HHS Public Access

Author manuscript

Circ Res. Author manuscript; available in PMC 2015 November 07.

Published in final edited form as:

Circ Res. 2014 November 7; 115(11): 919–928. doi:10.1161/CIRCRESAHA.115.305146.

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

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Abstract

Rationale—Loss-of function mutations in HERG potassium channels underlie long QT syndrome (LQTS) type 2 (LQT2), and are associated with fatal ventricular tachyarrhythmia. Previously, most studies focused on plasmamembrane-related pathways involved in arrhythmogenesis in LQTS, while pro-arrhythmic changes in intracellular Ca^{2+} handling remained unexplored.

Objective—We investigated the remodeling of Ca^{2+} homeostasis in ventricular cardiomyocytes derived from transgenic rabbit model of LQT2 in order to determine whether these changes contribute to triggered activity in the form of early afterdepolarizations (EADs).

Methods and Results—Confocal Ca^{2+} imaging revealed decrease in amplitude of Ca^{2+} transients and SR Ca^{2+} content in LQT2 myocytes. Experiments using SR-entrapped Ca^{2+} indicator demonstrated enhanced RyR-mediated SR Ca^{2+} leak in LQT2 cells. Western blot analyses showed increased phosphorylation of RyR in LQT2 myocytes vs. controls. Co-immunoprecipitation 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 Ca^{2+} releases and EADs, which were abolished by inhibition of CaMKII. Computer simulations showed that late

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DISCLOSURES

None.

aberrant Ca^{2+} releases caused by RyR hyperactivity promote EADs and underlie the enhanced triggered activity through increased forward mode of NCX1.

Conclusions—Hyperactive, hyperphosphorylated RyRs due to reduced local phosphatase activity enhance triggered activity in LQT2 syndrome. EADs are promoted by aberrant RyR-mediated Ca^{2+} releases that are present despite a reduction of sarcoplasmic reticulum (SR) content. Those releases increase forward mode NCX1, thereby slowing repolarization and enabling L-type Ca^{2+} current reactivation.

Keywords

Long QT syndrome; ryanodine receptor; computational model; cardiac arrhythmia; protein phosphatase; calcium regulators; phosphatase; Ca^{2+}-induced Ca^{2+}

INTRODUCTION

Sudden cardiac death due to 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. So far mutations in 13 genes were associated with congenital Long QT Syndrome (LQTS) including loss-of function mutations in KCNH2 encoding rapid rectifier K^+ channel which leads to significant prolongation of action potential (AP) (1). LQT2 associated with loss of I_{Kr} 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 Pointes identified in the electrocardiogram recordings are believed to originate at the cellular level from membrane oscillations during the repolarizing phase of AP called early afterdepolarizations (EADs) (3,4).

We have created a transgenic rabbit model of long QT syndrome type 2 which recapitulates human arrhythmia phenotype (5). 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) due to polymorphic ventricular tachycardia (5). The underlying mechanisms of substrate for arrhythmia in these rabbits studied using *ex vivo* optical mapping include a prominent spatial dispersion of action potential duration (APD) and discordant APD alternans (5,6), while triggered activity is present in the form of EADs under β-adrenergic stimulation (7).

The regular rhythmic cardiac cycling is maintained by the interplay between the action potential (AP) and Ca^{2+} release from the sarcoplasmic reticulum (SR) mediated by the ryanodine receptor channels (RyR). Thus, although LQTS phenotypically is an electrical disorder, possible pro-arrhythmic remodeling of Ca^{2+} signaling pathways is recognized as an important unresolved question in LQTS pathophysiology (8). We recently reported that sex hormones effectively modulate arrhythmic potential and SCD in LQT2 rabbits by modulating expression and/or 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 (9). This strongly implies that Ca^{2+}-mediated communication between RyRs and other Ca^{2+} transport complexes could play a role in EADs 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_{CaL} during the plateau phase of the AP (10,11). 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} (12,13). 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. Pro-arrhythmic increase in RyR activity was attributable to enhanced RyR phosphorylation due to dissociation of protein phosphatases 1 and 2 from the RyR macromolecular complex.

METHODS

The cellular, sub-cellular, 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) (5,7,8). 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 (14,15) at 37°C. Changes in protein expression, protein-protein interactions and protein phosphorylation were studied using standard techniques (14–16). Computer simulations exploring arrhythmic potential of LQT2-associated changes in Ca^{2+} handling were performed using an extension of a physiologically detailed multi-scale rabbit ventricular myocyte model (17,18). This model bridges the submicron scale of individual couplons of plasmalemmal L-type Ca^{2+} channels clusters and SR Ca^{2+} release units (CRUs) and the whole cell.

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

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 (8). 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 to littermate controls (LMCs). LQT2 myocytes preincubated with 50 nmol/L of β-adrenergic agonist isoproterenol (ISO) for 3–10 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 ISO 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 NCX1. However, measurements of time constant of decay of caffeine-induced Ca^{2+} transient which reflects NCX1 activity demonstrated no differences between experimental groups (Fig. 1C). 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^{2+} currents between LQT2 and LMC myocytes either under basal conditions or in the presence of ISO (Online Fig. 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 α1c (Online Fig. II) These data suggest that changes in SR Ca^{2+} content and cytosolic Ca^{2+} transients in LQT2 myocytes are likely due to impaired ability of SR to maintain Ca^{2+}.

Enhanced SERCA-mediated Ca^{2+} uptake and RyR-mediated Ca^{2+} leak in LQT2 myocytes

To investigate potential changes in function of RyR and SR Ca^{2+} ATPase (SERCA) in LQT2 cells without interference of plasmalemmal Ca^{2+} transporters we employed experimental system of saponine-permeabilized myocytes. Figure 2 demonstrates representative confocal line scan images of spontaneous Ca^{2+} sparks recorded using intracellular solution containing 100 nmol/L free [Ca^{2+}]; and averaged data for Ca^{2+} sparks parameters. LQT2 myocytes exhibited significant increase in spark dimensions and frequency suggestive of enhanced SR [Ca^{2+}] leak through RyR clusters. Application of 10 mmol/L caffeine showed moderate but significant increase in SR Ca^{2+} content in permeabilized LQT2 myocytes, indicating that not only SR Ca^{2+} leak but SERCA-mediated uptake is accelerated in these cells as well. Next in order to study leak and uptake separately we utilized experimental protocol with low affinity indicator Fluo-5N loaded into the SR. To assess SERCA-mediated Ca^{2+} uptake permeabilized myocytes with SR entrapped Fluo-5N were exposed to 10 mmol/L caffeine to fully empty Ca^{2+} stores, then caffeine was washed out with 0 [Ca^{2+}] solution and RyRs were blocked with RyR inhibitor Ruthenium Red (RuRed, 40 μmol/L). Subsequent reintroduction of 250 nmol/L [Ca^{2+}] into the cytosol

evokes time-dependent increase in Fluo-5N fluorescence reflective of the dynamics of SERCA-mediated re-sequestration of Ca^{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 representative recording and pooled data for the time constants of Fluo-5N fluorescent signals (Fig. 3B), SERCA-mediated SR Ca^{2+} uptake is significantly accelerated in LQT2 myocytes in comparison to LMCs. It has been recently demonstrated that not all SR Ca^{2+} leak is mediated by Ca^{2+} sparks, and substantial component of leak occur in the form of unitary RyR openings undetectable by cytosolic Ca^{2+} indicators (19). In order to assess total RyR-mediated SR Ca^{2+} leak Fluo-5N loaded cells were exposed to specific inhibitor of SERCA thapsigargin (Tg, 10 μmol/L). Application of Tg evoked time-dependent loss in fluorescence signal of the SR-entrapped Ca^{2+} indicator, which was ~2-fold faster in LQT2 myocytes than in LMCs (Fig. 3C and D). This data indicates 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 (PLB), an important regulator of SERCA activity (Online Fig. II). Previously we and others demonstrated in several large animal models of acquired cardiac disease and ageing that disease-related remodeling of Ca^{2+} handling involves posttranslational modifications of RyR and PLB causative of aberrant functioning of SR Ca^{2+} release channels and SERCA (14,15,20,21–24). To test the hypothesis that altered RyR and SERCA function in LQT2 is caused by RyR and PLB phosphorylation we performed analysis using custom-made phospho-specific antibodies against RyR p-Ser-2809 and p-Ser-2815, rabbit PKA and CAMKII sites respectively; and antibodies to detect phosphorylation of PLB 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, while RyR phosphorylation at CaMKII site is maximal even at basal conditions. Phosphorylation of PLB at PKA site Ser-16 is also increased in untreated LQT2 myocytes to a degree that pretreatment of myocytes with 50 nmol/L ISO fails to produce additional effects. In theory shift of phosphorylation-de-phosphorylation balance towards 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 PP1 and PP2A; two major Ser-Thr phosphatases that control phosphorylation state of RyR (25), were comparable in ventricular myocytes from LQT2 and LMC hearts (Fig. 5A and B). Furthermore, our experiments using phosphatase activity assays showed no differences in activity of PP1 and PP2A in samples from LQT2 and LMC ventricular myocytes (Fig. 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 co-immunoprecipitation (Co-IP) experiments and found significant reduction of PP1c and PP2Ac scaffolded to RyR (Fig. 5C and D). To gain further insights into how these changes manifest in LQT2 we carried out 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 while the levels of regulatory subunit PPP1R9B (or spinophilin, known to scaffold PP1c to RyR, 25) tended to be lower in LQT2 (Online Fig. IXA and B). By contrast, we did not find statistically significant differences in regulatory subunits of PP2A including PPP2R5A, PPP2R5D, PPP2R5E in LQT2 vs. LMC; while PPP2R3A, a PP2A regulatory subunit also known as PR130 that scaffolds PP2Ac to RyR (25) was upregulated (Online Fig. IXA and B). The latter data did not provide an explanation for the loss of PP2Ac from the SR Ca^{2+} release complex. Therefore, since the ability of PP2Ac to form complexes with regulatory subunits is controlled by posttranslational modifications, we tested whether phosphorylation and methylation levels of PP2Ac were altered in LQT2 (Online Fig. IXC-D). Western blot analysis using anti-phospho-Y307 and anti-methyl-L309 antibodies demonstrated a significant increase in PP2Ac phosphorylation, while methylation levels tended to be lower in LQT2 vs. LMC, providing the basis for the dissociation of PP2A activity from the RyR complex. Noteworthy, our experiments using radio-ligand binding assay showed no differences in activity of CaMKII in ventricular tissue samples from LQT2 and LMC rabbits (Online Fig. IIIA). Furthermore, Co-IP experiments revealed no changes in abundance and phosphorylation of CaMKII residing on RyR (Online Fig. IIIC and D). 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 Ca^{2+} transients in current clamped myocytes exposed to 50 nmol/L of ISO. 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 (Fig. 6A and BC). These changes in membrane potential were accompanied by a decrease in peak amplitude of Ca^{2+} transients, prolonged time to peak (Fig. 6A and EF), and a protracted tail component of Ca^{2+} transients with distinguishable disorganized Ca^{2+} sparks, miniwaves and more synchronized Ca^{2+} signals during EADs. As a parameter describing this component we chose the time of the decay to 25% of Ca^{2+} transient amplitude after the peak (Fig. 6G). The changes in the shape of Ca^{2+} signal suggest that hyperhosphorylated 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 Fig. 6A-G, exposure of ISO-treated LMC myocytes to 250 μmol/L caffeine reduced Ca^{2+} transient amplitude and evoked sustained tail component of Ca^{2+} 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 Ca^{2+} channels was similar for LQT2 cells and LMC myocytes treated with caffeine (Fig. 6A and D). 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, 5–10 min.) increased amplitude of and most importantly shortened Ca^{2+} transient which resulted in shortening of AP and

elimination of EADs (Fig. 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 formation under β-adrenergic stimulation. We compare in Figure 7 the steady-state voltage and Ca^{2+} activity of LQT2 myocytes paced at 0.25 Hz under the influence of ISO for the cases where RyRs are hyperactive (with increased open probability and shortened refractoriness) and "stabilized" (with the same normal open probability and refractoriness as LMC myocytes). The results for hyperactive RyRs (blue line in Fig. 7A) show EADs while those for stabilized RyRs show no EADs (red lines in Fig. 7A). Figure. 7B-E characterizes the effects of RyR hyperactivity on Ca^{2+} cycling. The Ca^{2+} transient amplitude is seen to be decreased approximately two fold (Fig. 7B) due to a significant reduction in SR load (from ~750 μmol/L for stabilized RyRs to ~500 μmol/L for hyperactive RyRs as shown in Fig. 7C). RyR hyperactivity shortens the refractoriness of CRUs, resulting in a significantly increased spark frequency and reduced inter-spark interval during the AP plateau as seen in the confocal linescan equivalents (Fig. 7D–E). 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 to 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 ISO compared to LQT2 myocytes also under the influence of ISO but with RyR hyperactivity stabilized by KN93. Figures 7F-I characterizes the effects of RyR hyperactivity on V_m dynamics (Fig. 7F) and Ca^{2+}-dependent sarcolemmal currents (Figs. 7G–I) and highlight 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 (Fig. 7E) than for stabilized RyRs (Fig. 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 (Fig. 7H) during a critical time interval (between approximately 200 and 300 ms after depolarization) when V_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 (i.e. an almost equal fraction of channels are in an inactivated state) prior to 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 due to 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 β-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 towards lower cytosolic Ca^{2+} concentration in LQT2 than LMC myocytes. This difference can be attributed to the fact that the depolarizing NCX1 current needs to overcome a larger repolarizing current in LMC (with I_{Kr}) than LQT2 myocytes during the AP plateau, thereby requiring a higher frequency of late Ca^{2+} releases.

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 in order to preserve its ability to provide sufficient circulation to meet metabolic demands of the body even under conditions of permanent stress like hypertension or post-infarct injury. 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 (8). 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 (Fig. 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 (Figs. 1–3, Online Fig. I). Additionally, 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 (Figs. 4,5).

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 hyperhosphorylation and subsequently increased function due to the loss of local phosphatase activity is a common mechanism which serves as a protection from cellular Ca^{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 (25), tends to decrease. Furthermore, we observed a robust (300%) increase in PPP1R3A (Online Fig. IXA and B), which targets PP1 to glycogen (39). PP1 has been established as a key regulator of glycogen metabolism via regulation of activities of glycogen synthase and phosphorylase (39). Our recent proteomics study showed the latter being upregulated in LQT2 rabbit hearts along with adaptive upregulation of multiple energy-producing enzymes (32). It is plausible that increased metabolic demand due to 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 Fig. IXA and B) suggesting the existence of alternative mechanisms responsible for the reduction of RyR-bound PP2Ac. Recent in-depth study of changes in PP2A 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 (40). Nevertheless, the authors uncover an important common phenomenon in human ischemic heart failure, human non-ischemic heart failure; and canine heart failure induced by rapid pacing. In all three models the authors report increased PP2A catalytic subunit phosphorylation at Y307 and its decreased methylation at L309, which can explain loss of PP2Ac from specific substrates (40,41). Likewise, prolongation of APD in LQT2 was accompanied by increased phosphorylation of PP2Ac, while PP2Ac methylation tended to be lower (Online Fig. IXC–E). Future studies are necessary to thoroughly delineate signaling pathways that link APD prolongation with aberrant regulation of phosphatases in the heart.

Increase in phosphorylation of PLB in disease settings is a less common observation than increased phosphorylation of RyR. Contrary to our findings in LQT2 rabbits, PLB was shown to be hypo-phosphorylated in human and several animal models of HF (20). Increased PLB phosphorylation was earlier reported in rabbit model of HF (21) and more recently in rabbit bradycardia-evoked acquired LQTS caused by chronic AV ablation (33) which was accompanied by an increase in SR Ca^{2+} content and amplitude of Ca^{2+} transients, suggesting that in this model SR Ca^{2+} leak-uptake relationship is shifted towards uptake, in contrast to congenital LQT2.

While accelerated loss of SR Ca^{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 pro-arrhythmic effect. Under β-adrenergic stimulation, when LTCC and SERCA functions are increased, further amplified spontaneous Ca^{2+} release evokes arrhythmogenic oscillations of membrane potential via electrogenic NCX1 (EADs and DADs). We demonstrate that in the presence of β-adrenergic agonist ISO cells derived from LQT2 hearts

exhibit EADs and prolonged AP plateau associated with sustained tail component of Ca^{2+} transient (Fig. 6). Recent modeling and experimental works provided ample evidence that formation of EADs involve a complex chain of events in addition to reduced repolarization, which include window Ca^{2+} current and/or depolarizing I_{NCX} driven by SR Ca^{2+} release through untimely re-activated RyRs (4,11,13). Similarity between LQT2 myocytes and healthy myocytes treated with RyR activator caffeine strongly supports the key role of hyperactive non-refractory RyR in increased arrhythmic potential in LQT2. Furthermore, we show that CaMKII inhibition effectively eliminates tail of Ca^{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 (10–13). However, those studies have focused primarily on alterations of sarcolemmal membrane currents. Furthermore, they have been carried out with so-called "common pool" models (e.g. 34,35), which are models where both the trigger Ca^{2+} flux through LTCCs and the release flux through RyRs feed into the same (whole cell) compartment, and Ca^{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^{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^{2+} transient amplitude. The apparent paradox stems from the fact that a smaller Ca^{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 (12). The present modeling results (Fig. 7 and Online Fig. IV) show that the NCX1 current is indeed less depolarizing with hyperactive than with stable RyRs early in phase 2 when the cytosolic Ca^{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 (12), Ca^{2+} transient amplitude. In addition, the mechanism of triggered activity we present explains the presence of EADs that persist after I_{CaL} and I_{Ks} are maximally potentiated by β-adrenergic stimulation. Hence, those EADs do not depend on the different rates of increases of I_{CaL} and I_{Ks} after ISO that has been invoked previously as a mechanism of transient EADs (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 two-fold reduction of Ca^{2+} transient amplitude. Furthermore, imaging of Ca^{2+} activity in both simulations and experiments reveals that the genesis of EADs is accompanied by similar patterns of asynchronous late Ca^{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^{2+} waves that have been found to promote EADs under the combined influence of Ca^{2+} overload and β-adrenergic stimulation (36). Ca^{2+} waves have also been traditionally invoked as a mechanism of delayed afterdepolarizations (DADs) (37,38). Hence, in this broader context of Ca^{2+}-triggered arrhythmias, a major novel finding of the present study is that abnormal Ca^{2+} handling can promote EAD formation under a condition where the SR load is dramatically reduced and aberrant Ca^{2+} releases are predominantly triggered by Ca^{2+} influx through LTCCs.

Conclusion

In conclusion, 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 L-type Ca^{2+} channels and generation of EADs. Targeting of hyperactive RyR hyperhosphorylated due to dissociation of phosphatases from the SR Ca^{2+} channel complex may present a rational strategy for treating hereditary arrhythmias associated with QT prolongation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

SOURCES OF FUNDING

This work was supported by NIH R01 HL0460005-23 and R01 HL110791-01A1 to GK; L.L.C. and W.L. were supported by NIH grant 5T32HL094300-05.

Nonstandard Abbreviations and Acronyms

LQT2	Long QT syndrome type 2
LMC	Littermate control
SCD	Sudden cardiac death
EAD	Early afterdepolarization
CICR	Ca^{2+}-induced Ca^{2+} release
SR	Sarcoplasmic reticulum
RyR	Cardiac ryanodine receptor type 2
SERCA	Sarcoplasmic reticulum ATPase type 2A
LTCC	L-type Ca^{2+} channel
NCX1	Na^{+}/Ca^{2+} exchanger type 1
CaMKII-	Ca^{2+}/calmodulin dependent protein kinase type 2

PKA	Protein kinase A
PP1	Serine-threonine protein phosphatase type 1
PP2A	Serine-threonine protein phosphatase type 2A
ISO	Isoproterenol
Tg	Thapsigargin
RuRed	Ruthenium Red
Co-IP	Co-Immunoprecipitation

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Novelty and Significance

What Is Known?

- Loss-of function mutations in HERG potassium channels underlie long QT syndrome (LQTS) type 2 (LQT2), and are associated with ventricular tachyarrhythmia and sudden cardiac death (SCD).
- Transgenic rabbit model of LQT2 overexpressing pore mutant of human KCNH2 (HERG-G628S) recapitulates human phenotype exhibiting prolonged QT interval and ~50% incidence of SCD.
- Triggered activity in LQT2 rabbit hearts is presented in the form of early after depolarizations (EADs), which have been traditionally attributed to reopening of L-type calcium channels due to prolonged repolarization.

What New Information Does This Article Contribute?

- In ventricular myocytes derived from LQT2 rabbit hearts the activity of sarcoplasmic reticulum (SR) Ca^{2+} release channels ryanodine receptors (RyRs) is abnormally high. This results in aberrant SR Ca^{2+} releases during a vulnerable phase of the action potential plateau.
- Using an advanced computer model we show that the experimentally observed reduction in refractoriness of SR Ca^{2+} release plays a key role in providing an ideal electrophysiological milieu for generation of EADs by facilitating the reopening of L-type calcium channels.
- The pro-arrhythmic 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 LQTS is an electrical disorder, potential maladaptive pro-arrhythmic changes in intracellular Ca^{2+} handling are considered an important unresolved question in pathogenesis of malignant arrhythmias in LQTS. In this study, we characterize abnormalities in intracellular Ca^{2+} homeostasis in a large animal model of hereditary LQTS Type 2 and demonstrate a key role of these abnormalities in arrhythmogenesis. Specifically, we found that RyR activity in LQT2 is abnormally high due to increased phosphorylation of the channel. The reduction in refractoriness of RyR-mediated SR Ca^{2+} release prolongs the repolarization phase of action potential thereby promoting the generation of EADs in LQT2. Targeting of hyperactive, hyperphosphorylated RyRs may present a novel therapeutic strategy for hereditary arrhythmias associated with QT prolongation.

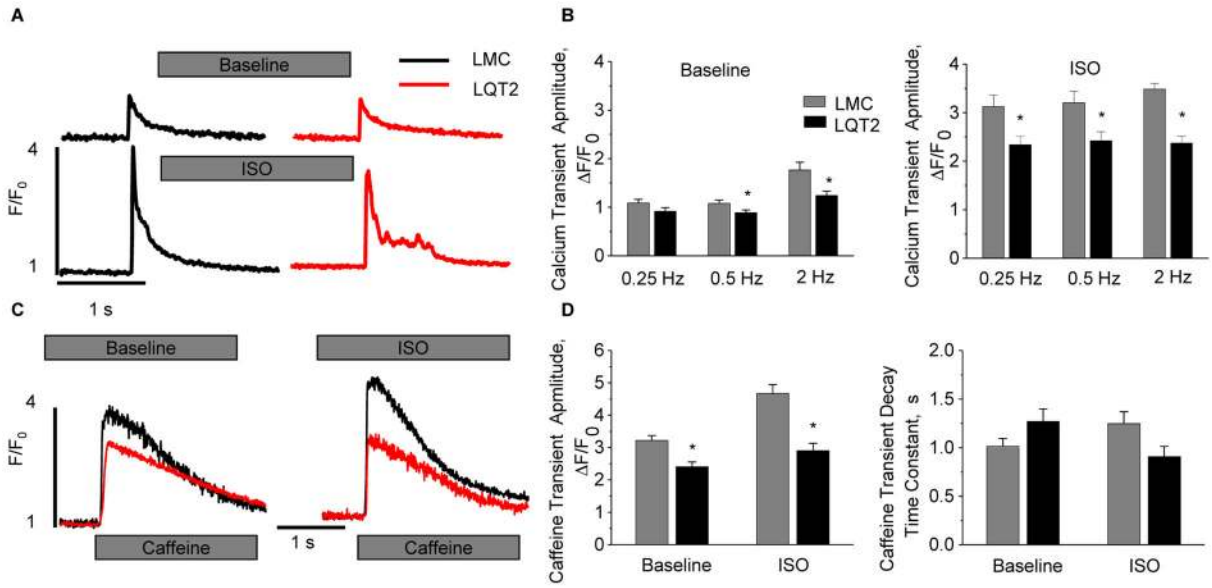

Figure 1. Decreased [Ca^{2+}] Transient Amplitude and SR [Ca^{2+}] Content in Intact LQT2 Myocytes

A, Representative Ca^{2+} transients recorded in intact 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 ISO. B, Bar Graphs present pooled data for Ca^{2+} transient amplitudes at different stimulation frequencies. Grey bars: LMC; black bars: LQT2.C, Representative traces of Ca^{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's *t*-test, n=6–33.

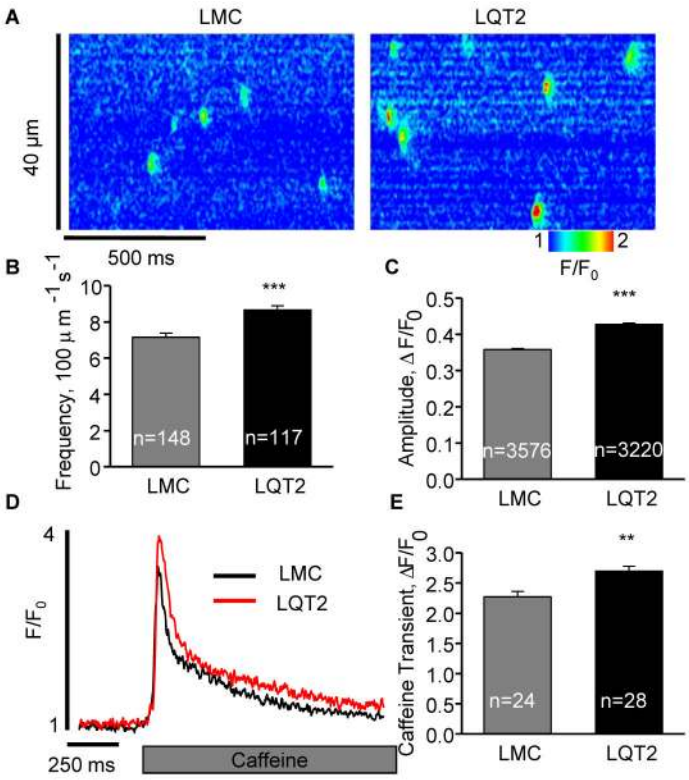

Figure 2. Increased SR [Ca^{2+}] Content and Increased Frequency and Amplitude of [Ca^{2+}] Sparks in Permeabilized LQT2 Myocytes

A, Representative line scan images of Ca^{2+} sparks recorded in permeabilized myocytes using intracellular solution with 100 nmol/L [Ca^{2+}]free. BC, Bar Graphs present averaged frequency and amplitude of Ca^{2+} sparks. D, Representative traces of Ca^{2+} release induced by 10 mmol/L caffeine application; and E, pooled data for Caffeine-induced Ca^{2+} transients. *p<0.05; **p<0.01. and ***p<0.001 vs. LMC, unpaired Student's *t* test.

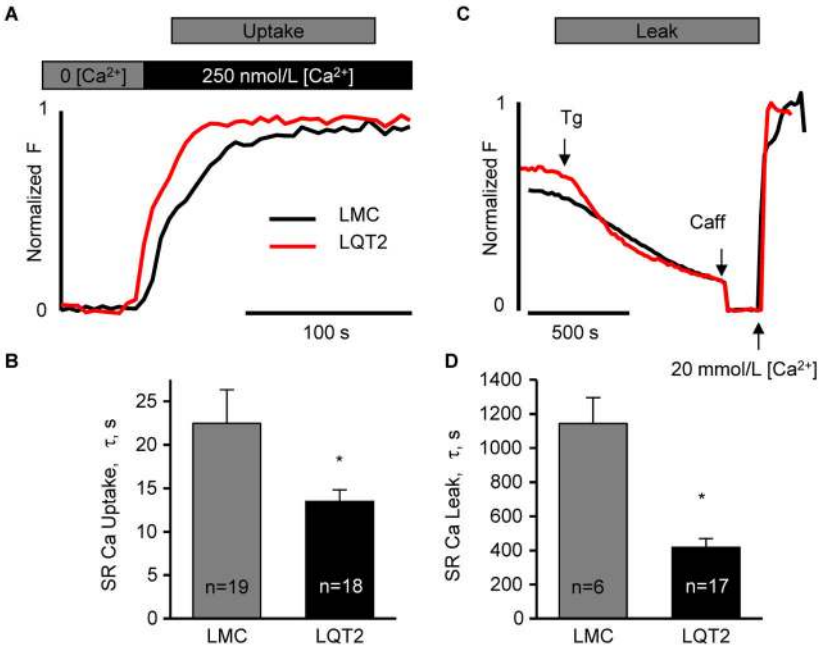

Figure 3. Accelerated SR [Ca^{2+}] Uptake and Leak in Permeabilized LQT2 Myocytes

SR Ca^{2+} uptake and leak was measured in myocytes with SR-entrapped low affinity Ca^{2+} indicator Fluo-5N. A, To measure SERCA-mediated SR Ca^{2+} uptake saponin-permeabilized myocytes were exposed to 10 mmol/L caffeine to exhaust Ca^{2+} stores, then RyRs were blocked with 40 μmol/L RuRed in Ca^{2+}-free solution. Time constant of increase in Fluo-5N fluorescence upon re-introduction of 250 nmol/L [Ca^{2+}] into cytosol was used as a measure of SERCA activity (B). C, RyR-mediated SR Ca^{2+} leak was unmasked by application of specific inhibitor of SERCA thapsigargin (Tg, 10 μmol/L). D, Pooled data for SR-Ca^{2+} leak. *p<0.05 vs. LMC, unpaired Student's *t* test.

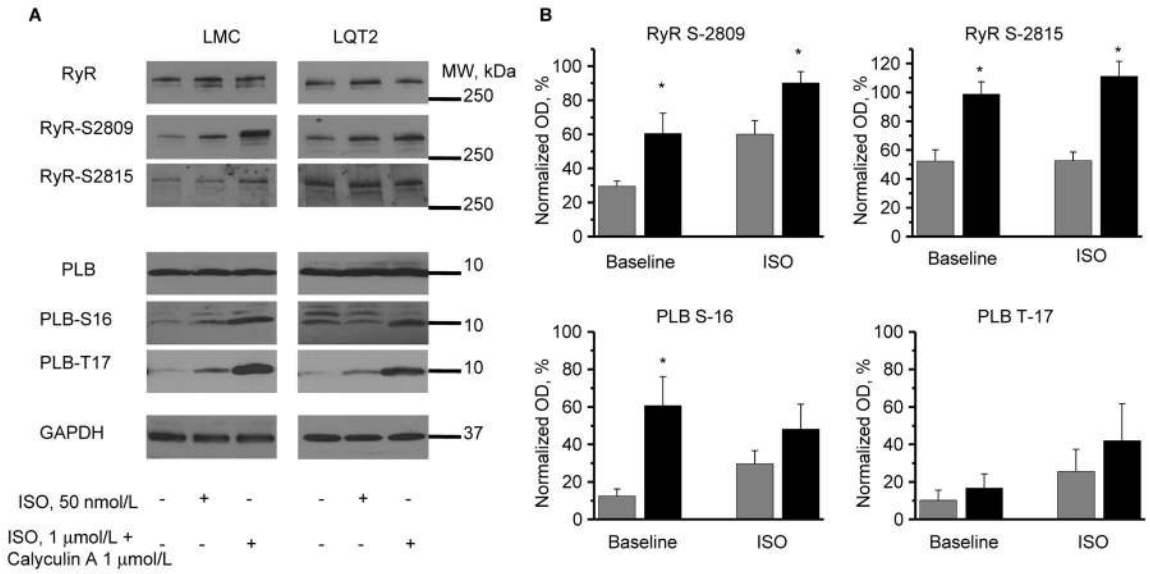

Figure 4. Changes in RyR and PLB Phosphorylation in LQT2 Myocytes

A, Representative Western blots for RyR and PLB phosphorylation at PKA and CaMKII sites. RyR2 phosphorylation at sites Ser-2815 (CaMKII) and Ser-2809 (PKA) and PLB phosphorylation at Ser-16 (PKA) and Thr-17 (CaMKII) was measured with phospho-specific antibodies. Total RyR/PLB protein content was measured in the same samples on a different blot and used as a control for loading. Ventricular cells isolated from 1 heart were split into 3 samples; one sample was kept at baseline conditions, second sample was exposed to 50 nmol/L ISO for 3 min., and third sample was treated with 1 μmol/L Calyculin A and 1 μmol/L ISO to achieve maximum phosphorylation and used for normalization of the signals. B, Data pooled from 6 experiments in LQT2 and 4 experiments in LMC cells. *$p<0.05$ vs. LMC, unpaired Student's *t* test.

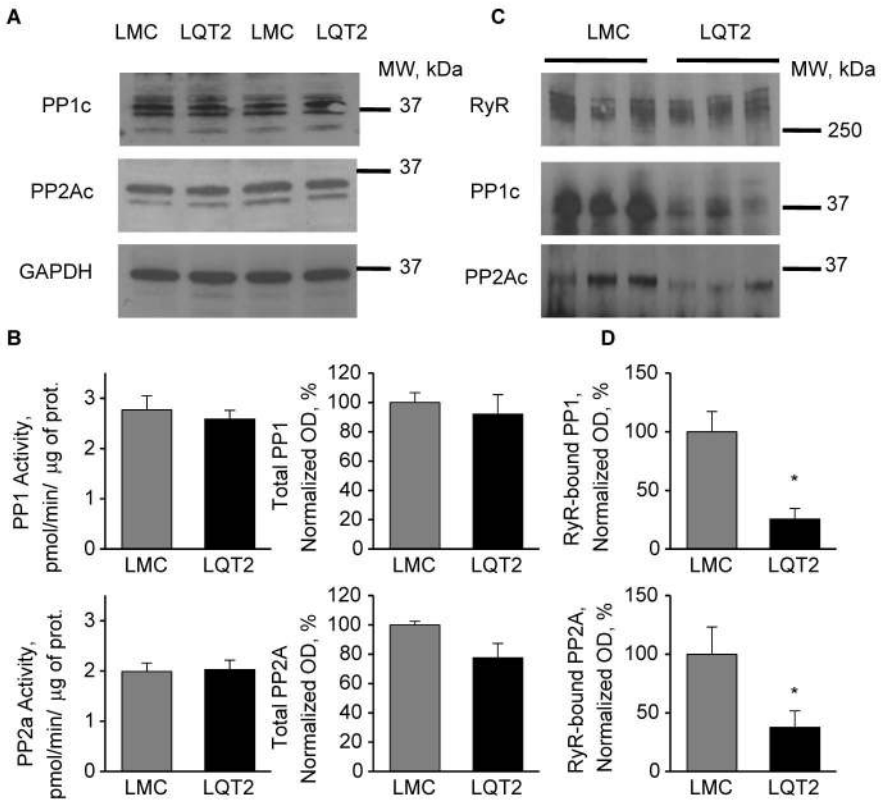

Figure 5. Reduced Levels of Phosphatases in RyR Macromolecular Complex in LQT2 Myocytes

A, Representative western blots of PP1 and PP2A catalytic subunits in samples from LV myocytes from LMC and LQT2 hearts. B, Pooled Data for PP1 and PP2A phosphatase activity of in samples from ventricular myocytes (n=3); and averaged normalized optical density (OD) for PP1 and PP2A catalytic subunits from cell lysates (n=7). C, Representative western blots of PP1c and PP2Ac immuno-precipitated with anti-RyR abs. E, Bar Graph demonstrates data for normalized OD for PP1c and PP2Ac subunit pooled down with RyRs, *p<0.05 vs. LMC, paired Student's *t* test, n=5.

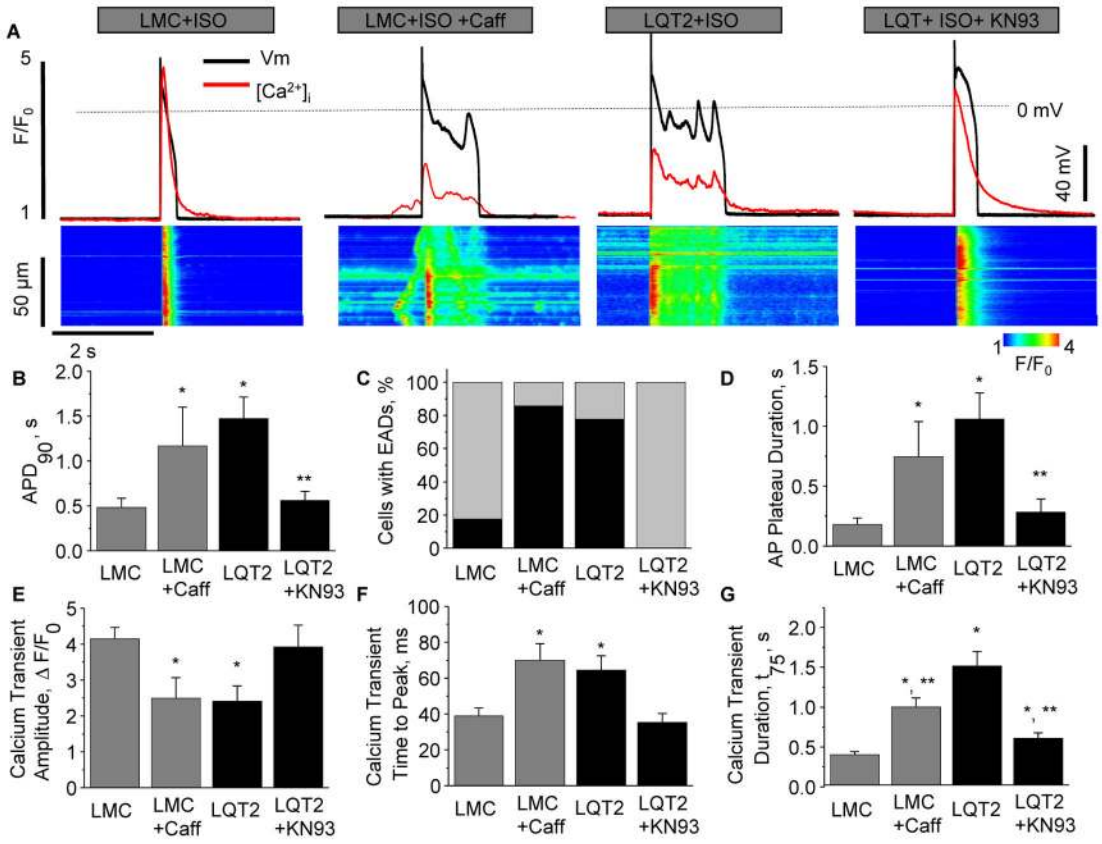

Figure 6. Inhibition of CaMKII Abolishes EADs in LQT2 Myocytes Exposed to ISO

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 LMC and LQT2 myocytes undergoing repetitive stimulation at 0.25 Hz in the presence of 50 nmol/L ISO. Application of RyR sensitizer caffeine (250 μmol/L) prolongs APD and Ca^{2+} transient, and promotes EADs in LMC cells, while preincubation of LQT2 myocytes with CaMKII inhibitor attenuates pro-arrhythmic Ca^{2+} mishandling. BCDEFG, 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's *t* test, n=4–18.

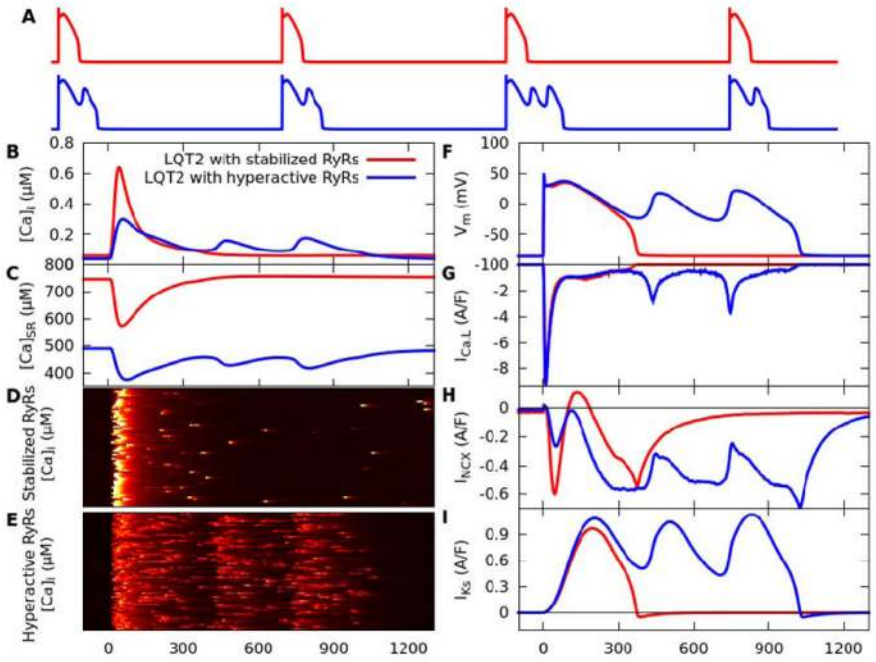

Figure 7. Causal Link between RyR Hyperactivity and EAD Formation in LQT2 Myocytes under β–Adrenergic Stimulation

Comparison of computer modeling results of LQT2 myocytes paced at 0.25 Hz under ISO stimulation with hyperactive (blue) and stabilized (red) RyRs highlighting the causal relationship of RyR hyperactivity to AP prolongation and EAD formation. Aberrant late Ca^{2+} releases in the hyperactive case increase the forward mode depolarizing NCX1 current thereby slowing repolarization and allowing more time for reactivation of LTCCs when the transmembrane voltage V_m traverses the window (i.e. the V_m range where the steady-state $I_{Ca,L}$ current is appreciable). A, 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 third beat in A of the cytosolic Ca^{2+} concentration (B) the SR Ca^{2+} concentration (C), confocal line scan equivalents for stabilized (D) and hyperactive (E) RyRs, V_m traces (F), and three key sarcolemmal currents including $I_{Ca,L}$ (G), I_{NCX} (H), and $I_{K,s}$ (I). The origin of time in B to I corresponds to the start of the third beat in A.