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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

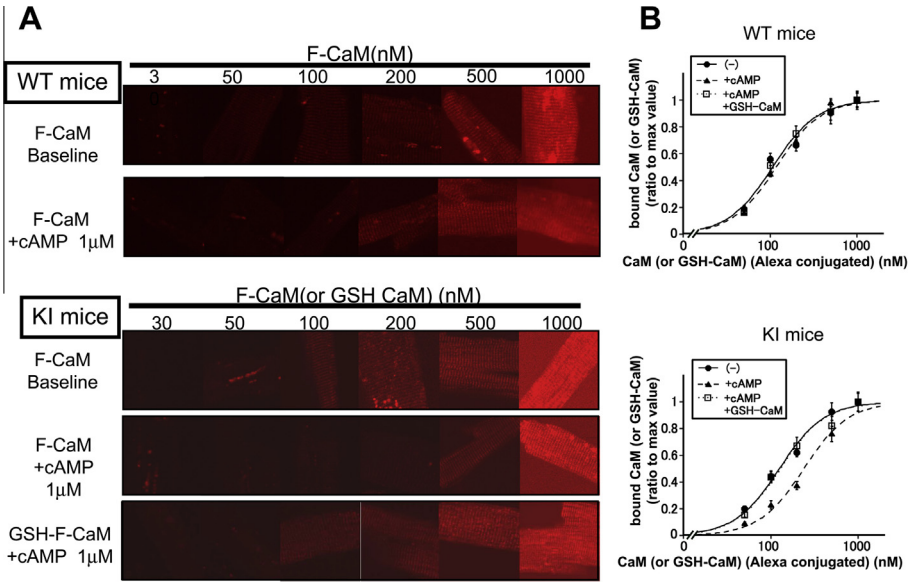

Fig. 1. The binding characteristics of exogenously introduced CaM in saponin-permeabilized cardiomyocytes. (A) Representative images of binding of F-CaM or F-GSH-CaM to RyR2 with or without cAMP (1 μmol/L). (B) Either F-CaM or F-GSH-CaM fluorescence was measured and expressed as the ratio to its maximum value (WT mice:B, KI mice:D). Each datum point per concentration represents mean ± SE of 8–13 cells from three to four hearts.

Instruments). Only quiescent, Ca^{2+}-tolerant, rod-shaped cells with clear cross striations were used for electrophysiological recordings. For action potential recording, myocytes were electrically stimulated by intracellular current injection through patch electrodes using depolarizing pulses with a duration of 3 ms and amplitude of 1.5 to 2.5 nA. All signals were acquired at 5 kHz (Digidata 1322A, Axon Instruments) and analyzed with the use of personal computer running pCLAMP version 9.2 software (Axon Instruments). Its amplitude was determined from the difference between the peak of the transient current and the mean of current just before and after the transient current. The CaMKII inhibitor KN93 was incorporated into myocytes in a Tyrode's solution [19].

2.8. Statistical analyses

Paired or unpaired *t*-tests were used for statistical comparisons of data obtained during the 2 different situations, while ANOVA with a post hoc Scheffe's test was used for statistical comparison of concentration-dependent data. Cross-tabulations with χ^2 were used as appropriate for event incidences.

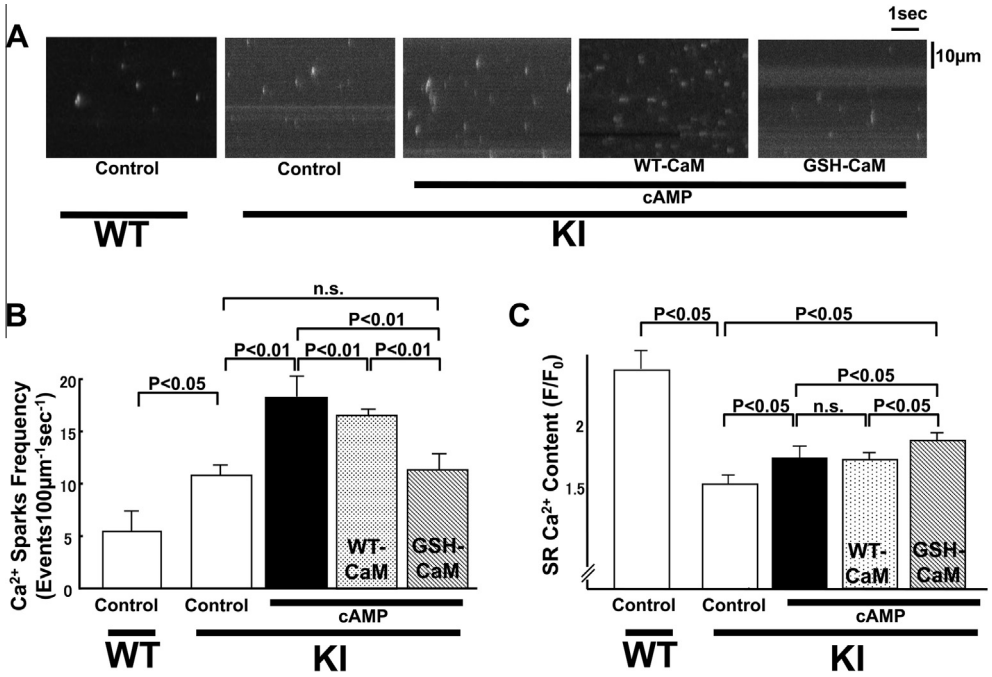

Fig. 2. Effect of WT-CAM (or GSH-CaM) (200 nmol/L) on Ca^{2+} SpF and SR Ca^{2+} content in (saponin-permeabilized) normal cardiomyocytes and KI cardiomyocytes treated with cAMP (1 μmol/L). SR Ca^{2+} content was measured by adding 10 mmol/L caffeine. Ca^{2+} spark images were obtained in the presence of the CaMKII inhibitor KN-93 (1 μmol/L). (A) Representative images of Ca^{2+} sparks. (B) Summarized data of Ca^{2+} SpF. (C) Summarized data of SR Ca^{2+} content. Data represent the mean ± SE of 8–16 cells from each of three to four hearts.

3. Results

In our previous study, we showed that CaM binding affinity of RyR is markedly decreased, thereby inducing spontaneous Ca^{2+} leak which leads to contractile dysfunction and arrhythmia [9,10,20]. Moreover, in failing hearts we found that CaM-(Gly-Ser-His), which shows higher binding affinity to the RyR1 than CaM [21], stabilized the RyR2 channel, inhibited Ca^{2+} leak, and improved myocyte function [10]. Here, we further examined whether CaM-GSH restores a normal channel function in the R2474S KI mouse model.

3.1. CaM binding to CPVT-RyR2, but not to WT-RyR, becomes weaker upon cAMP stimulation

To examine whether the CaM binding to the CPVT-RyR2 is altered in a physiological condition, we assessed the CaM binding to sub-cellular fractions by introducing CaM, fluorescently labeled with HiLyte Fluor 647 (F-CaM), into saponin-permeabilized CPVT-KI and WT cardiomyocytes (Fig. 1A). The fluorescence signal of exogenously introduced F-CaM and that of immuno-staining of RyR2 showed a periodical pattern associated with that of sarcomere, and importantly the staining patterns of both CaM and RyR2 showed an excellent match with each other (Supplemental figure). When we plotted the intensity of the Alexa fluorescence along the sarcomeres (see Section 2) as a function of the concentration of the introduced F-CaM or F-GSH-CaM in WT myocytes (Normal), the concentration dependence of GSH-CaM binding was unchanged relative to the dependence of WT-CaM binding regardless of the presence of cAMP (Fig. 1). In KI cardiomyocytes, the concentration dependence of WT-CaM binding was greatly shifted towards higher concentrations in response to cAMP, while the binding affinity of GSH-CaM appeared unchanged, even in the presence of cAMP (Fig. 1).

3.2. CaM-GSH inhibits spontaneous Ca^{2+} sparks and increases SR Ca^{2+} content in KI cardiomyocytes

To examine whether correction of the defective interaction of CaM to RyR2 inhibits spontaneous Ca^{2+} release events, we measured the Ca^{2+} sparks in saponin-permeabilized cardiomyocytes. In KI cardiomyocytes, the Ca^{2+} spark frequency was significantly increased in response to cAMP, while CaM-GSH (100 nM), but not WT-CaM (100 nM), inhibited the increase in SpF (Fig. 2A, B).

To assess the effect of exogenous CaM on SR Ca^{2+} content, we applied caffeine (10 mmol/L) to the saponin-permeabilized cardiomyocytes. As shown in Fig. 2C, in KI cardiomyocytes, SR Ca^{2+} content was decreased in response to cAMP, whereas it was

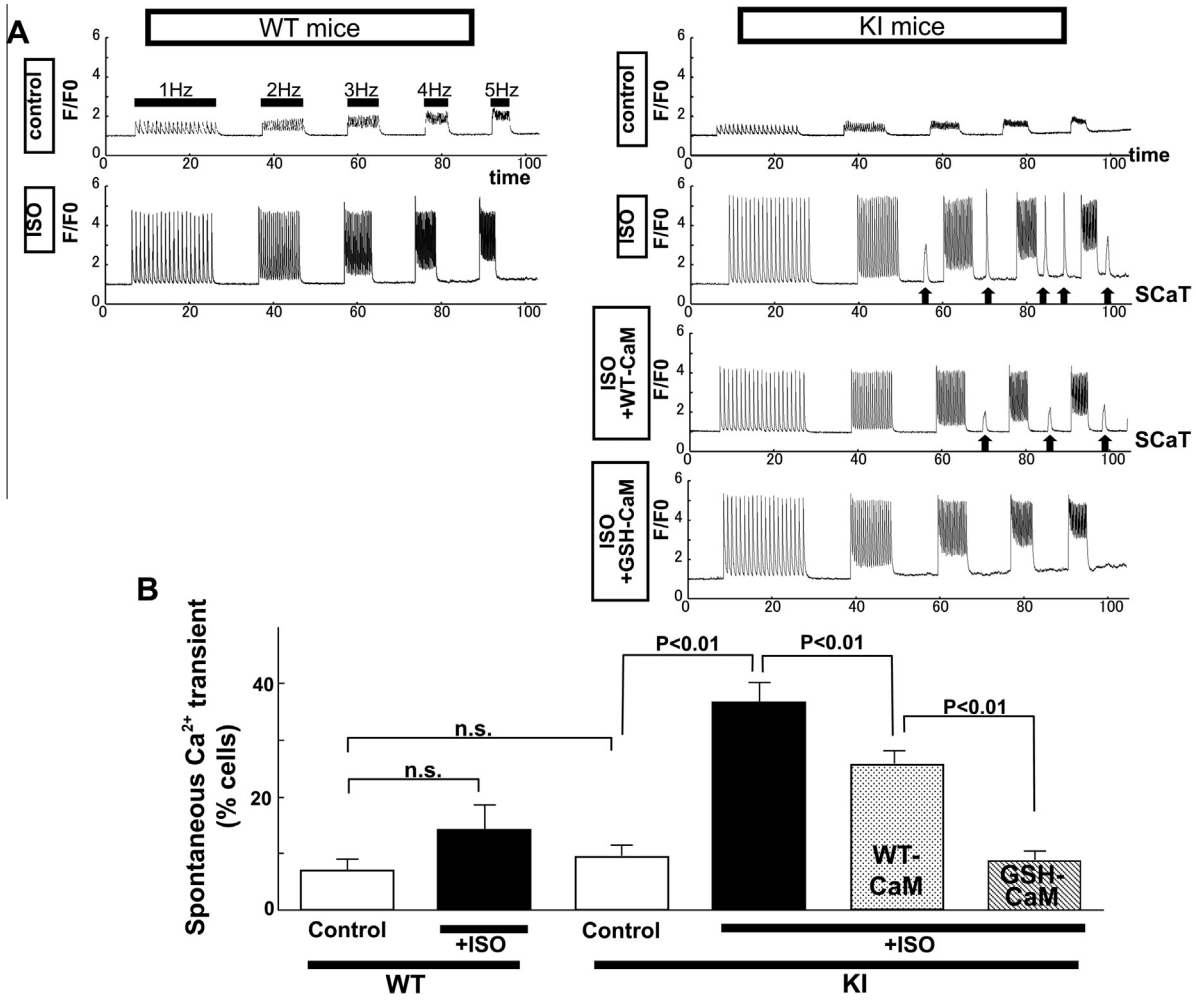

Fig. 3. Relative occurrence of spontaneous Ca^{2+} releases upon cessation of stimulation at 1, 2, 3, 4 and 5 Hz stimulation with or without isoproterenol in WT and KI cardiomyocytes. Higher pacing frequency increased the occurrence of spontaneous Ca^{2+} release. (A) Representative data of intracellular Ca during pacing. (B) Summarized data of % occurrence of spontaneous Ca^{2+} transient (SCaT). GSH-CaM prevented SCaT events induced by pacing and isoproterenol. Data represent the mean ± SE of 20–102 cells from each of three to four hearts.

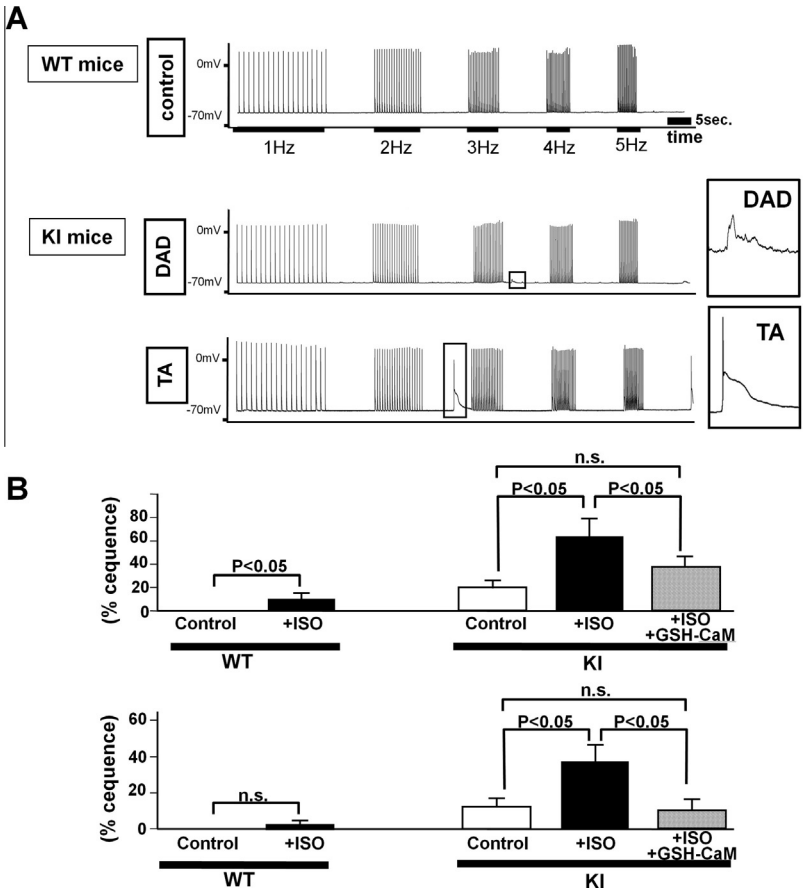

Fig. 4. Action potentials recorded from KI myocytes stimulated at 1 to 5 Hz. DADs develop when pacing is interrupted. (A) The figure shows the last 5 driven action potentials at each pacing frequency and DADs and triggered activity developing when pacing was discontinued. Note that DADs and triggered activity were observed after relatively faster pacing frequencies. (B) Summarized data of % occurrence of DAD or triggered activity. Data represent the mean ± SE of 10–15 cells from each of four to five hearts.

restored to a normal level by the addition of CaM-GSH (but not WT-CaM).

3.3. Anti-arrhythmogenic effects of enhanced CaM binbing affinity in the intact (non-permeabilized) R2474S/+ KI cardiomyocytes

WT cardiomyocytes showed no spontaneous Ca^{2+} transient in response to isoproterenol (30 nmol/L) (Fig. 3A). However, KI cardiomyocytes showed spontaneous Ca^{2+} transients in response to isoproterenol when the pacing rate increased from 1 to 5 Hz (Fig. 3). Importantly, the spontaneous Ca^{2+} transients disappeared by incorporation of CaM-GSH (with the use of protein delivery kit), but not WT-CaM (Fig. 3).

3.4. Distinctly different effects of WT-CaM and GSH-CaM on action potential, delayed-afterdepolarization (DAD) and triggered activity in WT and KI cardiomyocytes

Neither action potential amplitude nor resting potential differed significantly between WT cardiomyocytes ($n = 60$) and KI cardiomyocytes ($n = 49$) (amplitude, 116.9 ± 0.7 versus 116.2 ± 1.2 mV, n.s.1; resting potential, −70.5 ± 0.3 versus −70.4 ± 0.1 mV, n.s.).

As shown in Fig. 4, no spontaneous DADs were observed in WT myocytes at any pacing frequency, whereas DADs were observed in 20% of KI cardiomyocytes (11/55 $P = 0.001$ versus WT) and 12% (7/55) of KI cardiomyocytes also revealed triggered activity.

In response to 10 nmol/L isoproterenol, 11% of WT myocytes showed small DADs (5/45) but only one (2%, 1/45) triggered activity, whereas in KI myocytes the incidence of DADs and triggered activity further increased to 60% (24/40) of cells ($P = 0.01$ versus WT) and (15/40, 38%; $P = 0.01$ versus WT), respectively. Upon perfusion of GSH-CaM (100 nM), GSH-CaM inhibited DADs and triggered activity.

4. Discussion

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is an inherited form of lethal arrhythmia caused by single point mutation in RyR2 [22,23]. The fact that in vivo amino acid mutation of RyR2 in several knock-in mouse models [13,15,24,19] showed the inducible VT by exercise or epinephrine clearly indicates that single point mutation of RyR2 is a causative factor of CPVT. Nevertheless, it still remains unclear why single amino acid mutation out of about 5000 amino acids in RyR2 produces such a drastic change in the channel gating property leading to lethal arrhythmia. Using two different types of KI mouse models having R2474S or S2246L mutations, we have reported that these mutations produce defective inter-domain interaction (domain unzipping) between the N-terminal region:1–600 and the central region: 2000–2500 (designated as domain switch), which in turn sensitize the channel to agonists and reduce the threshold of luminal Ca^{2+} for activation [13,15]. We also have reported mutation-caused aberrant domain unzipping of the domain switch, via subsequent global conformational changes, induced dissociation of

calmodulin (CaM) from the RyR2, resulting in diastoic Ca^{2+} release leading to lethal arrhythmia[9].

The major findings of this study are as follows. First, decreased affinity of CaM binding to CPVT-RyR2 in response to cAMP is the cause of triggered activity and DAD. Second, enhancement of the CaM binding to RyR2 (by using CaM-GSH: much stronger RyR-binder than native CaM) prevented aberrant Ca^{2+} release, DaD, and triggered activity in cAMP-treated KI cardiomyocytes, in which CaM binding affinity to RyR2 is markedly decreased. In the light of the aforementioned concept of the involvement of coordinated/global conformational changes in different domains in the control of RyR channel functions, these findings suggest that in the CPVT-associated mutants, domain unzipping of the domain switch causes conformational disorder of the CaM binding domain in such a way that it weakens CaM binding activity, thereby predisposing to aberrant Ca^{2+} release and lethal arrhythmia. According to the report by Gangopadhyay and Ikemoto [25], an inter-domain interaction between the CaM binding domain (CaMBD) and the CaM-like domain (CaMLD) activates Ca^{2+} channel, and binding of CaM to the CaMBD interferes the inter-domain interaction, preventing over-activation of the channel. In the light of this concept, it seems that CPVT mutation in the domain switch causes aberrant tighter interaction of these two domains and aberrant channel activation via the aforementioned mechanism of coordinated global conformational change; this results in the reduced activity of CaM binding and abnormally excessive channel activation. Then, enhanced CaM binding to the CaMBD using CaM-GSH will suppress pathogenic overly strong CaMBD/CaMLD inter-domain interactions. Extending this view we can predict that any agents that specifically antagonize with the CaMBD/CaMLD interaction will be promising candidates for therapeutic treatment of CPVT.

We have previously reported that in pacing-induced failing hearts, domain unzipping of the domain switch is induced by either PKA phosphorylation of Ser2808 [26] or oxidative stress [27], which gives rise to aberrant Ca^{2+} release and subsequently contractile dysfunction of cardiomyocytes via reduced SR Ca^{2+} content. Recently, we found that domain unzipping seen in failing hearts also dissociates CaM as in the case of CPVT, and rebinding of CaM ameliorates the stability of the RyR2 [10,20]. These findings indicate that dissociation of CaM allosterically linked to domain unzipping plays a critical role in abnormal Ca^{2+} handling as a common pathogenic mechanism underlying CPVT and heart failure, by which aberrant Ca^{2+} release is induced. This notion is further supported by our previous findings: (1) that dantrolene restores normal operation of the domain switch from unzipping to zipping and prevents aberrant Ca^{2+} release that is seen commonly in CPVT and heart failure [28,29] and (2) that dantrolene effectively prevented dissociation of CaM in failing hearts [20] and also in R2474S CPVT-type KI hearts [13,29].

In conclusion, our results suggest that the reduction of CaM binding ability to the CPVT-RyR2 is triggered by PKA phosphorylation. This is the primary cause of arrhythmogenesis in the CPVT cardiomyocytes, as evidenced by the present finding that most of abnormal RyR channel functions caused by the CPVT mutations could be corrected by introducing chemically modified CaM that has significantly higher RyR-binding affinity than native CaM. Based upon the present finding we might suggest that a group of reagent, which have specific and high-affinity binding property to the CaM-binding domain of RyR, will serve as useful therapeutic agents to treat CPVT-linked arrhythmia.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.03.152.

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