
| G620T      | S209F      | Missense     | S3     |           | Napolitano et al., 2005 |
| G643A      | V215M      | Missense     | S3     |           | Napolitano et al., 2005 |

Amino acid numbering is based on Gene Bank cDNA:AF000571 and nucleotide numbering starts from ATG. RWS, Romano-Ward syndrome.
Significance was tested using Student’s unpaired t test or one-way ANOVA as appropriate.

To compare current densities at the respective $V_{1/2}$ for each mutant to that of WT (see Table II and Fig. 3 A), a correction factor was used to take into account the difference in driving force (DF) at each $V_{1/2}$. The current density (CD) at the $V_{1/2}$ for each mutant was corrected for driving force as follows. Corrected CD = CD at $V_{1/2} × (DF_{WT}/DF_{Mt})$, where DF = ($V_{1/2} − E_{rev}$). The reversal potential ($E_{rev}$) used was $−87.5 \text{ mV}$. An additional comparison of CD between mutants was made at $+100 \text{ mV}$, where the $P_o$ of channels is ≈1.0. These data are shown in Table II and are not corrected for DF.

Proteinase K and Western blot analysis

T7-tagged WT and mutant $KCNQ1$ channels were expressed in a $KCNE1$-expressing mouse $ltk^−$ stable line. At 24 h, cells from half the flask were removed by scraping and the remainder were exposed to proteinase K as described previously (Manganas and Trimmer, 2000) to cleave any surface-expressed channel. Total protein was determined using a modified Lowry assay (Thermo Fisher Scientific), and equal amounts of total protein from proteinase K–untreated and −treated samples were loaded onto SDS-PAGE gels and probed with anti-$KCNQ1$ antibodies (Santa Cruz Biotechnology, Inc.).

Figure 1. Reported LQT1-associated S3 mutants traffic to the plasma membrane in $ltk^−$ cells. (A) Western blot of WT and mutant $KCNQ1$ channels were expressed in a $KCNE1$-expressing mouse $ltk^−$ stable line. At 24 h, cells from half the flask were removed by scraping and the remainder were exposed to proteinase K as described previously (Manganas and Trimmer, 2000) to cleave any surface-expressed channel. Total protein was determined using a modified Lowry assay (Thermo Fisher Scientific), and equal amounts of total protein from proteinase K–untreated and −treated samples were loaded onto SDS-PAGE gels and probed with anti-$KCNQ1$ antibodies (Santa Cruz Biotechnology, Inc.).

**A**

|          | GFP | Wt | D202H | D202N | I204F | I204M | V205M | S209F | V215M |
|----------|-----|----|-------|-------|-------|-------|-------|-------|-------|
| PK       | -   | +  | -     | -     | -     | -     | -     | -     | +     |
| **KCNQ1**|     |    |       |       |       |       |       |       |       |
| Actin    |     |    |       |       |       |       |       |       |       |

**B**

**Closed**

|          | V215 | S209 | V205 | I204 | D202 |
|----------|------|------|------|------|------|

**Open**

|          | V215 | S209 | V205 | I204 | D202 |
|----------|------|------|------|------|------|
436 S3 LQT1 mutations modulate gating of \(i_{Ks}\)

Molecular modeling of WT and mutant channels
As described previously (Smith et al., 2007), models for the open and closed states of the channel domain (S1–S6) of human KCNQ1 were developed using a combination of homology-based modeling (using available Kv1.2 structures as templates) and ROSETTA-based structure prediction (Das and Baker, 2008) to define segments that could not be well-defined based on the Kv1.2 structures. These models are available online from Smith et al. (2007).

Models for the mutant channels were developed using the WT models noted above as the starting point and using RosettaDesign (Kuhlman and Baker, 2000). The mutated channels were then subjected to five cycles of side chain repacking and gradient energy minimization, by which point the energy and coordinates of the structures converged. Mutants were typically analyzed using “contact analysis” in which inter-residue van der Waals interactions involving a designated (often mutated) site were assessed by determining which other residues in the protein have at least

Table II

| Variant | \(V_{1/2}\) mV | 0 mV | 20 mV | \(n\) | 100 mV | \(n\) |
|---------|---------------|------|-------|-----|--------|-----|
| WT      | 17.7 ± 2.4    | 92.1 ± 15.4 | 215.3 ± 25.4 | 215.3 ± 25.4 | 47 | 488.3 ± 52.8 | 25 |
| D202H   | 34.3 ± 5.3    | 35.4 ± 7.0   | 88.9 ± 16.0   | 104.3 ± 18.6\* | 24 | 311.7 ± 56.2 | 15 |
| D202N   | 41.5 ± 2.9    | 9.4 ± 1.8    | 43.9 ± 9.3    | 98.6 ± 18.6\* | 20 | 326.6 ± 59.7 | 14 |
| I204F   | 71.0 ± 3.9    | 24.2 ± 11.4  | 49.8 ± 17.1   | 172.6 ± 32.8  | 22 | 424.1 ± 64.2 | 12 |
| I204M   | 55.8 ± 2.6    | 21.2 ± 7.2   | 72.8 ± 16.3   | 157.9 ± 26.2  | 21 | 337.3 ± 46.1 | 16 |
| V205M   | 37.0 ± 5.4    | 26.8 ± 4.4   | 78.4 ± 11.5   | 134.7 ± 17.8\* | 38 | 344.6 ± 56.5 | 21 |
| S209F   | −31.0 ± 2.3   | 59.1 ± 9.1   | 75.7 ± 11.4   | 54.1 ± 8.3\*  | 25 | 121.4 ± 17.8\* | 17 |
| V215M   | 37.9 ± 4.3    | 18.8 ± 3.9   | 82.7 ± 10.0   | 172.0 ± 41.5  | 22 | 450.1 ± 84.8 | 17 |
| V207M   | 24.8 ± 2.9    | 85.7 ± 17.6  | 200.1 ± 32.3  | 200.1 ± 32.3  | 20 | 486.9 ± 51.7 | 17 |

Current densities at \(V_{1/2}\) for each mutant channel were corrected for difference in driving force from WT (see Materials and methods). Only current densities at \(V_{1/2}\) and 100 mV were tested for significance.

\(^*P < 0.05\) compared to WT.
clamp experiments (Table II). Current densities were calculated at four potentials: at the $V_{1/2}$ of activation, as measured from 2-s activation relationships after correction for driving force (see Materials and methods and Fig. 3 A), and at 0 and +20 mV, which are two potentials relevant to the plateau of the cardiac AP, where changes in current density and gating are likely to have the most significant effects on repolarization. In addition, current

**RESULTS**

LQTS due to trafficking versus biophysical defects

The IAD lists several mutations in the S3 domain (which comprises approximately amino acids Ile 198 to Val 215) of KCNQ1 that have been linked to LQTS (Table I). These mutations span the entire length of the S3 domain (Figs. 1 B and 2), with six of the seven (including V205M; Arbour et al., 2008) predicted to line one face of the S3 helix in an open-state homology model of KCNQ1 (Smith et al., 2007). In addition, the IAD lists one S3 polymorphism, V207M, which in our models is predicted to face lipid (Fig. 2). We were interested in studying this mutant not only as it represented an opportunity to test the KCNQ1 model, but also, as noted previously, the literature was inconsistent as to the proper classification, polymorphism or pathogenic (Ackerman et al., 2003; Nishio et al., 2009).

Initially, we wanted to explore the possibility that these mutations lead to trafficking defective protein, so T7-tagged WT and mutant KCNQ1 channels were expressed in a KCNE1 (E1)-expressing mouse $ltk^{-}$ stable line. At 24 h, cells from half the flask were removed by scraping and the remainder exposed to proteinase K to cleave any surface-expressed channel. Western blots of untreated and proteinase K–treated samples show that WT and all mutant channels, D202H/N, I204F/M, V205M, S209F, and V215M, express significantly at the surface, as the majority of the 70-Kd KCNQ1 band is cleaved by proteinase K (Fig. 1 A). Densitometric analysis revealed that, normalized to actin, 10–25% of mutant protein was not digested by proteinase K, compared with 27% of WT protein ($n=3$ and $P=NS$), suggesting that the S3 mutations do not significantly affect channel trafficking per se. Although the cell surface ratio of the S209F mutant was similar to WT, this mutant consistently showed lower levels of total protein, suggesting that perhaps protein stability was an issue for this mutant. The Western blot data were generally supported by current density measurements from patch
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Unlike WT (Fig. 3 B), and this was accompanied by a positive shift in the $V_{1/2}$ of activation of 17 mV from WT (Fig. 3 C). The combination of the positive displacement of the activation relationship and the lower overall expression (Fig. 3 and Table II) resulted in much reduced peak currents in D202H in the physiological range of plateau potentials, 0 and +20 mV (Table II and Fig. 3 A).

In addition to modulating $I_{Ks}$ channel activation properties, the D202H mutation accelerated the rate of channel deactivation compared with WT (Fig. 4). The time constant of deactivation was determined by applying hyperpolarizing steps after a prepulse to +80 mV and fitting the tail currents between $-40$ and $-100$ mV to single-exponential functions (Fig. 4 B). It is clear that the D202H mutation accelerated deactivation at all the potentials tested compared with WT channels. These data suggest that the D202H mutation reduces $I_{Ks}$ channel availability by shifting the voltage dependence of activation to more depolarized potentials, in addition to moderately slowing activation at positive potentials and accelerating the rate of channel deactivation.

The electrophysiological data from a scan of other mutations at D202 are shown in Table III, along with residue properties such as volume, hydrophobicity, and charge. Charge reversal, as in D202H or D202K, resulted in channels that did not express as well as WT (Table S1) and suffered similar depolarizing shifts in...
the $V_{1/2}$ of activation to about +40 mV, without much change in the activation time constants at either +20 mV or at the $V_{1/2}$ activation, and a clearly accelerated deactivation. It is less obvious why the D202E charge-conserving mutation results in such an extreme shift in the $V_{1/2}$ activation (to +72 mV) and accelerated deactivation. The other naturally occurring mutant at this position in S3 results in neutralization of the charge, D202N. This mutation results in a channel that could express almost as well as WT (Table II) and has the effect of accelerating activation and showing extremely rapid deactivation kinetics (Table III). These data suggest that stability of both the open and the closed states may have been affected by the loss of charge (see below). In addition, in this mutant as was seen with D202H (Figs. 3 B and 4 B), both activation and deactivation curves have relatively little apparent voltage dependence (not depicted).

Representative data and summary tables for the electrophysiological properties of the other naturally occurring LQTS mutants, V205M, S209F, V215M, V207M, and I204F/M, are also shown in Figs. 2–4, Table III, and Fig. S1, as well as properties of other substitutions made at these sites. The mean changes in the activation and deactivation time constants relative to control are shown in Fig. 5. It can be seen that naturally occurring mutations, as well as those introduced during the study into the middle of the helix, at V205 and I204 cause the most slowing of channel activation, whereas the mutations toward the proximal and distal ends of S3, at D202 and V215, either have little effect on activation or speed it somewhat. In contrast, all the mutations, with the exception of those at S209, I204A, and V215A, accelerate deactivation, often substantially as seen at D202, V205, and V215. Little kinetic effect of the V207M mutation is apparent. All of the naturally occurring mutations except V207M and S209F caused a positive shift of the $V_{1/2}$ activation from WT, with values ranging from +37 and +38 mV in V205M and V215M, respectively, like the D202 mutants, to more extreme values of +54 and +71 mV in the I204M and I204F mutants, respectively (Fig. 5). At I204, mutations and substitutions (I204A/N) the $V_{1/2}$ of activation to about +40 mV, without much change in the activation time constants at either +20 mV or at the $V_{1/2}$ activation, and a clearly accelerated deactivation. It is less obvious why the D202E charge-conserving mutation results in such an extreme shift in the $V_{1/2}$ activation (to +72 mV) and accelerated deactivation. The other naturally occurring mutant at this position in S3 results in neutralization of the charge, D202N. This mutation results in a channel that could express almost as well as WT (Table II) and has the effect of accelerating activation and showing extremely rapid deactivation kinetics (Table III). These data suggest that stability of both the open and the closed states may have been affected by the loss of charge (see below). In addition, in this mutant as was seen with D202H (Figs. 3 B and 4 B), both activation and deactivation curves have relatively little apparent voltage dependence (not depicted).

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| Variant | Volume | Hydrophobicity | $\tau_{act}$ at $V_{1/2}$ | $\tau_{deact}$ | $V_{1/2}$ of activation | Slope |
|---------|--------|----------------|--------------------------|---------------|--------------------------|-------|
|         | $\AA^2$ |                | 20 mV                    | -50 mV        |                         |       |
| D202    | 111.1  | -3.5           | Negative                 | 729           | 126                      | 17.7 ± 2.4 | 17.2 ± 1.0 |
| D202N   | 114.1  | -3.5           | Neutral                  | 400           | 11                        | 41.5 ± 2.9 | 14.0 ± 1.5 |
| D202H   | 153.2  | -3.2           | Pos/Neu                  | 607           | 33                       | 34.3 ± 5.3 | 14.5 ± 2.0 |
| D202A   | 88.6   | 1.8            | Neutral                  | 644           | 44                       | 29.8 ± 2.9 | 20.0 ± 2.2 |
| D202E   | 138.4  | -3.5           | Negative                 | 728           | 42                       | 72.3 ± 3.4 | 17.2 ± 1.9 |
| D202M   | 162.9  | 1.9            | Neutral                  | 738           | 28                       | 41.2 ± 2.9 | 14.6 ± 1.5 |
| D202K   | 168.6  | -3.9           | Positive                 | 878           | 61                       | 39.5 ± 3.9 | 11.5 ± 1.4 |
| I204    | 166.7  | 3.8            |                         | 729           | 126                      | 17.7 ± 2.4 | 17.2 ± 1.0 |
| I204M   | 162.9  | 1.9            |                         | 845           | 82                       | 53.8 ± 2.6 | 16.2 ± 0.6 |
| I204F   | 189.9  | 2.8            |                         | 5,287         | 54                       | 71.0 ± 3.9 | 18.5 ± 2.0 |
| I204A   | 88.6   | 1.8            |                         | 1,13           | 170                      | 36.4 ± 3.3 | 12.0 ± 1.1 |
| I204N   | 114.1  | -3.5           |                         | 1,802         | 88                       | 50.6 ± 9.8 | 16.0 ± 1.9 |
| V205    | 140    | 4.2            |                         | 729           | 126                      | 17.7 ± 2.4 | 17.2 ± 1.0 |
| V205M   | 162.9  | 1.9            |                         | 1,081         | 53                       | 37.0 ± 5.4 | 17.3 ± 2.2 |
| V205L   | 166.7  | 3.8            |                         | 333           | 75                       | 0 ± 3.3 | 9.7 ± 0.6 |
| V205A   | 88.6   | 1.8            |                         | 915           | 52                       | 68.8 ± 7.7 | 12.3 ± 2.0 |
| S209    | 89     | -0.8           |                         | 729           | 126                      | 17.7 ± 2.4 | 17.2 ± 1.0 |
| S209F   | 189.9  | 2.8            |                         | 496           | 430                      | -31.0 ± 2.3 | 14.9 ± 1.2 |
| S209M   | 162.9  | 1.9            |                         | 617           | 420                      | -22.6 ± 3.7 | 15.0 ± 0.9 |
| S209A   | 88.6   | 1.8            |                         | 295           | 302                      | -16.3 ± 3.9 | 15.0 ± 1.2 |
| S209L   | 166.7  | 3.8            |                         | 803           | 352                      | -30.1 ± 4.7 | 15.7 ± 2.3 |
| V215    | 140    | 4.2            |                         | 729           | 126                      | 17.7 ± 2.4 | 17.2 ± 1.0 |
| V215M   | 162.9  | 1.9            |                         | 448           | 55                       | 37.9 ± 4.3 | 14.4 ± 0.6 |
| V215A   | 88.6   | 1.8            |                         | 501           | 331                      | 8.4 ± 2.3 | 14.0 ± 1.0 |
| V215L   | 166.7  | 3.8            |                         | 459           | 85                       | 27.9 ± 4.3 | 13.6 ± 1.5 |
| V215P   | 112.7  | -1.6           |                         | 593           | 29                       | 55.7 ± 4.4 | 12.2 ± 0.9 |

Hydrophobicity was based on the Kyte Doolittle scale, and other values were obtained as indicated in the legend to Fig. 2.

*Significant difference from WT (P < 0.01).

*Significant difference from WT (P < 0.05).

$^a$ $\tau_{act}$ for I204F activation was measured at 6 s rather than 2 s due to this process being very slow. Only $V_{1/2}$ values were tested for significance.
S209F is a gain-of-function mutant

Of the naturally occurring mutants in S3, S209F is a much bulkier and more hydrophobic residue than the WT serine, and it produced dramatic changes in channel kinetics (Figs. 3 and 4). Overall, current expression was reduced in this mutant (Fig. 3 A and Table II), but most interesting was the marked hyperpolarizing shift of channel activation ($V_{1/2} = -31.0$ mV; Figs. 3 C and 5). As a result of this shift, the activation time constants were accelerated at physiological potentials, but overall, opening was slowed as suggested by the greatly delayed activation time course at the $V_{1/2}$ activation (time constant was 1.9 s vs. 730 ms in WT). The deactivation was extremely slow for S209F, at $\leq 400$ ms, and was quite voltage independent (see Fig. 4 B). Similar kinetic properties were seen in the other substitutions at S209, with all showing $V_{1/2}$’s of activation in the negative range of potentials (Fig. 5) and very slow deactivation (Fig. 5 and Table III). Only S209A showed more normal activation kinetics, with activation time constants close to WT at the $V_{1/2}$, and it had a $V_{1/2}$ activation closer to the WT than other bulkier substitutions (Table III). Activation time constants were much faster when S209F was expressed alone (87.6 ± 5.0 ms at +20 mV compared with 496 ms for S209F+KCNE1; Fig. 6), despite the fact that the voltage dependence of activation was relatively unaffected by coexpression of S209F with KCNE1 (Fig. 6 C). The clear slowing of the activation time course (Fig. 6 A) and the slowed time constants indicate that the negative shift in activation was not a result of failure to coassemble with KCNE1.
higher up S3, like S209 and V215. To better understand the contribution of $I_{KS}$ with the S3 mutants to repolarization, currents were recorded from WT $I_{KS}$ and the naturally occurring mutant $I_{KS}$ channels D202N and S209F KCNQ1+E1 during simulated ventricular APs (Figs. 7 and 8). The clamp protocol (Fig. 7 A) at 2 Hz produced $I_{KS}$ currents during simulated cardiac APs.

Overall, these findings suggested that charge and volume were important regulators of activation and deactivation gating at D202 and hydrophobicity at V205 in the S3 of KCNQ1, but that size and/or polar/hydrophobicity were important regulators of gating at residues higher up S3, like S209 and V215. To better understand the contribution of $I_{KS}$ with the S3 mutants to repolarization, currents were recorded from WT $I_{KS}$ and the naturally occurring mutant $I_{KS}$ channels D202N and S209F KCNQ1+E1 during simulated ventricular APs (Figs. 7 and 8). The clamp protocol (Fig. 7 A) at 2 Hz produced...
a bell-shaped WT $I_{Ks}$ waveform in channels with relatively slow activation kinetics and a peak of ionic current during the repolarization phase of the AP. During the second and subsequent APs, currents were potentiated (Fig. 7 B) and the total charge movement, estimated by integrating currents during the AP (Fig. 7 D), increased by $\sim 40\%$ during pulsing to a steady state, compared with the first. In sharp contrast, D202N channels displayed little detectable ionic current due to $I_{Ks}$ during the AP (Fig. 7 C), with most of the current comprising leak currents; thus, total charge during the simulated AP was effectively 0% of that for WT channels.

The shifted $V_{1/2}$ of activation, slowed activation, and the accelerated deactivation in the D202N mutant all reduced the number of open $I_{Ks}$ channels during rapid AP firing. At 4 Hz, in the WT channels, the current amplitude at the beginning of the pulse was increased, as was the overall current envelope (Fig. 7 E), and the outward charge movement ($+338 \pm 7\%; n = 12$) increased from the first pulse (Fig. 7 G). In cells expressing D202N $I_{Ks}$, there were only small currents during APs (Fig. 7 F), and the outward charge did not increase at 4 Hz (Fig. 7 G; $n = 7$).

Similar results were found for the D202H, I204M/F, V205M, and V215M mutants (not depicted), as expected from their slowed activation and accelerated deactivation kinetics shown in prior figures. However, it was of interest to examine the effect of AP clamps on the S209F gain-of-function mutant (Fig. 8). Here, it is apparent that the rapid activation kinetics in the plateau range of potentials and the persistent activation can give rise to a significant potentiation of currents during 2-Hz stimulation (Fig. 8 C) compared with WT (Fig. 8 B). The rapid S209F kinetics also change the shape of currents during the AP so that it closely matches the waveform shape, rather than developing during the plateau and repolarization like the WT $I_{Ks}$. At 4 Hz, S209F $I_{Ks}$ currents summate much like they do at 2 Hz and like WT channels, so that the normalized charge is much the same in WT and mutant channels at high stimulus rates (Fig. 8 G). Notably, the shortened interpulse interval at 4 Hz negates the more rapid deactivation in WT channels compared with S209F. Still, the more rapid activation of S209F channels gives a current waveform biased to more depolarized potentials early in phases 1 and 2 of the AP, rather than phase 3, where the dominant repolarization current appears in the WT channels.

The V207M polymorphism, which shows no significant kinetic difference from WT $I_{Ks}$, is indistinguishable from WT $I_{Ks}$ during simulated AP clamps (Fig. S2).
DISCUSSION

S3 mutant subunits of I\textsubscript{\textkat Grip} express well at the cell surface

The majority of KCNQ1 mutations produce a loss of function by disrupting protein folding, assembly, and/or trafficking to the membrane (Shalaby et al., 1997; Dahimène et al., 2006). In our cell expression system, all the naturally occurring mutations expressed at the surface as determined by the proteinase K assay, with no significant changes in the surface/intracellular protein ratios. At +100 mV, only the S209F mutation showed a significantly decreased expressed current density over WT, although reduced current densities at the \( V_{1/2} \) of activation were found electrophysiologically for D202H/N, V205M, and S209F (Table II). It may be that the very slow activation in the V205M and D202H/N mutants (Figs. 2 and 3) leads to incomplete activation of currents at the most positive potentials, an underestimation of the \( V_{1/2} \) of activation in these mutants, and an apparently reduced current density at the \( V_{1/2} \). However, no mutation at the S3 sites, in addition to those reported in the IAD, resulted in channels with WT kinetic properties. This suggests that the major defects with the remaining LQTS-associated S3 mutants are kinetic, although we cannot rule out the possibility that trafficking efficiency is reduced in the native myocyte, perhaps with a larger proportion of channels undergoing ER-associated degradation due to increased folding times (Ellgaard and Helenius, 2003).

Altered kinetics of S3 mutants

The face of the KCNQ1 S3 helix lined by D202, V205, I204, S209, and V215 (Figs. 1 and 2) appears to be kinetically sensitive to mutation, which likely indicates extensive interaction with the other transmembrane domains of the voltage sensor. This was in contrast to the V207M mutant, predicted to face lipid in the model, which was identical to WT in its kinetic properties and current density (Fig. 5, Figs. S2 and S3, and Tables II and III), and consistent with its classification as a polymorphism in the IAD. All mutations made at S209 as well as V205L and V215A caused negative shifts in the \( V_{1/2} \) of activation, whereas every other single-point mutation caused a positive shift ranging from 12 to 55 mV (Fig. 5). The effects on deactivation followed this same grouping pattern: single mutations that shifted the \( V_{1/2} \) of activation to more negative potentials, now with the addition of I204A and excepting V205L, slowed deactivation with respect to WT. All of the other mutations sped deactivation at −50 mV (Fig. 5 and Table III). Similar results have been reported for the Q357R mutation in the S6 region (Boulet et al., 2006), R294H in S4, and R555C in the C terminus (Chouabe et al., 2000), but the effect of S3 mutations on the kinetics of I\textsubscript{\textkat Grip} channels reported here is much more severe.

Summation of I\textsubscript{\textkat Grip} at high heart rates

Ordinarily, the activation kinetics of WT I\textsubscript{\textkat Grip} channels are quite slow relative to the duration of a single AP, and the deactivation kinetics allow accumulation of I\textsubscript{\textkat Grip} channels in the open state during repetitive activity. This provides a “repolarizing reserve” to allow abbreviation of the cardiac AP and therefore systole, at high heart rates (Jost et al., 2005). I\textsubscript{\textkat Grip} current amplitude also increases during sympathetic stimulation (Stengl et al., 2006), and block of I\textsubscript{\textkat Grip} at this time lengthens the ventricular AP and prolongs the QT interval (Jost et al., 2005), suggesting that I\textsubscript{\textkat Grip} is critically important for the shortening of the QT interval during sympathetic drive and tachycardia.

Ionic currents during a cardiac AP voltage clamp at 2 Hz showed that changes in mutant channel kinetics can eliminate outward I\textsubscript{\textkat Grip} currents through D202N channels (Fig. 7). Although the current amplitude increased in WT channels stimulated with an AP waveform at 2 Hz, it was at 4 Hz that the ionic currents increased dramatically, and more importantly, the I\textsubscript{\textkat Grip} current was present throughout the AP due to the summation effect. This increase was not observed in D202N channels and provides evidence that the D202N and the other mutations that shift the opening equilibrium positive relative to WT channels (Fig. 7) must reduce the contribution of I\textsubscript{\textkat Grip} during the cardiac AP, an effect that is likely to be more pronounced at high heart rates and during stress. On the other hand, the kinetic gain-of-function mutant, S209F, causes a large increase in I\textsubscript{\textkat Grip} even at 2 Hz (Fig. 8), and so were it a more stable protein, it would presumably lead to short QT syndrome or perhaps atrial fibrillation, as was recently reported in carriers of an S209P mutation (Das et al., 2009).

S3 LQT mutations in the context of the KCNQ1 structural model

There is strong evidence that the S2 and S3 transmembrane regions of Kv channels play an important role in modulating channel gating (Papazian et al., 1995; Planells-Cases et al., 1995). These domains contain acidic residues in highly conserved positions, which interact specifically with basic residues in the S4 domain to regulate channel folding and gating (Tiwari-Woodruff et al., 2000). Although the role of noncharged hydrophobic residues in the S3 domain is not fully understood, structurally there are several differences between KCNQ1 and Kv1.2 and Shaker channels. In addition to having fewer charges in S4, it lacks the proline in S3 that divides the helix into 3a and 3b, and it has a shorter S3–S4 linker with no negative charges (Kv1.2 and Shaker have four and five, respectively). Although no crystal structure exists for KCNQ1, models for its open and closed states have been developed based on Kv1.2 (Smith et al., 2007), which provides some basis for interpreting our experimental data (Figs. 1 and 9–11). In general,
mutations at V205, I204, and V215 appear to track most closely with changes in size; therefore, it is expected that the mutations will lead to changes in packing between the helices, sometimes favoring the closed state without affecting the open state (I204A), or additionally destabilizing (I204M) the open state.

**Mutations at D202**
A few studies have looked at the importance of charge at equivalent positions to D202, in Shaker, Kv1.2 (Jogini and Roux, 2007), and KCa3.1 (Gao et al., 2008). In Shaker, the conserved aspartate (D316 in Shaker) is thought to be part of a network of long-range electrostatic interactions involving positive charges in S4 as well as additional negatively charged glutamates in S2 (Papazian et al., 1995). Thus, the negative charge at position 202 in KCNQ1 is expected to be important initially during the folding of the channel protein and then subsequently in the stabilization of the open and/or closed states. Mutation of the corresponding acidic residue in KCa3.1 to alanine results in rapid proteasomal degradation of the channel protein (Gao et al., 2008), and a D501A hERG mutant was also shown to express very poorly (Piper et al., 2008). It is therefore consistent that charge reversal, in the form of the D202H or D202K, results in channels that do not express as well and show accelerated deactivation (Table III and Figs. 3 A and 4). It is less obvious why the D202E charge-conserving mutation would result in such an extreme shift in the V1/2 of activation and accelerated deactivation, until one considers that in the closed-channel state it appears that the side chains for D202, R195 (S2–S3 linker), and Y171 (S2) all interact in what is likely an intricate salt bridge/hydrogen-bonding arrangement (Fig. 9). In the open state, the D202–Y171 interaction appears to persist, but R195 is no longer interacting with the other two (Fig. 9, C and D), and instead R174 at the base of S2 is now involved. The kinetic changes, coupled with a normal slope factor, favor a hypothesis in which this three-way closed-state interaction is further optimized when Asp is replaced with Glu. The strengthened interaction has stabilized the closed state of these mutations.

**Figure 9.** Molecular interactions at the cytoplasmic end of S2 and S3 are important for efficient gating of KCNQ1. D202/Y171 salt bridge/hydrogen bond with R195 in the closed state (A and B), but not in the open state (C and D). Instead, these residues now interact with R174 in the open state (C and D), an interaction not seen in the closed state (A and B). Key residues in this network of interactions are in space fill. Yellow, D202; green, Y171; magenta, R195; orange, R174. In B and D, the view is looking at the single voltage sensor domain from below.
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Preventing formation of these salt bridges might be expected to affect the stability of both states to varying degrees. In Shaker, D316N is able to rescue the nonfunctional K374Q mutant and results in a hyperpolarizing shift in the g-V curve, suggesting that this salt bridge is important for stabilizing the closed state (Papazian et al., 1995).

Mutations at V205
Additional mutations made at V205 (Fig. 10) proved interesting in that some resulted in channels with depolarizing shifts in $V_{1/2}$'s of activation (V205M and V205A; Figs. 3 C and 5) and one hyperpolarizing shift (V205L). Energy minimization after making the equivalent mutations at position 205 in the model resulted in subtle changes to the position in the backbones of the TMDs (Fig. 10), with V205A allowing S2 and S3 to shift apart slightly, whereas in V205L they appeared to be somewhat closer in the closed state. A comparison of the residues in contact with R195 and D202, sites involved in open state (Börjesson and Elinder, 2008). Preventing formation of these salt bridges might be expected to affect the stability of both states to varying degrees.

The other naturally occurring mutant at this position in S3 is a neutralization of the charge, D202N. This mutation has the effect of accelerating both activation and deactivation kinetics (Table III), indicating that both the open and the closed state may have been affected by the loss of charge and changes in hydrogen-bonding patterns. In addition, both activation and deactivation curves have relatively little apparent voltage dependence, supporting the notion that successive electrostatic interactions may normally be formed and broken as the channel opens and closes. Indeed, in addition to direct interactions between R195/Y171 in the closed state and R174/Y171 in the open state proposed above, D202 may also be involved in other longer-range interactions. For Kv1.2 and Shaker channels, the equivalent residues, D259 and D316, respectively, are thought to form long-range electrostatic interactions with arginines of S4, along with a glutamate in S2 in the closed state and with K307 (Kv1.2) or K374 (Shaker) in the open state (Börjesson and Elinder, 2008). Preventing formation of these salt bridges might be expected to affect the stability of both states to varying degrees.

Figure 10. Model predictions for the effect of mutations at V205 on voltage sensor transmembrane domain backbone structure. Mutations were made in the KCNQ1 homology models, and then subjected to side chain repacking and gradient energy minimization, including the WT channel. Resulting closed- (top row) and open-state (bottom row) mutant structures were then overlain on the WT channel backbone structure. WT channel is blue, and mutant channels are orange. The residue at position 205 is shown in bonds-only mode, as are F167 and Y171 in S2.
the salt bridge/hydrogen bond that stabilizes the closed state (Table S2), showed a pattern in which close interactions between the cytosolic ends of S2 and S3 as well as the S2–S3 linker, centered around the highly conserved, positively charged residue in this linker (R195 in KCNQ1), are associated with more hyperpolarized $V_{1/2}$'s of activation (V205L and V205). Loss of this network of interactions in V205A leads to a 50-mV depolarizing shift in the $V_{1/2}$ of activation for the mutant channel. This does not explain why V205M, which is not expected to disrupt this network, also has a right-shifted g-V curve. However, energy-minimized structures of this mutant show an additional interaction in the closed state not seen in the others. V205M is predicted to also interact with F167 in the middle of S2 (Table S2), an interaction that may serve to further stabilize the closed state and prevent S3 from moving as efficiently. In addition, this phenylalanine in S2, which is 99% conserved in Kv channels (Lee et al., 2009), in our model makes what are obviously functionally important contacts with S4 and S1 residues in the closed and open states. These F167 interactions with S1 (V129, I132, and V133) and S4 (Q234 and M238) are lost in the closed state of V205M, but the open-state interactions are maintained (V129, I132, V133, and H240). In the WT channel, V205 is predicted to interact with V171 in S2 (Fig. S3), but this interaction is lost in V205L, further supporting the concept that interhelical interactions at this position slow channel activation.

![Figure 11](image-url)

**Figure 11.** Molecular interactions for S209 and V215 from the KCNQ1 homology model. Polar S209 moves to a hydrophobic pocket in the open state. (A) Close-up view of the residues within van der Waals contact of S209 in the KCNQ1 closed- and (B) open-state models. S209 from the green subunit is in space fill. Residues determined to have side chains in van der Waals contact (see Materials and methods) with the side chain of S209 are labeled and have side chains shown in bonds-only mode. (C and D) The KCNQ1 models predict that V215M can be accommodated in the closed state but perturbs the open state. The V215M mutation was made in the KCNQ1 homology models, and the WT and mutant channel were then subjected to side chain repacking and gradient energy minimization. The WT channel is in blue, and V215M is in orange. The amino acids at 215 as well as A223, I227, I230, and L233 are shown in bonds-only mode.
Experiments involving neutralization of the Shaker S3 residue D316 to N316 (D202 in KCNQ1) resulted in a decrease of approximately two proton charges per channel (Seoh et al., 1996), indicating that neutralization either caused repositioning of S4 or that S3 is also contributing to gating. If S3 also moves during gating and S1–S2 are indeed the largely stationary component of the voltage sensor domain involved in bracing the moveable component (Lee et al., 2009), disrupting certain “bracing” interactions between S2 and S3 might affect coupling between depolarization and gating, as seen for the V205A mutant.

Mutations at S209
In the open state, S209 is deeply buried in the hydrophobic core (Fig. 11 B). This is the region, including a turn of the helix down at V205, where S3 makes its closest approach to S2 in the open state. It is not uncommon for a serine or alanine to be at such a position within TMD helix interfaces (Adamian and Liang, 2001). In the closed state, S209 is partially exposed to the protein surface, where it may be able to hydrogen bond with water. All the mutations at this position lead to strong bias toward channel opening at all potentials (Fig. 5 and Table III). All the mutations were to hydrophobic residues, but they varied greatly in size from similar to Ser (Ala), to much larger residues. Ser209Ala led to the smallest shift in $V_{1/2}$ and kinetics, whereas the introduction of bulkier and more hydrophobic side chains led to even greater negative shifts in the $V_{1/2}$ of activation. We suggest that the impact of the Ser209-to-apolar substitutions on the energy of the closed state is likely to destabilize the closed state as a result of loss of hydrogen bonding in this state. At the same time, Ser-to-apolar substitutions are likely to stabilize the open state by relieving energetic frustration that stems from burying a hydroxyl side chain in a hydrophobic environment without a hydrogen-bonding partner. By both destabilizing the closed state and stabilizing the open state, Ser-to-hydrophobic substitutions result in gain-of-function channel behavior.

Mutations at V215
The region of the channel including V215 in our models is quite interesting in that in the closed state, this region is somewhat unstructured, but when the channel opens it becomes part of the S3 helix (Fig. 11, C and D). Putting a proline (a helix breaker) at this position resulted in a 40-mV positive shift in the $V_{1/2}$ of activation but faster activation and deactivation in the physiological range (similar to V215M shown in Figs. 3 B, 4 B, and 5, and Table III), indicating that the proline has at least affected the stability of the open state. The V215M mutation results in a 20-mV positive shift in activation, and in the model it shows little change in the closed-state structure (Fig. 11 C). Although the closed state appears to accommodate the bulkier side chain of methionine, the open state is more perturbed, with methionine making more contacts with the S3–S4 linker and S4, creating subtle repositioning of S3 with respect to S2 and S4 (Fig. 11 D) and destabilizing the open state. On the other hand, the V215A mutation shifts the $V_{1/2}$ in the opposite direction slightly, suggesting that the less constrained S4 is by interactions with the carboxyl end of S3, the more stable the open state. Consistent with this, deactivation rates for V215A approach three times that of the WT channel (Fig. 5).

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