Arrhythmogenic Calmodulin Mutations Disrupt Intracellular Cardiomyocyte Ca\(^{2+}\) Regulation by Distinct Mechanisms

Guo Yin, MS,* Faisal Hassan, BS,* Ayman R. Haroun, MS; Lisa L. Murphy, BS; Lia Crotti, MD, PhD; Peter J. Schwartz, MD; Alfred L. George, Jr, MD; Jonathan Satin, PhD

Background—Calmodulin (CaM) mutations have been identified recently in subjects with congenital long QT syndrome (LQTS) or catecholaminergic polymorphic ventricular tachycardia (CPVT), but the mechanisms responsible for these divergent arrhythmia-susceptibility syndromes in this context are unknown. We tested the hypothesis that LQTS-associated CaM mutants disrupt Ca\(^{2+}\) homeostasis in developing cardiomyocytes possibly by affecting either late Na current or Ca\(^{2+}\)-dependent inactivation of L-type Ca\(^{2+}\) current.

Methods and Results—We coexpressed CaM mutants with the human cardiac Na channel (Na\(_V\)1.5) in tsA201 cells, and we used mammalian fetal ventricular cardiomyocytes to investigate LQTS- and CPVT-associated CaM mutations (LQTS- and CPVT-CaM). LQTS-CaM mutants do not consistently affect L-type Na current in heterologous cells or native cardiomyocytes, suggesting that the Na channel does not contribute to LQTS pathogenesis in the context of CaM mutations. LQTS-CaM mutants (D96V, D130G, F142L) impaired Ca\(^{2+}\)-dependent inactivation, whereas the CPVT-CaM mutant N54I had no effect on Ca\(^{2+}\)-dependent inactivation. LQTS-CaM mutants led to loss of Ca\(^{2+}\)-transient entrainment with the rank order from greatest to least effect: CaM-D130G–CaM-D96V>CaM-F142L. This rank order follows measured Ca\(^{2+}\)-CaM affinities for wild-type and mutant CaM. Acute isoproterenol restored entrainment for CaM-130G and CaM-D96V but caused irreversible cytosolic Ca\(^{2+}\) overload for cells expressing a CPVT-CaM mutant.

Conclusions—CaM mutations associated with LQTS may not affect L-type Na\(^{-}\) current but may evoke defective Ca\(^{2+}\)-dependent inactivation of L-type Ca\(^{2+}\) current. (J Am Heart Assoc. 2014;3:e000996 doi: 10.1161/JAHA.114.000996)

Key Words: calcium • calmodulin • cardiomyocyte • long QT Syndrome • L-type Ca\(^{2+}\) channel

C ongenital long QT syndrome (LQTS) and catecholaminergic polymorphic ventricular tachycardia (CPVT) are 2 genetic disorders of heart rhythm that may present during childhood and cause life-threatening cardiac arrhythmias.1 The molecular mechanisms underlying arrhythmia susceptibility have been inferred from studies of mutant gene products, which are largely ion channels or channel regulators. In LQTS, loss of function in potassium channels or gain of function in either Na or Ca\(^{2+}\) channels represent the major molecular mechanisms. In contrast to disturbances in plasma membrane ion channel function, CPVT arises from disordered intracellular Ca\(^{2+}\) regulation, most frequently because of mutation of the ryanodine receptor Ca\(^{2+}\)-release channel of the sarcoplasmic reticulum (SR). These advances have improved our understanding of arrhythmogenesis in general and have revealed new therapeutic targets.

Recently, recurrent cardiac arrest in infants with a severe form of LQTS was associated with mutations in CALM1 (D130G, F142L) or CALM2 (D96V), 2 genes encoding the ubiquitous Ca\(^{2+}\)-signaling protein calmodulin (CaM).2 Independently, 2 distinct CaM mutations (N54I, N98S) were discovered in subjects with a CPVT-like syndrome although one of these mutations (N98S) has also been identified in a subject with LQTS.3 Most of the mutations affect conserved amino acid residues that are known to participate in Ca\(^{2+}\) binding or in energetic coupling of ion binding to CaM activation. Biochemical

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studies revealed that most CaM mutations, except N54I, cause reduced Ca\(^{2+}\)-binding affinity.\(^2,4\) However, the cellular basis for arrhythmogenesis in the setting of CaM mutations has not been elucidated.

CaM controls a large number of enzymes, ion channels, and other proteins.\(^5\) In cardiomyocytes, key CaM-interacting proteins include those critically involved in beat-to-beat Ca\(^{2+}\) homeostasis. CaM is prebound to the cardiac L-type Ca\(^{2+}\) channel (LTCC), where it senses local Ca\(^{2+}\) influx and cytosolic Ca\(^{2+}\). The complex of Ca\(^{2+}\)-CaM promotes rapid inactivation of LTCC Ca\(^{2+}\) current (\(I_{Ca,L}\)).\(^6\) This process, known as Ca\(^{2+}\)-dependent inactivation (CDI), is a major determinant of cardiac excitability.\(^7\) Engineered CaM with carboxyl lobe (C-lobe) mutations abrogating C-lobe Ca\(^{2+}\) binding attenuated CDI and prolonged action potential (AP) duration.\(^7\) The majority of the z-line CaM also binds to ryanodine receptor 2 (RyR2),\(^8\) and CaM inhibits RyR2 openings independent of Ca\(^{2+}\) concentration.\(^9,10\) Disrupting CaM-RyR2 interaction has multiple effects including increased opening of RyR2, diminished \(I_{Ca,L}\), and increased cytosolic Ca\(^{2+}\) at negative membrane potentials, creating a molecular substrate for arrhythmogenic triggers.\(^11\)

Consequently, at the cellular level, we anticipate that CaM mutants targeting CDI will have distinct effects from those disrupting CaM-RyR2 interactions. Most recently, CPVT-CaMs were shown to cause arrhythmogenic Ca\(^{2+}\) disturbances in permeabilized cardiomyocytes that were distinct from LQT-CaM–provoked alterations.\(^12\)

In this study, we examined the effects of LQTS-associated CaM mutations on Na and Ca\(^{2+}\) channel currents to elucidate cellular and molecular mechanisms underlying arrhythmia predisposition. We found that CaM mutations had inconsistent effects on Na channels but caused impaired CDI of \(I_{Ca,L}\) in cardiomyocytes and dysregulation of cytosolic Ca\(^{2+}\).\(^13\) In parallel, we examined the impact of the CPVT-associated mutation, N54I, on \(I_{Ca,L}\) and observed no direct effects on LTCC current in cardiomyocytes; however, this mutation caused significant abnormalities in Ca\(^{2+}\) homeostasis. Our findings implicate different molecular substrates for arrhythmogenesis associated with CaM mutations.

**Methods**

All experimental procedures and protocols involving animals were approved by the animal care and use committee of the University of Kentucky and conform to the NIH Guide for Care and Use of Laboratory Animals. Isolated fetal ventricular cardiomyocytes (FVM) were prepared as previously described,\(^13\) except embryonic day 18 to 19 pregnant dams were used.

**Mutagenesis of Calmodulin**

Mutant CaMs were engineered by making one of the following amino acid substitutions: N54I, D96V, N98S, D130G, or F142L. A mammalian expression plasmid was used to drive expression of wild-type (WT) or mutant CaM by the immediate early CMV promoter along with the coding region for either green fluorescent protein or CD8 preceded by the IRES2 element as cotransfection markers.

**Heterologous Expression of Human Na\(_V\)1.5 With Calmodulin**

Plasmids encoding recombinant human cardiac Na channel isoforms (Na\(_V\)1.5) were described previously.\(^14\) For studies of Na channel function, WT cardiac Na channel cDNA (0.5 \(\mu\)g) and WT or mutant CaM (D130G, D96V, and F142L) cDNA (0.5 \(\mu\)g) in vectors encoding the cotransfection marker CD8 were transiently transfected into tsA201 cells using FuGene HD (Roche Diagnostics, Indianapolis, IN), then incubated for 48 hours at 37°C prior to electrophysiological measurements. Dynabeads-CD8 (Invitrogen Life Technologies, Carlsbad, CA) were added to cell culture media according to manufacturer’s instructions to mark cells transfected successfully. Cells selected for patch clamp recordings exhibited CD8 expression with Dynabeads attached to the cell surface and were easily distinguishable from non–CD8-expressing cells.

**Fetal Ventricular Myocyte Tissue Harvest or Cell Culture**

Hearts of embryonic day 18 to 19 (a fetal stage) mouse (ICR outbred strain; Harlan Laboratories) were dissected free of connective tissues, and ventricles were separated from atria. Cells were enzymatically dispersed and cultured as previously described.\(^15\) Briefly, about 10 fetuses were minced and quickly transferred to nominally Ca\(^{2+}\)-free digestion buffer containing 0.5 mg/mL collagenase (type II; Worthington Biochemical Corporation) and 1 mg/mL pancreatin for two 15-minute cycles. Digested tissue yielded a large fraction of single spontaneously beating cells in culture media consisting of DMEM plus 10% FBS. Cells were transfected in 24-well plates using Lipofectamine transfection reagent (Invitrogen Life Technologies). Cells were transfected with plasmids given above and used 24 to 48 hours after transfection. FVM were selected for study based on enhanced green fluorescent protein fluorescence unless otherwise noted.

**Electrophysiology**

Na currents were recorded at room temperature using the whole-cell patch clamp technique as described pre-
The extracellular bath solution contained the following (in millimoles per liter): 140 NaCl, 4 KCl, 1.8 CaCl₂, 1 MgCl₂, 10 HEPES, and 10 glucose, pH 7.35 (adjusted with NaOH). The low-Ca²⁺ intracellular pipette solution contained the following (in millimoles per liter): 10 NaF, 100 CsF, 20 CsCl, 20 BAPTA, 10 HEPES, 10 glucose, pH 7.35 (adjusted with CsOH). The high-Ca²⁺ (−1 μmol/L free) intracellular pipette solution contained the following (in millimoles per liter): 10 NaF, 100 CsF, 20 CsCl, 1 BAPTA, 0.9 CaCl₂, 10 HEPES, 10 glucose, pH 7.35 (adjusted with CsOH).¹⁷ Osmolarity was adjusted to 310 mOsm/L with sucrose for the bath solution and 300 mOsm/L for pipette solution. Data were acquired with an Axopatch 200B patch clamp amplifier and pClamp 10.2 software. Tetrodotoxin-sensitive persistent current was determined with 200-ms depolarization to −30 mV as the average current recorded between 190 and 200 ms and reported as a percentage of peak current following digital subtraction of currents recorded in the presence and absence of 30 μmol/L tetrodotoxin (Tocris Biosciences, Ellisville, MO). All data were analyzed with pClamp 10.2 (Axon Instruments, Inc, Sunnyvale, CA) and plotted using SigmaPlot 10 (IBM Corp, Armonk, NY) software. Unless otherwise noted, statistical comparisons were made by using a 1-way ANOVA and Bonferroni correction to WT Naᵥ1.5 coexpressed with WT CaM. Statistical significance was assumed for P<0.05. Results are presented as mean±SEM for all pooled data.

FVM IₘCa,L recordings were recorded in the whole-cell configuration of the patch clamp technique as previously described.¹⁸ All recordings were performed at room temperature (20 to 22°C). The pipette solution consisted of (in millimoles per liter): 125 Cs-methanesulfonate, 15 TEA-Cl, 1 MgCl₂, 0.5 EGTA, 5 HEPES, pH 7.2. Bath solution contained (in millimoles per liter): 150 NMDG, 2.5 CaCl₂, 1 MgCl₂, 10 glucose, 10 HEPES, 5 4-aminopyridine, pH 7.2. Activation voltage dependence parameters were obtained by fitting the current-voltage slope conductance transform to a Boltzmann distribution of the form G(V)=Gₘ₉/MM/(1+exp(V₁/V₆/k)) in which Gₘ₉ is the maximal conductance and V₁/V₆ is the activation midpoint. Results are presented as mean±SEM for all pooled data.

Cytosolic Ca²⁺ Imaging
Cardiac myocytes were loaded with 2 μmol/L fura-2-AM for 10 minutes in a 5% CO₂ incubator and then de-esterified in Tyrodes (140 NaCl, 1.8 CaCl₂, 1 MgCl₂, 10 HEPES [free acid], 5.4 KCl, 10 glucose, pH 7.4, NaOH) solution for ~20 minutes. Field-stimulated Ca²⁺ transients were recorded from the annulus of the photometry tube focused on a single cell or a cell cluster. All recordings were performed at 20 to 22°C. The cells were excited with light of 340-nm and 380-nm wavelengths. The images obtained at 340 and 380 nm were divided pixel by pixel, and the ratio data were reported. Data were collected and analyzed with IonOptix (Milton, MA) hardware and software. Additional offline transient analysis was performed with custom routines written in MatLab (Northampton, MA). Exponential fitting was performed using pClamp 9.2 (Axon Instruments, Union City, CA). The function used for exponential fitting of caffeine-induced Ca²⁺ transient relaxations was f(t)=A₁×exp (−t/τ₁)+C.

Statistical Analysis
Data were analyzed for Figures 5C, 6B, 7B, and 7D with the chi-square test. Figure 9A was analyzed with 1-way ANOVA with Dunn’s multiple comparison test. Figure 9A and the remaining data analysis passed the D’Agostino and Pearson test and the Shapiro-Wilk test for normality, and tests of statistical difference for the remaining data were performed using the t test with statistical significance correction of multiple comparisons and α=0.05 using the Holm-Sidak method in GraphPad Prism version 6.0 for Windows (GraphPad Software, La Jolla, CA).

Results
Effect of CaM Mutants on Cardiac Na Channels
Because CaM is known to regulate cardiac Na channel inactivation and a specific defect in Na channel inactivation (increased persistent Na current) occurs in LQTS,¹⁹ we investigated the effects of LQTS-CaM mutants on heterologously expressed Naᵥ1.5 as well as on native Na current. We performed heterologous coexpression of WT or mutant CaM with human Naᵥ1.5 in tsA201 cells and then measured electrophysiological properties of the expressed channels. When recording Na current with a nominally Ca²⁺-free intracellular solution, we did not observe differences in the level of persistent Na current (expressed as a percentage of peak current) for any of the CaM mutations compared with WT CaM. Consequently, we repeated these experiments with elevated intracellular calcium (~1 μmol/L free Ca²⁺) to provide a saturating concentration of Ca²⁺ to promote binding of CaM to Naᵥ1.5.²,¹⁷ Furthermore, because of the early age of onset of LQTS in the reported probands, we also tested the effects of CaM mutations on a fetal and neonatal-expressed Naᵥ1.5 splice variant.¹⁴ Cells coexpressing CaM-D130G with fetal Naᵥ1.5 exhibited 7.5-fold larger persistent Na current (1.5±0.4%) compared with cells coexpressing the fetal splice variant with WT CaM (0.2±0.1%; P<0.05) when recordings were made with high intracellular calcium (Figure 1A). This greater level of persistent current was not observed when CaM-D130G was coexpressed with the canonical (adult expressed) splice isofrom of Naᵥ1.5 or with low intracellular...
calcium (Table 1). In contrast, cells coexpressing fetal or canonical NaV1.5 splice isoforms with either CaM-D96V or CaM-F142L did not exhibit abnormal levels of persistent Na current under high calcium conditions (Table 1). Other Na channel properties including conductance-voltage relationship, voltage dependence of steady-state inactivation, inactivation kinetics, and recovery from inactivation were largely unaffected by CaM mutants (Table 2). Furthermore, expression of CaM-D130G in mouse FVM did not evoke detectable differences in the level of persistent Na current compared with cells transfected with WT CaM (Figure 1B). Given the inconsistent effect of CaM mutants on heterologous and native Na current, we concluded that NaV1.5 dysfunction was not the major cause of LQTS in the setting of CaM mutations.

**CDI is Slowed by LQTS-CaM But Not by a CPVT-Associated CaM Mutant**

We tested the hypothesis that LQTS-CaM mutations with impaired C-domain Ca\(^{2+}\) affinity will slow \(I_{Ca,L}\) decay in a native cardiomyocyte environment due to impaired CDI. Figure 2A illustrates representative current sweeps normalized to peak current for FVM expressing exogenous WT CaM superimposed on traces from cells expressing each of the LQTS-CaM mutants. All LQTS-CaM mutants slow \(I_{Ca,L}\) decay with the 2 relatively low Ca\(^{2+}\) \(K_d\) CaM-mutants with altered highly conserved aspartic acids that directly chelate the Ca\(^{2+}\) ion, showing relatively greater effect (Figure 2). Current density and voltage dependence of current activation were

<table>
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<tr>
<th>Persistent Current %</th>
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<tr>
<td>NaV1.5+apo WT CaM</td>
<td>0.3±0.2</td>
</tr>
<tr>
<td>NaV1.5+apo CaM-D130G</td>
<td>0.2±0.1</td>
</tr>
<tr>
<td>NaV1.5+WT CaM</td>
<td>0.3±0.1</td>
</tr>
<tr>
<td>NaV1.5+CaM-D130G</td>
<td>0.2±0.1</td>
</tr>
<tr>
<td>NaV1.5+CaM-D96V</td>
<td>0.2±0.1</td>
</tr>
<tr>
<td>NaV1.5+CaM-F142L</td>
<td>0.1±0.1</td>
</tr>
<tr>
<td>Fetal NaV1.5+apo WT CaM</td>
<td>0.4±0.1</td>
</tr>
<tr>
<td>Fetal NaV1.5+apo CaM-D130G</td>
<td>0.6±0.2</td>
</tr>
<tr>
<td>Fetal NaV1.5+WT CaM</td>
<td>0.2±0.1</td>
</tr>
<tr>
<td>Fetal NaV1.5+CaM-D130G</td>
<td>1.5±0.4</td>
</tr>
<tr>
<td>Fetal NaV1.5+CaM-D96V</td>
<td>0.4±0.1</td>
</tr>
<tr>
<td>Fetal NaV1.5+CaM-F142L</td>
<td>0.6±0.5</td>
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</table>

CaM indicates calmodulin; WT, wild type.

*apo WT CaM is Apocalmodulin (apo WT CaM is Ca\(^{2+}\)-free CaM; WT refers to the disease-associated sites identified in the probands\(^3\)).

\(^1P<0.05\) compared to fetal NaV1.5+WT CaM.

\(^2P<0.05\) compared to fetal NaV1.5+WT CaM with 10 \(\mu\)mol/L KN-93.

Data compared using Kruskal-Wallis 1-way ANOVA and Dunn’s Test.
Calmodulin Mutations Associated With Arrhythmias  Yin et al

Table 2. CaM Mutants Do Not Exhibit Major Biophysical Effects on Peak I_Na

<table>
<thead>
<tr>
<th>Voltage-Dependence of Activation</th>
<th>Steady-State Availability</th>
<th>Recovery From Inactivation</th>
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<tr>
<td></td>
<td>V_1/2 (mV)</td>
<td>k (mV)</td>
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<tr>
<td>Biophysical parameters</td>
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<tr>
<td>NaV_1.5+WT CaM</td>
<td>-45.0±2.1</td>
<td>6.6±0.5</td>
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<tr>
<td>NaV_1.5+CaM-D130G</td>
<td>-43.1±2.3</td>
<td>6.6±0.5</td>
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<tr>
<td>NaV_1.5+CaM-D96V</td>
<td>-42.6±1.1</td>
<td>7.8±0.3</td>
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<tr>
<td>NaV_1.5+CaM-F142L</td>
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<td>6.7±0.8</td>
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<td>Fetal NaV_1.5+WT CaM</td>
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<td>7.5±0.5</td>
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<tr>
<td>Fetal NaV_1.5+CaM-D130G</td>
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<td>7.2±0.5</td>
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<tr>
<td>Fetal NaV_1.5+CaM-D96V</td>
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<td>9.4±0.4^†</td>
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<tr>
<td>Fetal NaV_1.5+CaM-F142L</td>
<td>-35.4±1.7*</td>
<td>8.3±0.6</td>
</tr>
</tbody>
</table>

All recordings were performed with high intracellular calcium concentration. CaM indicates calmodulin; WT, wild type.

*p < 0.05 compared to NaV_1.5+WT CaM.

†p < 0.05 compared to fetal NaV_1.5+WT CaM.

‡p < 0.005 compared to NaV_1.5+WT CaM.

not different between FVM expressing WT or LQTS-CaM mutants (Figure 3; Table 3). In contrast, the CPVT-association CaM mutation N54I did not significantly affect CDI (Figure 4). These data suggest that attenuated CDI and resulting slower I_Ca,L decay can contribute to mutant CaM-associated LQTS but not CPVT.

Rate Dependence of Entrainment to Field Stimulation

Field stimulation of FVM causes a Ca^{2+}-induced Ca^{2+} release that follows a fire-diffusion-fire paradigm qualitatively similar to that of mature cardiomyocytes. Figure 5A illustrates representative Ca^{2+} transients from FVMs expressing either WT or mutant CaM continuously paced from quiescence to 2 Hz. For cells expressing WT CaM, there was a synchronous entrainment of field stimulation to Ca^{2+} transient (eg, 1:1 ratio; Figure 5A, top panel). Expression of CaM-F142L does not compromise entrainment of Ca^{2+} transients at low stimulation frequencies. The lower Ca^{2+}-affinity mutations CaM-D96V and CaM-D130G show frequent loss of entrainment, as shown by the presence of 2:1 pacing block (eg, 2 stimuli for 1 transient; Figure 5B). The loss of entrainment observed for LQTS-CaM mutants summarized as “number of escapes” follows the rank order of Ca^{2+}-CaM affinity, whereby cardiomyocytes expressing the weaker Ca^{2+}-affinity CaM mutants escaped from pacing at progressively lower frequencies (Figure 5C).

We categorized the Ca^{2+} transient phenotypes into those displaying alternans, and those exhibiting slower Ca^{2+} reuptake manifested as smaller amplitude, slower decaying, and elevated diastolic levels (Figure 6). The prevalence of pathological Ca^{2+}-transient phenotypes tracks with the rank order of Ca^{2+}-CaM affinity. The lowest affinity CaM mutations, D96V and D130G, uniquely displayed overload phenotypes (defined as diastolic Ca^{2+} >1 standard deviation above the WT-CaM diastolic fluorescence level), and cells expressing D96V and D130G also show a preponderance of reuptake anomalies (Figure 6). In summary, LQTS-CaM mutants induce arrhythmogenic activity in developing ventricular cardiomyocytes. The severity of the dysfunction induced by various LQTS-CaM mutations tracks with reported Ca^{2+}-CaM affinities.

LQTS-CaM Mutations Perturb Ca^{2+} Handling

We investigated whether elevation of cytosolic Ca^{2+} could drive the loss of entrainment induced by LQTS-CaM mutants (Figure 6). FVM expressing LQTS-CaM mutations have significantly higher twitch amplitude than cells expressing WT CaM (Figure 8A). Notably, the mean twitch amplitude for D130G is less than that for F142L and D96V. Similarly, diastolic Ca^{2+} is higher in cells expressing the lower Ca^{2+}-affinity CaM mutations D96V and D130G (Figure 8B). CaM has multiple sites of action with respect to Ca^{2+} homeostasis: CaM modulation of RyR2 and Ca-CaMKII regulation of SR Ca^{2+}.
stores. Consequently, we probed the contribution of LQTS-CaM mutation to SR Ca\(^{2+}\) load by assessing caffeine-releasable Ca\(^{2+}\) stores (Figure 8C). The mean SR Ca\(^{2+}\) appears greater in LQTS-CaM mutant expressing cardiomyocytes compared with cells expressing WT CaM, but the differences did not reach statistical significance (Figure 8D). Examination of the decay of caffeine-induced Ca\(^{2+}\) transient reveals no significant difference for any of the LQTS-CaM mutants (Figure 8E). This suggests that Na\(^+\)/Ca\(^{2+}\) exchange (NCX) function is not altered by LQTS-CaM mutations. These results are consistent with the model that LQTS-CaM mutant promoter increased trigger Ca\(^{2+}\) from the slowed decay of I\(_{\text{Ca, L}}\). NCX is the main surface membrane efflux pathway, thus more LTCC Ca\(^{2+}\) influx paired with unchanged NCX-based efflux could lead to elevated cytosolic Ca\(^{2+}\).

### Isoproterenol Restores Entrainment for LQTS-CaM Mutations

The LQTS-CaM mutants D96V and D130G cause abnormal Ca\(^{2+}\) transients at elevated stimulation frequencies (Figures 4 and 5) and exhibit elevated cytosolic Ca\(^{2+}\) levels (Figure 8). Higher Ca\(^{2+}\) levels could drive arrhythmic activity analogous to that seen for CPVT.\(^{21}\) Faster pacing rates protect against catecholamine-induced triggered activity in CPVT models\(^{22}\); however, Ca\(^{2+}\)–transient abnormalities are exacerbated at even modestly higher rates (≥1 Hz) in FVM expressing relatively low Ca\(^{2+}\)-affinity LQTS-CaM mutants (Figure 5). Isoproterenol (ISO) enhances the rate of Ca\(^{2+}\) reuptake to the SR in developing cardiomyocytes (Figure 9A and 9Ai), and this effect is preserved for cardiomyocytes.
expressing LQTS-CaM mutants (Figure 9A and 9Ai). Given the propensity of defective Ca\(^{2+}\) reuptake observed in D96V and D130G-CaM expressing cardiomyocytes, we tested the hypothesis that ISO treatment alleviates these LQTS-CaM mutant-induced Ca\(^{2+}\)-transient loss of entrainment. Figure 9B shows a representative D96V-CaM-expressing cardiomyocyte. Prior to ISO addition, a 2:1 block occurs (Figure 9B, upper left). ISO induces a partial rescue of 1:1 entrainment manifested as a spontaneous transition from 2:1 to 1:1 (stimulus to Ca\(^{2+}\) transient; Figure 9B, upper right). This type of behavior was scored as “partial rescue” because of spontaneous restoration and reversion of pathological Ca\(^{2+}\) transients. The relatively lower Ca\(^{2+}\) affinity D130G-CaM exhibited alternans at 0.5 Hz pacing frequency (Figure 9C, upper left). ISO rapidly results in full restoration of normal Ca\(^{2+}\) transients in this example (Figure 9C, upper right). Overall, ISO caused some degree of restoration of normal Ca\(^{2+}\) homeostasis in 65% of cells tested. For D96V-CaM-expressing cardiomyocytes, 9 and 2 of 17 cells showed partial and full restoration, respectively. For D130G-CaM-expressing cardiomyocytes, 4 and 3 of 11 cells showed partial and full restoration, respectively. In sharp contrast, acute ISO led to cell death in 6 of 9 CPVT-CaM mutant-expressing cardiomyocytes and had no beneficial effects in the remaining cells.

**Figure 3.** Peak current-voltage relationships for \(I_{\text{Ca,L}}\) in fetal ventricular cardiomyocytes. WT calmodulin data are repeated in each panel for clarity of presentation. No significant differences were noted in voltage dependence or amplitude. See Table 3 for Boltzmann distribution fitted parameters. WT indicates wild type.

**Table 3.** \(G_{\text{max}}, V_{1/2}\), and \(k\) in Fetal Cardiac Ventricular Myocytes Expressing WT or long QT CaM

<table>
<thead>
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<th></th>
<th>(G_{\text{max}})</th>
<th>(V_{1/2})</th>
<th>(k)</th>
<th>(n)</th>
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<tr>
<td>WT CaM nongreen</td>
<td>0.109±0.018</td>
<td>-7.2±0.6</td>
<td>6.6±0.2</td>
<td>13</td>
</tr>
<tr>
<td>WT CaM green</td>
<td>0.055±0.008*</td>
<td>-8.8±0.4</td>
<td>6.6±0.2</td>
<td>9</td>
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<tr>
<td>WT CaM combination</td>
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<td>-7.9±0.4</td>
<td>6.6±0.1</td>
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<tr>
<td>CaM-F142L</td>
<td>0.059±0.014*</td>
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<td>7.2±0.3</td>
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<tr>
<td>CaM-D96V</td>
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<td>-6.3±0.5</td>
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<tr>
<td>CaM-D130G</td>
<td>0.054±0.024</td>
<td>-9.8±1.5</td>
<td>6.3±0.1</td>
<td>6</td>
</tr>
</tbody>
</table>

CaM indicates calmodulin; WT, wild type.

*\(P<0.05\) compared with WT CaM nongreen.
tested. These data suggest that slowed Ca$^{2+}$ reuptake contributes to LQTS-CaM–associated arrhythmogenesis, and enhancing the rate of reuptake is a plausible mechanism for ameliorating LQTS-CaM dysfunction.

Discussion

The major new finding of this study is that CaM mutants associated with LQTS cause significantly slowed CDI of $I_{Ca,L}$.
Figure 6. Loss of Ca$^{2+}$ transient entrainment is dominated by faulty reuptake in myocytes expressing the lowest Ca$^{2+}$-affinity calmodulin (CaM) mutants. A, Representative Ca$^{2+}$ transients for 1-Hz stimulation frequency. WT CaM and F142L-CaM expressing cardiomyocyte Ca$^{2+}$ transients were entrained to 1-Hz stimuli. Pathological loss of entrainment categorized as overload (middle and lower left for D96V-CaM and D130G, respectively), reuptake defective (middle right for D130G-CaM), and alternans (lower right). The lower left D130G trace displaying overload (blue) is at a level greater than the WT diastolic level (upper left panel). Note that the representative alternans trace (lower right, red) is displaced by 1 fluorescent unit to illustrate an alternating pattern of relatively large- and small-amplitude transients. B, Summary of pathological Ca$^{2+}$-transient phenotypes. For statistical testing, we tested the hypothesis that long QT syndrome CaM mutants induced any pathological phenotype. N=20 cells for each group; $P<10^{-4}$. WT indicates wild type.
and alter Ca\(^{2+}\) homeostasis in FVM. We also excluded a major contribution of dysregulated Na current caused by LQTS-CaM mutations. In contrast, the CPVT-CaM mutant N54I had no effect on CDI but disrupted Ca\(^{2+}\) homeostasis. We conclude that LQTS-CaM evokes the novel mechanism of CDI slowing to initiate the sequence of cellular physiological events leading to ventricular arrhythmia.

This study shows a rank-order relationship between Ca\(^{2+}\)-CaM affinity and the propensity for abnormal Ca\(^{2+}\) homeostasis. This relationship holds for the 3 tested LQTS-CaM mutants and is distinct for CPVT-CaM mutant N54I. LQTS is driven by a prolongation of the ventricular cellular AP duration, and AP duration is determined by the sum of ionic conductance, principally net voltage-gated K\(^{+}\) channels and LTCC. CaM prebound to LTCC confers a direct relationship between CaM alterations of LTCC current and AP duration, whereby increased Ca\(^{2+}\) influx prolongs AP duration. LQTS-CaM mutations occur de novo in the carboxyl-terminus (C-lobe) of CaM.\(^2\) The C-lobe of LTCC-bound CaM senses Ca\(^{2+}\) entry through single LTCC channel openings, and this occurs in a restricted space where local [Ca\(^{2+}\)] can reach levels as high as 60 to 100 \(\mu\)mol/L.\(^{23,24}\) The LQTS-CaM mutation F142L C-lobe Ca\(^{2+}\)-affinity \(K_d\) is 15 \(\mu\)mol/L, well below the estimated local [Ca\(^{2+}\)], and thus may explain the relatively subtle effects observed with F142L. The D130G Ca\(^{2+}\) \(K_d\) is 150 \(\mu\)mol/L, which is consistent with a greater propensity to promote pathological Ca\(^{2+}\) transients among LQTS-CaM mutants tested. Recent studies also quantified the LQTS-CaM LTCC effects\(^{25}\) based on the conceptual framework of an enzyme-inhibitor-like tuning of LTCC-CaM signaling.\(^{26,27}\) In contrast, the CPVT-CaM mutation N54I is located on the N-lobe and shows sensitive, near normal Ca\(^{2+}\) affinity.\(^4\) This supports the contention that LQTS-CaM mutation primarily targets LTCC, whereas CPVT-CaM leaves LTCC function intact.

Examination of Ca\(^{2+}\) transients reveals mutant-CaM effects that are not predicted by examining LTCC function alone. LQTS-CaM slows CDI, but CaM-N54I has no effect on CDI; however, both classes of LQTS-CaM mutations D96V, D130G, and N54I result in abnormal Ca\(^{2+}\) transients. Recent work demonstrated that CPVT-CaM, but not LQTS-CaM, induces inappropriate SR Ca\(^{2+}\) release\(^{12}\) driven by aberrant CaM-RyR2 interactions (cf\(^{16}\)). In the present study, we uniquely show that the nature of the Ca\(^{2+}\)-transient phenotype differs in cardio-

Figure 7. Catecholaminergic polymorphic ventricular tachycardia calmodulin induces abnormal Ca\(^{2+}\) transients. A, Representative Ca\(^{2+}\) transients in cardiomyocytes expressing calmodulin-N54I. B, Loss of entrainment for frequencies \(\geq 1\) Hz; for 3 Hz, no entrainment was possible. \(P<10^{-4}\). C, Representative Ca\(^{2+}\) transient displaying alternans at 1 Hz. D, For 1 Hz, all calmodulin-N54I cardiomyocytes displayed abnormal Ca\(^{2+}\)-transient phenotypes; the majority of cells displayed alternans. \(P<10^{-4}\). WT indicates wild type.
myocytes expressing LQTS-CaM–associated mutations D96V and D130G compared with cells expressing CPVT-CaM mutants. The predominant pattern of pathological Ca2+-transients exhibited by cells expressing these 2 LQTS-CaM mutants was slowed SR Ca2+ reuptake, whereas cells expressing the CPVT-CaM mutant N54I showed a combination of alternans and 2:1 blockade. The latter finding is consistent with other models of CPVT.28 Moreover, in mouse models of CPVT, catecholamines trigger activity in the ventricular myocardium,29–31 and the catastrophic effect of ISO on CaM-N54I FVM is consistent with expectations of CPVT. A dominant contributor to twitch Ca2+-transient relaxation is phospholamban-SERCa function. In this vein, ISO is predicted to have 2 major effects: increasing the rate of SR Ca2+ reuptake through phospholamban-SERCa signaling and increasing Ca2+ influx by LTCC activation. Additional considerations reveal the benefit of assessing Ca2+ transients as an integrative measure of function. For example, an ISO-induced increase of K+ conductance simultaneously acts to shorten AP duration, blunting the time course of LTCC Ca2+ influx. ISO is well established to speed Ca2+-transient kinetics and increase SR Ca2+ load. If LQTS-CaM effects were limited only
to slowing of CDI, then we would have expected that ISO would not restore normal Ca\textsuperscript{2+} transients. The finding that ISO restored normal Ca\textsuperscript{2+} transients in LQTS reveals distinctions between CPVT-CaM and LQTS-CaM mutations and highlights the unexpected contribution of augmented Ca\textsuperscript{2+} reuptake as a future therapeutic direction for LQTS-CaM patients. Applying therapeutics involving increased SR Ca\textsuperscript{2+} reuptake has plausible future possibilities considering early successes of the Calcium Up-regulation by Percutaneous Administration of Gene Therapy in Cardiac Disease (CUPID) trial.\textsuperscript{32}

**Study Limitations**

For heterologous expression of Na\textsubscript{v}1.5 we selected 2 prominently expressed products of alternative splicing: the canonical form expressed in adult heart and a developmentally regulated splice variant that is expressed prominently in

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**Figure 9.** β-adrenergic stimulation speeds twitch duration and restores entrained rhythmicity in cardiomyocytes expressing relatively low Ca\textsuperscript{2+}-affinity long QT syndrome calmodulin (CaM) mutants. A, Stimulated twitch decay (1 Hz) at baseline and following 100 nmol/L ISO. *P<0.05. Ai, Regardless of exogenous CaM expression, the twitch decay rate was faster following ISO. Data from (A) replotted as cell-by-cell response to ISO. **P<0.001; ***P<10\textsuperscript{-4}; n=10 or 11. B, Representative Ca\textsuperscript{2+} transient for D96V-CaM expressing fetal ventricular myocyte. Left panel shows 1-Hz stimulation to illustrate baseline 2:1 block. Right panel shows an example of partial restoration of entrainment of Ca\textsuperscript{2+} transient to field stimulation. With ISO, the 2:1 activity spontaneously reverts to 1:1 activity. n=10. C, Representative Ca\textsuperscript{2+} transient for D130G-CaM expressing fetal ventricular myocyte. Left panel shows 0.5-Hz stimulation to illustrate baseline alternans. Right panel shows an example of recovery of entrainment following ISO. n=10. ISO indicates isoproterenol, WT, wild type.
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fetal and infant heart. Evidence exists for protein expression of both splice isoforms. Other SCN5A splice variants have been demonstrated at the mRNA level but not at the protein level, and their developmental regulation is uncertain. The level, and their developmental regulation is uncertain. The mRNA has been demonstrated at the mRNA level but not at the protein level, and their developmental regulation is uncertain. The most straightforward interpretation of our Na\(^+\)I.5 data is that augmentation of late I\(_{Na}\) is not to the main pathophysiological mechanism responsible for the LQTS-CaM phenotype; however, we cannot exclude that another unstudied splice variant conspires with mutant CaM to evoke a pathological phenotype. In contrast, our data on Ca\(^{2+}\) channel modulation argues effectively that delayed CDI is a more plausible mechanism for LQTS associated with CaM mutations.

Relatively late development stage FVM served as a cellular model to study the effects of LQTS-CaM–associated mutations. These late-stage FVM express a full complement of mature cardiomyocyte physiological responses including Ca\(^{2+}\)-induced Ca\(^{2+}\) release, and \(\beta\)-adrenergic receptor–mediated responses. As with any cellular system, there are limitations. First, although the LTCC multiprotein complex is fully functional, the same issues discussed above for expression of various channel protein isoforms applies. Second, this is a cellular model, and behaviors of dispersed cells may not report native physiological responses. Although this system allows us to use a common background to compare the influence of CaM mutations, it will be important to follow up studies in more complex in vivo settings.

In summary, LQTS- and CPVT-associated CaM mutations both disrupt Ca\(^{2+}\) homeostasis in murine cardiomyocytes, but there is a sharp dichotomy in pathways leading to pathological phenotypes. LQTS-CaM mutations occurring on the C-lobe of CaM reduce Ca\(^{2+}\) affinity and, consequently, cause slowing of LTCC kinetics by attenuating CDI. In contrast, CPVT-CaM mutant N54I has no effect on LTCC kinetics. As expected for CPVT, \(\beta\)-adrenergic stimulation irreversibly overloaded cytosolic Ca\(^{2+}\) but also partially restored entrainment. This suggests a potential proof of principle for genotype-specific treatment of “calmodulinopathies.”

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Disclosures
None.

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