Published in final edited form as:

Biochem Biophys Res Commun. 2010 April 9; 394(3): 660–666. doi:10.1016/j.bbrc.2010.03.046.

Defective calmodulin binding to the cardiac ryanodine receptor plays a key role in CPVT-associated channel dysfunction

Xiaojuan Xu^a, Masafumi Yano^{a,*}, Hitoshi Uchinoumi^a, Akihiro Hino^a, Takeshi Suetomi^a, Makoto Ono^a, Hiroki Tateishi^a, Tetsuro Oda^a, Shinichi Okuda^a, Masahiro Doi^a, Shigeki Kobayashi^a, Takeshi Yamamoto^a, Yasuhiro Ikeda^a, Noriaki Ikemoto^{b,c}, and Masunori Matsuzaki^a

^aDepartment of Medicine and Clinical Science, Division of Cardiology, Yamaguchi University Graduate School of Medicine, 1-1-1 Minamikogushi, Ube, Yamaguchi, 755-8505, Japan

^bBoston Biomedical Research Institute, Watertown, Massachusetts 02472, USA

^cDepartment of Neurology, Harvard Medical School, Boston, Massachusetts 02115, USA

Abstract

Calmodulin (CaM), one of the accessory proteins of the cardiac ryanodine receptor (RyR2), is known to play a significant role in the channel regulation of the RyR2. However, the possible involvement of calmodulin in the pathogenic process of catecholaminergic polymorphic ventricular tachycardia (CPVT) has not been investigated. In this study, we investigated the state of RyR2-bound CaM and channel dysfunctions using a knock-in (KI) mouse model with CPVT-linked RyR2 mutation (R2474S). Without added effectors, the affinity of CaM binding to the RyR2 was indistinguishable between KI and WT hearts. In response to cAMP (1 μ mol/L), the RyR2 phosphorylation at Ser2808 increased in both WT and KI hearts to the same extent. However, cAMP caused a significant decrease of the CaM binding affinity in KI hearts, but the affinity was unchanged in WT. Dantrolene restored a normal level of CaM-binding affinity in the cAMP-treated KI hearts, suggesting that defective inter-domain interaction between the N-terminal domain and the central domain of the RyR2 (the target of therapeutic effect of dantrolene) is involved in the cAMP-induced reduction of the CaM binding affinity. In saponin-permeabilized cardiomyocytes, the addition of cAMP increased the frequency of spontaneous Ca²⁺ sparks to a significantly larger extent in KI cardiomyocytes than in WT cardiomyocytes, whereas the addition of a high concentration of CaM attenuated the aberrant increase of Ca²⁺ sparks. In conclusion, CPVT mutation causes defective inter-domain interaction, significant reduction in the ability of CaM binding to the RyR2, spontaneous Ca²⁺ leak, and then lethal arrhythmia.

Keywords

catecholaminergic polymorphic ventricular tachycardia (CPVT); calcium; sarcoplasmic reticulum; calmodulin; ryanodine receptor

*Address for correspondence: Masafumi Yano, M.D., Ph.D., Department of Medicine and Clinical Science, Division of Cardiology, Yamaguchi University Graduate School of Medicine, 1-1-1 Minamikogushi, Ube, Yamaguchi, 755-8505, JAPAN, Tel: +81-836-22-2248 yanoma@yamaguchi-u.ac.jp.

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.

Introduction

Ryanodine receptor (RyR2), a Ca^{2+} release channel in the cardiac sarcoplasmic reticulum, plays a central role in cardiac excitation-contraction coupling, and its dysfunction has a severe impact on the cardiac function as seen in several forms of cardiac disease, such as an inherited form of lethal arrhythmia, catecholaminergic polymorphic ventricular tachycardia (CPVT) [1]. All these RyR2 mutations cluster into 3 regions of the RyR2 that correspond to the 3 malignant hyperthermia/central core disease mutation regions in the RyR1 (designated as N-terminal domain (aa 1-600), central domain (aa 2000-2500), and C-terminal channel-forming domain) [1]. Because single point mutation in either the N-terminal or the central domain in skeletal RyR1 was found to hyper-activate and/or hyper-sensitize the channel opening, Ikemoto and colleagues [2] have proposed that the two domains (N-terminal and central domain) interact with each other to act as the implicit on/off switch for channel opening and closing; zipping of the interacting domains closes the channel, and unzipping opens the channel. We have shown that defective inter-domain interaction in the RyR2 of failing heart, namely aberrant domain unzipping between the N-terminal and the central domains in an otherwise resting conditions, destabilizes the channel and causes diastolic Ca^{2+} leak [3,4]. Importantly, the addition of cardioprotective agent K201(JTV519) (1,4-benzothiazepine derivative) to the failing RyR2 corrected defective unzipped configuration to a normal zipped configuration, and stopped Ca^{2+} leak [3,4]. Furthermore, we have shown that dantrolene, a therapeutic agent for malignant hyperthermia (MH), also prevents abnormal Ca^{2+} leak by correcting the defective inter-domain interaction of RyR2 and improves the contractile function of pacing-induced failing hearts [5]. Since the N-terminal and the central mutable regions (located in domains 5 and 6 of the so-called clamp region, respectively) [6,7] are distant from the channel, there must be some mechanism by which the on/off conformational signal elicited in the clamp region is transmitted to the channel.

Calmodulin (CaM), one of the accessory proteins of RyR2, is known to regulate the channel function [8]. Namely, CaM inhibits RyR2 Ca^{2+} channels at physiological concentrations of Ca^{2+} [9]. Recently, Yamaguchi *et al.* [10] demonstrated that knock-in mouse model, in which several critical mutations were introduced in the CaM-binding domain (3583–3603) of RyR2 to make the RyR2 unable to bind CaM, showed a phenotype of dilated hypertrophic cardiomyopathy with severely impaired systolic function. This finding clearly indicates that the specific CaM binding to the RyR2 is essential for normal cardiac functions. Since the CaM binding site of RyR2 locates in the midway between the “clamp” region and the trans-membrane channel region in a 3D image of the RyR2 molecule [11], we hypothesize that the aforementioned on/off action of inter-domain interaction between the N-terminal and the central domains occurring in the clamp region is conveyed to the channel by mediation of a CaM-dependent mechanism.

Using knock-in mice harboring human CPVT-type mutation (R2474S), we recently demonstrated that upon catecholaminergic stimulation the single mutation causes the defective inter-domain interaction, hyper-sensitizes the channel to luminal Ca^{2+} activation, induces spontaneous Ca^{2+} leak, and in turn causes lethal arrhythmia [12]. In the present study, using the R2474S/+ knock-in mice we further investigated the role of the CaM bound to the RyR2 in the mutation-linked defective Ca^{2+} release.

Materials and methods

Animal model

Knock-in mice with the RyR2 R2474S Mutation were generated as described previously [12].

Preparation of cardiac homogenates

We prepared LV crude homogenates from mice LV, as described previously [12]. Left ventricles were homogenized in a solution containing 10 mmol/L Tris-malate, protease inhibitor cocktail (complete, EDTA-free, Roche), at pH 6.8. The homogenate was centrifuged at 4,000 g for 15 minutes and the resultant supernatant was centrifuged at 100,000 g for 30 minutes. The pellet was resuspended in a solution containing 0.15 mol/L NaCl, 50 mmol/L MOPS, 0.1 % CHAPS, protease inhibitor cocktail (complete, EDTA-free), at pH 6.8. This fraction was used for the CaM binding and phosphorylation assays.

Peptide used and peptide synthesis

We used the following domain peptide: DPc10 which harbors CPVT-type mutation site (R2474S), as described previously [3].

DPc10: DPc2460-2495

2460GFCPDHKAAMVFLDRVYGIEVQDFLLHLLGVGFLP2495

Peptides were synthesized on an Applied Biosystems model 431A synthesizer employing Fmoc (N-(9-fluorenyl)methoxycarbonyl) as the alpha-amino protecting group, as described previously [13].

Association of CaM to the RyR2

The mammalian CaM (mCaM) cDNA was kindly provided by Dr. Z. Grabarek (Boston Biomedical Institute, Boston, MA). Expression and purification of CaM were carried out as described previously [14]. For the assessment of CaM bound to the RyR2, the conventional pull-down assay (using the anti-RyR2 antibody, followed by detection of the bound CaM using the anti-CaM antibody) was impractical because of the marked non-specific CaM binding on Sepharose A or G even without the anti-RyR2 antibody. Therefore, we assessed the CaM binding on the RyR2 using a photoreactive crosslinker, Sulfosuccinimidyl-6-[4'-azido-2'-nitrophenylamino]hexanoate (Sulfo-SANPAH, PIERCE). First, CaM-SANPAH conjugate was formed by incubating 0.2 mmol/L CaM with 0.8 mmol/L SANPAH in a 20 mmol/L HEPES (pH 8) solution for 60 minutes at 22 °C in the dark. The reaction was quenched by 20 mmol/L Tris-HCl, at pH 7.5. Free Sulfo-SANPAH was removed using Sephadex G25 column. The CaM-SANPAH conjugate (32 nmol/L-1 μmol/L in a final concentration) was mixed with 0.5 mg/mL cardiac homogenates in the sample solution containing a 1 mmol/L EGTA/calcium buffer (0.3 μmol/L free $[Ca^{2+}]$) in the dark, and photolyzed with UV light in a Pyrex tube at 4 °C for 60 seconds. Then, the mixture was immunoblotted with a monoclonal anti-CaM antibody (MILLIPORE, CA) to detect the RyR2-bound CaM.

Isolation of cardiac cardiomyocytes

Cardiomyocytes were isolated from mice hearts as described previously [12,15]. In brief, after sacrifice, the LV myocardium was minced with scissors in a fresh collagenase-containing buffer and the rod-shaped adult mice cardiomyocytes were prepared by retrograde perfusion of the hearts with 95% O₂/5% CO₂-bubbled. The isolated mice cardiomyocytes were transferred to laminin-coated glass culture dishes, and incubated for 12 hours at 37°C in a 5% CO₂/95% O₂ atmosphere.

Analysis of Ca²⁺ sparks with laser scanning confocal microscopy

Ca²⁺ sparks were measured in saponin-permeabilized cardiomyocytes as previously described using a laser scanning confocal microscope (LSM-510, Carl Zeiss) [12,16]. In brief, ventricular myocytes were superfused with relaxing solution containing EGTA 0.1

mmol/L, ATP 5 mmol/L, HEPES 10 mmol/L, K-aspartate 150 mmol/L, MgCl₂ 0.25 mmol/L, and reduce-glutathione 10 mmol/L, at 22°C. The sarcolemma was permeabilized with saponin (50 µg/mL) for 30 seconds. 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 3 mmol/L; free [Mg²⁺] 1 mmol/L; reduced glutathione 10 mmol/L; free [Ca²⁺] 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 fluorescence was acquired at wavelengths of >560 nm. Ca²⁺ spark images were obtained from permeabilized ventricular myocytes in the presence of Ca²⁺/CaM-dependent protein kinase II (CaMKII) inhibitor KN-93 (1 µmol/L) and okadaic acid (1 µmol/L). 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).

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

The exogenous CaM, fluorescently labeled with Alexa Fluor 488 (Molecular Probes, OR), was introduced into the saponin-permeabilized WT and KI cardiomyocytes under the same condition as the aforementioned Ca²⁺ spark measurements. Then, the distribution of localized CaM was determined by densitometric measurement of Alexa-CaM fluorescence. Briefly, the fluorescently labeled cardiomyocytes were laser-scanned with the confocal microscope (LSM-510, Carl Zeiss). (numerical aperture, 1.3; excitation at 488 nm; emission 505-530 nm). The sarcomere-related periodical increase in the Alexa488 fluorescence intensity from baseline was integrated with respect to the longitudinally selected distance (~25 µm) and then divided the value by the distance. The Alexa-CaM labeled cardiomyocytes were also applied to the immunofluorescent staining by anti-RyR2 antibody, as follows. The cardiomyocytes were fixed with 4% paraformaldehyde in PBS for 5 minutes, washed three times with PBS, and permeabilized in 0.5% Triton X-100 and 1% BSA for 15 minutes. Then, the cardiomyocytes were incubated overnight at 4°C with a monoclonal anti-RyR antibody (Affinity Bioreagents, CO) in 1% BSA and 0.5 % Triton X-100, followed by labeling with an Alexa633-conjugated rabbit anti-mouse secondary antibody (Molecular Probes, OR). The cardiomyocytes were washed three times with PBS. Images for co-localization of Alexa-CaM and RyR were acquired at 488nm excitation wavelength using a BP 505-530 nm band-pass detection filter for Alexa-CaM, and 633 nm excitation wavelength with a LP 650 nm long pass filter for anti-RyR antibody. Secondary antibody labeling alone showed no detectable fluorescence pattern.

Determination of the binding of endogenous CaM to the RyR2 in intact cardiomyocytes

The isolated cardiomyocytes were plated on glass-based dishes, fixed with 4% paraformaldehyde in PBS for 5 minutes, washed three times with PBS, and permeabilized in 0.5% Triton X-100 and 1% BSA for 15 minutes. These cardiomyocytes were incubated overnight at 4°C with a monoclonal mouse anti-CaM antibody (MILLIPORE, CA) and polyclonal rabbit anti-RyR antibody (C-terminal Ab4963; cf. ref 4) in 1% BSA and 0.5 % Triton X-100, followed by each labeling with an Alexa488-conjugated goat anti-rabbit secondary antibody (Molecular Probes, OR) and Alexa633-conjugated rabbit anti-mouse secondary antibody (Molecular Probes, OR). Cardiomyocytes were then washed three times with PBS. For co-localization of anti-CaM antibody (Alexa633:red) and anti-RyR antibody (Alexa488:green) images were acquired at 633 nm and 488 nm excitation in conjunction with LP 650 filter and BP505-530 nm, respectively. Both secondary antibodies

showed no detectable fluorescence pattern and did not cross-react with either primary antibody. For co-localization analysis, the fluorescent images were acquired sequentially in order to prevent spectral cross-talk.

Antibodies and immunoblot analyses

Immunoblot analyses of CaM, RyR2, and P-Ser2808-RyR2 were performed by using an anti-CaM antibody (MILLIPORE, CA), anti-RyR antibody (C-terminal Ab4963), and the antibody against P-Ser2808-RyR2 (cf. ref 12), respectively. For the assessment of the specific CaM binding on the RyR2, the antibody against CaM binding domain (3583–3603), which was an order-made from Sigma, was used.

Statistical analyses

Unpaired t-test was used for statistical comparison of the data between two different situations. Data are expressed as means $\pm$ SE. We accepted a p value less than 0.05 as statistically significant.

Results

Triggered arrhythmia

As shown in our previous report [12], either injection of caffeine plus epinephrine (i.p.) or exercise with treadmill induced bidirectional or polymorphic VT in KI mice, but not in WT mice (cf. Fig. 2A in ref 12).

Upon PKA phosphorylation the affinity of CaM binding to the RyR2 was reduced in KI hearts, but not in WT hearts

Figure 1A shows immunoblot data of concentration dependent CaM-SANPAH binding to the RyR2. There was no difference in the concentration dependence of the CaM binding to the RyR2 at control condition between KI and WT hearts. In response to cAMP (1 μ mol/L), which induced domain unzipping between the N-terminal and the central domains only in KI mice [12], the affinity of the CaM binding to the RyR2 was reduced in KI hearts, but not in WT hearts. After the addition of cAMP, phosphorylation level of Ser2808 in the RyR2 increased in both WT and KI hearts to an equal extent (Figure 1B). In both WT and KI hearts, the CaM binding was markedly suppressed in the presence of the antibody raised against the CaM binding domain (3583–3603), indicating that this domain is the major target of the CaM binding site in RyR2 (Figure 1C). In WT hearts, the CaM binding to the RyR2 was unchanged in response to cAMP (1 μ mol/L), but it decreased in the presence of DPc10, which was previously shown to induce the domain unzipping mimicking R2474S mutation-linked disorder [3, 12] (Figure 1C). In KI hearts, DPc10 had no further effect on the cAMP-induced decrease in the CaM binding. Conversely, dantrolene, which has been shown to correct the domain unzipping and inhibit spontaneous Ca²⁺ leak in either pacing-induced canine hearts [5] or the R2474S KI mouse hearts [12], restored the CaM binding in both DPc10-treated WT hearts and KI hearts (Figure 1C). These findings strongly suggest that PKA-phosphorylation at Ser-2808 of RyR2 induces domain unzipping in the CPVT- (but not WT) RyR2 [12], in turn reduces the binding affinity of CaM to the CaM-binding domain (3583–3603) of RyR2. Addition of high concentration of CaM (1 μ mol/L) restored the CaM binding to the RyR2, regardless of the presence of DPc10 (in WT) or cAMP (in KI) (not shown).

To examine whether the CaM binding to the CPVT-associated RyR2 is altered in a more physiological condition, we assessed the CaM binding to sub-cellular fractions by introducing CaM, fluorescently labeled with Alexa Fluor 488, into saponin-permeabilized KI and WT cardiomyocytes. The fluorescence signal of exogenously introduced CaM-Alexa

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 (Figure 2A). In KI cardiomyocytes, addition of cAMP significantly decreased the CaM binding, whereas DPc10 had no further effect on the cAMP-induced decrease in the CaM binding (Figure 2B). In WT cardiomyocytes, the CaM binding was unchanged after the addition of cAMP, whereas it decreased in the presence of DPc10 (Figure 2B). In both WT and KI cardiomyocytes, dantrolene restored the CaM binding even after the addition of DPc10 and cAMP in WT and KI cardiomyocytes, respectively. Addition of high concentration of CaM (1 $\mu\text{mol/L}$) again restored the CaM binding to RyR2, regardless of the presence of DPc10 (in WT) or cAMP (in KI) (not shown).

To further assess the binding of endogenous CaM to the RyR2 in intact cardiomyocytes, we performed immunofluorescent staining using anti-CaM antibody (See Methods). As shown in Figure 3A, CaM was detected along with sarcomeres, showing an excellent co-localization with the RyR2. In WT cardiomyocytes, isoproterenol was without effect on the endogenous CaM binding (Figure 3B). In contrast, isoproterenol decreased the endogenous CaM binding in KI cardiomyocytes (Figure 3B).

CaM inhibits spontaneous Ca^{2+} sparks in KI cardiomyocytes

To investigate the role of CaM on the RyR2 channel opening, we measured Ca^{2+} sparks in saponin-permeabilized cardiomyocytes (Figure 4). In agreement with our previous report [12], the Ca^{2+} spark frequency was increased by addition of cAMP, whereas it was unchanged in WT cardiomyocytes (Figure 4A). However, addition of DPc10 increased the Ca^{2+} spark frequency in WT cardiomyocytes. Interestingly, co-addition of high concentration of CaM (1 $\mu\text{mol/L}$) with DPc10, which was found to bind to the RyR2 even in the presence of DPc10 or cAMP, decreased the Ca^{2+} spark frequency (Figure 4A). The profile of the dependence of Ca^{2+} spark frequency on the SR Ca^{2+} content is shown in Figure 4B. As shown, frequent Ca^{2+} sparks occur at much lower SR Ca^{2+} load in KI cardiomyocytes compared with WT cardiomyocytes. Addition of domain unzipping peptide DPc10 to WT mimicked the situation with KI (reduced threshold of SR Ca^{2+} load for activation), but the addition of CaM partially restored normal profile. Addition of cAMP to KI produced a significant increase in the spark frequency, but again the addition of CaM partially restored the situation with WT (increased threshold of SR Ca^{2+} load for activation) (Figure 4B). These data suggest that defective inter-domain interaction (aberrant diastolic domain unzipping), which has occurred in the KI RyR2 dissociates CaM from the RyR2 and produces increased spontaneous Ca^{2+} sparks even at a reduced level of SR Ca^{2+} load.

Discussion

CaM, one of the accessory proteins of RyR2, is known to have an inhibitory effect on the channel opening in a physiological range of intracellular $[\text{Ca}^{2+}]$ in normal RyR2 [8,9]. However, the pathogenic role that CaM may play in the defective channel opening in diseased hearts remains to be elucidated.

The major finding of this study is that in CPVT-associated KI mice the affinity of CaM binding to the RyR2 is reduced upon PKA phosphorylation, which seems to be a critical cause of spontaneous local Ca^{2+} release events. In our recent report [12], we demonstrated that PKA-phosphorylation induces defective inter-domain interaction between the N-terminal (aa 1-600) and central (aa 2000-2500) domains in the CPVT-associated RyR2, causing spontaneous Ca^{2+} release events, which may lead to lethal arrhythmia. Since in the 3D image of the RyR2, Ser2808, the site of PKA phosphorylation, has been localized in the vicinity of the boundary between the N-terminal domain (aa 1-600) and the central domain (aa 2000-2500) [18], PKA phosphorylation at Ser2808 may accelerate the domain unzipping

in the KI channel, where the domain unzipping has already been under the way [12]. Although the domain unzipping is most likely to be a primary cause of the hypersensitized channel gating in KI mice, it remains to be elucidated how the defective conformational signal is conveyed through the bulky cytoplasmic structure to the channel pore.

The present study addresses this question. We have shown here that defective inter-domain interaction caused by single point mutation (R2474S) reduces the affinity of CaM binding to the RyR2. This notion is supported by the following findings. (1) Addition of DPc10 (2460-2495), which induces domain unzipping in WT RyR2 mimicking ‘unzipped’ KI RyR2 [12], reduced the affinity of CaM binding to the WT RyR2. (2) The lowered binding affinity of CaM to the KI RyR2 can be restored in the presence of dantrolene. (3) The binding of exogenously introduced Alexa-CaM to the RyR2 in situ was decreased in either DPc10-treated WT cardiomyocytes or cAMP-treated KI cardiomyocytes; dantrolene restored the CaM binding in all of these situations. (4) The amount of the RyR2-bound endogenous CaM was indeed reduced in isoproterenol-treated KI cardiomyocytes.

It is well known that aberrant Ca^{2+} release events induce lethal arrhythmia through delayed afterdepolarization [19]. Therefore, inhibition of such spontaneous activity of the channel will be a new therapeutic strategy against lethal arrhythmia. As we reported previously, dantrolene corrects defective inter-domain interaction (aberrant unzipped state) to a normal zipped state, thereby inhibiting Ca^{2+} leak/sparks [5]. Since dantrolene has no appreciable effect on the normal Ca^{2+} transient, but inhibits only abnormal Ca^{2+} release [5], the drug seems to satisfy those requirements for safe application to the clinical treatment of heart failure and lethal arrhythmia.

In conclusion, CaM binding ability to the CPVT-RyR2 is markedly reduced in response to PKA phosphorylation, presumably via defective inter-domain interaction. This causes spontaneous diastolic Ca^{2+} sparks, then lethal arrhythmia. The present finding that the rebinding of CaM to the CPVT-mutated RyR2 channel partially inhibits the spontaneous Ca^{2+} release restoring normal SR Ca^{2+} load-dependence suggests that the CaM-binding domain is a new target for therapies of CPVT and heart failure.

Acknowledgments

This work was supported by grants-in-aid for scientific research from The Ministry of Education in Japan (grant Nos. 20390226 to MY, 20590868 to TY, 20591805 to SK, 19209030 to MM), a grant from Takeda Science Foundation (to MY), and a grant from the National Heart, Lung and Blood Institutes (HL072841 to NI). The authors declare no competing financial interests.

References

1. Yano M, Yamamoto T, Ikeda Y, et al. Mechanisms of Disease: ryanodine receptor defects in heart failure and fatal arrhythmia. *Nat Clin Pract Cardiovasc Med* 2006;3:43–52. [PubMed: 16391617]
2. Ikemoto N, Yamamoto T. Regulation of calcium release by interdomain interaction within ryanodine receptors. *Front Biosci* 2002;7:d671–d683. [PubMed: 11861212]
3. Oda T, Yano M, Yamamoto T, et al. Defective regulation of interdomain interactions within the ryanodine receptor plays a key role in the pathogenesis of heart failure. *Circulation* 2005;111:3400–3410. [PubMed: 15967847]
4. Yamamoto T, Yano M, Xu X, et al. Identification of target domains of the cardiac ryanodine receptor to correct channel disorder in failing hearts. *Circulation* 2008;117:762–72. [PubMed: 18227387]
5. Kobayashi S, Yano M, Suetomi T, et al. Dantrolene, a therapeutic agent for malignant hyperthermia, markedly improves the function of failing cardiomyocytes by stabilizing interdomain interactions within the ryanodine receptor. *J Am Coll Cardiol* 2009;53:1993–2005. [PubMed: 19460614]

6. Liu Z, Wang R, Zhang J, et al. Localization of a disease-associated mutation site in the three-dimensional structure of the cardiac muscle ryanodine receptor. *J Biol Chem* 2005;280:37941–37947. [PubMed: 16157601]
7. Wang R, Chen W, Cai S, et al. Localization of an NH₂-terminal disease-causing mutation hot spot to the “clamp” region in the three-dimensional structure of the cardiac ryanodine receptor. *J Biol Chem* 2007;282:17785–17793. [PubMed: 17452324]
8. Bers DM. Macromolecular complexes regulating cardiac ryanodine receptor function. *J Mol Cell Cardiol* 2004;37:417–429. [PubMed: 15276012]
9. Yamaguchi N, Xu L, Pasek DA, et al. Molecular basis of calmodulin binding to cardiac muscle Ca²⁺ release channel (ryanodine receptor). *J Biol Chem* 2003;278:23480–23486. [PubMed: 12707260]
10. Yamaguchi N, Takahashi N, Xu L, et al. Early cardiac hypertrophy in mice with impaired calmodulin regulation of cardiac muscle Ca release channel. *J Clin Invest* 2007;117:1344–1353. [PubMed: 17431507]
11. Samsó M, Wagenknecht T. Apocalmodulin and Ca²⁺-calmodulin bind to neighboring locations on the ryanodine receptor. *J Biol Chem* 2002;277:1349–1353. [PubMed: 11694536]
12. Uchinoumi H, Yano M, Suetomi T, et al. Catecholaminergic polymorphic ventricular tachycardia is caused by mutation-linked defective conformational regulation of the ryanodine receptor. *Circ Res.* in press.
13. Yamamoto T, Ikemoto N. Peptide probe study of the critical regulatory domain of the cardiac ryanodine receptor. *Biochem Biophys Res Commun* 2002;291:1102–1108. [PubMed: 11866478]
14. Tan RY, Mabuchi Y, Grabarek Z. Blocking the Ca²⁺-induced conformational transitions in calmodulin with disulfide bonds. *J Biol Chem* 1996;271:7479–7483. [PubMed: 8631777]
15. Zhou YY, Wang SQ, Zhu WZ, et al. Culture and adenoviral infection of adult mouse cardiac myocytes: Methods for cellular genetic physiology. *Am J Physiol* 2000;279:H429–436.
16. Terentyev D, Nori A, Santoro M, et al. Abnormal interactions of calsequestrin with the ryanodine receptor calcium release channel complex linked to exercise-induced sudden cardiac death. *Circ Res* 2006;98:1151–1158. [PubMed: 16601229]
17. Picht E, Zima AV, Blatter LA, et al. SparkMaster - automated calcium spark analysis with ImageJ. *Am J Physiol* 2007;293:C1073–1081.
18. Meng X, Xiao B, Cai S, et al. Three-dimensional localization of serine 2808, a phosphorylation site in cardiac ryanodine receptor. *J Biol Chem* 2007;282:25929–25939. [PubMed: 17606610]
19. Liu N, Priori SG. Disruption of calcium homeostasis and arrhythmogenesis induced by mutations in the cardiac ryanodine receptor and calsequestrin. *Cardiovasc Res* 2008;77:293–301. [PubMed: 18006488]

Fig. 1.

CaM binding to the RyR2 in cardiac homogenates. **(A) (Top)** Representative immunoblots of RyR2-bound CaM-SANPAH (a photoreactive crosslinker; N-Succinimidyl-6- [4 $\text{\AA}$ '-azido-2 $\text{\AA}$ '-nitrophenylamino]). The WT and KI cardiac homogenates were reacted with various concentrations of CaM-SANPAH (32 – 1024 nmol/L) in the presence or absence of cAMP (1 μ mol/L) and okadaic acid (1 μ mol/L). The CaM binding to the RyR2 was determined by immunoblotting with the anti-CaM antibody to detect the RyR2-bound CaM (See Methods). **(Bottom)** Summarized data of the CaM-binding to the RyR2 as a function of the concentration of CaM-SANPAH. The CaM binding was expressed as the ratio to the maximum binding at 1024 nmol/L CaM. Data represent means $\pm$ SE of 4 hearts. **(B)** Effect of

cAMP on the phosphorylation level of Serine2808-RyR2 (P-Ser2808-RyR2). In the presence of cAMP (1 $\mu\text{mol/L}$) and okadaic acid (1 $\mu\text{mol/L}$), KI and WT cardiac homogenates were incubated in a sample solution containing 0.15 mol/L NaCl, 50 mmol/L MOPS, 0.1 % CHAPS, at PH 6.8 for 30 minutes at 22 °C, followed by Western blotting with anti- P-Ser2808-RyR2-antibody. No significant difference was seen in the level of the P-Ser2808-RyR2 between WT and R2474S/+ KI hearts. Data are means $\pm$ SE of 3 hearts. **C.** Effect of cAMP (1 $\mu\text{mol/L}$), DPc10 (10 $\mu\text{mol/L}$), the antibody against CaM binding domain (3583–3603), and/or dantrolene (1 $\mu\text{mol/L}$) on the CaM-binding to the RyR2. The concentration of CaM-SANPAH was fixed at 256 nmol/L.

Fig. 2. Localization and the binding characteristics of exogenously introduced CaM in saponin-permeabilized cardiomyocytes. **(A) (Left)** Representative images of exogenously introduced CaM, which is co-localized with the RyR2 in WT cardiomyocytes. The CaM (200 nmol/L), fluorescently labeled with Alexa Fluor 488 (Molecular Probes, OR), was introduced into the cardiomyocytes. Left; CaM-Alexa Fluor 488 (green); middle; immuno-staining of RyR2 (red); right; merged image. **(Right)** Periodical increases in the Alexa fluorescence signals of either RyR2 (red) or CaM (green). **(B)** Effect of cAMP (1 μmol/L), DPc10 (10 μmol/L) and/or dantrolene (1 μmol/L) on the Alexa-CaM (200 nmol/L) binding in WT and KI

cardiomyocytes. Representative images (left) and the summarized data (right). Data represent the means $\pm$ SE of 24 cells from 3-4 hearts.

Fig. 3. Localization and the binding characteristics of the endogenous CaM in intact cardiomyocytes. **(A) (Left)** Representative images of the endogenous CaM, which is co-localized with the RyR2 in WT cardiomyocytes. Left; immuno-staining of CaM (red); middle; immuno-staining of RyR2 (green); right; merged image. **(Right)** Periodical increases in the Alexa fluorescence signals of either CaM (red) or RyR (green). **(B)** Effect of isoproterenol (10 nmol/L) on the endogenous CaM bound to the RyR2 in WT and KI cardiomyocytes. Representative images (left) and the summarized data (right). Data represent the means $\pm$ SE of 10 cells from 3 hearts.

Fig. 4.

Effect of DPc10 (10 μmol/L), cAMP (1 μmol/L), and/or CaM (1 μmol/L) on Ca²⁺ sparks in saponin-permeabilized WT and KI cardiomyocytes. **(A)** Representative images of Ca²⁺ sparks. **(B)** Relationship between Ca²⁺ spark frequency and SR Ca²⁺ content. The intracellular [Ca²⁺] was buffered at 30 nmol/L in both KI and WT cardiomyocytes. The SR Ca²⁺ content was measured by the amount of Ca²⁺ discharged from the SR by 10 mmol/L caffeine. Data represent the means±SE of 24- 45 cells prepared from 3-4 hearts.