Altered Na⁺ Channels Promote Pause-Induced Spontaneous Diastolic Activity in Long QT Syndrome Type 3 Myocytes

Sandra Fredj, Nicolas Lindegger, Kevin J. Sampson, Peter Carmeliet, Robert S. Kass

Abstract—Long QT syndrome (LQTS) type 3 (LQT3), typified by the ΔKPQ mutation (LQT3 mutation in which amino acid residues 1505 to 1507 [KPQ] are deleted), is caused by increased sodium entry during the action potential plateau resulting from mutation-altered inactivation of the Na₅.1.5 channel. Although rare, LQT3 is the most lethal of common LQTS variants. Here we tested the hypothesis that cellular electrical dysfunction, caused not only by action potential prolongation but also by mutation-altered Na⁺ entry, distinguishes LQT3 from other LQTS variants and may contribute to its distinct lethality. We compared cellular electrical activity in myocytes isolated from mice heterozygous for the ΔKPQ mutation (ΔKPQ) and myocytes from wild-type littermates. Current-clamp pause protocols induced rate-dependent spontaneous diastolic activity (delayed afterdepolarizations) in 6 of 7 ΔKPQ, but no wild-type, myocytes (n=11) tested. Voltage-clamp pause protocols that independently control depolarization duration and interpulse interval identified a distinct contribution of both depolarization duration and mutant Na⁺ channel activity to the generation of Ca⁺⁺-dependent diastolic transient inward current. This was found at rates and depolarization durations relevant both to the mouse model and to LQT3 patients. Flecainide, which preferentially inhibits mutation-altered late Na⁺ current and is used to treat LQT3 patients, suppresses transient inward current formation in voltage-clamped ΔKPQ myocytes. Our results demonstrate a marked contribution of mutation-altered Na⁺ entry to the incidence of pause-dependent spontaneous diastolic activity in ΔKPQ myocytes and suggest that altered Na⁺ entry may contribute to the elevated lethality of LQT3 versus other LQTS variants. (Circ Res. 2006;99:1225-1232.)

Key Words: ion channels ■ persistent current ■ long QT syndrome

Arrhythmias result from abnormalities in impulse initiation or impulse conduction or a combination of both.¹ One of the possible cellular mechanisms for impulse initiation is triggered activity caused by afterdepolarizations. There are 2 types of afterdepolarizations in heart. Early afterdepolarizations (EADs) are generated near action potential plateau voltages before repolarization is complete and are driven, at least in part, by L-type calcium channel activity.² A second type of arrhythmic disturbance, generally referred to as “delayed after depolarization” (DAD) because of the close coupling between preceding action potential activity and subsequent depolarization that follows with a delay,³ can occur over more negative voltages. Both EADs and DADs are calcium-dependent events that can be exacerbated by changes in action potential waveform.⁴,⁵ It is now clear that action potential prolongation, whether disease or drug induced, can lead to life-threatening cardiac arrhythmias, most likely through 1 or both of these pathways.⁶

The acquired and congenital long QT syndromes (LQTSs), caused either by drug- or mutation-induced changes in cardiac ion channel function, share a common phenotype of action potential (and hence concomitant QT interval) prolongation.⁷ Thus LQTS-associated arrhythmias are likely to occur via 1 or both of these Ca⁺⁺-dependent pathways. In most cases, action potential prolongation occurs in LQTSs (acquired and congenital) because of mutation or drug-induced loss of channel function. However, this is not the case for LQTS type 3 (LQT3), which is caused by mutations in SCN5A, the gene coding for the Na₅.1.5 sodium channel α subunit. Here, mutation-induced changes in Na⁺ channel gating provide additional inward Na⁺ channel current that delays cellular repolarization.⁸ Though rare, LQT3 mutations are thought to be the most lethal of the prevalent LQTS mutations.⁹

Previous in vivo¹⁰,¹¹ and in silico¹²,¹³ studies have provided support for the concept that LQTS-induced action potential prolongation can induce EAD-driven electrical activity, which is related in a gene or drug-dependent manner to heart rate and/or adrenergic input. However, little evidence has been presented to date that diastolic dysfunction may also be causally related to LQTS perturbation in electrical activity. Here we focused on a well-described LQT3 mutation in which amino acid residues 1505 to 1507 (KPQ) are deleted in the Nav1.5 channel (ΔKPQ mutation)¹⁴ that promotes persis-
tent or sustained Na\(^+\) channel current (\(I_{\text{NaL}}\)) during prolonged depolarization through mutation-altered inactivation.\(^{15}\) We took advantage of myocytes from a previously described mouse heterozygous for a knock-in KPQ deletion (\(\Delta\text{KPQ}\))\(^{11}\) to test the hypothesis that, in addition to previously reported proarhythmic behavior in the form of rate-dependent EAD driven dysfunction, myocytes isolated from these mice are also predisposed to spontaneous diastolic DAD arrhythmic activity caused by mutation-altered Na\(^+\) entry.

Our results support this hypothesis and provide evidence for the first time that although all LQTS mutations, independent of the mutated gene, have a common phenotype (delayed ventricular repolarization); our results demonstrate a marked additional contribution of mutation-altered Na\(^+\) entry to the incidence of pause-dependent spontaneous diastolic activity that is unique to LQT3 and suggest that altered Na\(^+\) entry may contribute to the elevated lethality of LQT3 versus other LQTS variants.

Materials and Methods

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org

Transgenic Mice and Isolation of Cardiac Ventricular Myocytes

Mice heterozygous for a knock-in KPQ deletion in SCN5A\(^{11}\) were genotyped by PCR analysis to confirm the expression of the SCN5A \(\Delta\text{KPQ}\) Na\(^+\) channels. Ventricular myocytes from adult mice (1 to 8 months of age) were dissociated as previously described.\(^{16,17}\) The institutional Animal Care and Use Committee at Columbia University approved the protocols for all animal studies.

Electrophysiology

Current- and voltage-clamp protocols and solutions are described in detail in the online data supplement. All experiments were carried out at room temperature (22°C).

Data Analysis

pClamp 8.0 (Axon Instruments Inc), Excel (Microsoft), and Origin (Microcal Software) were used for data acquisition and analysis. Data are presented as mean values±SEM. Two-tailed Student’s \(t\) test was used to compare 2 means; a value of \(P<0.05\) was considered statistically significant.

Results

The \(\Delta\text{KPQ}\) mutation disrupts Na\(^+\) channel inactivation such that during prolonged depolarization, channels harboring this mutation are characterized by a small component of noninactivating Na\(^+\) channel activity.\(^{15}\) Knock-in mice, heterozygous for Na\(^+\) channels with the \(\Delta\text{KPQ}\) mutation, express Na\(^+\) channel current with this phenotype that leads to prolongation of the ventricular action potential.\(^{11}\) We begin this study by illustrating the impact of the \(\Delta\text{KPQ}\) mutation on cellular action potentials and currents measured in myocytes isolated from \(\Delta\text{KPQ}\) heterozygous mice and their wild-type (WT) littermates under our recording conditions. Figure 1 illustrates the presence of \(I_{\text{NaL}}\) (Figure 1B) and subsequent action potential prolongation (Figure 1D) in myocytes isolated from \(\Delta\text{KPQ}\) knock-in compared with WT mice (Figure 1A and 1C) under our recording conditions. We found no significant differences in peak current density (\(I_{\text{NaP}}\)) in WT and \(\Delta\text{KPQ}\) (\(+/–\)) myocytes either in low-sodium solutions (illustrated in Figure 1E) or in full-sodium external solutions (data not shown). However, in the \(\Delta\text{KPQ}\) myocytes, a large component of \(I_{\text{NaL}}\) (1.35±0.17 pA/pF; \(n=12\)) was recorded (\(<10\) mV, 150 ms), which was significantly greater than \(I_{\text{NaL}}\) in WT myocytes (0.10±0.03 pA/pF; \(n=7\)). Furthermore, we compared total cell capacitance, which is a reflection of myocyte surface area, of WT (155.4±6.9 pF; \(n=7\)) versus \(\Delta\text{KPQ}\) (165.2±7.4 pF; \(n=12\)) myocytes and found no significant difference in this parameter. We also tested for but found no significant difference between L-type Ca\(^{2+}\) channel density in \(\Delta\text{KPQ}\) and WT myocytes (Figure IV in the online data supplement). Current-clamp action potential recordings for the \(\Delta\text{KPQ}\) myocytes are summarized in the Table 1. In comparison with recordings in WT myocytes, action potentials recorded in knock-in heterozygote myocytes displayed no significant differences in action potential peak voltage or resting membrane potential. However, the \(\Delta\text{KPQ}\) myocyte action potentials were substantially prolonged at 50% (APD\(_{50}\)) and 90% (APD\(_{90}\)) of depolarization. APD\(_{50}\) and APD\(_{90}\) values were measured respectively as 20.6±3.3 ms and 242.4±32.4 ms in \(\Delta\text{KPQ}\) myocytes as compared with \(\pm 0.4\) ms and 66.6±4.1 ms for WT myocytes (all \(P<0.001\), WT versus \(\Delta\text{KPQ}\) myocytes). Our results under our experimental conditions, thus, were consistent with those reported by Nuyens et al.,\(^{11}\) with the exception that we found no
significant difference in peak current expression in the −/+ myocytes.

Pause-Induced Spontaneous Depolarizations

We next performed a series of current-clamp studies to determine whether the ΔKPQ myocytes were more prone to pause induced depolarizations at diastole. In each experiment, a train of preconditioning pulses was delivered with 3-ms, near-threshold current pulses followed by a 500-ms pause, a single near-threshold current pulse, and prolonged diastole (see supplemental Figure I). Using this protocol and varying the frequency of the preconditioning train pulses, we observed a mutation- and frequency-dependent incidence of spontaneous diastolic depolarizations. Figure 2 shows that as ΔKPQ heterozygous myocytes are paced at increasing rates during conditioning trains, spontaneous events occur and become larger, more frequent, and less delayed after the final

Table 1. Mouse Action Potential Properties at 1 Hz

<table>
<thead>
<tr>
<th></th>
<th>Myocyte Electrophysiology (1Hz)</th>
<th>WT</th>
<th>Δ KPQ (+/−)</th>
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</thead>
<tbody>
<tr>
<td>Peak (mV)</td>
<td>49.4 ± 3.8</td>
<td>53.2 ± 9.4</td>
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<tr>
<td>RMP (mV)</td>
<td>−72.2 ± 0.6</td>
<td>−71.3 ± 1.5</td>
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<tr>
<td>APD_{50} (ms)</td>
<td>2.7 ± 0.3</td>
<td>20.6 ± 3.6</td>
<td></td>
</tr>
<tr>
<td>APD_{90} (ms)</td>
<td>62.3 ± 4.9</td>
<td>242.4 ± 32.4</td>
<td></td>
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</table>

For baseline electrophysiology, RMP is resting membrane potential, n=5 for ΔKPQ (+/−) myocytes and n=16 for WT myocytes. Data are shown as mean±SEM. Student’s t test: *P<0.001 WT vs ΔKPQ (+/−).

Figure 2. Pause-dependent afterdepolarizations in ΔKPQ myocytes. The traces shown include the last action potential from a train of 100 action potentials at 2 Hz (A and B), 5 Hz (C and D), and 10 Hz (E and F), followed by a 500-ms pause and single-triggered beat. Action potentials from cardiomyocytes isolated from WT mice (A, C, and E) elicited no spontaneous depolarizations, whereas ΔKPQ myocytes (B, D, and F) showed a frequency dependent incidence of after depolarizations.

stimulus. Only 1 of 5 ΔKPQ myocytes had spontaneous diastolic activity at 2 Hz and 2 of 5 at 5 Hz, whereas none of the WT myocytes displayed afterdepolarizations using this stimulus protocol. At 10 Hz, there is a significant difference (statistical power >95%, based on the difference and sample sizes) in DAD appearance comparing WT (0 of 13) versus ΔKPQ (5 of 7) myocytes (see the Table 2).

Voltage-Clamp Studies of Transient Inward Current

In the preceding current-clamp recordings, interpretation of the mechanism responsible for the afterdepolarizations was clouded by the concomitant increase in action potential duration (APD) present in myocytes with the LQT3 lesion as well as rate-dependent adaptation and cell-to-cell variability in APD. As a result, we designed voltage-clamp protocols and sonic conditions to mimic both the short and long action potentials recorded in WT and mutant myocytes. Similar to the previous experiments, we applied trains of 100 conditioning voltage waveforms (either short- or long-preconditioning pulses) followed by a 500-ms pause, 1 extra voltage pulse, and then diastole. The short pulse experiment used a voltage step from −75 to −10 mV for 2 ms followed by a 38-ms ramp back to −75 mV, whereas the longer pulse consisted of a voltage step from −75 to −10 mV for 20 ms followed by a 70 ms ramp to −75 mV (supplemental Figure II). The results shown in Figure 3 illustrate the effect of pulse duration, preconditioning stimulus rate, and the presence of the mutation on spontaneous activity, which, under voltage-clamp conditions, is observed as transient inward current (I_{TI}). Even in the case of WT myocytes, increasing the duration and pulse frequency of the conditioning train voltage pulses can induce I_{TI} (compare Figure 3 WT traces short- versus long-pulse data), but we consistently found ΔKPQ myocytes more susceptible to I_{TI} induction in response to the same conditioning protocols. To quantify the combined magnitude and prevalence of the I_{TI}, the time integral of the transient inward current (f_{I_{TI}}) is calculated for the 5-second interval following the last stimulus (see the expanded Materials and Methods section in the online data supplement). This represents the total charge transported during the spontaneous events, which correlates with generation of after depolarizations and extra systole under current-clamp conditions.18,19 This charge measurement is shown in Figure 3C and 3D, where it is evident that prolongation of the conditioning pulse and the presence of the ΔKPQ deletion mutation both predispose the myocytes to the generation of I_{TI}s.
Confocal imaging of Ca\(^{2+}\) transients in fluo 3-loaded myocytes indicated that transient inward currents correlated with measured spontaneous Ca\(^{2+}\) events at diastole following conditioning trains, and the transient currents were larger and more frequent in \(\Delta\)KPQ myocytes (\(\approx 2.5 \, \text{F/F}_0\) in 3 of 4 cells) than in WT myocytes (\(\approx 1.5 \, \text{F/F}_0\) in 2 of 4 myocytes) (supplemental Figure VI). These experiments indicate that the diastolic transient inward currents correlated with Ca\(^{2+}\) transients recorded at diastolic potentials following conditioning pulse trains. These are well-described properties of \(I_{\text{T}i}\)s generated by spontaneous sarcoplasmic reticulum Ca\(^{2+}\) release\(^{20}\) and carried largely by Na\(^{+}\)/Ca\(^{2+}\) exchange current.\(^{18}\)

If the \(I_{\text{T}i}\)s generated in the \(\Delta\)KPQ myocytes were caused by diastolic Ca\(^{2+}\) transients resulting from sarcoplasmic reticulum Ca\(^{2+}\) release, we reasoned that these events should be inhibited by increasing Ca\(^{2+}\) buffer capacity of intracellular recording solutions. In the preceding recordings, free [Ca\(^{2+}\)] in the pipette filling solution was buffered to 100 nmol/L using only 50 \(\mu\)mol/L EGTA (Materials and Methods) to minimize effects of EGTA on conditioning train-dependent changes in Ca\(^{2+}\). To test the role of Ca\(^{2+}\) buffering on \(I_{\text{T}i}\) recorded in these experiments, the short- and long-pulse protocols were repeated with another pipette solution in which free [Ca\(^{2+}\)] was again buffered to 100 nmol/L but with a high EGTA concentration (11 mmol/L). Figure 4 shows that high Ca\(^{2+}\) buffer suppresses \(I_{\text{T}i}\) formation. We found similar results in 5 of 5 \(\Delta\)KPQ myocytes tested, indeed suggesting a role for Ca\(^{2+}\) in the genesis of the spontaneous activity. After observing that the presence of \(I_{\text{T}i}\)s is greatly enhanced in the \(\Delta\)KPQ myocytes, we wanted to determine whether the mutation-induced changes in Na\(^{+}\) channel activity contributed to \(I_{\text{T}i}\) induction, providing a pathway for arrhythmia susceptibility that might be linked specifically to LQTS lesions. Therefore, voltage-clamp protocols were repeated in heterozygote and WT littermates in the absence or presence of tetrodotoxin (TTX) (30 \(\mu\)mol/L). Figure 5 shows current traces for both the short- and long-pulse voltage-clamp protocols in mutant and WT cells before and after treatment with TTX. In the \(\Delta\)KPQ myocytes, application of TTX greatly reduces the magnitude and number of the observed transient currents. Examining the time integral of the \(I_{\text{T}i}\), as shown in Figure 5, it is evident that TTX (30 \(\mu\)mol/L) significantly reduces the charge transported across the membrane. However, in WT myocytes, where \(I_{\text{T}i}\)s are observed with long-pulse conditioning trains, TTX application does not signif-

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**Figure 3.** The development of \(I_{\text{T}i}\)s as a function of depolarization duration, conditioning frequency, and the presence of the \(\Delta\)KPQ lesion. Current traces following 100 preconditioning pulses, at 5 and 10 Hz, and a single 500-ms pause using a short pulse (A) and long pulse (B). Complete stimulus protocol is given in the online data supplement. Bar graphs summarizing the time integral of the \(I_{\text{T}i}\)s recorded with short (C) and long (D) pulses for the 5 seconds at rest following the last depolarizing pulse.
preconditioning pulse. Figure 6 illustrates currents recorded in response to the 400-ms protocol at a preconditioning rate of 0.5 Hz for WT and ΔKPQ myocytes. For the ΔKPQ, but not WT myocytes, this protocol induces $I_{\text{TI}}$ (Figure 6B), which is suppressed by TTX (Figure 6D). Figure 6E shows summary data for the effects of TTX on $I_{\text{SI}}$ in WT and ΔKPQ myocytes. These protocols did not induce $I_{\text{SI}}$ in WT myocytes. In contrast, ΔKPQ myocytes were prone to $I_{\text{TI}}$ induction for both long- and short-pulse conditioning trains, and in all cases $I_{\text{TI}}$ was significantly reduced by TTX application.

$I_{\text{TI}}$ Suppression by Flecainide

The results with TTX implicate a role of altered Na$^+$ channel activity in $I_{\text{TI}}$ induction in ΔKPQ myocytes without implicating a role of nonactivating Na$^+$ channel activity in this process. Consequently, we tested the effects of flecainide on ΔKPQ myocyte $I_{\text{SI}}$ generated by conditioning trains. We chose to study flecainide because it not only preferentially inhibits late, nonactivating Na,1.5 channel activity, like other local anesthetic drugs, but also because it has been shown to be an effective therapeutic agent in the treatment of LQT3 mutation carriers. To examine this, the 100- and 400-ms depolarizing pulses were used again at 0.5 Hz and $I_{\text{TI}}$ was calculated for any events occurring before and after flecainide (3 μmol/L) application. We first confirmed that late current is approximately 5-fold more sensitive to flecainide block than peak current in ΔKPQ myocytes and that a 3 μmol/L flecainide concentration blocks almost 50% of late current, $I_{\text{SI}}$, while inhibiting less than 10% peak current (supplemental Figure III), and thus we chose to test this flecainide concentration on $I_{\text{TI}}$. Figure 7, which summarizes these experiments, shows that 3 μmol/L flecainide effectively inhibits $I_{\text{SI}}$ induced by 0.5-Hz conditioning trains of 100-ms (left panel) and 400-ms (right panel) pulses, as illustrated in Figure 7C and 7D compared with the drug free correlates with Figure 7A and 7B, respectively. The bar graphs in Figure 7E, which summarize data from multiple experiments for each protocol, plot mean charge carried by $I_{\text{TI}}$ as a function of preconditioning waveform. Flecainide significantly reduced the charge and subsequently the arrhythmogenic potential of these spontaneous events in response to both conditioning protocols.

Discussion

The results presented in this study suggest a role for mutation-enhanced $I_{\text{SI}}$ in the generation of spontaneous diastolic activity in LQT3. In myocytes isolated from mice heterozygous for a knock-in KPQ deletion (ΔKPQ), diastolic arrhythmogenic activity is significantly more prevalent than in myocytes isolated from WT littermates. Because pause-dependent arrhythmias were previously reported in ECG measurements in these mice, and because clinical, in vivo animal and computational studies have demonstrated correlation between the spontaneous arrhythmias in LQT3s following pauses in stimulation, we focused our studies on pause-dependent activity that might occur in myocytes. Our results show that even in WT myocytes, prolonged depolarization can lead to spontaneous activity ($I_{\text{SI}}$). Because $I_{\text{SI}}$s are known to be caused by sarcoplasmic reticulum calcium overload, our results indicate that even in WT myocytes
that are stimulated at sufficiently high frequencies and pulse durations, sarcoplasmic reticulum overload can occur. Our data indicate that these \( I_{\text{TI}} \)s are not TTX-sensitive and are consistent with effects of action potential duration on intracellular calcium dynamics via non–Na\(^+\)/H\(^+\) channel pathways such as L-type calcium channels.\(^2\) When the same protocols are applied to \( \Delta \)KPQ myocytes, spontaneous diastolic activity is more pronounced and is markedly suppressed by Na\(^+\)/H\(^+\) channel blockade. We suggest not only that the induction of diastolic activity by prolonged depolarization is a potential contributor to arrhythmogenesis for all LQTS mutations but also that its further increase and sensitivity to Na\(^+\) channel blockade in \( \Delta \)KPQ myocytes reveals a key role of altered Na\(^+\) channels in this process, which is unique to LQT3 mutations. This finding thus will impact therapeutic management of LQT3 patients and will provide additional strategies to develop treatments that are specific for distinct LQTS variants.

**Clinical Significance**

The influence of genotype on the clinical course of the LQTS has been the subject of multiple important and informative investigations,\(^9\,2\,20\,3\,29\,3\,32\) and, although clear patterns such as gene-related lethality and arrhythmia triggers are suggested, in part because of the relatively small numbers of cases available for study, relationships between genetic background and risk stratification remain complex. This is particularly relevant to studies of LQT3 patients, in which the number of patients investigated is the smallest of the major LQT variants. Here, studies suggest that high lethality is associated with arrhythmic events in LQT3\(^9\,2\) and that, compared with LQT1 and LQT2 patients, arrhythmia risk is higher during sleep for LQT3 patients, but elevated for LQT1 patients during exercise (elevated sympathetic nerve activity).\(^2\) However, a close look at the available data indicate that, even in the case of LQT3, other factors may also exacerbate arrhythmia risk because in the previous study, it was found that of the studied triggers in LQT3 patients: 39\% were during sleep, 13\% during exercise, 19\% during emotional events, and 29\% by unknown causes.\(^2\) Furthermore, during sleep, heart rate and sympathetic nerve traffic decrease progressively in non-rapid eye movement sleep, but both can increase significantly and transiently during rapid eye movement sleep.\(^3\) Extraction of a single risk factor for LQT3 sleep during sleep clearly is complex. Thus, it is important to investigate potential mutation-specific mechanisms that might contribute to arrhythmogenic activity under a range of conditions that include, but are not limited to, slow heart rate (stimulation rate) and prolonged action potential (voltage-pulse) duration.

In the case of our experimental analysis of \( I_{\text{TI}} \) induction in \( \Delta \)KPQ myocytes, conditioning pulse stimulation rate and dura-
tion increase the likelihood of \( I_{\text{NaL}} \) induction. However, it must be remembered that in these experiments, in contrast to physiological conditions in LQT3 patients, conditioning pulse duration was constant, whereas pulse rate (equivalent to heart rate) was varied. When voltage is not feedback controlled in LQT3 mutation carriers, QT interval (APD) will shorten with increasing heart rate and thus reduce, according to our experiments, the impact on subsequent \( I_{\text{T}} \) induction.\(^{13,34,35}\) In contrast, at slower heart rates, both the amplitude of \( I_{\text{NaL}} \) and APD are expected to increase, and hence our experiments predict that in LQT3 patients, it is under these conditions that \( I_{\text{NaL}} \) induction would be most likely to occur. Because LQT3 mutation carriers are most susceptible to cardiac events during bradycardia and low SNS,\(^{21}\) our results suggest that diastolic spontaneous activity can be a contributory factor to the initiation of arrhythmias in these patients.

**\( I_{\text{NaL}} \) and DADs**

That \( I_{\text{NaL}} \) induction is more sensitive to TTX inhibition in ΔKPQ than in WT myocytes implicates a role of altered Na\(^+\) channel activity in this phenomenon, but the potent effects of flecainide on \( I_{\text{T}} \) in ΔKPQ myocytes, supports the hypothesis that mutation-enhanced \( I_{\text{NaL}} \) is a major pathway contributing to these events. This result has several implications that add to our understanding of the therapeutic mechanisms of Na\(^+\) channel blockade in LQT3 and raise the possibility of these mechanisms and therapies in other cardiovascular disorders. First, for LQT3, where flecainide and other Na\(^+\) channel blockers have been shown to be effective clinically, our data suggest that this efficacy has at least 2 pathways. Because \( I_{\text{NaL}} \) is more sensitive to flecainide and other previously tested Na\(^+\) channel blockers, these drugs shorten LQT3 altered APD (and QT interval) as has been demonstrated in vivo, in vitro, and in silico.\(^{13,24,25,36,37}\) However, in addition to the direct effects of correction of APD, particularly at slow heart rates, these drugs have the added benefit of reducing the contributions to \( I_{\text{T}} \) induction of mutation-altered Na\(^+\) entry during the prolonged QT intervals of mutation carriers. This distinction is made clearly in the experiments of Figure 7, in which flecainide was found to effectively inhibit \( I_{\text{T}} \) despite the fact that conditioning pulse (similar to QT) duration was constant. Thus, our results predict that development of compounds that maximize the selectivity for block of \( I_{\text{NaL}} \) versus \( I_{\text{NaP}} \) would lead to improved therapeutic potential of future compounds. Furthermore, because there is growing evidence that more common cardiovascular pathologies, such as bradycardia and impaired SNS, share features with LQT3, the potential of these mechanisms and therapies in other cardiovascular disorders is promising.
as heart failure38 and ischemia,39 may also promote increases in \( I_{\text{NaL}} \); our results suggest that augmented \( I_{\text{NaL}} \) and its inhibition by selective blockers may contribute to the genesis and subsequent management of arrhythmias in these diseases as well.

**Sources of Funding**

This work was supported by grants from the NIH (P01 HL067849-05 and HL-56810-09 to R.S.K.) and from the Swiss National Science Foundation (fellowship PBBEA-111201 to N.L.).

**Disclosures**

None.

**References**

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*Circ Res.* 2006;99:1225-1232; originally published online November 2, 2006; doi: 10.1161/01.RES.0000251305.25604.b0
*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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Supplemental Methods

Confocal imaging

Ca$^{2+}$ events were monitored using laser-scanning confocal microscopy (Nikon PCM2200, lens = 40X, iris = 22 mm). The Ca$^{2+}$ dye fluo3 was excited at 488 nm (25mW) using an argon ion laser (model 263C and controller 361C) and its emission collected at 515 nm. In order to increase the temporal resolution, data were acquired using the linescan mode of the confocal microscope (SimplePCI Compix Inc., 1.2 sec/image). ROIs were self-ratioed using ImageSXM (http://www.liv.ac.uk/~sdb/ImageSXM) and analyzed using IgorPro (www.wavemetrics.com).

$I_{\text{T1}}$ area measurement

Charge carried by transient inward currents was measured in Origin (OriginLab, Northampton, MA). Briefly, $I_{\text{T1}}$ area was calculated by defining a baseline diastolic current and measuring the area under the curve for the difference between this baseline and any diastolic events. The total charge for each myocyte was calculated as the sum of all diastolic events occurring in the 5 seconds of diastole following the pause protocol. Statistical data analysis was assessed with Student’s $t$-test for simple comparisons: differences at $p<0.05$ were considered to be significant.
Current clamp studies

Action potentials were recorded in the current clamp mode with the following solutions in mM (1) internal: 110 KCl, 18 CsOH, 5 Na2ATP, 0.05 EGTA, 10 HEPES, 0.03 CaCl2, and 1 MgCl2, at pH 7.3 adjusted with KOH; (2) external: 132 NaCl, 4.8 KCl, 2 CaCl2, 1.2 MgCl2, 5 Glucose, 10 HEPES, and 5 glucose (pH 7.4 with NaOH). The pipette solution free Ca2+ concentration was calculated to be 100 nM while keeping a relatively low Ca2+ buffer capacity.

Cells were stimulated in current clamp mode with rectangular (3 ms) depolarizing current pulses, 120% threshold amplitude, at room temperature (22°C). Action potentials were low pass filtered at 2 kHz and sampled at 20 kHz using a Digidata 1200 A/D card (Axon Instruments Inc., Foster City, California, USA). The stimulation protocol consists in a 100 pre-conditioning current pulses delivered with a pulse duration of 3 ms at 2, 5 and 10 Hz followed by a 500 ms pause, a single current pulse and a prolonged diastole (see Supplemental Figure 1).

Voltage clamp studies

Membrane currents were measured using whole-cell procedures with an Axopatch 200B amplifier (Axon Instruments Inc.). Capacity current and series resistance compensation were carried out using analogue techniques according to the amplifier manufacture (Axon Instruments Inc.). Micropipette electrode resistance was 1.5 to 2.5 MΩ when micropipettes were filled with the internal
solution. Membrane currents were low pass filtered at 2 kHz and sampled at 20 kHz. All measurements were obtained at room temperature (22°C). Tetrodotoxin (Sigma Chemical Co, St. Louis, MO)-containing solutions were prepared from 50 mM stock solution in H₂O. Iₐ₉ was measured 150 ms after depolarization to -10 mV. The holding potential was -75 mV. The block of Iₐ₉ by flecainide was measured at 1 Hz after steady state was achieved in the presence of the drug (2 to 3 minutes). Flecainide (Sigma Chemical Co, 3 mM) solution was made from 10 mM stock solution in H₂O. Estimation of peak Na⁺ current densities from TTX-sensitive current-voltage relationship recordings were performed in low Na⁺ conditions.

Unless otherwise noted, Na⁺ current was recorded using the following solutions (in mM) (1) internal: 50 aspartic acid, 60 CsCl, 5 Na₂ATP, 0.05 EGTA, 10 HEPES, 0.0215 CaCl₂, and 1 MgCl₂, at pH 7.2 adjusted with CsOH; (2) external: 130 NaCl, 2 CaCl₂, 5 CsCl, 1.2 MgCl₂, 10 HEPES, 0.5 BaCl₂ and 5 glucose (pH 7.4 with CsOH). Free [Ca²⁺] in the pipette filling solution was buffered as above. High concentrations of tetrodotoxin (TTX, Sigma Chemical Co, St. Louis, MO, 30 and 50 µM) were used to block Na⁺ channel currents and reveal background currents that were digitally subtracted. Flecainide (Sigma Chemical Co, 3 µM) was used to block Iₐ₉. Estimation of peak Na⁺ current densities were performed in low Na⁺ conditions with the following solutions shown in mM (1) internal: CsAsp 124, Na⁺-ATP 2.5, EGTA 11, CaCl₂ 1, HEPES 10 and MgCl₂ 2 (pH 7.3
Protocols used to mimic mouse action potentials were performed using internal and external solutions used for action potentials recordings to determine whether membrane currents underlying spontaneous activity could be detected under the same ionic conditions used during current clamp experiments. In the experiments designed to mimic human physiology, free [Ca$^{2+}$] in the pipette filling solution was buffered as above 1, but currents were recorded using the internal and external solutions used for whole cell Na$^+$ channel recordings. Control experiments (not shown) indicate that these solutions did not affect induction of Ca$^{2+}$-dependent events using the mouse action potential protocols described above.

Stimulation protocols consisted of trains of 100 conditioning voltage waveforms (either short or long pulses) followed by a 500 ms pause, one single voltage pulse and a prolonged diastole. During the pre-conditioning train, the short pulse is a voltage step from –75 mV to –10 mV for 2 ms followed by a 38 ms ramp back to –75 mV whereas the long pulse consisted of a voltage step from –75 mV to –10 mV for 20 ms followed by a 70 ms ramp back to –75 mV. For the human length protocols, the procedure is repeated with 10 pre-conditioning pulses and either a 100 or 400 ms depolarization followed by a 100 ms repolarizing ramp (see Supplemental Figure 2).

References
Supplemental Figure 1

Current clamp pacing protocol

n = 100 pulses
2, 5 and 10 Hz

100 pre-conditioning near-threshold current pulses are delivered with a pulse duration of 3 ms at 2, 5 and 10 Hz followed by a 500 ms pause, and then a single near-threshold current and a prolonged diastole.
**Supplemental Figure 2**

**Voltage clamp protocols**

**Mouse AP mimicking Protocols**

**Figures 3-6**

**Short Pulse Protocol**

- Voltage step from -75 mV to -10 mV for 2 ms followed by a 38 ms ramp back to -75 mV.

**Long pulse Protocol**

- Voltage step from -75 mV to -10 mV for 20 ms followed by a 70 ms ramp back to -75 mV.

**Human AP mimicking Protocols**

**Figures 7-9**

**100ms Protocol**

- Voltage step from -75 mV to -10 mV for 100 ms followed by a 500 ms ramp back to -75 mV.

**400ms Protocol**

- Voltage step from -75 mV to -10 mV for 400 ms followed by a 500 ms ramp back to -75 mV.

For the voltage clamp protocols designed to mimic mouse APs (left column), a train of 100 conditioning voltage waveforms (either short or long pulses) are applied followed by a 500 ms pause, one single voltage pulse and a prolonged diastole. During the preconditioning train, the short pulse is a voltage step from -75 mV to -10 mV for 2 ms followed a 38 ms ramp back to -75 mV whereas the long pulse consisted of a voltage step from -75 mV to -10 mV for 20 ms followed by a 70 ms ramp back to -75 mV. For the human length protocols (right column), the procedure is repeated with 10 preconditioning pulses and either a 100 or 400 ms depolarization followed by a 100 ms repolarizing ramp.
Supplemental Figure 3

Effect of Flecainide on ΔKPQ myocytes

Current traces recorded upon a depolarizing step (200 ms at -10 mV, pulse frequency 1Hz) in cardiomyocytes isolated from mice expressing ΔKPQ channels shown at high (A) and low (B) gain before and after steady state block of the late $I_{Na}$ ($I_{NaL}$, A) and peak $I_{Na}$ ($I_{NaP}$, B) by flecainide (3 μM). C, Bar graphs summarize the effect of flecainide (3 μM) on late (filled bars) and peak (open bars) $I_{Na}$ measured in cardiomyocytes expressing ΔKPQ channels. Fractional block was determined as the percentage of fraction of the pulse current normalized to control current reduced by the drug. Shown are means ± SEM data. **, p<0.01
Supplemental Figure 4

L-type calcium current in ΔKPQ myocytes

A, Barium current traces recorded upon a depolarizing step (200 ms at +10 mV, holding potential -40 mV) in cardiomyocytes isolated from mice expressing WT (black trace) and ΔKPQ (red trace) channels. B, Bar graphs summarize the peak barium current density measured in cardiomyocytes expressing WT (open bars) and ΔKPQ (filled bars) channels. Shown are mean ± SEM data. Ns, non significant.
Supplemental Figure 5

Extension to human rates and depolarization durations

\( \Delta \) KPQ myocytes

Extension to human rates and depolarization durations. Steady-state TTX sensitive currents are shown at high gain recorded during 100 ms (A) and 400 ms (B) depolarizations to -10 mV, each with a 100 ms ramp repolarization, with a pulse frequency of 0.5 Hz. All current traces shown are from \( \Delta \) KPQ myocytes (peak currents are off-scale).
Supplemental Figure 6

The 2 last depolarizations of a 10Hz long-pulse preconditioning train protocol and the following resting period are shown for WT and ΔKPQ myocytes in panel A and D. Note the presence of ITIs in both recording (D) and its lower incidence in the WT recording (A). The resultant Ca^{2+} transients were recorded using the linescan mode of the confocal microscope for higher temporal resolution. Self-ratioed images are shown in panels B and E for WT and ΔKPQ respectively. Means (B and E) are depicted in panels C and F. Transient depolarizations correlated with the spontaneous Ca^{2+} events and were larger and more frequent in ΔKPQ cells (about 2.5 F/F₀ in 3 out of 4 cells) than in WT myocytes (about 1.5 F/F₀ in 2 out of 4 myocytes).