Intracellular Calcium Attenuates Late Current Conducted by Mutant Human Cardiac Sodium Channels

Franck Potet, PhD; Thomas M. Beckermann, PhD; Jennifer D. Kunic, BS; Alfred L. George Jr, MD

**Background**—Mutations of the cardiac voltage-gated sodium channel (SCN5A gene encoding voltage-gated sodium channel [Na\textsubscript{v}1.5]) cause congenital long-QT syndrome type 3 (LQT3). Most Na\textsubscript{v}1.5 mutations associated with LQT3 promote a mode of sodium channel gating in which some channels fail to inactivate, contributing to increased late sodium current (I\textsubscript{Na,L}), which is directly responsible for delayed repolarization and prolongation of the QT interval. LQT3 patients have highest risk of arrhythmia during sleep or during periods of slow heart rate. During exercise (high heart rate), there is elevated steady-state intracellular free calcium ([Ca\textsuperscript{2+}]) concentration. We hypothesized that higher levels of intracellular Ca\textsuperscript{2+} may lower arrhythmia risk in LQT3 subjects through effects on I\textsubscript{Na,L}.

**Methods and Results**—We tested this idea by examining the effects of varying intracellular Ca\textsuperscript{2+} concentrations on the level of I\textsubscript{Na,L} in cells expressing a typical LQT3 mutation, delKPQ, and another SCN5A mutation, R225P. We found that elevated intracellular Ca\textsuperscript{2+} concentration significantly reduced I\textsubscript{Na,L} conducted by mutant channels but not wild-type channels. This attenuation of I\textsubscript{Na,L} in delKPQ expressing cells by Ca\textsuperscript{2+} was not affected by the CaM kinase II inhibitor KN-93 but was partially attenuated by truncating the C-terminus of the channel.

**Conclusions**—We conclude that intracellular Ca\textsuperscript{2+} contributes to the regulation of I\textsubscript{Na,L} conducted by Na\textsubscript{v}1.5 mutants and propose that, during excitation–contraction coupling, elevated intracellular Ca\textsuperscript{2+} suppresses mutant channel I\textsubscript{Na,L} and protects cells from delayed repolarization. These findings offer a plausible explanation for the lower arrhythmia risk in LQT3 subjects during fast heart rates.

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**Key Words:** calcium ■ electrophysiology ■ long QT syndrome ■ Na\textsuperscript{+} current ■ Na\textsubscript{v}1.5 voltage-gated sodium channel ■ SCN5A protein, human

Inward sodium current (I\textsubscript{Na}) in cardiac myocytes produced primarily by voltage-gated sodium channel (Na\textsubscript{v}1.5) is responsible for the rapid upstroke of atrial and ventricular action potentials, as well as rapid propagation of depolarization throughout the heart. Genetic alterations of Na\textsubscript{v}1.5 gating in which I\textsubscript{Na} fails to completely inactivate can cause long-QT syndrome type 3 (LQT3), which predisposes to life-threatening ventricular arrhythmias.1 Cardiac events in LQT3 occur primarily during periods of rest or sleep and much less commonly during exertion. Faster heart rates appear protective of arrhythmia in LQT3 but the mechanism responsible for this protection is unknown.

Several studies have demonstrated that intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]) can regulate sodium channel inactivation by direct and indirect effects.2 Furthermore, >20% of known LQT3 mutations (source: www.fsm.it/cardmoc/) are localized to Na\textsubscript{v}1.5 domains implicated as parts of a Ca\textsuperscript{2+}-regulatory apparatus (DIII-DIV linker, C-terminal EF-hand and IQ motifs). Most of these mutations cause increased late sodium current as a common molecular mechanism underlying arrhythmia susceptibility. These prior observations led us to speculate that intracellular Ca\textsuperscript{2+} may modulate Na\textsubscript{v}1.5 inactivation differently in the context of LQT3 mutations.

Here, we provide evidence that [Ca\textsuperscript{2+}]\textsubscript{i} regulates delKPQ channel inactivation and suppresses I\textsubscript{Na,L}. We also provide evidence that [Ca\textsuperscript{2+}]\textsubscript{i}, by a different mechanism involving CaMKII and the C-terminus of the channel, can regulate delKPQ whole-cell inactivation kinetics. Moreover, we show that I\textsubscript{Na,L} evoked by another disease-associated mutation (R225P) is also sensitive to [Ca\textsuperscript{2+}]\textsubscript{i}. Our data demonstrate that intracellular Ca\textsuperscript{2+} participates in the regulation of I\textsubscript{Na,L} conducted by Na\textsubscript{v}1.5 mutants and elevated intracellular free Ca\textsuperscript{2+} may contribute to suppression of I\textsubscript{Na,L} conducted by mutant channels and partially protect cells from delayed repolarization. These findings provide an explanation for the lower arrhythmia risk during exercise and higher risk during sleep of LQT3 patients.3

**Methods**

**Plasmid Constructs, Cell Culture, and Transfection**

Plasmids encoding human Na\textsubscript{v}1.5 mutant delKPQ and delKPQ/S1885X were subcloned into an IRES-DsRed vector for coexpression of the channel protein with a red fluorescent protein. Human wild-type...
WHAT IS KNOWN

- Mutations in SCN5A encoding the cardiac voltage-gated sodium channel (Na$_1$,$\alpha_{1.5}$) are associated with congenital long-QT syndrome type 3 (LQT3).
- LQT3 mutations cause impaired sodium channel inactivation contributing to increased late sodium current, which is directly responsible for impaired repolarization and prolongation of the QT interval.
- LQT3 patients have highest risk of arrhythmia during sleep or during periods of slow heart rate when diastolic intracellular Ca$^{2+}$ concentration is lowest.

WHAT THE STUDY ADDS

- We demonstrated that intracellular Ca$^{2+}$ contributes to the regulation of late sodium current conducted by mutant Na$_1$,$\alpha_{1.5}$ channels. Based on this finding, we propose that during rapid heart rates, elevated diastolic intracellular Ca$^{2+}$ suppresses mutant channel late sodium current and diminishes the impact of LQT3 mutations on repolarization.
- Our findings offer a plausible explanation for the lower arrhythmia risk in LQT3 subjects during fast heart rates.

Electrophysiology

Sodium currents were recorded at room temperature 24 to 48 hours after transfection using the whole-cell patch clamp technique. Patch-clamp pipettes were pulled from thin wall borosilicate glass (optical density, 1.5 mm, Warner Instrument Corp., Hamden, CT) on a P-97 multistage Flaming-Brown micropipette puller (Sutter Instruments, Novato, CA) and fire polished to a resistance between 1.0 and 2.5 MΩ. To avoid the time-dependent shift of the $I_{\text{Na}}$ availability curve commonly observed during patch-clamp experiments, voltage-dependent inactivation was assessed within 2 minutes after rupture of the membrane. Specific voltage-clamp protocols are depicted as figure insets. The pipette solution used to approximate zero intracellular Na$^+$ concentration was (in mmol/L): 145 NaCl, 4 KCl, 1 MgCl$_2$, 10 HEPES, and 1.8 CaCl$_2$, adjusted to pH 7.35 with CsOH. Series resistance was compensated at 80%. Data acquisition was performed using an Axopatch 200B patch clamp amplifier and pCLAMP 10.0 software (Molecular Devices, Sunnyvale, CA). A Boltzmann function ($I=\frac{I_{\text{max}}}{1+\exp(Vt-V_{1/2}/k)}$) was fitted to the availability curves to determine the membrane potential eliciting half-maximal inactivation ($V_{1/2}$), where $k$ is the slope factor. All data were analyzed using pCLAMP 10.0 or Microsoft Excel 2007 and plotted using SigmaPlot 10.0 (Systat Software, Inc, San Jose, CA).

Experiments examining late $I_{\text{Na}}$ and ramp currents used tetrodotoxin (Tocris Bioscience, Bristol, United Kingdom) to allow for the determination of tetrodotoxin-sensitive sodium current. Tetrodotoxin was added to the bath solution from a stock solution of tetrodotoxin (3 mmol/L in water) to a final concentration of 10 µmol/L. Tetrodotoxin-sensitive current was determined by offline digital subtraction. Calmodulin-dependent protein kinase II (CaMKII) inhibitor KN93 (Sigma-Aldrich, St. Louis, MO; dissolved in water) was used at a final concentration of 10 µmol/L in the patch-clamp pipette solution.

Data Analysis

Patch-clamp measurements are presented as the mean±SEM. Comparisons were made using Student t test or 2-way ANOVA (a Holm-Sidak method was used for the multiple comparison procedure), with $P<0.05$ considered significant.

Results

Intracellular Ca$^{2+}$ Stabilizes Na$_1$.$\alpha_{1.5}$ delKPQ Inactivation

We examined the effects of intracellular Ca$^{2+}$ on the inactivation of both WT and mutant Na$_1$.$\alpha_{1.5}$ channels. As observed for most LQTS-associated Na$_1$.$\alpha_{1.5}$ mutations, delKPQ caused an inactivation defect manifested as increased late sodium current ($I_{\text{NaL}}$). This phenomenon has been demonstrated under different recording conditions with various [Ca$^{2+}$]$_i$ with $I_{\text{NaL}}$ amplitude varying from 0.25 to 2.1% of the peak current.$^{4,11}$ We hypothesized that variable levels of [Ca$^{2+}$]$_i$ could explain this wide range of reported late current amplitudes. To test this hypothesis, we compared the effect of high and low [Ca$^{2+}$]$_i$, on $I_{\text{NaL}}$ in cells expressing either WT-Na$_1$.$\alpha_{1.5}$ or delKPQ channels. We observed that the level of $I_{\text{NaL}}$ exhibited by delKPQ expressing cells recorded in high [Ca$^{2+}$]$_i$ was significantly lower when compared with the low [Ca$^{2+}$]$_i$ condition. There was no comparable effect observed in cells expressing WT-Na$_1$.$\alpha_{1.5}$ (Figure 1A and 1B; Table 1). High [Ca$^{2+}$]$_i$ also significantly hastened the whole-cell inactivation of delKPQ but did not affect WT inactivation kinetics (Figure 1C and 1D; Table 1). These effects of [Ca$^{2+}$]$_i$ on delKPQ $I_{\text{NaL}}$ and inactivation kinetics were not dependent on the presence of the β1 subunit. In the presence of the β1 subunit, delKPQ $I_{\text{NaL}}$ was also lower and inactivation kinetics faster in high [Ca$^{2+}$]$_i$, (low [Ca$^{2+}$]$_i$: $I_{\text{NaL}}$=$2.5±0.43\%$, $\tau_{\text{Fast}}$=20±5 ms, $n=25$; high [Ca$^{2+}$]$_i$: $I_{\text{NaL}}$=0.9±0.08%, $\tau_{\text{Fast}}$=0.9±0.09 ms, $n=27$; $P<0.05$). Analysis of a concentration-response curve of delKPQ $I_{\text{NaL}}$ to [Ca$^{2+}$]$_i$ yielded a half maximal inhibitory concentration ($C_{50}$) of 38 mmol/L [Ca$^{2+}$]$_i$ (Figure 2). This value is consistent with physiologically relevant Ca$^{2+}$ regulation of Na$_1$.$\alpha_{1.5}$ late current in cardiomyocytes.

To ensure that the difference in BAPTA concentrations between the low [Ca$^{2+}$]$_i$ (20 mmol/L BAPTA) and high [Ca$^{2+}$]$_i$ (1 mmol/L BAPTA) intracellular solutions were not responsible for these effects on inactivation, we examined the effects of a high [Ca$^{2+}$]$_i$, pipette solution that included 20...
mmol/L BAPTA. We observed that \( I_{\text{NaL}} \) and inactivation kinetics exhibited by delKPQ in high \([Ca^{2+}]_i\) with 20 mmol/L or 1 mmol/L BAPTA were not significantly different (20 mmol/L BAPTA: \( I_{\text{NaL}} = 0.70 \pm 0.11\% \), \( \tau_{\text{Fast,−20mV}} = 0.70 \pm 0.08 \) ms, \( n \geq 5 \); 1 mmol/L BAPTA: \( I_{\text{NaL}} = 0.58 \pm 0.07\% \), \( \tau_{\text{Fast,−20mV}} = 0.82 \pm 0.05 \) ms, \( n \geq 11 \); \( P = 0.32 \) and 0.29, respectively). In addition, chelating \( Ca^{2+} \) with HEDTA instead of BAPTA did not alter the effect (Figure I in the Data Supplement). These data indicate that \([Ca^{2+}]_i\) was most likely responsible for the observed differences in delKPQ \( I_{\text{NaL}} \) and kinetics of inactivation.

We also examined the effects of intracellular \( Ca^{2+} \) on the voltage dependence of inactivation for WT-NaV1.5 and delKPQ channels. As previously reported,\(^{12}\) we found that the voltage dependence of WT-NaV1.5 steady-state inactivation was shifted to more depolarized potentials when measured in the presence of elevated \([Ca^{2+}]_i\) compared with nominally intracellular \( Ca^{2+} \)-free conditions (Table 1, Figure IIA in the Data Supplement). This phenomenon was not influenced by the type of \( Ca^{2+} \) chelator used for the intracellular solution (BAPTA versus HEDTA; Figure IIA in the Data Supplement). By contrast, the voltage dependence of steady-state inactivation observed in cells expressing delKPQ channels was not different between low and high \([Ca^{2+}]_i\) (Table 1; Figure IIB in the Data Supplement).

The effect of \([Ca^{2+}]_i\) on inactivation of WT and delKPQ channels were further probed by examining responses to slow voltage ramps. Comparing delKPQ ramp currents in low and high \([Ca^{2+}]_i\), revealed a significant difference in magnitude of evoked currents (Figure 3, Table 1). The net charge movement during voltage ramps was 4-fold greater for delKPQ in low \([Ca^{2+}]_i\) than in high \([Ca^{2+}]_i\) (13.4±1.9 pC/nA in low \([Ca^{2+}]_i\), versus 3.5±0.7 pC/nA in high \([Ca^{2+}]_i\); \( n = 13 \) and 11 respectively; \( P < 0.001 \)). By contrast, ramp currents measured in cells expressing WT-NaV1.5 were not sensitive to changes in \([Ca^{2+}]_i\) (Figure 3; Table 1). These results are consistent with effects of elevated \([Ca^{2+}]_i\) on \( I_{\text{NaL}} \) for delKPQ channels.

### Intracellular \( Ca^{2+} \) Effects on \( Na_{1.5}\)-R225P

The SCN5A mutation R225P has been associated with pre-natal arrhythmias and impaired cardiac contractility.\(^{13}\) This mutation affects a residue in the domain 1 voltage sensing S4 segment and causes profound abnormalities in activation and inactivation resulting in a larger window current, slower inactivation kinetics, and increased \( I_{\text{NaL}} \). Similar to delKPQ, elevated \([Ca^{2+}]_i\) stabilized R225P inactivation as evidenced by faster whole-cell inactivation kinetics and suppression of \( I_{\text{NaL}} \) (Figure 4A and 4B; Table 1). Comparison of normalized
ramp currents for R225P in low and high [Ca\(^{2+}\)], also revealed a large difference in magnitude of evoked currents (Figure 4C; Table 1). The net charge movement during voltage ramps was 2.3-fold smaller in high [Ca\(^{2+}\)] than in low [Ca\(^{2+}\)], (10.5±1.8 pC/nA in low [Ca\(^{2+}\)] versus 4.6±1.3 pC/nA in high [Ca\(^{2+}\)]; n=6; P<0.05). Our observations with R225P suggest that effects of intracellular Ca\(^{2+}\) on mutant Na\(_V1.5\) inactivation may be a more generalizable phenomenon.

CaMKII and [Ca\(^{2+}\)], Regulation of Late Current in Mutant Na\(_V1.5\)

We considered plausible mechanisms to explain the modulation of delKPQ \(I_{\text{NaL}}\) by [Ca\(^{2+}\)]. Calcium and calmodulin-dependent protein kinase II (CaMKII) has been previously shown to phosphorylate Na\(_V1.5\) and cause increased \(I_{\text{NaL}}\). Therefore, we examined whether CaMKII mediates the [Ca\(^{2+}\)], modulation of \(I_{\text{NaL}}\) in cells expressing delKPQ. We measured \(I_{\text{NaL}}\) and inactivation kinetics in low and high [Ca\(^{2+}\)] for cell expressing delKPQ in the presence of intracellular KN93 (a specific inhibitor of CaMKII that competes for the binding site of CaM on the kinase\(^{19}\)). As illustrated in Figure 5A and Table 2, KN93 did not attenuate the effect of elevated [Ca\(^{2+}\)] on delKPQ \(I_{\text{NaL}}\). Further, comparison of normalized ramp currents for delKPQ in presence of KN93 in low and high [Ca\(^{2+}\)] revealed that the net charge movement during voltage ramps was 2-fold smaller in high [Ca\(^{2+}\)] than in low [Ca\(^{2+}\)] (Figure 5B; Table 2), similar to what we observed in the absence of CaMKII inhibitor. By contrast, KN93 appeared to inhibit the modification of delKPQ inactivation kinetics by [Ca\(^{2+}\)] (Figure 5C; Table 2). These data suggest that CaMKII may be involved in the Ca\(^{2+}\)-

### Table 1. Effects of [Ca\(^{2+}\)] on WT, delKPQ, and R225P Channels

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>delKPQ</th>
<th>R225P</th>
</tr>
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<tbody>
<tr>
<td><strong>Inactivation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(V_{1/2}), mV</td>
<td>−85.1±1.9</td>
<td>−77.8±1.5*</td>
<td>−87.7±1.5</td>
</tr>
<tr>
<td>(k)</td>
<td>−8.2±0.4</td>
<td>−8.8±0.4</td>
<td>−7.1±0.3</td>
</tr>
<tr>
<td>(n)</td>
<td>14</td>
<td>13</td>
<td>18</td>
</tr>
<tr>
<td><strong>Inactivation decay (−20 mV)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(A_1), %</td>
<td>89.4±2.6</td>
<td>89.5±3.0</td>
<td>87.4±2.6</td>
</tr>
<tr>
<td>(\tau_{\text{fast}}), ms</td>
<td>0.81±0.06</td>
<td>0.84±0.11</td>
<td>1.11±0.09</td>
</tr>
<tr>
<td>(A_2), %</td>
<td>10.6±2.6</td>
<td>10.5±3.0</td>
<td>12.6±2.6</td>
</tr>
<tr>
<td>(\tau_{\text{slow}}), ms</td>
<td>8.0±1.6</td>
<td>9.2±2.3</td>
<td>11.8±1.6</td>
</tr>
<tr>
<td>(I_{\text{NaL}})</td>
<td>12</td>
<td>9</td>
<td>21</td>
</tr>
<tr>
<td>% peak</td>
<td>0.25±0.05</td>
<td>0.19±0.03</td>
<td>2.35±0.28</td>
</tr>
<tr>
<td>(n)</td>
<td>11</td>
<td>9</td>
<td>13</td>
</tr>
<tr>
<td><strong>Ramp current</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Area (pC/nA)</td>
<td>1.9±0.4</td>
<td>1.9±0.4</td>
<td>13.4±1.9</td>
</tr>
<tr>
<td>(n)</td>
<td>10</td>
<td>8</td>
<td>13</td>
</tr>
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</table>

Low [Ca\(^{2+}\)] vs high [Ca\(^{2+}\)]. \(I_{\text{NaL}}\) indicates late sodium current; and WT, wild-type.

*P<0.05.
regulation of delKPQ inactivation kinetics, but not in the Ca\textsuperscript{2+}-dependent modulation of \(I_{\text{NaL}}\). Collectively, these data suggest that the suppression of \(I_{\text{NaL}}\) in delKPQ expressing cells by elevated [Ca\textsuperscript{2+}] is mediated by a distinct mechanism from that mediating the effects on inactivation kinetics.

C-Terminal Truncation Attenuates Effect of [Ca\textsuperscript{2+}]\textsubscript{i} on delKPQ Inactivation

In human Na\textsubscript{v}1.5, 2 regions have been identified that interact with CaM: an IQ motif in the C-terminus and distinct structures within the DIII-DIV linker.\textsuperscript{12,20-23} There is speculation that CaM modulates the interaction of the Na\textsubscript{v}1.5 C-terminus with the DIII-DIV linker, stabilizing the inactivation gate in a closed position and diminishing \(I_{\text{NaL}}\).\textsuperscript{22,23} More recent studies proposed the CaM-IQ motif complex as the Na\textsubscript{v}1.5 Ca\textsuperscript{2+} sensor.\textsuperscript{12,24,25} Truncation of the IQ domain as in the Na\textsubscript{v}1.5-S1885X mutation (engineered premature stop codon at amino acid 1885) has been shown to cause significantly greater levels of \(I_{\text{NaL}}\) than WT channels.\textsuperscript{26} Similar to WT-Na\textsubscript{v}1.5, \(I_{\text{NaL}}\) and inactivation kinetics exhibited by Na\textsubscript{v}1.5-S1885X were not sensitive to [Ca\textsuperscript{2+}]. (low [Ca\textsuperscript{2+}]: \(I_{\text{NaL}}=0.55\pm0.07\%\), \(\tau_{\text{fast}}[-20\text{mV}]=0.88\pm0.05\text{ ms}, n=9; \text{high [Ca\textsuperscript{2+}]}:\ I_{\text{NaL}}=0.56\pm0.06\%\), \(\tau_{\text{fast}}[-20\text{mV}]=0.79\pm0.06\text{ ms, n=7; } P=0.32 \text{ and 0.51, respectively; data not shown}).

To test if the C-terminal domain is necessary for the effect of intracellular Ca\textsuperscript{2+} on delKPQ, we examined the effect of varying [Ca\textsuperscript{2+}]. on a Na\textsubscript{v}1.5 channel that combined delKPQ and S1885X mutations (delKPQ/S1885X). As illustrated in Figure 6A and 6B, we observed that elevated [Ca\textsuperscript{2+}] significantly suppressed \(I_{\text{NaL}}\) and voltage ramp-evoked currents in cells expressing delKPQ/S1885X. However, the level of residual \(I_{\text{NaL}}\) in the presence of high [Ca\textsuperscript{2+}] was significantly greater for delKPQ/S1885X than delKPQ alone (ANOVA, \(P<0.005\)), even though the difference between \(I_{\text{NaL}}\) in low [Ca\textsuperscript{2+}] was not significant (\(P=0.2\)). By contrast to delKPQ, we observed that delKPQ/S1885X inactivation kinetics were not sensitive to [Ca\textsuperscript{2+}], (Figure 6C; Table 2). These results suggest that the distal portion of Na\textsubscript{v}1.5 channel C-terminal domain, containing the IQ motif, contributes to the regulation of delKPQ inactivation kinetics by [Ca\textsuperscript{2+}]. Furthermore, the blunted Ca\textsuperscript{2+}-dependent suppression of \(I_{\text{NaL}}\) in the absence of the distal C-terminus suggests that this domain is partially responsible for this phenomenon.

Discussion

The effects of intracellular Ca\textsuperscript{2+} on several Na\textsubscript{v} channels have been described.\textsuperscript{2,12,20,21,24,25,27-39} These include direct and indirect

Figure 4. Intracellular Ca\textsuperscript{2+} suppresses late sodium current (\(I_{\text{NaL}}\)) and hastens inactivation kinetics of voltage-gated sodium channel (Na\textsubscript{v}1.5) R225P. 

A (Left), Average traces of \(I_{\text{Na}}\) from cells expressing R225P Na\textsubscript{v}1.5 in low (black) and high (red) intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]). 

B (Left), Representative tetrodotoxin (TTX)-subtracted \(I_{\text{Na}}\) from cells expressing Na\textsubscript{v}1.5 R225P in low (black) and high (red) [Ca\textsuperscript{2+}]. 

C, Normalized and averaged TTX-subtracted ramp currents (0.09 mV/ms) of Na\textsubscript{v}1.5 R225P as a percentage of peak \(I_{\text{Na}}\) at −20 mV in low and high [Ca\textsuperscript{2+}]. All data are presented as mean±SEM. *\(P<0.05\) and †\(P<0.001\). Holding potential in all experiments was −120 mV.
effects on the onset of slow inactivation or voltage-dependence and kinetics of inactivation. The results from our study strongly suggest a new phenomenon in which intracellular Ca\(^{2+}\) regulates Na\(_V\)1.5 \(I_{\text{NaL}}\) in disease-associated mutant channels. Specifically, we observed that elevated intracellular Ca\(^{2+}\) concentration, within a physiological range, suppresses \(I_{\text{NaL}}\) evoked by the LQT3 mutation delKPQ and the unrelated SCN5A mutation R225P. By contrast, \(I_{\text{NaL}}\) conducted by WT-Na\(_V\)1.5 was not sensitive to intracellular Ca\(^{2+}\). Although we found that 2 unrelated mutations exhibiting large \(I_{\text{NaL}}\) were sensitive to intracellular Ca\(^{2+}\), we cannot reliably predict this behavior for other mutations. These findings help do explain the wide range of \(I_{\text{NaL}}\) amplitudes reported previously for delKPQ by different laboratories.\(^{4-11}\) Furthermore, this phenomenon may also explain the lower arrhythmia risk in LQT3 during physiological circumstances associated with faster heart rates.

**Pathophysiological Significance of Ca\(^{2+}\) Suppression of Late Current**

In cardiac myocytes, a complex balance of inward and outward currents maintains the action potential plateau. Repolarization ensues when outward currents exceed inward currents. The increase in \(I_{\text{NaL}}\) observed with LQT3 mutations explains prolongation of the action potential plateau by tipping this balance toward inward current. Action potential prolongation is a necessary conditioning event for the generation of torsades de pointes.\(^{40}\)

Intracellular Ca\(^{2+}\) participates in the regulation of numerous key physiological processes in cardiomyocytes and therefore, its level is subjected to tightly controlled homeostasis. This homeostatic control allows for acute and sizeable changes in [Ca\(^{2+}\)] in [Ca\(^{2+}\)] through Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores during systole and reuptake into these stores during diastole.\(^{41}\) During exercise (high heart rate), there is an increase in the steady-state intracellular free Ca\(^{2+}\) concentration. For instance, when the pacing rate is increased from 0.2 to 3 Hz, diastolic [Ca\(^{2+}\)] increases from 50 to 90 nmol/L in isolated rabbit ventricular myocytes.\(^{37}\) Our results show that within this small window of [Ca\(^{2+}\)], there is a wide range of \(I_{\text{NaL}}\) amplitude evoked by delKPQ channels which could in turn affect action potential duration (Figure 2). Suppression of \(I_{\text{NaL}}\) has been shown to improve function and reduce arrhythmogenic activity in ventricular, atrial, and nodal cardiac tissues by reducing spatial dispersion of repolarization and refractoriness.\(^{40}\)
A rate-dependent reduction of $I_{NaL}$ was previously described, but we observed that this rate-dependent reduction was not sensitive to [Ca$^{2+}$] (Figure III in the Data Supplement). Collectively, our results show that the previously described rate-dependent reduction of $I_{NaL}$, which is [Ca$^{2+}$]-independent, and higher levels of intracellular Ca$^{2+}$ that stabilize NaV1.5 inactivation are contributing to suppression of $I_{NaL}$ and protecting myocytes from delayed repolarization. In the future, experiments using induced pluripotent stem cell–derived cardiomyocytes from LQT3 patients might clarify to what extent these [Ca$^{2+}$]-dependent and independent mechanisms contribute to the reduction of $I_{NaL}$. By inference, our findings provide a plausible explanation for increased arrhythmia risk in LQT3 during slow heart rates.

Variation in [Ca$^{2+}$] has also been shown to influence the voltage-dependence of steady-state inactivation (SSI) of WT-NaV1.5 with an EC$_{50}$ value of 175 nmol/L, a value more than 4-fold greater than what we observed for suppression of NaV1.5 delKPQ evoked $I_{NaL}$. (IC$_{50}$=38 nmol/L). The mechanism by which [Ca$^{2+}$] modifies the SSI is still unclear, as well as the physiological significance of this phenomenon. In this study, we demonstrated that [Ca$^{2+}$] suppresses $I_{NaL}$ generated by delKPQ channels but does not affect SSI. One potential mechanism we considered to explain Ca$^{2+}$ regulation of $I_{NaL}$ involves CaMKII, which has been previously shown to phosphorylate NaV1.5 and increase $I_{NaL}$ of WT channels. Here, we show that the specific inhibitor of CaMKII

### Table 2. Effects of [Ca$^{2+}$] on delKPQ in Presence of KN93 and on delKPQ/S1885X

<table>
<thead>
<tr>
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<th>delKPQ+10 μmol/L KN93</th>
<th>delKPQ/S1885stop</th>
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<tbody>
<tr>
<td></td>
<td>Low Ca$^{2+}$</td>
<td>High Ca$^{2+}$</td>
</tr>
<tr>
<td>Inactivation decay (−20 mV)</td>
<td></td>
<td></td>
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<tr>
<td>$A_1$, %</td>
<td>93.4±1.1</td>
<td>96.7±1.3</td>
</tr>
<tr>
<td>$\tau_{fast}$, ms</td>
<td>0.8±0.1</td>
<td>0.8±0.1</td>
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<tr>
<td>$A_2$, %</td>
<td>6.6±1.1</td>
<td>3.3±1.3</td>
</tr>
<tr>
<td>$\tau_{slow}$, ms</td>
<td>13.7±2.8</td>
<td>7.8±1.7</td>
</tr>
<tr>
<td>$I_{NaL}$ % peak</td>
<td>2.2±0.2</td>
<td>0.8±0.3*</td>
</tr>
<tr>
<td>n</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Ramp current</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Area (pC/nA)</td>
<td>9.3±0.6</td>
<td>3.9±1.9*</td>
</tr>
<tr>
<td>n</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

Low [Ca$^{2+}$] vs high [Ca$^{2+}$], $I_{NaL}$ indicates late sodium current; and WT, wild-type. *P<0.05.
(KN93) blocks the effect of [Ca²⁺], on delKPQ whole-cell inactivation kinetics but not on \( I_{\text{NaL}} \). Together, our data suggest that distinct mechanisms are responsible for Ca²⁺ regulation of SSI and \( I_{\text{NaL}} \) and that CaMKII is not involved in the Ca²⁺-dependent suppression of \( I_{\text{NaL}} \).

Structural Basis for Ca²⁺ Regulation of \( I_{\text{NaL}} \)

For \( \text{Na} \) channels, fast inactivation is at least partly mediated by the cytoplasmic linker between domains III and IV (DIV–DIII linker). This domain has been described as a hinged lid that covers the channel pore from the cytoplasmic side (inactivation gate). Fast inactivation is a complex process that involves many structural domains. Several studies suggest that a region in the DIII–DIV linker consisting of only 3 hydrophobic amino acids isoleucine, phenylalanine, and methionine (IFM region, amino acids 1484–1486) seems to interact with multiple sites (S4–S5 linker of domain III and IV) in the channel to mediate pore occlusion. Interactions of the DIII–DIV linker with the C-terminus may also be required to stabilize the inactivation gate. The interaction between the C-terminus and the DIII–DIV linker may require CaM making this protein an essential component in the stabilization of the inactivation gate. Our data show that the absence of the distal C-terminal part of the channel, containing the IQ-domain, can blunt the Ca²⁺-dependent suppression of \( I_{\text{NaL}} \) and suppress the Ca²⁺ effect on delKPQ inactivation kinetics. These results suggest a partial role for the IQ domain in the regulation of \( I_{\text{NaL}} \) by intracellular calcium raising the possibility that other channel domains might also be responsible for the stabilization of the channel inactivation by intracellular calcium. This other domain could be the DIII-DIV linker. CaM, as an extrinsic calcium sensor with a role in mediating the interaction between the C-terminus and the DIII-DIV linker is a good candidate for mediating calcium inhibition of \( I_{\text{NaL}} \).

It has previously been shown that the C-terminal domain partly influences \( \text{Na}_{1.5} \) inactivation kinetics. We observed that the absence of the distal C-terminal part rendered the delKPQ inactivation kinetics insensitive to intracellular calcium (Tables 1 and 2) confirming that this protein region contributes to the regulation of delKPQ inactivation kinetics. We think that such truncation slows the rate of inactivation by preventing the stabilization of the channel inactivation by calcium. In summary, our data suggest that the distal portion of \( \text{Na}_{1.5} \) channel C-terminal domain, containing the IQ motif, contributes to the stabilization of the inactivation gate and block of \( I_{\text{NaL}} \) amplitude by [Ca²⁺], providing a mechanism for lower arrhythmia risk during exercise.

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This work was supported by a Scientist Development Grant (11SDGS5330006) from the American Heart Association (Dr Potet) and National Institutes of Health grant HL083374 (Dr George).

Disclosures

None.

References


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Fig. S1. HEDTA does not influence delKPQ current. A. Representative TTX-subtracted $I_{Na}$ at -20 mV from cells expressing $Na_v1.5$ delKPQ in high [Ca$^{2+}$], using BAPTA (red) or HEDTA (blue) as a chelator. $I_{NaL} = 0.58 \pm 0.07 \%$; $n = 11$ using BAPTA and $I_{NaL} = 0.61 \pm 0.06 \%$; $n = 8$ using HEDTA; $P = 0.75$. B. Voltage dependence of inactivation time constant (fast component) from cells expressing $Na_v1.5$ delKPQ, in high [Ca$^{2+}$], using BAPTA (red) or HEDTA (blue) as a chelator. Inactivation kinetics at -20 mV exhibited by delKPQ $Na_v1.5$ in high [Ca$^{2+}$], were not significantly different using BAPTA ($\tau_{Fast} = 0.82 \pm 0.05$ ms; $n = 17$) or HEDTA ($\tau_{Fast} = 0.88 \pm 0.03$ ms; $n = 5$; $P = 0.56$).
Fig. S2. delKPQ mutant voltage-dependent inactivation is not sensitive to intracellular Ca\textsuperscript{2+}. A. Voltage-dependence of inactivation in cells expressing Na\textsubscript{v}1.5 WT in low (black) or high [Ca\textsuperscript{2+}]\textsubscript{i} using BAPTA (red) or HEDTA (blue) as a chelator. V\textsubscript{1/2} was -85.1 ± 1.9 mV (n = 14) in low [Ca\textsuperscript{2+}]\textsubscript{i} and in high [Ca\textsuperscript{2+}]\textsubscript{i} -77.8 ± 1.5 mV (n = 13) using BAPTA and -77.2 ± 1.4 mV (n = 8) using HEDTA. B. Voltage-dependence of inactivation in cells expressing Na\textsubscript{v}1.5 delKPQ in low and high [Ca\textsuperscript{2+}]\textsubscript{i}. V\textsubscript{1/2} was -87.7 ± 1.5 mV (n = 18) in low [Ca\textsuperscript{2+}]\textsubscript{i}, and -87.6 ± 0.9 mV (n = 12) in high [Ca\textsuperscript{2+}]\textsubscript{i}, P = 0.71. Inset: voltage clamp protocol used to assess the voltage-dependent inactivation. Current amplitude measured during an activating pulse to -20 mV (arrow) as a function of the preceding membrane potential (from -160 mV to -20 mV) were used to construct the steady-state inactivation curve (clamp pulses of 50 msec were utilized to exclude slowly-developing inactivation processes that involve extracellular domains). All data are presented as mean ± S.E.M.
Fig. S3. Rate-dependent reduction of $I_{\text{Na}}$ is not sensitive to intracellular $\text{Ca}^{2+}$. **A.** Normalized peak current ($I_{\text{NaP}}$) from cells expressing delKPQ in low and high $[\text{Ca}^{2+}]_i$ using stimulation of different cycle lengths. The amplitude of the 50th pulse was normalized to the amplitude of the 1st pulse. Currents were elicited by a train of 100 ms steps from -120 to -20 mV using 3 different inter-pulse durations (500, 300 and 200 ms). Compared with pulse 1, the relative amplitude of $I_{\text{NaP}}$ at pulse 50 using inter-pulse durations of 500, 300 and 200 ms were 96 ± 2, 91 ± 2 and 87 ± 2 % in low $[\text{Ca}^{2+}]_i$ and 95 ± 2, 88 ± 2 and 85 ± 2 % in high $[\text{Ca}^{2+}]_i$, respectively; $P = 0.45, P = 0.33$ and $P = 0.35$ ($n > 6$). **B.** Normalized TTX-sensitive $I_{\text{NaL}}$ from cells expressing delKPQ in low and high $[\text{Ca}^{2+}]_i$ using stimulation of different cycle lengths (same protocol as described for panel A). The amplitude of the 50th pulse was normalized to the amplitude of the 1st pulse. Compared with pulse 1, the relative amplitude of $I_{\text{NaL}}$ at pulse 50 using inter-pulse durations of 500, 300 and 200 ms were 78 ± 4, 65 ± 4 and 54 ± 6 % in low $[\text{Ca}^{2+}]_i$ and 74 ± 5, 58 ± 4 and 50 ± 6 % in high $[\text{Ca}^{2+}]_i$; $P = 0.54, P = 0.23$ and $P = 0.59$ ($n > 6$). Data symbols and error bars represent the mean ± S.E.M.