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Original article

Impaired calcium-calmodulin-dependent inactivation of $Ca_v1.2$ contributes to loss of sarcoplasmic reticulum calcium release refractoriness in mice lacking calsequestrin 2

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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 $Ca_v1.2$, which contributes to 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*.

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 ($Ca_v1.2$), 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 $Ca_v1.2$ 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].

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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 $Ca_v1.2$ 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 $Ca_v1.2$ 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 $Ca_v1.2$ 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.

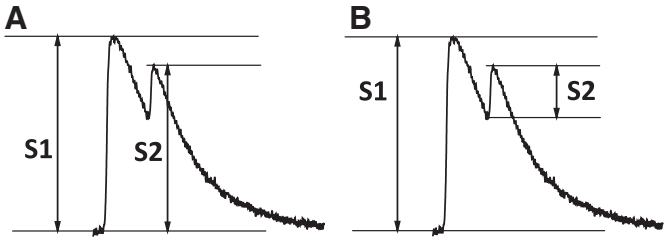

Fig. 1. Calculation of S2 amplitude. To calculate the restitution curve of Ca^{2+} release, we included the Ca^{2+} that still remains in the cytosol for measuring the amplitude of the premature S2 beat (A), because that Ca^{2+} has not been taken up into the SR and hence cannot be released. Previous 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.

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

Ventricular myocytes from 12- to 16-week-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 (~23 °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 $[Ca^{2+}]_i$ 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 the following (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 the following (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 $[Ca^{2+}]_i$ 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 $Ca_v1.2$ current

For measurements of $Ca_v1.2$ 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 2 mM $BaCl_2$. 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 100g 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