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Calsequestrin 2 deletion shortens the refractoriness of Ca^{2+} release and reduces rate-dependent Ca^{2+} -alternans in intact mouse hearts

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1. Introduction

Calsequestrin 2 (Casq2) is the major Ca^{2+} -binding protein located in sarcoplasmic reticulum (SR) of cardiomyocytes [1]. Casq2, together with the cardiac ryanodine receptor (RyR2), triadin, and junctin forms the so-called junctional complex of Ca^{2+} release units (CRU) [2]. These CRU play a key role in regulating the phenomenon known as Ca^{2+} -induced Ca^{2+} release (CICR). CICR controls the massive Ca^{2+} efflux from the SR in response to the activation of RyR2 by Ca^{2+} ions entering the cell through voltage-dependent L-type Ca^{2+} channels during action potential (AP) [3,4].

As a low-affinity, high-capacity Ca^{2+} -binding protein [5], Casq2 was initially thought to be a Ca^{2+} -storage protein (for review [6]). Nevertheless, more recent studies suggested that Casq2 can also be involved in controlling CICR by regulating sites at the luminal side of the RyR2 complex [7-10]. The pathophysiological importance of Casq2 has been revealed by the identification of mutations in Casq2 gene that have been associated with catecholaminergic polymorphic ventricular tachycardia (CPVT) in humans [11-13].

Interestingly, cardiac contractile function is not affected in mice lacking Casq2 or in humans displaying changes in Casq2 properties [13, 14]. Moreover, Ca^{2+} storage and release measured in isolated cardiac myocytes have been shown to be largely unaltered [14, 15].

In order to consider Casq2 as a potential target for clinical treatment, it is important to identify the role of Casq2 in the regulation of the cardiac function. Although the role of Casq2 has been studied in cardiac ventricular myocytes [10, 16, 17], a further assessment of subcellular Ca^{2+} dynamics in intact hearts of transgenic mice lacking this protein [14] will help us to understand its physiological role during the cardiac cycle. The goal of our experimental approach was to evaluate the role of Casq2 in the regulation of Ca^{2+} release in intact hearts. Specifically, we tested the following idea: if Casq2 directly regulates RyR2, the ablation of this protein should change the time-dependency of recovery of Ca^{2+} release

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(restitution) and consequently the dependency of Ca^{2+} transients on heart rate. Additionally, as intracellular Ca^{2+} release modifies the repolarization of the AP, transmural changes of the AP repolarization across the free ventricular wall could lead to proarrhythmic electrical events detectable on an electrocardiographic recording. This concept of an intra-organelle protein controlling the electrical activity of the plasma membrane will enlighten novel possible regulatory mechanisms of the cardiac function.

To evaluate this mechanism, we conducted experiments on intact hearts under conditions similar to *in vivo* to preserve the integrity of the cardiac tissue. Alterations in the Ca^{2+} transients' restitution, the time course of the SR Ca^{2+} replenishment, heart rate dependency of the Ca^{2+} transients and modification of the electrocardiographic profile were observed. Thus, we conclude that the ablation of Casq2 not only defines the refractoriness of contractility but also alters the electrocardiographic activity under tachycardic conditions.

2. Materials and Methods

2.1. Transgenic KO animals

Transgenic mice lacking Casq2 (Casq2^{-/-}) and wild-type (Casq2^{+/+}) animals [14] were used for all studies. All animal studies were approved by the UC Merced institutional animal care and use committee (IACUC) and performed in accordance with NIH guidelines.

2.2. Heart preparation

Hearts were obtained from young (3-7 week-old) animals. The aorta was cannulated on a horizontal Langendorff apparatus and perfused with Tyrode solution (2 mM CaCl_2 , 140 mM NaCl, 5.4 mM KCl, 1 mM MgCl_2 , 0.33 mM Na_2HPO_4 , 10 mM HEPES, 10 mM glucose, pH=7.4). All reagents and chemicals were purchased from Sigma-Aldrich Chemical Company unless otherwise indicated. Temperature of extracellular solutions was controlled with a Peltier unit. All solutions were equilibrated with 100% oxygen.

2.3. Fluorophores loading

Mag-fluo-4 AM was used to measure the intra-SR free $[\text{Ca}^{2+}]$. The dye was dissolved in 45 μl of DMSO/pluronic acid and 1 ml of normal Tyrode (final concentration 61.2 μM). The loading was performed as described before (see [18,19] for additional information on this technique). Previously results published by our laboratory demonstrated that, despite relatively high concentration of Ca^{2+} in SR, mag-fluo-4 inside the SR does not become saturated under our experimental conditions and can be effectively used to monitor intra-SR Ca^{2+} dynamics [19]. Moreover, there was no evidence of the dye saturation on the amplitude or the kinetics of the fluorescence signals recorded with mag-fluo-4 in the hearts of the transgenic mice lacking Casq2.

Rhod-2 AM (final concentration 44.5 μM) and the potentiometric dye di-8-ANEPPS (final concentration 1.6 μM) were used to measure cytosolic Ca^{2+} signals and membrane potential respectively. These dyes were prepared in a similar way as mag-fluo-4 AM. For measurements with di-8-ANEPPS intact hearts were immobilized by adding blebbistatin (7.5 μM), into Tyrode solution. A number of studies have shown that, at this concentration, blebbistatin has no significant side effects on either Ca^{2+} transients or AP [20-23]. All dyes were purchased from Invitrogen, USA.

2.4. Optical setup

A modified version of our custom-made setup for Pulsed Local-Field Fluorescence microscopy [24] was employed to measure fluorescent signals from the intact heart. Two solid-state Nd-YAG lasers, blue (473 nm) and green (532 nm), were used as illuminating

sources (Enlight Technologies, USA). Both lasers were optically multiplexed by ferroelectric modulators (Newport, USA).

The excitation light was focused into a multimode optical fiber and the emitted light was carried back through the same fiber, filtered to eliminate the reflected excitation component, and focused on an avalanche diode connected to an integrating I-V converter controlled by Digital Signal Processor (DSP 320, Texas Instruments). Fluorescence signals were digitized at 5 MHz and filtered to 500 kHz.

One end of the optic fiber was gently placed on the tissue to attenuated the motion artifacts. After electrically ablating the pacemaker cells with an ophthalmic bipolar pencil (Mentor Ophthalmics, USA), the hearts were paced at various rates by means of an electrical stimulator ISOSTIM A320R (WPI, USA) controlled by a PC. A set of bipolar pacing platinum electrodes was positioned at the base of the left ventricle.

2.5. Whole heart electrophysiological measurements

APs were recorded from the epicardial layer of the left ventricle. The signals were recorded using glass microelectrode filled with 3 M KCl (10-20 MΩ resistance) and an electrometric amplifier Duo 773 (WPI, USA). The electrical signals were filtered at 500 kHz, digitally at 5 MHz and then digitally resampled.

Electrocardiographic recordings were performed by placing one Ag-AgCl micropellet inside the left ventricle and a second pellet outside the left ventricle. Signals were amplified by a custom-made DC-coupled instrumentation amplifier and were digitally sampled identically to the AP recordings.

2.6. Restitution protocols

Restitution curves were obtained by applying an additional stimulation pulse (S2) at different times (described as S1-S2 coupling intervals) with respect to the regular pacing pulses (S1). By changing the time interval between electrical stimulations (S1-S2), we were able to analyze the kinetics of the recovery of different variables such as Ca^{2+} -transients, intra-SR Ca^{2+} signals and AP triggering. Fractional recovery ratios (A_2/A_1) were calculated from the amplitudes of the variables at the S2 (extrasystolic) and S1 (regular baseline pacing) stimulations. Restitution curves were built using data from several independent experiments (hearts) and presented as semilog plots to better illustrate the exponential nature of the recovery process.

2.7. Statistical Analysis

Data are presented as Means $\pm$ Standard Deviations (SD). The total number of animals used in this study was 87 (44 Casq2^{-/-} and 43 Casq2^{+/+}). Normality test (Shapiro-Wilk) was performed prior to application of one-way ANOVA. Differences were considered to be significant if the value of p was less than 0.05.

3. Results

3.1. Cytosolic Ca^{2+} -transients from the epicardial layer of Casq2 KO mice

Casq2 ablation is expected to modify intracellular Ca^{2+} dynamics. We evaluated this hypothesis by measuring cytosolic Ca^{2+} transients in hearts from Casq2^{+/+} and Casq2^{-/-} mice loaded with rhod-2 AM. Unexpectedly, the Ca^{2+} transients recorded from both genotypes did not reveal significant differences between them (Fig.1 A). The time to peak and the decay time constants were similar at 2 mM extracellular $[\text{Ca}^{2+}]$ (Table 1) most likely

due to morphological factors, namely changes in the SR volume [14] or homeostatic conditions such as a divalent saturation of Casq2.

This latter possibility was tested by reducing the intra-SR $[Ca^{2+}]$. For example, when extracellular Ca^{2+} was reduced from 2 mM to 0.5 mM, mag-fluo-4 diastolic fluorescence level decreased by $16 \pm 11\%$ ($n=8$, $p=0.001$, $37^\circ C$, 4 Hz).

As expected, the amplitude of Ca^{2+} -transients for both genotypes declined as a result of a reduction in the extracellular $[Ca^{2+}]$ (Figs. 1B and 1C). A summary of the effect of extracellular Ca^{2+} on the amplitude of the Ca^{2+} transients is presented in Fig. 1D. The differences are significant for different extracellular $[Ca^{2+}]$ but not between both genotypes at the same extracellular $[Ca^{2+}]$.

Additionally, the rise time of the Ca^{2+} transients increased as extracellular Ca^{2+} decreased. This effect was more prominent in Casq2 $^{-/-}$ than in the control animals. Lower extracellular Ca^{2+} (0.1 mM) significantly increased the time to peak and the rise time of Ca^{2+} transients, 1.6-fold and 2-fold respectively for Casq2 $^{-/-}$ animals in comparison to Casq2 $^{+/+}$ (Table 1). Summarizing, the experiments presented in this section showed that at physiological extracellular Ca^{2+} , the absence of Casq2 did not affect the kinetics of cytosolic Ca^{2+} transients of beating hearts stimulated with low (bradycardic) frequency.

3.2. Refractoriness of Ca^{2+} release from the epicardial layer of Casq2 KO mice

The refractoriness of Ca^{2+} release is defined as the time-dependent recovery (restitution) of Ca^{2+} release. This strongly depends on how fast the SR can be reloaded and become ready to release Ca^{2+} in addition to the refractoriness of the trigger (I_{Ca}). A significant change in the SR Ca^{2+} buffer capacity should introduce a modification in the refractoriness of Ca^{2+} release [19]. Thus, we generated restitution curves by applying an additional extrasystolic stimulation pulse (*see section 2.6*) (Fig. 2A and 2B). The fractional recovery of Ca^{2+} release was calculated as the ratio between the amplitude of the Ca^{2+} transient after an extrasystolic stimulation (A2 on Fig. 2C) and the amplitude of the Ca^{2+} transient during regular pacing (A1). The largest differences were observed at shorter S1-S2 coupling intervals (Fig. 2D). Our experiments revealed noticeably reduced refractoriness of Ca^{2+} release in Casq2 $^{-/-}$ mice.

3.3. Effect of reducing intraluminal free Ca^{2+} content on Ca^{2+} dynamics in Casq2 KO mice

We have already shown that the perfusion of hearts with EGTA-AM diminished the level of free Ca^{2+} in the SR by impairing the activity of the SR Ca^{2+} ATPase (SERCa2a) [14]. The idea tested here was to evaluate the intracellular Ca^{2+} dynamics when the SR Ca^{2+} content was modified without changing the extracellular $[Ca^{2+}]$.

The comparison of the normalized Ca^{2+} transients recorded with rhod-2 in presence of EGTA-AM for the two genotypes is shown in Fig. 3A. The exogenous buffer modified the relaxation kinetics of the cytosolic Ca^{2+} transients [14, 25] revealing a fast (τ_1) and slow (τ_2) component (Fig. 3A). The kinetics of the cytosolic Ca^{2+} -transients was similar for Casq2 $^{+/+}$ and Casq2 $^{-/-}$ mice for the first 150 ms after the peak (Fig. 3A). No significant differences between Casq2 $^{+/+}$ and Casq2 $^{-/-}$ were found in τ_1 (20 ± 2 ms and 23 ± 4 ms, respectively, at $37^\circ C$; $n=6$, $p=0.075$), or in the time to 50% relaxation (Fig. 3B). This effect can be explained by the presence of EGTA-AM, which accelerates the initial phase of the relaxation of Ca^{2+} transients by competing with the Ca^{2+} dye. On the contrary, the second component of the relaxation kinetics (τ_2) was significantly faster for Casq2 $^{-/-}$ than for Casq2 $^{+/+}$ (Fig. 3B).

In addition, the recovery of Ca^{2+} release (Fig. 3D, typical recording during a double-pulse experiment is shown in Fig. 3C) was also faster for $\text{Casq2}^{-/-}$ in the presence of EGTA-AM. Time to 50% peak recovery was determined at room temperature (21°C, 1 Hz pacing) and showed a faster recovery for $\text{Casq2}^{-/-}$ than for $\text{Casq2}^{+/+}$ mice (285 ± 27 ms and 150 ± 20 ms, respectively, $n=6$, $p<0.001$).

3.4. How does the presence of Casq2 modify the intra-SR Ca^{2+} dynamics?

We hypothesized that the ablation of Casq2 will induce profound changes in the intra-SR Ca^{2+} dynamics. To evaluate this hypothesis we used an experimental approach described in our previous publications [19, 23]. The hearts were loaded with mag-fluo-4, which was used as a low affinity Ca^{2+} indicator to provide us with direct measurements of Ca^{2+} dynamics inside the SR in intact whole-heart preparations [19].

The relaxation kinetics of the intra-SR signal was found to be slightly faster for the hearts of $\text{Casq2}^{-/-}$ mice (Fig. 4A). The cytosolic Ca^{2+} and intra-SR Ca^{2+} -transients were fitted with a double exponential function. The time constant of the fast component and the time to 50% relaxation were statistically different between both genotypes (Fig. 4B). The data presented in Fig. 3 and Fig. 4 indicate that both cytosolic and intra-SR Ca^{2+} -transients had faster relaxation kinetics for the $\text{Casq2}^{-/-}$ than for $\text{Casq2}^{+/+}$ mice during the time period between 150 and 300 ms. Thus, the removal of Casq2 leads to a faster reload of the Ca^{2+} stores. Such an effect can be attributed to the action of Casq2 as a Ca^{2+} buffer.

As shown in Fig. 4D, the restitution of Ca^{2+} depletion was faster for $\text{Casq2}^{-/-}$ mice. Furthermore, this effect was also observed for time to 50% of the fractional recovery of intra-SR Ca^{2+} -transients measured under the same conditions (243 ± 3 ms and 172 ± 10 ms for $\text{Casq2}^{+/+}$ and $\text{Casq2}^{-/-}$, respectively, $n=5-6$, $p=0.001$). Interestingly, Fig. 4C illustrates that, when the second stimulation occurred at the same level of intra-SR $[\text{Ca}^{2+}]$, $\text{Casq2}^{-/-}$ hearts displayed a larger Ca^{2+} depletion than the $\text{Casq2}^{+/+}$. Although Casq2 as a buffer can potentially influence the restitution of Ca^{2+} release by changing the free Ca^{2+} dynamics in the SR, the fact that the amplitude of Ca^{2+} release (depletion) in absence of Casq2 was larger for the same free intra-SR $[\text{Ca}^{2+}]$ favors the idea that Casq2 acts also as a local regulator that modifies the SR Ca^{2+} release.

3.5. Frequency dependency of Ca^{2+} -transients: effect of Casq2 on Ca^{2+} -alternans

The dependence of cardiac contractility on heart rate is a key factor governing the mechanical functionality of the heart. Additionally, the differences in Ca^{2+} -transient restitution could influence the frequency dependency of Ca^{2+} transients. Thus, it is reasonable to expect that both genotypes could display differences in the amplitude of the Ca^{2+} transients as function of the heart rate. Indeed, this is the case for the $\text{Casq2}^{-/-}$ as shown in supplemental material Fig. 1 where the KO animals were able to maintain the amplitude of Ca^{2+} -transients at higher heart rates ($n=5$).

Not only the mechanical behavior of the heart is regulated by heart rate but also instabilities of the electrical activity can be triggered during tachycardia. Previously, we have shown that an acceleration of Ca^{2+} transients restitution can shift the appearance of Ca^{2+} -alternans towards higher frequencies suggesting a close relationship between these two phenomena [19]. To test this idea we recorded cytosolic Ca^{2+} transients in $\text{Casq2}^{+/+}$ and $\text{Casq2}^{-/-}$ mouse hearts at different heart rates. Typical cytosolic Ca^{2+} transients recorded at 6 Hz (21°C) with rhod-2 are shown in Fig. 5A (no EGTA was added into loading solution). The comparison of two genotypes reveals dramatic difference in the amplitude of Ca^{2+} alternans calculated as the ratio $(A_{\max} - A_{\min}) / A_{\max}$, where $A_{\max}$ and $A_{\min}$ are the maximal and the minimal the amplitudes of Ca^{2+} transients during regular pacing at a given frequency. This

ratio was plotted as function of the basic cycle length (Fig. 5 B). Compared to wild-type, *Casq2*^{-/-} exhibited delayed onset of Ca^{2+} -alternans with significantly smaller amplitudes. For instance, at the basic cycle length of 143 ms, which corresponds to the stimulation frequency of 7 Hz, the amplitude of alternans was 0.72 ± 0.08 for control animals and 0.24 ± 0.07 for knockouts ($p=0.01$, $n=3$). This result is consistent with the idea that the onset and amplitude of cardiac alternans depends on the refractoriness of Ca^{2+} release.

3.6. Effects of *Casq2* on epicardial electrical activity

The amplitude of Ca^{2+} release does not merely depend on the free SR Ca^{2+} and the functional properties of RyR2. It also depends on the electrical activity of cardiomyocytes, namely, the time-course of the AP, which determines Ca^{2+} influx via voltage-dependent Ca^{2+} channels that triggers CICR. Thus, the refractoriness of Ca^{2+} release could be tightly controlled by the restitution of the AP (AP triggering recovery) and the voltage-dependent Ca^{2+} channels. In order to rule out this possibility, we conducted AP measurements for *Casq2*^{+/+} and *Casq2*^{-/-} animals using di-8-ANEPPS, a dye sensitive to membrane potential. Typical optically recorded AP traces are shown in Fig. 6A. The time-course of AP, absolute and relative refractory periods for the different genotypes were not significantly different. The duration of the absolute refractory period was 61 ± 5 ms and 55 ± 11 ms for *Casq2*^{+/+} and *Casq2*^{-/-} animals, respectively ($p=0.477$, $n=3-6$). The duration of the relative refractory period was 102 ± 15 ms and 103 ± 26 ms for both genotypes ($p=0.910$, $n=3-6$). The AP triggering recovery was analyzed by plotting the ratio of the amplitude of premature S2 AP to the amplitude of the S1 AP appearing as a result of regular pacing. This result suggests that the modification in the restitution of the Ca^{2+} release in *Casq2* knockouts was not caused by an altered AP triggering recovery.

The AP triggering recovery is a reasonable way to distinguish if the absence of Ca^{2+} release during the extrasystolic stimulus was due to the absence of an AP. However, it is not a robust test to evaluate the restitution of the Ca^{2+} influx across the plasmalemma. To identify the rate limiting step that defines the refractoriness of Ca^{2+} release we performed experiments in which SR Ca^{2+} release was impaired by a combination of pharmacological tools. Ryanodine ($15 \mu\text{M}$) and thapsigargin ($4 \mu\text{M}$) were used to lock the RyR2 in a sub conductance state and inhibit the transport mediated by the SERCA2 pump. Under these conditions, there were no significant differences (Fig. 6 C and 6D) in the Ca^{2+} transient restitutions between both genotypes. This indicates that the rate-limiting step defining the restitution differences between both genotypes is the Ca^{2+} release from the SR.

3.7. Effects of *Casq2* on T-wave alternans

We already stated that during tachycardia the heart can develop electrical instabilities like T-wave alternans (TW-Alt). TW-Alt is usually observed as alternating beat-to-beat changes in the amplitude of the T-wave of the electrocardiogram (ECG) and constitutes an important arrhythmogenic mechanism that can lead to sudden cardiac death [26]. The likelihood of developing TW-Alt increases with tachycardia. This phenomenon is thought to be associated with abnormalities in intracellular Ca^{2+} handling and/or cellular metabolism [27-29]. Actually, Ca^{2+} -alternans can trigger alternans in the repolarization of the AP. Data supporting this idea is presented in Fig. 2 of supplemental material where alternations in Ca^{2+} transients amplitude and in the duration of the AP are shown. Furthermore, *Casq2*^{-/-} hearts present a shift in the frequency at which Ca^{2+} transients alternate and a very similar shift in the frequency at which the repolarization of the AP alternates. These experiments demonstrate the relationship between Ca^{2+} -alternans and AP alternans. Additionally, they illustrate that the *Casq2*^{-/-} animals are less prone to display alternans in the AP repolarization.

Transmural differences in the alternations in the duration of the AP can not only lead to electrical instabilities but also can generate TW-Alt. To illustrate this idea we performed transmural electrocardiographic recordings in both genotypes and evaluated the frequency dependency of the T-wave amplitude. At a heart rate of 4 Hz, there are no significant differences between wild type and KO animals (Fig. 7A and 7B). At higher heart rates (9 Hz), TW-Alt occur in both models. However, the Casq^{-/-} mice display a smaller TW-Alt than wild type animals (Fig. 7C and 7D). Furthermore, when a complete frequency scan was performed, the KO hearts displayed a smaller TW-Alt appearing at a higher heart rate (Fig. 7E). These results clearly demonstrate that the ablation of an intra-organelle protein not only modifies the organellar Ca²⁺ dynamics but also change the whole electrical activity of the heart.

4. Discussion

4.1. Epicardial Ca²⁺-transients in Casq2 KO mice

In this paper, we evaluate for the first time the role of Casq2, the major SR Ca²⁺ buffering protein, in the intracellular Ca²⁺ dynamics in the intact heart, under physiological conditions (i.e. body temperature and physiological heart rate). Interestingly, no significant differences between Casq2^{+/+} and Casq2^{-/-} mice were found in the kinetics and the amplitude of Ca²⁺-transients recorded at the epicardial layer (Fig 1A). These experimental findings are in agreement with Ca²⁺-transients recorded on enzymatically isolated ventricular cardiac myocytes from animals having the same genotypes [14]. A possible interpretation of these results is that the increase in the SR volume in myocytes lacking Casq2 [14] compensates the decrease in the buffer capacity caused by the ablation of Casq2. However, when decreased levels of Casq2 in cardiac myocytes were achieved by means of the injection of recombinant adenoviruses with coding regions of canine Casq2 in antisense orientation [30,31], an approach that prevents the adaptive changes in the SR volume, myocytes depleted of Casq2 displayed cytosolic Ca²⁺-transients with smaller amplitudes than control ones. The effect was attributed to the duration of CICR rather than the modification of the trigger (L-type Ca²⁺ current, I_{Ca}).

Alternatively, it is possible that under physiological conditions the free SR [Ca²⁺] of an intact heart was high enough to saturate all the Ca²⁺ binding sites on Casq2, thereby reducing the dynamic buffer capacity of this protein. In order to test this latter possibility, we performed experiments at different extracellular [Ca²⁺] (Fig. 1 D). Although this should set the free SR Ca²⁺ to different values, no differences were found between Casq2^{+/+} and Casq2^{-/-} mice indicating that even under conditions where Casq2 should not be fully saturated with Ca²⁺ the amplitude of Ca²⁺ transients remained the same. However, when extracellular [Ca²⁺] was reduced to 0.1 mM (Table 1), the time to peak of Ca²⁺ transients measured in Casq2^{-/-} animals was significantly slower than in the control ones. This suggests that under these experimental conditions the Ca²⁺ release process was activated for a longer time or that the recruitment of local Ca²⁺ release sites (i.e. Ca²⁺ sparks) was less efficient in Casq2^{-/-} animals.

4.2. Casq2 regulates refractoriness of SR Ca²⁺ release during the cardiac cycle

Exogenous Ca²⁺ buffers can alter the properties of Ca²⁺ sparks by increasing the intra-SR Ca²⁺ buffer capacity [33]. Additionally, myocytes virally deprived of Casq2 display changes in the rhythmicity of Ca²⁺-transients and restitution behavior of Ca²⁺-release sites [30, 31]. In our present work, we demonstrate that a premature AP can induce a higher release of Ca²⁺ from the SR when Casq2 is not present (Fig. 2). This effect can have important consequences on the refractoriness of cardiac contractility but also may be responsible for premature spontaneous Ca²⁺ release events that lead to afterdepolarizations.

Ca^{2+} termination and recovery has been extensively studied by others [32-37]. Differences between those experiments and the ones presented in this paper might be due differences in the experimental conditions. For example, in previous studies, the cells were maintained at room temperature where the rate of Ca^{2+} transport by the SERCa2a pump is highly impaired leading to possible changes in intra-SR $[\text{Ca}^{2+}]$ [31]. Additionally, in most of these papers the cells were not continuously stimulated to mimic the complex Ca^{2+} dynamics in myocytes during a regular cardiac cycle.

The acceleration of the Ca^{2+} -transient restitution process in $\text{Casq2}^{-/-}$ (Fig. 2B) can be controlled by several factors like a faster rise in free SR $[\text{Ca}^{2+}]$ and/or a direct regulation of RyR2 kinetic activity. The faster SR Ca^{2+} replenishment, mediated by the SERCa2a pump activity, could be attributed to a decrease in the total SR Ca^{2+} buffer capacity. Furthermore, in the absence of Casq2, the diffusion of Ca^{2+} from the “uptaking” sites (longitudinal SR) to the “releasing” sites (terminal cistern) could be speeded up by the absence of immobile binding sites along the Ca^{2+} diffusional path. On the other hand, if the refilling time is the main factor setting the differences in refractoriness between $\text{Casq2}^{+/+}$ and $\text{Casq2}^{-/-}$, the larger the degree of SR depletion the larger the Casq2 effect will be. Moreover, under conditions where the SR Ca^{2+} load (free SR Ca^{2+}) was modified by adding EGTA-AM as an exogenous buffer the differences in the Ca^{2+} restitution were still present. This indicates that in a wider range of intra SR Ca^{2+} the presence of Casq2 still exerts a control of the Ca^{2+} transient restitution (Fig. 3D). Finally, as shown in Fig. 6 the differences in the restitution of Ca^{2+} -transients between genotypes are not due differences in AP triggering recovery or the Ca^{2+} influx through the plasma membrane.

4.3. Ablation of Casq2 modifies intra-SR Ca^{2+} dynamics

Although the kinetics of cytosolic Ca^{2+} transients is tightly coupled with the intra-SR Ca^{2+} , the only direct way to discriminate between the different hypotheses related to the role of Casq2 in the regulation of the ventricular SR Ca^{2+} signaling is by directly assessing the intra-SR Ca^{2+} dynamics. Fig. 4A and 4B show that the rate of rise of the free intra-SR $[\text{Ca}^{2+}]$ is significantly faster in hearts from animals lacking Casq2. This faster rise in free intra-SR $[\text{Ca}^{2+}]$, both at room and at physiological temperatures, is consistent with the idea that there is a decrease in the luminal Ca^{2+} buffer capacity in absence of Casq2. This result is also in agreement with the outcome of the restitution experiments (Fig. 2 and 3) where the restitution of the Ca^{2+} -transients is faster in $\text{Casq2}^{-/-}$.

Additionally, restitution of SR Ca^{2+} depletion is also much faster in hearts from KO animals (Fig. 4D). However, these intra-SR Ca^{2+} restitution experiments show that for the same fractional free $[\text{Ca}^{2+}]$ within the SR the amplitude of Ca^{2+} depletion during a second pulse is considerably larger in KO animals (Fig. 4C). This result suggests that in absence of Casq2 the Ca^{2+} release process is less inhibited than in the presence of Casq2. This demonstrates for the first time that under physiological conditions Casq2 is not only buffering Ca^{2+} but, perhaps more important, it also regulates the release machinery as well. Moreover, a decrease in Casq2 content is also known to be associated with an acceleration of the relaxation kinetics of intra-SR Ca^{2+} waves in isolated canine cardiac myocytes [38]. Furthermore, the faster Ca^{2+} replenishment of the SR has been suggested to be involved in the genesis of ventricular tachycardia, which is caused by mutations that either decrease the level of Casq2 expression or impair the Ca^{2+} binding ability of the protein [6].

4.4. Role of Casq2 in arrhythmogenesis

Tachycardia, an increase in heart rate, is known to induce several pathophysiological syndromes. The increase in the heart rate can trigger TW-Alt which is detected as alternating beat-to-beat changes in the T-wave and represents an important arrhythmogenic mechanism

that can trigger transmural reentry arrhythmias. In general, tachycardia will limit the time for SR Ca^{2+} refilling. This will reduce SR Ca^{2+} load resulting in less Ca^{2+} -bound Casq2 and causing less Ca^{2+} release from the SR. Smaller release will leave a higher residual intra-SR Ca^{2+} level and a subsequent SR refilling (on top of residual) will result in higher SR load and Ca-Casq2 levels. This will promote a larger release leaving a lower intra-SR residual level and the cycle starts again. This alternating Ca^{2+} dynamics (Ca^{2+} -alternans) is shown in Fig. 5A. Finally, Ca^{2+} -alternans will induce differences in Na^+ - Ca^{2+} exchanger activity leading to differences in AP repolarization (see Fig. 2 supplemental material) and this will be manifested in the ECG waveform as TW-Alt (Fig. 7). In the present work, we show that the amplitude of Ca^{2+} -alternans was significantly reduced in the absence of Casq2 (Fig. 5B). The shift in the frequency dependency of alternans in Casq2 knockouts was most likely the result of acceleration of the restitution of Ca^{2+} release, which was consistently faster in the animals lacking Casq2 when measured at several stimulation frequencies. In other words, it appears that the fast recovery of Ca^{2+} release allows the intact hearts of Casq2 $^{-/-}$ mice to function without Ca^{2+} alternans at higher frequencies in comparison to Casq2 $^{+/+}$ animals. This supports the idea that Casq2 is a key element in the genesis of Ca^{2+} alternans and TW-Alt. Our results also suggest that reduction or loss of Casq2 will diminish TW-Alt as shown in Fig. 7E. At the same time, the accelerated restitution and loss of Ca^{2+} release refractoriness due to loss of Casq2 will increase the likelihood of spontaneous premature Ca^{2+} release events that will lead to delayed afterdepolarizations and triggered arrhythmias. Clinically, the loss of Ca^{2+} release refractoriness results in a phenotype of catecholaminergic polymorphic ventricular tachycardia caused by focal triggered activity originated in the ventricle [39].

5. Conclusion

In conclusion, our experiments revealed that Casq2 ablation modified the Ca^{2+} dynamics in the cardiac myocytes of the intact mouse hearts. The faster rise in SR $[\text{Ca}^{2+}]$ in Casq2 $^{-/-}$ hearts during Ca^{2+} reuptake led to an acceleration of the restitution of Ca^{2+} release. As a result, the Casq2 $^{-/-}$ hearts were less sensitive to frequency-induced alternans. This defines the role of Casq2 in the genesis of TW-Alt. The data are in accordance with the hypothesis that Casq2 plays an important and dual role in the regulation of CICR acting as a Ca^{2+} chelator and as a Ca^{2+} -sensing factor modulating the Ca^{2+} release process.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Research highlights

- Ablation of calsequestrin accelerates restitution of Ca^{2+} release from SR.
- The ablation of calsequestrin speeds up the rate of change of Ca^{2+} within the SR.
- The lack of calsequestrin does not affect the restitution Ca^{2+} influx through the plasma membrane.
- Calsequestrin regulates the appearance of Ca^{2+} alternans and AP repolarization alternans.
- Calsequestrin ablation modifies TW-alternans.

Figure 1.

Typical Ca²⁺-transients recorded with the cytosolic Ca²⁺ indicator (rhod-2 AM) in the intact hearts of Casq2+/+ (black line) and Casq2-/- (red line) mice at 37°C and at a stimulation frequency of 4 Hz (A). Cytosolic Ca²⁺ transients (rhod-2 AM) recorded from the intact

hearts of Casq2^{+/+} (B) and Casq2^{-/-} (C) at different concentrations of CaCl₂ in Tyrode solution (37°C and 4 Hz). Panel D shows the effect of low extracellular Ca²⁺ on the amplitude of Ca²⁺ release in control (empty columns) and knockout (filled columns) animals. Data is expressed as percentage of the initial amplitude determined at 2 mM CaCl₂. Data are means ± standard deviations (n=3-5). *p* values located above the pairs of columns represent the results of the comparison Casq2^{+/+} *versus* Casq2^{-/-} for that particular pair of columns.

Figure 2.

Typical Ca^{2+} -transients recorded with a cytosolic Ca^{2+} indicator (rhod-2 AM) from epicardial layers of intact hearts of $\text{Casq2}^{+/+}$ (A) and $\text{Casq2}^{-/-}$ (B) mice. The levels of rhod-2 fluorescence were normalized to the amplitude of the first peak and the normalized traces were superimposed. Panel C compares typical traces recorded when the S1-S2 coupling intervals were similar for $\text{Casq2}^{+/+}$ and $\text{Casq2}^{-/-}$. Restitution of Ca^{2+} release (D) was analyzed using the ratio A_2/A_1 , where A_1 is the amplitude of Ca^{2+} release during regular pacing (S1 stimulus) and A_2 is the amplitude of the Ca^{2+} release in response to an premature (S2) stimulation. The recordings were obtained at 37°C and at stimulation frequency 3 Hz ($n=3-4$). Semilogarithmic plots in panel D, contain the averaged values of the A_2/A_1 ratio calculated by combining the data from the selected time periods (bins) corresponding to S1-S2 coupling intervals. The time constants for the exponential restitution process were 182 ± 32 ms for the $\text{Casq2}^{+/+}$ and 111 ± 22 ms for the $\text{Casq2}^{-/-}$. See Results section for more details.

Figure 3.

Cytosolic Ca^{2+} -transients recorded from hearts of *Casq2*^{+/+} (black line) and *Casq2*^{-/-} (red line) mice using rhod-2 AM (A, C). Measurements were conducted at 21°C and at stimulation frequency of 1 Hz (71 μM EGTA-AM) was present in the loading solution). The rate constant of the slow component (τ_2) for rhod-2 signal as well as time to 50% relaxation of cytosolic Ca^{2+} transients are shown in panel B. *p*-values are given for each pair of the columns (*Casq2*^{+/+} versus *Casq2*^{-/-}, *n*=6). Panel C represents typical traces obtained with double-pulse protocol used for estimation of the restitution of Ca^{2+} release. The restitution was analyzed using the ratio between the amplitudes of normal (regular pacing) and premature (in response to S_2 stimulation) Ca^{2+} releases. Semilogarithmic plots in panel D contain the averaged values of the ratio calculated using the data from certain time periods (S_1 - S_2 coupling intervals), *n*=5-6.

Figure 4.

Intra-SR Ca^{2+} transients recorded from hearts of *Casq2*^{+/+} (black line) and *Casq2*^{-/-} (red line) mice using mag-fluo-4 AM (A, C). Measurements were conducted at 21°C and at a stimulation frequency of 1 Hz (71 μM EGTA-AM was added into the loading solution). The rate constant of the fast component of the signal relaxation (τ_1) for mag-fluo-4 signal as well as time to 50% relaxation of the intra-SR Ca^{2+} -transients are shown in panel B. *p*-values are given for each pair of the columns (*Casq2*^{+/+} versus *Casq2*^{-/-}, *n*=6). Panel C represents typical traces obtained with double-pulse protocol used for estimation of the restitution of Ca^{2+} release. The recordings were selected to compare Ca^{2+} releases starting from the same normalized level of depletion. Transients were normalized to the amplitude of nadir. Restitution was analyzed using the ratio between the amplitudes of normal (regular pacing) and premature (in response to S2 stimulation) Ca^{2+} releases/depletions. Semilogarithmic plots in panel D contain the averaged values of the ratio A_2/A_1 calculated using the data from certain time periods (S_1 - S_2 coupling intervals), *n*=5-6.

Figure 5.

(A) Typical cytosolic Ca^{2+} transients (rhod-2 fluorescence, 21°C, 6 Hz) showing alternans, which is more pronounced in Casq2^{+/+} (red line). (B) Dependence of the Ca^{2+} -alternans on basic cycle length during regular pacing of intact hearts from Casq2^{+/+} and Casq2^{-/-} mice at 21°C. The cytosolic Ca^{2+} transients were recorded using rhod-2 AM. No EGTA was added into the loading solution. The data are means $\pm$ standard deviations (n=3).

Figure 6.

AP triggering recovery of AP recorded in the presence of blebbistatin using potentiometric dye di-8-ANEPPS at 37°C and at a stimulation frequency of 4 Hz. The typical traces are shown in panel A. The ratio of the peaks of APs in response to the additional (A2) and normal (A1) stimulation pulse was used to estimate the recovery of the AP's peak. The values of A2/A1 ratio were grouped according to the time periods (bins). Then averaged values were plotted against $\ln(t)$, where t is the time between regular and extrasystolic stimulations. The data were collected from $n=3-5$ independent experiments (B). Restitution of Ca^{2+} transients in the cytosol during the AP stimulation in the presence of 15 μM ryanodine and 4 μM thapsigargin (C). Semilogarithmic plots in panel D contain the averaged values of the ratio calculated using the data from certain time periods (S1-S2 coupling intervals), $n=5$. The dotted lines in plots B and D correspond to the Ca^{2+} transients restitution plots shown in Fig. 2D. All the experiments were performed at 37°C.

Figure 7.

Effect of Casq2 ablation on TW-Alt. Panels A and B show a comparison of electrocardiographic recordings obtained from *Casq2*^{+/+} and *Casq2*^{-/-} when the hearts were paced at 4 Hz. QRS complex and T-wave can clearly be observed, none of the T-waves for each genotype show a TW-Alt. Upon increase in the heart rate to 9 Hz it is possible to observe alternanses in the T-wave, however the amplitude of the TW-Alt is smaller in the KO hearts (C and D). A complete frequency scan for both genotypes is presented in Panel E.

The amplitude of the TW-Alt is significantly larger for the wild type animals n=4 for Casq2^{+/+} and n=9 for Casq2^{-/-}. The isoelectric line is used as a reference to measure the TW-amplitudes. All the experiments were done at 37°C.

Effect of extracellular Ca^{2+} on Ca^{2+} dynamics in the hearts of control animals ($\text{Casq2}^{+/-}$) and transgenic mice lacking Casq2 ($\text{Casq2}^{-/-}$). The experiments were conducted at 37°C and 4 Hz. The decay time constant was determined as the time between 10% and 90% of the relaxation divided by 2.2. Rise time is the time between 10% and 90% of increase in the signal during Ca^{2+} release. Data are means $\pm$ SD.

Table 1

Parameter	Concentration of $[\text{Ca}^{2+}]$ in Tyrode solution			
	2 mM		0.1 mM	
	$\text{Casq2}^{+/-}$	$\text{Casq2}^{-/-}$	$\text{Casq2}^{+/-}$	$\text{Casq2}^{-/-}$
Number of Experiments (Hearts)	9	7	8	7
Time to peak (ms)	16 $\pm$ 2	16 $\pm$ 1	31 $\pm$ 7	51 $\pm$ 8
Rise time (ms)	7 $\pm$ 1	8 $\pm$ 1	15 $\pm$ 2	30 $\pm$ 6
Decay time constant (ms)	59 $\pm$ 4	53 $\pm$ 9	64 $\pm$ 5	60 $\pm$ 5
				<i>p</i> value
				0.001
				0.001
				0.131