Clinical Research

Novel Arrhythmogenic Mechanism Revealed by a Long-QT Syndrome Mutation in the Cardiac Na\(^+\) Channel

Hugues Abriel, Candido Cabo, Xander H.T. Wehrens, Ilaria Rivolta, Howard K. Motoike, Mirella Memmi, Carlo Napolitano, Silvia G. Priori, Robert S. Kass

Abstract—Variant 3 of the congenital long-QT syndrome (LQTS-3) is caused by mutations in the gene encoding the \(\alpha\) subunit of the cardiac Na\(^+\) channel. In the present study, we report a novel LQTS-3 mutation, E1295K (EK), and describe its functional consequences when expressed in HEK293 cells. The clinical phenotype of the proband indicated QT interval prolongation in the absence of T-wave morphological abnormalities and a steep QT/R-R relationship, consistent with an LQTS-3 lesion. However, biophysical analysis of mutant channels indicates that the EK mutation changes channel activity in a manner that is distinct from previously investigated LQTS-3 mutations. The EK mutation causes significant positive shifts in the half-maximal voltage \((V_{1/2})\) of steady-state inactivation and activation \((+5.2\) and \(+3.4\) mV, respectively). These gating changes shift the window of voltages over which Na\(^+\) channels do not completely inactivate without altering the magnitude of these currents. The change in voltage dependence of window currents suggests that this alteration in the voltage dependence of Na\(^+\) channel gating may cause marked changes in action potential duration because of the unique voltage-dependent rectifying properties of cardiac K\(^+\) channels that underlie the plateau and terminal repolarization phases of the action potential. Na\(^+\) channel window current is likely to have a greater effect on net membrane current at more positive potentials (EK channels) where total K\(^+\) channel conductance is low than at more negative potentials (wild-type channels), where total K\(^+\) channel conductance is high. These findings suggest a fundamentally distinct mechanism of arrhythmogenesis for congenital LQTS-3. (Circ Res. 2001;88:740-745.)

Key Words: long-QT syndrome  ■  Na\(^+\) channel  ■  genetics  ■  arrhythmias

Electrical activity in the heart is the result of a complex interaction of a large number of ion channels, pumps, and exchange mechanisms that serve unique physiological roles in anatomically distinct regions.\(^1\)\(^-\)\(^2\) The action potential duration (APD) of ventricular cells is determined by a long-lasting (hundreds of milliseconds) depolarization or plateau phase that is controlled by a fine balance between small inward and outward ionic currents and during which there is little change in membrane potential.\(^3\) Key to an energetically favorable maintenance of depolarization are the voltage-dependent rectification properties of at least two types of potassium channel currents, \(I_{K1}\) and \(I_{Ko}\), which restrict outward movement of potassium during the plateau but permit large outward currents during the terminal phases of repolarization as the membrane potential becomes more negative.\(^4\)\(^-\)\(^5\)

Physiological insight into the roles of ion channels in the control of the human cardiac action potential (AP) plateau phase has grown rapidly during the past 5 years from studies of the congenital long-QT syndrome (LQTS), an inherited cardiac arrhythmia, which is clinically characterized by prolongation of the ECG QT interval, syncope, and sudden death.\(^6\)\(^-\)\(^12\) The unexpected importance of Na\(^+\) channel activity to the control of QT intervals has been revealed by studies of LQTS-3, which is caused by mutations in the SCN5A gene, which codes for the \(\alpha\) subunit of the cardiac Na\(^+\) channel.\(^8\)\(^-\)\(^10\)\(^13\)

Expression of LQTS-3 mutant channels in heterologous systems has revealed mutation-induced channel activity that either directly\(^14\)\(^-\)\(^16\) or indirectly\(^17\)\(^-\)\(^20\) causes a small increase in net inward current over the voltage range and time course of the AP plateau. Computational analysis has shown that this increase in inward current is sufficient to explain the cellular phenotype of APD prolongation.\(^17\)\(^21\) Nevertheless, not all LQTS-3 mutations alter Na\(^+\) channel functional properties in the same manner, and distinction in mutation-induced changes in channel properties is important to document, not only because of the possibility of mutation-specific clinical phenotypes but also because such changes may have implications for therapeutic intervention.

In the present study, we report the biophysical consequences of a novel LQTS-3 mutation that change a conserved negative into a positive amino acid (E1295K [EK]) in a region immediately adjacent to the extracellular portion of the S4 segment of channel domain III (DIIIS4). Expression of mutant channels in a mammalian cell line indicates that the
primary effect of this mutation is to cause small positive shifts in the voltage dependence of both activation and inactivation gating of the channel, which, in turn, shift the window of voltages over which noninactivating Na\(^+\) channel activity can be measured.\(^{22-24}\)

These small changes in the voltage dependence of Na\(^+\) channel gating occur over the plateau range of membrane potentials for which two cardiac K\(^+\) channel currents, \(I_{K1}\) and \(I_{K2}\), show strong inward rectification.\(^{4,25-32}\) Window current that flows during the terminal phase of repolarization (wild-type [WT] channels), where rectification of \(I_{K1}\) and \(I_{K2}\) is being relieved, is likely to be less effective than window current that flows over more positive voltages (EK channels) where the conductance of these two channels is minimal. The linkage of these small changes in Na\(^+\) channel gating to delay in ventricular repolarization through LQTS-3 confirms the important principle of balance of currents necessary to maintain the AP plateau phase and the role of membrane input impedance in determining the effects of small changes in ion channel currents on cellular electrical activity.\(^3\)

Thus, our findings are important because they further support the concept of phenotypic heterogeneity of LQTS-3 that has to be taken into account when developing new therapeutic strategies for this disorder.

## Materials and Methods

### Molecular Screening

Genomic DNA was extracted from peripheral blood lymphocytes by standard techniques. The coding region of the SCN5A was screened using single-strand conformation polymorphism (SSCP) on polymerase chain reaction (PCR)-amplified genomic DNA samples. The abnormal conformers were directly sequenced using an ABI310 genetic analyzer or cloned (TopoTA cloning, Invitrogen) and sequenced using plasmid-specific oligonucleotides. SSCP shifts were also checked against a panel of genomic DNA from 300 (600 chromosomes) healthy reference individuals. The presence of a second mutation in the remaining LQTS-related genes (ie, \(KCNQ1\), \(HERG\), \(KCNE1\), and \(KCNE2\)) was excluded by molecular analysis.

### Mutagenesis and Expression of Recombinant Na\(^+\) Channels

The E1295K mutation of SCN5A was engineered into WT cDNA cloned in pcDNA3.1 (Invitrogen) using standard PCR techniques. Transient transfections of WT and mutant Na\(^+\) channels in HEK293 cells were carried out with equal amounts of Na\(^+\) channel \(\alpha\) subunit, with \(h\beta_1\) and/or \(h\beta_2\) subunits cDNA subcloned individually into the pcDNA3.1 (Invitrogen) vector (total cDNA 2.5 \(\mu\)g) using a previously described procedure.\(^3\) We found no difference in the properties of expressed channels with or without cotransfection of \(h\beta_2\).

### Electrophysiology

Membrane currents were measured using whole-cell patch-clamp procedures, with Axopatch 200B amplifiers (Axon Instruments). Recordings were made at room temperature (22°C) using an internal solution containing (mmol/L) CsCl 60, cesium aspartate 80, EGTA 11, MgCl\(_2\) 1, CaCl\(_2\) 1 (effective free calcium 100 nmol/L), HEPES 10, and Na\(_2\)-ATP 5, pH adjusted to 7.2 with CsOH. See the online data supplement available at http://www.circresaha.org for details of external solutions and software used. Holding potentials were –80 mV unless otherwise indicated. Time-dependent shifts in the voltage dependence of gating processes were minimized by the use of our internal solution and by performing time-matched recordings when no significant shifts were observed (see online data supplement for details). Data are represented as mean±SEM. Two-tailed Student’s \(t\) test was used to compare means; \(P<0.05\) was considered statistically significant.

An expanded Materials and Methods section can be found in an online data supplement available at http://www.circresaha.org.

## Results

### Description of a New LQTS-3 Mutation in a Patient With QTc Prolongation

The patient, an 18-year-old white man, was referred to us because of the documentation of QT interval prolongation at medical checkup. No history of syncope and cardiac arrest was present. Family history was negative for syncope and sudden death. The 12-lead ECG showed normal sinus rhythm and normal atrioventricular and intraventricular conduction; a QT interval prolongation (QTc D2: 480 ms), in the absence of T-wave morphological abnormalities, was also observed. No ventricular ectopies were found at 24-hour Holter recording or elicited at the exercise stress test. Interestingly, QTc interval was shorter during relative tachycardia (both at Holter and exercise stress test, Figures 1A and 1B), indicating a steep QT/R-R relationship. Figure 1C, which compares the relationship between QT and R-R intervals for this proband

![Figure 1. ECG recordings of the EK carrier during relative tachycardia (A) and bradycardia (B), showing the increase of QT corrected for heart rate with Bazett's formula (QTc) at slower heart rates. C, Analysis of the QT versus R-R interval relationship in the EK carrier. QT and R-R intervals were measured in lead D2 during 12-lead Holter recording at different heart rates for the mutation carrier and for a healthy control subject (the mother of the proband). The QT/R-R slopes were 0.254 and 0.145 for the EK patient and the control subject, respectively, indicating the increased propensity to excessive QT prolongation at slow heart rate for the proband.](http://circres.ahajournals.org/DownloadedFrom/abriel-et-al-novel-lqts-3-mutation-741)
and a noncarrier indicates almost a doubling of the QT/R-R slope for the proband. These data show an increased propensity to excessive QT prolongation at slow heart rate for the carrier of the EK mutation and suggest increased risk of arrhythmia during bradycardia. This abnormal adaptation of the QT interval has been reported in carriers of LQTS-3 mutations but not in carriers of LQTS-1 or LQTS-2 mutations or in control subjects. Thus, the clinical phenotype suggested the possibility of an LQTS-3 lesion.

Analysis of the DNA of the proband by SSCP in fact revealed an abnormal conformer in the exon 22 of the SCN5A gene. Subsequent DNA sequence analysis of this exon with specific primers for the regions of introns 21 and 22 flanking exon 22 showed a single nucleotide transition from G to A at the first base of the codon 1295 leading to the missense mutation Glu1295Lys, changing the charge at this location from negative to positive (Figure 2). Because this change in charge occurs close to the extracellular extremity of DIIIS4, a critical contributor to channel gating, we next sought to determine whether functional changes accompany this amino acid change.

**Biophysical Characterization of the EK Mutation in HEK293 Cells**

We performed a detailed characterization of mutant EK channels to look for biophysical properties that may explain the observed clinical phenotype. HEK293 cells transiently transfected with EK cDNA expressed Na⁺ currents similar in amplitude and time course to WT channels (Figure 3A). Mean peak current density was not different in cells expressing WT or EK channels: 254±21 pA/pF (n=13) and 259±31 pA/pF (n=10), respectively. Figure 3B shows that, in contrast to most of the previously reported LQTS-3 mutations, we did not measure any mutation-induced increase in sustained current in response to voltage pulses applied near the expected peak of the I/V relationship (−10 mV). The measured fractional tetrodotoxin (TTX)-sensitive current after 50 ms was 0.29±0.09% (n=9) of peak current with WT channels and 0.24±0.07% (n=5) with mutant channels.

We next fitted the time course of the onset of current inactivation with a monoeXponential decay function, because it is possible that changes in inactivation kinetics may contribute to control of APD. In some cells, this fit could be slightly improved when a biexponential model was used, but in these fits, the slower component of inactivation never exceeded 10% of the total weight (data not shown). Therefore, we used a monoeXponential model for simplicity. The effect of the EK mutation on kinetics was restricted to the voltage range between −35 and −20 mV where it significantly slowed the onset of inactivation (Figure 4A). We then measured the time course of recovery from inactivation (RFI) (after a 100-ms conditioning pulse to −10 mV) and observed a mutation-induced speeding of this process (Figure 4B). We next evaluated the effects of the EK mutation on the voltage dependence of steady-state activation and inactiva-
Figure 5. Steady-state activation and inactivation relationships were measured with protocols presented in the insets. For activation measurements, peak currents were normalized to driving force to determine conductance. Fits to experimental data yielded the resulting Boltzmann parameters (WT and EK). Inactivation (mV): \( I_{1/2} = -65.2 \pm 0.2 \); \( V_{1/2} = -60.0 \pm 0.02 \); \( K = 6.1 \pm 0.2 \); \( 6.1 \pm 0.1 \) (n=13 for WT and n=10 for EK); activation: \( V_{1/2} = -21.5 \pm 0.3 \); \( -18.6 \pm 0.3 \); \( 6.9 \pm 0.3 \); \( 6.7 \pm 0.2 \) (n=6 for WT and n=7 for EK). All differences between inactivation and activation \( V_{1/2} \) are statistically significant at \( P<0.01 \).

Mutation-Induced Shift in Window Current

Are these effects on the voltage dependence of activation and inactivation related to the cellular phenotype: APD prolongation? Close inspection of the effects of the EK curves in Figure 5 suggests that the mutation changes the window of voltages where these two curves overlap. To test for this, we used a slow-voltage ramp protocol to measure window currents. Such slowly rising voltage ramps promote inactivation of transient currents and have proven useful in the measurement of the voltage dependence of noninactivating \( Na^+ \) current for other LQTS-3 mutations. We applied this protocol before and after the application of 30 \( \mu \)mol/L TTX to first measure and then subtract background currents. As illustrated in Figure 6, we measured small \( Na^+ \) channel window current for WT and EK channels. Current density of the window current was not modified by the mutation. However, the peak of the window current is shifted. The average voltage of the peak was shifted by about +10 mV by the EK mutation: \( WT = -44.5 \pm 1.3 \) mV (n=8) and \( EK = -35.5 \pm 1.0 \) mV (n=10); \( P<0.001 \). The \( I/V \) relationship obtained from this protocol indicates a very restricted range of voltages over which noninactivating \( Na^+ \) current is expressed for the EK channel, a range of voltages that coincides only with the window of overlap between the two gating curves (Figure 5). Furthermore, this voltage dependence distinguishes the activity of EK mutant channels from other LQTS-3 mutant channels that conduct sustained channel activity over a broader voltage range and have a fundamentally distinct response to voltage-ramp protocols as illustrated by the \( \Delta KPQ \) mutation.

Discussion

Novel Mechanism of APD Prolongation Linked to the LQTS-3 Syndrome

The main findings of this study are that a novel SCN5A mutation, which is linked to a clinical phenotype of LQTS-3, changes the biophysical properties of expressed channels in a manner that is distinct from previously reported LQTS-3 mutations. In contrast with other previously investigated LQTS-3 mutations such as the \( \Delta KPQ \) mutation, the EK mutation promotes sustained channel activity only over a very narrow window of voltages that differs from WT channels only in the voltage range of the window. To significantly alter net membrane current, the mutation-induced window current must flow when currents through repolarizing channels are small.

Heterogeneity in the Pathological Mechanisms in LQTS-3

Thus far, seven mutations linked with LQTS-3 have been functionally studied. Most of these mutations alter the fast inactivation properties of the channel-inducing sustained \( Na^+ \) current on prolonged membrane depolarization over a wide range of voltages.
range of plateau voltages. Such a defect is consistent with an increase in inward current during the entire plateau phase of the AP, which consequently prolongs the APD. This is not the only mechanism by which LQTS-3–linked Na\(^+\) channel mutations are able to prolong cellular APD. Recent studies\(^{37,38}\) showed that an LQTS-3 mutation in the carboxy-terminus region of the channel (D1790G), which does not produce persistent current different from WT channels, prolongs APD indirectly through effects on other ion channels. In the present work, we report yet a third mechanism by which an LQTS-3 mutation is likely to prolong the cellular AP.

**Roles of K\(^+\) Channel Rectification in EK Channel Cellular Phenotype**

It has been known for almost 50 years that the plateau phase of the cardiac AP is a period in which the cellular input impedance is maximal\(^3\). Consequently, during this phase, small changes in net ionic current will cause large changes in membrane potential. Repolarization of the AP normally begins when outward current through delayed rectifier (I\(_{\text{Ks}}\) and I\(_{\text{Kr}}\)) channels is sufficient to just balance and eventually exceed the total flow of inward current across the membrane. As repolarization begins, the cellular membrane potential reenters the voltage range for which WT Na\(^+\) channels may again open to contribute as window current. This voltage range, however, coincides with voltages at which rectification of inward rectifier (I\(_{\text{K1}}\)) and HERG channels\(^{32,41,42}\) is relieved. Consequently, the effect of WT Na\(^+\) channel window current is minimal. In the case of cells expressing EK channels, the range of voltages over which window current can flow is more positive, and the contribution of these channels is more pronounced because window current through them is activated over a voltage range in which rectification of I\(_{\text{Ks}}\) and I\(_{\text{Kr}}\) channels minimizes their contribution to total membrane current.

Note that because the functional effects of this mutation are most pronounced when background K\(^+\) channel currents are minimized, it is likely that the cellular (and hence systemic) effects of the mutation may be more pronounced in cells with smaller outward repolarizing current such as M cells.\(^{43}\) If one assumes heterogeneity of repolarizing current across the ventricular wall,\(^{44}\) then it is likely that the effect of the EK mutation will be blunted in cells with higher density of outward currents. A difference between repolarization in different cell types might therefore exacerbate heterogeneity in APD within the myocardial wall and increase the likelihood of an arrhythmic event.\(^{45}\)

**Structure-Function Relationship**

Glutamic acid (E1295), mutated into a positively charged lysine in this patient, is found in a 4-amino acid extracellular loop connecting DIII S3 with DIII S4. E1295 is the amino acid closest to DIII S4, which is one of the voltage sensors for activation gating process of the channel.\(^{46}\) The presence of a negative residue just external to the DIII S4 segment is not only conserved among cardiac channels of different species but also among all different isoforms of the Na\(^+\) channel. We postulate that this mutation-induced change in charge alters the voltage dependence of activation and/or inactivation gating sensor and that this is reflected by the recorded shifts in steady-state activation and inactivation curves. Window currents are found at voltages where there is overlap of the activation and inactivation curves, and as there is a concomitant shift of both, window currents are shifted accordingly (Figure 6). The EK mutation hastens the RFI after a 100-ms inactivating pulse. This observation is consistent with recent observations\(^{47,48}\) showing that movement of DIV S4 and DIV S4 is coupled to fast inactivation. These changes in channel kinetics may also have to be taken into account when considering the basis of frequency-dependent changes in cellular electrical activity induced by the EK mutation.

**Clinical and Electrophoretic Implications**

This study provides a further illustration that the LQTS-3 type of LQTS is aggravated by bradycardia (Figure 1). Beta-blockers are the mainstay in the management of the LQTS; however, the efficacy of this treatment in LQTS-3 patients has been questioned\(^{49}\) mainly because the decrease in heart rate that accompanies this treatment can enhance QT prolongation in LQTS-3 carriers. Because the present study provides evidence that bradycardia markedly aggravates the phenotype (prolonged QT) in the case of the EK mutation, our results predict that beta-blockers may worsen the phenotype carriers of this mutation by slowing heart rate. Clearly, our results emphasize the importance of identifying the genotype of LQTS patients in general and LQTS-3 patients in particular, before an optimum therapeutic regimen can be planned. For example, in the case of the EK mutation, alternative strategies such as pacing (which prevents bradycardia) or administration of mexiletine (which by shifting the inactivation curve to more negative voltages may normalize the voltage dependence of the window current) might prove to be more effective in preventing arrhythmias.

**Implications for Drug-Induced LQTS**

Our investigation into the functional consequence of the EK mutation on Na\(^+\) activity provides one more example of the delicate interplay of small inward and outward currents that control the plateau phase of the cardiac AP. In the case of the EK mutation, there is no net increase in the magnitude of inward current flowing through Na\(^+\) channels; instead, there is a shift in the voltages over which channels can reopen. Such small changes in the voltage-dependent properties of inward Na\(^+\) channel current can have marked effects on APD only if they occur in the presence of a highly nonlinear background of outward K\(^+\) channel currents.\(^{50}\) By inference, these results suggest that alteration in background K\(^+\) channel activity against different patterns of Na\(^+\) channel activity would be expected to have distinct effects on APD. It is interesting to speculate that subtle changes in Na\(^+\) channel gating caused by coding changes in the α subunit, insufficient to cause substantial changes in cellular APD by themselves, may lead to excessive changes in APD in the face of inhibition of one of these key K\(^+\) channels and thus contribute to some forms of drug-induced LQTS. Experiments are underway to test for this possibility.

**Acknowledgments**

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Novel Arrhythmogenic Mechanism Revealed by a Long-QT Syndrome

Mutation in the Cardiac Na⁺ Channel

Hugues Abriel, Candido Cabo, Xander H.T. Wehrens, Ilaria Rivolta, Howard K. Motoike, Mirella Memmi, Carlo Napolitano, Silvia G. Priori, and Robert S. Kass

Corresponding author: R.S. Kass
**Expanded Materials and Methods**

**Molecular screening**

Genomic DNA was extracted from peripheral blood lymphocytes by standard techniques. The coding region of the *SCN5A* gene encoding the cardiac Na\(^+\) channel \(\alpha\) subunit was screened using single strand conformation polymorphism (SSCP) on PCR amplified genomic DNA samples. The abnormal conformers were directly sequenced using an ABI310 genetic analyzer or cloned (TopoTA cloning, Invitrogen) and sequenced using plasmid-specific oligonucleotides. SSCP shifts were also checked against a panel of genomic DNA from 300 (600 chromosomes) healthy reference individuals. The presence of a second mutation in the remaining LQTS-related genes (i.e. *KCNQ1*, *HERG*, *KCNE1* and *KCNE2*) was excluded by molecular analysis.

**Mutagenesis and expression of recombinant Na\(^+\) channels**

The E1295K (EK) mutation of *SCN5A* was engineered into WT cDNA cloned in pcDNA3.1 (Invitrogen) by overlap extension using mutation-specific primers and Quick Change Site-Directed Mutagenesis Kit (Stratagene). The presence of the mutation was confirmed by sequence analysis. WT and mutant Na\(^+\) channels were expressed in HEK293 cells. Briefly, transient transfections were carried out with equal amounts of Na\(^+\) channel \(\alpha\) subunit, with h\(\beta_1\), and/or h\(\beta_2\) subunits cDNA subcloned individually into the pcDNA3.1 (Invitrogen) vector (total cDNA 2.5 \(\mu\)g) using a previously described lipofection procedure\(^1\). Control
Online Data-supplement experiments (data not shown) indicated no significant differences in channel activity for these subunit combinations.

**Electrophysiology**

![Graphs showing voltage dependence of inactivation and activation](image)

**Figure 1 supplement.** Stability of recordings with intracellular solution used in this study. **Inactivation (A) and activation (B) curves** determined as in Methods recorded at the indicated times. We detect no significant change in the voltage-dependence of inactivation or activation over the recording periods used in this study.

Membrane currents were measured using whole cell patch-clamp procedures, with Axopatch 200B amplifiers (Axon Instruments, Foster City, CA). Recordings were made at room temperature (22 °C) using an internal solution containing...
Online Data-supplement

(mmol/L) CsCl 60, Cs Aspartate 80; EGTA 11, MgCl₂ 1, CaCl₂ 1 (effective free calcium 100 nmol/L), HEPES 10, and Na₂-ATP 5, pH adjusted to 7.2 with CsOH. External solution consisted of (mmol/L) NaCl 130, CsCl 5, CaCl₂ 2, MgCl₂ 1.2, HEPES 10, glucose 5, pH 7.4. In experiments recording I/V curves, external Na⁺ was reduced to 30 mmol/L using n-methyl-glucamine as a Na⁺ substitute. The use of these solutions in our experiments allowed us to make very stable recordings with minimal time-dependent changes in voltage-parameters of activation and inactivation.

We have carried out control experiments for both voltage dependence of activation and inactivation. After an initial dialysis time of 1 to 2 minutes, we determined that there are no statistically significant changes in the voltage-dependence of activation or inactivation for periods at least as long as 12 minutes (Fig. 1 Supplement). However, in order to ensure reproducibility, we systematically timed each experiment and thus restrict recording times between 3 min and 8 min after rupturing the membrane to establish whole conditions. pClamp7 (Axon Instruments, Foster City, CA); Excel (Microsoft, Seattle, WA); and Origin (Microcal Software, Northampton, MA) were used for data acquisition and analysis. Protocols used have been described previously by us²³. Holding potentials were –80 mV unless noted otherwise. Control experiments of voltage-dependence of activation and inactivation with –100 mV holding potentials did not yield significantly different values. Data are represented as mean ± SEM.
Two-tailed Student t-test was used to compare means; p < 0.05 was considered statistically significant.
Reference List

