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Abnormal Interactions of Calsequestrin With the Ryanodine Receptor Calcium Release Channel Complex Linked to Exercise-Induced Sudden Cardiac Death

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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 1 in the online data supplement). Analysis of the domain structures of

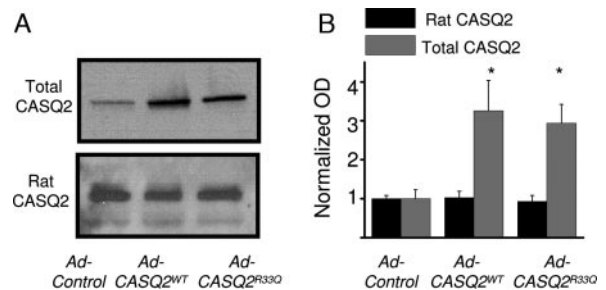


Figure 1. Immunoblot analysis of calsequestrin levels in myocytes infected with *Ad-Control*, *Ad-CASQ2^{WT}*, and *Ad-CASQ2^{R33Q}* vectors. A, Representative Western blot of total CASQ2 (rat and human) (top) and rat CASQ2 alone (bottom). B, Normalized optical density for rat and total CASQ2. Comparisons 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 CASQ2^{R33Q}, was assessed from the amplitude of the Ca^{2+} transients and the integral of Na/Ca^{2+} exchange current (I_{NCX})

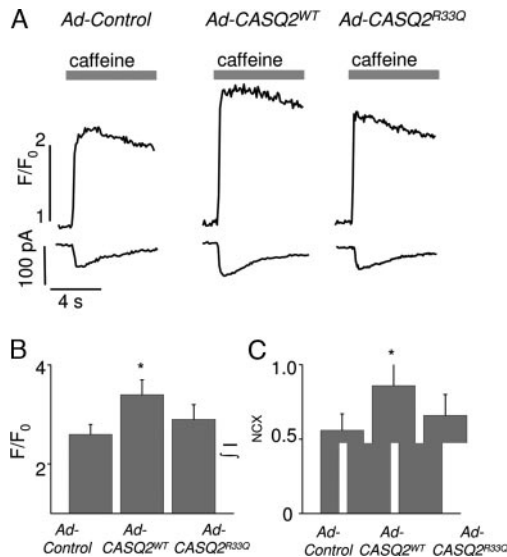


Figure 2. Effects of expression of CASQ2^{WT} or CASQ2^{R33Q} on myocyte SR Ca²⁺ content. A, Representative traces of caffeine-induced Ca²⁺ transients (upper traces) and NCX currents (lower traces) in myocytes infected with *Ad-Control*, *Ad-CASQ2^{WT}*, and *Ad-CASQ2^{R33Q}* vectors. B and C, Pooled data for caffeine-induced Ca²⁺ 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 $P < 0.05$.

evoked by the application of caffeine. Although, ectopic expression of the WT protein resulted in a dramatic increase in SR Ca²⁺ content, no statistically significant changes in SR Ca²⁺ content were observed with expression of CASQ2^{R33Q} (Figure 2A and 2B).

Next, the effects of overexpression of CASQ^{WT} and CASQ^{R33Q} on Ca²⁺ release during EC coupling were compared in myocytes undergoing voltage clamp stimulation. The amplitude of the I_{Ca} -induced Ca²⁺ 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 Ca²⁺ transients was slowed in CASQ^{WT}-overexpressing cells, Ca²⁺ transient rise was accelerated in CASQ^{R33Q}-expressing myocytes (supplemental Table I). Additionally, expression of CASQ^{WT} and CASQ^{R33Q} had opposite effects on the gain of CICR (ie, Ca²⁺-release rate for a given Ca²⁺ trigger and a given SR Ca²⁺ content), a term that characterizes the efficiency of I_{Ca} to elicit Ca²⁺ 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 CASQ^{R33Q} enhanced the functional activity of the Ca²⁺-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 Ca²⁺-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 Ca²⁺-release process.

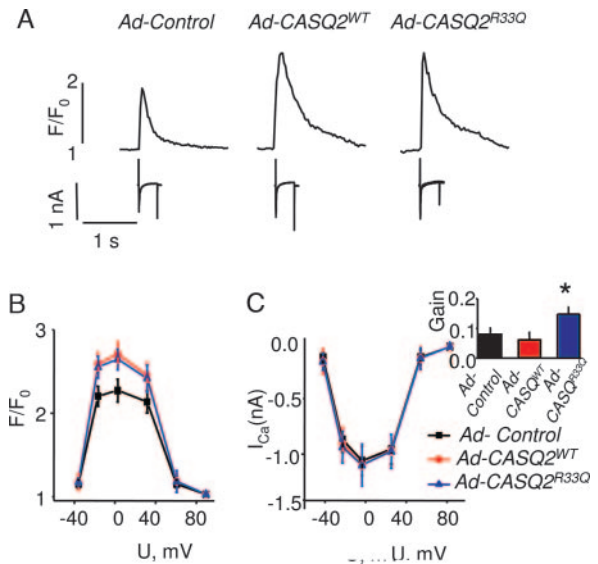


Figure 3. Effects of expression of CASQ2^{WT} or CASQ2^{R33Q} on I_{Ca} and Ca²⁺ transients in cardiac myocytes. A, Representative recordings of I_{Ca} (lower traces) and intracellular Ca²⁺ transients (upper traces) evoked by depolarizing steps from a holding potential of -50 to 0 mV in cardiomyocytes infected with *Ad-CASQ2^{WT}*, *Ad-CASQ2^{R33Q}*, and *Ad-Control* vectors. B and C, Voltage dependencies of Ca²⁺ 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 $d(F/F_0)/dt/I_{\text{Ca}}/(F_{\text{Ca}}/F_0)$, where F_{Ca} —Ca²⁺ release, and Ca²⁺ current were measured on depolarization to 0 mV. Data are mean ± SE from 3 to 10 experiments performed in myocytes from 8 heart preparations.

Periodic Pacing

CPVT is associated with ventricular tachycardia, particularly in response to adrenergic stimulation, and thus we next examined whether CASQ^{R33Q} expression would affect electrical and intracellular Ca²⁺ signals in rhythmically paced cardiac myocytes. Periodic Ca²⁺ transients and action potentials (APs) were compared in myocytes overexpressing CASQ^{WT} and CASQ^{R33Q} undergoing rhythmic stimulation in the absence or presence of isoproterenol (ISO). In control and CASQ^{WT}-overexpressing myocytes, we observed stable, rhythmic Ca²⁺ transients and APs both in the absence and presence of ISO ($1 \mu\text{mol/L}$; 8 and 6 experiments, respectively; not shown). In the absence of ISO, CASQ^{R33Q} myocytes also showed only regular AP-induced Ca²⁺ transients. However, following exposure to 0.01 to $1 \mu\text{mol/L}$ ISO, these cells developed characteristic disturbances in Ca²⁺ release and electrical activity manifested as extrasystolic Ca²⁺ 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 $0.01 \mu\text{mol/L}$ to a maximum of 80% at 0.2 to $1 \mu\text{mol/L}$ ISO).

Ca²⁺ Sparks and Waves

To further understand the effects of CASQ^{R33Q} on the Ca²⁺-release mechanism, we measured spontaneous local (sparks) and global (waves) Ca²⁺ signals in saponin-permeabilized myocytes maintained at a constant cytosolic [Ca²⁺]

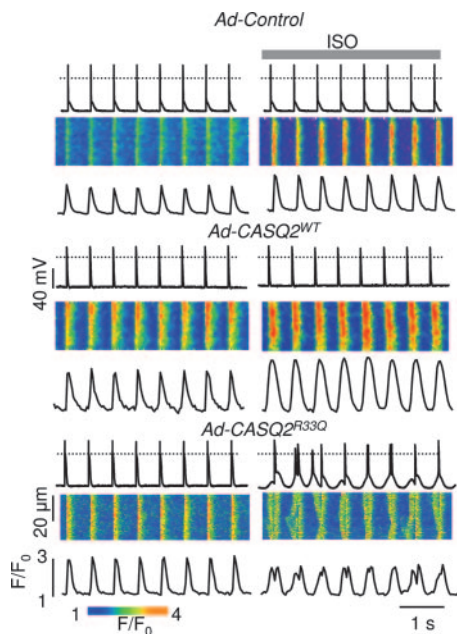


Figure 4. Arrhythmogenic disturbances in Ca^{2+} cycling in myocytes expressing $\text{CASQ2}^{\text{R33Q}}$. Recordings of membrane potential (upper traces), along with line-scan images (middle traces) and averaged temporal profiles (lower traces) of fluo-3 fluorescence in myocytes infected with *Ad-Control*, *Ad-CASQ2^{WT}*, and *Ad-CASQ2^{R33Q}* vectors. The myocytes were stimulated at 2 Hz in the presence of 1 $\mu\text{mol/L}$ 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 $\text{CASQ2}^{\text{R33Q}}$ 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 $\text{CASQ2}^{\text{R33Q}}$ 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 $\mu\text{mol/L}$ EGTA. Under these conditions, overexpression of CASQ2^{WT} dramatically reduced wave frequency, whereas overexpression of $\text{CASQ2}^{\text{R33Q}}$ 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 $\text{CASQ2}^{\text{R33Q}}$ -overexpressing myocytes, and the kinetics of Ca^{2+} transients were slowed in CASQ2^{WT} but accelerated in $\text{CASQ2}^{\text{R33Q}}$ cells (Figure 5E and supplemental Table III). Of note, expression of $\text{CASQ2}^{\text{R33Q}}$ also resulted in an increase in the frequency of Ca^{2+} sparks and waves in intact myocytes loaded with fluo-3 acetoxy-methyl ester (fluo-3 AM) (supplemental Table VI and supplemental Figure II), indicating that the observed $\text{CASQ2}^{\text{R33Q}}$ -induced changes in Ca^{2+} signals were not attributable to myocyte permeabilization. These results suggest that expression of $\text{CASQ2}^{\text{R33Q}}$ 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 ($[\text{Ca}^{2+}]_{\text{SR}}$). 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

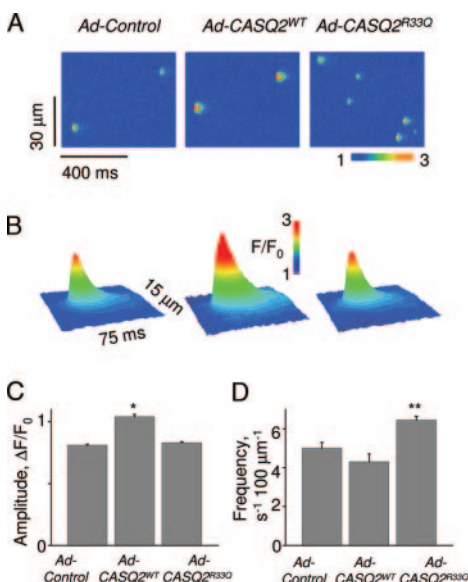


Figure 5. Effects of expression of CASQ2^{WT} or $\text{CASQ2}^{\text{R33Q}}$ on properties of spontaneous Ca^{2+} sparks and waves in saponin-permeabilized myocytes. A and B, Representative line-scan images of Ca^{2+} sparks (A) and averaged sparks surface plots (B) in cells infected with *Ad-Control*, *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 \pm 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 as $*P < 0.001$ 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 $\mu\text{mol/L}$ EGTA).

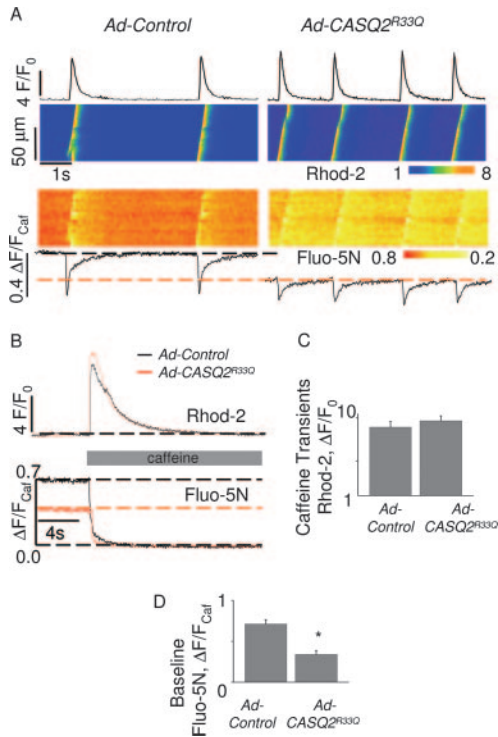


Figure 6. Effects of expression CASQ2^{R33Q} on properties of intra-SR Ca²⁺ waves in permeabilized myocytes. A, Representative line-scan images along with time-dependent profiles of rhod-2 (cytosolic) and fluo-5N (intra-SR) fluorescence in permeabilized myocytes infected with Ad-Control and Ad-CASQ2^{R33Q}. B, Caffeine-evoked cytosolic Ca²⁺ transients (top) and the associated SR Ca²⁺-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 Ca²⁺ transients recorded with Rhod-2. D, Pooled data showing the changes in steady-state [Ca²⁺]_{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 Ca²⁺ indicator fluo-5N loaded into the SR. The cytosolic Ca²⁺ signal was recorded simultaneously using the Ca²⁺ dye rhod-2. In comparison with control cells, the basal [Ca²⁺]_{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 Ca²⁺-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 Ca²⁺ store by caffeine (supplemental Table IV), indicating that the changes in fluo-5N fluorescence in Ca²⁺-loaded SR reflected true changes in [Ca²⁺]_{SR}. The reduced [Ca²⁺]_{SR} in CASQ2^{R33Q} myocytes suggests that SR Ca²⁺ leak was enhanced in these cells, consistent with the increased frequency of spontaneous Ca²⁺ sparks. Similar to intact myocytes (Figure 2), the amplitude of the cytosolic spontaneous and caffeine-induced Ca²⁺ transients was preserved in permeabilized CASQ2^{R33Q} myocytes despite the reduction of [Ca²⁺]_{SR} (Figure 6B through 6D). The ability of the CASQ2^{R33Q} myocytes to maintain the amplitude of their Ca²⁺ transients despite the reduced [Ca²⁺]_{SR} is attributable to increased intrastore Ca²⁺

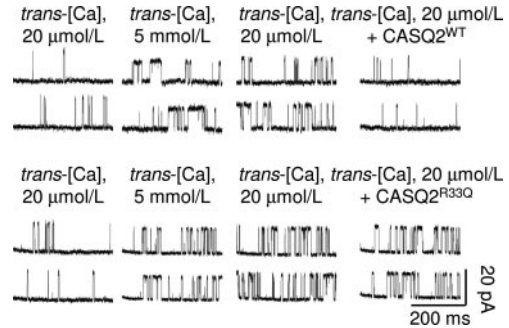


Figure 7. Activity of native RyR2 channels is modulated by CASQ2^{WT} and CASQ2^{R33Q}. Representative single-channel traces illustrating the irreversibility of the effects of 5 mmol/L luminal Ca²⁺ and restoration of initial low activity by CASQ2^{WT} but not CASQ2^{R33Q} added to the *trans* chamber in native RyRs. The P_o values were 0.06 ± 0.02 for low *trans* [Ca²⁺] (20 μmol/L); 0.34 ± 0.09 for high *trans* [Ca²⁺] (5 mmol/L); 0.36 ± 0.10 reverting to low *trans* [Ca²⁺] (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 Ca²⁺-binding sites that can bind and release Ca²⁺ on discharge of the store) in myocytes overexpressing CASQ2^{R33Q}. Collectively, these results suggest that CASQ2^{R33Q} expression resulted in both increased leak of Ca²⁺ through the RyR2 and increased intra-SR Ca²⁺-buffering capacity.

Effects of CASQ^{WT} and CASQ^{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 Ca²⁺. This treatment promoted efficient dissociation of CASQ2, as evidenced by the enhanced RyR2 activity that persisted after the [Ca²⁺] 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 Ca²⁺ release.

Determination of Ca²⁺-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 Ca²⁺-binding properties of the mutant protein. We, therefore, tested whether the CASQ2^{R33Q} protein displayed altered Ca²⁺-binding affinities when compared with CASQ2^{WT} using Ca²⁺ overlay experiments.¹⁹ Two kinetic parameters of Ca²⁺ binding, the Ca²⁺ 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 Ca²⁺ binding.

Discussion

Genetic defects in the SR Ca²⁺-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 Ca²⁺ leak from the SR and enhancing the propensity of SR Ca²⁺ release to be spontaneously activated.

Molecular Mechanisms of R33Q

The potentiatory effects of CASQ2 on the Ca²⁺-release channels were evidenced by the following findings. Expression of CASQ2^{R33Q} resulted in a shortening of the activation kinetics of Ca²⁺ transients, and increased CICR gain compared with control myocytes or myocytes overexpressing CASQ2^{WT}. Additionally, the frequency of spontaneous Ca²⁺ sparks and waves were increased in myocytes expressing CASQ2^{R33Q}. These changes in focal and global cytosolic Ca²⁺ transients were accompanied by a dramatic decrease in intra-SR [Ca²⁺], consistent with an increase in the leak of Ca²⁺ through RyR2s in CASQ2^{R33Q}-expressing cells. The consequences of expressing CASQ2^{R33Q} on Ca²⁺ 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 Ca²⁺ release and SR Ca²⁺ content.^{16,17} These effects were attributed to disruptions of the CASQ2 polymerization¹⁶ that is required for high-capacity Ca²⁺ 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 Ca²⁺-binding capacity of CASQ2. The free [Ca²⁺] in the SR lumen at steady state is determined by the balance of Ca²⁺ leakage and uptake across the SR

membrane and should not be influenced by the concentration of Ca²⁺-binding sites inside the SR. Therefore, the reduced [Ca²⁺]_{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 Ca²⁺-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 Ca²⁺ content (judged from the size of caffeine-induced Ca²⁺ transients) was preserved in cells expressing CASQ2^{R33Q}, indicating that the concentration of Ca²⁺-binding sites in the SR increased, as would be expected if the mutant protein maintained its Ca²⁺-binding function. Similarly, the mutation did not affect the ability of CASQ2 to bind Ca²⁺ in vitro. Thus, it appears that the R33Q mutation alters intracellular Ca²⁺ handling by compromising interactions of CASQ2 with the RyR2 complex without affecting CASQ2 Ca²⁺-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 Ca²⁺ stores followed by DADs and extrasystolic action potentials (Figure 4). Spontaneous SR Ca²⁺ release in cardiac myocytes is commonly associated with increased SR Ca²⁺ load^{23–25} and stimulatory effects of high luminal [Ca²⁺] 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 Ca²⁺.

It is interesting to note that although expression of CASQ2^{R33Q} produced clear changes in Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ 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 gating²⁹ (but see George et al³⁰) 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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