Published in final edited form as:

J Mol Cell Cardiol. 2015 May ; 82: 75–83. doi:10.1016/j.yjmcc.2015.02.027.

Impaired calcium-calmodulin-dependent inactivation of $\text{Ca}_v1.2$ contributes to loss of sarcoplasmic reticulum calcium release refractoriness in mice lacking calsequestrin 2

Dmytro O. Kryshchal¹, Oleksiy Gryshchenko^{1,2}, Nieves Gomez-Hurtado¹, and Bjorn C. Knollmann¹

¹Department of Medicine, Vanderbilt University, Nashville, TN, USA

²Department of General Physiology of Nervous System, Bogomoletz Institute of Physiology, Kyiv, Ukraine

Abstract

Aims—In cardiac muscle, Ca^{2+} release from sarcoplasmic reticulum (SR) is reduced with successively shorter coupling intervals of premature stimuli, a phenomenon known as SR Ca^{2+} release refractoriness. We recently reported that the SR luminal Ca^{2+} binding protein calsequestrin 2 (Casq2) contributes to release refractoriness in intact mouse hearts, but the underlying mechanisms remain unclear. Here, we further investigate the mechanisms responsible for physiological release refractoriness.

Methods and Results—Gene-targeted ablation of Casq2 (Casq2 KO) abolished SR Ca^{2+} release refractoriness in isolated mouse ventricular myocytes. Surprisingly, impaired Ca^{2+} -dependent inactivation of L-type Ca^{2+} current (I_{Ca}), which is responsible for triggering SR Ca^{2+} release, significantly contributed to loss of Ca^{2+} release refractoriness in Casq2 KO myocytes. Recovery from Ca^{2+} -dependent inactivation of I_{Ca} was significantly accelerated in Casq2 KO compared to wild-type (WT) myocytes. In contrast, voltage-dependent inactivation measured by using Ba^{2+} as charge carrier was not significantly different between WT and Casq2 KO myocytes. Ca^{2+} -dependent inactivation of I_{Ca} was normalized by intracellular dialysis of excess apo-CaM (20 μM), which also partially restored physiological Ca^{2+} release refractoriness in Casq2 KO myocytes.

Conclusions—Our findings reveal that the intra-SR protein Casq2 is largely responsible for the phenomenon of SR Ca^{2+} release refractoriness in murine ventricular myocytes. We also report a novel mechanism of impaired Ca^{2+} -CaM-dependent inactivation of $\text{Ca}_v1.2$, which contributes to

© 2015 Published by Elsevier Ltd.

Correspondence: Björn C. Knollmann, MD, PhD, Professor of Medicine and Pharmacology, Division of Clinical Pharmacology, Vanderbilt University School of Medicine, Medical Research Building IV, Rm. 1265, 2215B Garland Ave, Nashville, TN 37232-0575, Phone: (615) 343-6493, bjorn.knollmann@vanderbilt.edu.

Conflict of Interest

The authors declare no conflict of interest

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

the loss of SR Ca^{2+} release refractoriness in the Casq2 KO mouse model and, therefore, may further increase risk for ventricular arrhythmia *in vivo*.

Keywords

Calsequestrin; calmodulin; calcium release restitution; L-type calcium channel; sarcoplasmic reticulum

1. Introduction

Precise control of the release of Ca^{2+} from intracellular stores of sarcoplasmic reticulum (SR) in cardiac myocytes is important for normal contractility and functioning of cardiac muscle [2]. On the other hand, spontaneous Ca^{2+} release from ryanodine receptor (RyR2) SR Ca^{2+} release channels is thought to be one of the underlying mechanisms responsible for ventricular arrhythmia and sudden cardiac death. Both acquired (such as acute myocardial infarction and heart failure) and inherited (e.g. catecholaminergic polymorphic ventricular tachycardia) arrhythmia syndromes are accompanied by abnormalities in Ca^{2+} handling in cardiac myocytes [2]. Published reports suggest that spontaneous Ca^{2+} release from the SR, particularly due to alterations in RyR2 activity, may serve as a trigger for ventricular arrhythmia in heart failure [3–6]. Moreover, interventions that decrease sudden cardiac death and increase survival in patients with heart failure also normalize cardiac Ca^{2+} handling [7, 8]. In aggregate, these data suggest that altered myocyte Ca^{2+} release and ventricular arrhythmias are closely linked.

Calsequestrin 2 (Casq2) is a high-capacity Ca^{2+} -binding protein located in the junctional sarcoplasmic reticulum (jSR) of cardiac muscle [9–11]. Casq2 binds to the RyR2, either directly or via two other jSR proteins, junctin and triadin [12, 13] which together form the SR Ca^{2+} release unit (CRU) [14]. This protein complex is responsible for Ca^{2+} release from the SR, triggered by increase in cytosolic Ca^{2+} concentration due to activation of L-type Ca^{2+} channels ($\text{Ca}_v1.2$), also known as Ca^{2+} -induced Ca^{2+} release (CICR) [15, 16]. In this way, CRUs are responsible for the precise control of Ca^{2+} release during excitation-contraction coupling in cardiac tissue [17]. Functionally, Ca^{2+} entering through $\text{Ca}_v1.2$ during an action potential triggers Ca^{2+} release from the SR [15]. In case of two consecutive stimuli, the amplitude of the resulting Ca^{2+} transient depends on the diastolic interval that preceded the second stimulus – a phenomenon known as Ca^{2+} release refractoriness [18].

Casq2, the major intra-SR Ca^{2+} buffering protein [18], has been suggested to modulate activity of RyR2 Ca^{2+} -release channels [19, 20] regulating its sensitivity to intra-SR luminal Ca^{2+} . We recently found that Casq2, acting both as intra-SR Ca^{2+} buffer and as a regulator of sensitivity of RyR2 to luminal Ca^{2+} , importantly governs Ca^{2+} transient restitution in intact mouse hearts [1]. Here, we further investigate the role of Casq2 in regulating SR Ca^{2+} release refractoriness in isolated ventricular myocytes under experimental condition that allow control of the physiological trigger of Ca^{2+} release, the $\text{Ca}_v1.2$ current.

2. Methods

2.1. Ethics

All experiments were approved by the institutional animal care and use committees at Animal Care and Use Committees of Vanderbilt University and performed in accordance with NIH guidelines. Mouse heart harvest was performed under general anesthesia (inhalation of 3% isoflurane vapor) and animals euthanized by exsanguination.

2.2. Restitution protocols

Restitution curves were obtained by introducing an additional stimulation pulse (S2) at different time intervals (S1–S2 coupling intervals) with respect to the regular pacing pulses (S1) as shown in Fig. 2A. The same experimental protocol was used in all the experiments studying refractoriness of either cytosolic Ca^{2+} transients or $\text{Ca}_v1.2$ currents. To analyze the kinetics of the recovery of cytosolic Ca^{2+} transients, recovery ratios were calculated from the amplitudes of S2 (extrasystolic) and S1 (regular baseline pacing) beat. To plot restitution curves, we expressed either the S2 Ca^{2+} release transient or the amplitude of $\text{Ca}_v1.2$ currents during S2 stimulus as a function of the S1–S2 coupling interval, using the following formula:

$$1 - \frac{S1 - S2}{S1} \times 100\%$$

Our approach for analyzing SR Ca^{2+} restitution is different from the one that was used in our previous reports [1]. To better reflect that Ca^{2+} reuptake is not complete and hence the SR is still depleted during the very premature S2 beats, we use the value of the peak of the S2 transient rather than the S2 transient amplitude (see Fig. 1 for details). This approach takes into account the Ca^{2+} in the cytosol that is not yet taken up when the premature S2 stimulus is delivered and obviously cannot be released. We posit that this is a more accurate approach for measuring RyR2 release refractoriness in the intact myocyte.

2.3. Myocyte isolation and cytosolic Ca^{2+} transient measurements

Ventricular myocytes from 12 to 16 weeks old male and female Casq2 KO or wild-type (WT) mice from the same C57BL/6 strain were isolated using a modified collagenase/ protease method as previously described [21]. All experiments on field-stimulated myocytes were conducted in Tyrode's solution (TS) containing (in mM): NaCl 134, KCl 5.4, MgCl_2 1, CaCl_2 2, glucose 10, HEPES 10, adjusted to pH 7.4 with NaOH. After isolation, myocytes were loaded with Fura-2 acetoxymethyl ester, Fura-2 AM as described previously [22]. Briefly, myocytes were incubated with 2 μM Fura 2 AM for 6 minutes at room temperature to load the indicator in the cytosol. Myocytes were then washed twice for 10 minutes with TS containing 250 μM probenecid to retain the indicator in the cytosol. A minimum of 30 min was allowed for de-esterification before imaging the cells.

Fura-2-loaded healthy rod-shaped isolated ventricular myocytes were placed into the experimental chamber, field stimulated, and superfused with TS. Intracellular Ca^{2+} transients were measured using a dual-beam excitation fluorescence photometry setup

(IonOptix Corp.) utilizing the protocol shown in Fig. 2A. After that, myocytes were exposed for 4 seconds to TS containing 10 mmol/l caffeine using a rapid concentration-clamp system. The amplitude of the caffeine-induced Ca^{2+} transient was used as an estimate of total SR Ca^{2+} content [18]. All experiments were conducted at room temperature ($\sim 23^\circ\text{C}$). Ca^{2+} transients were analyzed using specialized data analysis software (IonWizard, IonOptixCorp.). Excitation wavelengths of 360 and 380 nm were used to monitor the fluorescence signals of Ca^{2+} -bound and Ca^{2+} -free fura-2, and $[\text{Ca}^{2+}]_i$ measurements are reported as fluorescence ratios (F_{ratio}).

For the measurements of cytosolic Ca^{2+} transients in voltage-clamp mode, cells were loaded with fluo-4 pentapotassium salt (final concentration 100 μM), added into pipette solution from stock. Pipette solution contained (in mM): CsCl 125, MgATP 5, MgCl_2 1, glutathione (GSH) 5, cAMP 0.05, HEPES 20, adjusted to pH 7.25 with CsOH. External K-free solution contained (in mM): NaCl 134, CsCl 5, MgCl_2 1, CaCl_2 2, glucose 10, HEPES 10, adjusted to pH 7.4 with NaOH. The same S1–S2 stimulation protocol, only applied in voltage-clamp mode, was used. Fig. 3A demonstrates representative examples of membrane currents and corresponding $[\text{Ca}^{2+}]_i$ transients from the cytosol in response to S1 and S2 voltage stimuli. Again, in the end of experiment each cell was exposed to TS containing 10 mmol/l caffeine to estimate total SR Ca^{2+} content. All chemicals, unless otherwise specified, were obtained from Sigma (St. Louis, MO).

2.4. Measurements of $\text{Ca}_v1.2$ current

For measurements of $\text{Ca}_v1.2$ current, freshly isolated murine ventricular myocytes were whole-cell patched in Tyrode's solution and then solution was changed to K^+ -free solution (described above) containing either 2 mM CaCl_2 or 2mM BaCl_2 . In all experiments, myocytes were pre-incubated for 30 min in 50 μM Ryanodine + 10 μM Thapsigargin + 30 μM TTX to eliminate SR Ca^{2+} release and block sodium currents. The pipette solution contained (in mM): CsCl 110, MgCl_2 1, MgATP 5, cAMP 0.2; EGTA 14; Fluo-4 0.1, Hepes 20; pH=7.25 (CsOH). For experiments testing effect of calmodulin (CaM) on inactivation of I_{Ca} , CaM purified from bovine testes (Sigma) was added into pipette solution for a final concentration of 20 μM . All experiments were carried out at room temperature.

2.5. Western Blot

Ventricular cardiomyocytes obtained from wild-type and Casq2 KO mice were homogenized and centrifuged at 100 g for 1 min at 4°C to eliminate the cellular debris. The supernatants were used for immunoblotting. The extracted proteins were separated on SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% milk and incubated overnight with primary antibodies: Calmodulin (Cell signaling antibodies) and GAPDH (Ambion). Specific bands were detected using Pierce ECL protein detection system. Western blot analyses were performed using ImageJ software (NIH).

2.6. Statistical analysis

Differences between groups were assessed using a one-way analysis of variance (ANOVA). If statistically significant differences were found, individual groups were compared with

Student's two-sided t test. Results were considered statistically significant if the P value was <0.05 . Unless otherwise indicated, results are expressed as arithmetic means $\pm$ SE.

3. Results

3.1. Refractoriness of SR Ca^{2+} release is eliminated in ventricular myocytes lacking Casq2

Previous studies have shown that ventricular myocytes isolated from Casq2 KO mice exhibit elevated rates of premature spontaneous Ca^{2+} releases, delayed after depolarizations and triggered beats, and catecholamine-induced ventricular arrhythmias *in vivo* [21, 22]. We hypothesized that the arrhythmogenic potential of loss of Casq2 may be related to how quickly a secondary Ca^{2+} release can be elicited in cardiac myocyte. To investigate this hypothesis, we compared refractoriness of SR Ca^{2+} release in intact ventricular myocytes from WT and Casq2 KO mice using a field stimulation protocol (Fig. 2A). Under these conditions, myocytes from WT mice exhibit robust time-dependent refractoriness of Ca^{2+} release from the SR, i.e., the cytosolic Ca^{2+} transients in response to premature S2 stimuli were significantly smaller compared to the transients elicited during regular S1 pacing, especially at the shortest S1–S2 interval (Fig. 2B). Unlike WT, myocytes from Casq2 KO animals exhibited near complete absence of SR Ca^{2+} release refractoriness, even at very short S1–S2 coupling intervals (Fig. 2C).

Average restitution curves were plotted for each group (Fig. 2D), demonstrating an almost complete lack of SR Ca^{2+} release refractoriness in Casq2 KO myocytes. At the same time, SR Ca^{2+} content, estimated by the amplitude of Ca^{2+} transient as response to rapid application of caffeine (10 mM/L), was similar for both models (Fig. 2E). Our results indicate that Casq2 protein is responsible for SR Ca^{2+} refractoriness observed in isolated ventricular myocytes. Our data are in agreement with our previous report, where dramatic acceleration of Ca^{2+} release recovery was found at the whole heart level [1].

3.2. Accelerated recovery of $\text{Ca}_v1.2$ current contributes to the loss of SR Ca^{2+} release refractoriness in Casq2 KO myocytes

There are several factors that may contribute to the refractoriness of SR Ca^{2+} release in cardiac muscle. Since $\text{Ca}_v1.2$ current serves as the trigger for Ca^{2+} release from the SR during the cardiac action potential, refractoriness of $\text{Ca}_v1.2$ channels can be one of potential contributors to SR Ca^{2+} release refractoriness. To test this hypothesis, we next used an experimental protocol in which voltage clamp was used instead of field stimulation (Fig. 3A). $\text{Ca}_v1.2$ tail currents were used to activate Ca^{2+} release from the SR. This approach allows maintaining the Ca^{2+} current trigger essentially constant and hence eliminating possible refractoriness of $\text{Ca}_v1.2$ channels. Fig. 3B and C demonstrate representative recordings from voltage-clamped WT and Casq2 KO myocytes in response to the S1–S2 protocol shown above. Comparison of respective Ca^{2+} transient records from two approaches (field stimulation vs. voltage clamp) demonstrates the importance of $\text{Ca}_v1.2$ refractoriness for SR Ca^{2+} release, because refractoriness of SR Ca^{2+} release was accelerated substantially in voltage-clamped compared to field-stimulated cells for both WT and Casq2 KO myocytes.

Next, we compared the restitution kinetics of $\text{Ca}_v1.2$ currents in WT vs Casq2 KO myocytes (Fig. 4 A). Myocytes were pre-incubated with ryanodine (50 μM) and thapsigargin (10 μM) for 30 min to eliminate SR Ca^{2+} release. 200 μM cAMP was present in the pipette solution to fully phosphorylate the channels and to model conditions of adrenergic stimulation under which CPVT-related arrhythmias usually occur. Both groups had decreased I_{Ca} at short S1–S2 intervals, but surprisingly, I_{Ca} recovered significantly faster in Casq2 KO vs WT myocytes. At the same time, current-voltage relationships were not significantly different between the two groups (Fig. 4 B). Thus, recovery of $\text{Ca}_v1.2$ in mice lacking Casq2 is accelerated and could contribute to loss of SR Ca^{2+} release refractoriness in this model.

3.3. Ca^{2+} -dependent inactivation of $\text{Ca}_v1.2$ to cytosolic Ca^{2+} is suppressed in Casq2 KO myocytes

$\text{Ca}_v1.2$ channels exhibit both voltage- and Ca^{2+} -dependent inactivation [23, 24]. A plausible explanation for the accelerated recovery of I_{Ca} in Casq2 KO myocytes is that $\text{Ca}_v1.2$ channel inactivation is impaired. Therefore, we next investigated the rate of decay of I_{Ca} using a longer depolarizing step of 200 ms. The declining phase of I_{Ca} (=inactivation) was well fitted by a single-exponential function. The rate of inactivation was substantially slower in Casq2 KO myocytes ($\tau=93\pm11$ ms, $n=10$) compared to WT ($\tau=45\pm4$ ms, $n=10$, $p<0.005$), as shown in Fig. 5A. To distinguish between voltage- and Ca^{2+} -dependent inactivation, we next replaced Ca^{2+} in the external solution with equimolar Ba^{2+} . Ba^{2+} currents are nearly insensitive to Ba^{2+} -dependent inactivation of $\text{Ca}_v1.2$ channels [25]. Consequently, inactivation of I_{Ba} was much slower than that of I_{Ca} (compare Fig. 5A and 5B). Importantly, the difference in τ between WT and Casq2 KO myocytes was eliminated (WT: $\tau=145\pm19$ ms, Casq2 KO: $\tau=145\pm20$ ms, $n=10$ for each group). Moreover, substituting Ba^{2+} for Ca^{2+} also abolished the differences in the $\text{Ca}_v1.2$ current restitution curves in Casq2 KO and WT myocytes (Fig. 5C). Taken together, these results indicate that Ca^{2+} -dependent inactivation of $\text{Ca}_v1.2$ is defective in Casq2 KO myocytes, which is responsible for the difference between $\text{Ca}_v1.2$ current restitution curves of WT and Casq2 KO myocytes.

3.4. Apo-Calmodulin rescues the inactivation defect of $\text{Ca}_v1.2$ current and partially recovers refractoriness of SR Ca^{2+} release in Casq2 KO myocytes

The Ca^{2+} -free form of CaM (apo-CaM) is tightly bound to the C-terminus of $\text{Ca}_v1.2$ channels and serves as Ca^{2+} sensor for Ca^{2+} -dependent inactivation [26, 27]. To test if CaM-dependent regulation of $\text{Ca}_v1.2$ channels was altered, we added apo-CaM (20 μM) to the pipette solution and repeated the I_{Ca} measurements. Apo-CaM significantly accelerated inactivation of I_{Ca} in Casq2 KO myocytes (Fig. 6A), rendering the I_{Ca} inactivation kinetics almost identical to that of WT myocytes (Casq2 KO + CaM: $\tau=46\pm3$ ms, $n=7$, vs. WT: $\tau=45\pm4$ ms for WT, $n=10$, $p=0.8$). In contrast, adding CaM had no significant effect on the rate of I_{Ca} inactivation in WT myocytes (Fig. 6B). Fig. 6C and Table 1 compare tau values for all the experiments. Addition of CaM also normalized the I_{Ca} restitution curve of Casq2 KO (Fig. 6D). At the same time, the total amount of CaM protein was not different between WT and Casq2 KO myocytes (Supplemental Fig. 1).

Finally, we studied the effect of apo-CaM on refractoriness of SR Ca^{2+} release in Casq2 KO and WT myocytes. For these experiments, we used the S1–S2 protocol in voltage-clamp

mode, similar to that used in Fig. 3, but L-type Ca^{2+} currents were elicited by 30-ms depolarizing step to 0mV from holding potential of -70 mV rather than using tail currents to activate SR Ca^{2+} release (to allow physiological Ca^{2+} -induced inactivation of L-type Ca^{2+} channels). AIP ($1 \mu\text{M}$) was added to intracellular solution to prevent CaMKII activation by CaM, because activated CaMKII will phosphorylate RyR2 and could accelerate SR Ca^{2+} release refractoriness [28]. Under these conditions, addition of CaM resulted in a partial restoration of SR Ca^{2+} release refractoriness in Casq2 KO myocytes (Fig. 7). Ca^{2+} transient decay during the S1 beat was significantly slower in Casq2 KO compared to WT myocytes, which was completely normalized by adding CaM in Casq2KO myocytes (WT + CaM: $\tau=226\pm25$ ms; Casq2 KO + CaM: $\tau=201\pm11$ ms; Casq2 KO: $\tau=425\pm78$ ms, $p<0.05$ vs. both CaM groups; $n=9-14$ cells per group). This result suggests that impaired CaM-dependent $\text{Ca}_v1.2$ inactivation contributes to excess Ca^{2+} influx in Casq2 KO myocytes. Furthermore, because CaM addition completely normalizes LTCC Ca^{2+} refractoriness in Casq2 KO myocytes but has no effect on WT myocytes (Fig. 6), the results of Fig. 7D also indicate that the $\text{Ca}_v1.2$ inactivation defect is responsible for approximately 50% of the loss of Ca^{2+} release refractoriness found in Casq2 KO myocytes.

4. Discussion

4.1. Casq2 – a critical determinant for physiological SR Ca^{2+} release refractoriness

Structurally, the CRU of cardiac myocyte includes $\text{Ca}_v1.2$ channels located in the sarcolemma and juxtaposed RyR2 SR Ca^{2+} release channels in the junctional SR. RyR2 channels are associated with other SR proteins like triadin and junctin, and through them also with Casq2. During action potential, Ca^{2+} influx via $\text{Ca}_v1.2$ causes much bigger amount of Ca^{2+} being released from the SR. The amplitude of the resulting cytosolic Ca^{2+} transient depends on the diastolic interval between two stimuli (normally, the amplitude of the second Ca^{2+} release is lower when the diastolic interval is short – Fig. 2 and 3), a phenomenon described as Ca^{2+} release restitution. Release restitution is regulated and affected by number of different voltage-, time- and Ca^{2+} -dependent processes such as refractoriness (recovery from inactivation) of $\text{Ca}_v1.2$ channels, recovery of RyR2 channels and SR Ca^{2+} uptake kinetics [29]. Two previous studies have reported that accelerated restitution of SR Ca^{2+} release is associated with human arrhythmia diseases: Our earlier report demonstrating accelerated Ca^{2+} transient recovery in intact Casq2 KO hearts [1], which are a model of a rare genetic form of catecholaminergic polymorphic ventricular tachycardia (CPVT, discussed in more detail below), and a study in the canine model of post-myocardial infarction ventricular fibrillation [30]. The later study by Belevych et al. demonstrated that acceleration of Ca^{2+} release restitution of post-MI myocytes was attributable to RyR2 phosphorylation and oxidation. In current study, we investigate in more detail the role that Casq2 plays in Ca^{2+} release restitution in isolated ventricular myocytes, and also test $\text{Ca}_v1.2$ as one of potential contributors to the restitution of SR Ca^{2+} release. We show that not only is Casq2 a critical determinant for the restitution of Ca^{2+} release from the SR but also recovery of I_{Ca} is accelerated in mice lacking Casq2 and this, along with loss of intra-SR Ca^{2+} buffering and altered sensitivity of RyR2 to luminal Ca^{2+} [1], contributes to the loss of SR Ca^{2+} release restitution in Casq2 KO mice. We further demonstrate that accelerated recovery from inactivation of I_{Ca} in Casq2 KO model is due to impaired sensitivity of

Ca_v1.2 channel to intracellular Ca²⁺ and this altered Ca²⁺ sensitivity can be recovered by application of excess apo-CaM.

4.2. SR Ca²⁺ release refractoriness and ventricular arrhythmia

CPVT is a highly lethal form of inherited arrhythmogenic disease characterized by adrenergically mediated polymorphic ventricular tachycardia caused by mutations in genes that encode CRU-composing proteins [31, 32]. The most common form is caused by mutations in RyR2 [31, 33]. Much less common, CPVT can be caused by mutations in Casq2 [32, 34], triadin or calmodulin [35]. Unlike CPVT caused by RyR2 mutations, CPVT linked to Casq2 mutations is usually autosomal recessive, with complete absence of Casq2 predicted for patients homozygous for Casq2 nonsense mutations. Despite this fact, they display surprisingly normal cardiac contractile function [34]. *Ex vivo*, isolated ventricular myocytes from Casq2 KO mice display normal SR Ca²⁺ release and contractile function under basal conditions, but exposure to catecholamines causes increased diastolic SR Ca²⁺ leak, resulting in premature spontaneous SR Ca²⁺ releases and triggered beats [21], which are considered major hallmarks of CPVT phenotype and a culprit for arrhythmia onset *in vivo*. Even modest reduction in Casq2 is able to increase diastolic SR Ca²⁺ leak and cause spontaneous SR Ca²⁺ release [22]. Unlike Casq2 KO [21], heterozygous Casq2 +/- mice have normal level of all the other CRU-composing SR proteins (namely, triadin-1 and junctin), which makes Casq2 the prime candidate responsible for arrhythmogenic phenotype in this model. On the other hand, triadin is crucially important for maintaining the structural and functional integrity of the cardiac CRU, and its ablation reduces SR Ca²⁺ release and impairs negative feedback of SR Ca²⁺ release on Ca_v1.2 currents [36]. Recently, junctin itself, without (or in addition to) involvement of Casq2, was reported as one of important modulators of RyR2 sensitivity to luminal Ca²⁺ [37].

Studies have shown that altered Ca²⁺ handling and, in particular, premature spontaneous Ca²⁺ releases from the SR serve as a culprit for CPVT [38]. Our data demonstrate that in Casq2 KO murine ventricular myocytes refractoriness of Ca²⁺ release from the SR is accelerated compared to myocytes from WT animals, and this acceleration is not accompanied by alterations in SR Ca²⁺ content (Fig. 2 E). These results confirm on a cellular level the accelerated recovery of Ca²⁺ transients previously reported from the same mouse model on a whole-heart level [1]. Unchanged SR Ca²⁺ content despite dramatic loss of SR Ca²⁺ buffering might be explained by substantial increase in SR volume of Casq2 KO myocytes, which effectively compensates for the absence of Casq2 [21].

4.3. Accelerated Ca_v1.2 recovery from inactivation importantly contributes to loss of Ca²⁺ release refractoriness in Casq2 KO myocytes

Our study shows that, surprisingly, recovery from inactivation of Ca_v1.2 current is accelerated in Casq2 KO mouse model (Fig. 4A), providing enhanced triggering for CICR during premature stimulation. Along with faster rise in free intra-SR Ca²⁺ [1], this creates a potential for much more massive, compared to WT, SR Ca²⁺ release at very short S1–S2 intervals. What are the possible mechanisms for the accelerated recovery of Ca_v1.2 currents produced by ablation of Casq2? We found no difference in Ca_v1.2 current density (Fig. 4B), hence, it is unlikely that the difference in recovery from inactivation is due to changes in

Ca_v1.2 expression level. On the other hand, substituting Ca²⁺ with Ba²⁺ as charge carrier abolished the differences in Ca_v1.2 current inactivation and restitution (Fig. 5), suggesting that difference in Ca²⁺-dependent inactivation (CDI) of the channel is the most likely culprit.

We reported previously that inactivation of Ca_v1.2 is also impaired in ventricular myocytes lacking triadin [36]. Since expression of triadin is decreased in Casq2 KO mice [21], one could suggest that loss of triadin contributed to the accelerated recovery of Ca_v1.2 from inactivation. However, in triadin null myocytes, the difference in inactivation rate of Ca_v1.2 current was abolished by blocking RyR2 Ca²⁺ release with ryanodine [36]. Since all our Ca_v1.2 measurements in Casq2 KO myocytes were done in presence of ryanodine and thapsigargin, it seems unlikely that decrease in triadin can explain the accelerated recovery from inactivation of Ca_v1.2 in Casq2 KO myocytes.

4.4. Defective regulation by CaM mediates accelerated recovery of Ca_v1.2 in Casq2 KO myocytes

Studies have shown that Ca²⁺ binding to CaM is responsible for CDI [26, 27]. Acute overexpression of engineered, Ca²⁺-insensitive mutant CaM in rat ventricular myocytes can efficiently displace endogenous CaM on native Ca_v1.2 channels, resulting in strong inhibition of CDI without perturbation of other gating functions, such as voltage-dependent inactivation [27]. Since apo-CaM, the Ca²⁺-free form of CaM pre-associates with target molecules [39] such as L-type Ca²⁺ channels, whose function is subsequently modulated when an elevation of Ca²⁺ calcifies the associated CaM [26, 40, 41], any reduced availability of apo-CaM could contribute to impaired CDI, as has been shown experimentally for Ca_v1.3, where lack of pre-associated apo-CaM rendered Ca_v1.3 channels incapable of CDI while still being able to open normally [42]. Since there is extensive competition for CaM in the intracellular milieu (only one out of 100 is free), it seems possible that due to the increased SR Ca²⁺ leak and elevated diastolic Ca²⁺ in Casq2 KO myocytes [21, 22] available apo-CaM is reduced. Moreover, in *Xenopus* oocytes, injection of excess purified CaM protein was able to reduce dramatically the inhibitory effect of mutant, Ca²⁺-insensitive CaM₁₂₃₄ on CDI of Ca_v1.2 channels [43]. This effect presumably is based on the competition for the same binding site located in the proximal C terminus of the α_{1C} subunit of Ca_v1.2 that both “normal” CaM and mutant CaM₁₂₃₄ share. In brain, there is another Ca²⁺ sensor protein known to have overlapping binding sites with CaM in pCT of Ca_v1.2 – Ca-binding protein 1 (CaBP1) [43]. CaBP1 is structurally related to CaM but its effect on Ca_v1.2 is opposite to that of CaM: Addition of CaBP1 slows down CDI [44] and CaBP1 competes with CaM for aforementioned binding sites in Ca_v1.2 [43]. However, CaBP1 is not expressed in the heart [45], hence its involvement in attenuation of CDI in Casq2 KO ventricular myocytes seems doubtful. Another possibility would be that CaM protein expression is reduced in Casq2 KO myocytes. But our experiments show no difference in CaM protein between Casq2 KO and WT myocytes (suppl. Fig. 1).

4.5. Implication of defective Ca_v1.2 regulation by CaM for arrhythmogenesis

How could the impaired Ca²⁺-dependent inactivation of Ca_v1.2 and faster recovery of Ca²⁺ current contribute to the arrhythmogenesis in Casq2 KO mice? The impaired Ca/CaM-

dependent inactivation of $\text{Ca}_v1.2$ will result in greater Ca^{2+} influx during each beat, leading to further Ca^{2+} loading of the myocytes especially during catecholaminergic stimulation, which, in turn, increases the probability of spontaneous diastolic Ca^{2+} releases. Premature spontaneous Ca^{2+} release from the SR is generally accepted as the culprit for CPVT [38, 48]. Interestingly, it was reported that Ca^{2+} channel blocker verapamil provides added benefit in CPVT patients when administered along with β -blockers [46, 47], a finding that is consistent with our hypothesis that enhanced Ca^{2+} influx via $\text{Ca}_v1.2$ contributes to excess myocyte Ca loading and arrhythmogenesis in *Casq2* KO myocytes. Analogous to CPVT, increased SR Ca^{2+} leak and DADs have also been observed in animal models of heart failure [48, 49]. It is intriguing to speculate that impaired CaM dependent $\text{Ca}_v1.2$ may also contribute to arrhythmogenesis in heart failure and other heart diseases associated with increased SR Ca^{2+} leak, a hypothesis that should be tested in future studies.

One could argue that excess CaM used in our voltage clamp experiments can also inhibit RyR2 and in this way restore refractoriness of SR Ca^{2+} release. While CaM is a physiological inhibitor of RyR2, it binds with high affinity to RyR2 and the CaM inhibitory action on RyR2 is saturated at physiological free concentrations of 100nM. As such, excess CaM will not further inhibit RyR2 channels and hence is not expected to contribute to the normalization of Ca^{2+} release refractoriness.

5. Summary and Conclusions

In the presented study we investigated mechanisms responsible for SR Ca^{2+} release refractoriness in cardiac muscle. We find that gene-targeted ablation of *Casq2* results in complete loss of SR Ca^{2+} release refractoriness (Fig. 2&3). Surprisingly, accelerated recovery of I_{Ca} (Fig. 4) also contributed to the loss of SR Ca^{2+} release restitution in mice lacking *Casq2*. We found that the faster recovery of I_{Ca} in *Casq2* KO mice is due to impaired Ca^{2+} -dependent inactivation of $\text{Ca}_v1.2$ (Fig. 5), which could be reversed by excess apo-CaM (Fig. 6). Because excess CaM also partially restored release refractoriness in *Casq2* KO myocytes (Fig. 7), these results suggest that impaired Ca^{2+} -CaM-dependent inactivation of $\text{Ca}_v1.2$ independently contributes to loss of SR Ca^{2+} release refractoriness in *Casq2* KO mice. We conclude that impaired CaM-dependent $\text{Ca}_v1.2$ regulation may independently contribute to arrhythmogenesis in CPVT, a new concept that could also be relevant in other heart diseases (e.g. heart failure) associated with increased SR Ca^{2+} leak.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding

This work was partly supported by the United States National Institutes of Health [HL88635, HL71670 & HL108173 to B.C.K.]; by the American Heart Association [13IRG13680003 to B.C.K., 12POST12080080 to D.K.], and by the Australian National Health & Medical Research Council [APP1005974 to B.C.K.].

References

1. Kornyevev D, Petrosky AD, Zepeda B, Ferreira M, Knollmann B, Escobar AL. Calsequestrin 2 deletion shortens the refractoriness of Ca(2)(+) release and reduces rate-dependent Ca(2)(+)-alternans in intact mouse hearts. *J Mol Cell Cardiol.* 2012; 52:21–31. [PubMed: 21983287]
2. Bers DM. Cardiac excitation-contraction coupling. *Nature.* 2002; 415:198–205. [PubMed: 11805843]
3. Belevych A, Kubalova Z, Terentyev D, Hamlin RL, Carnes CA, Gyorke S. Enhanced ryanodine receptor-mediated calcium leak determines reduced sarcoplasmic reticulum calcium content in chronic canine heart failure. *Biophys J.* 2007; 93:4083–4092. [PubMed: 17827226]
4. Kubalova Z, Terentyev D, Viatchenko-Karpinski S, Nishijima Y, Gyorke I, Terentyeva R, da Cunha DN, Sridhar A, Feldman DS, Hamlin RL, Carnes CA, Gyorke S. Abnormal intrastore calcium signaling in chronic heart failure. *Proc Natl Acad Sci U S A.* 2005; 102:14104–14109. [PubMed: 16172392]
5. Marx SO, Reiken S, Hisamatsu Y, Jayaraman T, Burkoff D, Rosembliit N, Marks AR. PKA phosphorylation dissociates FKBP12.6 from the calcium release channel (ryanodine receptor): defective regulation in failing hearts. *Cell.* 2000; 101:365–376. [PubMed: 10830164]
6. Wehrens XH, Lehnart SE, Reiken S, Vest JA, Wronska A, Marks AR. Ryanodine receptor/calcium release channel PKA phosphorylation: a critical mediator of heart failure progression. *Proc Natl Acad Sci U S A.* 2006; 103:511–518. [PubMed: 16407108]
7. Plank DM, Yatani A, Ritsu H, Witt S, Glascock B, Lalli MJ, Periasamy M, Fiset C, Benkusky N, Valdivia HH, Sussman MA. Calcium dynamics in the failing heart: restoration by beta-adrenergic receptor blockade. *Am J Physiol Heart Circ Physiol.* 2003; 285:H305–315. [PubMed: 12649072]
8. Terracciano CM, Hardy J, Birks EJ, Khaghani A, Banner NR, Yacoub MH. Clinical recovery from end-stage heart failure using left-ventricular assist device and pharmacological therapy correlates with increased sarcoplasmic reticulum calcium content but not with regression of cellular hypertrophy. *Circulation.* 2004; 109:2263–2265. [PubMed: 15136495]
9. Jones LR, Cala SE. Biochemical evidence for functional heterogeneity of cardiac sarcoplasmic reticulum vesicles. *J Biol Chem.* 1981; 256:11809–11818. [PubMed: 6271762]
10. Cala SE, Jones LR. Rapid purification of calsequestrin from cardiac and skeletal muscle sarcoplasmic reticulum vesicles by Ca²⁺-dependent elution from phenyl-sepharose. *J Biol Chem.* 1983; 258:11932–11936. [PubMed: 6619149]
11. Campbell KP, MacLennan DH, Jorgensen AO, Mintzer MC. Purification and characterization of calsequestrin from canine cardiac sarcoplasmic reticulum and identification of the 53,000 dalton glycoprotein. *J Biol Chem.* 1983; 258:1197–1204. [PubMed: 6337133]
12. Kobayashi YM, Jones LR. Identification of triadin 1 as the predominant triadin isoform expressed in mammalian myocardium. *J Biol Chem.* 1999; 274:28660–28668. [PubMed: 10497235]
13. Jones LR, Zhang L, Sanborn K, Jorgensen AO, Kelley J. Purification, primary structure, and immunological characterization of the 26-kDa calsequestrin binding protein (junctin) from cardiac junctional sarcoplasmic reticulum. *J Biol Chem.* 1995; 270:30787–30796. [PubMed: 8530521]
14. Zhang L, Kelley J, Schmeisser G, Kobayashi YM, Jones LR. Complex formation between junctin, triadin, calsequestrin, and the ryanodine receptor. *Proteins of the cardiac junctional sarcoplasmic reticulum membrane.* *J Biol Chem.* 1997; 272:23389–23397. [PubMed: 9287354]
15. Endo M, Tanaka M, Ogawa Y. Calcium induced release of calcium from the sarcoplasmic reticulum of skinned skeletal muscle fibres. *Nature.* 1970; 228:34–36. [PubMed: 5456208]
16. Fabiato A, Fabiato F. Contractions induced by a calcium-triggered release of calcium from the sarcoplasmic reticulum of single skinned cardiac cells. *J Physiol.* 1975; 249:469–495. [PubMed: 809571]
17. Flucher BE, Franzini-Armstrong C. Formation of junctions involved in excitation-contraction coupling in skeletal and cardiac muscle. *Proc Natl Acad Sci U S A.* 1996; 93:8101–8106. [PubMed: 8755610]
18. Bers, DM. *Excitation-contraction coupling and cardiac contractile force.* Dordrecht; Boston: Kluwer Academic Publishers; 2001.

19. Ikemoto N, Ronjat M, Meszaros LG, Koshita M. Postulated role of calsequestrin in the regulation of calcium release from sarcoplasmic reticulum. *Biochemistry*. 1989; 28:6764–6771. [PubMed: 2790030]
20. Gyorke I, Hester N, Jones LR, Gyorke S. The role of calsequestrin, triadin, and junctin in conferring cardiac ryanodine receptor responsiveness to luminal calcium. *Biophys J*. 2004; 86:2121–2128. [PubMed: 15041652]
21. Knollmann BC, Chopra N, Hlaing T, Akin B, Yang T, Ettensohn K, Knollmann BE, Horton KD, Weissman NJ, Holinstat I, Zhang W, Roden DM, Jones LR, Franzini-Armstrong C, Pfeifer K. Casq2 deletion causes sarcoplasmic reticulum volume increase, premature Ca²⁺ release, and catecholaminergic polymorphic ventricular tachycardia. *J Clin Invest*. 2006; 116:2510–2520. [PubMed: 16932808]
22. Chopra N, Kannankeril PJ, Yang T, Hlaing T, Holinstat I, Ettensohn K, Pfeifer K, Akin B, Jones LR, Franzini-Armstrong C, Knollmann BC. Modest reductions of cardiac calsequestrin increase sarcoplasmic reticulum Ca²⁺ leak independent of luminal Ca²⁺ and trigger ventricular arrhythmias in mice. *Circ Res*. 2007; 101:617–626. [PubMed: 17656677]
23. Kass RS, Sanguinetti MC. Inactivation of calcium channel current in the calf cardiac Purkinje fiber. Evidence for voltage- and calcium-mediated mechanisms. *J Gen Physiol*. 1984; 84:705–726. [PubMed: 6096480]
24. Lee KS, Marban E, Tsien RW. Inactivation of calcium channels in mammalian heart cells: joint dependence on membrane potential and intracellular calcium. *J Physiol*. 1985; 364:395–411. [PubMed: 2411919]
25. Ferreira G, Yi J, Rios E, Shirokov R. Ion-dependent inactivation of barium current through L-type calcium channels. *J Gen Physiol*. 1997; 109:449–461. [PubMed: 9101404]
26. Peterson BZ, DeMaria CD, Adelman JP, Yue DT. Calmodulin is the Ca²⁺ sensor for Ca²⁺-dependent inactivation of L-type calcium channels. *Neuron*. 1999; 22:549–558. [PubMed: 10197534]
27. Alseikhan BA, DeMaria CD, Colecraft HM, Yue DT. Engineered calmodulins reveal the unexpected eminence of Ca²⁺ channel inactivation in controlling heart excitation. *Proc Natl Acad Sci U S A*. 2002; 99:17185–17190. [PubMed: 12486220]
28. Polakova E, Illaste A, Niggli E, Sobie EA. Maximal acceleration of Ca²⁺ release refractoriness by beta-adrenergic stimulation requires dual activation of kinases PKA and CaMKII in mouse ventricular myocytes. *J Physiol*. 2014
29. Liu OZ, Lederer WJ, Sobie EA. Does the Goldilocks Principle apply to calcium release restitution in heart cells? *J Mol Cell Cardiol*. 52:3–6. [PubMed: 22056316]
30. Belevych AE, Terentyev D, Terentyeva R, Ho HT, Gyorke I, Bonilla IM, Carnes CA, Billman GE, Gyorke S. Shortened Ca²⁺ signaling refractoriness underlies cellular arrhythmogenesis in a postinfarction model of sudden cardiac death. *Circ Res*. 2012; 110:569–577. [PubMed: 22223353]
31. Priori SG, Napolitano C, Tiso N, Memmi M, Vignati G, Bloise R, Sorrentino V, Danieli GA. Mutations in the cardiac ryanodine receptor gene (hRyR2) underlie catecholaminergic polymorphic ventricular tachycardia. *Circulation*. 2001; 103:196–200. [PubMed: 11208676]
32. Lahat H, Pras E, Olender T, Avidan N, Ben-Asher E, Man O, Levy-Nissenbaum E, Khoury A, Lorber A, Goldman B, Lancet D, Eldar M. A missense mutation in a highly conserved region of CASQ2 is associated with autosomal recessive catecholamine-induced polymorphic ventricular tachycardia in Bedouin families from Israel. *Am J Hum Genet*. 2001; 69:1378–1384. [PubMed: 11704930]
33. Priori SG, Napolitano C, Memmi M, Colombi B, Drago F, Gasparini M, DeSimone L, Coltorti F, Bloise R, Keegan R, Cruz Filho FE, Vignati G, Benatar A, DeLogu A. Clinical and molecular characterization of patients with catecholaminergic polymorphic ventricular tachycardia. *Circulation*. 2002; 106:69–74. [PubMed: 12093772]
34. Postma AV, Denjoy I, Hoorntje TM, Lupoglazoff JM, Da Costa A, Sebillon P, Mannens MM, Wilde AA, Guicheney P. Absence of calsequestrin 2 causes severe forms of catecholaminergic polymorphic ventricular tachycardia. *Circ Res*. 2002; 91:e21–26. [PubMed: 12386154]
35. Nyegaard M, Overgaard MT, Sondergaard MT, Vranas M, Behr ER, Hildebrandt LL, Lund J, Hedley PL, Camm AJ, Wettrell G, Fosdal I, Christiansen M, Borglum AD. Mutations in

- calmodulin cause ventricular tachycardia and sudden cardiac death. *Am J Hum Genet.* 2012; 91:703–712. [PubMed: 23040497]
36. Chopra N, Yang T, Asghari P, Moore ED, Huke S, Akin B, Cattolica RA, Perez CF, Hlaing T, Knollmann-Ritschel BE, Jones LR, Pessah IN, Allen PD, Franzini-Armstrong C, Knollmann BC. Ablation of triadin causes loss of cardiac Ca²⁺ release units, impaired excitation-contraction coupling, and cardiac arrhythmias. *Proc Natl Acad Sci U S A.* 2009; 106:7636–7641. [PubMed: 19383796]
 37. Altschafel BA, Arvanitis DA, Fuentes O, Yuan Q, Kranias EG, Valdivia HH. Dual role of junctin in the regulation of ryanodine receptors and calcium release in cardiac ventricular myocytes. *J Physiol.* 589:6063–6080. [PubMed: 22025663]
 38. Watanabe H, Knollmann BC. Mechanism underlying catecholaminergic polymorphic ventricular tachycardia and approaches to therapy. *J Electrocardiol.* 44:650–655. [PubMed: 21872879]
 39. Jurado LA, Chockalingam PS, Jarrett HW. Apocalmodulin. *Physiol Rev.* 1999; 79:661–682. [PubMed: 10390515]
 40. Erickson MG, Alseikhan BA, Peterson BZ, Yue DT. Preassociation of calmodulin with voltage-gated Ca(2+) channels revealed by FRET in single living cells. *Neuron.* 2001; 31:973–985. [PubMed: 11580897]
 41. Zuhlke RD, Pitt GS, Deisseroth K, Tsien RW, Reuter H. Calmodulin supports both inactivation and facilitation of L-type calcium channels. *Nature.* 1999; 399:159–162. [PubMed: 10335846]
 42. Ben Johny M, Yang PS, Bazzazi H, Yue DT. Dynamic switching of calmodulin interactions underlies Ca²⁺ regulation of CaV1.3 channels. *Nat Commun.* 4:1717. [PubMed: 23591884]
 43. Oz S, Benmocha A, Sasson Y, Sachyani D, Almagor L, Lee A, Hirsch JA, Dascal N. Competitive and non-competitive regulation of calcium-dependent inactivation in CaV1.2 L-type Ca²⁺ channels by calmodulin and Ca²⁺-binding protein 1. *J Biol Chem.* 288:12680–12691. [PubMed: 23530039]
 44. Zhou H, Yu K, McCoy KL, Lee A. Molecular mechanism for divergent regulation of Cav1. 2 Ca²⁺ channels by calmodulin and Ca²⁺-binding protein-1. *J Biol Chem.* 2005; 280:29612–29619. [PubMed: 15980432]
 45. Haeseleer F, Sokal I, Verlinde CL, Erdjument-Bromage H, Tempst P, Pronin AN, Benovic JL, Fariss RN, Palczewski K. Five members of a novel Ca(2+)-binding protein (CABP) subfamily with similarity to calmodulin. *J Biol Chem.* 2000; 275:1247–1260. [PubMed: 10625670]
 46. Swan H, Laitinen P, Kontula K, Toivonen L. Calcium channel antagonism reduces exercise-induced ventricular arrhythmias in catecholaminergic polymorphic ventricular tachycardia patients with RyR2 mutations. *J Cardiovasc Electrophysiol.* 2005; 16:162–166. [PubMed: 15720454]
 47. Rosso R, Kalman JM, Rogowski O, Diamant S, Birger A, Biner S, Belhassen B, Viskin S. Calcium channel blockers and beta-blockers versus beta-blockers alone for preventing exercise-induced arrhythmias in catecholaminergic polymorphic ventricular tachycardia. *Heart Rhythm.* 2007; 4:1149–1154. [PubMed: 17765612]
 48. Ai X, Curran JW, Shannon TR, Bers DM, Pogwizd SM. Ca²⁺/calmodulin-dependent protein kinase modulates cardiac ryanodine receptor phosphorylation and sarcoplasmic reticulum Ca²⁺ leak in heart failure. *Circ Res.* 2005; 97:1314–1322. [PubMed: 16269653]
 49. van Oort RJ, McCauley MD, Dixit SS, Pereira L, Yang Y, Respress JL, Wang Q, De Almeida AC, Skapura DG, Anderson ME, Bers DM, Wehrens XH. Ryanodine receptor phosphorylation by calcium/calmodulin-dependent protein kinase II promotes life-threatening ventricular arrhythmias in mice with heart failure. *Circulation.* 2010; 122:2669–2679. [PubMed: 21098440]

Highlights

- Ablation of Casq2 results in complete loss of SR Ca^{2+} release restitution.
- Accelerated recovery of I_{Ca} contributes to this phenomenon in Casq2 KO mice.
- I_{Ca} recovers faster in Casq2 KO cells due to impaired Ca^{2+} -dependent inactivation of $\text{Ca}_v1.2$.
- Accelerated recovery of I_{Ca} in Casq2 KO mice can be reversed by excess apo-CaM.
- Excess apo-CaM partially restores SR Ca^{2+} release restitution in Casq2 KO myocytes.

Fig. 1. Calculation of S2 amplitude

To calculate the restitution curve of Ca^{2+} release, we included the Ca^{2+} that still remains in the cytosol for measuring the amplitude of the premature S2 beat (A), because that Ca^{2+} has not been taken up into the SR and hence cannot be released. Previous approaches [1] measured S2 as the height of the premature S2 (B), which results in a restitution curve that mostly reflects the rate of SR Ca^{2+} uptake, and not the intrinsic refractoriness of the RyR2 Ca^{2+} release channel complex.

Fig. 2. Refractoriness of SR Ca²⁺ release in field-stimulated myocytes

A - Experimental protocol used to assess refractoriness of SR Ca²⁺ release in a cytosol of murine LV myocytes using electric field stimulation. Premature extra stimuli (S2) were applied at successively shorter S1–S2 coupling intervals following a 1 Hz conditioning train (S1 stimuli). **B** and **C** - representative examples of Ca²⁺ transient recorded in WT and Casq2 KO myocytes, respectively in response to the S1–S2 protocol shown in **A**. Note the complete loss of Ca²⁺ release refractoriness in the Casq2 KO myocyte. **D** - Average S2 Ca²⁺ release fraction plotted as a function of varying the S1–S2 coupling interval (n=7 for WT, open diamonds; n=6 for Casq2 KO, black diamonds; **P<0.005, *P<0.05). **E** - SR Ca²⁺ content measured by rapid caffeine application (n = 5–6 myocytes per group).

Fig. 3. Refractoriness of SR Ca^{2+} release in voltage-clamped myocytes

A –voltage clamp protocol (bottom trace), corresponding membrane currents (middle trace) and $[Ca^{2+}]_i$ (top trace) during an S1 and S2 stimuli used to assess Ca^{2+} release refractoriness in voltage-clamped myocytes. To maintain constant Ca^{2+} trigger, SR Ca^{2+} release was activated with I_{Ca} tail currents that elicited maximal Ca^{2+} release during the S1 train. **B** and **C** - representative examples of Ca^{2+} transients recorded in WT and Casq2 KO myocytes, respectively, in response to the S1–S2 voltage clamp protocol shown in **A**. **D** - S2 Ca^{2+} release fraction changes as a result of varying the S1–S2 coupling interval (n=12 for WT, open circles and Casq2 KO, black circles; ** $P < 0.005$, * $P < 0.05$). **E** – SR Ca^{2+} content measured by rapid caffeine application (n = 8 myocytes per group).

Fig. 4. Restitution of $\text{Ca}_v1.2$ current is accelerated in Casq2 KO myocytes

A – Average $\text{Ca}_v1.2$ current restitution curve for WT (white circles, $n=12$) and Casq2 KO (black circles, $n=12$) myocytes. Inset shows representative examples of $\text{Ca}_v1.2$ current records for each group and the voltage protocol. $\text{Ca}_v1.2$ currents were elicited by a 50-ms depolarizing steps to 0 mV from holding potential -70 mV. Myocytes were pre-incubated in $50 \mu\text{M}$ Ryanodine + $10 \mu\text{M}$ Thapsigargin + $30 \mu\text{M}$ TTX for 30 min to deplete the SR and eliminate I_{Na^+} . $**P < 0.005$, $*P < 0.05$. **B** – current-voltage relationships of $\text{Ca}_v1.2$ peak currents of WT (white circles, $n=6$) and Casq2 KO (black circles, $n=6$). There was no significant difference in voltage dependence or $\text{Ca}_v1.2$ current density between the two mouse models.

Fig. 5. Ca^{2+} -dependent inactivation of $\text{Ca}_v1.2$ current is defective in Casq2 KO myocytes
A – Representative $\text{Ca}_v1.2$ current for WT (light gray) and Casq2 KO (black) myocytes elicited by 200-ms depolarizing step to 0 mV from holding potential -70 mV in bath solution containing 2 mM Ca^{2+} . To illustrate the difference in $\text{Ca}_v1.2$ current inactivation, current traces were normalized to peak amplitude value for each myocyte. **B** – $\text{Ca}_v1.2$ currents recorded in bath solution containing 2 mM Ba^{2+} . Note that substituting Ca^{2+} with Ba^{2+} completely abolished the difference in $\text{Ca}_v1.2$ current inactivation. **C** – Average restitution curves and representative records (inset) of Ba^{2+} current for WT (white circles, $n=13$) and Casq2 KO (black circles, $n=13$) myocytes. The same experimental conditions and voltage protocol as in Fig. 4 were used.

Fig. 6. Apo-CaM accelerates inactivation kinetics of $\text{Ca}_v1.2$ current only in Casq2 KO myocytes
A– Addition of apo-calmodulin (CaM, 20 μM) to the intracellular dialysis solution accelerated inactivation of $\text{Ca}_v1.2$ Ca^{2+} currents in Casq2 KO myocytes. **B** – Addition of CaM had no effect on inactivation kinetics of $\text{Ca}_v1.2$ in WT myocytes. Voltage protocol is shown below. The declining phase of $\text{Ca}_v1.2$ current was well fitted by a single exponential function. **C** – Average inactivation time constant (τ) for all 4 groups ($n=7-10$ cells per group, $**P<0.005$). **D** – Average restitution curves of $\text{Ca}_v1.2$ currents for WT (white circles, $n=12$) and Casq2 KO myocytes in the presence (black/white circles, $n=6$) and absence (black circles, $n=12$) of CaM (20 μM) in the pipette solution. Myocytes were pre-incubated in 50 μM Ryanodine + 10 μM Thapsigargin + 30 μM TTX for 30 min. $**P<0.005$, $*P<0.05$ for Casq2 KO compared to Casq2 KO + CaM.

Fig. 7. Apo-CaM partly restores SR Ca²⁺ release refractoriness in Casq2 KO myocytes
 Representative examples of Ca²⁺ transient restitution in Casq2 KO myocytes in the absence (A) and in the presence of CaM (20 μM) in the pipette solution (B), as well as in WT myocytes in the presence of CaM (C). D - S2 Ca²⁺ release fraction plotted as a function of the S1-S2 coupling interval (n=8-10; Casq2 KO w/o CaM - black circles; Casq2 KO+CaM - grey circles, *P < 0.05 (vs. Casq2 KO w/o CaM); WT+CaM - light-grey diamonds, #P < 0.05 (vs. Casq2 KO+CaM)). E - No significant difference in SR Ca²⁺ content was detected between three experimental groups.

Table 1

Tau values for I_{Ca} and I_{Ba} inactivation.

	N	Current density (pA/pF)	Tau (ms)	<i>P-value</i> (for tau vs WT)
WT	10	15.8	45	
Casq2 KO	10	14.8	93	0.002 (vs. WT)
Casq2 KO + CaM	7	17.9	46	0.002 (vs. Casq2 KO) 0.8 (vs. WT)
WT + CaM	7	15.4	41	0.4 (vs. WT)
WT (Ba²⁺)	10	11.2	145	
Casq2 KO (Ba²⁺)	10	9.2	145	0.99 (vs. WT (Ba ²⁺))