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Background—Inherited long-QT syndrome is characterized by prolonged QT interval on the ECG, syncope, and sudden death caused by ventricular arrhythmia. Causative mutations occur mostly in cardiac potassium and sodium channel subunit genes. Confidence in mutation pathogenicity is usually reached through family genotype-phenotype tracking, control population studies, molecular modeling, and phylogenetic alignments; however, biophysical testing offers a higher degree of validating evidence.

Methods and Results—By using in vitro electrophysiological testing of transfected mutant and wild-type long-QT syndrome constructs into Chinese hamster ovary cells, we investigated the biophysical properties of 9 *KCNQ1* missense mutations (A46T, T265I, F269S, A302V, G316E, F339S, R360G, H455Y, and S546L) identified in a New Zealand–based long-QT syndrome screening program. We demonstrate through electrophysiology and molecular modeling that 7 of the missense mutations have profound pathological dominant-negative loss-of-function properties, confirming their likely disease-causing nature. This supports the use of these mutations in diagnostic family screening. Two mutations (A46T, T265I) show suggestive evidence of pathogenicity within the experimental limits of biophysical testing, indicating that these variants are disease-causing via delayed- or fast-activation kinetics. Further investigation of the A46T family has revealed an inconsistent cosegregation of the variant with the clinical phenotype.

Conclusions—Electrophysiological characterization should be used to validate long-QT syndrome pathogenicity of novel missense channelopathies. When such results are inconclusive, great care should be taken with genetic counseling and screening of such families, and alternative disease-causing mechanisms should be considered. **(*Circ Arrhythmia Electrophysiol*. 2009;2:417-426.)**

Key Words: long QT ■ mutations ■ arrhythmia ■ ion channels ■ sudden cardiac death

Long-QT syndrome (LQTS) is a potentially fatal arrhythmic syndrome typically associated with a prolonged QT interval on the surface 12-lead ECG. It represents a diverse range of disorders associated with prolonged ventricular repolarization.[1] Current estimates are that LQTS mutation carriers can be present in 1 in 1000 to 5000.[2,3] Recent advances in the molecular basis of LQTS have revealed a greater than expected incidence within the population caused by the extent of incomplete penetrance within families with causative potassium and sodium cardiac ion channel genes.[4,5] More recently, LQTS and the allelic *SCN5A* disorder, Brugada syndrome, have been strongly associated with a proportion of sudden unexpected death syndrome cases, including in infancy.[6]

Clinical Perspective on p 426

To date, 10 genes have proven association with LQTS: LQT1 (*KCNQ1*) and LQT2 (*HERG*) encode α-subunits of the voltage-gated K^+ channel, I_{Ks} and I_{Kr}, respectively; LQT3 (*SCN5A*) encodes the α-subunit of a voltage-gated Na^+

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channel; LQT5 (*KCNE1*) and LQT6 (*KCNE2*) code for the β-subunit of I_{Ks} and I_{Kr}, respectively; and LQT4 (*Ankyrin-B*) is a member of a membrane adapter protein family.[7] Recently, the Andersen (*KCNJ1*) and Timothy (*Cav1.2*) syndrome genes have been suggested as loci for LQT7 and LQT8, respectively.[8,9] Voltage-gated K^+ channels require 4 α-subunits with 6 transmembrane domains (S1–S6), a voltage sensor (S4), a pore loop containing a conserved K^+-selective signature sequence between S5 and S6, and 1 β-subunit to form a functional ion channel.

Several LQTS genetic screening studies have been published identifying more than 600 mutations that have revealed important pathophysiological mechanisms of arrhythmogenesis.[3,10,11] Pathological mutations in these channel subunits can reduce the depolarizing/repolarizing cardiac current through several mechanisms, including allelic haploinsufficiency, heterotetrameric dominant-negative mechanisms, nonsense-mediated decay, and trafficking defects.[12,13] Although the outcome of mutations that cause frameshift RNA messages and truncated polypeptides are generally unequivocal, the novel and recurrent missense variants are more problematic, as observed in many other genetic disorders.[14] It is imperative that genotype pathogenicity is convincing to assign a degree of assurance that family cascade testing is truly informative. This has led to a trend in clinical and scientific best practice in which variants are determined to be mutations of pathological significance by using both larger control cohorts with ethnic stratification and electrophysiological testing of missense mutations. In response to this, we tested the biophysical properties of 5 novel and 4 literature-recurrent *KCNQ1* mutations that were detected in a genetic screening program.[15] We demonstrate that 7 of the missense mutations we assayed and modeled have pathological dominant-negative properties that abolish I_{Ks} dynamics. Two novel mutations that are phylogenetically conserved and excluded from the control population have alternative explanations in which we present suggestive data on the mechanism of abnormal receptor activity.

Methods

Patients and Cases

The cases were referred to tertiary cardiac arrhythmia clinics for a variety of reasons. Most patients had experienced syncope or resuscitated sudden cardiac death. This study also included 8 patients previously diagnosed with epilepsy who had exhibited prolonged or borderline QT_c intervals on ECG examination and 4 surviving parent-pairs of young children who were victims of sudden cardiac death. Informed consent for genetic testing was obtained in all cases as established in the multicenter ethics approval protocols covering this project (Auckland Regional Ethics Committee). DNA was extracted from blood samples using standard phenol-chloroform extraction.

Mutation Detection

The mutations have been described previously[15,16] and were discovered by denaturing high-performance liquid chromatography (dHPLC) analysis of LQTS genes in index cases. Any sample that displayed a variant chromatogram profile was selected for further analysis by DNA sequencing. Purified DNA fragments were sequenced using Big Dye Terminator kits and an ABI 3100 automated sequencer (Applied Biosystems, Foster City, Calif) at the Centre for Gene Technology, University of Auckland. Population frequency of single-nucleotide polymorphisms or mutations were assessed in 300 control chromosomes as well as data from other screening studies and database sites. Various Genebank databases were aligned with LQTS gene families and homologs at the point of mutation to assess the degree of evolutionary conservation.

Site-Directed *KCNQ1* Mutagenesis and Transient Transfection in Chinese Hamster Ovary Cells

The human *KCNQ1* DNA was originally provided by Dr Mark Keating (currently at Novartis Institute of Biomedical Research, Cambridge, Mass). Individual *KCNQ1* mutation constructs were made using a QuikChange XL site-directed mutagenesis kit and manufacturer instructions (Stratagene Inc, La Jolla, Calif). The human I_{Ks} channel auxiliary subunit *KCNE1*-IRES-pEGFP construct was a gift from Dr Al George at Vanderbilt University. All inserts were sequenced to ensure that only the desired mutation was obtained. Wild-type or mutated *KCNQ1* constructs and *KCNE1* (at 1:1 μg ratio) were transiently cotransfected into cultured Chinese hamster ovary cells with FuGENE6 transfection reagent (Roche Applied Science, Indianapolis, Ind). A plasmid encoding the enhanced green fluorescent protein (pEGFP) linked to *KCNE1* was used to identify transfected cells for the voltage clamp studies. Cells were grown for 48 hours after transfection before study.

Whole-Cell Voltage Clamp Studies and Solutions

Whole-cell voltage clamping was performed at room temperature with 3 to 5 mol/LΩ patch microelectrodes and an Axopatch 200A amplifier (Molecular Devices, Sunnydale, Calif). The cell chamber (extracellular) solution contained (in mmol/L) NaCl 145, KCl 4.0, $MgCl_2$ 1.0, $CaCl_2$ 1.8, glucose 10, and HEPES 10; the pH was 7.4, adjusted with NaOH. The pipette (intracellular) solution contained (in mmol/L) KCl 110, $MgCl_2$ 1.0, ATP-K_2 5.0, BAPTA-K_4 5.0, and HEPES 10; the pH was 7.2, adjusted with KOH. Data acquisitions were done by use of pClamp9.2 (Axon Instruments Inc), sampled at 1 kHz, and low-pass–filtered at 5 kHz. Activating current was elicited with 5-second depolarizing pulses to +60 mV from a holding potential of −80 mV at a 10-mV increments, and tail current was recorded on return to −40 mV. Pulses were delivered every 30 seconds. To mimic a "physiological" action potential duration, a single 400-ms pulse to +20 mV and back to −40 mV for another 100 ms from the holding potential of −80 mV was used to compare the initial I_{Ks} magnitudes in wild-type and several particular *KCNQ1* mutant channels in some experiments. To determine the membrane potential of the channels activated, I-V relationships were established by fitting data to the Boltzmann equation: $I=I_{max}/\{1+\exp[(V_t-V_{0.5})/k]\}$, where I_{max} is the maximal current, V_t is the testing potential, $V_{0.5}$ is the membrane potential at which 50% of the channels are activated, and k is the slope factor. Current densities (pA/pF) were obtained after normalization to cell surface area calculated by Membrane Test (OUT 0) in pClamp9.2. The steady-state activating current at the end of a 5-second depolarizing pulse to +60 mV and the peak deactivating tail current at −40 mV were measured for comparisons of wild type (WT) with mutated I_{Ks} densities. The time constants (Tau, ms) for activating I_{Ks} currents in WT and any mutations with obvious channel gating changes were obtained by using the Chebyshev method to fit individual activating current traces to the monoexponential function: A1*exp{−(t-*k*)/tau}+C.

Statistical Analysis

Data are expressed as mean±SEM. For comparisons among means of several groups, ANOVA was used, with post hoc pairwise comparisons by Duncan test if significant differences among means were detected. If only 2 groups were being compared, Student's *t* test was used. A probability value of <0.05 is considered statistically significant.

Structural Modeling

The locations of the newly identified *KCNQ1* mutations were investigated using the recently published homology model of the transmembrane regions of the open and closed *KCNQ1* channel in

Table. *KCNQ1* Gene Variants Characterized in the Study

Sequence Variants and Amino Acid Changes	Protein Position	Age, y	Sex	Ethnicity	Syncope	SCD of First-Degree Relative	RSCD	Identified Trigger/s	QT_c, ms
136G>A: A46T	N-terminal	57	F	European	Y	Y		Stress	620
794C>T: T265I	S5	40	F	European	Y		Y	Rest/sibutramine	660
887T>C: F296S	S5/pore	14	F	European					490
905C>T: A302V	Pore	12	F	Maori	Y			Exercise/stress	490
947G>A: G316E	Pore	8	M	European	Y		Y*	Exercise (water)	560
1016T>C: F339S	S6	9	M	European	Y	Y		Exercise	480
1078A>G: R360G	C-terminal	15	F	European	Y			Exercise (water)	600
1363C>T: H455Y	C-terminal	38	F	European		Y			470
1637C>T : S546L	C-terminal	9	M	European	Y			Exercise (water)	480

Representation of novel/recurrent *KCNQ1* variants and phenotypic presentation of the index patients (refer to Reference 15).[15] The patients presented with syncope, resuscitated sudden cardiac death (RSCD), or as surviving parents of sudden cardiac death (SCD) in young children. All were subsequently identified as having Romano-Ward Syndrome (RWS) by presentation of QT-interval elongation and mutations in *KCNQ1* (LQT1).

*DC cardioversion required.

tetrameric form.[17] All models were visualized using the molecular graphics program Chimera (http://www.cgl.ucsf.edu/chimera/).

Results

LQTS Cases

All 9 patients with 5 novel and 4 literature-recurrent *KCNQ1* mutations selected for functional analysis had Romano-Ward syndrome, an autosomal dominant hereditary disorder characterized by a prolonged QT interval on the ECG, syncope, and sudden death. The heart rate–corrected QT interval (QT_c) ranged from 480 to 660 ms (average, 534±20 ms). Eight patients were of European descent, and 1 was of Maori descent. The presenting clinical features are indicated in Table 1. Three cases were identified from follow-up of sudden unexpected death in a first-degree family member.

Novel *KCNQ1* Mutations in LQTS

Between 2001 and 2005, 48 gene-positive probands were identified in an LQTS screening program in New Zealand[15]: 25 had *KCNQ1* mutations, 9 of which (in 2004) had not been reported in the literature (see Table 1). Subsequently, 4 of the variants (A46T, A302V, G316E, and S546L) have been reported by other LQTS screening programs but without any supporting electrophysiological data, whereas there are alternative amino acid substitutions or frameshifts recorded at positions 265 (T265fs +22X), 302 (A302T), and 360 (R360T).[10,18] Therefore, all 9 variants described here are novel observations based on receptor properties recorded by in vitro functional electrophysiology. Sequence analysis revealed that these mutations are located in a variety of domain regions in *KCNQ1* (Figure 1): the N terminus (A46T), S5 (T265I), the S5 and P loop linker (F296S), the P loop (A302V and G316E), S6 (F339S), and the C terminus (R360G, H455Y, and S546L). Population studies confirmed that these sequence variations were not represented in unrelated controls, and phylogenetic alignment of *KCNQ1* homologs confirmed that the mutations have occurred at conserved sites. Mutation constructs of *KCNQ1* were prepared using a QuikChange XL site-directed mutagenesis kit and submitted for electrophysiological characterization.

Seven *KCNQ1* Mutations Are Loss-of-Function Channels

To compare the biophysical properties of normal I_{Ks} with those of the *KCNQ1* subunit mutations, we used single 5-second pulsing to +60 mV from a holding potential of −80

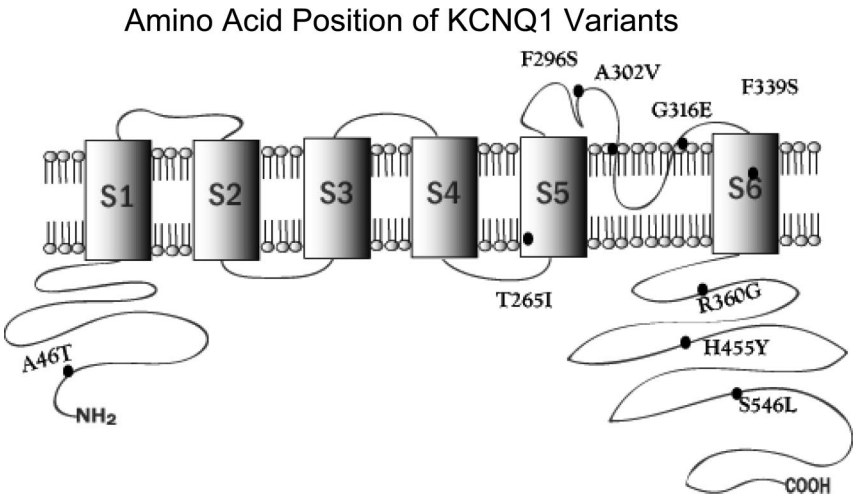


Figure 1. Positioning of the *KCNQ1* variants on a 2-D representation of the KCNQ1 channel protein subunit domains.

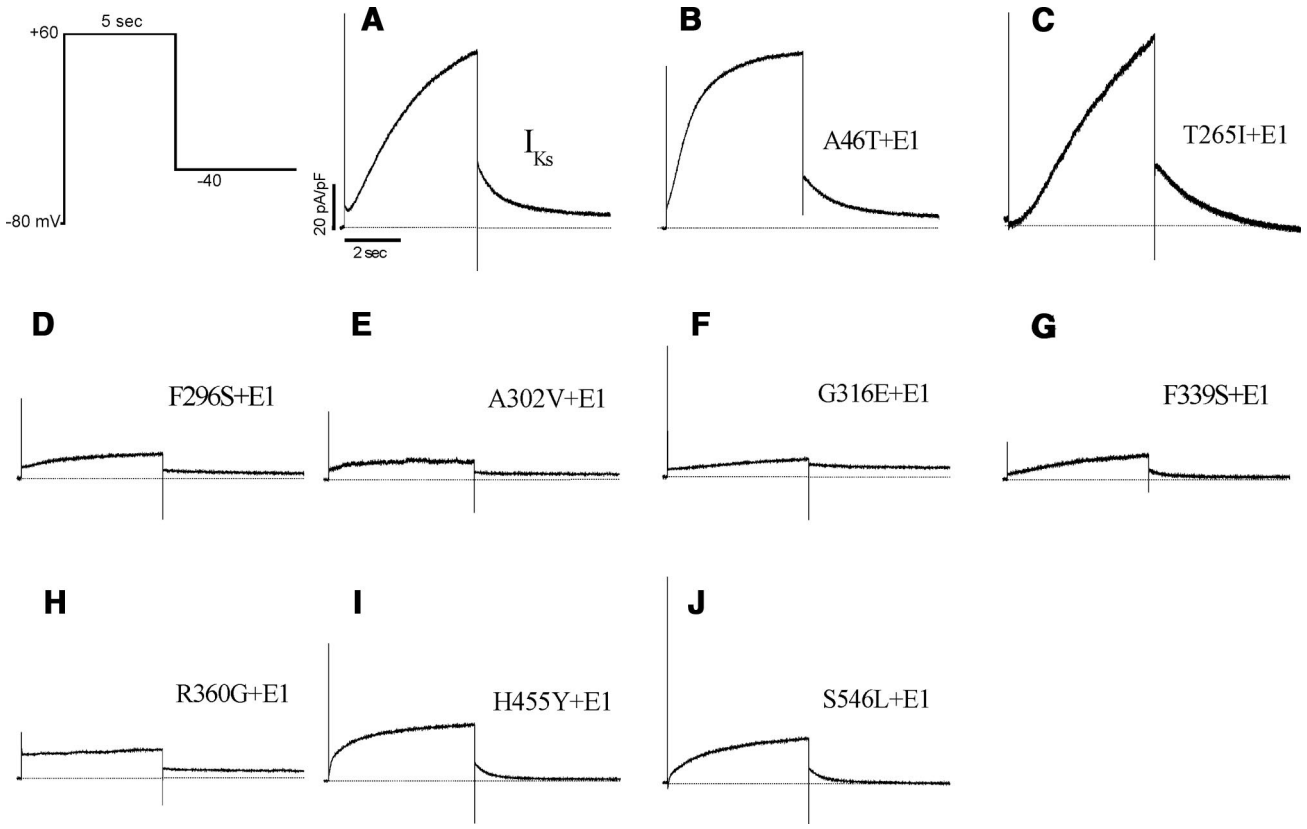


Figure 2. I_{Ks} for WT (A) and mutated *KCNQ1* constructs (B through J) in the presence of *KCNE1* in Chinese hamster ovary cells. Raw I_{Ks} traces were obtained by using the voltage-clamp protocol at room temperature. Cells were clamped to a holding potential of −80 mV. Activating I_{Ks} was elicited with a 5-second pulse from −80 to +60 mV, and deactivating tail current was recorded on repolarization to −40 mV for another 5 seconds.

mV to elicit the I_{Ks} current. The tail current was recorded on return to −40 mV. WT *KCNQ1* and the auxiliary subunit *KCNE1* were first coexpressed to generate an outward potassium current typical to cardiac I_{Ks} current (Figure 2A). Individual *KCNQ1* mutations were then studied with *KCNE1* coexpression for comparison. As illustrated in Figure 2 and Figure 3A and B, 7 of the 9 *KCNQ1* mutations generated a reduced I_{Ks} current when coexpressed with *KCNE1*. The I_{Ks} magnitudes by the other 2 mutations (A46T and T265I) were similar to normal I_{Ks}, except that A46T-I_{Ks} showed a fast activation (Figure 2B): the time constants for activation at +60 mV were 1717.3±192.4 ms (WT) and 977.6±68.9 ms (A46T-I_{Ks}, $P<0.01$, n=6 cells for each group), respectively. The half-maximal voltage ($V_{0.5}$) of I_{Ks} activation was not different among WT, A46T, T265I, G316E F339S, and H455Y ($P>0.05$) (Figure 3C). However, the $V_{0.5}$ for the mutation F296S was ≈10 mV more negative than WT-I_{Ks} ($P<0.05$), whereas the $V_{0.5}$ for S546L was significantly shifted toward a more positive direction (+20 mV greater than WT-I_{Ks}, $P<0.01$). Because of very small I_{Ks} magnitude from A302V and R360G, the current-voltage curves for the 2 mutations were not established to generate the $V_{0.5}$ values.

Enhanced Initial I_{Ks} Currents and Loss of the Delay Phase of the I_{Ks} in Particular *KCNQ1* Mutations

In our experiments with a 5-second pulse protocol, we found that 2 *KCNQ1* mutations (A46T and H445 years) activated more rapidly without an initial delay at the very beginning phase of the I_{Ks} (Figure 2, B and I). In addition, T265I appears to have a longer delay before the current activation (Figure 2C). Accordingly, we applied a single 500-ms pulse protocol that mimics a physiological action potential duration. In this protocol, a 400-ms depolarization pulse to +20 mV with a 100-ms repolarization to −40 mV was used. The current magnitudes at 100th- and 400th-ms points were measured for comparisons. As illustrated in Figure 4A, WT-I_{Ks} showed an initial delay at first ≈50-ms before activation (rising phase in dotted box). The mutations A46T-I_{Ks} and H455Y-I_{Ks} lost this initial current delay with a rapid activation/rising phase (Figure 4, B and E). However, the mutation T265I-I_{Ks} displayed a longer delay (≈150 ms) before activation (Figure 4D). The summarized data on the current magnitudes at 100th- and 400th-ms points are presented in Figure 4, C and F.

Given the fact that the I_{Ks} channel is highly upregulated by β-adrenergic stimulation and that the mutation A46T was located in the N terminus of the KCNQ1 channel, we further compared the effects of a β-receptor agonist isoproterenol (1 μmol/L) on WT-I_{Ks} and this mutant. The experimental results in Figure 4 (G through I) showed that the β-stimulation increased the current in both WT and A46T to a similar extent, implying that the A46T mutation did not influence the β-stimulation–mediated phosphorylation of the *KCNQ1* channel. Previously, we and others have already

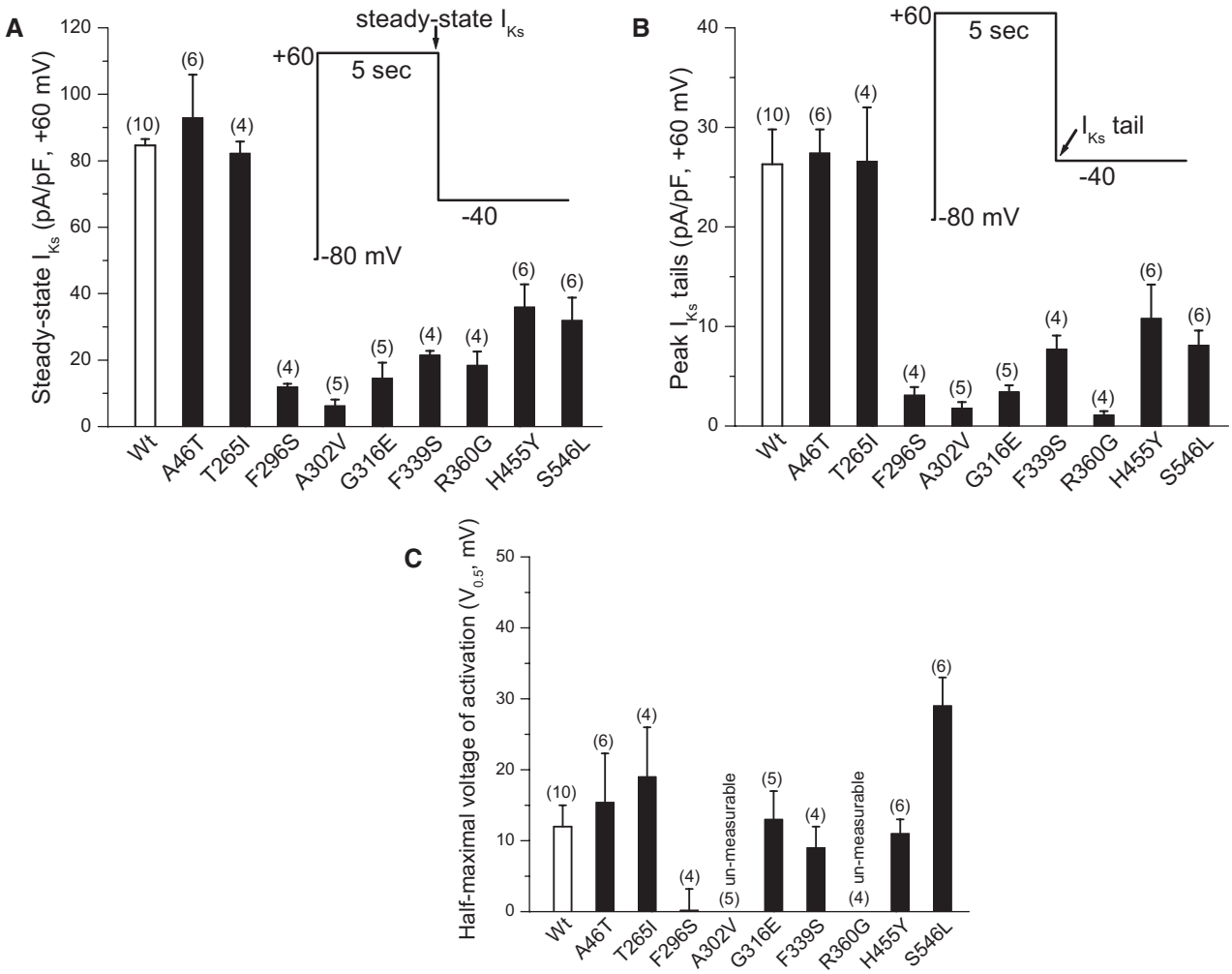


Figure 3. Summarized data for WT and mutated I_{Ks} currents. A, Steady-state I_{Ks} current levels for WT and mutations. Currents were measured at the end of a 5-second depolarizing pulse. B, Peak I_{Ks} tail current for WT and mutations. Peak tails were measured after a 5-second repolarizing pulse to −40 mV. C, Half-maximal voltage of I_{Ks} activation ($V_{0.5}$, mV) for WT and mutations. After current-voltage relations were obtained, the $V_{0.5}$ values were obtained after fitting individual data points to the Boltzmann equation: $I=I_{max}/\{1+\exp[(V_t-V_{0.5})/k]\}$. Two mutations (A302V and R360G) expressed tail currents too small to generate a $V_{0.5}$ value.

demonstrated that several amino acid residues in the N terminus (S27) and C terminus (S468 and T470) are critical phosphorylation sites of the *KCNQ1* channel.[19,20]

Two Mutations Have Suggestive Outcomes

There is inconclusive but suggestive evidence for functional consequences of 2 variants in this study (A46T and T265I) representing challenges for the clinical interpretation of the genotype. The mutation C794T (with resulting T265I) was found in a 40-year-old woman who presented after resuscitated sudden death during sleep (Figure 5A). She had been taking sibutramine, a medication to aid with weight loss, for approximately 3 weeks before her presentation, and her case has been previously reported.[16] Her QT_c was 600 to 660 ms, and she went on to have an intracardiac cardioverter-defibrillator inserted. A previous ECG was also abnormal, with prolonged QT_c of 500 ms. Subsequent ECGs have had QT_c values in the upper normal range. She has 4 children who all have prolonged QT_c intervals, 1 of whom has a history of syncope on exertion. Cascade testing in 3 younger children has identified the same mutation as the proband, whereas the oldest child has declined genetic testing. The proband's mother and 3 siblings have all had ECGs with normal QT_c intervals. Her father has a prolonged QT_c of 0.48 seconds and is gene-positive.

The mutation G136A (with resulting A46T) has previously been reported by Napolitano et al.[10] In our cohort, it was detected in a 57-year-old woman after presentation with acute syncope while taking cisapride (Figure 5B). She had a history of syncope at times of stress. Her initial QT_c interval was 620 ms, whereas subsequent ECGs have revealed QT_c intervals of 400 to 470 ms. Her presenting episode of collapse occurred in the days after the unexpected death of her son, who had died suddenly, during gentle activity, at 37 years of age. He also had a history of syncope, but there were no prior ECGs. The postmortem examination revealed no cause, but no DNA was available for posthumous genetic screening. Despite the proband's presentation, the acutely prolonged QT_c interval, and family history of young sudden death, further investigation of the family to date lends some doubt as to the

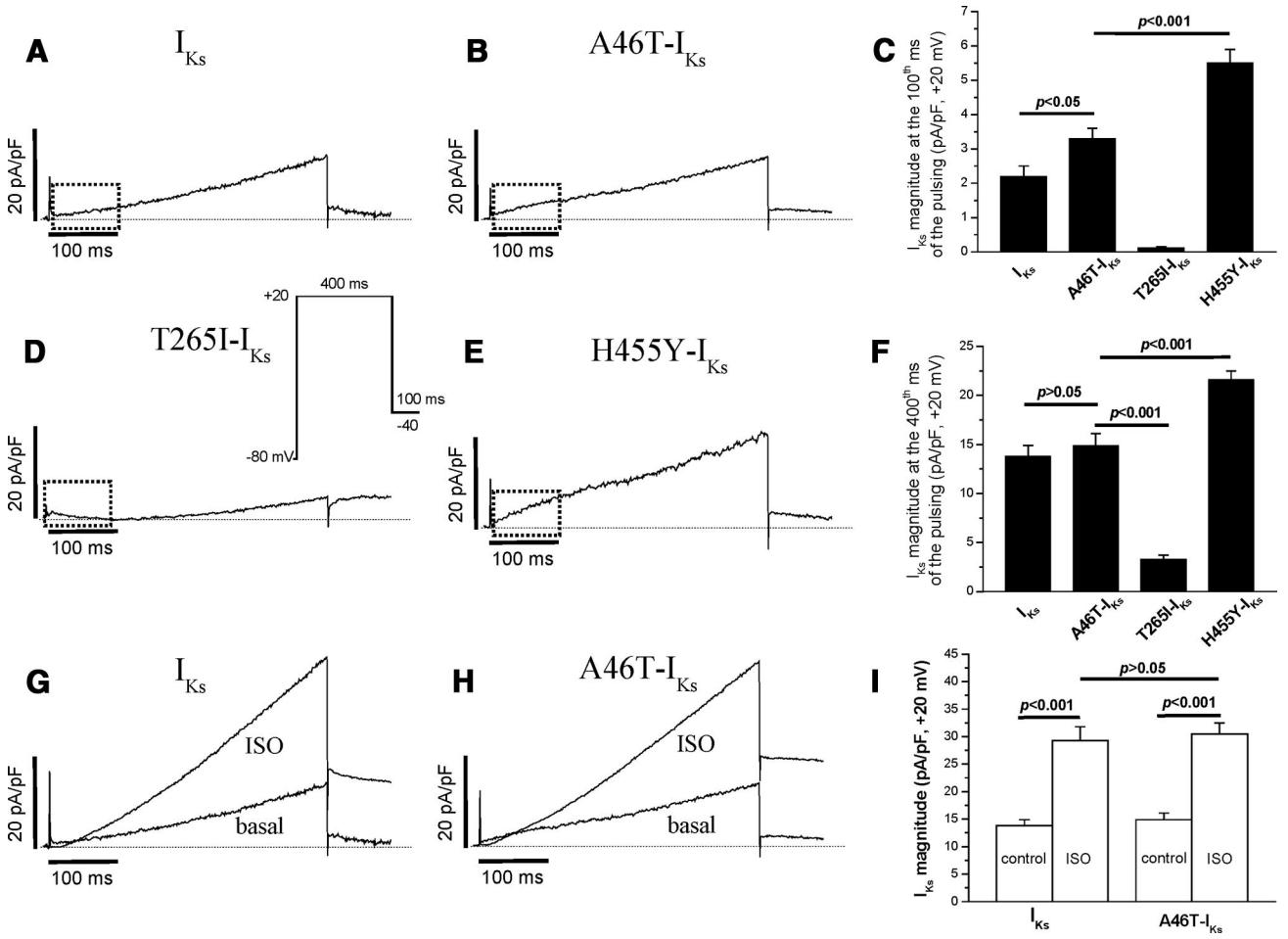


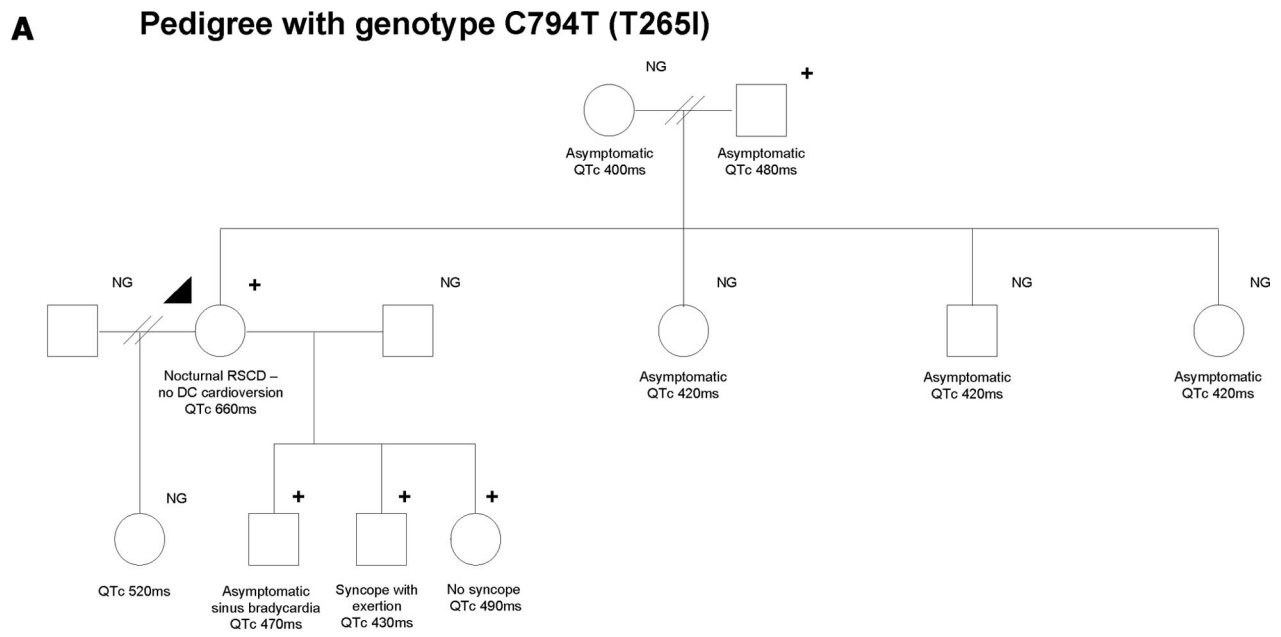
Figure 4. Five hundred–millisecond short pulse–elicited currents and responses to isoproterenol (ISO, 1 μmol/L). In several particular *KCNQ1* mutations, a voltage-clamp pulsing protocol to +20 mV for 500 ms (corresponding to the plateau membrane potential and duration of a cardiac action potential, inset) was used to compare further the very beginning (initial) currents. Responses of WT and mutation A46T to the β-adrenergic stimulation by isoproterenol were also compared.

malignancy of this mutation. The proband has 3 other children who all have a history of syncope and have undergone cascade genetic screening. One son (QT_c, 380 to 420 ms) and 1 daughter (QT_c, 420 to 480 ms) tested positive for this mutation; the other daughter (QT_c, 420 to 440 ms) had negative cascade testing. The proband's 2 living siblings have not presented for assessment but are reportedly asymptomatic. Another sibling died in an accident at age 20 years. With regard to the proband's parents, her father died at age 71 years, and her mother's genetic screening was negative. As a result of the poor cosegregation of the mutation with clinical findings in this family, together with the in vitro electrophysiological testing result, we are not using cascade screening as a definitive test for the presence or absence of disease in this family.

Molecular Modeling of the *KCNQ1* Mutations

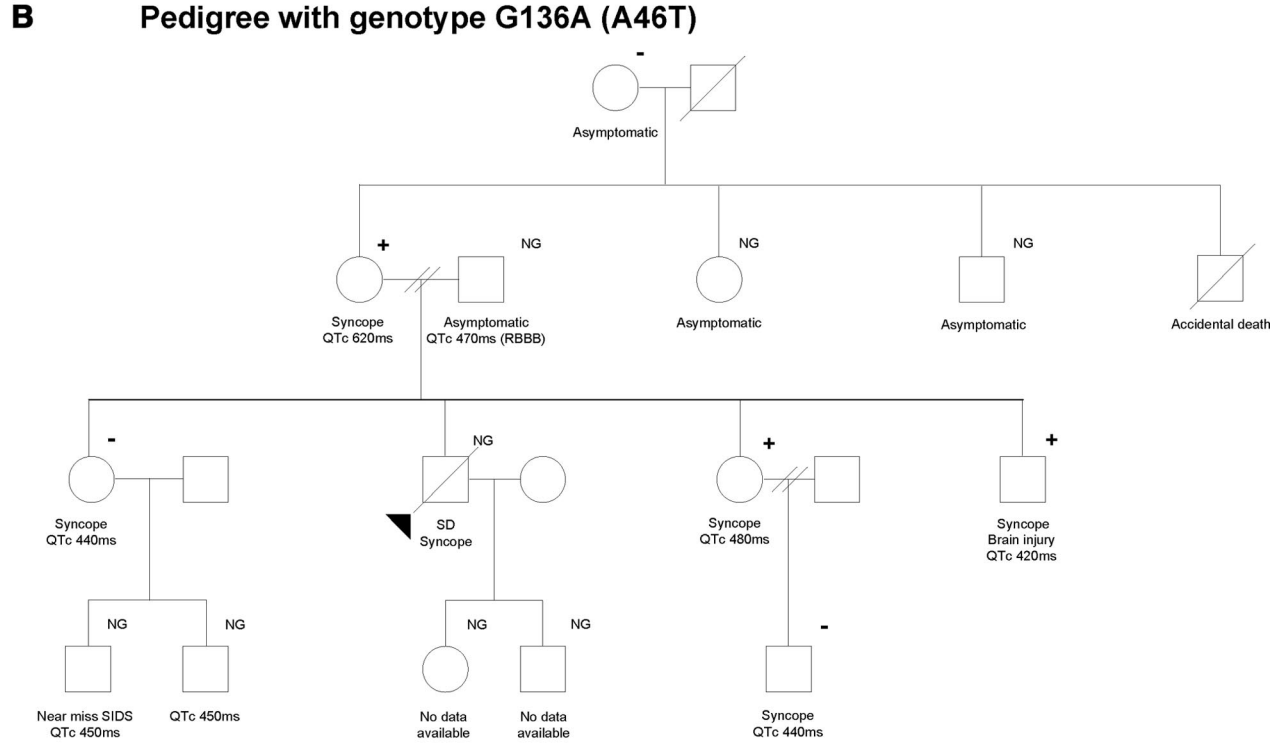
The tertiary positions of mutations T265I, F296S, A302V, G316E, and F339S were examined in relation to the recently published homology model of the transmembrane regions of the protein.[17] The N-terminal A46T mutation and the C-terminal R360G, H455Y, and S546L fell outside the current coverage of published models and crystal structures. A crystal structure for the C-terminal region of human *KCNQ1* has recently been elucidated,[21] but this is restricted to residues 584 to 621 of the C terminus.

As shown in Figure 6A, in the closed state, T265 and F339 are closely located and likely to interact by means of intrachain hydrophobic interactions between S5 and S6. F296 and A302 are also closely located, but on the extramembrane portion of the P loop. Comparison of the closed and open states of the channel (Figure 6A and B) reinforces the importance of the mutated positions. In the open state, the interactions between the residues of each pairing are less intimate, suggesting that engagement and loss of specific hydrophobic interactions are central to the conformational changes in the transmembrane and loop regions required for activity of the channel. We speculate that mutations involving these residues disrupt the fine architecture of the channel and preclude normal opening and closure. G316 is located on the P loop, squarely at the entry to the pore (Figure 6C). The G316E mutation directly restricts the aperture of the pore by introducing a much larger and negatively charged glutamic acid residue to this critical gating position (Figure 6D). It is also possible that the mutation may bring about misfolding of



Key

+	Genotype positive
-	Genotype negative
NG	Not genotyped
QTc	Corrected QT interval using Bazett formula (seconds)
RSCD	Resuscitated sudden cardiac death



Key

+	Genotype positive
-	Genotype negative
NG	Not genotyped
QTc	Corrected QT interval using Bazett formula (seconds)
RBBB	Right bundle branch block

Figure 5. Pedigrees and clinical data of the gene-positive families with uncertain, ambiguous outcomes. A, Family with *KCNQ1* A46T and associated clinical presentation and incidences. B, Family with T265I and associated clinical presentation and incidences. Refer to the figure for the key guide.

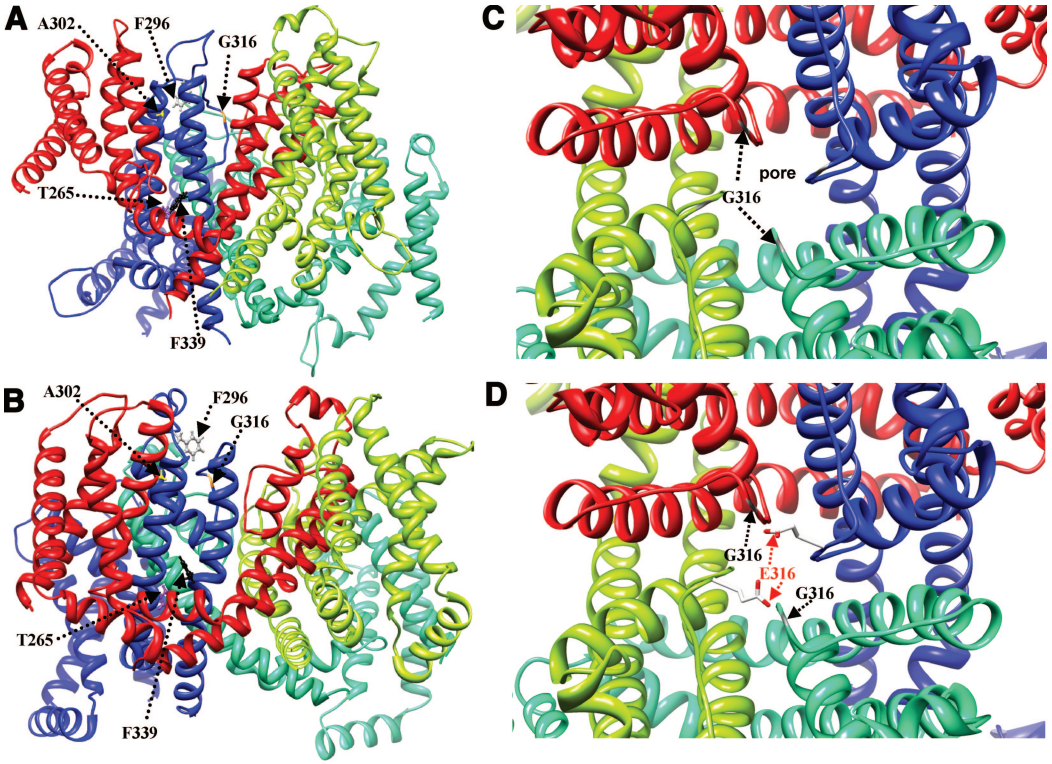


Figure 6. The chains of the tetramer shown in blue (a), green (b), lime (c), and red (d). Residues involved in mutations are shown in ball and stick. A, Closed: The 2 pairings, F296 (gray) and A302 (yellow); T265 (purple) and F339 (black) in close proximity; G316 (orange), on P loop, projected outward into pore of channel. B, Open: The 2 pairings are drawn apart, and G316 (orange), on P loop, is withdrawn from the pore of the channel. C, *KCNQ1* in open conformation shows the pore region. The G316 WT, with positions of glycine residues, which possess no side chain, are shown in gray, leaving the channel unobstructed. D, *KCNQ1* in open conformation shows restriction of the pore by the G316E mutation. E316, on P loop, is shown in orange for chains a and c, projected outward into the pore. For illustration, WT G316, shown in yellow on P loop of chains b and d, leaves the channel unobstructed. All models were visualized using the molecular graphics program Chimera (http://www.cgl.ucsf.edu/chimera/).

the protein, which may subsequently affect its trafficking or tetramerization, although homology modeling of the E316 variant (data not shown) suggests that this is unlikely.

Discussion

Many *KCNQ1* mutations and rare variants have now been identified in a variety of *KCNQ1* domains and regions (http://www.fsm.it/cardmoc), some of which are recurrent in populations whereas some remain private to 1 index case or extended family. This represents a dilemma in the clinical setting because it is difficult to assign pathogenicity to novel variants without several levels of validating evidence, the best of which is the demonstration of biophysical deficits by in vitro electrophysiological experiments. This is a highly specialized and challenging technology platform that is unlikely to cross over to the diagnostic domain; nevertheless, it can provide definitive evidence and clinical confidence that a gene variant can translate into a functional mutation.

For this reason, we describe in this study the biophysical characterization of 9 *KCNQ1* variants in patients with Romano-Ward syndrome.[15,16] The gene-positive patients had unequivocally prolonged QT_c intervals, and all but 1 had typical presentation scenarios such as syncope during exercise, a history of sudden death in the family, or resuscitated cardiac arrest. Within the *KCNQ1* subunit structure, these mutations are located in different regional domains: the N terminus (A46T), S5 (T265I), the S5 and P loop linker (F296S), P loop (A302V and G316E), S6 (F339S), and the C terminus (R360G, H455Y, and S546L). Four variants (A46T, A302V, G316E, and S546L) have subsequently been detected by other LQTS screening programs but without any supporting electrophysiological data, whereas there are alternative amino acid substitutions or frameshifts recorded at positions 265 (T265fs+22X), 302 (A302T), and 360 (R360T).[10,18] Whatever the status and origin of the KCNQ1 variants, the biophysical investigation of their pathogenicity has not been investigated.

The value of discovering the physiological properties of channel variants has contributed much to the understanding of the pathophysiological mechanisms of LQTS.[12–14] Cardiac slowly activating delayed rectifier K^+ current (I_{Ks}) is generated by coassembly of the α-subunit *KCNQ1* (*KvLQT1*) and its auxiliary subunit *KCNE1* (minK). The I_{Ks} current does not play a major role in regulation of cardiac repolarization under normal physiological conditions. However, mutations in the *KCNQ1* gene could cause inherited prolongation of the QT interval by reducing the quality of I_{Ks} current, thereby affecting cardiac repolarization. Expression studies have demonstrated that LQTS mutations of genes related with K^+ currents can produce loss of channel function by either a net current reduction by altering the channel gating and kinetic

properties, prevention of assembly of functional channel protein, or an abnormal intracellular protein trafficking.

We examined the electrophysiological properties of the 9 *KCNQ1* mutations in the presence *KCNE1* by heterologous expression in Chinese hamster ovary cells and by using a whole-cell voltage clamp. We found 7 KCNQ1 mutations causing a reduced I_{Ks} current density with coexpression of *KCNE1*, indicating the loss of function in the heterotetrametric state channel. The electrophysiological data for the 7 *KCNQ1* mutations with decrease the I_{Ks} density suggest several possible mechanisms: (1) a positive shift of the channel activation voltage could lead to a reduction of the current (this effect is more obvious in the mutation S546L); (2) the dominant-negative effect; and (3) failure of the channel protein expression within the cell membrane. The studies relating to the latter 2 effects are separate projects that are being pursued, but irrespective of the specific mechanism involved, it is now possible to label these 7 LQTS genotypes as definitive pathological mutations.

Two other mutations (A46T and T265I) displayed no alterations in the current density, although there is evidence that T265I-I_{Ks} displayed a longer delay (≈150 ms) before activation, and A46T-I_{Ks} (as well as H455Y-I_{Ks}) lost initial current delay with a rapid activation/rising phase. In the mutation T265I, a longer initial 100-ms current delay and small current magnitude would be responsible for prolonged QT intervals. Studies have demonstrated that the initial delay phase of I_{Ks} channel activation is caused by its β-subunit *KCNE1* via moving the channel through multiple closed states before opening during a depolarization,[22,23] thereby decreasing the initial current activation. In the cases of A46T-IKs and H455Y-IKs, whether the interaction between the mutation and *KCNE1* is affected to some degree remains to be studied further. The mutation-caused loss of delay of the initial I_{Ks} activation could be caused by channel accumulation in open states between depolarization pulses. The mutation-caused loss of delay of the initial I_{Ks} activation could be associated with more channel accumulations in Zone 1 of closed states that are near-open states in the Markov model of the I_{Ks} channel kinetics. The channel accumulation in Zone 1 of closed states between cardiac action potentials provides an "available reserve"[24] that represents an important mechanism for I_{Ks} participation in repolarization and its dependence on rate. During the action potential repolarization, especially at fast heart rates, these readily available channels can very quickly open on demand to cause rapid I_{Ks} activation and rise, an effect to shorten cardiac action potential duration. Again, the current magnitude at the first 100 ms of I_{Ks} activation was larger in the mutation A46T than in WT current (Figure 4C). Therefore, these alterations in I_{Ks} properties could explain failure to observe a prolonged QT interval in the A46T patient.

Structural modeling helps us to conceptualize the different functional effects of particular mutations. Mutation of T265 and F339 affect interactions between S5 and S6. This is consistent with the T265I mutation displaying a longer delay (≈150 ms) before activation (Figure 4D) as the normal movements of the S5 helix required for efficient opening and closure are impeded by the substitution of threonine by the larger more hydrophobic isoleucine. F339S, on the other hand, involves substitution by a smaller more polar residue, which is less physically impeding but nevertheless remains pathogenic as indicated by the functional assays (Figure 2G).

Although the A302V substitution involves residues of similar size, the observed functional effects may be caused by the external aqueous environment being less forgiving of changes in hydrophobic interactions. Similarly, changes in polarity of exposed extramembrane residues on mutation affect $V_{0.5}$ values, resulting accordingly in more negative (F296S, change to polar) or more positive S546L (change to hydrophobic) values. The G316E mutation appears to obstruct directly the normal aperture of the pore by introducing a much larger and negatively charged glutamic acid side chain to one of the most sensitive gating positions. Modeling and comparison of the heteromeric mutation of 2 of the 4 chains (Figure 6D) with the WT (Figure 6C) reveal the extent to which the pore is likely to be obstructed. It is interesting to note that the mutation also disrupts the second glycine residue in a combinatorial sequence pattern [S/T]xx[S/T]xG[F/Y]G that has been identified in 90% of 134 potassium channel reentrant loops analyzed,[25] which suggests that this glycine residue may also be important for the stable insertion of the P loop within the membrane.

In summary, the biophysical characterization of these 9 *KCNQ1* missense mutations has provided unequivocal heterogeneous proof of pathogenicity in 8 variants to support the various clinical and genetics studies. We remain uncertain as to the pathogenicity of A46T, given that the suggestive biophysical evidence and cosegregation studies are inconclusive in part because of resistance to wider family screening. In this family it is possible that the genetic basis of the QT prolongation lies elsewhere, and further molecular screening of the index cases must remain an option. Although cellular electrophysiological testing is unlikely to move easily into the clinical diagnostic area, its clinical value is very important and not in question, given the high proportion of novel genetic variants being discovered presenting a challenge to the clinical and genetic counseling teams. For the time being, a collaborative multidisciplinary approach is needed between clinical and research domains to permit relative and informative proof for cardiologists and genetic counseling teams.

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Disclosures

None.

References

1. Ackerman MJ. The long QT syndrome: ion channel diseases of the heart. *Mayo Clin Proc*. 1998;73:250–269.
2. Yang P, Kanki H, Drolet B, Yang T, Wei J, Viswanathan PC, Hohnloser SH, Shimizu W, Schwartz PJ, Stanton M, Murray KT, Norris K, George ALJ, Roden DM. Allelic variants in long-QT disease genes in patients

with drug-associated torsades de pointes. *Circulation*. 2002;105:1943–1948.
3. Tester DJ, Will ML, Haglund CM, Ackerman MJ. Compendium of cardiac channel mutations in 541 consecutive unrelated patients referred for long QT syndrome genetic testing. *Heart Rhythm*. 2005;2:507–517.
4. Gouas L, Bellocq C, Berthet M, Potet F, Demolombe S, Forhan A, Lescasse R, Simon F, Balkau B, Denjoy I, Hainque B, Baro I, Guicheney P. New KCNQ1 mutations leading to haploinsufficiency in a general population: defective trafficking of a KvLQT1 mutant. *Cardiovasc Res*. 2004;63:60–68.
5. Wehrens XHT, Vos MA, Doevendans PA, Wellens HJJ. Novel insights in the congenital long QT syndrome. *Ann Intern Med*. 2002;137:981–992.
6. Vincent GM. The long QT and Brugada syndromes: causes of unexpected syncope and sudden cardiac death in children and young adults. *Semin Pediatr Neurol*. 2005;12:15–24.
7. Mohler PJ, Schott J-J, Gramolini AO, Dilly KW, Guatimosim S, duBell WH, Song L-S, Haurogne K, Kyndt F, Ali ME, Rogers TB, Lederer WJ, Escande D, Le Marec H, Bennett V. Ankyrin-B mutation causes type 4 long-QT cardiac arrhythmia and sudden cardiac death. *Nature*. 2003;421:634–639.
8. Tristani-Firouzi M, Jensen JL, Donaldson MR, Sansone V, Meola G, Hahn A, Bendahhou S, Kwiecinski H, Fidzianska A, Plaster N, Fu Y-H, Ptacek LJ, Tawil R. Functional and clinical characterization of KCNJ2 mutations associated with LQT7 (Andersen syndrome). *J Clin Invest*. 2002;110:381–388.
9. Splawski I, Timothy KW, Sharpe LM, Decher N, Kumar P, Bloise R, Napolitano C, Schwartz PJ, Joseph RM, Condouris K, Tager-Flusberg H, Priori SG, Sanguinetti MC, Keating MT. Ca(V)1.2 calcium channel dysfunction causes a multisystem disorder including arrhythmia and autism. *Cell*. 2004;119:19–31.
10. Napolitano C, Priori SG, Schwartz PJ, Bloise R, Ronchetti E, Nastoli J, Bottelli G, Cerrone M, Leonardi S. Genetic testing in the long QT syndrome: development and validation of an efficient approach to genotyping in clinical practice. *JAMA*. 2005;294:2975–2980.
11. Splawski I, Shen J, Timothy KW, Lehmann MH, Priori S, Robinson JL, Moss AJ, Schwartz PJ, Towbin JA, Vincent GM, Keating MT. Spectrum of mutations in long QT syndrome genes. *Circulation*. 2000;102:1178–1185.
12. Huang L, Bitner-Glindzicz M, Tranebaerg L, Tinker A. A spectrum of functional effects for disease causing mutations in the Jervell and Lange-Nielsen syndrome. *Cardiovasc Res*. 2001;51:670–680.
13. Anderson CL, Delisle BP, Anson BD, Kilby JA, Will ML, Tester DJ, Gong Q, Zhou Z, Ackerman MJ, January CT. Most LQT2 mutations reduce Kv11.1 (hERG) current by a class 2 (trafficking-deficient) mechanism. *Circulation*. 2006;113:365–373.
14. Wilson AJ, Quinn KV, Graves FM, Bitner-Glindzicz M, Tinker A. Abnormal KCNQ1 trafficking influences disease pathogenesis in hereditary long QT syndromes (LQT1). *Cardiovasc Res*. 2005;67:476–486.
15. Chung SK, MacCormick JM, McCulley CH, Crawford J, Eddy CA, Mitchell EA, Shelling AN, French JK, Skinner JR, Rees MI. Long QT and Brugada syndrome gene mutations in New Zealand. *Heart Rhythm*. 2007;4:1306–1314.
16. Harrison-Woolrych M, Clark DW, Hill GR, Rees MI, Skinner JR. QT interval prolongation associated with sibutramine treatment. *Br J Clin Pharmacol*. 2006;61:464–469.
17. Smith JA, Vanoye CG, George AL Jr, Meiler J, Sanders CR. Structural models for the KCNQ1 voltage-gated potassium channel. *Biochemistry*. 2007;46:14141–14152.
18. Choi G, Kopplin LJ, Tester DJ, Will ML, Haglund CM, Ackerman MJ. Spectrum and frequency of cardiac channel defects in swimming-triggered arrhythmia syndromes. *Circulation*. 2004;110:2119–2124.
19. Marx SO, Kurokawa J, Reiken S, Motoike HK, D'Armiento J, Marks AR, Kass RS. Requirement of a macromolecular signaling complex for β adrenergic receptor modulation of the KCNQ1-KCNE1 potassium channel. *Science*. 2002;295:496–499.
20. Yang T, Kanki H, Roden DM. Phosphorylation of the I_{Ks} channel complex inhibits drug block: novel mechanism underlying variable anti-arrhythmic drug actions. *Circulation*. 2003;108:132–134.
21. Wiener R, Haitin Y, Shamgar L, Fernandez-Alonso MC, Martos A, Chomsky-Hecht O, Rivas G, Attali B, Hirsch JA. The KCNQ1 (Kv7.1) COOH terminus, a multitiered scaffold for subunit assembly and protein interaction. *J Biol Chem*. 2008;283:5815–5830.
22. Balser JR, Bennett PB, Roden DM. Time-dependent outward current in guinea pig ventricular myocytes: gating kinetics of the delayed rectifier *J Gen Physiol*. 1990;96:835–863.
23. Silva J, Rudy Y. Subunit interaction determines IKs participation in cardiac repolarization and repolarization reserve. *Circulation*. 2005;112:1384–1391.
24. Rudy Y, Silva JR. Computational biology in the study of cardiac ion channels and cell electrophysiology. *Q Rev Biophys*. 2006;39:57–116.
25. Lasso G, Antoniw JF, Mullins JG. A combinatorial pattern discovery approach for the prediction of membrane dipping (re-entrant) loops. *Bioinformatics*. 2006;22:e290–e297.

CLINICAL PERSPECTIVE

Long-QT syndrome (LQTS) is characterized by prolonged QT interval on the ECG, syncope, and sudden death caused by ventricular arrhythmia. Inherited LQTS is caused by mutations in cardiac ion channel genes, and mutation screening in LQTS is now a well-established part of genetic service programs around the world. Identification of mutations that are known to cause arrhythmias is useful. The significance of novel mutations is often unclear. Is such a mutation a rare natural variation in the human genetic code or a pathophysiological entity predisposing to sudden death? This study supports the use of biophysical testing to provide evidence for functional pathogenicity of such mutations. The protein coded for by the mutated gene can be expressed in vitro to allow electrophysiological characterization of the resulting ion channels, providing an indication of the mechanisms by which the clinical phenotype is produced. Electrophysiology analysis identified dominant negative/loss-of-function properties in 7 of 9 novel LQTS mutations evaluated in this study, supporting the disease-causing nature of these mutations. These findings support the use of electrophysiological characterization to potentially validate LQTS pathogenicity of novel missense mutations in ion channels. This approach may be helpful in assessing risk and providing genetic counseling and screening of families with novel mutations.