Abnormal Calcium Signaling and Sudden Cardiac Death Associated With Mutation of Calsequestrin

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Abstract—Mutations in human cardiac calsequestrin (CASQ2), a high-capacity calcium-binding protein located in the sarcoplasmic reticulum (SR), have recently been linked to effort-induced ventricular arrhythmia and sudden death (catecholaminergic polymorphic ventricular tachycardia). However, the precise mechanisms through which these mutations affect SR function and lead to arrhythmia are presently unknown. In this study, we explored the effect of adenoviral-directed expression of a canine CASQ2 protein carrying the catecholaminergic polymorphic ventricular tachycardia–linked mutation D307H (CASQ2D307H) on Ca2+ signaling in adult rat myocytes. Total CASQ2 protein levels were consistently elevated ~4-fold in cells infected with adenoviruses expressing either wild-type CASQ2 (CASQ2WT) or CASQ2D307H. Expression of CASQ2D307H reduced the Ca2+ storing capacity of the SR. In addition, the amplitude, duration, and rise time of macroscopic ICa-L-induced Ca2+ transients and of spontaneous Ca2+ sparks were reduced significantly in myocytes expressing CASQ2D307H. Myocytes expressing CASQ2D307H also displayed drastic disturbances of rhythmic oscillations in [Ca2+]i, and membrane potential, with signs of delayed afterdepolarizations when undergoing periodic pacing and exposed to isoproterenol. Importantly, normal rhythmic activity was restored by loading the SR with the low-affinity Ca2+ buffer, citrate. Our data suggest that the arrhythmogenic CASQ2D307H mutation impairs SR Ca2+ storing and release functions and destabilizes the Ca2+-induced Ca2+ release mechanism by reducing the effective Ca2+ buffering inside the SR and/or by altering the responsiveness of the Ca2+ release channel complex to luminal Ca2+.

These results establish at the cellular level the pathological link between CASQ2 mutations and the predisposition to adrenergically mediated arrhythmias observed in patients carrying CASQ2 defects. (Circ Res. 2004;94:471-477.)

Key Words: excitation-contraction coupling | calcium-induced calcium release
- catecholaminergic polymorphic ventricular tachycardia
- sarcoplasmic reticulum
- calsequestrin

Catecholaminergic polymorphic ventricular tachycardia (CPVT; Online Mendelian Inheritance in Man database, No. 604772) is a familial arrhythmogenic disorder characterized by adrenergically mediated polymorphic ventricular tachyarrhythmias leading to syncope and sudden cardiac death.1 Clinical manifestations of the disease typically occur in young children during physical activity or emotional stress. It has been suggested that arrhythmias in CPVT are mediated by delayed afterdepolarizations (DADs),2,3 oscillations of the membrane potential associated with Ca2+ overload, but no conclusive evidence exists to support this hypothesis.

Two genetic variants of CPVT have been identified, one transmitted as an autosomal dominant trait caused by mutations in the gene encoding the cardiac ryanodine receptor (RyR2)3,4 and one recessive form caused by mutations in the cardiac-specific isoform of the calsequestrin gene (CASQ2).5,6 RyR2 and CASQ2 are key components of the excitation-contraction (EC) coupling machinery. Both proteins are part of a supramolecular Ca2+ signaling complex in the junctional sarcoplasmic reticulum (SR) that also contains triadin 1 and junction, among other proteins.7-9 RyR2 serves as a Ca2+ release channel in the SR. During the EC coupling process, RyR2 channels are activated by Ca2+ that enters the cell through voltage-dependent L-type Ca2+ channels, causing the release of Ca2+ from the SR into the cytosol, a mechanism known as Ca2+-induced Ca2+ release (CICR).10,11 Increased cytosolic Ca2+ levels activate the contractile apparatus. Ca2+ release is terminated when SR luminal [Ca2+] falls below a threshold level, causing a decline in RyR2 activity via a mechanism termed luminal Ca2+-dependent deactivation.12,14 CASQ2 is a high-capacity Ca2+-binding protein whose primary function is to store the releasable Ca2+ within the SR.11,13 Additionally, CASQ2 seems to play an important role in...
The recessive form of CPVT was positionally mapped in several Bedouin families to the region of chromosome 1 (1p13-21) in which the \( \text{CASQ2} \) gene is located. Subsequent sequence analysis of \( \text{CASQ2} \) genes from these individuals identified a missense point mutation in a highly conserved region of \( \text{CASQ2} \). This mutation (referred to here as \( \text{CASQ2}_{D307H} \)) converts a negatively charged aspartic acid into a histidine in a putative \( \text{Ca}^{2+} \) chelating region of \( \text{CASQ2} \). Lahat et al.\(^5\) proposed that this mutation exerts its effects by disrupting \( \text{Ca}^{2+} \) binding to \( \text{CASQ2} \), but the specific mechanisms whereby the D307H mutation causes CPVT remain unknown. In the present study, we used an adenoviral-mediated gene transfer strategy to express a canine \( \text{CASQ2}_{D307H} \) protein in cardiac myocytes and explored the effects of this mutation on intracellular \( \text{Ca}^{2+} \) handling using \( \text{Ca}^{2+} \) imaging and patch-clamp techniques. Our results establish a pathological link between the expression of \( \text{CASQ2}_{D307H} \) and the clinical phenotype observed in patients carrying this mutation.

**Materials and Methods**

**Construction of Recombinant Adenoviruses**

The construction of \( \text{Ad-CSQ2}\) and \( \text{Ad-Control} \) was described previously.\(^{14}\) The D307H mutation was introduced into the full-length canine \( \text{CASQ2} \) cDNA using the Quikchange Site-Directed Mutagenesis Kit (Stratagene). The \( \text{CASQ2}_{D307H} \) coding region was transferred into the Adeno-X Viral DNA, and recombinant adenoviruses were generated according to the instructions of the manufacturer (Clontech).

**Adenoviral Gene Transfer**

Ventricular myocytes were obtained from adult male Sprague-Dawley rat hearts by enzymatic dissociation, infected with adenoviruses at a multiplicity of infection of 100, and maintained in a \( \text{CO}_2 \) incubator at 5% \( \text{CO}_2 \) and 95% air at 37°C. Dawley rat hearts by enzymatic dissociation, infected with adenovirus and the adenoviral constructs.

**Western Blotting**

Normal and mutant \( \text{CASQ2} \) protein levels were determined by immunoblot analysis as described previously.\(^{14}\) Briefly, 10 μg of cell lysate proteins was subjected to 12% SDS-PAGE, blotted onto PVDF membranes (Santa Cruz Biotechnology, Inc), and probed with antibodies specific for \( \text{CASQ2} \) (1:2500, PA1-913, Affinity Bioreagents). Blots were developed with Super Signal West Pico (PIERCE) and quantified using a Visage 2000 Blot Scanning and Analysis system (BioImage Systems Corporation).

**Electrophysiological Recordings**

Whole-cell patch-clamp recordings of transmembrane ionic currents were performed as described previously.\(^{12}\) The voltage-clamp protocol consisted of 400-ms-long voltage pulses to specified membrane potentials applied from a holding potential of \(-50\) mV at 1-minute intervals. The external solution contained (in mmol/L) \( \text{NaCl} \) 140, \( \text{KCl} \) 5.4, \( \text{CaCl}_2 \) 1.0, \( \text{MgCl}_2 \) 0.5, \( \text{HEPES} \) 10, and glucose 5.6, pH 7.3. Patch pipettes with tip resistance of 1 to 3 \( \Omega \cdot \text{M} \) were pulled from borosilicate glass (Sutter Instrument Co) and filled with a solution that contained (in mmol/L) \( \text{Cs-aspartate} \) 90, \( \text{CsCl} \) 50, \( \text{Na}_2\text{ATP} \) 3, \( \text{MgCl}_2 \) 3.5, \( \text{HEPES} \) 10, and \( \text{Fluo-3} \) \( \text{K}^+ \)-salt 0.05, pH 7.3. In some experiments, the cells were stimulated periodically (2 Hz) and the membrane potential was recorded in the current-clamp configuration. In these experiments the pipette solution contained \( \text{K}^+ \)-aspartate instead of Cs-aspartate. Citrate \( \text{K}^+ \)-salt (5 mmol/L) was added into the pipette solution by replacing osmotically equivalent amounts of \( \text{K}^+ \)-aspartate. Rapid applications of caffeine were used to measure \( \text{SR} \) \( \text{Ca}^{2+} \) content (10 mmol/L). The amount of \( \text{Ca}^{2+} \) released was assessed by integration of the Na\(^+-\text{Ca}^{2+} \) exchange current and from the peak amplitude of the caffeine-induced \( \text{Ca}^{2+} \) transients.

**Confocal \( \text{Ca}^{2+} \) Measurements**

Intracellular \( \text{Ca}^{2+} \) imaging was performed using a Bio-Rad Laser Scanning Confocal System (Bio-Rad MRC-1024ES interfaced to an Olympus IX-70 inverted microscope and equipped with an Olympus 60×1.4 NA oil objective), as described.\(^{12}\) Fluo-3 was excited by the 488-nm beam of an argon-ion laser, and the fluorescence was acquired at wavelengths >515 nm in the line scan mode of the confocal system at rate of 2 or 6 ms per scan. The magnitude of fluorescent signals was quantified in terms of \( F/F_0 \), where \( F_0 \) is baseline fluorescence. Assuming that the basal cytosolic \( \left[\text{Ca}^{2+}\right]_c \) is 100 mmol/L and a \( K_b \) for Fluo-3 \( \text{Ca}^{2+} \) binding of 1.1 μmol/L, the theoretical maximum for \( F/F_0 \) is 12. \( \text{Ca}^{2+} \) spark parameters were quantified with a detection/analysis computer algorithm.\(^{12}\)

**Statistics**

All values were expressed as mean ± SEM. Statistical comparisons were made using unpaired Student’s \( t \) test or 2-way ANOVA for repeated measurements (\( P<0.05 \)).

**Results**

**Adenoviral-Mediated Expression of Mutant \( \text{CASQ2} \) in Isolated Rat Myocytes**

The dog and human \( \text{CASQ2} \) proteins display 91% sequence identity overall, and the D307H mutation is located in a region that is highly conserved among \( \text{CASQ2} \) orthologues from various vertebrate species (Figures 1A and 1B). Interestingly, this residue is also located within a highly conserved region of \( \text{CASQ1} \) proteins, suggesting that it may be crucial for a shared function of \( \text{CASQ2} \) proteins (Figure 1B). The D307H mutation was introduced into the coding sequence of canine \( \text{CASQ2} \) by site-directed mutagenesis. The canine \( \text{CASQ2}_{D307H} \) coding sequence was inserted into an adenoviral vector to generate \( \text{Ad-CSQ2}_{D307H} \) for subsequent gene transfer into adult rat ventricular myocytes. In addition, two adenoviruses containing the WT canine \( \text{CASQ2} \) (\( \text{Ad-CSQ2}^{WT} \)) and the \( \text{CASQ2} \) coding sequence with a stop codon inserted after amino acid 70 (\( \text{Ad-Control} \)) were used as controls for viral infection and \( \text{CASQ2} \) expression. Our infection protocol results in infection efficiencies of nearly 100% at a multiplicity of infection of 100.\(^{14}\) Expression of both endogenous and recombinant proteins was examined in infected myocytes by Western blotting (Figures 1C and 1D). Infection of myocytes with either \( \text{Ad-CSQ2}^{WT} \) or \( \text{Ad-CSQ2}_{D307H} \) consistently resulted in equivalent ~4-fold increases in total \( \text{CASQ2} \) protein, and \( \text{CASQ2} \) levels were unchanged in cells infected with \( \text{Ad-Control} \).

**\( \text{CASQ2}_{D307H} \) Overexpression Decreases the \( \text{SR} \) \( \text{Ca}^{2+} \) Storage Capacity**

Caffeine applications (10 mmol/L) were used to assess the changes in the total \( \text{SR} \) \( \text{Ca}^{2+} \) content in each group of adenovirus-infected myocytes. The relative amounts of \( \text{Ca}^{2+} \) released from the \( \text{SR} \) after caffeine administration were assessed from changes in both Fluo-3 fluorescence and Na\(^+-\text{Ca}^{2+} \) exchange current (\( I_{\text{NCX}} \)) in myocytes dialyzed with...
the Ca\textsuperscript{2+} indicator Fluo-3 (Figure 2). The amplitude of caffeine-induced Ca\textsuperscript{2+} transients increased 2.2-fold in CASQ2 WT-overexpressing myocytes compared with control cells. In contrast, expression of CASQ2 D307H reduced the amplitude of the caffeine-induced fluorescence signal to 41% of control (Figure 2B). These changes in fluorescence signals in Ad-CASQ2 WT and Ad-CASQ2 D307H myocytes were paralleled by changes in the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange current. The integral of IC\textsubscript{NCX} was 2.3-fold higher in Ad-CASQ2 WT myocytes and decreased to 36% of control in Ad-CASQ2 D307H myocytes (Figure 2C). Thus, ectopic expression of CASQ2 D307H suppressed the ability of SR to store Ca\textsuperscript{2+}.

Macroscopic Ca\textsuperscript{2+} Transients and IC\textsubscript{Ca}

The effects of expressing CASQ2\textsuperscript{WT} and CASQ2\textsuperscript{D307H} on IC\textsubscript{Ca} and intracellular [Ca\textsuperscript{2+}] transients in patch-clamped myocytes are illustrated in Figure 3. There were no apparent changes in the parameters of IC\textsubscript{Ca} in myocytes expressing either CASQ2\textsuperscript{WT} or CASQ2\textsuperscript{D307H} (Figure 3A, bottom, and Figure 3B; Table 1). The peak amplitude of IC\textsubscript{Ca} was nearly identical for all groups of cells (Table 1). In addition, the time course of IC\textsubscript{Ca} decay was similar (Table 1). Thus, expression of CASQ2\textsuperscript{WT} or CASQ2\textsuperscript{D307H} did not change the characteristics of the Ca\textsuperscript{2+} trigger for Ca\textsuperscript{2+} release from the SR.

Overexpressing CASQ2\textsuperscript{WT} caused a dramatic increase in the magnitude and overall duration of Ca\textsuperscript{2+} transients (Figure 3A). The peak amplitude of IC\textsubscript{Ca} was nearly identical for all groups of cells (Table 1). In addition, the time course of IC\textsubscript{Ca} decay was similar (Table 1). Thus, expression of CASQ2\textsuperscript{WT} or CASQ2\textsuperscript{D307H} did not change the characteristics of the Ca\textsuperscript{2+} trigger for Ca\textsuperscript{2+} release from the SR.

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TABLE 1. Parameters of IC\textsubscript{Ca} and Ca\textsuperscript{2+} Transients

<table>
<thead>
<tr>
<th></th>
<th>IC\textsubscript{Ca}</th>
<th>Ca\textsuperscript{2+} Transients</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Peak Amplitude, pA</td>
<td>t\textsubscript{rise}, ms</td>
</tr>
<tr>
<td>Ad-Control</td>
<td>-951 ± 69</td>
<td>16.1 ± 4.3</td>
</tr>
<tr>
<td>Ad-CASQ2\textsuperscript{WT}</td>
<td>-1002 ± 79</td>
<td>17.5 ± 5.9</td>
</tr>
<tr>
<td>Ad-CASQ2\textsuperscript{D307H}</td>
<td>-920 ± 64</td>
<td>12.4 ± 2.2</td>
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*P<0.01 vs control (n=5 to 18).
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longing the duration of the Ca\textsuperscript{2+} control). Thus, expressing CASQ2\textsubscript{D307H} resulted in focal briefer and had rise times shorter than in control (73% of multiple individual events\textsuperscript{12} acquired in the same three groups

Furthermore, the rise time of Ca\textsuperscript{2+} in myocytes expressing CASQ2\textsubscript{D307H}, expression of CASQ2\textsubscript{WT} and CASQ2\textsubscript{D307H} on parameters of myocytes are illustrated in Figure 4B. The impact of

Effects of expressing the WT and mutant CASQ2 on properties of spontaneous Ca\textsuperscript{2+} sparks in permeabilized myocytes. A, Representative line-scan images of Ca\textsuperscript{2+} sparks acquired in myocytes infected with Ad-Control, Ad-CASQ2\textsubscript{WT}, and Ad-CASQ2\textsubscript{D307H} vectors. B and C, Voltage dependence of I\textsubscript{Ca} (B) and Ca\textsuperscript{2+} transients (C) in myocytes infected with Ad-Control, Ad-CASQ2\textsubscript{WT}, and Ad-CASQ2\textsubscript{D307H} vectors (n for each point ranged from 5 to 18).

3A, top, and Figure 3C; Table 1). Importantly, the duration of the rising phase was increased by 90%, consistent with the hypothesis that CASQ2 modulates SR Ca\textsuperscript{2+} release by prolonging the duration of the Ca\textsuperscript{2+} flux from the SR to the cytosol.\textsuperscript{14} In contrast, in myocytes expressing CASQ2\textsubscript{D307H}, the Ca\textsuperscript{2+} transients were drastically reduced in size and duration. Furthermore, the rise time of Ca\textsuperscript{2+} transients in Ad-CASQ2\textsubscript{D307H} myocytes was shortened significantly compared with control (by 39%). Thus, expressing CASQ2\textsubscript{D307H} inhibited active SR Ca\textsuperscript{2+} release, apparently by shortening Ca\textsuperscript{2+} release duration.

Ca\textsuperscript{2+} Sparks in Permeabilized Myocytes

We next examined the impact of expressing CASQ2\textsubscript{D307H} on properties of focal fluorescence signals, ie, Ca\textsuperscript{2+} sparks, in permeabilized myocytes. Myocytes were permeabilized with saponin, and Ca\textsuperscript{2+} sparks were recorded at a constant cytosolic [Ca\textsuperscript{2+}] of \( \sim 100 \) mmol/L.\textsuperscript{18} Representative line-scan images of sparks acquired in myocytes infected with Ad-Control, Ad-CASQ2\textsubscript{WT}, and Ad-CASQ2\textsubscript{D307H} are shown in Figure 4A, and surface plots of sparks obtained by averaging multiple individual events\textsuperscript{12} acquired in the same three groups of myocytes are illustrated in Figure 4B. The impact of expression of CASQ2\textsubscript{WT} and CASQ2\textsubscript{D307H} on parameters of Ca\textsuperscript{2+} sparks is documented in Table 2. Overexpression of CASQ2\textsubscript{WT} resulted in a dramatic increase in the overall magnitude and spatiotemporal spread of sparks. In addition, the duration of the rising phase of sparks was increased in Ad-CASQ2\textsubscript{WT} myocytes (172% of control). However, when CASQ2\textsubscript{D307H} was expressed, the Ca\textsuperscript{2+} sparks were smaller and briefer and had rise times shorter than in control (73% of control). Thus, expressing CASQ2\textsubscript{D307H} resulted in focal release events of reduced size and abbreviated duration.

\begin{table}[h]
\centering
\caption{Parameters of Ca\textsuperscript{2+} Sparks}
\begin{tabular}{lccccc}
\hline
& F/F\textsubscript{0} & Rise Time, ms & DHA,† ms & FWHM,‡ & µm\
\hline
Ad-Control & 1.62±0.02 & 7.9±0.1 & 14.9±0.2 & 2.45±0.02 & \\
Ad-CASQ2\textsubscript{WT} & 2.26±0.04* & 13.6±0.2* & 24.3±0.3* & 3.07±0.03* & \\
Ad-CASQ2\textsubscript{D307H} & 1.46±0.02* & 6.2±0.1* & 11.4±0.2* & 1.93±0.02* & \\
\hline
\end{tabular}
\end{table}

\footnotesize{\textsuperscript{*}P<0.01 vs control (n=1414 to 1525).
\textsuperscript{†}Spark duration at half-amplitude; \textsuperscript{‡}spark width at half-maximum.}

Ca\textsuperscript{2+} Cycling in Rhythmically Paced Myocytes

The effects of isoproterenol (ISO) treatment (1 µmol/L) on periodic Ca\textsuperscript{2+} transients in control myocytes and in myocytes expressing either CASQ2\textsubscript{WT} or CASQ2\textsubscript{D307H} is illustrated in Figure 5. The myocytes were stimulated at 2 Hz, and membrane potential (MP) changes were recorded in the current-clamp mode. In control myocytes, exposure to ISO caused an increase in the amplitude of Ca\textsuperscript{2+} transients without any apparent disturbances in periodic Ca\textsuperscript{2+} cycling (results are representative of six myocytes, Figure 5A). In myocytes overexpressing CASQ2\textsubscript{WT}, the amplitude of Ca\textsuperscript{2+} transients was increased with respect to control, consistent with measurements under resting conditions. ISO treatment caused an additional augmentation of Ca\textsuperscript{2+} signals, again without disrupting rhythmicity (data are representative of nine myocytes, Figure 5B). In myocytes expressing CASQ2\textsubscript{D307H}, the amplitude of Ca\textsuperscript{2+} transients was reduced with respect to control, consistent with measurements in resting myocytes. ISO application caused profound disturbances in Ca\textsuperscript{2+} cycling manifested by extrasystolic, spontaneous Ca\textsuperscript{2+} transients. As seen in the line-scan images (Figure 5C), spontaneous release usually originated locally and then propagated through the cell as a regenerative Ca\textsuperscript{2+} wave. Importantly, the MP traces showed clear signs of DADs at the time of spontaneous Ca\textsuperscript{2+} cycling (results are representative of nine myocytes, Figure 5D). Similar results were obtained in five other myocytes. These results indicate that expression of CASQ2\textsubscript{D307H} not only reduces the amount of Ca\textsuperscript{2+} released from the SR but also destabilizes the Ca\textsuperscript{2+} release mechanism, leading to spontaneous, premature discharges of SR Ca\textsuperscript{2+} stores in myocytes undergoing periodic pacing.
Restoration of Normal Periodic Ca\(^{2+}\) Transients by Increasing SR Ca\(^{2+}\) Buffering Capacity With Low-Affinity Ca\(^{2+}\) Buffers

We have recently proposed that CASQ2 regulates the functional size and stability of SR Ca\(^{2+}\) stores by serving as a buffer for Ca\(^{2+}\) in the SR lumen.\(^{14}\) To test the hypothesis that disruption of Ca\(^{2+}\) cycling observed in cells expressing CASQ2 D307H is attributable to abnormal intra-SR Ca\(^{2+}\) buffering, we carried out experiments using the low-affinity exogenous Ca\(^{2+}\) buffer, citrate. Citrate was loaded into the SR of CASQ2 D307H-expressing myocytes by dialyzing them with a citrate-containing pipette solution.\(^{12}\) Sequestration of citrate into the SR occurs substantially slower (20 to 30 minutes) than equilibration of Fluo-3 into the cytosol (5 to 10 minutes),\(^{12}\) thus permitting determination of the effects of intra-SR citrate on Ca\(^{2+}\) cycling in the same individual myocytes (Figure 6). Again, application of ISO produced characteristic disturbances in the periodic Ca\(^{2+}\) transients and MP in CASQ2 D307H-expressing myocytes (Figure 6, left and middle). Continuous dialysis of the myocytes with citrate-containing solution for 20 minutes led to a substantial increase in the magnitude of Ca\(^{2+}\) transients. Importantly, citrate dialysis also normalized Ca\(^{2+}\) cycling by eliminating the spontaneous, extrasystolic Ca\(^{2+}\) transients (Figure 6, right). Similar results were obtained in all five independent cells examined.

Discussion

It has been reported recently that certain recessive forms of CPVT are associated with mutations in the cardiac CASQ2 gene;\(^{5,6}\) however, the mechanisms by which CASQ2 mutations cause the clinical phenotype have not been established. The objective of the present study was to investigate the functional characteristics of the missense mutation D307H identified in the first large pedigree affected by CPVT linked to mutations in CASQ2.\(^{5}\) Adenoviral-mediated expression of CASQ2 D307H diminished the Ca\(^{2+}\) storing and releasing capabilities of the SR in rat ventricular myocytes, thus resulting in pronounced dominant-negative effects on SR Ca\(^{2+}\) handling. Relevant to the arrhythmogenic consequence of the D307H mutation, our data demonstrated that myocytes expressing CASQ2 D307H develop abnormal intracellular [Ca\(^{2+}\)] oscillations that cause DADs specifically during \(\beta\)-adrenergic stimulation. Therefore, within the limits of an in vitro cell model, we establish for the first time a pathological link between a specific, clinically relevant mutation in the CASQ2 gene and arrhythmogenic behavior underlying CPVT. The dominant-negative effects of the mutant protein on intracellular Ca\(^{2+}\) signaling were unexpected considering the recessive mode of inheritance of the disease. They provide new clues for understanding the structure-functional relationships within the junctional Ca\(^{2+}\) signaling complex.

Effects of D307H on CASQ2 Function

Based on the analysis of the amino acid sequence and crystal structure of calsequestrin, Asp307, which harbors this arrhythmogenic mutation, is localized to a putative Ca\(^{2+}\) binding region between the second and third thioredoxin-like domains...
of the protein.\(^5,21\) Consequently, it has been hypothesized that the pathology of CPVT may involve disrupted Ca\(^{2+}\) binding by CASQ2.\(^5\) Our finding that expression of CASQ2\(^{D307H}\) diminished SR Ca\(^{2+}\) releasing and storing capabilities despite the presence of normal levels of the endogenous wild-type protein suggests that the effect of this mutation on CASQ2 function may be more complex than merely altering Ca\(^{2+}\) binding by CASQ2 monomers. Previous studies performed with both the skeletal and cardiac isoforms of the protein demonstrated that calsequestrin oligomerizes in a Ca\(^{2+}\)-dependent fashion and that the formation of calsequestrin polymers is important for high-capacity Ca\(^{2+}\) binding.\(^22,23\) Although the precise mechanisms of Ca\(^{2+}\)-dependent aggregation of calsequestrin monomers and of Ca\(^{2+}\) sequestration by the calsequestrin complex are not known, it has been proposed that calsequestrin polymers provide an electrostatically charged surface onto which Ca\(^{2+}\) can be absorbed.\(^21,23\) Thus, structural defects in individual CASQ2 monomers could reduce high-capacity Ca\(^{2+}\) binding by disrupting Ca\(^{2+}\)-dependent CASQ2 polymerization. This possibility is consistent with reduced SR Ca\(^{2+}\) storing capacity of myocytes expressing CASQ2\(^{D307H}\) (Figure 2). It is also possible that the effects of the mutation are attributable to abnormal interactions of CASQ2 with other components of the SR Ca\(^{2+}\) release machinery. CASQ2 has been proposed to be actively involved in regulation of Ca\(^{2+}\) release through protein-protein interactions with RyR2, junctin, and triadin.\(^8,24\) Recent results obtained in our laboratory suggest that RyR2 complexed with junctin and triadin is inhibited by CASQ2 at low luminal [Ca\(^{2+}\)] and that this inhibition is relieved at high luminal [Ca\(^{2+}\)].\(^15\) If the D307H mutation were to affect the ability of CASQ2 to interact with the RyR2 complex, this could lead to RyR2 channels with abnormally high activity. In this case, the diminished SR Ca\(^{2+}\) storing and releasing functions in CASQ2\(^{D307H}\)-expressing myocytes could reflect the compromised ability of the SR to retain Ca\(^{2+}\) due to hyperactive, leaky, RyR2 channels. Regardless of the specific molecular alterations, our results indicate that Asp\(^92\) resides in a region of the protein that is critical for normal function of CASQ2 in cardiac SR.

It is interesting to note that whereas the CASQ2\(^{D307H}\) protein exerted clear dominant-negative effects in infected myocytes in our experiments, in the clinical setting, this mutation causes an abnormal phenotype only when 100% of the CASQ2 protein is abnormal (ie, in homozygous carriers of the mutation).\(^5\) This apparent discrepancy could be ascribed to relative levels of the wild-type and mutant CASQ2 proteins in our experiments, where the ratio of mutant to wild-type protein was \(\approx\)3:1 in myocytes infected with Ad-CASQ2\(^{D307H}\) (Figure 1). Indeed, when the mutant CASQ2 was expressed at levels similar to those of the endogenous protein (ie, at a ratio of 1:1), the myocytes showed no reduction in the amplitude of depolarization- and caffeine-induced Ca\(^{2+}\) transients with respect to control (see the online data supplement, available at http://circres.ahajournals.org). Because a \(\approx\)2-fold increase in total CASQ2 level did not lead to any gain in function, our results still imply that the function of CASQ2\(^{D307H}\) should be impaired in heterozygous carriers of this mutation (50% of normal protein present). It is likely, however, that the clinical phenotype of D307H is influenced by various adaptive changes in cellular Ca\(^{2+}\) handling mechanisms such as increased expression of other luminal Ca\(^{2+}\) binding proteins (eg, calreticulin) or CASQ1 isoform transition.

**Molecular Mechanisms of CPVT**

Our results provide a plausible explanation for the cellular mechanism by which the D307H mutation of CASQ2 causes catecholaminergic tachycardia. Exposure of CASQ2\(^{D307H}\) myocytes to \(\beta\)-adrenergic stimulation induces extrasystolic, spontaneous Ca\(^{2+}\) transients and resulted in the development of DADs (Figure 5C). It is known that generation of DADs involves Ca\(^{2+}\)-dependent inward Ca\(^{2+}\) currents and that these deflections of the membrane potential can trigger arrhythmias.\(^19,25-28\) Using the same experimental system as in the present study (ie, adult rat ventricular myocytes), we recently demonstrated that CASQ2 plays an important role in termination and restitution of CICR by influencing luminal Ca\(^{2+}\)-dependent gating of the RyR2 channels.\(^14\) Furthermore, reduction of CASQ2 levels to \(\approx\)30% of wild-type levels (achieved using an antisense RNA approach) led to profound disturbances in rhythmic Ca\(^{2+}\) cycling attributable to an accelerated functional recovery of the release sites from a luminal Ca\(^{2+}\)-dependent refractory state. The effects of expressing CASQ2\(^{D307H}\) on specific parameters of both cell-averaged Ca\(^{2+}\) transients and Ca\(^{2+}\) sparks (eg, amplitude and rise-time duration; Figures 2 through 4 and Tables 1 and 2) were similar to those we observed on reducing CASQ2 protein levels.\(^14\) Thus, our previous results and present findings suggest a mechanism whereby reduced buffering of Ca\(^{2+}\) in the SR lumen by CASQ2 (and/or disrupted interactions of CASQ2 with the RyR2 channel complex) leads to altered regulation of the Ca\(^{2+}\) release mechanism by luminal Ca\(^{2+}\). Within this mechanistic framework, the role of adrenergic stimulation in promoting the pathologic signs of disease can be ascribed to an accelerated recharging of the SR Ca\(^{2+}\) store (as a result of enhanced SR Ca\(^{2+}\) uptake by the CaATPase) and hence further contributing to the premature functional restitution of the RyR2s.\(^14\) Our finding that normal Ca\(^{2+}\) cycling in CASQ2\(^{D307H}\)-expressing myocytes can be restored by loading their SR with low-affinity exogenous Ca\(^{2+}\) buffers (Figure 6) provides a strong support for the proposed role of CASQ2 and luminal Ca\(^{2+}\) in the pathogenesis of CPVT.

Our results are also relevant for understanding the cellular mechanisms of genetically distinct forms of CPVT with similar clinical manifestations. Recent studies have indicated that RyR2 mutations associated with CPVT result in abnormal RyR2 channel activity, although the precise mechanisms underlying these changes remain controversial.\(^29-31\) Given our findings that abnormal luminal Ca\(^{2+}\) regulation of SR Ca\(^{2+}\) release accounts for the arrhythmic behavior of myocytes, it is logical to propose that CPVT associated with RyR2 channel mutations\(^3,4\) is a consequence of the compromised ability of the channel to sense or respond to changes in luminal Ca\(^{2+}\). In addition, it is possible that some forms of CPVT are caused by mutations in proteins such as junctin and triadin that are also part of the RyR2 complex\(^8\) and might be involved in sensing or communicating the luminal Ca\(^{2+}\) signal to the RyR2.
Conclusions

In conclusion, we have established a pathological link between the D307H mutation in the CASQ2 gene and the clinical phenotype observed in CPVT patients carrying this mutation. The pathological chain seems to involve the following steps. First, the mutation in Asp^{307} compromises the ability of CASQ2 to form high-capacity Ca^{2+}-binding oligomers and/or to interact with the RyR2 channel complex. Second, altered free [Ca^{2+}] dynamics near the luminal phase of the RyR2 (as a result of impaired intra-SR Ca^{2+} buffering) and/or disrupted ability of the channel to respond to changes in luminal [Ca^{2+}] (as a result of abnormal interactions of the mutant proteins with the RyR2 channel) cause premature functional restitution of the RyR2 Ca^{2+} release channels after each release. Third, premature functional recovery of the RyR channels leads to spontaneous, extrasystolic Ca^{2+} release.

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Abnormal Calcium Signaling and Sudden Cardiac Death Associated With Mutation of Calsequestrin

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Effects of CASQ2<sup>D307H</sup> expression on CICR and SR Ca content at 24 hrs after infection of the myocytes with the Ad-CASQ2<sup>D307H</sup> vector. A, Western blots of CASQ2 before and at different times (12, 20 and 48 hrs) after infection of the myocytes with Ad-CASQ2<sup>D307H</sup>. The intensities of the bands were measured by densiometry and are expressed relative to control. Data represent mean±SEM from 4 independent experiments. B, representative traces of I<sub>Ca</sub> (lower traces) and cytosolic Ca transients (upper traces) induced by depolarization from -50 to 0 mV in control myocytes and myocytes infected with the Ad-CASQ2<sup>D307H</sup> vector at 24 and 48 hrs. The peak amplitude of the depolarization-induced Ca transients measured in 4 independent experiments was 2.4±0.16, 2.2±0.15, and 1.5±0.2 for the control, 24 and 48 hrs of infection, respectively. C, Representative traces of Ca transients (upper traces) and I<sub>NCX</sub> (lower traces) induced by application of caffeine (10 mmol/L) in control myocytes and myocytes infected with the Ad-CASQ2<sup>D307H</sup> vector at 24 and 48 hrs. The peak amplitude of the caffeine-induced Ca transients measured in 3 independent experiments was 2.9±0.3, 2.8±0.4, and 1.7±0.2 for the control, 24 and 48 hrs of infection, respectively.