Hyperphosphorylation of RyRs Underlies Triggered Activity in Transgenic Rabbit Model of LQT2 Syndrome

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Rationale: Loss-of-function mutations in human ether go-go (HERG) potassium channels underlie long QT syndrome type 2 (LQT2) and are associated with fatal ventricular tachyarrhythmia. Previously, most studies focused on plasma membrane–related pathways involved in arrhythmogenesis in long QT syndrome, whereas proarrhythmic changes in intracellular Ca2+ handling remained unexplored.

Objective: We investigated the remodeling of Ca2+ homeostasis in ventricular cardiomyocytes derived from transgenic rabbit model of LQT2 to determine whether these changes contribute to triggered activity in the form of early after depolarizations (EADs).

Methods and Results: Confocal Ca2+ imaging revealed decrease in amplitude of Ca2+ transients and sarcoplasmic reticulum Ca2+ content in LQT2 myocytes. Experiments using sarcoplasmic reticulum–entrapped Ca2+ indicator demonstrated enhanced ryanodine receptor (RyR)–mediated sarcoplasmic reticulum Ca2+ leak in LQT2 cells. Western blot analyses showed increased phosphorylation of RyR in LQT2 myocytes versus controls. Coimmunoprecipitation experiments demonstrated loss of protein phosphatases type 1 and type 2 from the RyR complex. Stimulation of LQT2 cells with β-adrenergic agonist isoproterenol resulted in prolongation of the plateau of action potentials accompanied by aberrant Ca2+ releases and EADs, which were abolished by inhibition of Ca2+/calmodulin–dependent protein kinase type 2. Computer simulations showed that late aberrant Ca2+ releases caused by RyR hyperactivity promote EADs and underlie the enhanced triggered activity through increased forward mode of Na+/Ca2+ exchanger type 1.

Conclusions: Hyperactive, hyperphosphorylated RyRs because of reduced local phosphatase activity enhance triggered activity in LQT2 syndrome. EADs are promoted by aberrant RyR-mediated Ca2+ releases that are present despite a reduction of sarcoplasmic reticulum content. Those releases increase forward mode Na+/Ca2+ exchanger type 1, thereby slowing repolarization and enabling L-type Ca2+ current reactivation. (Circ Res. 2014;115:919-928.)

Key Words: arrhythmias, cardiac ■ long QT syndrome ■ protein phosphatase ■ ryanodine receptor ■ calcium release

Sudden cardiac death (SCD) because of malignant arrhythmias remains the major factor of mortality worldwide. Prolongation of QT interval is recognized as an important risk factor for SCD in acquired cardiac diseases including heart failure and myocardial infarction and has a significant genetic component as well. To date mutations in 13 genes were associated with congenital LQTS including loss-of-function mutations in KCNQ1 encoding rapid rectifier K+ channel, which leads to significant prolongation of action potential (AP).1 Long QT syndrome type 2 (LQT2) associated with loss of IKr accounts for a large fraction and a high rate of mortality of congenital LQTS.1,2 Most of the deaths of LQT2 patients occur as a result of triggered ventricular tachycardia and ventricular fibrillation evoked by emotional stress, exercise, or sudden startle.1-3 Triggered activity and torsade de points identified in the ECG recordings are thought to originate at the cellular level from membrane oscillations during the repolarizing phase of AP called early after depolarizations (EADs).3,4
We have created a transgenic rabbit model of long QT syndrome type 2, which recapitulates human arrhythmia phenotype. These rabbits overexpress a pore mutant of the human gene KCNH2 (HERG-G628S) in the heart to eliminate I_{Kr} currents and as a result exhibit prolonged QT interval and high incidence of SCD (>50% at 1 year of age) because of polymorphic ventricular tachycardia. The underlying mechanisms of substrate for arrhythmia in these rabbits studied using ex vivo optical mapping include a prominent spatial dispersion of AP duration (APD) and discordant APD alternans, whereas triggered activity is present in the form of EADs under β-adrenergic stimulation.

The regular rhythmical cardiac cycling is maintained by the interplay between the AP and Ca^{2+} release from the sarcoplasmic reticulum (SR) mediated by the ryanodine receptor (RyR) channels. Thus, although LQTS phenotypically is an electric disorder, possible proarrhythmic remodeling of Ca^{2+} signaling pathways is recognized as an important unresolved question in LQTS pathophysiology. We recently reported that sex hormones effectively modulate arrhythmic potential and SCD in LQT2 rabbits by modulating expression and function of important Ca^{2+} transport complexes including L-type Ca^{2+} channel (LTCC) and SR Ca^{2+} ATPase, which underscores a key role of intracellular Ca^{2+} handling in LQT2-associated arrhythmia. This strongly implies that Ca^{2+}-mediated communication between RyRs and other Ca^{2+} transport complexes could play a role in EAD formation in LQT2. However, possible maladaptive changes in Ca^{2+} homeostasis and the exact mechanisms underlying EADs in LQT2, or congenital LQTS in general, remain unresolved.

Most computer modeling studies to date have associated EADs with an instability of membrane voltage dynamics driven by reactivation of the L-type Ca^{2+} current I_{Ca,L} during the plateau phase of the AP. The role of RyR-mediated Ca^{2+} release in EAD formation has only been modeled in the setting of pharmacologically induced LQT2 with a generic block of I_{Kr}. Therefore, its role in hereditary LQT2 remains unexplored.

To further characterize the mechanism of triggered activity and SCD of LQT2 rabbits, we used a combination of cellular electrophysiology and confocal Ca^{2+} imaging to demonstrate that RyR activity in LQT2 is abnormally high, which resulted in diminished SR Ca^{2+} content and reduced Ca^{2+} transient amplitude in myocytes derived from LQT2 hearts. RyR hyperactivity disrupts timely cessation of SR Ca^{2+} release during AP in LQT2 myocytes. Furthermore, we have used computer modeling to show that this reduction in refractoriness of SR Ca^{2+} release plays a key role via the Na^{+}/Ca^{2+} exchanger current in maintaining membrane potential in the range of voltages ideal for reactivation of LTCCs leading to EADs under β-adrenergic stimulation. Proarrhythmic increase in RyR activity was attributable to enhanced RyR phosphorylation because of dissociation of protein kinases 1 and 2 from the RyR macromolecular complex.

### Methods

The cellular, subcellular, and molecular effects of hereditary LQT2 on Ca^{2+} homeostasis were studied in left ventricular myocytes isolated from the rabbit hearts expressing dominant-negative mutant of human gene KCNH2 (HERG-G628S) and from the hearts of wild-type littermate controls (LMC). Cytosolic and intra-SR Ca^{2+} changes were monitored using confocal microscopy, and membrane potential and whole cell currents were recorded with the patch-clamp technique at 37°C. Changes in protein expression, protein–protein interactions, and protein phosphorylation were studied using standard techniques. Computer simulations exploring arrhythmic potential of LQT2-associated changes in Ca^{2+} handling were performed using an extension of a physiologically detailed multiscale rabbit ventricular myocyte model. This model bridges the submicron scale of individual couplings of plasmalemmal LTCCs clusters and SR Ca^{2+} release units and the whole cell.

An expanded Materials and Methods section can be found in the Online Data Supplement.

### Results

#### Decrease in SR Ca^{2+} Content Underlies Diminished Ca^{2+} Transient Amplitudes in LQT2 Myocytes

Prolongation of AP by pharmacological inhibition of K^{+} channels is thought to increase intracellular [Ca^{2+}] by increasing Ca^{2+} influx through plasmalemmal voltage-dependent Ca^{2+} channels. To assess whether it happens in congenital LQT2, we performed measurements using confocal imaging of Ca^{2+} transients in intact myocytes undergoing periodic field stimulation at pacing frequencies from 0.25 to 2 Hz. As presented in Figure 1, the amplitude of Ca^{2+} transients in LQT2 myocytes at higher stimulation frequencies was significantly reduced in basal conditions in comparison with LMCs. LQT2 myocytes preincubated with 50 mmol/L of β-adrenergic agonist isoproterenol for 3 to 10 minutes exhibited ≈25% reduction in Ca^{2+} transient amplitude compared with LMC at all frequencies tested. The decrease in Ca^{2+} transient amplitude was accompanied by a significant reduction in SR Ca^{2+} load assessed by rapid application of 10 mmol/L caffeine after 20-s stimulation at 1 Hz. Interestingly, application of isoproterenol failed to increase SR Ca^{2+} content in LQT2 myocytes in sharp contrast to LMCs. The reduction in SR Ca^{2+} content can be potentially explained by reduced [Ca^{2+}] influx through LTCCs or enhanced extrusion of cytosolic [Ca^{2+}] by Na^{+}/Ca^{2+} exchanger type 1 (NCX1). However, measurements of time constant of...
decay of caffeine-induced Ca\textsuperscript{2+} transient, which reflects NCX1 activity, demonstrated no differences between experimental groups (Figure 1C and 1D). Likewise, whole cell voltage clamp experiments in the absence of EGTA in the pipette revealed no significant differences in current densities of L-type Ca\textsuperscript{2+} currents between LQT2 and LMC myocytes either under basal conditions or in the presence of isoproterenol (Online Figure I). In line with the lack of functional changes, Western blot analysis failed to demonstrate differences in expression levels of NCX1 and pore-forming subunit of LTCC α\textsubscript{1c} (Online Figure II). These data suggest that changes in SR Ca\textsuperscript{2+} content and cytosolic Ca\textsuperscript{2+} transients in LQT2 myocytes are likely because of impaired ability of SR to maintain Ca\textsuperscript{2+}.

Enhanced SERCA-Mediated Ca\textsuperscript{2+} Uptake and RyR-Mediated Ca\textsuperscript{2+} Leak in LQT2 Myocytes

To investigate potential changes in function of RyR and SR Ca\textsuperscript{2+} ATPase (SERCA) in LQT2 cells without interference of plasmalemmal Ca\textsuperscript{2+} transporters, we used experimental system of saponine-permeabilized myocytes. Figure 2 demonstrates representative confocal line images scan images of spontaneous Ca\textsuperscript{2+} sparks recorded using intracellular solution containing 100 nmol/L free [Ca\textsuperscript{2+}] and averaged data for Ca\textsuperscript{2+} sparks parameters. LQT2 myocytes exhibited significant increase in spark dimensions and frequency suggestive of enhanced SR [Ca\textsuperscript{2+}] leak through RyR clusters. Application of 10 nmol/L caffeine showed moderate but significant increase in SR Ca\textsuperscript{2+} content in permeabilized LQT2 myocytes, indicating that not only SR Ca\textsuperscript{2+} leak but SERCA-mediated uptake is accelerated in these cells as well. Next, to study leak and uptake separately, we used experimental protocol with low-affinity indicator fluo-5N loaded into the SR. To assess SERCA-mediated Ca\textsuperscript{2+} uptake, permeabilized myocytes with SR-entrapped fluo-5N were exposed to 10 nmol/L caffeine to fully empty Ca\textsuperscript{2+}-stores, and then caffeine was washed out with 0 [Ca\textsuperscript{2+}] solution and RyRs were blocked with RyR inhibitor ruthenium red (40 μmol/L). Subsequent reintroduction of 250 nmol/L [Ca\textsuperscript{2+}] into the cytosol evokes time-dependent increase in fluo-5N fluorescence reflective of the dynamics of SERCA-mediated resquestration of Ca\textsuperscript{2+} into the SR. Figure 3A depicts representative traces of cell-averaged fluo-5N signals obtained from permeabilized LQT2 and LMC myocytes. As evident from Figure 1. Decreased [Ca\textsuperscript{2+}] transient amplitude and sarcoplasmic reticulum [Ca\textsuperscript{2+}] content in intact long QT syndrome type 2 (LQT2) myocytes. A. Representative Ca\textsuperscript{2+} transients recorded in intact littermate control (LMC; black) and LQT2 (red) ventricular myocytes undergoing repetitive stimulation at 0.25 Hz at baseline conditions and in the presence of 50 nmol/L isoproterenol (ISO). B. Bar graphs present pooled data for Ca\textsuperscript{2+} transient amplitudes at different stimulation frequencies. Gray bars, LMC; black bars, LQT2. C. Representative traces of Ca\textsuperscript{2+} transients induced by application of 10 mmol/L caffeine. D. Bar graphs depict averaged amplitudes and decay time constants for caffeine transients. *P<0.05, unpaired Student t test (n=6–33).

Figure 2. Increased sarcoplasmic reticulum [Ca\textsuperscript{2+}] content and increased frequency and amplitude of [Ca\textsuperscript{2+}] sparks in permeabilized long QT syndrome type 2 (LQT2) myocytes. A. Representative line scan images of Ca\textsuperscript{2+} sparks recorded in permeabilized myocytes using intracellular solution with 100 nmol/L [Ca\textsuperscript{2+}] free. B and C. Bar graphs present averaged frequency and amplitude of Ca\textsuperscript{2+} sparks. D. Representative traces of Ca\textsuperscript{2+} release induced by 10 nmol/L caffeine application. E. Pooled data for caffeine-induced Ca\textsuperscript{2+} transients. *P<0.05; **P<0.01; ***P<0.001 vs littermate control (LMC), unpaired Student t test.
representing the data and pooled data for the time constants of fluo-5N fluorescent signals (Figure 3B), SERCA-mediated SR Ca\(^{2+}\) uptake is significantly accelerated in LQT2 myocytes in comparison with LMCs. It has been demonstrated recently that not all SR Ca\(^{2+}\) leak is mediated by Ca\(^{2+}\) sparks, and substantial component of leak occurs in the form of unitary RyR openings undetectable by cytosolic Ca\(^{2+}\) indicators. 19 To assess total RyR-mediated SR Ca\(^{2+}\) leak, fluo-5N–loaded cells were exposed to specific inhibitor of SERCA thapsigargin (10 \(\mu\)mol/L). Application of thapsigargin evoked time-dependent loss in fluorescence signal of the SR-entrapped Ca\(^{2+}\) indicator, which was \(\approx 2\)-fold faster in LQT2 myocytes than in LMCs (Figure 3C and 3D). These data indicate that RyR activity in LQT2 myocytes is abnormally high.

**Enhanced RyR Phosphorylation in LQT2 Myocytes Is Determined by the Loss of Local Phosphatase Activity**

Western blot analysis revealed no differences in expression levels of RyR, SERCA, and phospholamban, an important regulator of SERCA activity (Online Figure II). Previously, we and others demonstrated in several large animal models of acquired cardiac disease and aging that disease-related remodeling of Ca\(^{2+}\) handling involves post-translational modifications of RyR and phospholamban causative of aberrant functioning of SR Ca\(^{2+}\) release channels and SERCA. 14,15,20–24 To test the hypothesis that altered RyR and SERCA function in LQT2 is caused by RyR and phospholamban phosphorylation, we performed analysis using custom-made phospho-specific antibodies against RyR p-Ser-2809 and p-Ser-2815, rabbit protein kinase A (PKA) and Ca\(^{2+}/\)calmodulin-dependent protein kinase type 2 (CaMKII) sites, respectively, and antibodies to detect phosphorylation of phospholamban at PKA site Ser-16 and CaMKII site Thr-17. Data presented in Figure 4 demonstrate that RyR in LQT2 myocytes is significantly phosphorylated at its PKA site, whereas RyR phosphorylation at CaMKII site is maximal even at basal conditions. Phosphorylation of phospholamban at PKA site Ser-16 is also increased in untreated LQT2 myocytes to a degree that pretreatment of myocytes with 50 nmol/L isoproterenol fails to produce additional effects. In theory, shift of phosphorylation–dephosphorylation
balance toward higher phosphorylation levels may result from either increased activity of kinases or diminished activity of phosphatases. Western blot analysis showed that the expression levels of catalytic subunits of serine-threonine protein phosphatase type 1 (PP1) and serine-threonine protein phosphatase type 2A (PP2A), two major Ser-Thr phosphatases that control phosphorylation state of RyR,25 were comparable in ventricular myocytes from LQT2 and LMC hearts (Figure 5A and 5B). Furthermore, our experiments using phosphatase activity assays showed no differences in activity of PP1 and PP2A in samples from LQT2 and LMC ventricular myocytes (Figure 5B). Previous studies suggested that changes in kinase or phosphatase activity localized to specific substrates including RyR may be more important than the differences in total activity of enzymes in the cell.16,22,26,27 To test this possibility, we performed a series of coimmunoprecipitation experiments and found significant reduction of PP1c and PP2Ac scaffolded to RyR (Figure 5C and 5D). To gain further insights into how these changes manifest in LQT2, we performed Western blot analysis interrogating possible changes in the expression levels of regulatory subunits of phosphatases that tether catalytic subunits PP1 and PP2A to specific substrates. We found a significant 3-fold increase in PP1 regulatory subunit PPP1R3A, whereas the levels of regulatory subunit PPP1R9B (or spinophilin, known to scaffold PP1c to RyR25) tended to be lower in LQT2 (Online Figure IXA and IXB). By contrast, we did not find statistically significant differences in regulatory subunits of PP2A including PPP2R5A, PPP2R5D, PPP2R5E in LQT2 versus LMC, whereas PPP2R3A, a PP2A regulatory subunit also known as PR130 that scaffolds PP2Ac to RyR,25 was upregulated (Online Figure IXA and IXB). The latter data did not provide an explanation for the loss of PP2Ac from the SR Ca2+ release complex. Therefore, because the ability of PP2Ac to form complexes with regulatory subunits is controlled by post-translational modifications, we tested whether phosphorylation and methylation levels of PP2Ac were altered in LQT2 (Online Figure IXC and IXD). Western blot analysis using anti–phospho-Y307 and anti–methyl-L309 antibodies demonstrated a significant increase in PP2Ac phosphorylation, whereas methylation levels tended to be lower in LQT2 versus LMC, providing the basis for the dissociation of PP2A activity from the RyR complex. Noteworthy, our experiments using radioligand binding assay showed no differences in activity of CaMKII in ventricular tissue samples from LQT2 and LMC rabbits (Online Figure IIIA). Furthermore, coimmunoprecipitation experiments revealed no changes in abundance and phosphorylation of CaMKII residing on RyR (Online Figure IIIC and IIID). Taken together, these results corroborate the theory that changes in function of RyR in LQT2 are caused by dissociation of phosphatases from RyR complex.

**Reduced Refractoriness of Hyperphosphorylated RyRs Contributes to AP Prolongation in LQT2 Myocytes Under β-Adrenergic Stimulation**

To gain further insights into the role of hyperactive RyRs in LQT2, we recorded Ca2+ transients in current clamped myocytes exposed to 50 nmol/L of isoproterenol. In contrast to LMCs, β-adrenergic stimulation evoked significant prolongation of AP and EADs in LQT2 myocytes undergoing low frequency periodic stimulation at 0.25 Hz (Figure 6A–6C). These changes in membrane potential were accompanied by a decrease in peak amplitude of Ca2+ transients, prolonged time to peak (Figure 6A, 6E, and 6F), and a protracted tail component of Ca2+ transients with distinguishable disorganized Ca2+ sparks, miniwaves and more synchronized Ca2+ signals during EADs. As a parameter describing this component we chose the time of the decay to 25% of Ca2+ transient amplitude after the peak (Figure 6G). The changes in the shape of Ca2+ signal suggest that hyperphosphorylated RyRs lose their ability to transit into refractory state and are prone to premature reactivation in myocytes from LQT2 hearts. To assess the possible role of augmented RyR function in shaping AP, we examined the effects of low doses of caffeine to sensitize RyRs in LMC myocytes. As demonstrated in Figure 6A–6G, exposure of isoproterenol-treated LMC myocytes to 250 μmol/L caffeine reduced Ca2+ transient amplitude and evoked sustained tail component of Ca2+ transient, resulting in prolongation of AP and generation of EADs, rendering LMC myocytes similar to LQT2 cells. The time during plateau in a range of potentials between +10 and −50 mV suitable for reactivation of plasmalemmal Ca2+ channels was similar for LQT2 cells and LMC myocytes treated with caffeine (Figure 6A and 6D). Furthermore, the take-off potential for EADs in both groups of cells was the same: −18.3±1.5 mV and −18.5±1.6 mV for LQT2 (n=36 in 17 cells) and LMC+Caff (n=9 in 7 cells), respectively. Pretreatment of LQT2 myocytes with CaMKII inhibitor KN93 (500 nmol/L,
Figure 6. Inhibition of Ca\[^{2+}\]/calmodulin-dependent protein kinase type 2 (CaMKII) abolishes early after depolarizations (EADs) in long QT syndrome type 2 (LQT2) myocytes exposed to isoproterenol (ISO). A. Membrane potential traces (black) and corresponding averaged time-dependent profiles (red) and confocal line scan images of Ca\[^{2+}\] transients recorded in current clamped littermate control (LMC) and LQT2 myocytes undergoing repetitive stimulation at 0.25 Hz in the presence of 50 nmol/L ISO. Application of ryanodine receptor sensitizer caffeine (250 μmol/L) prolongs action potential duration (APD) and Ca\[^{2+}\] transient and promotes EADs in LMC cells, whereas preincubation of LQT2 myocytes with CaMKII inhibitor attenuates proarrhythmic Ca\[^{2+}\] mishandling. B to G, Bar graphs present pooled data for APD (B), incidence of EADs (C), duration of AP plateau from +10 to −50 mV (D), amplitude (E), time to peak (F), and time of decay of Ca\[^{2+}\] transients to 25% of peak amplitude (G). **P<0.05 vs LMC and LQT2, respectively, unpaired Student t test (n=4–18).

5–10 minutes.) increased amplitude of and most importantly shortened Ca\[^{2+}\] transient, which resulted in shortening of AP and elimination of EADs (Figure 6). Collectively, these results provide a strong support for the critical role of RyR phosphorylation by CaMKII in shortened refractoriness of SR Ca\[^{2+}\] release, which shapes the AP waveform rendering LQT2 myocytes prone to generation of arrhythmogenic EADs.

**Causal Link Between RyR Hyperactivity and Triggered Activity at the Whole Cell Level**

Our computer modeling studies of LQT2 and LMC myocytes recapitulate the main experimental findings. Furthermore, by dissecting the contributions of individual Ca\[^{2+}\]-dependent sarcolemmal currents, they shed light on the ionic mechanisms by which RyR hyperactivity causes AP prolongation and EAD colemmal currents, they shed light on the ionic mechanisms by which RyR hyperactivity causes AP prolongation and EADs. The Ca\[^{2+}\] transient amplitude is seen to be decreased by stimulation with 500 μmol/L ISO. Application of ryanodine receptor sensitizer caffeine (250 μmol/L) prolongs action potential duration (APD) and Ca\[^{2+}\] transient and promotes EADs in LMC cells, whereas preincubation of LQT2 myocytes with CaMKII inhibitor attenuates proarrhythmic Ca\[^{2+}\] mishandling. The net effect of numerous late Ca\[^{2+}\] releases with smaller fluxes is a longer time to peak of the Ca\[^{2+}\] transient for hyperactive (+60 ms) compared with stabilized (+40 ms) RyRs as well as a slower decay rate of the Ca\[^{2+}\] transient in the hyperactive case. Those results are in good overall agreement with experimental observations reported in Figure 6, which show a longer time to peak and slower decay of the Ca\[^{2+}\] transient in LQT2 myocytes under the influence of isoproterenol compared with LQT2 myocytes also under the influence of isoproterenol but with RyR hyperactivity stabilized by KN93. Figure 7F to 7I characterizes the effects of RyR hyperactivity on the V\(_{\text{m}}\) dynamics (Figure 7F) and Ca\[^{2+}\]-dependent sarcolemmal currents (Figure 7G–7I) and highlights its causal link with triggered activity. As seen in the confocal line scans, late SR Ca\[^{2+}\] release persist in a much larger number during the plateau of the AP for hyperactive (Figure 7E) than for stabilized RyRs (Figure 7D). This increased number of aberrant releases significantly increases the subsarcolemmal Ca\[^{2+}\] concentration, despite a reduction of SR load, thereby increasing the magnitude of the forward mode depolarizing NCX1 current (Figure 7H) during a critical time interval (between ≈200 and 300 ms after depolarization) when V\(_{\text{m}}\) enters the window for reactivation of LTCCs. The increased depolarizing NCX1 current for hyperactive RyRs slows down repolarization during this time shown in Figure 7C). RyR hyperactivity shortens the refractoriness of Ca\[^{2+}\] release units, resulting in a significantly increased spark frequency and reduced interspark interval during the AP plateau as seen in the confocal linescan equivalents (Figure 7D and 7E). The net effect of numerous late Ca\[^{2+}\] releases with smaller fluxes is a longer time to peak of the Ca\[^{2+}\] transient for hyperactive (+60 ms) compared with stabilized (+40 ms) RyRs as well as a slower decay rate of the Ca\[^{2+}\] transient in the hyperactive case. Those results are in good overall agreement with experimental observations reported in Figure 6, which show a longer time to peak and slower decay of the Ca\[^{2+}\] transient in LQT2 myocytes under the influence of isoproterenol compared with LQT2 myocytes also under the influence of isoproterenol but with RyR hyperactivity stabilized by KN93. Figure 7F to 7I characterizes the effects of RyR hyperactivity on the V\(_{\text{m}}\) dynamics (Figure 7F) and Ca\[^{2+}\]-dependent sarcolemmal currents (Figure 7G–7I) and highlights its causal link with triggered activity. As seen in the confocal line scans, late SR Ca\[^{2+}\] release persist in a much larger number during the plateau of the AP for hyperactive (Figure 7E) than for stabilized RyRs (Figure 7D). This increased number of aberrant releases significantly increases the subsarcolemmal Ca\[^{2+}\] concentration, despite a reduction of SR load, thereby increasing the magnitude of the forward mode depolarizing NCX1 current (Figure 7H) during a critical time interval (between ≈200 and 300 ms after depolarization) when V\(_{\text{m}}\) enters the window for reactivation of LTCCs. The increased depolarizing NCX1 current for hyperactive RyRs slows down repolarization during this time...
interval, thereby allowing sufficient time for LTCC reactivation. In contrast, for stabilized RyRs, the smaller NCX1 depolarizing current leads to a faster rate of repolarization that prevents reactivation of LTCCs. Importantly, statistical analysis of individual channel states at the whole cell level reveals that LTCCs for stabilized and hyperactive RyRs are equally inactivated (ie, an almost equal fraction of channels is in an inactivated state) before entering the window. This shows that the slower repolarization rate that promotes EAD formation for hyperactive RyRs is predominantly caused by the increased forward mode NCX1 current because of increased frequency of late Ca\(^{2+}\) releases and not by differences in Ca\(^{2+}\)-dependent inactivation of LTCCs.

Analogous results to Figure 7 are shown in the Online Figure IV for LMC myocytes under the effect of \(\beta\)-adrenergic stimulation. Addition of low-dose caffeine, which makes RyRs hyperactive, is shown to induce aberrant late Ca\(^{2+}\) releases that increase the depolarizing NCX1 current thereby slowing repolarization and allowing more time for reactivation of LTCCs within the window. By varying the degree of RyR hyperactivity in the computer model, we have found additionally that EAD formation requires a smaller shift of the RyR open probability toward lower cytosolic Ca\(^{2+}\) concentration because of increased frequency of late Ca\(^{2+}\) releases and not by differences in Ca\(^{2+}\)-dependent inactivation of LTCCs.

Discussion

Rapidly accumulating evidence suggests that clinical manifestations of arrhythmia in congenital arrhythmia syndromes may involve additional factors beyond causative single autosomal dominant mutations in genes encoding ion channels or accessory regulatory proteins.28-30 In the present report, using clinically relevant transgenic rabbit model of LQT2, we provide the first evidence for hereditary LQTS-induced adaptive remodeling of intracellular Ca\(^{2+}\) homeostasis. Our findings implicate hyperactive RyR as a key contributor to arrhythmogenesis in LQT2. Furthermore, we identified LQTS-related loss of phosphatases scaffolded to RyR as an underlying molecular basis for enhanced RyR phosphorylation and thereby increased activity.

Heart muscle possesses a substantial potential for plasticity to preserve its ability to provide sufficient circulation to meet metabolic demands of the body even under conditions of permanent stress like hypertension or postinfarct arhythmia. Likewise, patients affected by mutations linked to prolongation of QT interval do not exhibit profound changes in cardiac function under basal conditions indicative of adaptive remodeling.1-3 At the single cell level acute pharmacological inhibition of K\(^{+}\) channels resulting in prolongation of AP is known to lead to intracellular Ca\(^{2+}\) overload, which one would expect to result in substantial changes in mechanical properties of the whole heart.4 We demonstrate that in ventricular myocytes isolated from congenital LQT2 hearts cellular Ca\(^{2+}\) overload is not present and Ca\(^{2+}\) transients are in fact smaller than in controls (Figure 1) despite dramatic prolongation of AP. Given the lack of changes in LTCC and NCX1 function along with upregulated SERCA, our results implicate hyperactive RyR as a critical determinant of diminished dependence of systolic Ca\(^{2+}\) release on APD in LQT2 myocytes under basal conditions (Figures 1-3; Online Figure I). In addition, detected increase in RyR-mediated SR Ca\(^{2+}\) diastolic leak, which is expected to interfere with relaxation, provides mechanistic insight into recently discovered diastolic dysfunction in LQT2.31

Our present work demonstrates that phosphorylation of RyR in LQT2 is increased at both its PKA and CaMKII sites and this increase is associated with a loss of the resident phosphatases PP1 and PP2A from the RyR multimolecular complex (Figures 4 and 5). Interestingly, qualitatively similar results were obtained in rabbit and canine models of heart failure and in humans.16,22,27 Collectively, these findings imply that RyR hyperphosphorylation and subsequently increased function
because of the loss of local phosphatase activity is a common mechanism, which serves as a protection from cellular Ca\textsuperscript{2+} overload in broad range of cardiac diseases including hereditary LQTS. The redistribution of localized phosphatase activity can be attributed to disease-related changes in expression patterns of regulatory subunits of phosphatases that scaffold catalytic subunits to specific substrates. In LQT2 it seems to be the case for PP1. In LQT2 rabbit hearts we observed that spinophilin, a regulatory subunit that tethers PP1c to RyR,\textsuperscript{25} tends to decrease. Furthermore, we observed a robust (300\%) increase in PPP1R3A (Online Figure IXA and IXB), which targets PP1 to glycogen.\textsuperscript{32} PP1 has been established as a key regulator of glycogen metabolism via regulation of activities of glycogen synthase and phosphorylase.\textsuperscript{32} Our recent proteomics study showed the latter being upregulated in LQT2 rabbit hearts along with adaptive upregulation of multiple energy-producing enzymes.\textsuperscript{33} It is plausible that increased metabolic demand because of prolonged APD can lead to intracellular redistribution of PP1 to reinforce control of energy production. On the contrary, Western blot analysis of possible changes in the expression levels of regulatory subunits of PP2A in LQT2 provided negative results (Online Figure IXA and IXB), suggesting the existence of alternative mechanisms responsible for the reduction of RyR-bound PP2Ac. Recent in-depth study of changes in PP2Ac complexes in several cardiac disease models accompanied by prolonged APD demonstrated that the expression patterns of PP2A regulatory subunit differ greatly depending on the specific model.\textsuperscript{14} Nevertheless, the authors uncover an important common phenomenon in human ischemic heart failure, human nonischemic heart failure, and canine heart failure induced by rapid pacing. In all 3 models the authors report prolongation with aberrant regulation of phosphatases in the heart. Increase in phosphorylation of phospholamban in disease settings is a less common observation than increased phosphorylation of RyR. In contrary to our findings in LQT2 rabbits, phospholamban was shown to be hypophosphorylated in human and several animal models of HF.\textsuperscript{20} Increased phosphorylation of phospholamban was earlier reported in rabbit model of HF\textsuperscript{22} and more recently in rabbit bradycardia-evoked acquired LQTS caused by chronic A-V ablation,\textsuperscript{36} which was accompanied by an increase in Ca\textsuperscript{2+} content and amplitude of Ca\textsuperscript{2+} transients, suggesting that in this model SR Ca\textsuperscript{2+} leak–uptake relationship is shifted toward uptake, in contrast to congenital LQT2. Although accelerated loss of SR Ca\textsuperscript{2+} during diastole via hyperactive RyRs may play some positive role at basal conditions, in conditions of stress it is invariably known to produce detrimental proarrhythmic effect. Under β-adrenergic stimulation, when LTCC and SERCA functions are increased, further amplified spontaneous Ca\textsuperscript{2+} release evokes arrhythmogenic oscillations of membrane potential via electrogenic NCX1 (EADs and delayed after depolarizations). We demonstrate that in the presence of β-adrenergic agonist isoproterenol cells derived from LQT2 hearts exhibit EADs and prolonged AP plateau associated with sustained tail component of Ca\textsuperscript{2+} transient (Figure 6). Recent modeling and experimental works provided ample evidence that formation of EADs involves a complex chain of events in addition to reduced repolarization, which include window Ca\textsuperscript{2+} current and depolarizing I\textsubscript{NCX} driven by SR Ca\textsuperscript{2+} release through untimely reactivated RyRs.\textsuperscript{6,11,13} Similarity between LQT2 myocytes and healthy myocytes treated with RyR activator caffeine strongly supports the key role of hyperactive nonrefractory RyR in increased arrhythmogenic potential in LQT2. Furthermore, we show that CaMKII inhibition effectively eliminates tail of Ca\textsuperscript{2+} transient, shortening APD and abolishing EADs in LQT2 myocytes. Taken together, our data indicate that reduction in CaMKII-dependent phosphorylation of RyR as a way to stabilize channel activity is sufficient to interrupt malignant cross-talk cycling between hyper-responsive RyRs and prolonged depolarization.

Previous computer modeling studies have elucidated many important ionic mechanisms of EAD formation.\textsuperscript{10–13} However, those studies have focused primarily on alterations of sarcoplasmic membrane currents. Furthermore, they have been performed with so-called common pool models,\textsuperscript{37,38} which are models where both the trigger Ca\textsuperscript{2+} flux through LTCCs and the release flux through RyRs feed into the same (whole cell) compartment, and Ca\textsuperscript{2+} cycling is described by a deterministic set of equations. Here, in contrast, we have used a multiscale ventricular myocyte model to directly probe how alterations of stochastic RyR activity at the single channel level affect the Ca\textsuperscript{2+} and voltage dynamics at the whole cell level. This model has allowed us to explain the present paradoxical experimental observation that RyR hyperactivity promotes EAD formation while reducing the Ca\textsuperscript{2+} transient amplitude. The apparent paradox stems from the fact that a smaller Ca\textsuperscript{2+} transient amplitude has been traditionally associated with a smaller forward mode NCX1 depolarizing current during phase 2 of the AP, which shortens the AP and tends to suppress EADs as predicted in a computer modeling study of EAD formation in pharmacological LQT2.\textsuperscript{12} The present modeling results (Figure 7; Online Figure IV) show that the NCX1 current is indeed less depolarizing with hyperactive than with stable RyRs early in phase 2 when the cytosolic Ca\textsuperscript{2+} concentration peaks. However, the NCX1 current becomes more depolarizing in the hyperactive case later in phase 2, within a critical interval of time during which the voltage traverses the window for voltage-dependent reactivation of LTCCs. Hence, the present study associates EAD formation with a smaller, as opposed to a larger, Ca\textsuperscript{2+} transient amplitude. In addition, the mechanism of triggered activity we present explains the presence of EADs that persist after I\textsubscript{Ca} and I\textsubscript{Ks} are maximally potentiated by β-adrenergic stimulation. Hence, those EADs do not depend on the different rates of increases of I\textsubscript{Ca} and I\textsubscript{Ks} after isoproterenol that has been invoked previously as a mechanism of transient EADs.\textsuperscript{7,13}

The experimental results of Figure 6 are in good overall agreement with the computer modeling results of Figure 7 for LQT2 and the Online Figure IV for LMC. Both sets of results demonstrate that the combination of RyR hyperactivity, either resulting from adaptation in LQT2 or induced by caffeine in LMC, and β-adrenergic stimulation promotes EADs despite...
a 2-fold reduction of Ca\textsuperscript{2+} transient amplitude. Furthermore, imaging of Ca\textsuperscript{2+} activity in both simulations and experiments reveals that the genesis of EADs is accompanied by similar patterns of asynchronous late Ca\textsuperscript{2+} releases during the AP plateau, which are explicitly shown by modeling to slow repolarization during a vulnerable time window for LTCC reactivation. Importantly, those releases occur in a situation where the SR load is significantly decreased. This is in contrast to spontaneously generated Ca\textsuperscript{2+} waves that have been found to promote EADs under the combined influence of Ca\textsuperscript{2+} overload and α-adrenergic stimulation.\textsuperscript{39} Ca\textsuperscript{2+} waves have also been traditionally invoked as a mechanism of delayed afterdepolarizations.\textsuperscript{84,81} Hence, in this broader context of Ca\textsuperscript{2+}-triggered arrhythmias, a major novel finding of the present study is that abnormal Ca\textsuperscript{2+} handling can promote EAD formation under a condition where the SR load is dramatically reduced and aberrant Ca\textsuperscript{2+} releases are predominantly triggered by Ca\textsuperscript{2+} influx through LTCCs.

Conclusions

Our study shows that reduced refractoriness of RyR in LQT2 contributes to prolonged maintenance of AP plateau at the voltage range critical for reactivation of LTCCs and generation of EADs. Targeting of hyperactive RyR hyperphosphorylation because of dissociation of phosphatases from the SR Ca\textsuperscript{2+} channel complex may present a rational strategy for treating hereditary arrhythmias associated with QT prolongation.

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Disclosures

None.

References

Using an advanced computer model, we show that the experimentally observed reduction in refractoriness of sarcoplasmic reticulum Ca\(^{2+}\) release plays a key role in providing an ideal electrophysiological milieu for generation of early after depolarizations by facilitating the reopening of L-type calcium channels.

The proarrhythmic increase in RyR activity is attributable to enhanced RyR phosphorylation secondary to dissociation of protein phosphatases 1 and 2 from the RyR macromolecular complex.

Although long QT syndrome is an electric disorder, potential mal-adaptive proarrhythmic changes in intracellular Ca\(^{2+}\) handling are considered an important unresolved question in pathogenesis of malignant arrhythmias in long QT syndrome. In this study, we characterize abnormalities in intracellular Ca\(^{2+}\) homeostasis in a large animal model of hereditary long QT syndrome type 2 and demonstrate a key role of these abnormalities in arrhythmogenesis. Specifically, we found that RyR activity in LQT2 is abnormally high because of increased phosphorylation of the channel. The reduction in refractoriness of RyR-mediated sarcoplasmic reticulum Ca\(^{2+}\) release prolongs the repolarization phase of action potential, thereby promoting the generation of early after depolarizations in LQT2. Targeting of hyperactive, hyperphosphorylated RyRs may present a novel therapeutic strategy for hereditary arrhythmias associated with QT prolongation.
Hyperphosphorylation of RyRs Underlies Triggered Activity in Transgenic Rabbit Model of LQT2 Syndrome

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Supplemental Material

Methods

Cell Isolation

All procedures were approved by The Rhode Island Hospital Institutional Animal Care and Use Committee and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Ventricular myocytes were isolated from the hearts of transgenic LQT2 (n=10) and LMC (n=10) NZW rabbits of either sex (3.5–5.5 kg) using standard enzymatic digestion procedures. Briefly, the heart was removed from euthanized rabbits and perfused for 5 to 7 minutes with a nominally Ca\(^{2+}\)-free solution containing (in mmol/L): 140 NaCl, 4.4 KCl, 1.5 MgCl\(_2\), 0.33 NaH\(_2\)PO\(_4\), 16 taurine, 5 HEPES, 5 pyruvic acid, and 7.5 glucose. Subsequently, the heart was perfused for 10 to 15 minutes with the same solution to which 0.65% collagenase type II (Worthington Biochemical), and 0.1% BSA were added. The LV was cut off and minced, and the cells were dispersed with a glass pipette for 3 to 5 minutes in a solution containing (in mmol/L): 45 KCl, 65 K-glutamate, 3 MgSO\(_4\), 15 KH\(_2\)PO\(_4\), 16 taurine, 10 HEPES, 0.5 EGTA, and 10 glucose and 1% BSA (pH 7.3). The cell suspension was filtered through a 100-μm nylon mesh, and plated on laminin coated coverslips in medium M199, and used within 6 to 8 hours.

Cell Electrophysiology and Ca\(^{2+}\) Imaging

Calcium Currents and Action potentials (APs) were recorded using the whole-cell patch clamp technique at 35±2°C. The patch-clamp system was based on Axopatch 200B amplifier and Digidata 1322A interface (Axon Instruments, CA). The external solution consisted of (mmol/L): 140 NaCl, 5.4 KCl, 1.85 CaCl\(_2\), 0.5 MgCl\(_2\), 10 HEPES, and 5.6 glucose (pH 7.4). Patch pipettes were filled with the following solution (mmol/L): 90 K-aspartate, 50 KCl, 5 MgATP, 5 NaCl, 1 MgCl\(_2\), 0.1 Tris GTP, 10 HEPES, and 0.1 Rhod-2 (Molecular Probes, OR) (pH 7.2). For calcium currents recording membrane potential was held at -50 mV and voltage steps of 400 ms were applied in 10 s intervals in 10 mV increment between -40 and +40 mV. The APs were evoked by application of 3-ms-long voltage pulses with amplitude 20% above the threshold level. Intracellular Ca\(^{2+}\) imaging was performed using Leica SP5 confocal system in a line scan mode. Fluo-3 and Fluo-5N was excited by the 488 nm line of an argon-ion laser, and the fluorescence was acquired at 500-530 nm wavelengths. Rhod-2 was excited by 543 nm laser and the fluorescence was acquired at 590–690 nm wavelengths. Calcium transients were recorded at 35±2°C in intact cells loaded with Fluo-3-AM using Grass field stimulator. Calcium sparks were studied using Fluo-3 and SR-calcium uptake and leak were studied using Fluo-5N-AM in saponin-permeabilized myocytes at room t°(Life Technologies, Grand Island, NY) and the following intracellular solution: (mmol/L) 120 potassium aspartate, 20 KCl, 3 MgATP, 0.81 MgCl\(_2\), 10 phosphocreatine, 5 U ml\(^{-1}\) creatine phosphokinase, 0.5 EGTA (pCa 7) and 20 HEPES (pH 7.2). Calcium transients, Ca\(^{2+}\) sparks, and SR Ca\(^{2+}\) leak and uptake were analyzed using Leica Software, Origin 8.2 (OriginLab, Northampton,MA) and Image J (NIH, Bethesda, MA)

Western Blotting and Immunoprecipitation

Western blot analyses were performed as previously described (1) using RIPA buffer supplemented with phosphatase, calpain and protease inhibitors (Sigma, St. Louis MO). The levels of proteins involved in Ca\(^{2+}\) handling and their phosphorylation were assessed by immunoblot analysis using 40 μg of homogenates from left ventricular cell samples as described
previously. Primary antibodies used were: anti-RyR2, anti-Cav1.2α1c, anti-CaMKII and anti-CaMKII-phospho-Ser 286 from ThermoScientific (Waltham, MA), anti-SERCA2a from Sigma-Aldrich (St.Louis, MO), anti-phospholamban (PLB) and anti-PP1 catalytic subunit from Upstate (Lake Placid, NY), anti-phospho-PLB-T17 and anti-PPP2R5A from Santa Cruz (Dallas, TX); anti-phospho-PLB-S16, anti-Na+/Ca2+ exchanger(NCX1) and anti-PPP1R9B (spinophilin) from Millipore (Billerica, MA); anti-PP2A catalytic subunit from BD Biosci (San Jose, CA). Custom-made rabbit anti-phospho-RyR2-S2809 and anti-phospho-RyR2-S2815 were from Phosphosolutions (Aurora, CO); anti-PPP1R3A, anti-PPP2R3A, anti-PPP2R5D, anti-PPP2RE, anti-PP2A-alpha (phospho Y307), anti-PP2A-alpha (methyl L309) and anti-GAPDH antibody were from Abcam (Cambridge, MA). Phosphospecific anti-RyR pSer-2809 (PKA site) and anti RyR pSer-2815 (CaMKII site) were validated using tissue samples from S2808A and S2808A/S2815A double mutant mice (Online Fig. VIII) kindly provided by Dr. Hector Valdivia (Univ. of Michigan, Ann Arbor, MI). Expression levels of RyR, SERCA, PLB, Cav1.2α1c, PP1c, PP2Ac, NCX1, and regulatory subunits of phosphatases PP1 and PP2A were assessed after normalization to the loading control, GAPDH. Phosphorylation levels of RyR and PLB were analyzed following normalization to RyR or PLB protein levels assessed from gels run in parallel and presented as a percentile of maximum phosphorylation achieved by incubation of cell samples with 1 μmol/L β-adrenergic agonist Isoproterenol and 1 μmol/L phosphatase inhibitor Calyculin A for 15 min. For co-immunoprecipitation cell or tissue samples were incubated with protein A Sepharose beads at 4°C overnight, after which the beads were washed three times with buffer. Protein bands were visualized using the Super Signal West Pico kit (Pierce, IL) and quantified using ImageJ (National Institutes of Health) and Origin 8 (OriginLab, Northampton, MA) software.

Phosphatase and Kinase Activity Assays

Protein Phosphatase 2A and 1 activities was determined in LV and septum ventricular myocytes using the serine/threonine phosphatase assay system microplate assay kit (Promega, Madison, WI), which determines the amount of free phosphate generated in a reaction by measuring the absorbance of a molybedate: malachite green: phosphate complex. Appropriate phosphate standards were diluted in phosphate-free water, 5× PPase reaction mix and 1 mmol/L phosphopeptide were added to a flat-bottom 96-well plate (total volume 50 μL). Reaction was initiated by adding protein sample (15–30 μg) following incubation at room temperature for 15–20 min. The reaction was stopped by adding equal volume (50 μl) of Molybedate dye, followed by incubation for 15–30 min at room temperature. Optical density of the molybedate: malachite green: phosphate complex was read using a plate reader with a 630 nm filter. Ca2+/calmodulin dependent and independent CaMKII kinase activities were determined in LV tissue samples using method reported by Abraham et al.(2). Briefly, The Ca2+/Calmodulin-dependent kinase II activity in the lysates was determined in 25 μl containing (in mmol/L) 10 MOPS, pH 7.4, 10 MgCl2, 3 EGTA, 4 CaCl2 chloride, 0.0004 calmodulin, 0.2 [γ-32p] ATP (500 -1000 cpm/pmol/L), 0.025 autocamtide-2 peptide as substrate, and 15-20 μg of total lysate protein. In order to determine the Ca2+/CaM-independent kinase II activity in the same lysates, CaCl2 and Calmodulin were omitted from the assay mixture. The reaction was carried out at 30°C for 5 min. The reaction was terminated by precipitation of the phosphorylated peptide on P-81 phospho cellulose paper (Millipore) discs. The paper discs were washed thoroughly (4 x 5 min) in 75 mmol/L phosphoric acid, and the radioactivity was determine by liquid scintillation counter. The kinase activity was expressed as pico moles of phosphate incorporated into the substrate /min/mg of lysate protein.

Statistical Analysis
Data are presented as Mean±S.E.M. Statistical significance was evaluated using either Student's paired or unpaired t test. The proportion of cells displaying EADs was compared using Fisher's exact test. A p value of <0.05 was considered significant.

Computer Modeling

We use a physiologically detailed ventricular myocyte model of Ca$^{2+}$ cycling coupled to membrane voltage ($V_m$) dynamics (3,4) to investigate the ionic mechanisms of EAD formation and elimination in LQT2 myocytes under different conditions corresponding to the experiments. This multi-scale model bridges the submicron scale of individual Ca$^{2+}$ release units (CRUs) and the whole cell by simulating a realistically large number of 20,000 diffusively-coupled CRUs spatially distributed throughout the cell, with 4 LTCCs collocated with 100 RyRs in each CRU. It accounts for the stochastic nature of both the trigger and release Ca$^{2+}$ fluxes by using a Markov description of channel kinetics for both LTCCs and RyRs. In addition, it describes the bi-directional coupling of Ca$^{2+}$ and $V_m$ dynamics by incorporation of a full set of sarcolemmal currents including $I_{\text{Ca,L}}$, $I_{\text{NCX}}$, $I_{\text{Ks}}$, $I_{\text{to,t}}$, $I_{\text{K1}}$, and $I_{\text{NaK}}$, and also including (excluding) $I_{\text{Kr}}$ for LMC (LQT2) myocytes. This model has therefore the unique capability to explore the effects of alteration of RyR activity at the single channel level on EAD formation at the whole cell level.

We have implemented several improvements of the original model of Restrepo et al. (3,4) that include a higher spatial resolution of the diffusive coupling between CRUs, a more quantitative description of Ca$^{2+}$ buffers (including time-dependent binding and inclusion of sarcolemmal buffers in the dyadic cleft), and a 16 state Markov model of LTCCs. This model reduces at the whole cell level to a Hodgkin-Huxley formulation with voltage- and calcium-dependent inactivation of $I_{\text{Ca,L}}$. This Markov model of LTCCs provides more flexibility for fitting whole cell experimental measurements of $I_{\text{Ca,L}}$ including a larger window current area. We have also modified the calcium-dependent activation on the NCX current to be time dependent.

To model the effect of caffeine on LMC myocytes, we increase the Ca$^{2+}$-dependent rate of transition of RyR channels from closed to open states. This causes the open probability of RyRs as a function of local Ca$^{2+}$ in the dyadic cleft to shift towards a lower Ca$^{2+}$ concentration. In addition to increasing RyR open probability, we also model hyperactivity by shortening RyR refractoriness.

To model LQT2 myocytes, we exclude $I_{\text{Kr}}$ from the list of sarcolemmal currents and make additional changes to take into account the remodeling of Ca$^{2+}$ cycling proteins inferred from experimental measurements. Firstly, we increase the rate of SERCA uptake without ISO by 25% to reproduce the more rapid rate of Ca$^{2+}$ uptake in LQT2 myocytes compared to LMC. Secondly, we model RyR hyperactivity in a similar fashion as the effect of caffeine in LMC myocytes, by both shifting the open probability of RyRs towards lower Ca$^{2+}$ and shortening RyR refractoriness. The increase in open probability is consistent with lipid bilayer studies of hyperphosphorylated RyRs (5) and the observed increased frequency of Ca$^{2+}$ sparks in permeabilized LQT2 myocytes. Without β-adrenergic stimulation, the AP duration of LQT2 myocytes was increased by 40% compared to LMC myocytes consistent with experimental observations.

To model the effect of KN93 on LQT2 myocytes, we have deliberately chosen to only include the stabilizing effect of CaMKII inhibition on RyR activity. While KN93 may potentially influence other sarcolemmal currents, we are primarily interested in testing the hypothesis that RyR stabilization alone suffices to eliminate EADs in the setting of AP prolongation. We therefore model the effect of KN93 by simulating LQT2 myocytes with normal RyR gating. Hence those
myocytes only differ from LMC myocytes by the removal of \( I_{Kr} \). We refer to those model myocytes as LQT2 myocytes with "stabilized RyRs" to highlight that they only differ from LQT2 myocytes by RyR stabilization.

To model the effect of \( \beta \)-adrenergic stimulation with isoproterenol (ISO), we use a fit to experimental data that results in an increase in the SERCA uptake rate as well as increases in \( I_{Ca,L} \) and \( I_{Ks} \) currents. We use the same SERCA uptake rate for both LMC and LQT2 myocytes with ISO. We model the effect of \( \beta \)-adrenergic stimulation under steady-state conditions since we are interested in modeling non-transient EADs that persist on an experimental time scale longer than the time scales of ISO-induced \( I_{Ca,L} \) and \( I_{Ks} \) phosphorylation kinetics.

Computer simulations were carried out by pacing myocytes at 0.25 Hz until a steady state was reached. We recorded during pacing whole cell cytosolic and SR Ca\(^{2+}\) concentrations denoted by \([\text{Ca}]_i\) and \([\text{Ca}]_{SR}\), respectively, \(V_m\), and individual sarcolemmal currents. In addition, experimental confocal linescans were emulated by recording the local cytosolic calcium concentration along a longitudinal row of CRUs passing through the center of the myocyte and parallel to its long axis.

The pacing protocol was applied to LMC and LQT2 myocytes under \( \beta \)-adrenergic stimulation by ISO for the following four cases: (i) LQT2 with hyperactive RyRs, (ii) LQT2 with stabilized RyRs, (iii) LMC, (iv) LMC with Caff. The results of simulations of (i) and (ii) are compared in Figure 7 of the main text. The results of (iii) and (iv) are compared in Online Figure IV of this supplement.

We list below all the changes to the model of Restrepo et al (3,4). We use the same notation for all variables unless otherwise specified.

**Cytosolic calcium buffers:**

We modify the cytosolic calcium buffers to be more physiologically accurate. Buffers are now realistically spatially distributed, with sarcolemmal buffers existing only in the proximal and submembrane compartments. Buffering rates and concentrations are as in the Shannon et al model (9).

All buffers are modeled in a time-dependent manner, except in the proximal volume, in which we use the rapid buffering approximation (10). Because of the importance of buffers within the proximal volume, we no longer adiabatically equilibrate the proximal calcium concentration, it is now governed by the equation

\[
\dot{c}_p = \beta_p(c_p) \left( I_r + I_{Ca} + \frac{c_s - c_p}{\tau_{ps}} + \frac{c_{i,1} + c_{i,2} - 2c_p}{2\tau_{pi}} \right)
\]

\[
\beta_p(c_p) = \left[ 1 + \frac{B_{SL}K_{SL}}{(c_p + K_{SL})^2} + \frac{B_{SLH}K_{SLH}}{(c_p + K_{SLH})^2} \right]^{-1}
\]

**Cell architecture**

We use a finer grid spacing in order to increase the spatial resolution of the diffusive coupling between CRUs. More specifically, the cytosol is divided in equal size compartments with CRUs spaced two compartments apart along x, y and z. As a result, the number of cytosolic and NSR compartments is increased by a factor of 8 (i.e. there is now one CRU for every 8 compartments as shown in Online Figure V A).

The new time constants of diffusion between adjacent volumes are given below. To be more physiologically accurate, we now only allow submembrane diffusion parallel to the t-tubules.
within the z-plane. We make the simplifying assumption that all t-tubules run along a single transverse direction (Online Figure V B). Because we only have one proximal compartment and one submembrane compartment per CRU, we diffusively couple each compartment to only 2 cytosolic compartments (Online Figure V A,C).

Since the repeating building block of the cell architecture consists of 8 cytosolic compartments, we use the labels i,j,k to denote the position of each building block on a cubic lattice with the superscript indices i,j and k running along x,y and z, respectively, and the subscript index n=1, 2,...8 to locate the cytosolic compartments within each building block. The cytosolic concentration in each compartment is labeled accordingly as \( c_{i,j,k}^{i,j,k} \). A single building block is depicted schematically in Online Figure V A where we have omitted the superscript i,j,k on the labels of the concentration in each compartment. The new calcium cycling is given by

\[
\begin{align*}
\dot{c}_{i,n}^{i,j,k} &= I_{\text{leak},n}^{i,j,k} - I_{\text{up},n}^{i,j,k} - I_{\text{buff},n}^{i,j,k} + I_{d_{si,n}}^{i,j,k} \frac{v_s}{v_i} + I_{d_{pi,n}}^{i,j,k} \frac{v_p}{v_i} + I_{c_{i,n}}^{i,j,k} \\
\dot{c}_s^{i,j,k} &= I_{d_{ps}}^{i,j,k} \frac{v_p}{v_s} + I_{\text{NCX}}^{i,j,k} + I_{c_s}^{i,j,k} - I_{d_{si,1}}^{i,j,k} - I_{d_{si,2}}^{i,j,k} 
\end{align*}
\]

We incorporate a new diffusive current between the cytosolic and proximal compartments, \( l_{dpi} \), to distribute the \( \text{Ca}^{2+} \) flux from the dyadic cleft into both the submembrane and cytosolic compartments. This new current is given by

\[
I_{dpi,n}^{i,j,k} = \begin{cases} 
\frac{c_p^{i,j,k} - c_{i,n}^{i,j,k}}{2\tau_{dpi}}, & n = 1, 2 \\
0, & n \geq 3 
\end{cases}
\]

The following are the new equations for the diffusive current between cytosolic and submembrane compartments

\[
I_{d_{si,n}}^{i,j,k} = \begin{cases} 
\frac{c_s^{i,j,k} - c_{i,n}^{i,j,k}}{2\tau_{si}}, & n = 1, 2 \\
0, & n \geq 3 
\end{cases}
\]

between submembrane and proximal compartments,

\[
I_{d_{ps}}^{i,j,k} = \frac{c_p^{i,j,k} - c_s^{i,j,k}}{\tau_{ps}}
\]

and between nearest neighbor cytosolic compartments
The equations for SR calcium cycling are modified likewise where the new JSR refilling current is given by (8).

\[
I_{\text{JSR}}^{i,j,k} = \beta_{\text{CSQN}} (c_{\text{JSR}}^{i,j,k}) \left( I_{\text{tr},1}^{i,j,k} + I_{\text{tr},2}^{i,j,k} - I_{\text{r}}^{i,j,k} \frac{v_i}{v_{\text{JSR}}} \right)
\]  

\[
I_{\text{NSR},n}^{i,j,k} = (I_{\text{up},n}^{i,j,k} - I_{\text{leak},n}^{i,j,k}) \frac{v_i}{v_{\text{NSR}}} - I_{\text{tr},1}^{i,j,k} \frac{v_{\text{JSR}}}{v_{\text{NSR}}} + I_{\text{r}}^{i,j,k} v_{\text{NSR},n}
\]

where the new JSR refilling current is given by (9).

\[
I_{\text{tr},n}^{i,j,k} = \begin{cases} 
I_{\text{up},n}^{i,j,k} - I_{\text{leak},n}^{i,j,k}, & n = 1, 2 \\
0, & n \geq 3 
\end{cases}
\]

and the nearest neighbor diffusive currents between NSR compartments, \(I_{\text{cNSR},n}^{i,j,k}\), have the same form as the diffusive currents between cytosolic compartments given by Eq. (8). Finally, the diffusive current between submembrane compartments is given by (12).

\[
I_{\text{c}^{i,j,k}} = \left( c_{i,j,k}^{s} + c_{i,j,k}^{s-1} - 2c_{i,j,k}^{s} \right) \frac{\tau_{\text{c}^{i,j,k}}}{\tau_{\text{c}^{i,j,k}}}
\]

**Online Table I: Diffusive Timescales**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Value (ms)</th>
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<tbody>
<tr>
<td>(\tau_{\text{c}})</td>
<td>Transverse cytosolic</td>
<td>0.66</td>
</tr>
<tr>
<td>(\tau_{\text{c}})</td>
<td>Longitudinal cytosolic</td>
<td>1.4</td>
</tr>
<tr>
<td>(\tau_{\text{NSR}})</td>
<td>Transverse NSR</td>
<td>1.8</td>
</tr>
<tr>
<td>(\tau_{\text{NSR}})</td>
<td>Longitudinal NSR</td>
<td>6</td>
</tr>
</tbody>
</table>
Time-dependent calcium activation of $I_{Ks}$

We modified the calcium-dependent activation of $I_{Ks}$ to have the form

$$\frac{dQ_{Ks}}{dt} = \frac{(Q_{Ks,\infty} - Q_{Ks})}{\tau_{Q_{Ks}}}$$

(13)

$$Q_{Ks,\infty} = 0.2 \left( 1 + 0.8/\left( 1 + \left( \frac{0.28}{C_s} \right)^3 \right) \right)$$

(14)

where the time constant of activation is $\tau = 1$ s.

16-state Markov model of $I_{Ca,L}$

The open probability of the L-type Ca channels (LTCCs) is governed by the 16-state Markov model illustrated in Online Figure VI. The 16 states represent combinations of 4 independent binary gating mechanisms: 2 transitions modeling activation, one modeling voltage-dependent inactivation, and one modeling calcium-dependent activation. There are 4 states of activation O, C, C', and CC'. The voltage-dependent component of activation represents transitions between O and C (or C' and CC') with rates given by $\alpha_d$ and $\beta_d$. The voltage-independent component of activation represents transitions between O and C' (or C and CC') with rates given by $r_1$ and $r_2$. For each state of activation, an LTCC can additionally be in a voltage-inactivated state ($IV$), a calcium-inactivated state ($IC$) or both ($IVIC$). The rates of transitions to and from voltage inactivated states are given by $\alpha_f$ and $\beta_f$ respectively, and the rates of transitions to and from calcium inactivated states are given by $\alpha_{fc}$ and $\beta_{fc}$ respectively.

This model can be reduced to a simple 3-gate Hodgkin-Huxley form

$$P_O = C d f f_c$$

(15)

with a constant prefactor $C = r_1/(r_1+r_2)$. d and f are standard gating parameters for voltage-dependent activation and inactivation respectively. The fraction of non-calcium-inactivated channels ($f_c$), however, cannot be expressed with a simple mean-field ODE because $c_p$ is in general different in each CRU.

The front-most face of the lower left-hand cube in Online Figure V is the Markov construction of the normal d and f gates and, alone, leads to $P_O=df$. Transitions from any point in this face to the complementary point in the face behind represent Ca$^{2+}$-dependent inactivation, and so the cube as a whole yields $P_O=df f_c$. Transitions from states at the corner of this cube to the complementary states at the corners of the upper right-hand cube represent voltage-independent channel closings, and yield the additional constant prefactor C in Eq. (15).
The window current of LTCC occurs during repolarization of the action potential, typically peaking between 0 and -30 mV. The recovery from inactivation typically has sufficient time to almost reach to steady state during this period, such that the window current is proportional to the product \(d_\infty(V)f_\infty(V)\). Experimental measurements of LTCC under ISO show a wider window current, peaking at a lower voltage, in addition to a larger total current, and longer mean open time of individual channels. By constructing the model with only mutually orthogonal transitions between states we have the freedom to change each gate independently. For instance, the parameters \(d_\infty\) and \(\tau_d\) are chosen to match experimental measurements of voltage-dependence of LTCC activation, while the parameters \(r_1\) and \(r_2\) are chosen to match experimental measurements of peak fraction of open channels (~5%), and mean open lifetime (~0.3 ms). This flexibility also allows us to change the current to more precisely match experimental measurements under ISO. We alter the parameters of \(d_\infty\) and \(f_\infty\) to match the size of the window current under ISO, and decrease \(r_2\) to match measurements of longer open time of single channels and increased peak current.

The rates of transitions between states (in ms\(^{-1}\)) are given by:

\[
\begin{align*}
\alpha_d &= \frac{d_\infty}{\tau_d} \\
\beta_d &= \frac{1 - d_\infty}{\tau_d} \\
\alpha_f &= \frac{f_\infty}{\tau_f} \\
\beta_f &= \frac{1 - f_\infty}{\tau_f} \\
d_\infty &= \frac{1}{1 + \exp\left(-\frac{(V-d_{50})}{8}\right)} \\
\tau_d &= \frac{0.035(V - d_{50})}{1 - \frac{1}{1 + \exp\left(-\frac{(V-d_{50})}{9.1}\right)}}d_\infty \\
f_\infty &= \frac{1}{1 + \exp\left(-\frac{V-60}{12}\right)} \\
\tau_f &= \frac{0.02 - 0.007 \exp\left(-0.0337(V + 10.5)^2\right)}{0.175} \\
\beta_f &= \frac{1}{1 + \frac{25}{c_p}}
\end{align*}
\]

The parameters for LTCC gating are given in Online Table II. The parameters are altered under the effect of ISO to match experimental measurements of increased peak magnitude, longer open duration and larger window current. Representative time traces of \(I_{\text{Ca,L}}\) in response to different step increases of voltage are shown in Online Figure VII with ISO (A) and without ISO (B). (C) shows the results of simulations that mimic the experiments of Puglisi et al. (6). Those experiments investigated the \(\text{Ca}^{2+}\) dependence of LTCC inactivation by recording \(I_{\text{Ca,L}}\) at different beat numbers from a myocyte paced at constant cycle length with an AP clamped waveform after SR depletion by a caffeine pulse. The simulations reproduce the experimental finding that \(I_{\text{Ca,L}}\) becomes more inactivated by \(\text{Ca}^{2+}\) as the SR refills with increasing beat number after depletion. More importantly for the present study, similar traces recording with ISO stimulation (results not shown) demonstrate that the magnitude of \(I_{\text{Ca,L}}\) is ostensibly independent of calcium transient amplitude during the critical time period when \(V_m\) reenter the window for LTCC reactivation, for the range of amplitudes corresponding to LQT2 myocytes with hyperactive and stabilized RyRs.
### Online Table II  LTCC Gating parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$r_1$</td>
<td>0.22 ms$^{-1}$</td>
</tr>
<tr>
<td>$\alpha_{fc}$</td>
<td>0.006 ms$^{-1}$</td>
</tr>
</tbody>
</table>

**Without ISO**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$r_2$</td>
<td>3 ms$^{-1}$</td>
</tr>
<tr>
<td>$d_{50}$</td>
<td>5 mV</td>
</tr>
<tr>
<td>$f_{50}$</td>
<td>-22.8 mV</td>
</tr>
</tbody>
</table>

**With ISO**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$r_2$</td>
<td>2 ms$^{-1}$</td>
</tr>
<tr>
<td>$d_{50}$</td>
<td>0 mV</td>
</tr>
<tr>
<td>$f_{50}$</td>
<td>-28 mV</td>
</tr>
</tbody>
</table>

### Time-dependent calcium activation of NCX

We modify the allosteric calcium activation of NCX based on recent work showing that it is time-dependent (7). We modify the activation prefactor to be time-dependent as follows:

$$\frac{dA_{NCX}}{dt} = \frac{A_{NCX,\infty} - A_{NCX}}{\tau_{NCX}}$$  \hspace{1cm} (25)

$$A_{NCX,\infty} = \frac{1}{1 + \left(\frac{0.3}{c_s}\right)^3}$$  \hspace{1cm} (26)

We use a time constant of activation of $\tau_{NCX} = 150$ ms with the same magnitude of Ca$^{2+}$ background leak from the extracellular medium to the submembrane compartments. Simulations performed with larger values of $\tau_{NCX}$ showed no qualitative difference in the mechanism of EAD as long as $\tau_{NCX}$ was shorter than the pacing cycle length.

### Hyperactive and non-hyperactive gating of RyR

Hyperactivity in both the LMC model and LQT with caffeine model is modeled with an increase in the calcium-dependent rate of individual RyR opening. We model this shift based on lipid bilayers recordings of RyR hyperactivity under the effect of caffeine (8) for the dosage (250 $\mu$mol/L) corresponding to the present experiments, and a smaller dosage (150 $\mu$mol/L) to model the LQT2 hyperactivity. These experiments show an increased maximum open probability, and reduced dissociation constant under the influence of caffeine. In the 4-state Markov model of RyRs (Fig. 2a in Restrepo et al. (3)), RyR refractoriness is modeled by distinguishing closed and open states where calsequestrin (CSQN) is bound to, acting as a brake, or unbound from the RyR/Triadin/Junctin complex. Refractoriness was therefore shortened by increasing the transition rates of RyRs from both CSQN-bound and CSQN-unbound closed states to their corresponding open states. A dependence of those rates on $C_{JSR}$ was also included to model a luminal brake separate from CSQN binding. Eqs. (5) and (6) of the Restrepo et al. model (3) become:
The single RyR release flux, and time-constants of CSQN binding are also modified based on previous work by Sato et al. (11).

**Online Table III  RyR gating parameters**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{JSR}$</td>
<td>5000</td>
<td>$\mu$mol/L</td>
</tr>
<tr>
<td>$J_{max}$</td>
<td>0.15</td>
<td>$\mu$m$^3$ ms$^{-1}$</td>
</tr>
<tr>
<td>$\tau_u$</td>
<td>1100</td>
<td>ms</td>
</tr>
<tr>
<td>$\tau_b$</td>
<td>1</td>
<td>ms</td>
</tr>
</tbody>
</table>

**Stable**

| $K_u$    | 8.25  | ms$^{-1}$   |
| $K_b$    | 0.23  | ms$^{-1}$   |
| $K_p$    | 9.8   | $\mu$mol/L |

**LQT2**

| $K_u$    | 10.5  | ms$^{-1}$   |
| $K_b$    | 2.33  | ms$^{-1}$   |
| $K_p$    | 4.2   | $\mu$mol/L |

**250 $\mu$mol/L Caffeine**

| $K_u$    | 12.75 | ms$^{-1}$   |
| $K_b$    | 4.25  | ms$^{-1}$   |
| $K_p$    | 3.25  | $\mu$mol/L |
Uptake current under ISO for LQT2 myocytes

Due to homeostatic regulation of SERCA uptake rate in LQT2 myocytes, we increase the maximum SERCA uptake current \( (v_{up}) \) in the LQT model. We also increase the maximum uptake current due to pharmacological phosphorylation of PLB under ISO in both LQT2 and LMC models. The parameter under each condition is given in **Online Table IV**.

**Online Table IV**  Maximum uptake current

<table>
<thead>
<tr>
<th>Condition</th>
<th>( v_{up} ) (( \mu \text{mol/L ms}^{-1} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMC</td>
<td>0.3</td>
</tr>
<tr>
<td>LMC with ISO</td>
<td>0.525</td>
</tr>
<tr>
<td>LQT2</td>
<td>0.375</td>
</tr>
<tr>
<td>LQT2 with ISO</td>
<td>0.525</td>
</tr>
</tbody>
</table>

Supplement Figure Legends.

**Online Figure I.** Unchanged Voltage-Dependent Ca\(^{2+}\) Current in LQT2 Myocytes under Baseline Conditions and in the Presence of ISO. A, Representative traces of \( I_{Ca} \) recorded in voltage clamped LMC (black) and LQT2 (red) myocytes at basal conditions and in the presence of 50 nmol/L ISO; HP -50 mV, steps -20; 0 and +20 mV. B, Pooled data for Current-Voltage relationship curves; no significant differences at \( p<0.05 \), \( n=7-23 \).

**Online Figure II.** Unchanged Levels of Ca\(^{2+}\) Handling Proteins in LQT2 Myocytes. A, Representative western blots for important Ca\(^{2+}\) handling proteins including RyR, pore-forming subunit of L-type Ca\(^{2+}\) channel \( \alpha_{1c} \), NCX, SERCA and PLB. B, Pooled data for normalized optical density. Paired Student’s \( t \) test showed no significant differences, \( n=5-7 \).

**Online Figure III.** Unchanged CaMKII Activity in LQT2 Myocytes. A, Bar graphs demonstrate pooled data for Ca\(^{2+}\)/calmodulin dependent and independent activities of CaMKII in LV tissue samples from LQT2 and LMC hearts (\( n=3 \)) assessed using radio-ligand binding assay. B, Representative western blots of CaMKII and phospho-CaMKII immuno-precipitated with anti-RyR antibodies. C, Pooled data for RyR-bound CaMKII and a ratio of p-CaMKII to RyR-tethered CaMKII. Not significant at \( p<0.05 \), paired Student’s \( t \) test, \( n=5 \).

**Online Figure IV.** Causal link between RyR hyperactivity and EAD formation in LMC myocytes under \( \beta \)-adrenergic stimulation. Comparison of computer modeling results of LMC myocytes paced at 0.25 Hz under ISO stimulation with hyperactive RyRs emulating addition of 250 \( \mu \text{mol/L} \) caffeine (blue) and normal RyRs (red) highlighting a similar causal relationship between RyR hyperactivity and EAD formation as in LQT2 myocytes (Fig. 7 of main text). As in A, transmembrane voltage \( V_m \) traces for 4 consecutive beats in steady-state demonstrating elimination of EADs by RyR stabilization. B-I shows a detailed comparison for the second beat in A of various key quantities including the cytosolic Ca\(^{2+}\) concentration (B) the SR Ca\(^{2+}\) concentration (C), confocal line scan equivalents for the stabilized (D) and hyperactive cases (E), \( V_m \) traces (F), and key sarcolemmal currents including \( I_{Ca,L} \) (G), \( I_{NCX} \) (H), \( I_{Ks} \) and \( I_{Kr} \) (I). The origin of time in B to I corresponds to the start of the second beat in A.

**Online Figure V.** Cell architecture of in silico myocyte model. A, Three-dimensional layout of spatially repeating unit where each unit consists of 8 cytosolic compartments with a t-tubule passing through 2 out of 8 compartments and a single dyad (green circle) at the boundary of
those two compartments. The myocyte consists of 16,120 of those units arranged in a 62 x 26 x10 lattice with the position of each unit inside this lattice labeled by the indices i, j, and k. B. Myocyte cross section at constant index i corresponding to a z-plane showing the layout of t-tubules and the location of dyads (green circles). Each z-plane consists of 26 parallel t-tubules aligned in the z-direction, each containing 10 dyads. C. Longitudinal myocyte cross-section showing the structure of the elementary Ca²⁺ release unit (CRU). For each dyad, there is an associated proximal, submembrane and JSR compartment, and 8 cytosolic and NSR compartments. Calcium transport channels (LTCC, RyR, NCX and SERCA) are labeled in purple.

Online Figure VI. 16-State LTCC Markov model. Schematic Illustration of different channel states that include one open state O and combinations of closed states (C or C’) and voltage- and calcium-dependent inactivated states (Ivak and IC, respectively). All 8 states located at the corners of the lower left-hand cube are connected to the corresponding states at the corners of the upper right-hand cube by gray and cyan arrows with constant forward and backward transition rates r₁ and r₂, respectively. Only transitions between two corners of each cube are shown for clarity.

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Online Figure VIII. Validation of Custom-Made Phosphospecific anti RyR-pS2809 and -pS2815 antibodies. A, Representative Western blots of phospho-RyRs probed with custom made anti-pSer-2809 and -pSer-2815 in tissue samples from WT, S2808A and double mutant S2808A/S2814A mouse hearts. B, C, Detection of ISO-mediated increase in RyR Ser-2808 phosphorylation levels in tissue samples from WT mouse hearts. Ventricular tissue samples were prepared from isolated hearts Langendorf-perfused with control Tyrode solution or 100 nmol/L β-adrenergic agonist isoproterenol for 10 min. Please note that mouse S2808 and S2814 correspond to rabbit S2809 and S2815 respectively.

Online Figure IX. Molecular Determinants of PP1cand PP2Ac Displacement from the RyR Macromolecular Complex in LQT2. A, Representative Western blots of regulatory subunits of PP1 and PP2A and corresponding pooled data for optical density (%). B, C, Representative Western blots of PP2Ac and phospho-Y307 and methyl-L309 of PP2Ac. D,E, Normalized optical density for PP2Ac phospho-Y307 and methyl-L309 respectively (%). *p<0.05, Paired Student’s t-test, n=4-6.

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