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Abstract—Catecholaminergic polymorphic ventricular tachycardia (CPVT) is a familial arrhythmogenic disorder associated with mutations in the cardiac ryanodine receptor (*RyR2*) and cardiac calsequestrin (*CASQ2*) genes. Previous in vitro studies suggested that RyR2 and CASQ2 interact as parts of a multimolecular Ca^{2+}-signaling complex; however, direct evidence for such interactions and their potential significance to myocardial function remain to be determined. We identified a novel CASQ2 mutation in a young female with a structurally normal heart and unexplained syncopal episodes. This mutation results in the nonconservative substitution of glutamine for arginine at amino acid 33 of CASQ2 (R33Q). Adenoviral-mediated expression of $CASQ2^{R33Q}$ in adult rat myocytes led to an increase in excitation–contraction coupling gain and to more frequent occurrences of spontaneous propagating (Ca^{2+} waves) and local Ca^{2+} signals (sparks) with respect to control cells expressing wild-type CASQ2 ($CASQ2^{WT}$). As revealed by a Ca^{2+} indicator entrapped inside the sarcoplasmic reticulum (SR) of permeabilized myocytes, the increased occurrence of spontaneous Ca^{2+} sparks and waves was associated with a dramatic decrease in intra-SR [Ca^{2+}]. Recombinant $CASQ2^{WT}$ and $CASQ2^{R33Q}$ exhibited similar Ca^{2+}-binding capacities in vitro; however, the mutant protein lacked the ability of its WT counterpart to inhibit RyR2 activity at low luminal [Ca^{2+}] in planar lipid bilayers. We conclude that the R33Q mutation disrupts interactions of CASQ2 with the RyR2 channel complex and impairs regulation of RyR2 by luminal Ca^{2+}. These results show that intracellular Ca^{2+} cycling in normal heart relies on an intricate interplay of CASQ2 with the proteins of the RyR2 channel complex and that disruption of these interactions can lead to cardiac arrhythmia. **(*Circ Res*. 2006;98:1151-1158.)**

Key Words: calsequestrin ■ ryanodine receptor ■ sarcoplasmic reticulum ■ Ca^{2+}-induced Ca^{2+} release ■ catecholaminergic polymorphic ventricular tachycardia

Catecholaminergic polymorphic ventricular tachycardia (CPVT) (Online Mendelian Inheritance in Man no. 604772) is a familial arrhythmogenic disorder characterized by adrenergically mediated polymorphic ventricular tachyarrhythmias, leading to syncope and sudden cardiac death in individuals with structurally normal hearts.[1] The episodes of tachyarrhythmia are typically triggered by physical exercise or emotional stress. Two genetic variants of the disease have been described: a recessive form associated with homozygous mutations in the gene encoding the cardiac isoform of calsequestrin (*CASQ2*)[2,3] and a second form transmitted as an autosomal dominant trait associated with mutations in the gene encoding the cardiac ryanodine receptor (*RyR2*).[4,5]

The contractile machinery of cardiac myocytes becomes activated when Ca^{2+} enters the sarcoplasmic reticulum (SR) via L-type Ca^{2+} channels and triggers a process termed Ca^{2+}-induced Ca^{2+} release (CICR) from the SR.[6] Whereas CICR controls the release process from the cytosolic side, a second Ca^{2+}-dependent mechanism controls the activity of the Ca^{2+}-release channels from the SR lumen. Specifically, the decline of intra-SR [Ca^{2+}] that accompanies the Ca^{2+}-release process contributes to Ca^{2+}-release termination, a mechanism referred to as luminal Ca^{2+}-dependent deactivation.[7–9] The Ca^{2+}-release channel is present in the junctional SR membrane in the form of a quaternary complex composed of RyR2, triadin, junctin, and CASQ2.[10,11] The integral membrane proteins triadin and junctin physically interact

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with RyR2 and link the Ca^{2+}-binding protein CASQ2 to the complex. Ca^{2+}-dependent interactions of CASQ2 with the RyR2–triadin complex are thought to provide a molecular basis for regulation of RyR2 channel by luminal Ca^{2+}.[12,13] In addition, CASQ2 monomers can form polymers with high Ca^{2+}-binding capacities that are essential for the Ca^{2+} storage function of the SR.[14,15]

To date, 4 homozygous sequence variations in the *CASQ2* gene have been identified in patients with CPVT (see Inherited Arrhythmias Database at http://pc4.fsm.it:81/cardmoc).[2,3] The precise molecular basis for the alterations in Ca^{2+} handling in cells expressing CPVT-linked CASQ2 mutants remains to be determined. To date, the effect of only 1 of these mutations on CASQ2 activity and function has been examined.[16,17] These studies focused on a CASQ2 mutant protein in which aspartate 307 is changed to histidine ($CASQ2^{D307H}$) and suggested that the $CASQ2^{D307H}$ protein is compromised in its ability to facilitate the Ca^{2+} storing and releasing functions of the SR. These effects may be a consequence of a reduction in the Ca^{2+}-binding capacity of the mutant protein or altered interactions between $CASQ2^{D307H}$ and components of the RyR2 channel complex.[16,17] Furthermore, it is unknown how independent mutations in the *CASQ2* and *RyR2* genes result in similar clinical manifestations in CPVT. In the present study, we report the identification of a new mutation in the *CASQ2* gene in a patient with CPVT. We also demonstrate that this mutation alters the functional interactions between CASQ2 and the RyR2 channel complex, resulting in abnormal luminal Ca^{2+}-dependent regulation of the RyR2 channel.

Materials and Methods

CPVT in human patients was diagnosed using standard cardiologic tests. Genetic analyses of the CPVT patients were performed using a combination of methods of PCR, single-strand conformation polymorphism (SSCP) analysis, and denaturing high-performance liquid chromatography (DHPLC) (Wave Transgenomics). The cellular effects of the newly identified CPVT-linked CASQ2 mutation were studied in isolated adult rat ventricular myocytes infected with adenoviruses for expression of either the wild-type (WT) or mutant forms of CASQ2. Cytosolic and intra-SR [Ca^{2+}] changes were monitored using confocal microscopy, and whole cell currents were recorded with the patch-clamp technique. In vitro single-RyR2 channel recordings and CASQ2 Ca^{2+}-binding measurements were performed.

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

Results

Identification of a Novel CPVT-Associated Mutation in CASQ2

The CASQ2 coding sequence from a patient diagnosed with CPVT revealed the presence of a previously unidentified sequence alteration (online data supplement). This alteration changed codon 33 of CASQ2 from CGA to CAA and resulted in the nonconservative substitution of glutamine for arginine (R33Q). This residue is located within a conserved region of CASQ2, and this position in the related CASQ1 protein is also occupied by arginine in all CASQ2 and CASQ1 sequences available in public databases (Figure I in the online data supplement). Analysis of the domain structures of

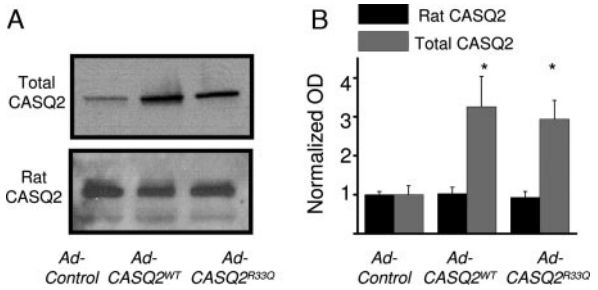

Figure 1. Immunoblot analysis of calsequestrin levels in myocytes infected with *Ad-Control*, *Ad-CASQ2^{WT}*, and *Ad-CASQ2^{R33Q}* vectors. A, Representative Western blot of total CASQ2 (rat and human) (top) and rat CASQ2 alone (bottom). B, Normalized optical density for rat and total CASQ2. Comparisons were performed by using 1-way ANOVA. *Significance was defined at $P<0.05$ (n=8 and 5 for total and rat CASQ2, respectively). The measurements were performed 48 to 56 hours after infection of myocytes with the Adv constructs.

CASQ2 and particularly CASQ1 suggest that this residue is located in a domain involved in protein–protein interactions that may participate in the formation of CASQ polymers or interactions with other components of the junctional complex.[10,15,18] Interestingly, a different mutation in this codon was previously reported that resulted in a stop codon in place of the arginine residue,[3] suggesting it may represent a relatively frequently mutated genomic location.

Electrophysiological Recordings and Intracellular Ca^{2+} Transients in Myocytes Expressing $CASQ2^{R33Q}$

CASQ2 is a major intracellular Ca^{2+}-binding protein that plays a key role in cardiac excitation–contraction (EC) coupling. To test whether the R33Q substitution in CASQ2 caused substantial changes in EC coupling and intracellular Ca^{2+} handling, we examined the effects of overexpressing the $CASQ2^{R33Q}$ protein on a series of electrophysiological and intracellular Ca^{2+}-handling parameters in rat ventricular myocytes. In these experiments, cultured myocytes were infected with adenoviral vectors engineered to direct the expression of either human $CASQ2^{WT}$ or $CASQ2^{R33Q}$. An adenovirus containing a nontranslatable fragment of CASQ2 sequence was used as an infection control. We have previously used this experimental strategy to characterize the effects of a different CPVT-associated CASQ2 mutant protein on myocyte function.[16] In agreement with our earlier studies, immunoblot analysis revealed that this infection protocol resulted in a ≈3-fold increase in total CASQ2 protein levels in cells infected with either the $CASQ2^{WT}$ or $CASQ2^{R33Q}$ adenovirus, whereas the control virus did not affect CASQ2 levels (Figure 1). The increase in total CASQ2 abundance was caused by expression of the mutant protein because endogenous protein levels remained unchanged in $CASQ2^{R33Q}$ cells, as determined by an antibody that recognizes the rat but not the human form of CASQ2.

Initially, the effects of $CASQ2^{R33Q}$ expression on SR Ca^{2+} handling and release were tested. The total SR Ca^{2+} content of control myocytes, or myocytes expressing $CASQ2^{WT}$ or $CASQ^{2R33Q}$, was assessed from the amplitude of the Ca^{2+} transients and the integral of Na/Ca^{2+} exchange current (I_{NCX})

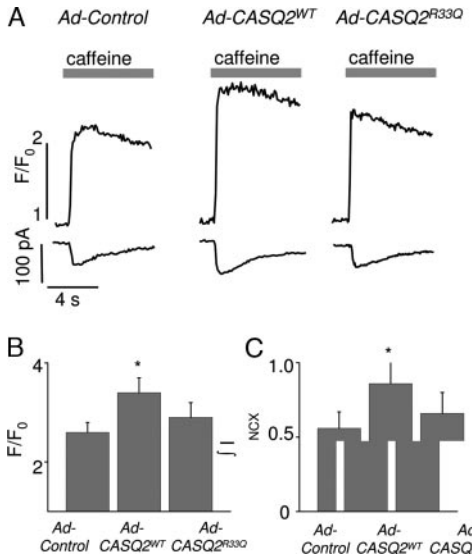

Figure 2. Effects of expression of CASQ2^{WT} or CASQ2^{R33Q} on myocyte SR Ca^{2+} content. A, Representative traces of caffeine-induced Ca^{2+} transients (upper traces) and NCX currents (lower traces) in myocytes infected with *Ad-Control*, *Ad-CASQ2^{WT}*, and *Ad- CASQ2^{R33Q}* vectors. B and C, Pooled data for caffeine-induced Ca^{2+} transients (B) and I_{NCX} integrals (C) for the 3 groups of cells. Data are mean±SE from 5 to 7 experiments in myocytes from 6 heart preparations. Comparisons were performed by using 1-way ANOVA. *Significance was defined at $P<0.05$.

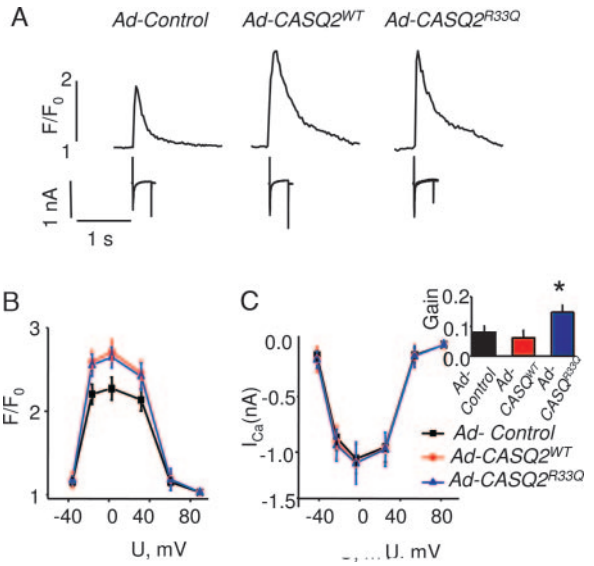

Figure 3. Effects of expression of CASQ2^{WT} or CASQ2^{R33Q} on I_{Ca} and Ca^{2+} transients in cardiac myocytes. A, Representative recordings of I_{Ca} (lower traces) and intracellular Ca^{2+} transients (upper traces) evoked by depolarizing steps from a holding potential of −50 to 0 mV in cardiomyocytes infected with *Ad-CASQ2^{WT}*, *Ad-CASQ2^{R33Q}*, and *Ad-Control* vectors. B and C, Voltage dependencies of Ca^{2+} transients (B) and I_{Ca} (C) in myocytes infected with *Ad-Control* (black), *Ad-CASQ2^{WT}*, (red), or *Ad-CASQ2^{R33Q}* (blue) vectors. C (inset), Gain of CICR for the same 3 groups of cells. Gain was assessed from the equation $d(F/F_0)/dt/I_{Ca}/(F_{caf}/F_0)$, where F, I_{Ca}–Ca^{2+} release, and Ca^{2+} current were measured on depolarization to 0 mV. Data are mean±SE from 3 to 10 experiments performed in myocytes from 8 heart preparations.

evoked by the application of caffeine. Although, ectopic expression of the WT protein resulted in a dramatic increase in SR Ca^{2+} content, no statistically significant changes in SR Ca^{2+} content were observed with expression of CASQ2^{R33Q} (Figure 2A and 2B).

Next, the effects of overexpression of CASQ^{WT} and CASQ2^{R33Q} on Ca^{2+} release during EC coupling were compared in myocytes undergoing voltage clamp stimulation. The amplitude of the I_{Ca}-induced Ca^{2+} transients was similarly increased ≈30% in myocytes overexpressing both forms of CASQ2 (Figure 3 and supplemental Table I). However, whereas the duration of the rising phase of the Ca^{2+} transients was slowed in CASQ2^{WT}-overexpressing cells, Ca^{2+} transient rise was accelerated in CASQ2^{R33Q}-expressing myocytes (supplemental Table I). Additionally, expression of CASQ2^{WT} and CASQ2^{R33Q} had opposite effects on the gain of CICR (ie, Ca^{2+}-release rate for a given Ca^{2+} trigger and a given SR Ca^{2+} content), a term that characterizes the efficiency of I_{Ca} to elicit Ca^{2+} release. Whereas overexpression of WT CASQ2 resulted in a decreased gain of CICR, expression of the mutant form of the protein increased CICR gain (Figure 3C, inset). Therefore, expression of CASQ2^{R33Q} enhanced the functional activity of the Ca^{2+}-release mechanism with respect to both control myocytes and myocytes overexpressing the WT form of the protein. Because the potentiating effects of R33Q on the Ca^{2+}-release mechanism occurred on the background of a full set of native CASQ, they can be qualified as "dominant positive" effects. In general, these effects strongly suggest that the mutant protein disrupts protein–protein interactions involved in control of the SR Ca^{2+}-release process.

Periodic Pacing

CPVT is associated with ventricular tachycardia, particularly in response to adrenergic stimulation, and thus we next examined whether CASQ2^{R33Q} expression would affect electrical and intracellular Ca^{2+} signals in rhythmically paced cardiac myocytes. Periodic Ca^{2+} transients and action potentials (APs) were compared in myocytes overexpressing CASQ2^{WT} and CASQ2^{R33Q} undergoing rhythmic stimulation in the absence or presence of isoproterenol (ISO). In control and CASQ2^{WT}-overexpressing myocytes, we observed stable, rhythmic Ca^{2+} transients and APs both in the absence and presence of ISO (1 μmol/L; 8 and 6 experiments, respectively; not shown). In the absence of ISO, CASQ2^{R33Q} myocytes also showed only regular AP-induced Ca^{2+} transients. However, following exposure to 0.01 to 1 μmol/L ISO, these cells developed characteristic disturbances in Ca^{2+} release and electrical activity manifested as extrasystolic Ca^{2+} transients, delayed afterdepolarizations (DADs), and irregular APs (Figure 4 and supplemental Figure IV). The percentage of cells exhibiting such disturbances increased with increasing ISO concentration (from ≈30% at 0.01 μmol/L to a maximum of 80% at 0.2 to 1 μmol/L ISO).

Ca^{2+} Sparks and Waves

To further understand the effects of CASQ2^{R33Q} on the Ca^{2+}-release mechanism, we measured spontaneous local (sparks) and global (waves) Ca^{2+} signals in saponin-permeabilized myocytes maintained at a constant cytosolic [Ca^{2+}]

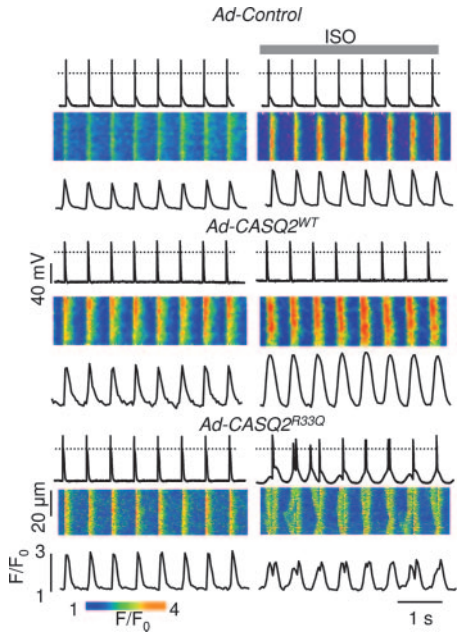

Figure 4. Arrhythmogenic disturbances in Ca^{2+} cycling in myocytes expressing $CASQ2^{R33Q}$. Recordings of membrane potential (upper traces), along with line-scan images (middle traces) and averaged temporal profiles (lower traces) of fluo-3 fluorescence in myocytes infected with *Ad-Control*, *Ad-CASQ2*^{WT}, and *Ad-CASQ2*^{R33Q} vectors. The myocytes were stimulated at 2 Hz in the presence of 1 μmol/L ISO.

(≈75 nmol/L). Consistent with earlier results, overexpression of $CASQ2^{WT}$ resulted in an increase in the magnitude and slowing of the kinetics of Ca^{2+} sparks without significantly changing the frequency of events (Figure 5A through 5D and supplemental Table II). In contrast, although overexpression of $CASQ^{R33Q}$ did not alter the amplitude of Ca^{2+} sparks, it did increase their frequency (Figure 5A through 5D). The kinetics of the local events in R33Q myocytes, however, did not change with respect to control cells (Figure 5 and supplemental Table II).

Next, the consequences of overexpression of $CASQ2^{WT}$ and $CASQ2^{R33Q}$ on the periodic occurrence of spontaneous Ca^{2+} waves were compared in myocytes incubated in a bathing solution containing 75 nmol/L Ca^{2+} and 100 μmol/L EGTA. Under these conditions, overexpression of $CASQ2^{WT}$ dramatically reduced wave frequency, whereas overexpression of $CASQ2^{R33Q}$ caused an increase in Ca^{2+} wave occurrence (Figure 5E and supplemental Table III). As with I_{Ca}-induced Ca^{2+} transients (see Figure 3 and supplemental Table I), the amplitude of spontaneous Ca^{2+} transients was increased in both $CASQ2^{WT}$ and $CASQ2^{R33Q}$-overexpressing myocytes, and the kinetics of Ca^{2+} transients were slowed in $CASQ2^{WT}$ but accelerated in $CASQ2^{R33Q}$ cells (Figure 5E and supplemental Table III). Of note, expression of $CASQ2^{R33Q}$ also resulted in an increase in the frequency of Ca^{2+} sparks and waves in intact myocytes loaded with fluo-3 acetoxymethyl ester (fluo-3 AM) (supplemental Table VI and supplemental Figure II), indicating that the observed $CASQ2^{R33Q}$-induced changes in Ca^{2+} signals were not attributable to myocyte permeabilization. These results suggest that expression of $CASQ2^{R33Q}$ enhances the propensity for spontaneous Ca^{2+} release from the SR, apparently by increasing the functional activity of the RyR2 channels.

Intra-SR $[Ca^{2+}]$

Given the 2 potential functions of CASQ2 (ie, as a Ca^{2+}-binding protein and as a modulator of the RyR2 channel), expression of the R33Q mutant could influence the total amount of Ca^{2+} stored in the SR by changing SR Ca^{2+} buffering and/or by affecting Ca^{2+} leak through RyR2s. To distinguish between these mechanisms, we performed measurements of free $[Ca^{2+}]$ inside the SR ($[Ca^{2+}]_{SR}$). The total resting SR luminal $[Ca^{2+}]$ is determined by both Ca^{2+} transport across the SR membrane and Ca^{2+} binding to luminal buffers. On the other hand, owing to the finite nature of the SR Ca^{2+} store, the steady-state free SR $[Ca^{2+}]$ is independent of the concentration of intra-SR Ca^{2+}-binding sites and is solely governed by a balance between Ca^{2+} leak and Ca^{2+} uptake across the SR membrane. Therefore, potential changes in free basal $[Ca^{2+}]_{SR}$ should provide good indications for altered RyR2 activity. $[Ca^{2+}]_{SR}$ was monitored

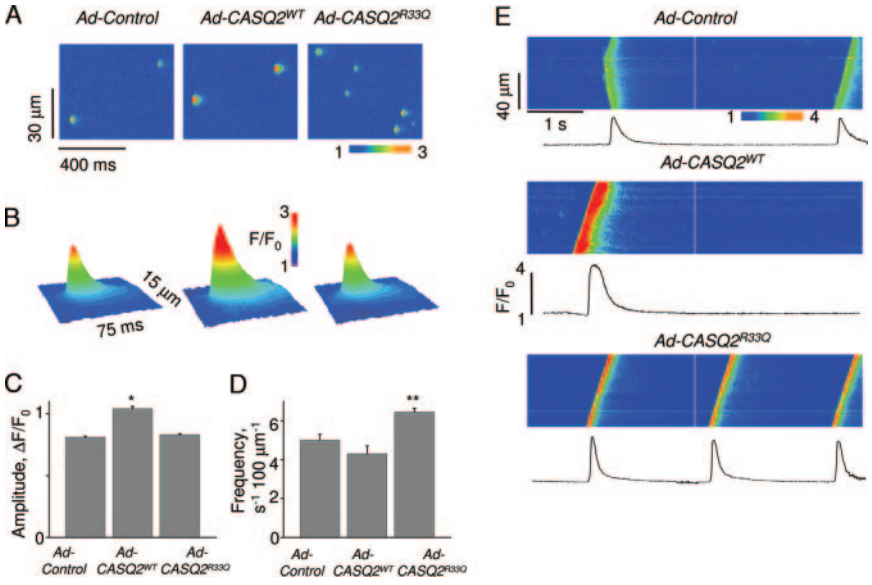

Figure 5. Effects of expression of $CASQ2^{WT}$ or $CASQ2^{R33Q}$ on properties of spontaneous Ca^{2+} sparks and waves in saponin-permeabilized myocytes. A and B, Representative line-scan images of Ca^{2+} sparks (A) and averaged sparks surface plots (B) in cells infected with *Ad-Control*, *Ad-CASQ2*^{WT}, and *Ad- CASQ2*^{R33Q} vectors. C and D, Bar graphs of pooled values of spark amplitude (C) and the frequency (D) for the same groups of cells. Data are mean±SE (based on analysis of 794 to 1544 sparks from 23 to 32 myocytes from 6 heart preparations). Comparisons were performed by using 1-way ANOVA. Significance was defined at $^{*}P<0.001$ or $^{**}P<0.05$. E, Representative line-scan images along with time-dependent profiles of spontaneous Ca^{2+} waves acquired in permeabilized myocytes infected with *Ad-Control*, *Ad-CASQ2*^{WT}, and *Ad-CASQ2*^{R33Q} vectors (as indicated) maintained in a bath solution with reduced Ca^{2+}-buffering strength (100 μmol/L EGTA).

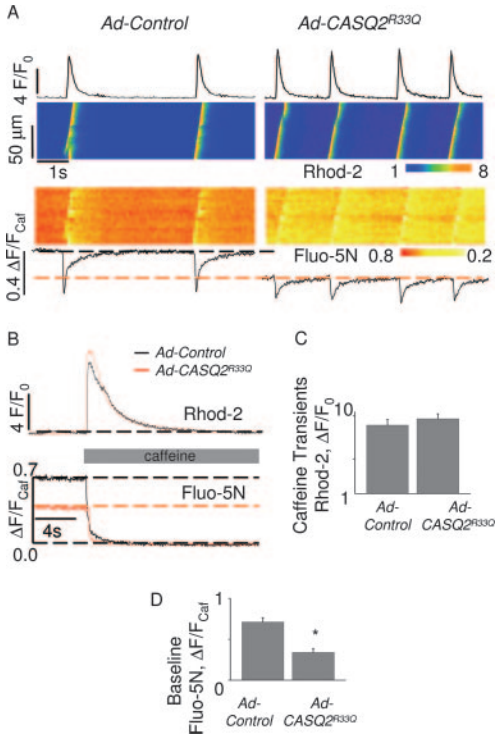

Figure 6. Effects of expression CASQ2^{R33Q} on properties of intra-SR Ca^{2+} waves in permeabilized myocytes. A, Representative line-scan images along with time-dependent profiles of rhod-2 (cytosolic) and fluo-5N (intra-SR) fluorescence in permeabilized myocytes infected with *Ad-Control* and *Ad-CASQ2^{R33Q}*. B, Caffeine-evoked cytosolic Ca^{2+} transients (top) and the associated SR Ca^{2+}-depletion signals (bottom) recorded in myocytes infected with *Ad-Control and Ad-CASQ2^{R33Q}* vectors. C, Pooled data showing the lack of effects on the amplitude of caffeine-induced Ca^{2+} transients recorded with Rhod-2. D, Pooled data showing the changes in steady-state $[Ca^{2+}]_{SR}$ measured with SR-entrapped fluo-5N. The data are presented as mean±SE (n=14 to 21). *Significantly different from control at $P<0.05$ (1-way ANOVA).

by the low-affinity Ca^{2+} indicator fluo-5N loaded into the SR. The cytosolic Ca^{2+} signal was recorded simultaneously using the Ca^{2+} dye rhod-2. In comparison with control cells, the basal $[Ca^{2+}]_{SR}$ was significantly reduced in CASQ2^{R33Q} myocytes, as evidenced by the reduced intensity of the SR-entrapped fluo-5N (Figure 6). Additionally, the amplitudes of the Ca^{2+}-depletion signals during waves were diminished in CASQ2^{R33Q}-expressing myocytes (Figure 6 and supplemental IV). Importantly, fluo-5N fluorescence in both cell types was similar after depletion of the SR Ca^{2+} store by caffeine (supplemental Table IV), indicating that the changes in fluo-5N fluorescence in Ca^{2+}-loaded SR reflected true changes in $[Ca^{2+}]_{SR}$. The reduced $[Ca^{2+}]_{SR}$ is CASQ2^{R33Q} myocytes suggests that SR Ca^{2+} leak was enhanced in these cells, consistent with the increased frequency of spontaneous Ca^{2+} sparks. Similar to intact myocytes (Figure 2), the amplitude of the cytosolic spontaneous and caffeine-induced Ca^{2+} transients was preserved in permeabilized CASQ2^{R33Q} myocytes despite the reduction of $[Ca^{2+}]_{SR}$ (Figure 6B through 6D). The ability of the CASQ2^{R33Q} myocytes to maintain the amplitude of their Ca^{2+} transients despite the reduced $[Ca^{2+}]_{SR}$ is attributable to increased intrastore Ca^{2+}

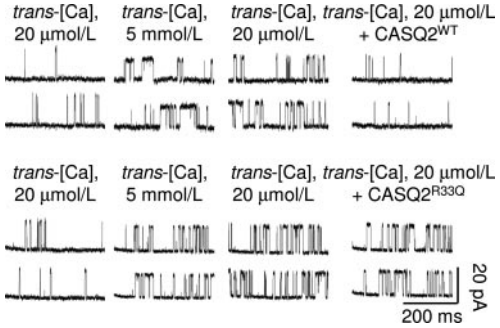

Figure 7. Activity of native RyR2 channels is modulated by CASQ2^{WT} and CASQ2^{R33Q}. Representative single-channel traces illustrating the irreversibility of the effects of 5 mmol/L luminal Ca^{2+} and restoration of initial low activity by CASQ2^{WT} but not CASQ2^{R33Q} added to the *trans* chamber in native RyRs. The P_o values were 0.06±0.02 for low *trans* $[Ca^{2+}]$ (20 μmol/L); 0.34±0.09 for high *trans* $[Ca^{2+}]$ (5 mmol/L); 0.36±0.10 reverting to low *trans* $[Ca^{2+}]$ (20 μmol/L); and 0.04±0.02 (n=5) vs 0.40±0.06 (n=6) after application of 5 to 20 μg/mL *trans* CASQ2^{WT} or CASQ2^{R33Q}, respectively. The data are presented as mean±SE. *Significantly different from WT at $P<0.05$ (1-way ANOVA).

buffering (ie, an increased concentration of Ca^{2+}-binding sites that can bind and release Ca^{2+} on discharge of the store) in myocytes overexpressing CASQ2^{R33Q}. Collectively, these results suggest that CASQ2^{R33Q} expression resulted in both increased leak of Ca^{2+} through the RyR2 and increased intra-SR Ca^{2+}-buffering capacity.

Effects of CASQ^{WT} and CASQ2^{R33Q} on Single-RyR2 Channel Activity

CASQ2 has been shown to inhibit the functional activity of the RyR2 channel complex.[12] To directly examine the effect of the R33Q mutation on the ability of CASQ2 to influence RyR2 behavior, we performed single-RyR2 channel recordings using the planar lipid bilayer technique (Figure 7). Cardiac SR vesicles were incorporated into planar lipid bilayers, and the activity of single-RyR2 channels was measured using Cs^+ as the charge carrier.[12] In these experiments, single RyR2s were stripped of endogenous CASQ2 by exposing the luminal side of the channel to 5 mmol/L Ca^{2+}. This treatment promoted efficient dissociation of CASQ2, as evidenced by the enhanced RyR2 activity that persisted after the $[Ca^{2+}]$ in the *trans* (luminal) chamber was reduced to the initial low level. Consistent with our previous studies,[12] addition of CASQ2^{WT} to the RyR2 complex resulted in a reduction in RyR2 activity. In contrast, addition of CASQ2^{R33Q} to the RyR2 complex did not produce a similar inhibitory effect. Interestingly, subsequent addition of CASQ2^{WT} (in the continuous presence of CASQ2^{R33Q}) did not restore RyR2 open probability (P_o) to that observed with CASQ2^{WT} alone (supplemental Figure V). Therefore, the substitution of glutamine for arginine at amino acid 33 of CASQ2 appears to compromise the ability of the protein to modulate the functional activity of the RyR2 channel. Moreover, the R33Q mutant seems to impair the functional interactions of the WT protein with the RyR2 complex, consistent with the dominant positive effects of the mutant on SR Ca^{2+} release.

Determination of Ca^{2+}-Binding Affinities of Recombinant $CASQ2^{WT}$ and $CASQ2^{R33Q}$

In principle, the pathological effects of the R33Q mutation could be attributable to alterations in the Ca^{2+}-binding properties of the mutant protein. We, therefore, tested whether the $CASQ2^{R33Q}$ protein displayed altered Ca^{2+}-binding affinities when compared with $CASQ2^{WT}$ using Ca^{2+} overlay experiments.[19] Two kinetic parameters of Ca^{2+} binding, the Ca^{2+} affinity (K_d) and capacity (B_{max}), were calculated and are shown in supplemental Table V. Both values were comparable for the two proteins and were in agreement with previous values reported for native $CASQ2^{WT}$.[20] Thus the effects of the R33Q mutation in CASQ2 function appears to be unrelated to changes in Ca^{2+} binding.

Discussion

Genetic defects in the SR Ca^{2+}-handling proteins RyR2 and CASQ2 have been linked to CPVT, a familial disease that predisposes young individuals with structurally normal hearts to sudden cardiac death. In this study, we report on a novel CPVT-linked mutation in CASQ2 that results in the nonconservative substitution of glutamine for arginine at amino acid 33. Using a combination of cellular and in vitro techniques, we demonstrate that ectopic expression of the mutant protein in cardiac myocytes increased the functional activity of the RyR2 channel, thereby increasing the rate of Ca^{2+} leak from the SR and enhancing the propensity of SR Ca^{2+} release to be spontaneously activated.

Molecular Mechanisms of R33Q

The potentiatory effects of CASQ2 on the Ca^{2+}-release channels were evidenced by the following findings. Expression of $CASQ2^{R33Q}$ resulted in a shortening of the activation kinetics of Ca^{2+} transients, and increased CICR gain compared with control myocytes or myocytes overexpressing $CASQ2^{WT}$. Additionally, the frequency of spontaneous Ca^{2+} sparks and waves were increased in myocytes expressing $CASQ2^{R33Q}$. These changes in focal and global cytosolic Ca^{2+} transients were accompanied by a dramatic decrease in intra-SR $[Ca^{2+}]$, consistent with an increase in the leak of Ca^{2+} through RyR2s in $CASQ2^{R33Q}$-expressing cells. The consequences of expressing $CASQ2^{R33Q}$ on Ca^{2+} handling were clearly different from the effects of expressing the $CASQ2^{D307H}$ mutant protein, the only other CPVT-linked CASQ2 mutation that has been characterized at the cellular and molecular level thus far.[16,17] In those earlier studies, ectopic expression of $CASQ2^{D307H}$ in myocytes led to decreases in both active SR Ca^{2+} release and SR Ca^{2+} content.[16,17] These effects were attributed to disruptions of the CASQ2 polymerization[16] that is required for high-capacity Ca^{2+} binding, although in vitro binding studies also indicated that the mutant protein interacted more weakly with triadin and junctin.[17]

Several key pieces of experimental data from our study suggest that $CASQ2^{R33Q}$ exerts its effects by disrupting protein–protein interactions within the RyR2 complex rather than by compromising the Ca^{2+}-binding capacity of CASQ2. The free $[Ca^{2+}]$ in the SR lumen at steady state is determined by the balance of Ca^{2+} leakage and uptake across the SR membrane and should not be influenced by the concentration of Ca^{2+}-binding sites inside the SR. Therefore, the reduced $[Ca^{2+}]_{SR}$ combined with the increased spark frequency observed in $CASQ2^{R33Q}$-expressing myocytes strongly suggests that RyR2 activity was enhanced independent of any changes in the intra SR Ca^{2+}-buffering capacity. Planar lipid bilayer experiments provided further evidence for altered interactions of $CASQ2^{R33Q}$ with the RyR2 channel complex. In this system, the inclusion of $CASQ2^{WT}$ decreases the open probability of RyR2 channels, presumably via interactions with triadin or junctin (present study and others[12,21]). However, the R33Q mutation abolished the ability of CASQ2 to inhibit RyR2 activity.

At the same time, the total SR Ca^{2+} content (judged from the size of caffeine-induced Ca^{2+} transients) was preserved in cells expressing $CASQ2^{R33Q}$, indicating that the concentration of Ca^{2+}-binding sites in the SR increased, as would be expected if the mutant protein maintained its Ca^{2+}-binding function. Similarly, the mutation did not affect the ability of CASQ2 to bind Ca^{2+} in vitro. Thus, it appears that the R33Q mutation alters intracellular Ca^{2+} handling by compromising interactions of CASQ2 with the RyR2 complex without affecting CASQ2 Ca^{2+}-binding function. Consistent with this conclusion, the N-terminal region of CASQ2, which contains a high proportion of negatively and positively charged amino acids, has been proposed to interact with KEKE motifs in triadin and/or junctin by forming "polar zippers."[10,18]

Implications for Pathophysiology of CPVT

Similar to other genetic forms of CPVT,[16,22] the cellular mechanisms of arrhythmia caused by the R33Q mutation involved spontaneous discharges of the SR Ca^{2+} stores followed by DADs and extrasystolic action potentials (Figure 4). Spontaneous SR Ca^{2+} release in cardiac myocytes is commonly associated with increased SR Ca^{2+} load[23–25] and stimulatory effects of high luminal $[Ca^{2+}]$ on the open probability of RyR2 channels.[26] Our results indicated that in $CASQ2^{R33Q}$-expressing myocytes the predisposition of SR to spontaneous discharges was increased because of enhanced responsiveness of the release mechanism to luminal Ca^{2+}.

It is interesting to note that although expression of $CASQ2^{R33Q}$ produced clear changes in Ca^{2+} handling and electrical activity in myocytes expressing the full set endogenous CASQ2, CPVT does not develop in the heterozygous carriers of the R33Q mutation; in fact, none of the heterozygous carriers in the study developed ventricular arrhythmias. This lack of a clinical phenotype in the heterozygous carriers could be attributable to the lower ratio of $CASQ2^{R33Q}$ to the WT protein in these human subjects (presumably ≈1:1) when compared with our myocyte experiments (≈2:1), leading to less-profound changes in Ca^{2+} handling than in myocytes. In support of this notion, expression of the mutant protein at levels similar to those of the endogenous protein (ie, at a ratio of 1:1; supplemental Figure III) did not result in changes in Ca^{2+} handling observed with higher mutant expression. However, we note that our rat myocyte model can be taken as only an approximate representation of the results of mutant protein expression during human disease. Species-related differences

in intracellular Ca^{2+} handling and membrane excitability, the likely presence of compensatory mechanisms in human disease but not during the acute myocyte experiments, and differences in adrenergic stimulation are only some of the factors that may complicate such a comparison.

Abnormal Modulation of RyR2 Channels by Luminal Ca^{2+} as a Common Mechanism for Various Genetic Forms of CPVT

To date, 4 mutations in the *CASQ2* gene have been linked to CPVT.[2,3] In addition, a number of mutations in the *RyR2* gene have been reported to be associated with CPVT.[27] Although the primary molecular alterations caused by the various genetic defects differ, they are likely to converge on a common pathogenic pathway to cause CPVT. Growing evidence indicates that abnormal modulation of RyR2 by luminal Ca^{2+} might be a common pathogenic factor in these genetically distinct forms of CPVT; however, clear proof of such a common mechanism is lacking. Mutations in CASQ2 that compromise either CASQ2 expression or its Ca^{2+}-binding ability reportedly act on RyR2 indirectly by altering the dynamics of free Ca^{2+} in the vicinity of the channel, hence accelerating the channel recovery from a luminal Ca^{2+}-dependent refractory state.[8,16,28] The effects of CPVT-associated RyR2 mutations have been ascribed to either dissociation of FKBP12.6 from the RyR2 causing changes in RyR2 gating[29] (but see George et al[30]) or, more recently, to changes in RyR2 sensitivity to luminal Ca^{2+}.[31,32] Our present findings clearly show that the R33Q mutation disrupts interactions of CASQ2 with the RyR2 complex, thereby sensitizing the release mechanism to activation by luminal Ca^{2+}. We propose that CPVT can be caused by genetic defects in any component of the luminal Ca^{2+}-signaling pathway, including steps involved in (1) controlling and sensing free Ca^{2+} in the vicinity of RyR2, (2) transmitting the luminal Ca^{2+} change signal to RyR2, and (3) RyR2-gating conformations. Our results strongly support a concept of abnormal luminal regulation as a common mechanism for genetically-distinct forms of CPVT.

Conclusions

In conclusion, our results show that substitution of glutamine for arginine at amino acid 33 of CASQ2 is a naturally occurring mutation that leads to CPVT in homozygous carriers. The underlying molecular mechanism of this mutation appears to involve disrupted interactions of CASQ2 with the proteins of the RyR2 Ca^{2+}-release complex, resulting in enhanced sensitivity of the RyR2 channel to activation by luminal Ca^{2+}. The enhanced responsiveness of RyR2s to luminal Ca^{2+} in turn leads to the generation of extrasystolic spontaneous Ca^{2+} transients, DADs, and arrhythmogenic action potentials in myocytes expressing $CASQ2^{R33Q}$. These results show that intracellular Ca^{2+} cycling in the normal heart relies on an intricate interplay of CASQ2 with the proteins of the RyR2 channel complex and that disruption of these interactions can lead to cardiac arrhythmias.

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ONLINE DATA SUPPLEMENT

ABNORMAL INTERACTIONS OF CALSEQUESTRIN WITH THE RYANODINE RECEPTOR CALCIUM RELEASE CHANNEL COMPLEX LINKED TO EXERCISE-INDUCED SUDDEN CARDIAC DEATH

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MATERIAL AND METHODS

Clinical evaluation

An 11-year old girl with unexplained syncopal episodes that occurred during exercise was referred to our center for clinical and molecular evaluation. Cardiological evaluations were performed included resting ECG, exercise stress testing, echocardiogram and Holter recording. Genetic counseling was performed and the parents of the proband provided written informed consent for clinical and genetic evaluation. The protocol was approved by the institutional review board of the Fondazione Salvatore Maugeri.

Genetic Analysis

DNA was extracted from peripheral blood lymphocytes using a standard phenol-chloroform method. The complete coding regions of genes associated with long QT syndrome (*KCNQ1*, *KCNH2*, *SCN5A*, *KCNE1*, *KCNE2)* and CPVT *(CASQ2 and RyR2)* genes were amplified by polymerase chain reaction (PCR) using exon-flanking intronic primers. All 104 exons of RyR2 were analysed by single-strand conformation polymorphism (SSCP) while PCR products encompassing the 11 exons of the *CASQ2* gene (NM_001232) and each of the genes associated with long QT syndrome were analyzed by denaturing high-performance liquid chromatography (DHPLC-Wave Transgenomics). All PCR products displaying abnormal SSCP patterns or DHPLC elution profiles were sequenced with a BigDye terminator sequencing kit (Applied Biosystems) on an ABI Prism 310 Genetic Analyzer^{tm}. These sequences were compared to sequences of 600 alleles from a reference group of healthy subjects to exclude sequence differences that represent rare polymorphisms rather than disease-associated mutations.

Construction of human CASQ2 expression vectors

The full length coding sequence of human *CASQ2* gene was amplified directly from pools of a human heart cDNA library (Stratagene) using the Gene Amp XL-PCR Kit (Roche). The PCR primers used were Cas1F and Cas11R, which anneal within the 5'- and 3'-untranslated regions, respectively. The 1.2 Kb PCR product was cloned into the pGEM-dT Easy Vector (Promega) and sequenced to ensure that no unintended changes were inserted during amplification. The codon for the arginine residue at position 33 was changed to glutamine by site-directed mutagenesis using the QuikChange Mutagenesis Kit (Stratagene). The introduction of the desired mutation was confirmed by sequencing the entire coding region. The wild-type ($CASQ2^{WT}$) or $CASQ2^{R33Q}$ cDNAs were subcloned in pENTR-4 Vector (Invitrogen) and

subsequently transferred into the Adenoviral Expression pAD/DEST Vector (Invitrogen) using the Gateway System, according to the manufacturer instructions.

Adenoviral infection of ventricular myocytes

Ventricular myocytes were obtained from adult male Sprague-Dawley rat hearts by enzymatic dissociation and infected with adenoviruses at a multiplicity of infection of 100 as described previously[1]. The cells were incubated at 37°C in a 5% CO_2/95% air environment and experiments were performed 48 to 56 hours after infection of myocytes with the adenoviral constructs.

Electrophysiological Recordings

Electrophysiological analyses were performed in cells incubated in an external solution containing (in mmol/L): 140 NaCl, 5.4 KCl, 1 $CaCl_2$, 0.5 $MgCl_2$, 5.6 glucose and 10 HEPES (pH 7.3). Whole-cell patch-clamp recordings of transmembrane ionic currents were performed with an Axopatch 200B amplifier (Axon Instruments). Patch pipettes (tip resistance of 1 to 3 MΩ) were filled with a solution that contained (in mmol/L): 90 Cs-aspartate, 50 CsCl, 3 Na_2ATP, 3.5 $MgCl_2$, 10 HEPES (pH 7.2) and 0.05 Fluo-3 K-salt. The myocytes were stimulated by application of 400-ms–long voltage pulses to specified membrane potentials from a holding potential of –50 mV at 1-minute intervals. For current clamp recordings Cs in the pipette solution was substituted with K.

Confocal Ca Measurements

Intracellular Ca imaging was performed using an Olympus Fluoview 1000 Laser Scanning Confocal microscope equipped with an Olympus 60x 1.4 N.A. oil objective. Fluo-3 was excited by the 488-nm line of an argon-ion laser and the fluorescence was acquired at wavelengths >510 nm in the line-scan mode of the confocal system at a rate of 2-5 ms per line. The internal solution for recording spontaneous Ca sparks and waves in permeabilized myocytes contained (in mmol/L): 120 K-aspartate, 20 KCl, 3 MgATP, 10 phosphocreatine, 5 U ml^{-1} creatine phosphokinase, 0.03 Fluo-3 K-salt, 0.5 or 0.1 EGTA ([Ca]~75 nmol/L) and 20 HEPES (pH 7.2).

Intra-SR and cytosolic Ca levels were simultaneously monitored by initially loading myocytes with Fluo-5N AM (10 μmol/L for 8-9 hours at 37^0C) and subsequently saponin-permeabilizing the myocytes to remove Fluo-5N from the cytoplasm, and to introduce Rhod-2 (30 μmol/L) to the internal solution, for cytosolic Ca measurements. Calcium waves were initiated by lowering the Ca^{2+} buffering capacity in the internal solution from 0.5 to 0.1 mmol/L EGTA (free Ca ~75 nmol/L^2). Fluo-5N and Rhod-2 were excited by 488- and 543-nm laser lines and fluorescence was measured at wavelengths 500-530 and >560nm, respectively. Line-scan images were obtained by scanning the myocytes in the longitudinal direction at a rate of 5 ms per line. To avoid contamination of the Rhod-2 signal by Fluo-5N fluorescence, a sequential line by line scanning mode was used. F_{max} for Fluo-5N was assessed by application of 20 μmol/L ionomycin, 10 mmol/L $CaCl_2$ and 100 mmol/L BDM to avoid movement. For quantitative studies, the temporal dynamics in fluorescence were expressed as $\Delta F/F_{Caf}$ $(F-F_{Caf})/F_{Caf})$, where F_{Caf} represents the fluorescence level of the cells after the application of 10 mmol/L caffeine.

Single RyR2 Channel Recordings

Heavy SR microsomes were isolated from canine left ventricular tissue. Single RyR2-containing channels were reconstituted by fusing SR microsomes into planar lipid bilayers and single channel currents were recorded as described previously[3]. Experimental solutions contained 350 mmol/L $CsCH_3SO_3$, 0.07 mmol/L $CaCl_2$, 3 mmol/L MgATP, 20 mmol/L HEPES (pH 7.4) on the cytosolic (cis) side of the bilayer, and 20 mmol/L $CsCH_3SO3$, 0.0001-2 mmol/L $CaCl_2$, 0.1 mmol/L EGTA, 20 mmol/L HEPES (pH 7.4) on the luminal (*trans*) side of the bilayer. Single channel currents were recorded at room temperature (21 to 23°C) with an Axopatch 200A (Axon Instruments) patch-clamp amplifier. Data were digitized at 5 to 10 kHz and filtered at 2 kHz. Acquisition and analysis of data were performed using pClamp 6.01 software (Axon Instruments). Recombinant purified wild-type and mutant CASQ2 were added to the trans side at concentrations of 5-20 μg/mL.

Western Blotting

The levels of CASQ2 were determined by immunoblot analysis. Cell lysate proteins (10 μg) were subjected to 4% to 20% SDS-PAGE, blotted onto nitrocellulose membranes (Bio-Rad Labs). Anti-CASQ2 antibodies were from Affinity Bioreagents (PAI-913; for detection of both rat and human CASQ2) and from Upstate (06-382; for detection of rat CASQ2 alone). Blots were developed with Super Signal West Pico (PIERCE) and quantified using a Visage 2000 Blot Scanning and Analysis system (BioImage Systems Corporation).

Expression and purification of recombinant CASQ2 proteins

Recombinant CASQ2 proteins were expressed from pET-5a-based plasmids in BL21 (DE3) E. *coli* cells (Novagen). For production of large quantities of recombinant proteins, fresh colonies carrying the different constructs were inoculated into 2 mL of Luria broth in the presence of ampicillin (100 μg/μL) and 1% glucose and grown overnight with shaking at 37°C. 100 μL of the saturated culture was diluted 1:1000 in Luria broth and grown at 37 °C to an OD_{600} of 0.6. Expression of recombinant proteins was induced by growing cells for three hours at 37 °C in the presence of 0.5 mmol/L isopropyl-β-D-thiogalactopyranoside with constant shaking. Cells were harvested and washed with cold phosphate-buffered-saline and re-suspended in 50 mL lysis buffer containing 50 mmol/L Tris-Cl (pH 7.5), 5 mmol/L DTT, 1 mmol/L EDTA and 0.1 mg/mL lysozyme for subsequent phenyl-Sepharose Purification[4], in the presence of aprotinin (1μg/μL), leupeptin (2μg/μL) and benzamidine (1 mmol/L). The cells were lysed by sonication (B. Brown Biotech International) on ice with three 20-s strokes. The cells were centrifuged at 50,000g for 30 min at 4 °C and the the supernatant was collected and incubated with 16 ml of phenyl-Sepharose resin (pre-washed and equilibrated in binding buffer (20 mmol/L MOPS (pH 7.2), 5 mmol/L DTT, 1 mmol/L EGTA, and 0.5 mol/L NaCl). The resin was washed two times with binding buffer and recombinant CASQ2 proteins were eluted with 1 column volume of elution buffer (10 mmol/L $CaCl_2$ in 20 mmol/L MOPS (pH 7.2), 1 mmol/L DTT, and 0.5 mol/L NaCl). Concentrations of eluted proteins were determined either using Bradford or Lowry assays[5,6]. Recombinant CASQ2 proteins were dialysed against water and stored at -20°C prior to use in ^{45}Ca overlay and bilayer experiments.

Measurement of binding affinities of recombinant CASQ2 proteins

2-3 μg of recombinant $CASQ2^{WT}$ or $CASQ2^{R33Q}$ was electroblotted onto nitrocellulose membranes in the absence of SDS. ^{45}Ca ligand overlay[7] experiments were performed in a binding buffer containing 5 mmol/L $MgSO_4$, 60 mmol/L KCl, 5 mmol/L imidazole(pH 7.4), and

0.6-6 μmol/L ^{45}Ca (specific activity 5-50 mCi/mg Ca). The membranes were cut into single lanes which were incubated at room temperature for 20 min at total Ca^{2+} concentrations ranging from 10 μmol/L to 6 mmol/L. The strips were washed twice with 30% ethanol and the regions containing the recombinant CASQ2 proteins were excised and radioactivity was measured in a scintillation counter. Background values for each lane were obtained by obtaining radioactivity measurements for a separate piece of nitrocellulose membrane having an area equivalent to that of the CASQ2 region.

RESULTS

Clinical Phenotype

The proband DB (born: 03-03-1986) was referred for clinical investigation after two syncopal spells with spontaneous recovery occurred while running at age 9 and 11 years. Family history was positive for sudden death of a 10 year-old brother who experienced syncope and died after he was transported to the emergency room of the local hospital with documented VT and VF. The proband's parents were asymptomatic with unremarkable clinical history and ECG.

The proband's baseline ECG showed normal sinus rhythm, normal QT interval and normal ST-T morphology, but frequent ventricular ectopic beats and runs of non-sustained VT were elicited during exercise stress test. Beta blocker therapy (propranolol) was initiated and an implantable cardioverter defibrillator (ICD) was positioned.

After ICD implant the patient had four appropriate shocks from the device (one at age 11 and three at age 12) always while she was performing sports activity (swimming or soccer). During the following 5 years of follow-up, propranolol was increased to 160mg/kg/day with no further events.

Genetic Analysis

No coding sequence abnormalities in known LQTS genes or the cardiac RyR2 gene were detected. An abnormal elution profile was observed in the exon 1 of the CASQ2 gene. Subsequent DNA sequencing analysis showed a single nucleotide transition (G98A) leading to a non-conservative substitution of glutamine for arginine at amino acid 33 of CASQ2 (R33Q). The mutation was present on both alleles (homozygous) and genetic analysis on the parents' DNA revealed that both of them harbored the R33Q in the heterozygous state.

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FIGURE LEGENDS

Figure 1. Online Data Supplement. Identification of CASQ2 mutation in CPVT. A) The domain structure of human CASQ2 is diagrammed to show the position of the signal sequence (SS), two putative protein-protein interaction domains (A and B, based on studies on CASQ1) and an Asp/Glu-rich region at the C-terminus. The amino acid coordinates are based on the precursor protein and reflect sequence homologies with the canine CASQ2 protein as previously described[8]. The underlined region is shown at the sequence level in B. B) Location of the altered amino acid in the CASQ2^{R33Q} mutant protein. The amino acid sequences from the indicated CASQ proteins were obtained from public databases and aligned using the AlignX component of the Vector NTI software package (Invitrogen, Carlsbad, CA). Amino acids that are identical in all proteins are highlighted in yellow and the R33Q substitution is shown in red. Accession numbers for each protein are listed. C. el.: *Caenorhabditis elegans*.

Figure 2. Online Data Supplement. Expression of *Ad-CASQ2^{R33Q}* increases Ca spark frequency in intact cardiac myocytes. Representative images of Ca sparks recorded in intact myocytes after 48 hours of infection with *Ad-Control*, *Ad-CASQ2^{WT}* or *Ad-CASQ2^{R33Q}*. Cells were loaded with Fluo 3-AM (5 μmol/L) for 25-30 min and sparks were recorded in the line scan mode of the confocal microscope at a rate of 2 ms per line. Extracellular Tyrode's solution contained 2 mmol/L $CaCl_2$.

Figure 3. Online Data Supplement. Expression of WT and mutant CASQ2 at a ratio ~1:1 does not result in the altered Ca handling observed at higher mutant expression. A) Representative Western blot of total CASQ2 levels in myocytes infected with *Ad-CASQ2^{R33Q}* vectors at different times after infection. B) Normalized optical density (OD). Comparisons were performed by using one way ANOVA, *significance was defined at $P < 0.05$ (n=5). C) Representative recordings of I_{Ca} (lower traces) and intracellular Ca transients (upper traces) evoked by depolarizing steps from a holding potential of –50 mV to 0 mV in cardiomyocytes infected with *Ad-CASQ2^{R33Q}* vector at baseline and at 24 and 48 hrs after infection. The values of peak amplitudes of the Ca transients were 2.1±0.2, 2.1±0.2 and 2.6±0.3 at baseline, 24 and 48 hrs, respectively (n=4-12). None of the 4 myocytes at 24 hrs exhibited the signs of arrhythmic behavior observed in the presence 1 μmol/L ISO in most myocytes at 48 hrs (Fig. 4 of the Article).

Figure 4. Online Data Supplement. Ca cycling in myocytes expressing CASQ2^{R33Q} in the presence of low doses of ISO. Examples of an arrhythmic behavior (lower panel) and a lack of it (upper panel) in CASQ2^{R33Q} myocytes exposed to 10 nmol/L ISO. Recordings of membrane potential (upper traces) along with line-scan images (middle traces) and averaged temporal profiles (lower traces) of fluo-3 fluorescence in CASQ2^{R33Q} myocytes stimulated at 2 Hz before and after addition of 10 nmol/L ISO. Two out of 9 myocytes showed arrhythmic behavior. At concentrations of 0.2-0.5 μmol/L ISO led to arrhythmic behaviour in 8 out of 10 cells.

Figure 5. Online Data Supplement. WT CASQ2 is unable to modulate single RyR2 channel activity in the presence of the R33Q CASQ2 mutant. Single channel recordings were carried out as described for Figure 7 of the article. Po values were 0.40±0.04 and 0.37±0.03 before and after addition of 10 μg/mL $CASQ2^{WT}$ to the *trans* chamber containing 20 μg/mL $CASQ2^{R33Q}$. Results are representative of 3 experiments.

TABLES

Online Table 1. Parameters of I_{Ca} and Ca Transients.

	I_{Ca}			Ca transients			N
	Peak Amplitude (nA)	τ_{fast} (ms)	τ_{slow} (ms)	F/F_0	Rise Time (ms)	τ_{decay} (ms)	
Ad-Control	-1.10±0.14	16.5±4.3	80±27	2.0±0.2	30±4	320±24	14
Ad-CASQ^{WT}	-1.12±0.20	16.8±4.7	86±26	2.7±0.2*	43±4*	407±35*	8
Ad-CASQ^{R33Q}	-1.12±0.19	16.3±4.8	91±36	2.6±0.3*	21±3*	382±44*	12

Data presented as Mean±SE; * P<0.01 *vs*. Control (One Way ANOVA).

Online Table 2. Parameters of Spontaneous Ca Sparks in Permeabilized Myocytes.

	Amplitude $\Delta F/F_0$	Rise Time (ms)	Half Time (ms)	Width, (μm)	Frequency (s^{-1} 100 μm^{-1})	N of Sparks	N of Cells
Ad-Control	0.81±0.01	8.0±0.1	16.3±0.2	2.57±0.02	5.0±0.3	1241	29
Ad-CASQ^{WT}	1.05±0.02*	11.9±0.3*	21.4±0.3*	2.86±0.03*	4.3±0.4	793	23
Ad-CASQ^{R33Q}	0.83±0.01	8.3±0.1	16.7±0.1	2.57±0.02	6.4±0.2**	1544	32

Data presented as Mean±SE; * P<0.001 *vs*. Control; ** P<0.05 *vs*. Control (One Way ANOVA).

Online Table 3. Parameters of Spontaneous Ca Waves in Permeabilized Myocytes.

	Amplitude, Fluo-3 $\Delta F/F_0$	Wave Period (s)	Time at Half Amplitude (ms)	N
Ad-Control	4.99±0.19	5.13±0.27	255±23	16
Ad-CASQ^{WT}	10.48±0.26*	6.5±0.32*	319±14*	18
Ad-CASQ^{R33Q}	6.77±0.22*	2.5±0.42*	205±18*	16

Data presented as Mean±SE; * P<0.05 *vs*. Control (One Way ANOVA).

Online Table 4. Spatiotemporal properties of intra-SR Ca during Ca waves.

	Baseline, $\Delta F/F_{Caf}$	Wave Amplitude $\Delta F/F_{Caf}$	Recovery Half Time (ms)	F_{Caf} (a.u.)	N
Ad-Control	0.71±0.05	0.34±0.02	343±21	296±16	11-14
Ad-CASQ^{R33Q}	0.34±0.05*	0.09±0.01*	423±22**	312±21	10-15

Data presented as Mean±SE; * P<0.001 *vs*. Control; ** P<0.05 *vs*. Control (One Way ANOVA).

Online Table 5. Calcium binding properties of recombinant CASQ2^{WT} and CASQ2^{R33Q}.

Recombinant CASQ2	Kd (mmol/L)	Bmax (pmol/µg)	N
WT	2.15±0.197 S^2=0,039	789±71.8 S^2=5194.9	7
R33Q	2.03±0.163 S^2=0,035	771±40.03 S^2= 1602.8	7

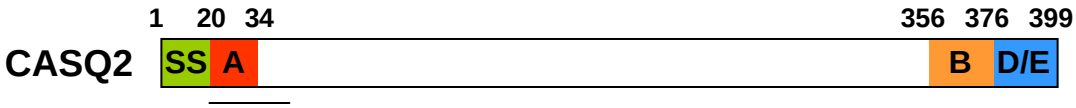
Kd and Bmax values are expressed as mean of N experiments ± S.D: and respective variance (S^2). For variance analysis, F-test was applied. For α=0.05, Kd and Bmax values are F= 1.10 and F=3.2, respectively, where homoscedasticity is significant for F<4.28. For comparison of Ca binding parameters, the unpaired T test was used; differences are considered significant when P < 0.05.

Online Table 6. Parameters of Spontaneous Ca Sparks in Intact Myocytes.

	Amplitude $\Delta F/F_0$	Rise Time (ms)	Half Time (ms)	Width, (µm)	Frequency (s^{-1} 100 μm^{-1})	N of Sparks	N of Cells
Ad-Control	1.19±0.04	10.4±0.5	23.5±0.9	2.24±0.05	2.1±0.3	170	41
Ad-CASQ^{WT}	1.49±0.06*	14.3±0.9*	34.6±1.9*	2.85±0.11*	2.3±0.3	186	35
Ad-CASQ^{R33Q}	1.23±0.06	11.4±0.4	26.3±0.7	2.41±0.05	4.4±0.5*	379	49

Data presented as Mean±SE; * P<0.05 *vs*. Control (One Way ANOVA).

A



B

Human	CASQ2	NP 001223	21	EGLNFPTYDGKDRVVSLSEKNFKQVLKKYDLL
Human	CASQ2	NP 001223	21	EGLNFPTYDGKDQVVSLSEKNFKQVLKKYDLL
Dog	CASQ2	P12637	21	EGLNFPTYDGKDRVVSLTEKNFKQVLKKYDVL
Mouse	CASQ2	NP 033944	22	EGLNFPTYDGKDRVVSLSEKNLKQMLKRYDLL
Rat	CASQ2	NP 058827	21	EGLNFPTYDGKDRVVSLSEKNLKQVLKRYDLL
Rabbit	CASQ2	P31235	21	EGLNFPTYDGKDRVVSLSEKNFKQILKKYDLL
Xenopus	CASQ2	AAH41283	42	EGLHFPTYDGKDRVLELGEKNYRQLMKKHKVF
Human	CASQ1	NP 001222	30	EGLDFPEYDGVDRVINVNAKNYKNVFKKYEVL
Mouse	CASQ1	NP 033943	30	DGLDFPEYDGVDRVINVNAKNYKNVFKKYEVL
Rabbit	CASQ1	P07221	30	EGLDFPEYDGVDRVINVNAKNYKNVFKKYEVL
Rana	CASQ1	P31231	24	DGLDFPEYDGEDRVIHISLKNYKAALKKYEVL
Xenopus	CASQ1	AAH46947	24	DGLDFPEYDGEDRVININLKNYKAALKKYEVL
Chicken	CASQ	CAA68743	21	EGLNFPTYDGKDRVIDLNEKNYKHALKKYDML
C. el	CASQ	NP 510438	31	LGYPDLEYDGFDRTEVLTEKNFNRTVFAEDTK

Figure 1 , Online Data Supplement

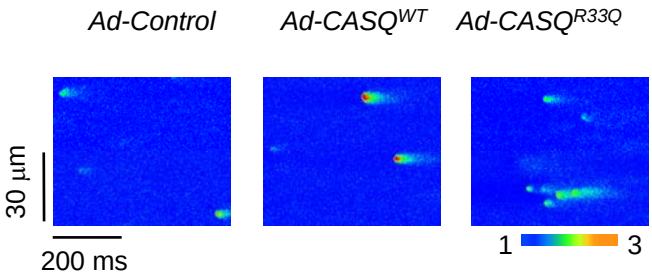
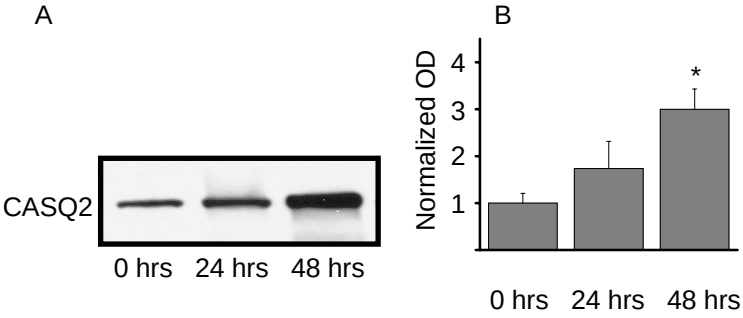


Figure 2 , Online Data Supplement



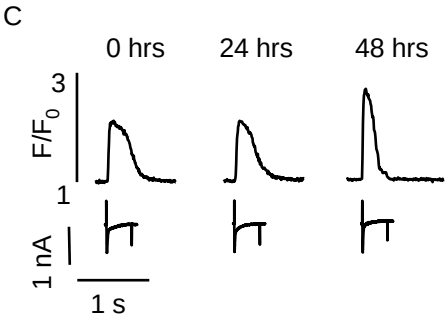


Figure 3 , Online Data Supplement

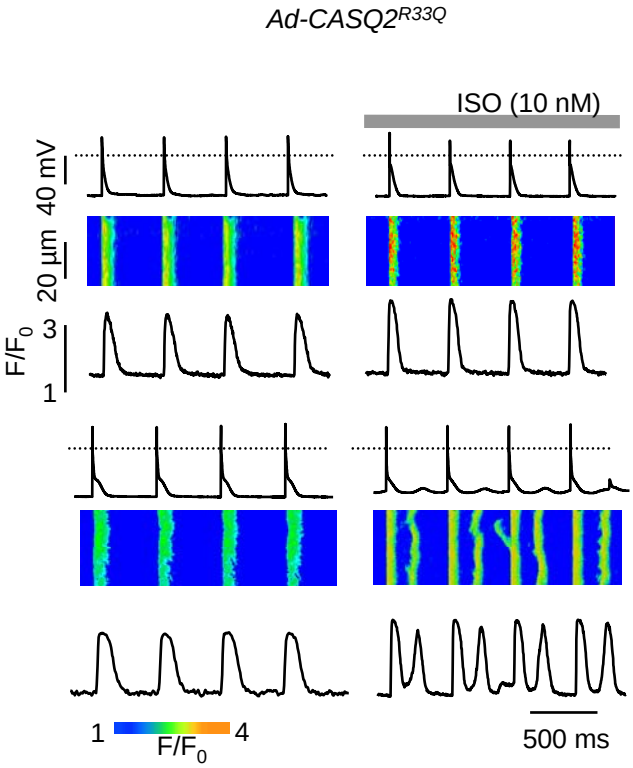
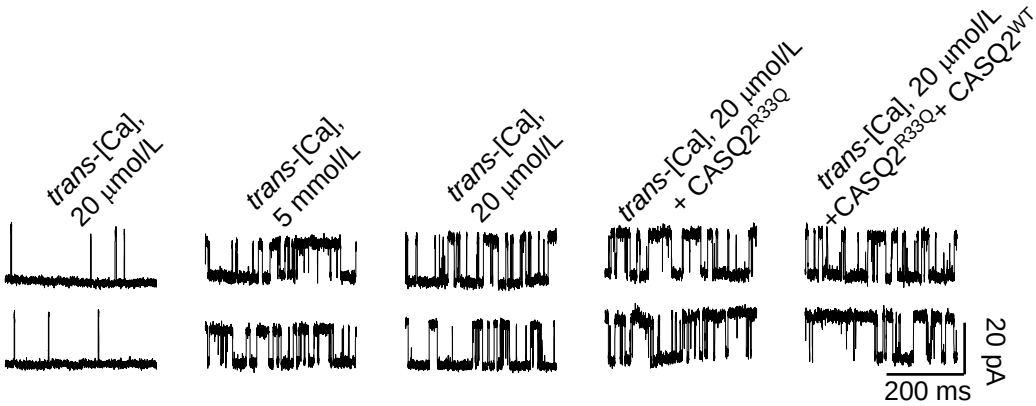


Figure 4 , Online Data Supplement



Online Supplement, Figure 5