Modest Reductions of Cardiac Calsequestrin Increase Sarcoplasmic Reticulum Ca\(^{2+}\) Leak Independent of Luminal Ca\(^{2+}\) and Trigger Ventricular Arrhythmias in Mice

Nagesh Chopra, Prince J. Kannankeril, Tao Yang, Thinn Hlaing, Izabela Holinstat, Kristen Ettenson, Karl Pfeifer, Brandy Akin, Larry R. Jones, Clara Franzini-Armstrong, Björn C. Knollmann

Abstract—Cardiac calsequestrin–null mice (Casq2\(^{-/-}\)) display catecholaminergic ventricular tachycardia akin to humans with CASQ2 mutations. However, the specific contribution of Casq2 deficiency to the arrhythmia phenotype is difficult to assess because Casq2\(^{-/-}\) mice also show significant reductions in the sarcoplasmic reticulum (SR) proteins junctin and triadin-1 and increased SR volume. Furthermore, it remains unknown whether Casq2 regulates SR Ca\(^{2+}\) release directly or indirectly by buffering SR luminal Ca\(^{2+}\). To address both questions, we examined heterozygous (Casq2\(^{+/+}\)) mice, which have a 25% reduction in Casq2 but no significant decrease in other SR proteins. Casq2\(^{-/-}\) mice (n=35) challenged with isoproterenol displayed 3-fold higher rates of ventricular ectopy than Casq2\(^{+/+}\) mice (n=31; P<0.05). Programmed stimulation induced significantly more ventricular tachycardia in Casq2\(^{-/-}\) mice than in Casq2\(^{+/+}\) mice. Field-stimulated Ca\(^{2+}\) transients, cell shortening, L-type Ca\(^{2+}\) current, and SR volume were not significantly different in Casq2\(^{-/-}\) and Casq2\(^{+/+}\) myocytes. However, in the presence of isoproterenol, SR Ca\(^{2+}\) leak was significantly increased in Casq2\(^{-/-}\) myocytes (Casq2\(^{-/-}\) \(0.18\pm0.02\) F\(_{ratio}\) versus Casq2\(^{+/+}\) \(0.11\pm0.01\) F\(_{ratio}\), n=57, 60; P<0.01), resulting in a significantly higher rate of spontaneous SR Ca\(^{2+}\) releases and triggered beats. SR luminal Ca\(^{2+}\) measured using Mag-Fura-2 was not altered by Casq2 reduction. As a result, the relationship between SR Ca\(^{2+}\) leak and SR luminal Ca\(^{2+}\) was significantly different between Casq2\(^{-/-}\) and Casq2\(^{+/+}\) myocytes (P<0.01). Thus, even modest reductions in Casq2 increase SR Ca\(^{2+}\) leak and cause ventricular tachycardia susceptibility under stress. The underlying mechanism is likely the direct regulation of SR Ca\(^{2+}\) release channels by Casq2 rather than altered luminal Ca\(^{2+}\). (Circ Res. 2007;101:617-626.)

Key Words: calsequestrin • ventricular arrhythmia • SR Ca\(^{2+}\) leak • SR free luminal Ca\(^{2+}\) • catecholaminergic polymorphic ventricular tachycardia

Cardiac calsequestrin (Casq2) is a low-affinity high-capacity Ca\(^{2+}\)-binding protein\(^1\)\(^-\)\(^2\) located in the junctional sarcoplasmic reticulum (jSR) of mammalian myocardium.\(^3\) The jSR in heart serves as the principal site for Ca\(^{2+}\) storage and release,\(^4\) where Casq2 appears as a densely staining protein in the lumen of the jSR on electron micrographs.\(^5\) At this site, Casq2 forms a quaternary complex\(^6\) with the sarcoplasmic reticulum (SR) Ca\(^{2+}\) release channel (cardiac ryanodine receptor [RyR2]) and with the jSR membrane proteins triadin-1\(^-\) and junctin.\(^8\) Casq2 has been thought to play a pivotal role in the regulation of cardiac Ca\(^{2+}\) storage and release required for excitation–contraction coupling in mammalian hearts.\(^8\) Surprisingly, our recent studies in Casq2-null (Casq2\(^{-/-}\)) mice,\(^10\) and observations from homozygous nonsense CASQ2 mutations in humans who are presumed to lack CASQ2,\(^11\) show that Casq2 is not essential for survival. Nevertheless, exercise-challenged Casq2\(^{-/-}\) mice display polymorphic ventricular arrhythmias, and Casq2\(^{-/-}\) ventricular myocytes have increased SR Ca\(^{2+}\) leak, causing dysfunctional SR Ca\(^{2+}\) release and triggered beats.\(^10\) Likewise, humans homozygous for nonsense CASQ2 mutations exhibit catecholaminergic polymorphic ventricular tachycardia (CPVT).\(^11\)

Controversy remains as to how loss of Casq2 causes SR Ca\(^{2+}\) leak and CPVT. SR Ca\(^{2+}\) release is strongly regulated by [Ca\(^{2+}\)]\(_{l}\) in the lumen of the SR, with the propensity of Ca\(^{2+}\) release increasing in steep nonlinear fashion with increasing luminal [Ca\(^{2+}\)].\(^12,13\) Thus, decreased luminal SR Ca\(^{2+}\) buffer-
ing by loss of Casq2 may increase SR luminal [Ca\(^{2+}\)] and could explain the phenotype of Casq2-null mice. On the other hand, bilayer studies have demonstrated that removal of Casq2 increased RyR2 open probability at fixed luminal [Ca\(^{2+}\)]. These data suggest that Casq2 may directly regulate the RyR2 Ca\(^{2+}\) release complex, independent of any effects on SR Ca\(^{2+}\) buffering. Unfortunately, complete ablation of Casq2 in mice induces compensatory responses that include a significant increase in the SR volume and near absence of the Casq2-binding proteins triadin-1 and junctin, making it difficult to use this model for studying the regulation of SR Ca\(^{2+}\) release by Casq2. Furthermore, it is possible that these adaptations also contribute to the arrhythmia phenotype. To address these 2 issues, we studied Casq2 heterozygous (Casq2\(^{+/−}\)) mice, which have reduced Casq2 but no significant changes in triadin-1, junctin, or other SR Ca\(^{2+}\) binding proteins. Our studies show that heterozygous Casq2\(^{+/−}\) mice have increased diastolic SR Ca\(^{2+}\) leak, resulting in spontaneous SR Ca\(^{2+}\) releases and triggered beats in Casq2\(^{+/−}\) myocytes, and ventricular ectopy and inducible ventricular tachycardia (VT) in vivo. Importantly, when plotting SR Ca\(^{2+}\) leak as a function of free [Ca\(^{2+}\)] in the SR lumen (to correct for any differences in SR Ca\(^{2+}\) buffering in Casq2\(^{+/−}\) myocytes), leak remained significantly higher in Casq2\(^{+/−}\) compared with Casq2\(^{+/+}\) myocytes. We conclude that even a modest reduction in Casq2 protein increases arrhythmia susceptibility. The underlying mechanism seems to be a direct modulation of the RyR2 SR Ca\(^{2+}\) release complex by Casq2 rather than increased [Ca\(^{2+}\)] in the SR lumen.

### Materials and Methods

#### Animal Model
The use of animals was approved by the Animal Care and Use Committees of Georgetown and Vanderbilt Universities and by the National Institute of Child Health and Human Development intramural research program. A total of 172 age- (2 to 4 months) and sex-matched (68 Casq2\(^{+/−}\), 70 Casq2\(^{+/+}\), 34 Casq2\(^{−/−}\)) mice were used for the experiments.

#### Protein Analysis, Electron Microscopy, Surface ECG, Intracardiac Electrophysiology, Ca\(^{2+}\) Fluorescence, Myocyte Shortening, and Voltage Clamp Experiments
Experiments were performed as described (for details, see the online data supplement at http://circres.ahajournals.org).

#### Ca\(^{2+}\) Indicator Loading for Intra-SR Ca\(^{2+}\) Measurements
Isolated cardiomyocytes were incubated with 5 μmol/L of the low-affinity Ca\(^{2+}\) indicator Mag-Fura-2 acetoxymethyl ester (AM) (Molecular Probes Inc, Eugene, Ore) for 30 minutes at 37°C. Cells were then washed twice for 10 minutes with 1.2 mmol/L Ca\(^{2+}\) Tyrode solution and kept at 37°C for another 1 hour for washout of the indicator from the cytosol. Cells were used within a 3-hour time period for experiments. With this protocol, 60% to 80% of the myocytes consistently exhibited down-going twitch and caffeine transients (Figure 8A and 8C), indicating that the fluorescence signal originated preferentially from SR.

### Measurement of Diastolic SR Ca\(^{2+}\) Leak
Diastolic SR Ca\(^{2+}\) leak was measured using the protocol described previously. Fura-2–loaded cardiac myocytes were field stimulated at 1 Hz until they reached a steady-state Ca\(^{2+}\) transient height. Stimulation was then switched off, and the external solution was quickly changed to Tyrode solution (0Ca\(^{2+}\), 0Na\(^{+}\) to eliminate transsarcolemmal Ca\(^{2+}\) fluxes, resulting in a new steady-state [Ca\(^{2+}\)]. When RyR2 channels are inhibited by 1 mmol/L tetracaine (in Tyrode solution, 0Ca\(^{2+}\), 0Na\(^{+}\)), Ca\(^{2+}\) shifts from the cytosol into the SR. The tetracaine-induced drop in diastolic Fura-2 fluorescence ratio was used as an estimate of SR Ca\(^{2+}\) leak, which is insensitive to changes in SR Ca\(^{2+}\) uptake. The myocytes were exposed for 4 seconds to 0Ca\(^{2+}\), 0Na\(^{+}\) Tyrode solution containing 10 mmol/L caffeine and 20 mmol/L 2,3-butanediol monoxime (to prevent myocyte hypercontracture). The amplitude of caffeine-induced Ca\(^{2+}\) transient was used as an estimate of total [Ca\(^{2+}\)], which included the Ca\(^{2+}\) leak (Figure 7A). The “load–leak” relationship shown in Figure 7C was constructed by grouping cells with similar total [Ca\(^{2+}\)].

To measure the relationship between free Ca\(^{2+}\) in the lumen of the SR ([Ca\(^{2+}\)]\(i\)) and diastolic SR leak, we used a similar protocol in myocytes loaded with the low-affinity Ca\(^{2+}\) indicator Mag-Fura-2 AM (Figure 8A and 8C). In these cells, when tetracaine application shifted Ca\(^{2+}\) from the cytosol into the SR, the Mag-Fura-2 fluorescence ratio increased (Figure 8C). This tetracaine-induced rise in diastolic free [Ca\(^{2+}\)]\(i\) was used as a measure of SR Ca\(^{2+}\) leak. The depth of caffeine-induced Ca\(^{2+}\) transient was used as a measure of free [Ca\(^{2+}\)]\(i\). To create the free [Ca\(^{2+}\)]\(i\) versus leak graph (Figure 8D), SR Ca\(^{2+}\) content was modulated by exposing the cells to Tyrode solution containing different Ca\(^{2+}\) concentrations (0.2, 2, 5 mmol/L). The composition of Tyrode solution (0Ca\(^{2+}\), 0Na\(^{+}\)) was (in mmol/L): LiCl 134, KCl 5.4, MgCl\(_2\) 1, EGTA 1, glucose 10, and HEPES 10, pH adjusted to 7.4 with LiOH.

#### Statistical Analysis
All experiments were done in random sequence with respect to the genotype, and measurements were taken by a single observer who was blinded to the genotype. Differences between groups were assessed using a 1-way ANOVA (for normally distributed parameters) or by Kruskal–Wallis test (for incidence of premature ventricular contractions [PVCs]). It statistically significant differences were found, individual groups were compared by using Student’s t test or nonparametric tests as indicated in the text. Results were considered statistically significant if the probability value was less than 0.05. Unless otherwise indicated, results are expressed as arithmetic means and SEM.

#### Results

### Heterozygous Casq2\(^{+/−}\) Hearts Have a Twenty-Five Percent Decrease in Casq2 Protein
We previously noted a modest decrease in Casq2 protein in heterozygous Casq2\(^{+/−}\) hearts. To more accurately determine Casq2 protein expression in heterozygous Casq2\(^{+/−}\) hearts, Casq2 was independently quantified by immunoblot of whole-heart homogenates and SR microsomes and by \(^{45}\)Ca\(^{2+}\)-binding assay of the SR microsomes (Figure 1A). These experiments consistently demonstrated a 25% reduction of Casq2 protein in Casq2\(^{+/−}\) compared with Casq2\(^{+/+}\) hearts. We have previously shown that the 3 Casq2 protein partners located in the JSR, RyR2, triadin-1, and junctin were not significantly different from wild-type Casq2\(^{+/+}\) littermates. Calreticulin is the major Ca\(^{2+}\)-binding protein in the endoplasmic reticulum of nonmuscle cells. To test whether calreticulin was changed to compensate for the decrease in Casq2, we next quantified calreticulin in whole-heart homogenates of fetal (15 days post conception) and adult (12 weeks) mice. Although easily detectable in fetal hearts, calreticulin...
A Modest Decrease in Casq2 Does Not Affect SR Volume

Ablation of the Casq2 gene causes a detectable decrease in the density of the lumen of the jSR cisternae because of absence of Casq2. Despite complete loss of the major Ca\(^{2+}\) buffering protein, SR Ca\(^{2+}\) content was largely maintained in Casq2\(^{-/-}\) myocytes, apparently by a 50% increase in the SR volume fraction. Here, the effect of a more modest reduction in Casq2 protein expression on SR volume was examined using quantitative electron microscopic analysis of heterozygous Casq2\(^{+/-}\) hearts. There were no differences in dyad structure between the 2 groups: Casq2 was readily visible within the flat jSR cisternae and had the characteristic “beaded” appearance (Figure 1C). The SR volume fraction (SR volume per total myocyte volume, percentage) was not statistically different between Casq2\(^{+/-}\) and Casq2\(^{-/-}\) hearts (Casq2\(^{+/-}\) 1.42±0.58, n=141 images, 47 cells, 3 mice, versus Casq2\(^{-/-}\) 1.35±0.53, n=177 images, 59 cells, 3 mice; *P=0.45; data are mean±SD). Similarly, the relative SR surface area (SR surface area per total myocyte volume, micrometers squared per micrometers cubed) was also unchanged in Casq2\(^{+/-}\) hearts (Casq2\(^{+/-}\) 0.31±0.12, n=141 images, 47 cells, 3 mice, versus Casq2\(^{-/-}\) 0.32±0.13, n=177 images, 59 cells, 3 mice; **P=0.25; data are mean±SD).

Together, these data indicate that the modest reduction of Casq2 protein in Casq2\(^{-/-}\) mice does not trigger a compensatory increase in the SR volume.

Casq2\(^{-/-}\) Mice Have an Increased Incidence of Ventricular Ectopy on Catecholamine Challenge

Casq2\(^{-/-}\) mice display normal cardiac contractility without any evidence of heart failure, hypertrophy, or myocardial fibrosis and have normal ECG parameters including QTc interval. To test whether Casq2\(^{-/-}\) mice display an arrhyth-
mia phenotype, we next recorded ECGs in anesthetized mice after isoproterenol challenge. Isoproterenol caused frequent PVCs (Figure 2A) and rarer episodes of nonsustained VT (Figure 2B) in anesthetized Casq2/−/− mice. On average, Casq2/−/− mice had a significantly higher rate of PVCs compared with Casq2/+/+ mice and a lower rate compared with Casq2+/− mice (Figure 2C).

To further quantify arrhythmia susceptibility, we subjected 7 Casq2/−/− mice and 6 Casq2/+/+ littermates to programmed stimulation, a technique recently used to demonstrate the arrhythmia phenotype of another murine CPVT model.17 There were no significant differences in heart rate, PR interval, Wenckebach cycle length, atrioventricular node effective refractory period, or ventricular effective refractory period. Interestingly, programmed ventricular stimulation induced VT in 3 of 7 Casq2/−/− mice, even in the absence of isoproterenol (Figure 3A). In the presence of isoproterenol, ventricular effective refractory period was significantly shorter in Casq2/−/− compared with Casq2/+/+ mice (Casq2/−/−, 32±4.9 ms [n=7], versus Casq2/+/+, 47±3.6 ms [n=6]; Figure 3B).

Figure 3. Casq2/−/− mice display inducible VT by programmed ventricular stimulation. A, Representative example of a tracing showing polymorphic VT induced by programmed stimulation in the presence of isoproterenol. B and C, Histograms of VT episodes induced at baseline (−ISO) and after isoproterenol (+ISO) in Casq2/−/− and Casq2+/− mice. Casq2/−/−, n=6; Casq2+/−, n=7.

Figure 4. Casq2/−/− myocytes have normal contractility, Ca2+ transient amplitudes, and kinetics. A, Representative examples of intracellular Ca2+ transients (top trace) and cell shortening (bottom trace) recorded from Fura-2 AM–loaded, field-stimulated myocytes at 1 Hz from Casq2/−/− (left) and Casq2+/− (right). Application of 1 μmol/L isoproterenol (ISO) significantly increased Ca2+ transient heights and cell shortening in both groups of myocytes. B, Example of spontaneous premature beats recorded in a different Casq2/−/− myocyte after isoproterenol application. Such premature beats occurred in a significant number of Casq2/−/− myocytes but were rare in Casq2+/− myocytes. C and D, Comparisons of peak Ca2+ transient height and τ in the absence and presence of isoproterenol. Only records without premature beats were analyzed (Casq2/−/− myocytes, n=46 [baseline, −ISO] and n=19 [after isoproterenol, +ISO]; Casq2+/− myocytes, n=37 [baseline] and n=24 [after isoproterenol]). Peak height indicates difference between diastolic F ratio and F ratio at peak Ca2+ transient; τ, time constant for Ca2+ transient decay.
VT was induced in 6 of 7 Casq2+/− mice (11 to 34 per mouse; 122 total). In Casq2+/+ mice, VT was induced significantly less frequently compared with Casq2+/− mice (Casq2+/+, 2±1.5 episodes per mouse [n=6], versus Casq2+/−, 17±1.6 episodes per mouse [n=7]; P=0.007). Episodes longer than 10 seconds were observed in only Casq2+/− mice. Figure 3B (before isoproterenol) and 3C (after isoproterenol) illustrates the differences between Casq2+/− and Casq2+/+ mice.

These data demonstrate that modest reductions in Casq2 cause a CPVT phenotype in the absence of changes in triadin-1, junctin, SR volume, or any other detectable myocardial disease.

**Casq2+/− Myocytes Display Normal SR Ca2+ Release and Myocyte Contractility but Reduced SR Ca2+ Content**

To examine the effect of reduced Casq2 protein on myocyte Ca2+ handling, we measured [Ca2+]i in isolated, field-stimulated (1 Hz) ventricular myocytes (Figure 4A). Twitch Ca2+ transient amplitude (Figure 4C) and Ca2+ transient decay kinetics (Figure 4D) were not significantly different between the 2 groups of myocytes. Average diastolic Ca2+ and myocyte contractile parameters (eg, fractional shortening) were also not significantly different between the 2 groups with and without isoproterenol (data not shown). However, time to peak Ca2+ was slightly prolonged in Casq2+/− myocytes in the presence of isoproterenol (Casq2+/−, 40±4 ms [n=19], versus Casq2+/+, 53±4 ms [n=24]; P=0.02). Interestingly, in the presence of isoproterenol, a significantly larger fraction of Casq2+/− compared with Casq2+/+ myocytes displayed 1 or more spontaneous premature beats (Figure 4B) during the 1-Hz pacing protocol (38% versus 12%; P<0.01).

We next examined the SR Ca2+ storage capacity using the height of the caffeine-induced Ca2+ transient as a measure of total SR Ca2+ content (Figure 5A). SR Ca2+ content of Casq2+/− myocytes was not statistically different without...
isoproterenol stimulation (Figure 5B). In the presence of isoproterenol, however, SR Ca\(^{2+}\) content was significantly decreased in Casq2\(^{-/-}\) compared with Casq2\(^{+/+}\) myocytes (Figure 5B). Fractional SR Ca\(^{2+}\) release (Figure 5C) and Ca\(^{2+}\) current (Figure 5D) were not significantly different between the 2 groups of myocytes. Na/Ca exchanger function was estimated by the decay of cytosolic Ca\(^{2+}\) during caffeine application\(^{18}\) and also was not significantly different between the 2 groups (\(\tau\), Casq2\(^{+/+}\) 1.8±0.4 second [\(n=37\)]; Casq2\(^{-/-}\), 1.9±0.2 second [\(n=37\)]; \(P=0.7\)).

**Casq2\(^{-/-}\) Myocytes Display Premature Spontaneous SR Ca\(^{2+}\) Releases and Triggered Beats**

We further quantified spontaneous SR Ca\(^{2+}\) release events (SCRs) and triggered beats noted during the 1-Hz pacing experiments (Figure 4B) in a larger number of field-stimulated ventricular myocytes loaded with Fura-2 AM. Figure 6A shows examples of SCRs and triggered beats recorded in a Casq2\(^{-/-}\) myocyte exposed to isoproterenol. Among the 3 groups, in the absence of isoproterenol, there was no difference in rates of SCRs per minute (Casq2\(^{+/+}\), 1.5±1.1 [\(n=28\)]; Casq2\(^{-/-}\), 1.24±1.3 [\(n=29\)]; Casq2\(^{-/-}\), 2.3±1.1 [\(n=47\)]; \(P=NS\) among all 3 groups) or triggered beats per minute (Casq2\(^{+/+}\) 1.1±0.8 [\(n=28\)], Casq2\(^{-/-}\) 1.2±1.3 [\(n=29\)]; Casq2\(^{-/-}\) 0.89±0.36 [\(n=47\)]; \(P=NS\) among all 3 groups). However, on isoproterenol exposure, the rates of SCRs or triggered beats per minute were lowest for Casq2\(^{+/+}\) followed by Casq2\(^{-/-}\) and highest for Casq2\(^{-/-}\) (Figure 6B and 6C). These results are consistent with the arrhythmia phenotype observed in vivo (compare Figure 2C).

**Diastolic SR Ca\(^{2+}\) Leak Is Increased in Casq2\(^{-/-}\) Myocytes**

The decreased SR Ca\(^{2+}\) content and increased rate of SCRs suggests that RyR2 channels are sensitized to effects of Ca\(^{2+}\) on isoproterenol exposure in Casq2\(^{-/-}\) myocytes. To test this idea more directly, we next estimated diastolic SR Ca\(^{2+}\) leak using the tetracaine method,\(^{10,19}\) as illustrated in Figure 7A. In the presence of isoproterenol, SR Ca\(^{2+}\) leak was significantly higher in Casq2\(^{-/-}\) myocytes compared with Casq2\(^{+/+}\) myocytes (Figure 7A and 7B). Moreover, when diastolic SR Ca\(^{2+}\) leak was prevented by application of tetracaine, the SR Ca\(^{2+}\) content was no longer different between the two groups (Casq2\(^{+/+}\) 1.45±0.05 [\(n=61\)], versus Casq2\(^{-/-}\), 1.44±0.05 [\(n=58\)]; \(P=0.9\)). Thus, the lower SR Ca\(^{2+}\) content of heterozygous mice (Figure 5B) appears to be the result of increased SR Ca\(^{2+}\) leak.

It is well recognized that the rate of SR Ca\(^{2+}\) leak increases in a quasieponential fashion with increase in SR Ca\(^{2+}\) content, likely as a result of the strong SR luminal Ca\(^{2+}\) dependence of RyR2 channels.\(^{19}\) Thus, the next experiment examined the effect of changes in SR Ca\(^{2+}\) content (SR...
Ca\textsuperscript{2+} buffering by reduced Casq2. We found that the relationship between free [Ca\textsuperscript{2+}]\textsubscript{sr} and SR Ca\textsuperscript{2+} leak was significantly different between Casq2\textsuperscript{−/−} and Casq2\textsuperscript{+/−} myocytes: SR leak was similar at low free [Ca\textsuperscript{2+}]\textsubscript{sr}, but as the free [Ca\textsuperscript{2+}]\textsubscript{sr} increased, the leak became significantly higher in Casq2\textsuperscript{+/−} than Casq2\textsuperscript{−/−} myocytes for the same free [Ca\textsuperscript{2+}]\textsubscript{sr} (Figure 8D). This result provides strong evidence for the direct regulation of RyR2 Ca\textsuperscript{2+} release channels by Casq2 in intact myocytes.

**Discussion**

We have previously reported that deletion of Casq2 causes premature spontaneous SR Ca\textsuperscript{2+} release, increased SR Ca\textsuperscript{2+} leak, and CPVT.\textsuperscript{10} However, the almost complete loss of triadin-1 and junctin and SR volume increase observed in the Casq2\textsuperscript{−/−} mice could contribute to their CPVT phenotype.\textsuperscript{10} Here, we demonstrate that even a modest reduction (25%) in Casq2 protein in the presence of normal levels of triadin-1 and/or junctin is not necessary for a CPVT phenotype. Studies in adult rat myocytes that were acutely transfected with adenoviral vector containing antisense canine calsequestrin cDNA displayed reduced Casq2 levels by >50%. This resulted in a 30% reduction in caffeine-releasable SR Ca\textsuperscript{2+} content and an increased incidence of spontaneous SR Ca\textsuperscript{2+} releases on isoproterenol exposure.\textsuperscript{20} Our results are also consistent with recent work demonstrating that increasing SR

The Relationship Between Free [Ca\textsuperscript{2+}]\textsubscript{sr} and SR Ca\textsuperscript{2+} Leak Is Altered in Casq2\textsuperscript{+/−} Myocytes

It remains unclear whether Casq2 inhibits SR Ca\textsuperscript{2+} release in intact myocytes by direct regulation of RyR2 channel complexes or indirectly by decreasing free Ca\textsuperscript{2+} in the SR lumen ([Ca\textsuperscript{2+}]\textsubscript{sr}). Thus, we next measured free [Ca\textsuperscript{2+}]\textsubscript{sr} in myocytes using the low-affinity Ca\textsuperscript{2+} indicator Mag-Fura-2 AM. Free [Ca\textsuperscript{2+}]\textsubscript{sr} was quantified as the decrease in the fluorescence ratio during caffeine exposure (Figure 8A). We found no significant difference in free [Ca\textsuperscript{2+}]\textsubscript{sr} between Casq2\textsuperscript{+/−} and Casq2\textsuperscript{−/−} myocytes (Figure 8B). Increasing extracellular [Ca\textsuperscript{2+}] to 5 mmol/L increased free [Ca\textsuperscript{2+}]\textsubscript{sr} to the same extent in both groups (Figure 8B), confirming that the Ca\textsuperscript{2+} indicator Mag-Fura-2 was not saturated in our experimental conditions.

To exclude the possibility that the Mag-Fura-2 loading caused excessive intra-SR buffering and thereby abolished the differences between the 2 groups of myocytes, we simultaneously measured SR Ca\textsuperscript{2+} leak and free [Ca\textsuperscript{2+}]\textsubscript{sr} using the protocol described in Figure 8C and plotted SR Ca\textsuperscript{2+} leak as a function of free [Ca\textsuperscript{2+}] in the SR lumen (Figure 8D). This would also correct for any differences in SR Ca\textsuperscript{2+} leak as a function of free [Ca\textsuperscript{2+}] in the SR lumen (Figure 8D). This would also correct for any differences in SR Ca\textsuperscript{2+} leak as a function of free [Ca\textsuperscript{2+}] in the SR lumen (Figure 8D). This would also correct for any differences in SR Ca\textsuperscript{2+} leak as a function of free [Ca\textsuperscript{2+}] in the SR lumen (Figure 8D). This would also correct for any differences in SR Ca\textsuperscript{2+} leak as a function of free [Ca\textsuperscript{2+}] in the SR lumen (Figure 8D).
Ca\[^{2+}\] leak only produces Ca\[^{2+}\] waves if SR Ca\[^{2+}\] content is maintained by maneuvers such as β adrenergic stimulation.\(^{21}\) Based on our results in heterozygous Casq2\(^{-/-}\) mice, humans with only 1 functional CASQ2 allele will likely have reduced cardiac CASQ2 protein. Interestingly, whereas most humans heterozygous for CASQ2 mutations appear asymptomatic, CPVT has been described in an individual heterozygous for the nonsense R33X CASQ2 mutation.\(^{11}\) Two of relatives of the patient (eg, maternal grandfather and uncle) had a milder CPVT phenotype detectable only during exercise tests.\(^{11}\) Based on our data from the heterozygous Casq2\(^{-/-}\) mouse model that mimics the human disease, CPVT with exercise testing.\(^{11}\) Based on our data from the heterozygous Casq2\(^{-/-}\) mouse model, which also displays CPVT with programmed stimulation in the presence of isoproterenol but has no sustained VT after isoproterenol or exercise challenge.\(^{17}\) Similar to our results, the R176Q RyR2 mutation also causes SR Ca\[^{2+}\] leak and spontaneous SR Ca\[^{2+}\] releases at high SR load, suggesting that in mice, the Casq2\(^{-/-}\) heterozygous phenotype is comparable to the RyR2 mutation in causing CPVT.\(^{23}\) Although the role of invasive electrophysiological testing in CPVT is not established in humans, our data suggest that heterozygous carriers of CASQ2 nonsense mutations may have an increased risk for inducible arrhythmias.

At high SR Ca\[^{2+}\] loads, Ca\[^{2+}\] leak increases proportional to the degree of Casq2 reduction (Figures 7 and 8 and Knollmann et al\(^{10}\)). Interestingly, at low SR Ca\[^{2+}\] load, SR Ca\[^{2+}\] leak is not different among Casq2\(^{+/+}\), Casq2\(^{-/-}\), and Casq2\(^{-/-}\) myocytes. Collectively, these findings demonstrate the importance of Casq2 as a modulator of SR Ca\[^{2+}\] release at higher SR Ca\[^{2+}\] content. Moreover, these data also suggest that Casq2 is not as important in modulating SR Ca\[^{2+}\] release at relatively low SR Ca\[^{2+}\] content.

The exact mechanism whereby Casq2 inhibits SR Ca\[^{2+}\] release at high SR Ca\[^{2+}\] load is controversial. It has been hypothesized that luminal SR Ca\[^{2+}\] buffering by Casq2 may decrease free [Ca\[^{2+}\]]\(_{\text{SR}}\). Given the strong luminal Ca\[^{2+}\] dependence of RyR2 channels,\(^{12,13,24}\) open probability will be
reduced.\textsuperscript{25} Alternatively, Casq2 protein may directly or in conjunction with triadin-1 and/or junctin modulate the open probability of the RyR2 channel. Consistent with the latter hypothesis, reports suggest that Casq2 dissociates from the triadin/junctin/RyR2 complex at high SR luminal [Ca\textsuperscript{2+}], resulting in a dramatically increased open probability of RyR2 Ca\textsuperscript{2+} release channels.\textsuperscript{14} Our present results support the latter hypothesis and provide evidence in intact myocytes that Casq2 inhibits SR Ca\textsuperscript{2+} release channels directly and not only through modulation of free [Ca\textsuperscript{2+}]\textsubscript{i}.

We cannot exclude that other mechanisms (eg, changes in K\textsuperscript{+} currents or electrogentic ion exchangers) contribute to the arrhythmia phenotype of Casq2\textsuperscript{-/-} mice. However, Na/Ca exchanger function was not statistically different between the 2 groups as estimated by the decay kinetics of caffeine transients. Given the normal QTc interval, contractile function, and heart size of the Casq2\textsuperscript{-/-} mice,\textsuperscript{10} the possibility of K\textsuperscript{+} channel dysfunction causing CPVT also appears much less likely. Another limitation is that we used the tetracaine-induced drop in diastolic Fura-2 fluorescent ratio as an estimate of diastolic SR Ca\textsuperscript{2+} leak (Figure 7A). The nonlinear relationship between Ca\textsuperscript{2+} and fluorescence ratio and the nonlinear buffering properties of the cytoplasm,\textsuperscript{26} our results cannot be directly compared with Ca\textsuperscript{2+} leak rates obtain in other studies.\textsuperscript{16,27}

The finding that even a modest decrease in Casq2 increases arrhythmia susceptibility has potential clinical implications. For example, a number of antidepressant, neuroleptic, and chemotherapeutic agents have been shown to bind to Casq2 in vitro.\textsuperscript{28} Based on the results from the Casq2\textsuperscript{-/-} mice, even a modest disruption of Casq2 function by drugs may result in clinically significant proarrhythmia. Thus, interactions with Casq2 may have contributed to the increased risk of sudden cardiac death associated with high doses of tricyclic antidepressants.\textsuperscript{29} Equally important, individuals with only 1 functional CASQ2 allele may be at increased risk for ventricular arrhythmia and sudden death.

We have previously suggested the role of Casq2 as a “molecular safety switch,” especially at high SR Ca\textsuperscript{2+} content, preventing spontaneous SR Ca\textsuperscript{2+} releases, Ca\textsuperscript{2+} oscillations, and CPVT.\textsuperscript{10} Our new results demonstrate the underlying mechanism: Casq2 regulates SR Ca\textsuperscript{2+} release channels independent of its function as a Ca\textsuperscript{2+} buffer in the SR lumen. As a result, even a 25\% reduction of Casq2 is sufficient to cause catecholamine-induced premature spontaneous SR Ca\textsuperscript{2+} releases in myocytes and predisposition to CPVT in vivo.

Acknowledgments
We thank Dan M. Roden, MD, for thoughtful suggestions after critically reviewing the manuscript.

Sources of Funding
This work was supported by NIH grants HL86635, HL71670, HL46681 (to B.C.K.); HL48093 (to C.F.A.); and HL28556 (L.R.J.); and funds from the National Institute of Child Health and Human Development Intramural Research Program (to K.P.), a Vanderbilt University School of Medicine Clinician Scientist Award (to P.K.), and American Heart Association grant 9865306B (to T.Y.).

Disclosures
None.

References


Modest Reductions of Cardiac Calsequestrin Increase Sarcoplasmic Reticulum Ca\(^{2+}\) Leak Independent of Luminal Ca\(^{2+}\) and Trigger Ventricular Arrhythmias in Mice

Nagesh Chopra, Prince J. Kannankeril, Tao Yang, Thinn Hlaing, Izabela Holinstat, Kristen Ettenson, Karl Pfeifer, Brandy Akin, Larry R. Jones, Clara Franzini-Armstrong and Björn C. Knollmann

*Circ Res.* 2007;101:617-626; originally published online July 26, 2007; doi: 10.1161/CIRCRESAHA.107.157552

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Copyright © 2007 American Heart Association, Inc. All rights reserved.

Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:

http://circres.ahajournals.org/content/101/6/617

Data Supplement (unedited) at:

http://circres.ahajournals.org/content/suppl/2007/07/26/CIRCRESAHA.107.157552.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation Research* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:

http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Circulation Research* is online at:

http://circres.ahajournals.org/subscriptions/
METHODS – ONLINE DATA SUPPLEMENT

Protein analysis

Mouse ventricular homogenates and microsomes enriched in SR vesicles were prepared as described. SDS-PAGE and immunoblotting was conducted with the antibodies to calsequestrin and calreticulin described in ref.1, 2 The calreticulin antibody was a gift from Dr. M. Michalak. Antibody-binding calsequestrin bands were visualized with 125I-protein A, and then quantified using a Bio-Rad Personal Fx phosphorimager. Antibody-binding calreticulin bands were visualized with fluorescence labeled secondary antibodies and quantified using the Odyssey infrared imaging system (Li-Cor Biosciences, Inc). 45Ca2+ overlay to detect Ca2+-binding proteins in mouse SR vesicles was performed as described.3

Electron microscopy

Hearts were processed, imaged by electron microscopy and estimates of relative surface areas and volumes of the total SR obtained as described.4 Note however that the formula relating surface area density to line intersections was corrected to C/2dPtest, rather than the C/dPtest previously used4, where C is the number of intersections, d is the spacing between the grid lines and Ptest is the number of grid intersections in the test area.

Surface ECG and intracardiac electrophysiology studies

Surface electrocardiography (ECG) recordings were done as described.4-6 Baseline ECG was recorded for five minutes followed by 20 minutes after intraperitoneal administration of isoproterenol (1.5 mg/kg). Intracardiac electrophysiology studies were performed as described.7

Myocyte isolation and Ca2+ indicator loading.

Single ventricular myocytes were isolated by a modified collagenase/protease method as described.4 All chemicals, unless otherwise specified were obtained from Sigma (St. Louis, MO).
All the experiments were conducted in Tyrode’s solution containing (in mmol/l): CaCl2 2-5, NaCl 134, KCl 5.4, MgCl2 1, glucose 10, and HEPES 10, pH adjusted to 7.4 with NaOH.

**Ca**^{2+} **fluorescence, myocyte shortening and whole cell voltage clamp studies.**

Fura-2AM loaded ventricular myocytes were used to measure Ca^{2+} fluorescence and myocyte shortening at room temperature (∼23°C) as described.^{4} Ca^{2+} transients and ventricular myocyte shortenings were analyzed at pacing rates of 1 Hz using IonWizard™ (IonOptix, Milton, MA). Only records without premature beats were included in the analysis. L-type Ca currents (ICa-L) were measured as described.^{4}

**Analysis of spontaneous Ca**^{2+} **release and triggered beats.**

Spontaneous Ca^{2+} releases defined as any spontaneous increase of greater than 0.07 ratiometric units (three times the average background noise) above diastolic F_ratio and triggered beat were counted during 20 second recording period and reported as average number of events/minute as described.^{4}

**REFERENCES**


