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The Cardiac Ryanodine Receptor Luminal Ca²⁺ Sensor Governs Ca²⁺ Waves, Ventricular Tachyarrhythmias, and Cardiac Hypertrophy in Calsequestrin Null Mice

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Abstract

Cardiac calsequestrin (CASQ2) is commonly believed to serve as the sarcoplasmic reticulum (SR) luminal Ca²⁺ sensor. Ablation of CASQ2 promotes spontaneous Ca²⁺ waves (SCWs) and catecholaminergic polymorphic ventricular tachycardia (CPVT) upon stress but not at rest. How SCWs and CPVT are triggered by stress in the absence of the CASQ2-based luminal Ca²⁺ sensor is an important unresolved question. Here we assessed the role of the newly identified RyR2-resident luminal Ca²⁺ sensor in determining SCW propensity, CPVT susceptibility, and cardiac hypertrophy in Casq2 knockout (KO) mice. We crossbred Casq2 KO mice with RyR2 mutant (E4872Q^{+/-}) mice, which lack RyR2-resident SR luminal Ca²⁺ sensing, to generate animals with both deficiencies. Casq2^{+/-} and Casq2^{-/-} mice showed stress-induced ventricular tachyarrhythmias (VTs), whereas Casq2^{+/-}/E4872Q^{+/-} and Casq2^{-/-}/E4872Q^{+/-} mice displayed little or no stress-induced VTs. Confocal Ca²⁺ imaging revealed that Casq2^{-/-} hearts frequently exhibited SCWs after extracellular Ca²⁺ elevation or adrenergic stimulation, while Casq2^{-/-}/E4872Q^{+/-} hearts had few or no SCWs under the same conditions. Cardiac hypertrophy developed and CPVT susceptibility increased with age in Casq2^{-/-} mice, but not in Casq2^{-/-}/E4872Q^{+/-} mice. However, the amplitudes and dynamics of voltage-induced Ca²⁺ transients in Casq2^{-/-} and Casq2^{-/-}/E4872Q^{+/-} hearts were not significantly different. Our results indicate that SCWs, CPVT and hypertrophy in Casq2 null cardiac muscle are governed by the RyR2-resident luminal Ca²⁺ sensor. This implies that defects in CASQ2-based luminal Ca²⁺ sensing can be overridden by

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the RyR2-resident luminal Ca^{2+} sensor. This makes this RyR2-resident sensor a promising molecular target for the treatment of Ca^{2+} -mediated arrhythmias.

Keywords

Sarcoplasmic reticulum; Ryanodine receptor; Calsequestrin; Ca^{2+} Waves; Ca^{2+} -triggered arrhythmias; cardiomyopathies

INTRODUCTION

In cardiac muscle cells, membrane depolarization induces the release of Ca^{2+} from the sarcoplasmic reticulum (SR) via the Ca^{2+} -induced Ca^{2+} release (CICR) mechanism. In this process, a small Ca^{2+} influx through voltage-dependent L-type Ca^{2+} channel activates the cardiac SR Ca^{2+} release channel/ryanodine receptor (RyR2). The resulting large SR Ca^{2+} release then drives muscle contraction [1-3]. However, SR Ca^{2+} release can also occur spontaneously under conditions of SR Ca^{2+} overload in the absence of membrane depolarization [4-9]. This SR Ca^{2+} overload triggered spontaneous Ca^{2+} release may evoke propagating Ca^{2+} waves that can result in delayed afterdepolarizations (DADs) and triggered arrhythmias [8,10-12]. These spontaneous Ca^{2+} wave (SCW)-triggered DADs are a major cause of ventricular tachyarrhythmias (VTs) in heart failure [12-14]. Such DADs are also thought to cause catecholaminergic polymorphic ventricular tachycardia (CPVT), which is linked to mutations in RyR2 and cardiac calsequestrin (CASQ2) [15]. Indeed, CPVT-linked RyR2 or CASQ2 mutations enhance the propensity for SCWs [16-23].

It is well established that when SR Ca^{2+} content reaches a critical level, RyR2 channels begin to open [24-27]. How elevating SR Ca^{2+} load opens the RyR2 channel and triggers SCWs is unclear. The RyR2 channel is thought to be regulated by an intra-SR Ca^{2+} sensing mechanism(s) that promotes RyR2 opening as SR Ca^{2+} content rises [28-32]. The molecular nature of the intra-SR Ca^{2+} sensor is debated. It is commonly thought that CASQ2, a low affinity high capacity SR luminal Ca^{2+} binding protein, serves as the SR luminal Ca^{2+} sensor [29,33]. At low SR Ca^{2+} levels, CASQ2 associates with the triadin/junctin/RyR2 complex and inhibits RyR2 activity. At high SR Ca^{2+} loads, CASQ2 dissociates from the complex, relieving the inhibition [29,34]. Interestingly, cardiomyocytes isolated from Casq2 knockout (KO) mice still respond to SR Ca^{2+} overload and display overload-induced SCWs [35]. These findings indicate that there are other non-CASQ2 based SR luminal Ca^{2+} sensing mechanisms operating in cardiac cells. Further, single purified native and recombinant CASQ2-free RyR2s in bilayers are still modulated by luminal Ca^{2+} [36-40]. These observations indicate that the RyR2 channel is also controlled by a non-CASQ2 based, perhaps a RyR2-resident luminal Ca^{2+} sensor.

We have recently identified a RyR2-resident luminal Ca^{2+} sensor on the channel protein itself [41]. We found that mutating the E4872 residue located in the RyR2's helix bundle crossing completely abolishes luminal, but not cytosolic, Ca^{2+} activation of RyR2. This residue is part of a network of salt-bridges involved in both luminal Ca^{2+} sensing and channel gating. Cardiomyocytes in heterozygous knock-in mouse hearts expressing the RyR2-E4872Q mutant were resistant to store-overload induced SCWs. Further, the E4872Q

heterozygous mutant mice are completely protected against stress-induced VTs [41]. Thus, this newly discovered RyR2-resident luminal Ca^{2+} sensor is a key determinant of store-overload induced SCWs and stress-induced VTs. The relationship between this RyR2-resident sensor and CASQ2-based sensing is entirely unexplored.

The absence of CASQ2 has little effect in unstressed cardiomyocytes, but enhances the propensity for store-overload induced SCWs and CPVT when cells are stressed [35]. An interesting and important question is how does elevated SR luminal Ca^{2+} promote the observed SCWs and CPVT in Casq2 KO mice when CASQ2-based luminal Ca^{2+} sensing is absent. In light of our recent finding [41], we hypothesize that the RyR2-resident luminal Ca^{2+} sensor controls the occurrence of SCWs and stress-induced CPVT in Casq2 KO mice. To test this hypothesis, we generated mice lacking both CASQ2-based and RyR2-resident Ca^{2+} sensing (Casq2^{-/-}/RyR2-E4872Q^{+/-}) by crossbreeding the Casq2 KO mice with the RyR2-E4872Q^{+/-} mutant mice. We found that the RyR2-E4872Q mutation markedly suppressed SR Ca^{2+} overload induced SCWs, stress-triggered VTs, and cardiac hypertrophy in Casq2 KO mice. Thus, defects in CASQ2-based luminal Ca^{2+} sensing can be overridden by manipulating the function of the RyR2-resident luminal Ca^{2+} sensor.

MATERIALS AND METHODS

Animal Studies

All animal studies were approved by the Institutional Animal Care and Use Committees at Rush University Medical Center and the University of Calgary, and were performed in accordance with NIH guidelines.

Generation of Casq2 knockout (KO), RyR2-E4872Q Mouse Model (Casq2^{-/-}/RyR2-E4872Q^{+/-})

RyR2-E4872Q^{+/-} mice [41] were crossbred with the Casq2 KO mice [35] to obtain the Casq2^{+/-}/RyR2-E4872Q^{+/-} mice and Casq2^{+/-} littermates. Casq2^{-/-}/RyR2-E4872Q^{+/-} mice were then generated by breeding between the Casq2^{+/-}/RyR2-E4872Q^{+/-} mice. The Casq2 KO mice were a kind gift from Dr. Bjorn Knollmann's laboratory at the Vanderbilt University School of Medicine, Division of Clinical Pharmacology, Nashville, TN.

Electrocardiogram Recording and Induction of Ventricular Tachyarrhythmias in Anesthetized Mice

Wild type (WT), Casq2^{+/-}, Casq2^{+/-}/RyR2-E4872Q^{+/-}, Casq2^{-/-}, Casq2^{-/-}/RyR2-E4872Q^{+/-} mice were assessed for their susceptibility to drug-induced arrhythmias using ECG recording. Briefly, mice were lightly anesthetized with isoflurane vapor (0.5-1%) and 95% O₂. Anesthetized mice were placed on a heating pad (27 °C), and needle electrodes were inserted subcutaneously into the right upper limb and left lower abdomen (BIOPAC MP System, Goleta, CA). The animals' ECG was continuously monitored. Once the heart rate stabilized, a 5-10 minute baseline ECG recording was made. Ventricular tachyarrhythmias (VTs) were induced by intraperitoneal (i.p.) injection of a mixture of epinephrine (1.6mg/kg) and caffeine (120 mg/kg). For assessing the age-dependent susceptibility to CPVT, VTs were induced by injection (i.p.) of epinephrine (1.6mg/kg)

alone. The ECG was continuously recorded for 30 minutes immediately after injection [42]. To determine the duration (in seconds) of VT, the 30 minute overall recording period was subdivided into 10 consecutive 3-min recording periods (i.e. 0-3, 3-6, 6-9 min,, 21-24, 24-27, 27-30 min). The VT duration (in %) within each 3-min period (as well as over the entire 30 min period) was then determined by calculating the percentage of time in VT (in seconds). VT was defined as 3 or more consecutive ectopic beats.

Post-Mortem Whole Heart Hypertrophy Measurements

The heart to body weight ratios of the *Casq2*^{-/-} and *Casq2*^{-/-}/*RyR2-E4872Q*^{+/-} mice at age of 4 to 6 months were determined. Animals were sacrificed by cervical dislocation after measuring the body weight. The hearts were rapidly excised and washed thoroughly in PBS (137 mM NaCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 2.7 mM KCl, pH7.4) solution and then dried by absorbant paper. Connective tissues and fat surrounding the hearts were removed and the heart weight was then measured.

Confocal Ca²⁺ Imaging of Intact Hearts In Situ

Excised hearts were loaded with Rhod-2 AM (0.3 mM) in Krebs-Henseleit's (KH) solution (in mM: 120 NaCl, 24 NaHCO₃, 11.1 glucose, 5.4 KCl, 2.0 CaCl₂, 1 MgCl₂, 0.42 NaH₂PO₄, 10 taurine, and 5 creatine, oxygenated with 95% O₂ and 5% CO₂) at room temperature for 40 min via retrograde Langendorff perfusion system [43]. After Rhod-2 loading, hearts were transferred to another Langendorff apparatus (37 °C) attached to a confocal microscope system. *In situ* confocal line-scan imaging of Ca²⁺ signals arising from epicardial myocytes was performed on hearts beating at sinus rhythm. To minimize motion artifacts during Ca²⁺ imaging, blebbistatin (3 μM, Sigma) with BDM (2, 3-butandion-monoxim, 10 mM, Sigma) or blebbistatin (5-10 μM) without BDM were added to the perfusion solution [44,45]. Line-scan images were acquired at a rate of 1.93 ms per line. In some experiments, hearts were perfused with KH solutions containing progressively higher Ca²⁺ levels (3, 4, 5, 6 and 7 mM) to induce spontaneous Ca²⁺ waves. In other experiments, hearts perfused with the KH solution were fast paced (6Hz) with 100 nM isoproterenol to mimic physiological stress conditions and induce spontaneous Ca²⁺ waves. The amplitude, time to peak, time to 50% decay (T50) of Ca²⁺ transients while pacing at 6 Hz were assessed.

Statistical Analysis

All values shown are mean ± SEM unless indicated otherwise. To test for differences between groups, we used Student's t test (2-tailed). Statistical analyses were carried out using SPSS V.15.0 (SPSS). A *P* value <0.05 was considered to be statistically significant.

RESULTS

The *RyR2-E4872Q*^{+/-} mutation suppresses CPVT in *Casq2* KO mice

We have recently reported that the *RyR2-E4872Q*^{+/-} mutation eliminates CPVT in the *RyR2-R4496C* CPVT mouse model [41]. Here, we tested whether the *RyR2-E4872Q*^{+/-} mutation also suppresses CPVT in the *Casq2* KO CPVT mouse model. *RyR2-E4872Q*^{+/-} mice were crossbred with *Casq2* KO mice (*Casq2*^{-/-}) to generate double mutant mice

heterozygous for both genetic attributes (i.e. Casq2^{+/-}/RyR2-E4872Q^{+/-}). These mice were then crossed to produce Casq2^{-/-}/RyR2-E4872Q^{+/-} mice entirely lacking CASQ2. We then tested CPVT susceptibility of (1) WT, (2) Casq2^{+/-}, (3) Casq2^{-/-}, (4) Casq2^{+/-}/RyR2-E4872Q^{+/-} and (5) Casq2^{-/-}/RyR2-E4872Q^{+/-} mice. CPVT susceptibility was determined from ECG measurements before and after injection (i.p.) of either a mixture of epinephrine (1.6 mg/kg) and caffeine (120mg/kg) (epi/caff) or epinephrine alone.

Figure 1 shows that epi/caff injection induced transient VTs in the Casq2^{+/-} mice, which peaked at ~6 min and disappeared after ~20 minutes (Fig.1A,C). The same epi/caff mixture also induced transient VTs in WT mice (Supplementary Fig. 1). However, The epi/caff injection induced little or no VTs in Casq2^{+/-}/RyR2-E4872Q^{+/-} mice (Fig. 1B). Figure 1C shows post-injection VT duration (% time in VT) summary results collected during 10 consecutive 3-min time segments. Figure 1D compares average VT duration over the entire 30 minute period. VT duration (over 30 min) in the Casq2^{+/-}/RyR2-E4872Q^{+/-} mice (0.8±0.6%) was greatly reduced compared to that in Casq2^{+/-} mice (32.8±4.8%) (P<0.001). WT mice showed 10.9±6.7% VT duration (over 30 min), which is significantly lower than that in Casq2^{+/-} mice (P<0.05).

Figure 2 shows analogous experiments on homozygous Casq2 KO mice. Sample ECG recordings from Casq2^{-/-} (Fig. 2A) and Casq2^{-/-}/RyR2-E4872Q^{+/-} (Fig. 2B) mice before and after epi/caff injection are shown. Casq2^{-/-} mice exhibit a more severe phenotype, compared to Casq2^{+/-} mice with long periods of VTs lasting over 30 min (Fig.2C, Supplementary Fig. 2). Casq2^{-/-}/RyR2-E4872Q^{+/-} mice displayed short periods of VTs that subsided after a few minutes (Fig. 2C, Supplementary Fig. 2). Figure 2D compares the average VT duration over the entire 30 minute post-injection recording period in Casq2^{-/-} and Casq2^{-/-}/RyR2-E4872Q^{+/-} mice. As was the case for Casq2^{+/-} mice (Fig. 1), the RyR2-E4872Q^{+/-} mutation significantly decreased VT duration in Casq2^{-/-} mice (83.2±8.6% to 6.6±4.2%; P<0.001). Thus, the RyR2-E4872Q^{+/-} mutation protects against stress-induced VTs in both heterozygous and homozygous Casq2 KO mice. These observations together with our recent findings [41] suggest that the RyR2-resident luminal Ca²⁺ sensor governs CPVT with or without CASQ2 present.

Age-dependent CPVT in Casq2^{-/-} mice is eliminated by the RyR2-E4872Q^{+/-} mutation

To determine whether there is an age-dependent progression of CPVT susceptibility in Casq2 KO mice, we performed stress tests in young and old Casq2^{-/-} mice (2-3 months and 5-6 months, respectively). Figure 3A-B shows that injection of epinephrine (1.6 mg/kg) alone (no caffeine) triggered sustained VTs in old Casq2^{-/-} mice but not when the old mice were harboring the RyR2-E4872Q^{+/-} mutation. Injection of epinephrine alone did not trigger VTs in WT mice (n=6) (data not shown). Unlike in old Casq2^{-/-} mice, VTs in young Casq2^{-/-} mice subsided about 20 minutes after the epinephrine injection (Fig. 3C, Supplementary Figs. 3 and 4). Figure 3D compares the average VT duration over the entire 30 minute post-injection recording period in the young and old Casq2^{-/-} mice (21.6±5.9% and 97.1±0.9%, respectively). The old Casq2^{-/-} mice were significantly (P<0.001) more susceptible to VT than young Casq2^{-/-} mice. When the RyR2-E4872Q mutation was present, there was no post-injection VT in Casq2^{-/-} mice at either age (Fig. 3C, 3D). Thus,

the RyR2-E4872Q^{+/-} mutation also limits age-dependent CPVT progression in Casq2 KO mice. Since the RyR2-E4872Q^{+/-} mutation protects against VTs induced by epinephrine alone (Fig.3) or by a mixture of epinephrine and caffeine (Figs.1, 2) in Casq2 KO mice, the protective effect of the RyR2-E4872Q mutation is not caffeine-dependent.

The RyR2-E4872Q^{+/-} mutation prevents cardiac hypertrophy in Casq2^{-/-} mice

Casq2^{-/-} mice are known to develop modest cardiac hypertrophy that increases their heart to body weight (HW/BW) ratio by ~10% [35]. Why the Casq2 KO induces cardiac hypertrophy is unknown. Since the RyR2-E4872Q mutation protects against CPVT in Casq2^{-/-} mice, it could also limit cardiac hypertrophy in Casq2^{-/-} mice. To test this possibility, we measured HW/BW ratio in WT, Casq2^{-/-}, and Casq2^{-/-}/RyR2-E4872Q^{+/-} mice. Consistent with the previous report [35], we found a slight but significant increase in the HW/BW ratio in Casq2^{-/-} mice compared to their WT littermates (4.9±0.1 vs. 4.0±0.1, P<0.001) (Fig. 4). Interestingly, Casq2^{-/-}/RyR2-E4872Q^{+/-} mice exhibited HW/BW ratios (4.1±0.1) similar to those observed with WT mice, but significantly different from those of the Casq2^{-/-} mice (P<0.001) (Fig. 4). Thus, the RyR2-E4872Q^{+/-} mutation (i.e. limiting SCWs and CPVT) prevents the development of cardiac hypertrophy in Casq2 KO mice. These data indicate that the RyR2-resident luminal Ca²⁺ sensor is also an important determinant of Casq2 KO induced cardiac hypertrophy.

The RyR2-E4872Q^{+/-} mutation does not alter the depolarization-induced Ca²⁺ transient

The amplitude and dynamics of Ca²⁺ release evoked by depolarization (6 Hz) in Casq2^{-/-} and Casq2^{-/-}/RyR2-E4872Q^{+/-} hearts were measured using confocal line-scan Ca²⁺ imaging. Figure 5 shows that there are no significant differences in Ca²⁺ transient amplitude (F/F₀; 1.09±0.03 vs. 1.05±0.05), time-to-peak (30.6±0.23 ms vs. 30.1±0.25 ms) or time-to-50% decay (T₅₀; 52.3±0.36 ms vs. 53.1±0.28 ms) in Casq2^{-/-} and Casq2^{-/-}/RyR2-E4872Q^{+/-} hearts, respectively. Therefore, the RyR2-E4872Q^{+/-} mutation does not significantly alter the amplitude and dynamics of depolarization-induced Ca²⁺ release in Casq2 KO hearts.

The RyR2-E4872Q^{+/-} mutation diminishes spontaneous Ca²⁺ waves in intact Casq2^{-/-} hearts

Enhanced propensity for SCWs is believed to cause CPVT in Casq2^{-/-} mice. Since RyR2-E4872Q mutation suppresses SR Ca²⁺ overload induced SCWs in WT hearts [41], it is possible that the mutation also suppresses SCWs (and consequently CPVT) in Casq2^{-/-} mice. To test this possibility, SCWs in intact Casq2^{-/-} hearts were induced by increasing the extracellular Ca²⁺ concentration (2 to 3, 4, 5, 6 or 7 mM). Intracellular Ca²⁺ dynamics were measured using confocal line-scan Ca²⁺ imaging of intact hearts. Figure 6A shows sample line-scan images from Casq2^{-/-} (top) and Casq2^{-/-}/RyR2-E4872Q^{+/-} (bottom) hearts at 4 mM extracellular Ca²⁺. Periodic Ca²⁺ transients occurred at the sinus rhythm, which varied from heart to heart. SCWs were evident (see arrows) in the Casq2^{-/-} heart but not in the Casq2^{-/-}/RyR2-E4872Q^{+/-} heart. Figure 6B shows an analogous experiment done with 7 mM extracellular Ca²⁺ present. In the 7 mM case, SCWs are observed in both model hearts but are less frequent with the RyR2-E4872Q^{+/-} mutation present (9.1±2.0 vs. 2.8±0.8 Hz/100µm; P<0.01). Figure 6C shows SCW frequency summary results. SCW frequency in

Casq2 KO hearts increased with extracellular Ca^{2+} but at all extracellular Ca^{2+} levels tested the RyR2-E4872Q^{+/-} mutation significantly decreased SCW frequency. To avoid motion artifacts, muscle contraction was inhibited by blebbistatin (3 μM) plus 2,3-butanedione-monoxim (BDM) (10 mM). Since BDM has been reported to be an RyR2 agonist, control studies were done without BDM present (Supplementary Fig. 5). The RyR2-E4872Q^{+/-} mutation suppressed SCWs in Casq2^{-/-} hearts in the presence or absence of BDM.

To eliminate the influence of the intrinsic sinus rhythm on SCW occurrence as well as to promote SR Ca^{2+} overload, we ablated the AV node by electro-cautery and then perfused hearts with 100 nM isoproterenol. AV node-ablated Casq2^{-/-} or Casq2^{-/-}/RyR2-E4872Q^{+/-} hearts were paced at 6 Hz to standardize the SR Ca^{2+} load and then the pace was reduced to 0.6 Hz. Figure 7A-B shows sample confocal line-scan images during the 0.6 Hz pacing period with SCW marked (see arrows). Figure 7C compares average SCW frequency in Casq2^{-/-} ($2.0 \pm 0.3\text{Hz}/100\mu\text{m}$) and Casq2^{-/-}/RyR2-E4872Q^{+/-} ($0.1 \pm 0.0\text{Hz}/100\mu\text{m}$) hearts. The presence of the RyR2-E4872Q^{+/-} mutation significantly ($P < 0.001$) reduced the occurrence of SCWs in Casq2 KO hearts. Taken together, these results show that the RyR2-resident luminal Ca^{2+} sensor is a key determinant of the occurrence of SCWs in Casq2 KO hearts.

DISCUSSION

A novel and important finding of the present study is that the RyR2-resident luminal Ca^{2+} sensor determines whether SCWs and Ca^{2+} -triggered arrhythmias occur in the absence of the CASQ2-based luminal Ca^{2+} regulation. We have recently shown that this RyR2 luminal Ca^{2+} sensor located in the helix bundle crossing (the proposed gate) of the channel is responsible for luminal Ca^{2+} activation of the RyR2 channel and the occurrence of SCWs in the heart. Further, we demonstrate that a RyR2 luminal Ca^{2+} sensing mutation, RyR2-E4872Q, completely abolishes stress-induced VTs in a mouse model harbouring a CPVT-linked RyR2 mutation R4496C [41]. In the present work, we show that the RyR2-E4872Q mutation diminishes SCWs and stress-induced VTs in Casq2 KO mice. Thus, manipulating the RyR2-resident luminal Ca^{2+} sensor can protect against stress-induced VTs that arise from either a RyR2 mutation or CASQ2 deficiency. Thus, the RyR2-resident luminal Ca^{2+} sensor represents a promising therapeutic target for suppressing Ca^{2+} -triggered arrhythmias.

Another novel finding is that the RyR2-E4872Q^{+/-} mutation prevented cardiac hypertrophy in Casq2 KO mice. The reason that Casq2 KO mice develop cardiac hypertrophy is not entirely clear. It is possible that CASQ2 deficiency enhances diastolic spontaneous SR Ca^{2+} leak, which could drive Ca^{2+} -dependent cardiac remodelling [46,47]. Thus, suppressing the RyR2-resident luminal Ca^{2+} sensor may prevent the cardiac remodelling by limiting diastolic SR Ca^{2+} leak. Another interesting finding is that Casq2 KO mice become more susceptible to CPVT with age. Again, the mechanism for this age-dependent progression of CPVT in Casq2 KO mice is unknown. It is also unclear whether or not CPVT progresses with age in humans. Unlike mice that do not usually die from CPVT, humans succumb to CPVT at a young age, making it difficult to study the age-dependent progression of CPVT in humans. Interestingly, CPVT progression in mice occurs concurrently with the development of cardiac hypertrophy, suggesting that the two may be associated. In any

event, manipulating the RyR2-resident luminal Ca^{2+} sensor limits both processes, and thus may generally be used to control Ca^{2+} -mediated cardiomyopathies.

It has long been recognized that the Ca^{2+} level inside the SR critically regulates SR Ca^{2+} release [24-27], but the molecular basis of that regulation is still debated. The RyR2 channel is likely to be modulated by SR Ca^{2+} load via some luminal Ca^{2+} sensing mechanisms [28-32]. CASQ2 is commonly believed to act as the luminal Ca^{2+} sensor responsible for the regulation of Ca^{2+} release by SR luminal Ca^{2+} [29,33]. However, cardiac SR Ca^{2+} release in Casq2 KO mice is still governed by SR Ca^{2+} load [35]. This implies that other intra-SR Ca^{2+} control mechanisms exist. The recently identified RyR2-resident luminal Ca^{2+} sensor [41] likely explains why the absence of CASQ2 does not eliminate regulation of RyR2 by SR luminal Ca^{2+} . Although ablating the CASQ2-based luminal Ca^{2+} sensor does not abolish luminal Ca^{2+} regulation of SR Ca^{2+} release, disabling the RyR2-resident luminal Ca^{2+} sensor eliminates SR luminal Ca^{2+} sensing with or without CASQ2 present. These observations indicate that even in the absence of the previously known luminal Ca^{2+} sensor (CASQ2), the RyR2-resident luminal Ca^{2+} sensor still works as an effective regulator of RyR2 and its absence leads to suppression of SCWs and CPVT. These data suggest that regulation of RyR2 by luminal Ca^{2+} may involve a hierarchy of intra-SR luminal Ca^{2+} sensors. In this hierarchy, the RyR2-resident luminal Ca^{2+} sensor overrides the CASQ2-based luminal Ca^{2+} sensor. This hierarchy of sensors is consistent with our observation that the RyR2-resident luminal Ca^{2+} sensor mutation, E4872Q^{+/-}, protects against both RyR2- and CASQ2-associated CPVT.

RyR2-linked CPVT in humans is inherited in an autosomal dominant fashion, whereas CASQ2-associated CPVT in humans is inherited in an autosomal recessive manner [15]. Consistent with human CPVT, stress-induced VTs can be readily observed in heterozygous CPVT-linked RyR2 mutant mice or in homozygous CPVT-linked Casq2-mutant or Casq2 KO mice. At a variance with human CPVT, heterozygous Casq2 KO mice also displayed stress-induced VTs [48]. Consistent with this previous observation, we found that heterozygous Casq2 KO mice are also susceptible to CPVT under our triggering conditions (i.e. with epinephrine and caffeine). CPVT sensitivity of heterozygous Casq2 KO mice has been explained by the modest reduction in CASQ2 protein expression [48]. It is thought that the reduced CASQ2 expression alters the CASQ2-RyR2 interaction and RyR2 function [48]. However, CASQ2 is also an intra-SR Ca^{2+} buffer and a reduction in CASQ2 expression could also alter intra-SR Ca^{2+} dynamics and, in turn, function of the RyR2-resident luminal Ca^{2+} sensor. CPVT-causing CASQ2 mutations typically result in a truncated CASQ2, reduced CASQ2 expression levels and/or altered CASQ2 Ca^{2+} binding capacity [15]. These diverse phenotypes may alter intra-SR Ca^{2+} buffering power and thus the SR Ca^{2+} dynamics that could influence the function of the RyR2-resident luminal Ca^{2+} sensor. Thus, the existence of the RyR2-resident luminal Ca^{2+} sensing mechanism makes defining the relative contributions of CASQ2 buffering and the CASQ2-RyR2 interaction more important than ever.

In summary, RyR2 function is likely governed by hierarchical operation of multiple intra-SR Ca^{2+} sensing mechanisms. Removal of RyR2-resident luminal Ca^{2+} sensing eliminates SCWs and CPVT with or without CASQ2 present. CASQ2 deficiency induces cardiac

hypertrophy and increases CPVT susceptibility with age in mice only when RyR2-resident luminal Ca^{2+} sensing is present. This dominant action of the RyR2-resident luminal Ca^{2+} sensor makes it a promising molecular target for the treatment of Ca^{2+} -triggered arrhythmias.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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THE ABBREVIATIONS USED

RyR2	cardiac ryanodine receptor
CASQ2	cardiac calsequestrin
CPVT	catecholaminergic polymorphic ventricular tachycardia
DAD	delayed afterdepolarization
SR	sarcoplasmic reticulum
SCWs	spontaneous Ca^{2+} waves

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Summary Statement

Suppressing ryanodine receptor-resident luminal Ca^{2+} sensing eliminates Ca^{2+} waves and wave-evoked arrhythmias with or without the calsequestrin-based luminal Ca^{2+} sensing mechanism, suggesting that the ryanodine receptor luminal Ca^{2+} sensor dominates luminal Ca^{2+} control of Ca^{2+} waves and Ca^{2+} triggered arrhythmias.

Figure 1. RyR2-E4872Q mutation suppresses CPVT in heterozygous Casq2^{+/−} mice

Representative ECG recordings (15s-trace at top, 2s-trace at bottom) of Casq2^{+/−} (A) and Casq2^{+/−}/E4872Q^{+/−} (B) mice before (top panels) and 3-6 min after (bottom panels) intraperitoneal injection of epinephrine (1.6mg/kg) and caffeine (120mg/kg). VTs occurred intermittently, and the percentage (%) time in VT (VT duration) was measured within each of 10 consecutive 3-min periods immediately post-injection (C). The percentage (%) time in VT (VT duration) over the entire 30-min recording period was also determined (D). Boxes indicate region that was time expanded below. Data shown are mean $\pm$ SEM from WT (n=6), Casq2^{+/−} (n=7), and Casq2^{+/−}/E4872Q^{+/−} (n=9) mice (* P <0.05, vs. Casq2^{+/−}; ** P <0.001, vs. Casq2^{+/−}).

Figure 2. RyR2-E4872Q mutation protects against CPVT in homozygous $Casq2^{-/-}$ mice
 Representative ECG recordings (15s-trace at top, 2s-trace at bottom) of $Casq2^{-/-}$ (A) and $Casq2^{-/-}/E4872Q^{+/-}$ (B) mice before (top panels) and 3-6 min after (bottom panels) intraperitoneal injection of epinephrine (1.6mg/kg) and caffeine (120mg/kg). VT occurred intermittently and % time in VT (VT duration) was measured in sequential 3-min periods post-injection (C). Average time in VT (VT duration) was measured (post-injection) over the entire 30-min recording period (D). Data shown are mean $\pm$ SEM from 8 mice for each group (** $P < 0.001$, vs. $Casq2^{-/-}$).

Figure 3. Age-dependent susceptibility to CPVT in Casq2^{-/-} but not in Casq2^{-/-}/E4872Q^{+/-} mice

Representative ECG recordings (15s-trace at top, 2s-trace at bottom) of 5-6 month-old Casq2^{-/-} mice (A) and Casq2^{-/-}/E4872Q^{+/-} (B) mice before (top panels) and 3-6 min after (bottom panels) intraperitoneal injection of epinephrine (1.6mg/kg) alone (no caffeine). VT duration (%) in 2-3 month-old and 5-6 month-old Casq2^{-/-} and Casq2^{-/-}/E4872Q^{+/-} mice was measured within each of 10 consecutive 3-min periods (C) as well as over the entire 30-min recording period (D). Note that epinephrine alone triggered long-lasting VTs in 5-6 month-old Casq2^{-/-} mice, but only short and transient VTs in 2-3 month-old Casq2^{-/-} mice. There were no VTs detected in Casq2^{-/-}/E4872Q^{+/-} mice of 2-3 month- or 5-6 month-old. Data shown are mean $\pm$ SEM from 6-9 mice for each group. (** $P < 0.001$, 2-3 months vs 5-6 months Casq2^{-/-} mice or Casq2^{-/-}/E4872Q^{+/-} vs. Casq2^{-/-} mice within the same age group).

Figure 4. RyR2-E4872Q mutation prevents cardiac hypertrophy in Casq2^{-/-} mice
Heart weight (HW, mg) to body weight (BW, g) ratio of 4-6 month old WT, Casq2^{-/-}, and Casq2^{-/-}/E4872Q^{+/-} mice. Note that the HW/BW ratio of Casq2^{-/-} mice was increased compared to that of WT mice, whereas the HW/BW ratio of Casq2^{-/-}/E4872Q^{+/-} mice was similar to that of WT mice. Data shown are mean $\pm$ SEM from WT (n=27), Casq2^{-/-} (n=28), and Casq2^{-/-}/E4872Q^{+/-} (n=20) mice (*P < 0.001 vs. WT or vs. Casq2^{-/-}/E4872Q^{+/-}).

Figure 5. RyR2-E4872Q mutation does not affect depolarization-induced Ca^{2+} transients in intact $\text{Casq2}^{-/-}$ hearts

Intact hearts isolated from $\text{Casq2}^{-/-}$ and $\text{Casq2}^{-/-}/\text{E4872Q}^{+/-}$ mice were loaded with Rhod-2-AM in KH solution and Langendorff-perfused with blebbistatin (5 μM) to inhibit muscle contraction. The perfused heart was paced at 6Hz, and Ca^{2+} transients were monitored by line-scan confocal Ca^{2+} imaging. Representative images/traces of $\text{Casq2}^{-/-}$ (A) and $\text{Casq2}^{-/-}/\text{E4872Q}^{+/-}$ hearts (B) are shown. Short bars to the left indicate cell boundaries within the intact heart. Average data of the amplitude (C), time to peak (D), and time to 50% decay (E) of Ca^{2+} transients in $\text{Casq2}^{-/-}$ and $\text{Casq2}^{-/-}/\text{E4872Q}^{+/-}$ hearts are shown. Data shown are mean $\pm$ SEM from 75 $\text{Casq2}^{-/-}$ and 60 $\text{Casq2}^{-/-}/\text{E4872Q}^{+/-}$ cardiomyocytes in 9 hearts for each group. Note that there are no significant differences in amplitude, time to peak, or time to 50% decay between $\text{Casq2}^{-/-}$ and $\text{Casq2}^{-/-}/\text{E4872Q}^{+/-}$ hearts.

Figure 6. RyR2-E4872Q mutation suppresses spontaneous Ca^{2+} waves induced by elevating extracellular Ca^{2+} in intact $\text{Casq2}^{-/-}$ hearts

Intact hearts isolated from $\text{Casq2}^{-/-}$ and $\text{Casq2}^{-/-}/\text{E4872Q}^{+/+}$ mice were loaded with Rhod-2-AM and Langendorff-perfused with KH solution containing extracellular Ca^{2+} ($[\text{Ca}^{2+}]_o$) from 2 to 7 mM to induce SR Ca^{2+} overload, and blebbistatin (3 μM) plus 2,3-butanedion-monoxim (BDM) (10 mM) to inhibit muscle contraction. Intracellular Ca^{2+} dynamics in epicardial ventricular myocytes in intact hearts under sinus rhythm was monitored by using line-scan confocal Ca^{2+} imaging. Representative line-scan Ca^{2+} images of $\text{Casq2}^{-/-}$ and $\text{Casq2}^{-/-}/\text{E4872Q}^{+/+}$ hearts at 4 mM (A) and 7 mM (B) $[\text{Ca}^{2+}]_o$ are shown. Arrowheads show the occurrence of SCWs. Short bars to the left indicate cell boundaries within the intact heart. (C) Frequency of SCWs at different $[\text{Ca}^{2+}]_o$ in $\text{Casq2}^{-/-}$ and $\text{Casq2}^{-/-}/\text{E4872Q}^{+/+}$ mouse hearts. Data shown are mean $\pm$ SEM from 4-5 mouse hearts for each group (* $p < 0.05$, ** $p < 0.01$, vs. $\text{Casq2}^{-/-}$).

Figure 7. RyR2-E4872Q mutation suppresses spontaneous Ca^{2+} waves induced by fast pacing and isoproterenol in intact $Casq2^{-/-}$ hearts

Intact hearts isolated from $Casq2^{-/-}$ and $Casq2^{-/-}/E4872Q^{+/-}$ mice were loaded with Rhod-2-AM and Langendorff-perfused with 2 mM extracellular Ca^{2+} , 100nM isoproterenol (ISO) and blebbistatin (10 μ M) in KH solution. Representative Ca^{2+} images of $Casq2^{-/-}$ heart (A) and $Casq2^{-/-}/E4872Q^{+/-}$ hearts (B) during 6Hz pacing and after switching the pacing from 6 Hz to 0.6 Hz in the presence of 100 nM ISO are shown. Arrowheads show the occurrence of SCWs. Short bars to the left indicate cell boundaries within the intact heart. (C) Frequency of SCWs after switching the pacing frequency from 6 Hz to 0.6 Hz in the presence of 100 nM isoproterenol (ISO). Data shown are mean $\pm$ SEM from 3-4 mouse hearts for each group (** $p < 0.001$, vs. $Casq2^{-/-}$).