Mechanisms of Abnormal Calcium Homeostasis in Mutations Responsible for Catecholaminergic Polymorphic Ventricular Tachycardia

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Abstract—Catecholaminergic polymorphic ventricular tachycardia is a heritable arrhythmia unmasked by exertion or stress and is characterized by triggered activity and sudden cardiac death. In this study, we simulated mutations in 2 genes linked to catecholaminergic polymorphic ventricular tachycardia, the first located in calsequestrin (CSQN2) and the second in the ryanodine receptor (RyR2). The aim of the study was to investigate the mechanistic basis for spontaneous Ca2+ release events that lead to delayed afterdepolarizations in affected patients. Sarcoplasmic reticulum (SR) luminal Ca2+ sensing was incorporated into a model of the human ventricular myocyte, and CSQN2 mutations were modeled by simulating disrupted RyR2 luminal Ca2+ sensing. In voltage-clamp mode, the mutant CSQN2 model recapitulated the smaller calcium transients, smaller time to peak calcium transient, and accelerated recovery from inactivation seen in experiments. In current-clamp mode, in the presence of β stimulation, we observed delayed afterdepolarizations, suggesting that accelerated recovery of RyR2 induced by impaired luminal Ca2+ sensing underlies the triggered activity observed in mutant CSQN2-expressing myocytes. In current-clamp mode, in a model of mutant RyR2 that is characterized by reduced FKBP12.6 binding to the RyR2 on β stimulation, the impaired coupled gating characteristic of these mutations was modeled by reducing cooperativity of RyR2 activation. In current-clamp mode, the mutant RyR2 model exhibited increased diastolic RyR2 open probability that resulted in formation of delayed afterdepolarizations. In conclusion, these minimal order models of mutant CSQN2 and RyR2 provide plausible mechanisms by which defects in RyR2 gating may lead to the cellular triggers for arrhythmia, with implications for the development of targeted therapy. (Circ Res. 2007;100:e22-e31.)

Key Words: catecholaminergic polymorphic ventricular tachycardia • delayed afterdepolarization • ryanodine receptor • calsequestrin • calcium handling • simulation
RyR2 channels show that under nonstimulated, resting conditions, CPVT-mutant RyR2 channels are indistinguishable from normal (wild-type) channels. However, several CPVT-linked mutant RyR2 channels display abnormal single-channel function following phosphorylation by protein kinase A. Although not universally demonstrated in all mutants, mutant RyR2 channels also have been shown to exhibit decreased affinity for the channel-stabilizing molecule FKBP12.6 (calstabin 2) compared with wild-type channels. The function of FKBP12.6 in vivo is likely to both structurally and functionally couple adjacent RyR2 subunits. One hypothesis that has been advanced is that the disruption of FKBP12.6–RyR2 interaction is responsible for hyperactive “leaky” release receptors, which promote DADs.

At present, the precise mechanistic basis for the genesis of DADs in CSQN2 and RyR2 mutations has not been determined. We therefore used a detailed model of excitation-contraction coupling to investigate the hypothesis that impaired luminal Ca$^{2+}$ sensing in CPVT promotes the spontaneous SR release underlying the DAD formation in this disease. Specifically, the aims of this study were (1) to evaluate the restitution properties of the RyR2 in CSQN2 mutations, (2) to examine the role of impaired FKBP12.6 binding in RyR2 mutations, and (3) to examine the role of each of these mechanisms in generating DADs at the whole-cell level under conditions that simulate stress.

Materials and Methods

We developed models for CSQN2 and RyR2 mutations that were comprehensive, yet relatively simple to understand. These models involved minimum order alterations of the normal model, yet recapturated the known experimental behavior of the mutants. All simulations were performed using a recently described biophysically detailed model of the human left ventricular myocyte.

Briefly, the whole-cell model is composed of a system of 67 coupled nonlinear differential equations that are integrated numerically. Numerical integration was performed using the Livermore Solver for Ordinary Differential Equations integration package, with $10^{-6}$ relative error tolerance. Use of these numerical methods ensures convergence of model equations and allows simulation of cell electrophysiological responses in real time.

The RyR2 Model

The RyR2 channel is represented using a model developed by Keizer and Levine. This model was developed to replicate open and dwell times of isolated RyR2 channels in vitro and in vivo, as well as measured peak and plateau open probabilities with Ca$^{2+}$ or cesium (Cs$^{+}$) as the charge carrier. The latter measurements describe the adaptive behavior of the RyR2 channel. As originally described, adaptation is a property of the RyR2 in which, after rapid activation by a step increase in Ca$^{2+}$, the channel undergoes a slow spontaneous decrease in open probability. Closing of the RyR2 has also been attributed to inactivation. In isolated bilayers, adaptation occurs within milliseconds, whereas inactivation occurs within a few seconds.

A state diagram of the RyR2 model is shown in Figure 1. This model has 2 open states (O1 and O2) and 2 closed states (C1 and C2). At rest, the channel resides primarily in the first closed state, C1. On an increase in Ca$^{2+}$, the channel switches briefly to the first open state, O1, allowing Ca$^{2+}$ to move through the channel, before it adapts by its transition to C2. On additional increases in Ca$^{2+}$, the channel reopens by its transition to state O2, displaying the adaptive behavior seen experimentally. In the original model, the charge carrier could be Ca$^{2+}$ or Cs$^{+}$, so that some transition rates depended on Ca$^{2+}$ in the subspace around the channel, whereas others depended on the bulk cytosolic Ca$^{2+}$. In the present model, the charge carrier is Ca$^{2+}$, so all rates depend on the Ca$^{2+}$ in the subspace.

Furthermore, the Keizer–Levine model assumes that the RyR2 can be exposed to peak [Ca$^{2+}$], values of $\approx 1.0 \mu$mol/L; in the present model, the RyR2 is located in the subspace (SS), where it is exposed to [Ca$^{2+}$]$_{ss}$ in excess of 10.0 $\mu$mol/L; therefore, the rates have been modified such that they are scaled to depend on [Ca$^{2+}$]$_{ss}$. In addition, because [Ca$^{2+}$]$_{ss}$ levels change more rapidly than [Ca$^{2+}$]$_{ss}$, resulting in saturating functions of the Ca$^{2+}$ following Michaelis–Menten kinetics of the Keizer–Levine model, the rate constants have been modified to adjust the channel sensitivity to Ca$^{2+}$ such that the channel functions properly in the appropriate Ca$^{2+}$ range. Transition rates between states are given by $k_i$ and are provided by Jafri et al.

The gating structure for the RyR2 channel is presented in Figure 1, and all parameter choices are shown in Table 1.

Incorporation of Luminal Ca$^{2+}$ Dependence of SR Ca$^{2+}$ Release

RyR2 channels are known to be activated by Ca$^{2+}$ concentration on the dyadic side of the protein during calcium-induced calcium release (CICR). Furthermore, RyR2 channels are also activated by free Ca$^{2+}$ on the luminal (infra-SR) side of the protein, as channel open probability has been shown to rise with increases in SR Ca$^{2+}$ in lipid bilayers. Recently, CSQN2 was shown to confer reduced open probability to partially assembled RyR2 release units, an inhibition that was relieved by increasing Ca$^{2+}$ concentration on the luminal side of the receptor.

To reflect luminal Ca$^{2+}$ dependence of RyR2 opening, the model C1→O1 transition was multiplied by a Hill function of SR Ca$^{2+}$ concentration, $k_{lumen}$ providing a graded increase in opening rate with increase in free SR Ca$^{2+}$:

$$k_{lumen} = \frac{H_{max} - H_{min}}{1 + \left(\frac{H_{50}}{[Ca^{2+}]_{SR}}\right)^n}$$

Furthermore, the mechanism(s) underlying termination of RyR2 release in the myocyte have not been precisely determined, but it appears that SR Ca$^{2+}$ depletion, in addition to adaptation (as implemented in the current model), may be involved. The role of SR Ca$^{2+}$ in termination of release is suggested by experiments in which the SR Ca$^{2+}$ content was modulated with low-affinity Ca$^{2+}$ buffers, resulting in altered Ca$^{2+}$ spark durations and amplitudes. These findings suggest that RyR2 channels might enter a refractory state more quickly on reductions in luminal Ca$^{2+}$, possibly by adjusting their responsiveness to cytosolic Ca$^{2+}$ (see Gyorke and

Figure 1. Schematic of gating structure for the RyR2 receptor. Channels are initially in closed state C1 and open by gating to conducting state O1. Adaptation is incorporated through closure of channels into state O2, from which the receptor recovers into state O1 and state O2. Addition of luminal Ca$^{2+}$ dependence is reflected by parameter $k_{lumen}$ in the C1→O1 and O1→C2 transitions (shown in red).
Györke\textsuperscript{35}). This behavior of the receptor was simulated by scaling the open—adapted transition (O1→C2) by 1/k\textsubscript{trans}, such that SR Ca\textsuperscript{2+} depletion accelerates termination of release.

Finally, restitution of RyR2 appears also to be dependent on restoration of SR load (reviewed elsewhere\textsuperscript{37}). Ventricular myocytes simultaneously loaded with imperatoxin A (an RyR2 activator\textsuperscript{38}), and intra-SR Ca\textsuperscript{2+} buffers showed increased spark frequency in the absence of SR buffering, suggesting enhanced recovery dynamics at higher luminal Ca\textsuperscript{2+}\textsuperscript{,39} This finding was recently supported by a study using paired pulses of photolysis of caged Ca\textsuperscript{2+}, in which by manipulating SR load, it was determined that SR Ca\textsuperscript{2+} directly determines the time course of restitution of the paired Ca\textsuperscript{2+} transient.\textsuperscript{39} To reproduce this important experimental behavior, the model RyR2 recovery from the adapted state (transition C2→O1) was also scaled by k\textsubscript{trans} (a parameter that controls the luminal Ca\textsuperscript{2+} sensitivity).

Initial parameter choices for k\textsubscript{trans} were taken from a recent simulation study\textsuperscript{40} and were adjusted to ensure physiological transition scaling rates to the range of wild-type human myocyte SR Ca\textsuperscript{2+} content (from 0.5 to 2 Hz range from 0.2 to 0.6 mmol/L) by pacing the model at 1 Hz; the final parameter choices are listed in Table 1.

### Modeling Impaired Luminal Ca\textsuperscript{2+} Sensing in CSQN2 Mutations

Regulation of RyR2 activity by luminal Ca\textsuperscript{2+} likely involves CSQN2 as a luminal Ca\textsuperscript{2+}“sensor” (reviewed elsewhere\textsuperscript{,41,42}). Recently, in a series of experiments, Györke and colleagues have shown that mutations in CSQN2\textsuperscript{41} or reduced CSQN2 expression levels\textsuperscript{11,16,41} affect properties of calcium release by altering the luminal Ca\textsuperscript{2+} dependent regulation of the RyR2. These changes were simulated in the mutant model through appropriate adjustments to k\textsubscript{trans}, the factor that confers luminal Ca\textsuperscript{2+} dependence to each RyR2 transition (see the online data supplement for details).

Viatchenko-Karpinski et al\textsuperscript{11} demonstrated that mutant CSQN2 expressed in rat ventricular myocytes was associated with smaller elemental Ca\textsuperscript{2+} sparks with reduced time to peak amplitude, consistent with accelerated termination of release in the mutant compared with wild-type myocytes. These findings were in line with experiments unpressuring CSQN2 in ventricular myocytes.\textsuperscript{16} Accelerated termination of release was therefore incorporated in the mutant model, by increasing maximal k\textsubscript{trans} for the O1→C2 adaptation transition.

Furthermore, Terentyev et al\textsuperscript{16} and Kubalova et al\textsuperscript{11} demonstrated that the kinetics of recovery of the RyR2 are also altered in CSQN2-underexpressing myocytes. These experiments showed (1) an increase in imperatoxin-induced spark frequency with mutant CSQN2\textsuperscript{16} and (2) shorter intervals between Ca\textsuperscript{2+} waves in myocytes expressing reduced CSQN2 levels,\textsuperscript{11} suggesting that the CSQN2 expression level might control the size of a reservoir of intra-SR Ca\textsuperscript{2+}, whose recharging by SR repletion controls the RyR2 recovery.

In this scheme, mutant CSQN2 or reduced levels of CSQN2 creates a lower threshold of SR Ca\textsuperscript{2+} repletion before store recharge.\textsuperscript{11,16,41} To emulate this process, mutant RyR2 recovery was accelerated in the model by reducing the H\textsubscript{m} “threshold” parameter (the SR Ca\textsuperscript{2+} concentration at which the magnitude of k\textsubscript{trans} is half-maximal), for the transition C2→O1.

Mutant CSQN2 has been shown to have reduced buffering capacity for SR Ca\textsuperscript{2+}\textsuperscript{,42} This was simulated by adjusting the rapid buffering approximation of Wagner and Keizer\textsuperscript{43} by scaling the effective CSQN2 concentration by the factor k\textsubscript{CSQN2}.

We used parametric analysis to determine physiologically relevant parameters for the mutant CSQN2 model (for further details, please see the online data supplement). Overall, in investigating the mechanisms of DAD formation in the mutant CSQN2 model, the selection of a well-defined specific value for each of the mutant models was not a goal of the study (a specific value itself may have little physiological significance). Instead, we aimed to identify processes that would reproducibly recapitulate the experimental data including DAD formation, for a physiologically reasonable range of the mutant parameters. Parameter adjustments reflecting the mutant CSQN2 are listed in Table 1.

### Modeling Altered RyR2 Gating in RyR2 Mutations

Several experiments investigating impaired calcium cycling in CPVT mutant RyR2 have demonstrated reduced FKBP12.6 (calstabin) binding to RyR2 on protein kinase A phosphorylation.\textsuperscript{9,12} FKBP12.6 is one component of the macromolecular complex that comprises the RyR2 receptor. FKBP12.6 binds adjacent RyR2 subunits and is responsible for the structural association of subunits and coupled gating between assembled channels,\textsuperscript{10,44} causing linked RyR2 channels to act as a synchronous Ca\textsuperscript{2+} release unit.\textsuperscript{45} Experiments using the immunophilin FK506 (which dissociates FKBP12.6 from assembled RyRs) have been shown to uncouple individual RyR2 channels functionally, but not structurally, as assessed by subconductance states unmasked with FK506 administration.\textsuperscript{19}

Both reduced coupled gating and altered intersubunit interaction in RyR2 mutants is modeled, as recently described,\textsuperscript{46,47} as a reduction in cooperativity of activation of RyR2, by scaling the parameters responsible for RyR2 cooperativity (m\textsubscript{coop} and n\textsubscript{coop}) by a constant k\textsubscript{coop}.

Similar to the mutant CSQN2 model, we used parametric analysis to determine physiologically relevant parameters for the mutant RyR2 model that would recapitulate the experimental data including the DAD formation.

### The Isoproterenol Model

The main effect of isoproterenol on Ca\textsuperscript{2+} cycling proteins was modeled as an increase in the density of the L-type calcium current...
and the rate of SR Ca\(^{2+}\) uptake by the SR Ca\(^{2+}\) ATPase by a factor \(k_\text{src}\). The choice not to incorporate changes in peak RyR2 Ca\(^{2+}\) flux and the sodium–calcium exchanger (NCX) current resulting from isoproterenol stimulation was made on the basis of better isolating changes to RyR2 gating imposed by the mutant gene. However, additional simulations that include the effect of a comprehensive isoproterenol model are shown in Figure II in the online data supplement, and did not change the conclusions drawn from this simpler isoproterenol stimulation model.

### Stimulation Protocols

To determine model parameters and facilitate comparisons with the experimental data,\(^{11,16}\) voltage-clamp simulations of the mutant CSQN2 model were conducted using 400-ms-long pulses from a holding potential of \(-40\) mV to a step potential of \(0\) mV, delivered at 0.5 Hz. Also, the virtual myocyte was stimulated at 2 Hz for all simulations in current-clamp mode, thus matching experimental pacing rates,\(^{11,16}\) which also correspond to the heart rate in humans, above which ventricular arrhythmias occur most commonly.\(^{12,48}\)

### Results

#### Luminal Ca\(^{2+}\) Dependence of RyR2 Gating

Figure 1 shows a schematic illustration of the RyR2 receptor model used in this study. Full details about the model state transitions and their dependence on luminal Ca\(^{2+}\), as well as the simulation protocols, are described in Materials and Methods.

We first incorporated SR Ca\(^{2+}\) dependence of RyR2 gating into the Keizer–Levine model\(^{23}\) and observed its effects on features of whole-cell Ca\(^{2+}\) cycling. In Figure 2, we present simulation results of the whole-cell model, stimulated in voltage-clamp mode at 0.5 Hz. Figure 2A shows Ca\(^{2+}\) transients ([Ca\(^{2+}\)\(_i\)]), produced by the original Keizer–Levine model without (black trace) and with (red trace) isoproterenol stimulation; Figure 2B shows [Ca\(^{2+}\)\(_i\)] produced by the modified Keizer–Levine model in which luminal Ca\(^{2+}\) dependence has been added, without (blue trace) and with (green trace) isoproterenol stimulation. The upgraded Keizer–Levine model achieves steady state after short-term pacing and reproduces [Ca\(^{2+}\)\(_i\)], with morphology and size typical of human voltage-clamped [Ca\(^{2+}\)\(_i\)].

In Figure 2C, we compare the behavior of the 2 models in voltage clamp and in the presence of simulated isoproterenol (red trace, the Keizer–Levine model + isoproterenol; green trace, the Keizer–Levine model with luminal Ca\(^{2+}\) dependence + isoproterenol). Incorporation of luminal Ca\(^{2+}\) dependence in the rate transitions between states (as shown in Figure 1) augments peak RyR2 open probability by 10%, consistent with the effect of higher SR Ca\(^{2+}\) on RyR2 activation. Furthermore, in the model that includes the luminal Ca\(^{2+}\) dependence, the initial depletion in SR Ca\(^{2+}\) results in earlier termination of Ca\(^{2+}\) release (accelerated transition from O1→O2). Thus, the functional significance of these results is that in the upgraded model presented here, SR Ca\(^{2+}\) depletion now becomes an important mechanism in terminating SR Ca\(^{2+}\) release.

#### CSQN2 Modulation of [Ca\(^{2+}\)\(_i\)]

The next set of simulations uses the mutant CSQN2 model to simulate the effect of CSQN2 under- and overexpression, as well as the altered RyR2 gating seen in D307H mutant CSQN2 gene transfer.\(^{11}\)

![Figure 2](http://circres.ahajournals.org/)

Figure 2. The RyR2 luminal Ca\(^{2+}\)-dependent model. Voltage-clamp simulations of the wild-type human cell model using the original Keizer–Levine RyR2 model (KL model) and the new model that includes luminal Ca\(^{2+}\) dependence; simulations were conducted by delivering 400-ms voltage steps to 0 mV from a holding potential of \(-40\) mV until steady state was achieved at 0.5-Hz stimulation frequency. A, Ca\(^{2+}\) transients ([Ca\(^{2+}\)\(_i\)]) of the original RyR2 model (black trace, KL model; red trace, KL model + ISO; ISO indicates isoproterenol). B, Ca\(^{2+}\) transients of the KL model with luminal Ca\(^{2+}\) dependence at baseline (blue trace) and in the presence of isoproterenol (green trace). C, Pooled RyR2 open probability (states O1 plus O2) in the presence of isoproterenol, for the KL model without (red trace) and with luminal Ca\(^{2+}\) dependence (green trace).

Figure 3A shows the overall evolution of [Ca\(^{2+}\)\(_i\)], to repetitive voltage-clamp stimulation at 0.5 Hz. As shown in the figure, [Ca\(^{2+}\)\(_i\)], is smaller in the mutant compared with the wild type, particularly in the initial several beats. On pacing to steady state, the difference in peak [Ca\(^{2+}\)\(_i\)], becomes smaller, a finding that is consistent with previous studies.\(^{40}\) Other experimental recordings of [Ca\(^{2+}\)\(_i\)], in CSQN2 mutants similarly show smaller [Ca\(^{2+}\)\(_i\)]; however, they remain small after long-term stimulation.\(^{11}\) Possible reasons for this experimental difference at steady state are discussed further in Discussion.

To closer examine the amplitude and kinetics of [Ca\(^{2+}\)\(_i\)], Figure 3B provides an expanded view of [Ca\(^{2+}\)\(_i\)], in the
vicinity of its peak amplitude. Compared with wild type, simulation of the mutant CSQN2 (in transition to steady state) shows a 36% smaller [Ca\(^{2+}\)], amplitude and reduced time to peak, with a similar rate of rise of [Ca\(^{2+}\)]. These results compare well with the faster time-to-peak and smaller amplitude [Ca\(^{2+}\)], measured in rat ventricular myocytes subjected to CSQN2 underexpression (compare with Terentyev et al\(^{16}\)) and gene transfer of D307H mutant CSQN2 (compare with Viatchenko-Karpinski et al\(^{11}\)).

The blue trace in Figure 3B shows the [Ca\(^{2+}\)] peak in simulated CSQN2 overexpression (simulated by reversing the

changes imposed in the mutant, ie, increasing CSQN2 buffering capacity, reducing peak adaptation rate, and shifting \(H_{50}\) for recovery to higher SR Ca\(^{2+}\); see Materials and Methods). Similar to the features of [Ca\(^{2+}\)], observed experimentally in CSQN2 overexpression,\(^{11,16}\) the simulation shows that peak [Ca\(^{2+}\)] is 130% larger than in the wild type, with a delayed, dome-like peak. The initial rate of rise of the [Ca\(^{2+}\)] is unchanged in the mutant and CSQN2 overexpression simulations, similar to the experiments.\(^{11,16}\)

To assess the time course of recovery of Ca\(^{2+}\) release, a paired voltage-clamp stimuli protocol was applied in mutant CSQN2 and wild-type simulations. The time course of recovery was accelerated in the mutant simulations (Figure 3C), providing confirmation that global Ca\(^{2+}\) release recovers more quickly in the mutant CSQN2 model.

Features of experimental and model-derived [Ca\(^{2+}\)], for mutant CSQN2 and CSQN2-underexpressing myocytes are presented in Table 2. Overall, the results presented in Figure 3 and Table 2 suggest that the mutant CSQN2 model reliably recapitulates the altered gating of the mutant CSQN2, reproducing the available experimental data in CSQN2-underexpressing and D307H mutant CSQN2-expressing ventricular myocytes.\(^{11,16}\)

**DADs in Mutant CSQN2**

It has been noted that rat ventricular myocytes expressing mutant CSQN2 demonstrate small-amplitude DADs on rhythmic pacing in the presence of isoproterenol.\(^{11}\) To examine whether the implemented changes in luminal Ca\(^{2+}\) sensing in the mutant CSQN2 model result in DADs, the model was stimulated at 2 Hz in current-clamp mode.
As shown in Figure 4, isoproterenol administration provokes DADs (Figure 4A). These DADs are caused by aberrant Ca\(^{2+}\) release, seen as spontaneous oscillations in intracellular Ca\(^{2+}\) (Figure 4B) and SR Ca\(^{2+}\) (Figure 4C). The response of the model to \(-\alpha\)-adrenergic stimulation and the shape and amplitude (5% of the action potential amplitude) of the DADs compare well with experimental results in rat ventricular myocytes expressing mutant CSQN2 (see Viatchenko-Karpinski et al.,\(^{11}\) in which the amplitude of the DADs is \(\approx 8\%\) of that of the action potential).

Use of a comprehensive isoproterenol model that also includes increases in the peak RyR2 Ca\(^{2+}\) flux, the density of delayed-rectifier K\(^+\) current (\(I_{Ks}\)) and the sodium–calcium exchanger current (see the online data supplement) results in triggered activity in the mutant myocyte, manifested by a premature action potential (see supplemental Figure II).

Similar to experiments in CSQN2-underexpressing myocytes,\(^{11}\) in which loading the SR with the low-affinity Ca\(^{2+}\) buffer citrate abrogated DADs, citrate simulated at a SR concentration of 5 mmol/L (using the Wagner–Keizer rapid-buffering approximation\(^{43}\) at a \(K_d\) of 0.47\(^{10}\)) produced no DADs, even in the presence of \(\beta\) stimulation (Figure 5). In these simulations, there is no evidence of aberrant Ca\(^{2+}\) release through pacing to steady state (action potential in Figure 5A and [Ca\(^{2+}\)]\(_i\) in Figure 5B). Thus, it appears that stabilization of the SR Ca\(^{2+}\) by the buffer reduces the probability of spontaneous Ca\(^{2+}\) release.

Interestingly, the wild-type model that included luminal Ca\(^{2+}\) dependence and reduced CSQN2 concentration (to 5% of baseline value) resulted in DADs in the presence of \(\beta\) stimulation. However, the same model in the presence of \(\beta\) stimulation exhibited no DADs in the absence of RyR2 luminal Ca\(^{2+}\) dependence. Finally, the mutant CSQN2 model, but with normal CSQN2 concentration, exhibited no DADs in the presence of \(\beta\) stimulation. These results suggest that because of the RyR2 luminal Ca\(^{2+}\) dependence, the buffering role of CSQN2 in the SR is critical in triggering DADs, as has been recently presented in a \(Casq2^{-/-}\) mouse model.\(^{49}\)

Thus in CSQN2 mutations, DADs form only in the presence of \(\beta\) stimulation. DADs arise with increases in SR Ca\(^{2+}\), and the buffering capacity of CSQN2 is critical in stabilizing the SR Ca\(^{2+}\) release process.

**DADs in Mutant RyR2**

In Figure 6A, one sees that on pacing the mutant RyR2 model in the presence of \(\beta\)-adrenergic stimulation at 2 Hz, a DAD is observed (after the third stimulus), which again corresponds to a spontaneous calcium release event.

The mechanism for the DAD becomes apparent from analysis of the RyR2 first open state O1 (Figure 6B), which exhibits higher initial and diastolic open probability and longer overall duration of release. In Figure 6B, at time 1400 ms during diastole, O1 is 0.0022 in the mutant model, which
is nearly double the 0.0012 value in the wild-type model. The results demonstrate that mutant channels, in the presence of isoproterenol, exhibit abnormally high open probability during diastole. This increased open probability results in progressive accumulation of subspace Ca\(^{2+}\)\(_{H11001}\), which leads to a regenerative opening of the RyR2 channels and a premature spontaneous Ca\(^{2+}\)\(_{H11001}\) release in the diastolic interval (Figure 6C).

Thus, in a mutant RyR2 model, stimulation induced reduction in coupled gating and intersubunit interaction, manifested in the model by a reduction in cooperativity of the RyR2 activation, results in DADs through an increase in diastolic open probability.

**Discussion**

This study investigated the mechanisms of DAD generation in CPVT using a mathematical model of human excitation-contraction coupling. It used a simple RyR2 model that incorporates explicit representations of activation, adaptation, and recovery properties of the channel. We have found (1) that the accelerated termination and recovery of RyR2 release in CSQN2 mutants result in DAD formation in the presence of \(\beta\) stimulation; (2) that the free SR Ca\(^{2+}\) content and rate of recovery from inactivation are the key parameters in controlling the stability of Ca\(^{2+}\) release from the RyR2s in CSQN2 mutations; and (3) that impaired FKBP12.6 binding is likely to generate DADs through hyperactive RyR2s that exhibit larger diastolic open probability. Overall, our results provide plausible mechanisms by which defects in RyR2 gating induced by CPVT-related gene mutations may lead to multiple cellular triggers of arrhythmias.

**SR Ca\(^{2+}\) and [Ca\(^{2+}\)\(_{H11001}\)] in the Mutant CSQN2 Model**

An important aspect of the present study is the ability to reproduce experimental recordings in myocytes reflecting impaired calcium handling secondary to CSQN2 mutations. A minimal order model was formulated to account for the effects of the mutant CSQN2 (Figure 1 and Table 1), based on a series of experimental studies aimed at modulating functional levels of CSQN2 and analyzing features of the resulting Ca\(^{2+}\) sparks and Ca\(^{2+}\) waves.\(^{11,16,41}\) Experiments using gene transfer of the mutant CSQN2 mutant D307H in rat ventricular myocytes show [Ca\(^{2+}\)]\(_{i}\) with smaller amplitude and faster time to peak (despite equivalent rise rates)\(^{11}\); these data were reproduced by our mutant CSQN2 model (Figure 3A and 3B of the current study and shown previously\(^{11,16}\)).

Our study shows that simulations in the mutant CSQN2 model, whereas in transition to steady state, [Ca\(^{2+}\)]\(_{i}\) amplitude is 36% smaller than in wild-type simulations (a 41% reduction has been observed experimentally in mutant CSQN2-expressing myocytes\(^{11}\)). However, at steady state, the simulated [Ca\(^{2+}\)]\(_{i}\) are only 10% smaller, whereas the experiments show larger persistent depressions in peak [Ca\(^{2+}\)]. Similar to our findings, simulations of CSQN2 overexpression\(^{40}\) and experiments using blockers of RyR2 release\(^{50}\) have revealed only transient effects on [Ca\(^{2+}\)]\(_{i}\), which abate on long-term pacing. The present study supports the idea of a type of “autoregulation” of SR Ca\(^{2+}\) release,\(^{40,50–52}\) whereby depressed SR Ca\(^{2+}\) release raises the SR Ca\(^{2+}\) content, which in turn increases SR Ca\(^{2+}\) release to baseline levels through an increase in luminal Ca\(^{2+}\) dependent RyR2 openings\(^{52}\) and a larger Ca\(^{2+}\) gradient across the SR membrane at the release site.

**Figure 6. Aberrant Ca\(^{2+}\) handling in RyR2 mutants in the presence of isoproterenol.** A, Action potentials for mutant RyR2. B, The RyR2 state O1 occupancy over time for the mutant (red trace) and the wild type (black trace). C, Ca\(^{2+}\) transients ([Ca\(^{2+}\)]\(_{i}\)) for the mutant RyR2. Arrows indicate DADs.
Although the reasons for the discrepancy in steady-state [Ca\textsuperscript{2+}]\textsubscript{i}\textsuperscript{11,16} are thus still unclear, it is likely that the stimulation frequency used experimentally is partially responsible.

The model was paced until steady state was achieved, at a cycle length of 2000 ms. However, in the experimental studies, myocytes were paced at a rate of 1 every 60 seconds. At this stimulation frequency, equilibration of intracellular Ca\textsuperscript{2+} pools may be difficult to achieve. Clearly, more experiments are needed to resolve the issue of steady-state Ca\textsuperscript{2+} dynamics in the CSQN2 mutant myocyte.

**Mechanisms of DADs in the Mutant CSQN2 Model**

A critical test of the mutant CSQN2 model is its ability to reproduce experimental data not are used to constrain the model. Thus, the demonstration that the mutant CSQN2 minimal order model (as a result of CSQN2-dependent altered RyR2 Ca\textsuperscript{2+} release termination and recovery) produced DADs only in the presence of isoproterenol provides evidence that the proposed mechanisms are plausible explanations for the aberrant Ca\textsuperscript{2+} release in the mutant.

How these DADs arise is a question that is particularly well suited for analysis using the model. To isolate the effect of each of the 2 modifications in the minimal model of CSQN2 mutant myocytes (ie, accelerated termination of release and accelerated recovery of release), additional simulations were conducted by (1) changing the rate of termination alone and holding the rate of recovery unchanged from that in the wild-type and (2) changing the rate of recovery from inactivation alone and holding the rate of termination at wild-type.

Simulations in which only termination of Ca\textsuperscript{2+} release was incrementally accelerated reveals that DADs could be elicited as SR Ca\textsuperscript{2+} reached a critical level. This phenomenon is likely to be a consequence of autoregulation, whereby smaller release events raise free SR Ca\textsuperscript{2+}, resulting in enhanced luminal Ca\textsuperscript{2+}-dependent aberrant RyR2 openings. Thus, these simulations add further support to the central role of free SR Ca\textsuperscript{2+} in the genesis of DADs.

One interpretation of these findings suggests that restoration of SR free Ca\textsuperscript{2+} may function to promote DADs by disinhibiting CSQN2-bound closed RyR2 (ie, reactivating the receptor or facilitating transition C1→O1 in Figure 1). Another possibility is that repletion of SR Ca\textsuperscript{2+} rescues RyR2 from a luminal Ca\textsuperscript{2+}-dependent refractory state (ie, from state C2). This possibility was tested in simulations that only modified the C2→O1, or recovery step, of the mutant. When the recovery step is accelerated sufficiently (in the absence of changes to the rate of termination of release), DADs result, as demonstrated in supplemental Figure IV. This finding suggests that primary modulation of RyR2 gating that provides premature recovery of the RyR2s from a luminal Ca\textsuperscript{2+}-dependent refractory state is a plausible mechanism for producing spontaneous Ca\textsuperscript{2+} release events. It also provides theoretical support to the scheme presented by Gyo¨rke and colleagues, who suggested that DADs in CSQN2 mutations might arise from altered RyR2 luminal Ca\textsuperscript{2+} sensitivity,\textsuperscript{11} possibly through premature recovery of receptors associated with accelerated recharge of a “smaller” functional SR Ca\textsuperscript{2+} store.\textsuperscript{16} In this scheme, β-adrenergic stimulation may unmask DADs by further enhancing the recharge of this smaller store. With either scenario (premature recovery from adaptation or premature reactivation) free SR Ca\textsuperscript{2+} content (or its disrupted sensing by RyR2) appears to be a critical determinant of DAD formation in CPVT. Alternatively, because of the RyR2 luminal Ca\textsuperscript{2+} dependence, the buffering capacity of CSQN2 alone appears to play a critical role in triggering DADs, as has been recently presented in a Casq2\textsuperscript{+} mouse model.\textsuperscript{49} Thus, interventions that reduce SR Ca\textsuperscript{2+} content, or alternatively that stabilize the release mechanism to respond at higher free SR Ca\textsuperscript{2+}, can be expected to minimize the effect of the mutation. This was demonstrated by simulations (Figure 5) and experiments\textsuperscript{14} in which citrate loaded into the SR stabilizes the release mechanism and restores rhythmic excitation in ventricular myocytes expressing mutant CSQN2.

**Mechanisms of DADs in the Mutant RyR2 Model**

The results of this investigation show that reduced RyR2 coupled gating as a consequence of reduced FKBP12.6 binding results in DADs (Figure 6). Common missense mutations linked to CPVT, when expressed in HEK293 cell lines, demonstrate reduced binding of FKBP12.6 to RyR2 in the presence of protein kinase A phosphorylation, as assessed by communoprecipitation.\textsuperscript{12} Most recently, FKBP12.6-null mouse myocytes were shown to exhibit higher diastolic SR Ca\textsuperscript{2+} leak and episodes of ventricular tachycardia.\textsuperscript{20} These experiments suggest that RyR2 CPVT mutants are likely to give rise to DADs through reduced FKBP12.6 binding.

Each RyR2 subunit in the tetrameric release assembly binds 1 FKBP12.6 protein, coupling RyR2 activity\textsuperscript{19,44} and stabilizing the closed conformation of the channel.\textsuperscript{46,47,53} Recent simulation studies\textsuperscript{37,53} have shown that increased coupling between adjacent RyR2 subunits in a “sticky cluster model” stabilizes the release mechanism, perhaps through a more synchronous activation of the subunits, thus shortening the overall release duration. Conversely, decreased RyR2 coupling increases heterogeneity of release across release sites, prolonging overall release duration, as confirmed by our study. We have found that in a whole-cell model of reduced FKBP12.6 binding that there was evidence of aberrant diastolic RyR2 openings, which are associated with increased diastolic open probability. This provides strong theoretical support to the idea that reduced FKBP12.6 binding in the presence of protein kinase A phosphorylation can lead to uncoupled, hyperactive RyR2 channels that are prone to triggered activity in CPVT.\textsuperscript{9,12,54}

It is likely that, given the genotypic heterogeneity of CPVT-linked mutations, there is also heterogeneity in the mechanisms whereby different mutations exert their effects.\textsuperscript{55} Indeed, some studies have not been able to demonstrate reduced FKBP12.6 binding in RyR2 mutations linked to CPVT.\textsuperscript{17,18,56} One explanation for this discrepancy is the use of different experimental preparations in these studies.\textsuperscript{7} The other possibility is that the DADs in these RyR2 mutants arise from FKBP12.6-independent mechanisms, such as impaired luminal Ca\textsuperscript{2+} sensing. Recently, Jiang et al proposed a scheme attributing DADs in RyR2 mutants to a reduced threshold for
functional recovery of receptors, a process they have termed "store overload–induced calcium release."118

Single-channel analysis in these mutants13 reveals that the mechanism for these changes is increased sensitivity of mutant channels to luminal Ca$^{2+}$, with little effect on features of cytosolic Ca$^{2+}$ activation. To test this hypothesis, simulations were performed in which the sensitivity of channels to luminal Ca$^{2+}$ was increased (by reducing the C2→O1 H$_{30}$ recovery threshold parameter to 0.5). These simulations (presented in supplemental Figure IV) produce DADs, providing support for the store overload–induced calcium release mechanism in RyR2 mutants. Such a scheme is furthermore attractive because it provides a common mechanistic framework for understanding different mutations in CSQN2 and RyR2 that lead to a similar phenotype, i.e., through enhanced sensitivity to luminal Ca$^{2+}$.

**Summary**

In conclusion, in this study, we explored the mechanistic basis for aberrant Ca$^{2+}$ homeostasis in 2 recently identified mutations linked to CPVT. Importantly, we developed minimal order models of mutant CSQN2 and RyR2 that recapitulated known experimental behavior of these gene products when expressed in ventricular myocytes. Using these models, we have demonstrated that CSQN2 mutations may give rise to DADs through accelerated recovery from inactivation and that free SR Ca$^{2+}$ is an important variable in determining the propensity for DADs. We have further demonstrated that reduced cooperativity of RyR2 gating, as might be found with reduced FKBP12.6–RyR2 binding in CPVT mutants, can also plausibly cause DADs associated with the mutant RyR2s via hyperactive release channels. These findings can potentially be used to guide further experiments in characterizing these mutants and assist in the development of targeted therapies.

**Limitations**

The current work provides demonstration that the proposed mechanisms are plausible explanations for the triggered activity observed in CPVT mutations. Unfortunately, the study is limited by the lack of published experimental characterization of calcium handling in human ventricular myocytes carrying the relevant mutations, eg, from biopsy specimens of human CPVT patients. Such data might permit more quantitative, rigorous constraining of parametric changes to the model.

Furthermore, the majority of DADs observed in this investigation are of small amplitude (=8% of the action potential peak amplitude), similar to those recorded experimentally.11 However, as we show in the online data supplement, triggered premature action potentials do occur in the mutant myocyte using the comprehensive β-stimulation model. Therefore, whereas the minimum-order model enabled us to relate alterations in the gating properties of the RyR2, to abnormal SR Ca$^{2+}$ release and DAD formation, the observations (1) that the β-stimulation effect in vivo is not precisely known and likely depends on the magnitude of the catecholaminergic surge in the exercising or stressed patient and (2) that β stimulation is likely to differentially affect each of the Ca$^{2+}$ regulatory genes (ie, L-type channel, RyR2, SERCA2a, and sodium–calcium exchanger) clearly provide plausible explanations about the variability in the DAD amplitude observed experimentally and in this study. Nevertheless, the current model serves as a valuable tool for unraveling mechanistic underpinnings of the disease and may become useful in assessing the effects of recently proposed therapeutic interventions.12,57

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**Disclosures**

None.

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Mechanisms of Abnormal Calcium Homeostasis in Mutations Responsible for Catecholaminergic Polymorphic Ventricular Tachycardia

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Results

Mutant CSQN2 and RyR2 model parameter selection

On the basis of the experimental data, we hypothesized that accelerated RyR2 release termination and accelerated recovery from refractoriness are likely to be mechanistically responsible for the DADs in mutant CSQN2, and that reduced cooperativity (reflecting the reduced functional coupling of the RyR2 channel) underlies the DADs in mutant RyR2. In this section we show the method of parameter selection for these processes.

In the Online Supplement Figure S1A, we show the effect of systematically varying the RyR2 release termination step (corresponding to the O1→C2 transition), by altering parameter $H_{\text{max}}$ from its baseline value of 0.5 to 0.05, while holding the recovery step fixed. As seen in the figure, peak $[\text{Ca}^{2+}]$ falls smoothly as this transition is incrementally decreased, a net reduction of 35% across the parameter range. In parallel, $[\text{Ca}^{2+}]$ rise time of these $\text{Ca}^{2+}$ tracings decreases, from a peak of 70 msec at baseline to 40 msec for the smallest parameter value (data not shown). A value of 0.2 was selected, in the middle of the range explored, to recapitulate the experimentally observed rise time and peak $[\text{Ca}^{2+}]$, during whole cell voltage clamp stimulation, with implementation of reduced CSQN2 buffering (which in the model reduces peak $[\text{Ca}^{2+}]$, by another 40% on average).
The Online Supplement Figure S1B, explores the effect of parameter variation for recovery from inactivation variable $H_{50}$, for the transition C2→O1. This parameter is incrementally decreased from its baseline value of 1 to 0.35, and restitution curves are generated in a two-pulse protocol. Although we do not know the true response of the recovery time constant in vivo, we can infer that the kinetics of whole cell Ca$^{2+}$ release recovery are accelerated in mutant cells from analysis of Ca$^{2+}$ sparks$^{1-4}$. For values of $H_{50}$ smaller than 0.3, spontaneous Ca$^{2+}$ release events are seen during the voltage clamp pulse at normal SR loads (behavior that is unlikely to occur in vitro). This suggests that recovery is too quick for that parameter choice. As shown in the panel, there is a smooth response of this variable as this variable is increased. Therefore, a parameter value in the middle of this range, 0.5, was selected to represent the mutant phenotype.

The RyR2 parameter variation was performed by decreasing the parameter $k_{coop}$ (which reflects the cooperativity of the channel) from 0.9 to 0.7. Online Supplement Figure S1C, shows the results of this simulation. As seen at the bottom panel, when $k_{coop}$ is 0.9, no DADs are observed. However, as $k_{coop}$ is incrementally decreased, a DAD occurs at sequentially earlier beats. DADs arise in the model through accumulation of RyR2’s in open states in diastole, after continued pacing from the initial conditions; once this accumulation reaches a threshold, a spontaneous Ca$^{2+}$ release occurs. In these simulations of parametric analysis, we observe that decreasing the $k_{coop}$ parameter speeds the accumulation of RyR2 in these permeable “ready to fire” states such that DADs occur earlier during stimulation.

Overall, in investigating the mechanisms of DAD formation in the mutant CSQN2 and RyR2 models, the selection of a well-defined specific value for each of the mutant models (a value which may have little physiological significance) was not a goal of the study. Instead we created hypotheses as to the likely mechanisms important in DAD formation in CPVT, and performed parameter variation over a reasonable range of mutant parameters to observe behavior. Indeed, the parameter selection process was particularly hampered by the lack of
experimental studies aimed to characterize specific features of disrupted Ca\(^{2+}\) homeostasis in the mutant myocytes. Thus, we believe that subtle variability in the chosen set of parameters for the mutant CSQN2 and RyR models, not only is likely to be physiologically relevant, but also does not alter the main conclusions drawn from this modeling study, namely that accelerated termination and recovery of RyR2 release and SR load promote DADs in mutant CSQN2, and uncoupling of release channels promote DADs in mutant RyR2.

Use of parametric analysis to constrain minimum order models of mutant CSQN2 and RyR2 allows for simple and meaningful conclusions to be drawn that recapitulate the experimental data, as opposed to using more complex models which might be under-constrained by experimental data and provide less clear mechanistic conclusions.

**Effect of isoproterenol model on DAD formation in the mutant CSQN2 and RyR2 models**

We chose to use a simple isoproterenol model, in order to keep the dimensions of complexity of the problem to a minimum, and to better isolate changes to RyR2 gating imposed by the mutant genes. The simple isoproterenol model does not include changes in (i) the peak RyR2 Ca\(^{2+}\) flux, (ii) the density of the slow component of the delayed rectifier potassium current \(I_{Ks}\), and (iii) the sodium calcium exchanger density.

However, to investigate the validity of this approach, we have performed additional simulations using a comprehensive isoproterenol model that included the components (i)-(iii) above. Thus, we have implemented a new isoproterenol model formulation for the most likely targets within the myocyte, that is: \(I_{CaL}\), SERCA2a, RyR2, NCX, and \(I_{Ks}\). Furthermore, to examine the additive effect of each of the new components alone we developed the new model in a step-wise fashion.

First, we examined the effect of an isoproterenol model in which, in addition to an increase in the magnitude of the L-type current by 50% and SR Ca\(^{2+}\) uptake by 50%, the peak RyR2 Ca\(^{2+}\) flux was also increased by 50%. We have found that the wild-type model in the
presence of isoproterenol did not exhibit delayed afterdepolarizations (DADs) through pacing to steady-state. However, the mutant CSQN2 model still exhibits small amplitude DADs without needing to modify the parameters describing the mutant CSQN2. Similar results were obtained in the mutant RyR2 model.

In the next model of β-adrenergic stimulation, in addition to an increase in the magnitude of the L-type current density by 50%, SR Ca^{2+} uptake by 50% and the peak RyR2 Ca^{2+} flux by 50%, the density of \( I_{Ks} \) was also increased (as described by Greenstein et al \(^5\)). We have found that at progressively increasing the \( I_{Ks} \) density of the AP duration and SR Ca^{2+} loads at 1 Hz, were progressively smaller. This is a natural consequence of reduced time-averaged Ca^{2+} entry into the cell, and causes a sustained reduction in SR load compared to the minimal model of isoproterenol. Consequently, this reduced range of SR loads required adjustment of the mutant model parameters in order to give rise to DAD. Consequently, we adopted a new set of parameters for the mutant CSQN2 model (parameters were reduced by 20% compared to the original mutant CSQN2 model), which when stimulated at 2 Hz, gave rise to DADs only under β-stimulation (in which the \( I_{Ks} \) density was increased by 200%, as described in experiments by Kathofer \(^6\) and simulations by Tanskanen \(^5\)). When we applied the same model of β-stimulation in the mutant RyR2 model (in which the relevant parameter kcoop was reduced by 20% compared to the baseline mutant RyR2 model), it also gave rise to DADs.

Finally, we developed a comprehensive β-adrenergic stimulation model in which, in addition to an increase in the magnitude of the L-type current by 50%, SR Ca^{2+} uptake by 50%, the peak RyR2 Ca^{2+} flux by 50%, the density of \( I_{Ks} \) by 200%, we also increased the NCX peak current by 100% \(^7\)\(^-\)\(^11\). Using this β-stimulation model, we have been able to demonstrate DADs when β-stimulation was applied to both the CSQN2 (parameters were decreased by 33% compared to the original CSQN2 mutant model) and RyR2 models (parameters were decreased by 20% compared to the original RyR2 mutant model). The response of the mutant CSQN2 and
mutant RyR2 models to this comprehensive representation of isoproterenol is demonstrated in Online Supplement Figure S2. As shown in panel A of this figure, the DADs in this comprehensive model are larger and give rise to triggered activity, which is manifested by a large membrane excitation that results in an action potential that occurs prior to the next regularly paced beat (at 10,500 sec).

For the simple isoproterenol model (which included changes only of $I_{CaL}$ and SR Ca$^{2+}$ ATPase) the APD$_{90}$ in control cells under $\beta$-stimulation was increased from 318 msec (control) to 341 msec (isoproterenol) at 1 Hz. In the comprehensive model, (including changes to $I_{CaL}$, SR Ca$^{2+}$ ATPase, RyR2, $I_{NaCa}$, and $I_{Ks}$) the APD$_{90}$ was reduced from 318 msec (control) to 306 msec under $\beta$-stimulation.

Overall, we have found that: (i) disrupted Ca$^{2+}$ homeostasis is a feature of the mutant models, but only in the presence of $\beta$-stimulation; (ii) physiologically appropriate adjustments of the mutant CSQN2 and RyR2 model parameters (required for different SR loads) give rise to DAD formation; and (iii) the mechanism of DADs in the comprehensive $\beta$-stimulation model appears to be similar, in that we observe spontaneous Ca$^{2+}$ release in the setting of enhanced SR Ca$^{2+}$ loading, which activates a transient inward current and depolarization of the membrane, with no evidence of EADs or reactivation of the L-type channels and no evidence of increased diastolic Ca$^{2+}$ compared to the wild-type model.

**Effect of membrane potential and stimulation rate on DAD formation in the mutant CSQN2 and RyR2 models**

To examine the role of the membrane potential on the DAD formation in the mutant CSQN2 and RyR2 models, in addition to the epicardial cell model, we developed endocardial and midmyocardial cell models, with altered $I_{o1}$, $I_{NaCa}$ and $I_{Ks}$ densities as previously described.
Models for each cell layer were constructed based on parameter adjustments shown in the table. The resulting wild-type APs at 1 Hz are shown in the Online Supplement Figure S3A.

To probe the effect of the mutants, the Online Supplement Figure S3B was generated by first pacing each myocyte type using identical initial conditions, generated by prolonged (~100 beats) pacing of the epicardial model at 2 Hz. The new cell types (endocardial and midmyocardial) were then paced for 20 beats in the absence of isoproterenol to ensure appropriate steady state behavior. No DADs were found during this stage of the simulation, either for wild-type (black traces) or mutant CSQN (red trace) myocytes.

Upon addition of isoproterenol (time 10,000 msec), the wild-type models show potentiated SR Ca\(^{2+}\) uptake and larger Ca\(^{2+}\) transients, but eventually reach a steady state and do not show DADs after long term pacing. In contrast, for all three cell layers, DADs are observed for the CSQN2 mutations in the presence of isoproterenol.

Similar results are obtained for the mutant RyR2 model (Online Supplement Figure S3C). For these simulations, since the mutant phenotype only arises in the presence of isoproterenol, the cells are paced at 2 Hz only in the presence of isoproterenol. For all cell layers, DADs emerge, while as demonstrated previously, wild-type models in each layer do not show DADs.

Certainly, the AP waveform might under some conditions lead to re-opening of the L-type channels and result to afterdepolarizations on this basis (early afterdepolarizations, or EADs). However, examination of the L-type current tracing in our model confirms that there are
no re-openings of LCC during these spontaneous release events. Thus, the NCX alone mediates the inward current responsible for the DAD formation.

Overall, the results of Online Supplement Figure S3A-3C suggest that membrane potential and APD, while important in shaping the magnitude of trigger current, may not be the primary trigger of DAD formation in the mutant CSQN2 and RyR2 myocytes. Rather, altered Ca\(^{2+}\) homeostasis arises more directly as a function of altered SR Ca\(^{2+}\) release.

For both mutant CSQN2 and RyR2 models, no DADs are observed at pacing frequencies 1 Hz and below. Similarly, there has been no experimental demonstration of DADs at these pacing frequencies. In the wild-type model no DADs are observed at high pacing rates (3 Hz) despite the increased SR Ca\(^{2+}\) load, even in the presence of isoproterenol. In contrast, in the mutant CSQN2 model, DADs are observed in the absence of \(\beta\)-stimulation at that high stimulation frequency. We believe that pacing at a very high frequency results in a higher SR load (giving rise to a similar effect to that of isoproterenol) and thus contributes to the genesis of spontaneous release events.

Effect of RyR2 accelerated recovery model on DAD formation in the mutant CSQN2 and RyR2 models

In the Online Supplement Figure S4, the wild-type model with luminal Ca\(^{2+}\) dependence of RyR2 gating was initially chosen. This model was modified such that the recovery of RyR2 from adaptation (transition C2→O1) was accelerated, by changing parameter H\textsubscript{50} from 1.0 mM to 0.45 mM, while all other transitions were left unchanged. With this “pure” accelerated recovery model, action potentials (panel A) and [Ca\(^{2+}\)]\(_i\) (panel B) show evidence of DADs in the presence of isoproterenol.
**Online Supplement Figure S1**  Use of parametric analysis to obtain parameters for the mutant CSQN2 and RyR2 models.  (A) Effect on Ca\(^{2+}\) transients ([Ca\(^{2+}\)]\(_i\)) of incremental decrease in parameter H\(_{\text{Max}}\) from 0.5 (wild-type value) to 0.05 with all other parameters held fixed.  (B) Effect on RyR2 open probability restitution curves of incremental decrease of parameter H\(_{50}\) from 1.0 mM (wild-type value) to 0.35, with all other parameters held fixed.  Arrows indicate DADs.

**Online Supplement Figure S2**  Mutant CSQN2 and RyR2 model responses to a comprehensive β-stimulation model.  This model includes an increase augments in the magnitude of the L-type current by 50%, SR Ca\(^{2+}\) uptake by 50%, the peak RyR2 Ca\(^{2+}\) flux by 50%, the density of I\(_{\text{Ks}}\) by 200%, and NCX peak current by 100%.  (A) Response of the mutant CSQN2 model – left, action potentials, middle, Ca\(^{2+}\) transients ([Ca\(^{2+}\)]\(_i\)), right, SR Ca\(^{2+}\) concentration.  The timing of isoproterenol administration is indicated by the solid bar.  Interestingly, we observe triggered activity manifested by excitation that occurs prior to the next regularly paced beat (at 10,500 sec).  (B) Response of the mutant RyR2 model – left, action potentials, middle, Ca\(^{2+}\) transients, right, SR Ca\(^{2+}\) concentration.  In this simulation, isoproterenol is present throughout, as the mutant RyR2 phenotype (ie, altered FKBP12.6 function) only emerges in vivo in the presence of β-stimulation.  Arrows indicate DADs.

**Online Supplement Figure S3**  Effect of membrane potential on DAD formation in the mutant CSQN2 and RyR2 models.  (A) Wild-type action potentials from endocardial, midmyocardial, and epicardial simulations.  (B) Wild-type (black traces) and mutant
CSQN2 (red traces) action potentials (top row), Ca\(^{2+}\) transients ([Ca\(^{2+}\)]; middle row), and SR Ca\(^{2+}\) concentration (bottom row). Responses are shown for the endocardial cell model (first column), midmyocardial cell model (middle column), and epicardial cell model (third column). The timing of isoproterenol administration is shown by the solid bar. (C) Mutant RyR2 action potentials (top row), Ca\(^{2+}\) transients (middle row), and SR Ca\(^{2+}\) concentration (bottom row). Responses are shown for the endocardial cell model (first column), midmyocardial cell model (middle column), and epicardial cell model (third column). Isoproterenol is present throughout these simulations as the mutant RyR2 phenotype (ie, altered FKBP12.6 function) only emerges in vivo in the presence of β-stimulation. Arrows indicate DADs.

**Online Supplement Figure 4** Test simulation of a hypothetical mutant in which only recovery from inactivation is accelerated (H50 of 0.45 mM), with no changes to rate of adaptation. (A) Action potentials. (B) Ca\(^{2+}\) transients ([Ca\(^{2+}\)]). Arrows indicate DADs
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Online Supplement Figure S2

A

CSQN2

membrane potential (mV)

Time (msec)

[Ca^{2+}]_i (nM)

Time (msec)

SR Ca^{2+} (mM)

Time (msec)

Triggered activity

+ISO

B

RyR2

membrane potential (mV)

Time (msec)

[Ca^{2+}]_i (nM)

Time (msec)

SR Ca^{2+} (mM)

Time (msec)

CSQN2 RyR2

Triggered activity

+ISO
Online Supplement Figure S3

A

-100 -80 -60 -40 -20 0 20 40 60 80 100

Time (msec)

membrane potential (mV)

- ENDO
- MID
- EPI
Online Supplement Figure S3

B

ENDO +ISO

MID +ISO

EPI +ISO

membrane potential (mV)

[Ca\textsuperscript{2+}]_i (nM)

SR Ca\textsuperscript{2+} (mM)

Time (msec)
Online Supplement Figure S3
Online Supplement Figure S4

A

membrane potential (mV)

Time (msec)

0 300 600 900 1200 1500

-100 -80 -60 -40 -20 0 20 40 60 80 100

[Ca^{2+}]_i (nM)

Time (msec)

0 300 600 900 1200 1500

0 200 400 600 800 1000 1200 1400

B

[Ca^{2+}]_i (nM)

Time (msec)

0 300 600 900 1200 1500