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Dissociation of FKBP12.6 from ryanodine receptor type 2 is regulated by cyclic ADP-ribose but not β-adrenergic stimulation in mouse cardiomyocytes

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Aims β-Adrenergic augmentation of Ca^{2+} sparks and cardiac contractility has been functionally linked to phosphorylation-dependent dissociation of FK506 binding protein 12.6 (FKBP12.6) regulatory proteins from ryanodine receptors subtype 2 (RYR2). We used FKBP12.6 null mice to test the extent to which the dissociation of FKBP12.6 affects Ca^{2+} sparks and mediates the inotropic action of isoproterenol (ISO), and to investigate the underlying mechanisms of cyclic ADP-ribose (cADPR) regulation of Ca^{2+} sparks.

Methods and results Ca^{2+} sparks and contractility were measured in cardiomyocytes and papillary muscle segments from FKBP12.6 null mice, and western blot analysis was carried out on sarcoplasmic reticulum microsomes prepared from mouse heart. Exposure to ISO resulted in a three- and two-fold increase in Ca^{2+} spark frequency in wild-type (WT) and FKBP12.6 knockout (KO) myocytes, respectively, and Ca^{2+} spark kinetics were also significantly altered in both types of cells. The effects of ISO on Ca^{2+} spark properties in KO cells were inhibited by pre-treatment with thapsigargin or phospholamban inhibitory antibody, 2D12. Moreover, twitch force magnitude and the rate of force development were not significantly different in papillary muscles from WT and KO mice. Unlike β-adrenergic stimulation, cADPR stimulation increased Ca^{2+} spark frequency (2.8-fold) and altered spark kinetics only in WT but not in KO mice. The effect of cADPR on spark properties was not entirely blocked by pre-treatment with thapsigargin or 2D12. In voltage-clamped cells, cADPR increased the peak Ca^{2+} of the spark without altering the decay time. We also noticed that basal Ca^{2+} spark properties in KO mice were markedly altered compared with those in WT mice.

Conclusion Our data demonstrate that dissociation of FKBP12.6 from the RYR2 complex does not play a significant role in β-adrenergic-stimulated Ca^{2+} release in heart cells, whereas this mechanism does underlie the action of cADPR.

1. Introduction

In cardiomyocytes, release of Ca^{2+} from intracellular Ca^{2+} stores to the cytoplasm is controlled by ryanodine receptors subtype 2 (RYR2). Activation and inactivation of RYR2 is regulated by a number of channel modulators, including FKBP12 and FKBP12.6,[1–4] calmodulin,[5] protein kinase A (PKA),[1,6,7] and Ca^{2+}/calmodulin-dependent kinase (CaMKII).[8–11] FKBP12.6, a 12.6 kDa protein, associates specifically with and regulates RYR2 in cardiomyocytes and smooth muscle.[12,13] Adult FKBP12.6 knockout (KO) mice develop abnormal cardiac function leading to either an enlarged heart[14] or exercise-induced cardiac arrhythmia and sudden death.[15] We demonstrated previously that FKBP12.6 KO mice exhibit a significant alteration in Ca^{2+} spark properties in both sexes.[13,14] These findings strongly suggest that FKBP12.6 is relevant for regulating cardiac function possibly via its association with cardiac RYR2.

It has been reported that PKA phosphorylation of RYR2 causes the dissociation of FKBP12.6 and affects the channel open probability, and hyperphosphorylation of

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RYR2 at a single amino acid residue, Ser-2809, leads to defective channel function in the failing heart.[16] Wehrens *et al.*[17] reported that disease-causing mutations in RYR2 reduce the binding affinity of FKBP12.6 for RYR2, and increase the channel leakage that can trigger fatal cardiac arrhythmias under physical and emotional stress. However, the theory proposed by Marks, Wehrens, and colleagues is challenged by other authors. Li *et al.*[18] demonstrated that PKA-mediated increase in Ca^{2+} spark frequency is due to phosphorylation of phospholyman (PLB) but not RYR2 in mouse ventricular myocytes. Jiang *et al.*[19] did not observe dissociation of FKBP12.6 from RYR2 in cardiac microsomal membranes treated with PKA. Stange *et al.*[20] made site-directed substitutions of RYR2 at Ser-2809 (with Asp or Ala) and showed that FKBP12.6 binding was not abolished nor were channel functional properties significantly changed in the mutant RYR2. Xiao *et al.*[7] found that FKBP12.6 binds to both the Ser-2809 (mouse) phosphorylated and non-phosphorylated forms of RYR2 and that a S2809D phosphomimetic mutant retains the ability to bind FKBP12.6. Furthermore, complete phosphorylation at Ser-2809 by exogenous PKA does not disrupt the FKBP12.6–RYR2 complex. An investigation of the three-dimensional location of the Ser 2030 and Ser 2809 PKA sites indicated that these PKA sites are not located close to the FKBP12.6-binding sites.[21,22] Thus the theory that PKA phosphorylation causes dissociation of FKBP12.6 proteins from the RYR2 complex in cardiomyocytes is highly controversial.

Cyclic ADP-ribose (cADPR), a metabolite of β-nicotinamide adenine dinucleotide, is a novel Ca^{2+} mobilizing second messenger. A number of reports over the last few years have suggested that cADPR has a patho-physiological role, as reviewed by Zhlang and Li.[23] It has been reported that cADPR, at micromolar concentrations, participates in the generation or modulation of intracellular Ca^{2+} sparks, Ca^{2+} waves or oscillations via RYRs,[24–26] despite the fact that the role and mechanisms of cADPR regulation of Ca^{2+} release remain unclear and very controversial. For instance, treatment of cardiomyocytes, smooth muscle, and pancreatic β-cells with cADPR increases Ca^{2+} spark frequency and Ca^{2+} release.[27–30] Growing evidence indicates that cADPR may function through its specific receptor protein FKBP12.6,[30,31] i.e. cADPR binds to FKBP12.6 and causes its dissociation from RYR2, resulting in the activation of RYR channels. However, some studies are inconsistent with the above observation. Copello *et al.*[32] reported that cADPR does not affect rabbit heart RYRs. A study on single channels indicates that cADPR does not activate single cardiac RYR channels in planar lipid bilayers.[33] Guo *et al.*[34] demonstrated that cADPR does not regulate sarcoplasmic reticulum (SR) Ca^{2+} release in intact cardiac myocytes at all. Although Lukyanenko *et al.*[27] reported that cADPR altered Ca^{2+} spark properties in rat cardiomyocytes, the underlying mechanism of cADPR modulation of SR Ca^{2+} release is via enhancement of Ca^{2+} uptake by SERCA instead of dissociation of the FKBP12.6 from RYR2.

We have used FKBP12.6 null mice to test the extent to which the dissociation of FKBP12.6 proteins mediates the Ca^{2+} spark and inotropic action of isoproterenol (ISO) and to investigate the underlying mechanism. Our results indicate that alteration of Ca^{2+} spark properties caused by PKA phosphorylation is not through dissociation of FKBP12.6 from the RYR2 complex and that cADPR alters Ca^{2+} spark properties by dissociating FKBP12.6 from the RYR2 complex.

2. Methods

2.1 Isolation of cardiomyocytes and electrophysiology

Single ventricular myocytes from adult mice of either sex were prepared as described previously.[14] Detailed procedures are described in Supplementary material online, Methods. All animal procedures described in this study were performed in adherence with 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), and with approval from the Institute of Biophysics Committee on Animal Care.

To test whether cADPR alters Ca^{2+} spark properties by enhancing SR Ca^{2+} ATPase activity and to dialyze cADPR or 2D12, cells were sealed and membranes were ruptured using a whole cell patch-clamp configuration (for detailed information see Supplementary material online).

2.2 Measurement of Ca^{2+} fluorescence

Single myocytes were incubated with 10 μM Fluo-4 a.m. (Molecular Probes) and Fluo-4 fluorescence was recorded using a laser scanning confocal head (BioRad Laboratories) attached to an inverted microscope (TE-300; Nikon; for detailed information see Supplementary material online).

2.3 Force measurement

The technique to measure force in isolated papillary muscles were similar to those previously described.[35] To eliminate the possibility that cardiac hypertrophy may have confounding effects on the force data measurements female (C57Bl/6 and FKBP12.6 KO) mice 10–15 weeks were used.[14] Muscles were slowly stretched until Lmax was obtained and stimulated at 4 Hz. The force was measured with an isometric force transducer (Radnoti, Monrovia, CA, USA) and data acquisition was carried out using Chart 3.4 (Adinstruments, Australia) installed on a microcomputer (for detailed information see Supplementary material online, Methods).

2.4 SR preparation and western blot analysis

SR microsomes from mouse heart were prepared[36] and western blot analysis was performed as previously described.[32] The detailed for this part, see Supplementary material online, Methods.

2.5 Data analysis

Image processing and data analyses were carried out using custom software written in MATLAB. Results were expressed as mean ± SEM. Significant differences were determined using the Student's *t* test. Data from three groups were compared by one-way, repeated measures ANOVA and significant differences between groups were determined by the Student–Newman–Keuls test for paired comparisons. Analysis and graphs for force measurement were completed with SigmaStat 3.0 and SigmaPlot 2000 (SPSS). Statistical significance ($P \leq 0.05$) was determined within groups before and after the addition of ISO using the Wilcoxon signed rank test while between groups the Mann–Whitney rank sum test was utilized.

3. Results

3.1 β-Adrenergic stimulation regulates Ca^{2+} sparks in FKBP12.6-deficient cardiomyocytes

Figure 1A shows confocal microscopy line-scan images obtained from wild-type (WT) mouse cardiomyocytes.

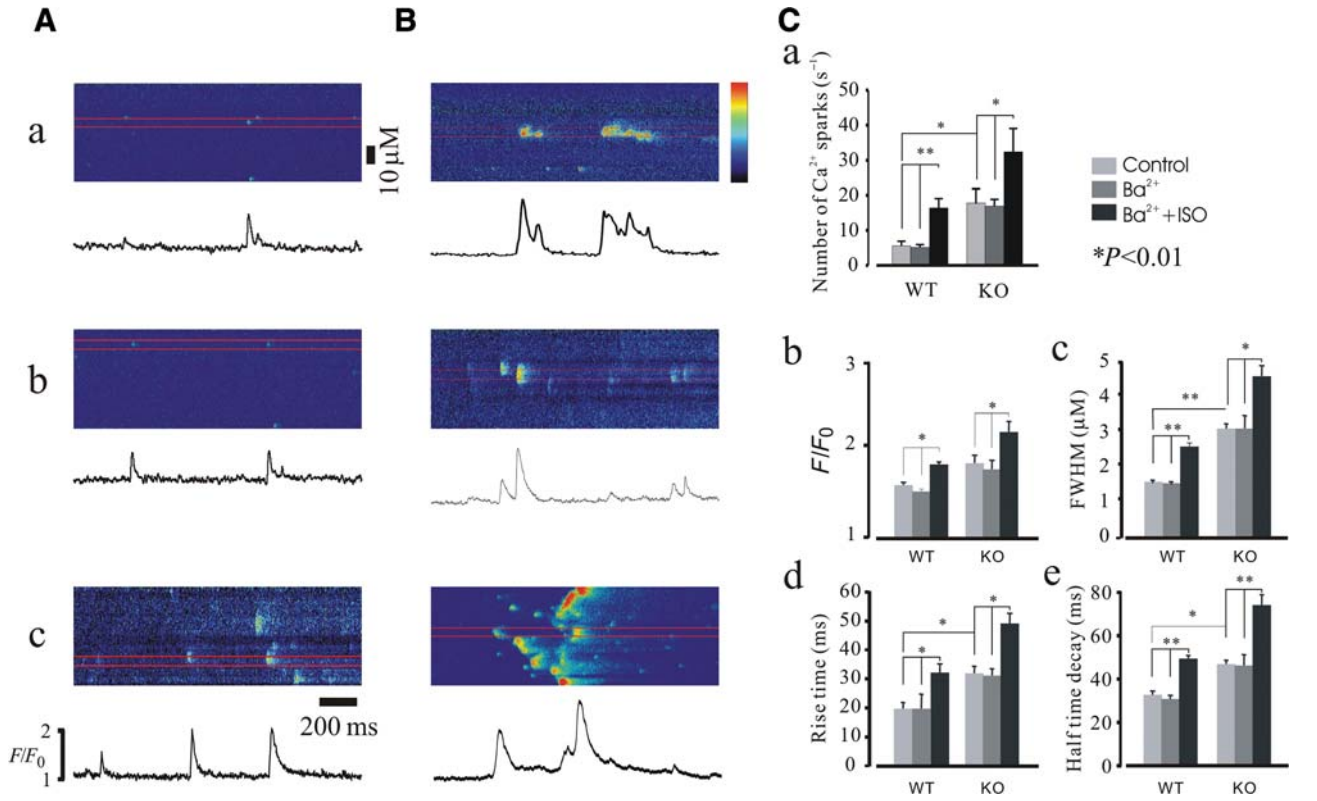

Figure 1 Isoproterenol altered Ca^{2+} spark properties. Confocal line-scan images showing spontaneous Ca^{2+} sparks collected in WT (*A*) and KO (*B*) cells. (*a*) normal control experiment; (*b*) Ba^{2+} extracellular solution control experiment; (*c*) in the presence of 1 μM ISO. It should be noted that the Ca^{2+} spark characteristics were already elevated under control conditions in KO myocytes compared with those of WT cells. (*C*) Summary data for properties of Ca^{2+} sparks. Frequency (*Ca*), F/F_0 (*Cb*), FWHM (*Cc*), RT (*Cd*), and half-time decay were dramatically altered by ISO in both WT and FKBP12.6 KO cardiomyocytes. The profiles (Ca^{2+} fluorescence ratio) shown below images were taken from the areas of the images indicated by red lines. $^*P < 0.05$ and $^{**}P < 0.01$.

When compared with controls (*Figure 1Aa* and *b*), addition of 1 μM ISO to bath solution increased the frequency of Ca^{2+} sparks by about three-fold [from 5.8 ± 2.57 to 17.2 ± 3.86 per 100 μm per second, $n = 286$ (sparks), $P < 0.05$]. The kinetics of Ca^{2+} sparks was also significantly altered by the addition of ISO. Ca^{2+} fluorescence ratio (F/F_0) was 1.52 ± 0.06 in controls and 1.71 ± 0.11 in the ISO group (*Figure 1Cb*, $n = 211$, $P < 0.05$). The full width at half maximum (FWHM) of Ca^{2+} sparks increased about 1.7-fold compared with controls (from 1.53 ± 0.24 μm to 2.51 ± 0.32 μm; *Figure1Cc*, $n = 211$, $P < 0.05$). The rise time (RT) and half-time decay were prolonged 1.65-fold (from 19.8 ± 3.67 ms to 32.6 ± 5.12 ms) and 1.5-fold (from 33.4 ± 3.34 ms to 48.7 ± 3.1 ms), respectively (*Figure 1Cd* and *e*, $n = 211$, $P < 0.01$). *Figure 1B* demonstrates results obtained from KO cardiomyocytes. ISO increased Ca^{2+} spark frequency about two-fold [to 32.6 ± 6.14 per 100 μm per second (*Figure 1Ca*, $n = 226$, $P < 0.05$) from 17.5 ± 3.54 per 100 μm per second, control]. F/F_0 was 1.72 ± 0.23 in controls and 2.21 ± 0.25 in the ISO group (*Figure 1Cb*, $n = 226$, $P < 0.05$). FWHM was increased about 1.5-fold compared with controls (from 3.1 ± 0.26 μm to 4.6 ± 0.25 μm; *Figure 1Cc*, $n = 226$, $P < 0.05$). RT and half-time decay of Ca^{2+} sparks were prolonged 1.7-fold (from 31.4 ± 3.6 ms to 49.3 ± 5.6 ms and from 46.6 ± 6.4 ms to 75.2 ± 6.8 ms, respectively; *Figure 1Cd* and *e*, $n = 226$, $P < 0.01$). These results indicate that Ca^{2+} spark properties were significantly altered by β-adrenergic stimulation in both WT and KO cardiomyocytes and suggest that modulation of Ca^{2+} release by ISO is not only via dissociation of FKBP12.6 from RYR2 but may also involve other mechanisms.

It is notable that the basal Ca^{2+} spark properties in KO cells are similar to those of control cells treated with ISO.[13,14] The effects of ISO on Ca^{2+} spark properties were also significantly different between WT and KO cells.

A further series of experiments was performed in KO cells to investigate the mechanism of Ca^{2+} spark property alteration induced by β-adrenergic stimulation in more detail. First, we sought to examine the effect of the SERCA inhibitor, thapsigargin, on the alteration of Ca^{2+} spark properties induced by ISO. As shown in *Figure 2*, as expected, thapsigargin alone exhibited an inhibitory effect on Ca^{2+} spark properties. In the presence of thapsigargin, the stimulatory effects of ISO on Ca^{2+} sparks were significantly reduced compared with the control (without thapsigargin).

Our next series of experiments was conducted to further probe the molecular mechanism of ISO augmentation of Ca^{2+} spark properties in KO mice. It is known that the PLB monoclonal antibody, 2D12, reverses the inhibitory effect of PLB on SERCA in cardiomyocytes.[36] So, we hypothesized that if β-adrenergic stimulation augments Ca^{2+} sparks via PKA phosphorylation of PLB as indicated by the above experiments, then ISO should not enhance Ca^{2+} sparks in KO cardiomyocytes in the presence of 2D12. After dialysis of cells with 2D12 (5 μm, through pipettes) for 15 min, Ca^{2+} spark frequency was increased 1.7-fold from 16.32 ± 2.6 (control) to 27.7 ± 3.2 (*Figure 3A* and *Ca*, $n = 257$, $P < 0.01$). The kinetics of Ca^{2+} sparks were also altered by 2D12, with the exception of peak Ca^{2+}. However, in the

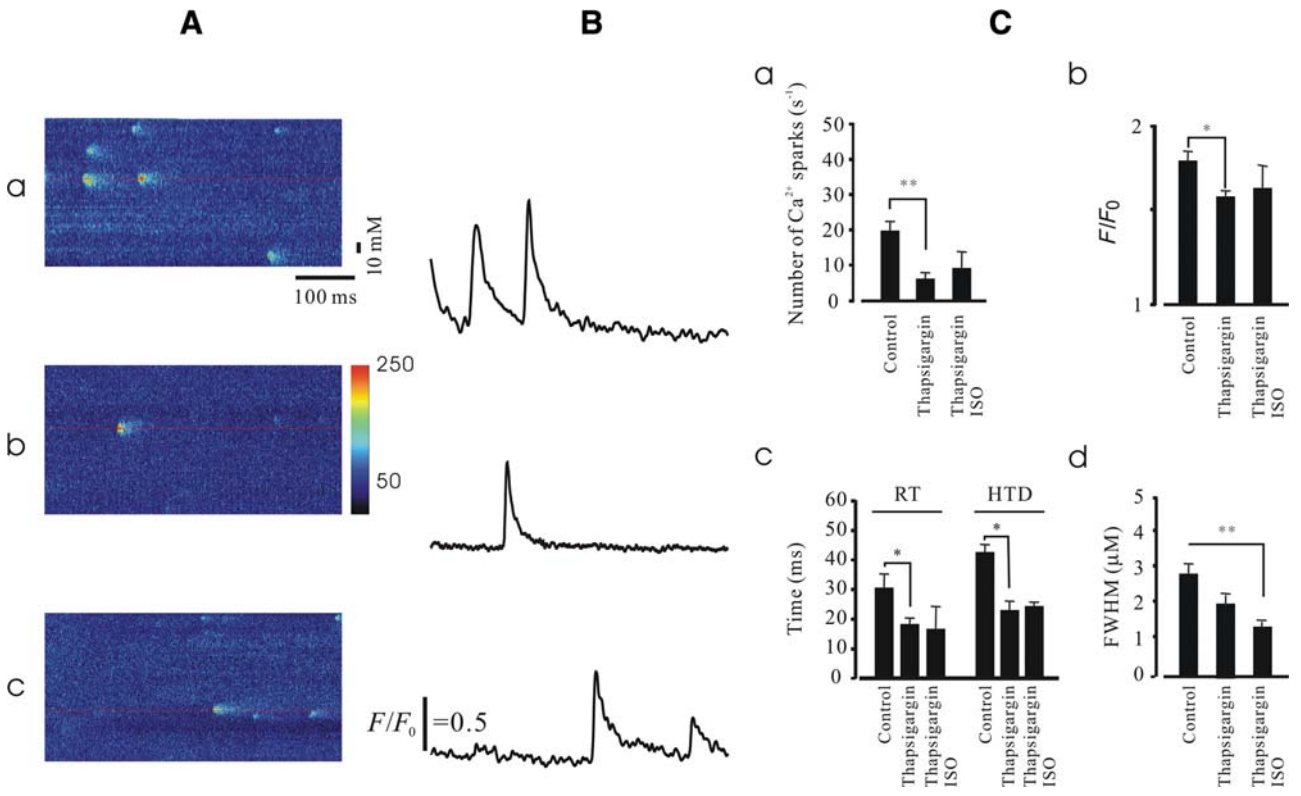

Figure 2 Blockage of the Ca^{2+} pump altered ISO-induced Ca^{2+} spark properties. The experiments were carried out in FKBP12.6 KO intact myocytes. (*A*) Representative line-scan images obtained from controls (*a*), in the presence of thapsigargin (10 μm, *b*), and in the presence of thapsigargin and ISO (*c*). (*B*) Profiles of relative Ca^{2+} fluorescence intensity taken from images (A) as indicated by red lines. (*C*) Summary data of Ca^{2+} spark properties. Note that the increased Ca^{2+} spark frequency (*a*), peak Ca^{2+} (*b*), RT/half time decay (*c*), and size (*d*) induced by ISO were significantly reduced by the application of thapsigargin ($n = 126$, $^*P < 0.05$ and $^{**}P < 0.01$).

presence of 2D12, ISO, as expected, could not increase Ca^{2+} spark frequency further compared with the control (*Figure 3Ac* and *Ca*, $P > 0.05$). Similarly, in the presence of 2D12 ISO also failed to change Ca^{2+} spark kinetics (*Figure 3C*).

Taken together, the alteration of Ca^{2+} spark properties via β-adrenergic stimulation by ISO in mouse cardiomyocytes occurred via phosphorylation of PLB, and probably not through dissociation of FKBP12.6.

3.2 Normal β-adrenergic stimulation of cardiac contraction in FKBP12.6 null cardiomyocytes

β-Adrenergic augmentation of cardiac contractility has been functionally linked to the phosphorylation-dependent dissociation of FKBP12.6 regulatory proteins from RYR2. If the disengagement of FKBP12.6 proteins from the RYR2 complex plays a significant role in inotropy, β-adrenergic stimulation would be expected to be less robust in hearts lacking FKBP12.6. Left ventricular papillary muscles from eight WT and eight FKBP12.6 KO female mice were stimulated at 4 Hz to assess cardiac contractility before and during β-adrenergic stimulation with ISO (1 μM). Baseline force was normalized to cross-sectional area, which was $0.36 \pm 0.05\ mm^2$ for WT and $0.33 \pm 0.07\ mm^2$ for FKBP12.6 KO mice (no significant difference, *Figure 4A* and *B*). Although the average magnitude of the twitch contraction ($1.2 \pm 0.2\ mN/mm^2$ vs. $0.8 \pm 0.1\ mN/mm^2$) and the rate of force development ($32 \pm 6\ mN/mm^2/s$ vs. $24 \pm 2\ mN/mm^2/s$) was larger in WT vs. KO mice, the differences were not significant (*Figure 4*, also see *Table 1*). Importantly, ISO stimulation increased the magnitude (45% vs. 43%) and rate of stress development (47% vs. 47%) to an equivalent degree in WT and KO mice (*Table 1*). Similarly, twitch relaxation time was increased to an equivalent degree in both genotypes (22% vs. 23%). These data demonstrate that β-adrenergic stimulation of contractility does not require dissociation of FKBP12.6 proteins, and that other mechanisms likely predominate in the inotropic effect.

3.3 cADPR altered Ca^{2+} spark properties only in WT cardiomyocytes

It is known that FKBP12.6 selectively associates with cardiac RYR2,[12] thus by using the FKBP12.6 KO mouse model we were able to examine the role of cADPR in both WT and FKBP12.6 KO cardiomyocytes in order to investigate the mechanism of cADPR regulation of Ca^{2+} release channels. Because the action of cADPR is temperature-dependent,[37] we carried out the experiments at 36°C. cADPR (1 μM) was dialyzed via patch pipettes in cells from both WT and KO mice. Representative line-scan images from WT myocytes are documented in *Figure 5A*. cADPR caused a 2.8-fold increase in Ca^{2+} spark frequency from 6.6 ± 1.4 per 100 μm per second (control) to 18.2 ± 2.6 per 100 μm per second (cADPR, $n = 168$, $P < 0.01$); cADPR increased F/F_0 1.2-fold from 1.51 ± 0.02 to 1.83 ± 0.08 ($n = 168$, $P < 0.05$); FWHM was 2.1 ± 0.08 μm in controls and 3.2 ± 0.14 μm in the cADPR group ($n = 168$, $P < 0.05$, *Figure 5Cd*); RT and half time decay of Ca^{2+} sparks were

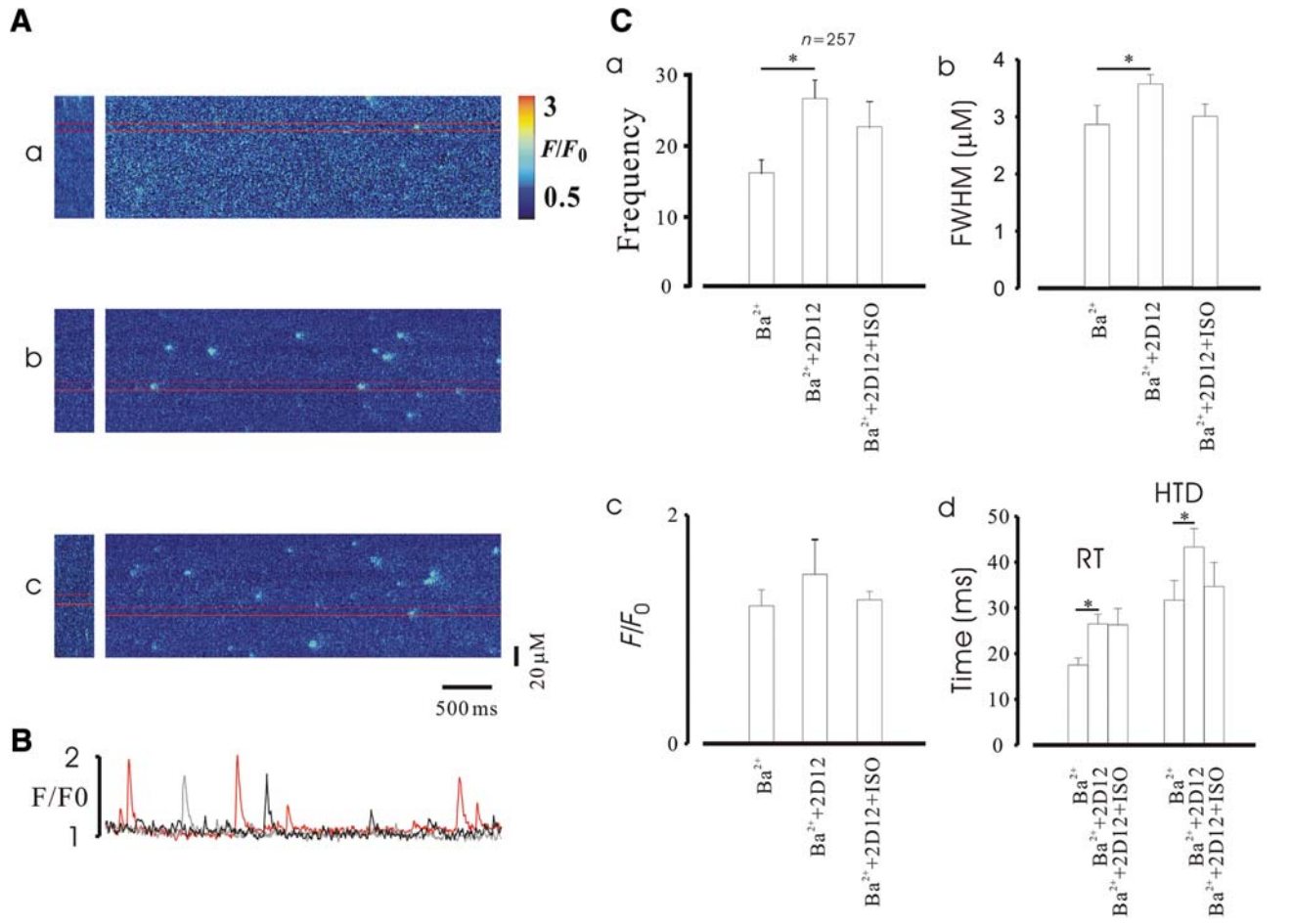

Figure 3 Phospholamban antibodies abrogated Ca^{2+} spark alteration caused by ISO in FKBP12.6 KO cardiomyocytes. (*A*) Representative line-scan images recorded under different conditions. (*Aa*) Image obtained in Ba^{2+} extracellular solution; (*Ab*) 2D12 altered Ca^{2+} spark properties significantly; (*Ac*) ISO failed to alter Ca^{2+} spark properties further in the presence of 2D12. (*B*) Profiles taken from the line-scan images (above) as indicated by gray (control), black (2D12), and red lines (2D12 + ISO). (*C*) Summary data for Ca^{2+} spark properties.

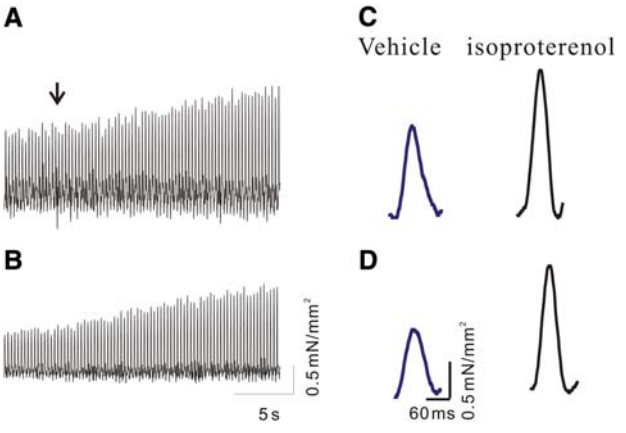

Figure 4 β-Adrenergic stimulation of cardiac contraction. (*A* and *B*) 20 s traces demonstrating force development during β-adrenergic stimulation in (A) WT and (B) FKBP12.6 KO mice. Notice in both WT and FKBP12.6 KO mice, the amplitude of the contraction nearly doubles from baseline to peak during ISO stimulation. (*C* and *D*) Individual representative contractions from WT (C) and FKBP12.6 KO mice (D) before (blue) and during (black) β-adrenergic stimulation with ISO (1 mM).

22.3 ± 2.1 ms vs. 38.1 ± 2.4 ms ($n = 168$, $P < 0.05$) and 36.4 ± 4.3 ms vs. 56.3 ± 3.8 ms (*Figure 5Ce* and *f*, $n = 168$, $P < 0.01$), respectively. As shown in *Figure 5B* and *C*, dialysis of KO cells with cADPR did not alter the Ca^{2+} spark properties significantly.

To determine whether the alteration of Ca^{2+} spark properties by cADPR is via enhancing the effect of SERCA as previously reported.[27] We tested the effect of cADPR on Ca^{2+} spark properties in the presence of thapsigargin in WT cells. As shown in *Figure 6*, spark characteristics were not markedly changed by cADPR in the presence of thapsigargin (10 μM) except for half time decay, as was also observed in the experiments described above. However, it should be noted that these parameters were altered by cADPR though differences were not statistically significant. We further examined the effects of cADPR on Ca^{2+} sparks in the presence of 2D12, and a similar result as that observed in thapsigargin experiments was obtained except for half time decay which was not significantly altered (data not shown) suggesting that the alteration of Ca^{2+} spark properties induced by cADPR was possibly not via action on the SR Ca^{2+} pump.

To test whether the increases in Ca^{2+} spark frequency/amplitude induced by cADPR were by enhancing SR Ca^{2+} ATPase activity, we analysed Ca^{2+} transient decay kinetics in voltage-clamped myocytes from WT mice. As shown in *Figure 7A*, cADPR increased peak Ca^{2+} from 1.71 ± 0.64 to 2.16 ± 0.16 [$P < 0.05$, $n = 189/12$ (sparks/cells)] by depolarizing membrane potential to 0 mV from a holding

Table 1 Isolated left ventricular anterior papillary muscle parameters in WT and FKBP12.6 KO mice

	$FKBP12.6^{+/+}$ Control	ISO	$FKBP12.6^{-/-}$ Control	ISO
Force (mN/mm^2)	1.2 ± 0.2	2.1 ± 0.2*	0.8 ± 0.1	1.5 ± 0.2*
% Change	45		43	
dF/dT_{max}	32 ± 6	63 ± 7*	24 ± 2	47 ± 5*
% Change	47		47	
Tau (ms)	36 ± 3	27 ± 2*	32 ± 2	24 ± 2*
% Change	22		23	

Force was normalized to cross-sectional area (mN/mm^2). Baseline was determined by averaging a total of 100 ms, 75 ms before, and 25 ms after the stimulus artefact. Peak amplitude (mN/mm^2) was baseline to maximal force development. dF/dT_{max} was the maximal rate of force development ($mN/mm^2/s$) during contraction. The time constant of decay, tau, was determined as the time to reach 37% of initial (maximal) value. Three to five consecutive contractions were averaged to obtain values of a representative contraction. Isoproterenol was added to a final concentration of 1 mM and values calculated at peak response. A total of eight experiments were performed for each group. Values are mean $\pm$ SE.

*$P \leq 0.02$ within a group. There was no statistical significance between groups.

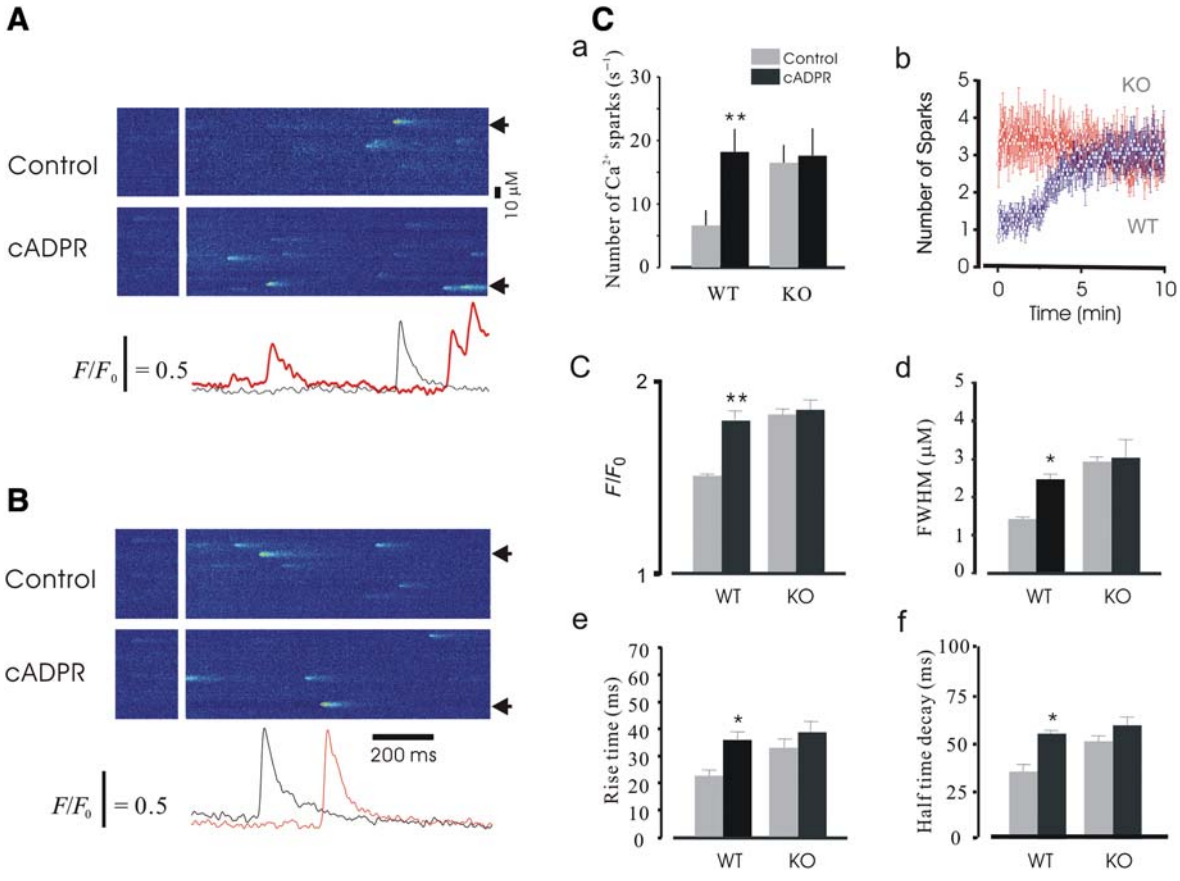

Figure 5 cADPR altered the properties of Ca^{2+} sparks in WT cardiomyocytes. Confocal line-scan images showing spontaneous Ca^{2+} sparks collected from cardiomyocytes. (*A*) Representative images obtained from a WT cell. cADPR significantly changed the properties of Ca^{2+} sparks. The locations from where profiles were taken are indicated by arrows at the bottom of the images. (*B*) Representative line-scan images obtained from a KO cardiomyocyte. It is noteworthy that Ca^{2+} spark properties were not significantly altered by cADPR. (*C*) Summary data for Ca^{2+} spark properties. cADPR greatly increased Ca^{2+} spark frequency (*a*, $P < 0.01$, $n = 168$), and the increase in the frequency of Ca^{2+} sparks occurred after dialysis of cADPR for 3 min and reached a maximum at ~7 min (*b*); peak Ca^{2+} (*c*, $P < 0.01$, $n = 168$), size (*d*, $P < 0.05$, $n = 168$), RT (*e*, $P < 0.01$, $n = 168$), and half-time decay ($P < 0.01$, $n = 168$) of Ca^{2+} sparks was also significantly altered by dialysis of cADPR in WT cardiomyocytes.

potential of −40 mV (100 ms duration), but RT and half-time decay were not significantly altered by cADPR in these voltage-clamped cells.

We also compared the effect of cADPR on SR Ca^{2+} load by measuring caffeine-induced Ca^{2+} release. 10 mM caffeine was puffed close to the cell for 200 ms using a pipette. As shown in *Figure 7C*, cADPR significantly reduced Ca^{2+} content (by 36.2%, $n = 13$) in WT cardiomyocytes. In contrast, there was no significant alteration of Ca^{2+} content observed in KO cells ($n = 16$). Although statistically there was no significant difference in SR Ca^{2+} content between WT and KO cells, the content of SR Ca^{2+} in KO cells was lower to some extent compared with that in WT cells, suggesting the existence of Ca^{2+} leakage due to the

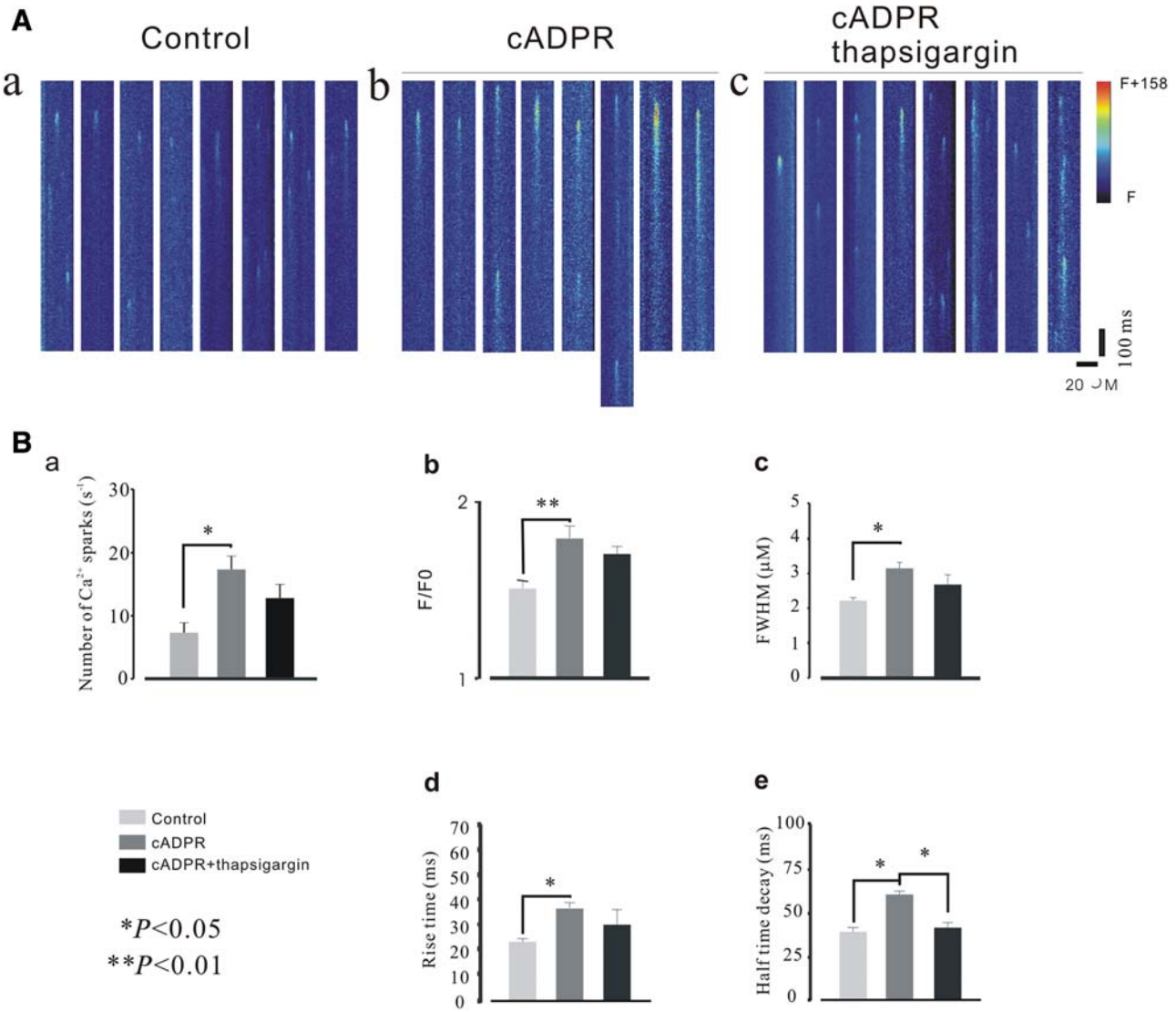

Figure 6 Effect of cADPR on Ca^{2+} spark properties in the presence of SERCA inhibitor. Spontaneous Ca^{2+} sparks were collected in a WT cell. (*A*) A representative line-scan image. (*Aa*) Control; (*Ab*) cADPR; (*Ac*) thapisgargin (10 μm)+ADPR. (*B*) Summary of Ca^{2+} spark properties. Except for half-time decay (*e*), Ca^{2+} spark frequency (*a*), peak Ca^{2+} (*b*), size (*c*), and RT (*d*) induced by dialysis of cADPR decreased, but were not abolished in the presence of thapsigargin. The number of Ca^{2+} sparks (*n*) was 186; * indicates $P < 0.05$, and ** $P < 0.01$, respectively.

deletion of the FKBP12.6.[16] Analysis of Ca^{2+} transient decay kinetics showed that there was no significant difference before or after application of cADPR.

Taken together, our results show that cADPR altered Ca^{2+} spark properties by causing dissociation of FKBP12.6 from RYR2 binding sites.

3.4 cADPR dissociates FKBP12.6 from SR

To further confirm that the effect of cADPR on Ca^{2+} spark properties is dependent on the dissociation of FKBP12.6 from SR, western blotting analysis was performed on SR fractions. As shown in *Figure 8*, endogenous FKBP12.6 was released from the SR fraction by incubation with cADPR, demonstrating that practically all of the FKBP12.6 was bound to the SR fraction in the pellet while no detectable 12.6 kDa protein band was observed in the supernatant.

By addition of FK506 almost all the FKBP12.6 was released from the SR fraction into the supernatant. A corresponding FKBP12.6 band was not detected in the pellet containing the SR fraction. Similar results were also observed after incubation of the SR fraction with cADPR. Most of the FKBP12.6 appeared in the supernatant and little remained associated with the SR fraction in the pellet. No obvious band was detected in the supernatant when the SR membrane was pre-treated with 8-Br-cADPR, a cADPR antagonist. These results suggest that cADPR significantly causes dissociation of FKBP12.6 from SR.

4. Discussion

Dissociation of FKBP12.6 protein from RYR2 caused by PKA phosphorylation is considered the leading mechanism underlying Ca^{2+} leakage in the failing heart.[16] However, there is increasing evidence to challenge this mechanism.[6,7,18–20,38] In the present study, we have used FKBP12.6 null mice to directly test the extent to which the dissociation of FKBP12.6 proteins mediates the Ca^{2+} sparks and inotropic action of ISO in cardiomyocytes. The present study shows that β-adrenergic stimulation increases Ca^{2+} spark frequency

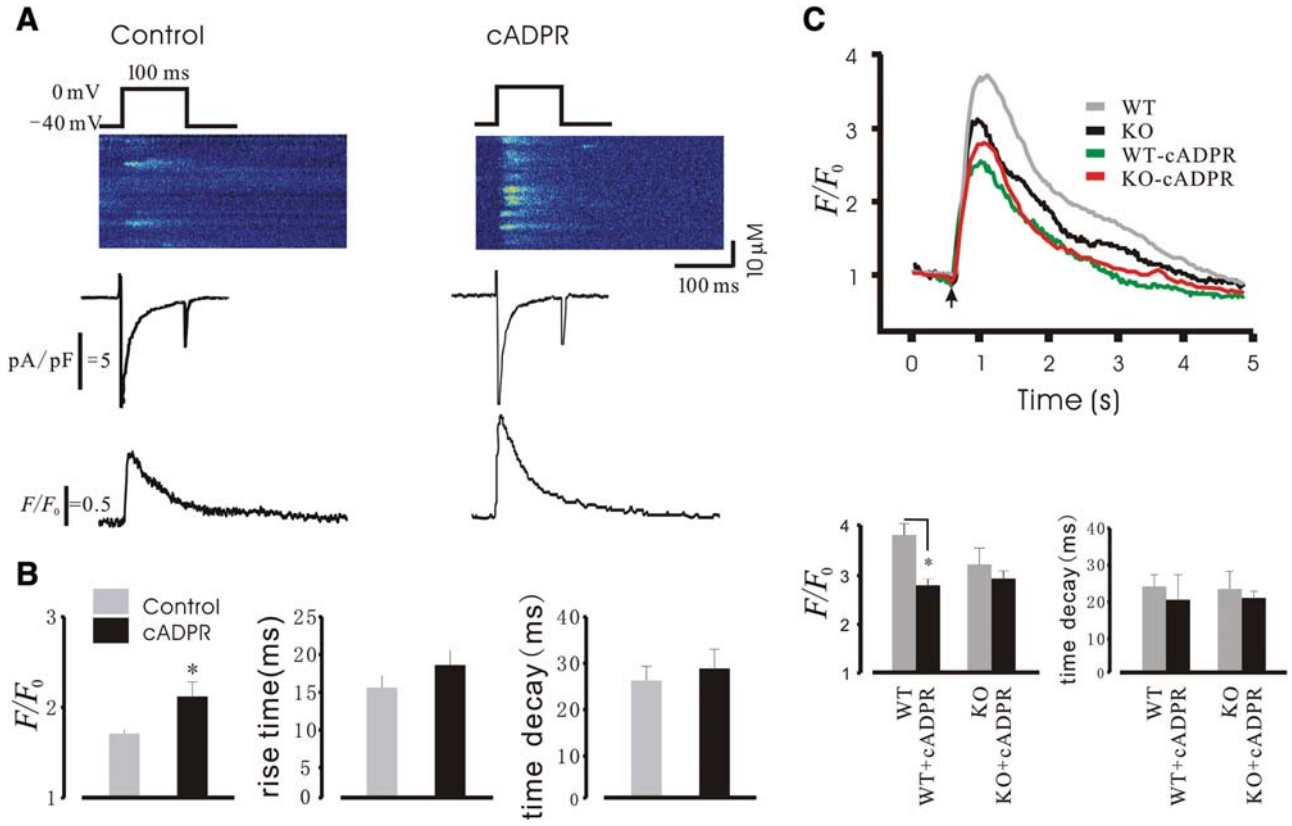

Figure 7 Effect of cADPR on voltage clamping-induced Ca^{2+} sparks and caffeine-induced Ca^{2+} release. (*A*) Sample of line-scan images collected in voltage-clamped cells from WT mice in the absence (left) and presence of cADPR (right). The protocol used to trigger Ca^{2+} sparks is shown above the images, whereas the currents (upper) and peak Ca^{2+} (lower) are shown below the images. (*B*) Summary data of Ca^{2+} spark properties induced by membrane depolarization. (*C*) Representative tracing of caffeine-induced Ca^{2+} transients (top panel). When compared with the control (gray), the Ca^{2+} transient was significantly reduced by cADPR (green, $n = 7$, $P < 0.05$); in contrast, cADPR did not reduce Ca^{2+} transients in FKBP12.6 KO cells (red). However, it is notable that the Ca^{2+} content was lower in FKBP12.6 null cells (black) compared with that in WT cells though not statistically significant ($n = 7$, $P = 0.06312$). The bottom panel shows the comparison of average peak values (left) and the time decay (right) of caffeine-induced Ca^{2+} transients or SR calcium load.

and significantly alters the kinetics of Ca^{2+} sparks in KO cardiomyocytes (*Figure 1*), suggesting that Ca^{2+} release from RYR2 does not occur through PKA phosphorylation-induced dissociation of FKBP12.6 from the RYR2 complex. This was further confirmed by the fact that ISO stimulation increased the magnitude of contraction, accelerated the rate of stress development and increased twitch relaxation time of left ventricular papillary muscles from FKBP12.6 KO mice (*Figure 4*). Thus, results from the present and earlier studies[6,7,16,19–21] do not support the hypothesis that phosphorylation of RYR2 by PKA causes dissociation of FKBP12.6 from the RYR2 complex and is followed by Ca^{2+} leakage.[39] Our later experiments showed that alteration of Ca^{2+} spark properties induced by ISO was prevented in the presence of thapsigargin, and a PLB antibody (*Figures 2* and *3*), suggesting that the modulation of Ca^{2+} release induced by PKA phosphorylation was caused by affecting PLB and the function of SERCA.[18,40] Taken together, our data do not support the theory that phosphorylation of RYR2 by PKA causes dissociation of FKBP12.6 from the RYR2 complex resulting in Ca^{2+} leakage.

cADPR is capable of inducing Ca^{2+} release from the SR by activation of RYRs in different tissues and cells.[27–30,41] There is growing evidence showing that cADPR induces Ca^{2+} release by causing the dissociation of FKBP12.6 from RYRs in pancreatic β-cells, smooth muscle and vascular endothelial cells,[20,30] however, that cADPR mediates Ca^{2+} release in cardiac myocytes is highly controversial. Prakash *et al.*[26] reported that cADPR induces Ca^{2+} release from the SR both through RYR channels and *via* a mechanism independent of RYR channels, and Lukyanenko *et al.*[27] have demonstrated that cADPR enhancement of Ca^{2+} sparks is mediated indirectly by increased accumulation of Ca^{2+} in the SR and subsequent luminal Ca^{2+}-dependent activation of RYRs. Here we demonstrated that cADPR altered Ca^{2+} spark properties in WT cardiomyocytes (*Figure 5A*) but not in FKBP12.6 null cells (*Figure 5B*), and that this effect of cADPR on Ca^{2+} spark frequency, amplitude, RT as well as FWHM was not significantly affected by either thapsigargin (*Figure 6*) or the PLB antibody, 2D12 (data not shown), suggesting that the effect of cADPR on Ca^{2+} spark properties in mouse cardiomyocytes is related to the dissociation of FKBP12.6 proteins from the RYR2 complex but not to PLB phosphorylation. One might argue that the increase in Ca^{2+} spark frequency/amplitude by cADPR may be by enhancing SR Ca^{2+} ATPase activity. However, experiments conducted in voltage-clamped myocytes demonstrated that cADPR increased peak Ca^{2+} without affecting RT and half-time decay of sparks (*Figure 7A*), suggesting that the effect of cADPR on Ca^{2+} spark properties was not mediated by affecting SR Ca^{2+} ATPase activity.

Indeed, this was further confirmed by experiments investigating FKBP12.6 release by cADPR (*Figure 8*) and by our recent

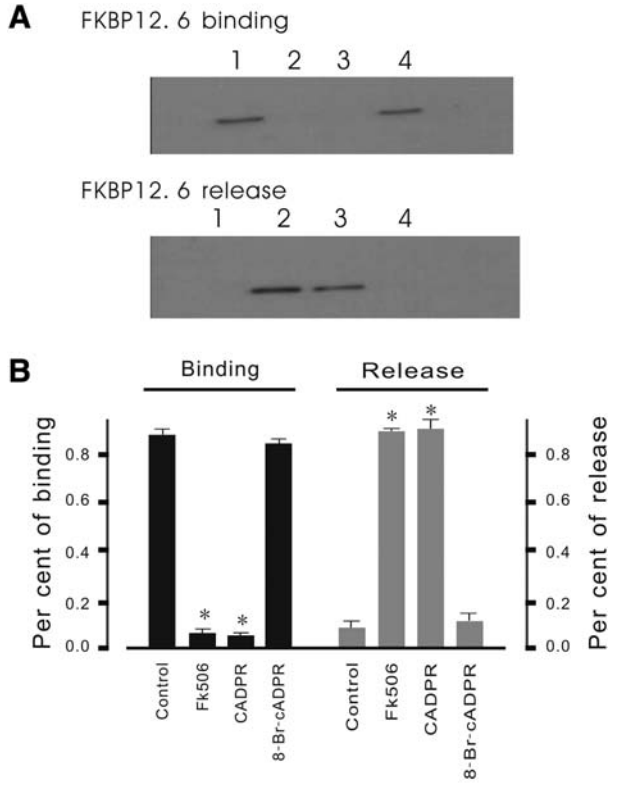

Figure 8 cADPR releases FKBP12.6 from mouse cardiac SR. (*A*) Western blotting analysis of FKBP12.6 binding and release. SR was incubated for 30 min at 37°C in control conditions or in the presence of cADPR, 8-Br-cADPR, or FK506. Released FKBP12.6 proteins appear in the supernatant (lower panel, lanes 1–4 are the control, FK506, cADPR, and 8-Br-cADPR, respectively) whereas bound FKBP12.6 remained in the pellets in control conditions and in the presence of 8-Br-cADPR (upper panel, lanes 1–4 are the control, FK506, cADPR, and 8-Br-cADPR, respectively). FK506 released almost all of the FKBP12.6 from the SR fraction. Similar results were obtained with cADPR though amounts of dissociated FKBP12.6 found in the supernatants were lower. Here, 8-Br-cADPR was used as a negative control. (*B*) Summary data from densitometry analysis. These results are from three independent experiments. The molecular weight of this protein is ~12 kDa. $^*P < 0.01$.

experiments that JTV-519, an RYR2 complex stabilizer, completely abrogated the alteration of Ca^{2+} spark properties induced by cADPR (data not shown) as well as by the observations that cADPR can directly bind to FKBP12.6 in islet microsomes[31] and that FKBP12.6 colocalizes with RYRs.[32] Further studies demonstrated that Ca^{2+} influx significantly decreased colocalization, and that this colocalization was reversed by 8-Br-cADPR, suggesting cADPR involvement in the dissociation of FKBP12.6 from RYRs.[31] Although it has been reported that the loss of FKBP12.6 has no significant effect on the conduction and activation of RYR2 or the propensity for spontaneous Ca^{2+} release,[42] the majority of studies have confirmed that FKBP12.6 is a RYR2 regulatory protein which closely associates with and regulates RYR2 Ca^{2+} release channels, and that the dissociation or deletion of FKBP12.6 from the RYR2 complex causes significant alteration in Ca^{2+} spark properties.[13–15,29,30,43,44]

In summary, our data demonstrate that (i) β-adrenergic stimulation of Ca^{2+} release and contractility does not require dissociation of FKBP12.6 from the RYR2 complex and (ii) cADPR alters Ca^{2+} spark properties in mouse cardiomyocytes by causing dissociation of FKBP12.6 proteins from the RYR2 complex.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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