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Enhanced binding of calmodulin to RyR2 corrects arrhythmogenic channel disorder in CPVT-associated myocytes

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ABSTRACT

Aims: Calmodulin (CaM) plays a key role in modulating channel gating in ryanodine receptor (RyR2). Here, we investigated (a) the pathogenic role of CaM in the channel disorder in CPVT and (b) the possibility of correcting the CPVT-linked channel disorder, using knock-in (KI) mouse model with CPVT-associated RyR2 mutation (R2474S).

Methods and results: Transmembrane potentials were recorded in whole cell current mode before and after pacing (1–5 Hz) in isolated ventricular myocytes. CaM binding was assessed by incorporation of exogenous CaM fluorescently labeled with HiLyte Fluor® in saponin-permeabilized myocytes. In the presence of cAMP (1 μM) the apparent affinity of CaM binding to the RyR decreased in KI cells (Kd: 140–400 nM), but not in WT cells (Kd: 110–120 nM). Gly-Ser-His-CaM (GSH-CaM that has much higher RyR-binding than CaM) restored normal binding to the RyR of cAMP-treated KI cells (140 nM). Neither delayed afterdepolarization (DAD) nor triggered activity (TA) were observed in WT cells even at 5 Hz pacing, whereas both DAD and TA were observed in 20% and 12% of KI cells, respectively. In response to 10 nM isoproterenol, only DAD (but not TA) was observed in 11% of WT cells, whereas in KI cells the incidence of DAD and TA further increased to 60% and 38% of cells, respectively. Addition of GSH-CaM (100 nM) to KI cells decreased both DADs and TA (DAD: 38% of cells; TA: 10% of cells), whereas CaM (100 nM) had no appreciable effect. Addition of GSH-CaM to saponin-permeabilized KI cells decreased Ca^{2+} spark frequency (+33% of WT cells), which otherwise markedly increased without GSH-CaM (+100% of WT cells), whereas CaM revealed much less effect on the Ca^{2+} spark frequency (+76% of WT cells). Then, by incorporating CaM or GSH-CaM to intact cells (with protein delivery kit), we assessed the *in situ* effect of GSH-CaM (cytosolic [CaM] = ~240 nM, cytosolic [GSH-CaM] = ~230 nM) on the frequency of spontaneous Ca^{2+} transient (sCaT, % of total cells). Addition of 10 nM isoproterenol to KI cells increased sCaT after transient 5 Hz pacing (37%), whereas it was much more attenuated by GSH-CaM (9%) than by CaM (26%) ($P < 0.01$ vs CaM).

Conclusions: Several disorders in the RyR channel function characteristic of the CPVT-mutant cells (increased spontaneous Ca^{2+} leak, delayed afterdepolarization, triggered activity, Ca^{2+} spark frequency, spontaneous Ca^{2+} transients) can be corrected to a normal function by increasing the affinity of CaM binding to the RyR.

1. Introduction

Calmodulin (CaM), one of the accessory proteins of RyR2, is known to be an intrinsic regulator of the channel [1,2]. Namely, CaM inhibits RyR2 Ca^{2+} channels at physiological concentrations of cytoplasmic Ca^{2+} [2]. Since the CaM binding site of RyR2 is located in the midway between the so-called 'clamp' region and the trans-membrane channel region of the RyR2 molecule [3–5], we hypothesized that the previously reported [6–8] on/off action of interacting pair of the N-terminal and the central domains (the so-called 'domain switch') located in the clamp region is conveyed to the channel by mediation of a CaM-dependent mechanism. In support of this idea, we have recently demonstrated that defective N-terminal/central inter-domain interaction caused by single point mutation (R2474S) reduces the affinity of CaM binding

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to the RyR2, resulting in spontaneous diastolic Ca^{2+} sparks, leading to lethal arrhythmia [9]. Further, using canine model of pacing-induced heart failure, we demonstrated that the defective inter-domain interaction of the domain switch reduced the binding affinity of CaM to RyR2, thereby causing spontaneous Ca^{2+} release in failing hearts, and that correction of the reduced CaM binding by adding exogenous CaM blocked aberrant Ca^{2+} release and restored normal Ca^{2+} transient and cell shortening [10]. The critical role of the RyR2-bound CaM in normal muscle function, and pathogenic role of CaM dissociation in the development of cardiac disorder, have also been shown in an earlier report by Meissner and his colleagues [11]. They generated a mouse with 3 amino acid substitutions in the CaM-binding domain (3583–3603) of the RyR2 to make the RyR2 unable to bind CaM, and found that the mutant mouse developed hypertrophic cardiomyopathy with severely impaired contractile function and early death [11]. This finding clearly indicates that the interaction of CaM with the RyR2 plays a key role in maintaining normal channel function, thus preventing from cardiac disorder such as hypertrophy.

In this study, we tested the hypothesis that a selective increase in the RyR-binding affinity of CaM will correct the aberrant spontaneous Ca^{2+} leak in CPVT. By using CaM-(Gly-Ser-His), which was previously reported to show higher binding affinity to the RyR1 than CaM [12], here we show that the high-affinity CaM has restored a normal function in the CPVT-RyR2 channel.

2. Methods

2.1. Animal model

This study conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The care of the animals and the protocols used were in accordance with the guidelines laid down by the Animal Ethics Committee of Yamaguchi University School of Medicine.

Knock-in mice with the RyR2 R2474S Mutation were generated as described previously [9,13].

2.2. Expression and purification of CaM and Gly-Ser-His-CaM

The mammalian CaM (mCaM) cDNA was kindly provided by Dr. Zenon Grabarek (Boston Biomedical Institute, Boston, MA). Human CaM cDNA was PCR amplified with oligonucleotide primers designated to include two restriction enzyme sites (the forward primer 5′-ACACAGGGGATCCCATATGGCTGAC-3′ and the reverse primer 5′-CAAGCTTGGCTCGAGTCACTTTGC-3′). The cDNA was inserted into a pGEX4T-1 vector. The expression vector was transformed into DH5a *Escherichia coli* (Nippongene). The strain was pre-incubated with Lysogeny Broth (LB) ampicillin for 16 h at 30 °C followed by 2 h incubation with 10 times the volumes of LB ampicillin at 37 °C.

2.3. Isolation of cardiac cardiomyocytes

Cardiomyocytes were isolated from mice hearts as described previously [13,14]. In brief, after sacrifice, the heart was quickly removed and retrograde perfusion was performed with 95%O_2/5%CO_2-bubbled collagenase-containing Minimal Essential Medium. The LV myocardium was minced with scissors in a fresh collagenase-containing buffer and the rod-shaped adult mice cardiomyocytes were prepared. The isolated mice cardiomyocytes were transferred to laminin-coated glass culture dishes, and incubated for 12 h at 37 °C in a 5%CO_2/95%O_2 atmosphere.

2.4. Analysis of Ca^{2+} sparks and SR Ca^{2+} content

Ca^{2+} sparks were measured in saponin-permeabilized cardiomyocytes as previously described using a laser scanning confocal microscope (LSM-510, Carl Zeiss) [13,15,16]. In brief, ventricular myocytes were superfused with a relaxing solution containing EGTA 0.1 mmol/L, ATP 5 mmol/L, HEPES 10 mmol/L, K-aspartate 150 mmol/L, $MgCl_2$ 0.25 mmol/L, and reduced glutathione 10 mmol/L, at 23 °C. The sarcolemma was permeabilized by treating with saponin (50 μg/mL) for 30 s. After permeabilization, cardiomyocytes were placed in an internal solution composed of: EGTA 0.5 mmol/L; HEPES 10 mmol/L; K-aspartate 120 mmol/L; ATP 5 mmol/L; free [Mg^{2+}] 1 mmol/L; reduced glutathione 10 mmol/L; free [Ca^{2+}] 30 nmol/L (calculated using MaxChelator (http://www.stanford.edu/~cpatton/webmaxcS.htm)), creatine phosphokinase 5 U/ml, phosphocreatine 10 mmol/L, dextran (Mr: 40,000) 4%; Rhod-2 0.02 mmol/L, pH 7.2. Rhod-2 was excited by 543 nm laser lines, and the fluorescence intensity was acquired at excitation wavelengths of >560 nm. Ca^{2+} spark images were obtained from permeabilized ventricular myocytes in the presence of Ca^{2+}/CaM-dependent protein kinase II (CaMKII) inhibitor KN-93 (1 μmol/L) and Okadaic acid (1 μmol/L). Experiments without KN93 and/or Okadaic acid were not done. Therefore, CaMKII inhibition by KN-93 or serine/threonine phosphatase inhibition by Okadaic acid was not measured directly.

Data were analyzed with SparkMaster, an automated analysis program which allows rapid and reliable spark analysis [17]. The analysis includes general image parameters (number of detected sparks, spark frequency) as well as individual spark parameters (Amplitude, FWHM: full width at half maximum, FDHM: full duration at half maximum) (see Supplemental figure). To assess SR Ca^{2+} content, caffeine (10 mmol/L) was rapidly perfused to discharge the SR-loaded Ca^{2+}.

2.5. Determination of the binding of exogenous CaM to the RyR2 in saponin-permeabilized cardiomyocytes

The fluorescently labeled CaM (F-CaM) with HiLyte Fluor 647 (AnaSpec Inc., CA), was introduced into the saponin-permeabilized WT and KI cardiomyocytes under the same condition as the aforementioned Ca^{2+} spark measurements. Then, the distribution of localized CaM was determined by densitometric measurement of F-CaM fluorescence. Briefly, the fluorescently labeled cardiomyocytes were laser-scanned with the confocal microscope (LSM-510, Carl Zeiss). (numerical aperture, 1.3; excitation at 633 nm; emission 640 nm). The sarcomere-related periodical increase in the HiLyte Fluor 647 fluorescene intensity from baseline was integrated with respect to the longitudinally selected distance (~25 μm) and then divided the value by the distance.

2.6. Monitoring of Ca^{2+} transients of cardiomyocytes

Isolated ventricular myocytes were incubated with 20 μM Fluo4 acetoxymethyl ester for 30 min at room temperature and washed twice with Tyrode's solution. All experiments were conducted at 35 °C. Intracellular Ca^{2+} measurements with cells stimulated by a field electric stimulator (IonOptix, MA, USA) were performed with a laser-scanning confocal microscope (LSM-510, Carl Zeiss) and fluorescent digital microscopy (BZ9000, Keyence, Japan). Relative occurrence of spontaneous Ca^{2+} releases upon cessation of stimulation at 1, 2, 3, 4 and 5 Hz stimulation was measured in WT and KI myocytes [18].

2.7. Electrophysiological recordings in isolated ventricular myocytes

Transmembrane potentials and currents were recorded in whole cell mode using a MultiClamp 700B amplifier (Axon