Removal of FKBP12.6 Does not Alter the Conductance and Activation of Cardiac Ryanodine Receptor and the Susceptibility to Stress-induced Ventricular Arrhythmias

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Running Title: Role of FKBP12.6 in RyR2 function and cardiac arrhythmia

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The 12.6 kDa FK506 binding protein (FKBP12.6) is considered to be a key regulator of the cardiac ryanodine receptor (RyR2), but its precise role in RyR2 function is complex and controversial. In the present study, we investigated the impact of FKBP12.6-removal on the properties of the RyR2 channel and the propensity for spontaneous Ca²⁺ release and the occurrence of ventricular arrhythmias. Single channel recordings in lipid bilayers showed that FK506-treatment of recombinant RyR2 co-expressed with or without FKBP12.6 or native canine RyR2 did not induce long-lived subconductance states. [³H]ryanodine binding studies revealed that co-expression with or without FKBP12.6 or treatment with or without FK506 did not alter the sensitivity of RyR2 to activation by Ca²⁺ or caffeine. Furthermore, single cell Ca²⁺ imaging analyses demonstrated that HEK293 cells co-expressing RyR2 and FKBP12.6 or expressing RyR2 alone displayed the same propensity for spontaneous Ca²⁺ release or store overload-induced Ca²⁺ release (SOICR). FK506 increased the amplitude and decreased the frequency of SOICR in HEK293 cells expressing RyR2 with or without FKBP12.6, indicating that the action of FK506 on SOICR is independent of FKBP12.6. As with recombinant RyR2, the conductance and ligand gating properties of single RyR2 channels from FKBP12.6-null mice were indistinguishable from those of single wild type channels. Moreover, FKBP12.6-null mice did not exhibit enhanced susceptibility to stress-induced ventricular arrhythmias, in contrast to previous reports (Wehren et al., Cell, 113, 829, 2003). Collectively, our results demonstrate that the loss of FKBP12.6 has no significant effect on the conduction and activation of RyR2 or the propensity for spontaneous Ca²⁺ release and stress-induced ventricular arrhythmias.

The cardiac Ca²⁺ release channel (ryanodine receptor, RyR2) governs the release of Ca²⁺ from the sarcoplasmic reticulum (SR) and is essential for excitation-contraction (EC) coupling in cardiac muscle (1). Naturally-occurring mutations in RyR2 have been linked to several forms of cardiac arrhythmias, including catecholaminergic polymorphic or bidirectional ventricular tachycardia, catecholaminergic idiopathic ventricular fibrillation, and arrhythmogenic right ventricular dysplasia type 2 (2-4). In addition to its involvement in inherited cardiac arrhythmias, defective RyR2 function has also been implicated in other cardiac abnormalities, including atrial fibrillation and heart failure (HF) (5-7). However, how the RyR2 channel is altered under these conditions is unclear and controversial.
An impaired interaction between RyR2 and the 12.6 kDa FK506 binding protein (FKBP12.6), which is tightly associated with the channel (8), has been proposed to be a major mechanism underlying cardiac dysfunction in HF (9). Marks and colleagues have shown that RyR2 is hyperphosphorylated in HF by the cAMP-dependent protein kinase A (PKA) at a single residue, ser-2809, which was originally identified as a unique phosphorylation site for the Ca\(^{2+}\) and calmodulin-dependent protein kinase II (CaMKII) (10). They also found that PKA-dependent phosphorylation of RyR2 at ser-2809 dissociated FKBP12.6 from the channel, which increased the sensitivity of single RyR2 channels to activation by Ca\(^{2+}\) and induced openings to subconductance levels (9, 11). In the heart, these FKBP12.6-dissociation-induced alterations in RyR2 function are believed to cause diastolic SR Ca\(^{2+}\) leak, which can result in delayed afterdepolarizations and triggered arrhythmias. This diastolic SR Ca\(^{2+}\) leak is also thought to contribute to the reduced SR Ca\(^{2+}\) content often observed in failing cardiac cells (12, 13). In line with this view, K201(JTV519), an experimental cardioprotective and anti-arrhythmic drug (14, 15), was found to stabilize the interaction between FKBP12.6 and RyR2 (16). These observations have led to the proposal that stabilizing the RyR2-FKBP12.6 interaction is a promising strategy for the treatment of HF and cardiac arrhythmias (16).

Many aspects of the theory on the modulation of RyR2 by FKBP12.6 have been called into question by contradictory findings. For example, K201(JTV519) has been shown to abolish spontaneous Ca\(^{2+}\) leak from SR vesicles containing little or no FKBP12.6 (17, 18). We explored further the action of K201 and found that K201 suppressed spontaneous Ca\(^{2+}\) release and \([\text{H}]\text{ryanodine binding to RyR2 irrespective of FKBP12.6 association (19). All these observations indicate that the inhibitory action of K201 on RyR2 and spontaneous Ca\(^{2+}\) leak is not mediated by FKBP12.6. The phosphorylation status of RyR2 in HF and its effect on RyR2-FKBP12.6 interaction are also controversial. Jiang et al showed that the phosphorylation level of RyR2 from failing hearts was indistinguishable from that of normal hearts (20). Similarly, we have shown that RyR2 is not hyperphosphorylated by PKA in HF (21). We have further demonstrated that RyR2 is phosphorylated by PKA at two major sites, Ser-2030 and Ser-2808 (21), and that stoichiometric phosphorylation of either recombinant or native RyR2 by PKA does not dissociate FKBP12.6 from RyR2 (22). These findings indicate that there is no correlation between PKA-dependent phosphorylation of RyR2 and the dissociation of FKBP12.6 (23). The impact of FKBP12.6 dissociation on the function of the RyR2 channel is also unclear. If the dissociation of FKBP12.6 can markedly alter the gating, conductance, and sensitivity of the RyR2 channel, one would expect that a complete ablation of FKBP12.6 in mice would have a detrimental impact on SR Ca\(^{2+}\) release and cardiac function. Surprisingly, FKBP12.6-null mice display no structural or functional abnormalities at rest in one case (11), and a mild, sex-dependent hypertrophy in the other (24). These observations are inconsistent with the dramatic impairment of single channel function of RyR2 after the dissociation of FKBP12.6 (9, 11), and raise an important question as to whether FKBP12.6 plays an essential role in RyR2 function and cardiac arrhythmias, as suggested.

To address this question, in the present study, we systematically investigated the impact of FKBP12.6-removal on the function of RyR2 at the molecular, cellular, and intact animal levels. We found that the absence or removal of FKBP12.6 does not induce long-lived subconductance states or alter the sensitivity of the RyR2 channel to activation by Ca\(^{2+}\) or caffeine. We also demonstrated that the presence or absence of FKBP12.6 does not affect the propensity for spontaneous Ca\(^{2+}\) release in HEK293 cells. Interestingly, treatment with FK506 slightly increased the amplitude and decreased the frequency of spontaneous Ca\(^{2+}\) release in HEK293 cells independent of FKBP12.6. Importantly, no stress-induced ventricular arrhythmias were detected in FKBP12.6-null mice. These results challenge the notion that FKBP12.6 is essential for the permeation and activation of the RyR2 channel.
EXPERIMENTAL PROCEDURES

Materials—FK506 was a gift from Astellas Pharma Inc. Soybean phosphatidylcholine was obtained from Avanti Polar Lipids, Inc (Alabaster, AL). [3H]ryanodine was from PerkinElmer Life Sciences. Anti-FKBP12.0/12.6 and anti-ryanodine receptor (34C) antibodies were purchased from Affinity BioReagents (Golden, CO). 3-(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS) and other reagents were purchased from Sigma (St. Louis, MO).

Generation of stable, inducible HEK293 cell lines—Stable, inducible HEK293 cell lines expressing RyR2 or RyR2 and FKBP12.6 were generated using the Flp-In T-REx Core Kit from Invitrogen as described previously (19). The double gene construct (RyR2/FKBP12.6) expresses both RyR2 and FKBP12.6, each under the control of a tetracycline operator and the CMV and SV40 promoters, respectively (19).

DNA transfection and preparation of cell lysate from HEK293 cells—HEK293 cells grown on 100-mm tissue culture dishes were transfected with RyR2 or co-transfected with RyR2 and FKBP12.6 cDNAs using Ca2+ phosphate precipitation as described previously (19, 22). After transfection for 24-26 hr, the cells were washed three times with PBS (137 mM NaCl, 8 mM Na2HPO4, 1.5 mM KH2PO4, 2.7 mM KCl) plus 2.5 mM EDTA and were harvested in the same solution by centrifugation for 8 min at 700x g in an IEC Centra-CL2 centrifuge. The cells were washed with PBS without EDTA and centrifuged again at 700x g for 8 min. The PBS-washed cells were solubilized in a lysis buffer containing 25 mM Tris/50 mM Hepes (pH 7.4), 137 mM NaCl, 1% CHAPS, 0.5% soybean phosphatidylcholine, 2.5 mM DTT, and a protease inhibitor mix (1 mM benzamidine, 2µg/ml leupeptin, 2µg/ml pepstatin A, 2µg/ml aprotinin, and 0.5 mM PMSF). The mixture was incubated on ice for 1 hr. Cell lysate was obtained by centrifugation at 16,000 x g for 30 min twice in a microcentrifuge at 4 °C to remove the unsolubilized materials.

[3H]Ryanodine binding—Equilibrium [3H]ryanodine binding to cell lysate was performed as described previously (25). Briefly, a binding mixture (300 µl) containing 30 µl of cell lysate (3-5 mg/ml), 25 mM Tris/50 mM Hepes (pH 7.4), 5 nM [3H]ryanodine, a protease inhibitor mix, and various concentrations of CaCl2 as indicated, was incubated at 37°C for 2.5-3.5 hours. The binding mixture was diluted with 5 ml of ice-cold washing buffer containing 25 mM Tris (pH 8.0), and 250 mM KCl, and immediately filtered through Whatman GF/B filters presoaked with 1% polyethylenimine. The filters were washed four times with 5 ml of ice-cold washing buffer and the radioactivity associated with the filters was determined by liquid scintillation counting. Nonspecific binding was determined by measuring [3H]ryanodine binding in the presence of 50 µM unlabeled ryanodine. All binding assays were done in duplicate. Data shown are mean ± SEM for n experiments. The curves for Ca2+ and caffeine dependent activation of [3H]ryanodine binding were obtained by fitting the data using the MacCurveFit program (Victoria, Australia). Statistical significance was evaluated using the Student's t test.

Single cell Ca2+ imaging of HEK 293 cells—Intracellular Ca2+ transients in stable, inducible HEK293 cells expressing RyR2 or RyR2/FKBP12.6 were measured using single-cell Ca2+ imaging and the fluorescent Ca2+ indicator dye fura-2 acetoxymethyl ester (fura-2 AM) as described previously (19). Cells grown on glass coverslips for 24 or 30 hours after induction by 1µg/ml tetracycline (Sigma) were loaded with 5 µM fura-2 AM in Krebs-Ringer-Hepes (KRH) buffer (125 mM NaCl, 5 mM KCl, 1.2 mM KH2PO4, 6 mM glucose, 1.2 mM, MgCl2, 25 mM Hepes, pH 7.4) plus 0.02% pluronic F-127 (Molecular Probes) and 0.1 mg/ml BSA for 20 min at room temperature (22°C). The coverslips were then mounted in a perfusion chamber (Warner Instruments, Hamden, CT) on an inverted microscope (Nikon TE2000-S) equipped with an S-Fluor 20x/0.75 objective. The cells were continuously perfused with KRH buffer containing 2 mM CaCl2 in the presence or absence of 5 µM FK506 at room temperature (22°C). 10 mM caffeine was applied at the end of each experiment to confirm the expression of functional RyR2 channels. Time-lapse images (0.33 frames s-1) were captured and analyzed with the Compix Inc. Simple PCI 6 software.
Fluorescence intensities were measured from regions of interest centered on individual cells. Only those cells that responded to caffeine were used in analyses.

**Isolation of adult rat ventricular myocytes** -- All studies with rats were approved by the Animal Care Committee of the University of Calgary and complied 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). Single rat ventricular myocytes were isolated as described previously (26). Isolated cells were stored at room temperature in a solution containing 20 mM taurine, 5 mg/ml albumin, and 0.5 mM CaCl₂ until used for single cell Ca²⁺ imaging studies.

**Single Cell Ca²⁺ Imaging of rat ventricular myocytes** -- Freshly isolated rat ventricular myocytes were placed on glass coverslips coated with 0.02% (w/v) gelatin and 10 µg/ml fibronectin, and loaded with 5 µM fluo-4-AM Ca²⁺ (Molecular Probes) plus 0.02% pluronic F-127 in KRH buffer with 1mM Ca²⁺ for 20 min at room temperature (22°C) (19). The coverslips were mounted in a perfusion chamber on an inverted microscope (Nikon TE2000-S) equipped with an S-Fluor 20x/0.75 objective. The [Ca²⁺] was then stepped to 3 mM for 5 minutes before a further increase to 6 mM. The cells were then continuously perfused with KRH buffer containing 6 mM CaCl₂ in the presence or absence of 5 µM FK506 at room temperature (22°C). Time-lapse images were captured every 0.4 sec and analyzed with the Compix Inc. Simple PCI 6 software. Fluorescence intensities were measured from regions of interest.

**Single channel recordings in planar lipid bilayers** -- Recombinant RyR2 proteins were partially purified from cell lysate by sucrose density gradient centrifugation (27). Heart phosphorylchololnolamine and brain phosphorylcholinerine (Avanti Polar Lipid), dissolved in chloroform, were combined in a 1:1 ratio (w/w), dried under nitrogen gas and suspended in 30 µl of n-decane at a concentration of 12 mg lipid/ml. Bilayers were formed across a 250-µm hole in a Delrin partition separating two chambers. The cis chamber (800 µl) was connected to the head stage input of an Axopatch 200A amplifier (Axon Instruments Inc.). The trans chamber (1.2 ml) was held at virtual ground. A symmetrical solution containing 250 mM KCl and 25 mM Hepes (pH 7.4), was used for all recordings, unless indicated otherwise. A 4 µl- aliquot (~1 µg of protein) of the sucrose density gradient-purified recombinant RyR2 protein was added to the trans chamber. Spontaneous channel activity was always tested for sensitivity to EGTA and Ca²⁺. The chamber to which the addition of EGTA inhibited the activity of the incorporated channel was presumed to correspond to the cytoplasmic side of the channel. The direction of single channel currents was always measured from the luminal to the cytoplasmic side of the channel, unless mentioned otherwise. Recordings were filtered at 2,500 Hz. Free Ca²⁺ concentrations were calculated using the computer program of Fabiato and Fabiato (28). Data analyses were carried out using the pClamp 8.1 software (Axon Instruments Inc.). For single channel recordings of native mouse RyR2 channels, ventricular sarcoplasmic reticulum microsomes from FKBP12.6+/+ and FKBP12.6−/− adult male mice were prepared. RyR2 channels from microsomes were reconstituted into bilayers as described previously (29, 30). In all experiments, the luminal (trans) solution contained 50 mM Ca²⁺/250mM HEPES. The cytosolic (cis) solution contained 118 Tris/250 HEPES (cis and trans, pH = 7.4). [Ca²⁺] in the cis solution was buffered with DiBromo BAPTA. Unless indicated otherwise, all experiments with native mouse RyR2 channels were carried out at a holding potential of 0 mV (transmembrane voltage). Bars at the side of the recordings indicate baseline levels. Open probabilities were obtained from the analysis of channel recordings of 4-8 minutes in length as described previously (29, 30).

**RyR2/FKBP12.6 pull-down assays and immunoblotting** -- Cell lysates (RyR2 or RyR2/FKBP12.6) or solubilized cardiac microsomes were incubated with protein G-Sepharose (20 µl) that was pre-bound with 1 µl anti-RyR antibody (34C) at 4 °C for 17-19 hours in the presence or absence of 5 µM FK506. The protein G/34C precipitates were washed with ice-cold lysis buffer containing a protease inhibitor mix three times, each time for 10 min. The
proteins bound to the Sepharose beads or 20 µl of cell lysates were then solubilized by the addition of 20 µl 2x Laemmli’s sample buffer (31) plus 5% β-mercaptoethanol and boiled for 5 min. The solubilized samples were then separated by 6.25% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (31). For the immunoprecipitation experiments, the sample volumes were adjusted to load a similar amount of RyR2 into each lane. The SDS-PAGE resolved proteins were transferred to nitrocellulose membranes at 45 V for 18-20 hours at 4 °C in the presence of 0.01% SDS according to the method of Towbin et al. (32). The nitrocellulose membranes containing the transferred proteins were blocked for 30 min with PBS containing 0.5% Tween-20 and 5% skim milk. The blocked membranes were then incubated with anti-RyR(34C) or anti-FKBP antibodies (both 1:1000) for 1-2 hour, and washed three times for 5 min each in PBS containing 0.5% Tween-20. The membrane was then incubated with the appropriate horseradish peroxidase conjugated secondary antibody (1:20,000) for 30 min. After washing 3 times for 5 min each in PBS containing 0.5% Tween-20, the RyR2 or FKBP12.6 proteins were detected by enhanced chemiluminescence (Pierce).

**Telemetric ECG recordings and stress tests** -- All studies with mice were approved by the Animal Care Committee of the University of Calgary and complied 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). ECG telemetry transmitters (Data Sciences International, St. Paul, MN, USA) were implanted in the abdominal cavity under general inhalant anesthesia. Body temperature was maintained at 37°C by use of a heating pad. After 5 days recovery from surgery, the mice (WT, n=9, FKBP12.6−/−, n=9) were injected with epinephrine (2 mg/kg ip) and caffeine (120 mg/kg ip). ECG was continuously monitored for 10 min before and 60-90 min after injection.

**RESULTS**

The removal of FKBP12.6 does not induce subconductance states in the recombinant or native canine RyR2 channels -- To investigate the role of FKBP12.6 in the conduction of the RyR2 channel, we expressed RyR2 wt alone or with FKBP12.6 in HEK293 cells. The expressed RyR2 channels associated with or without FKBP12.6 were then purified by sucrose-density gradient centrifugation and used for single channel analyses in planar lipid bilayers. We have previously shown that FKBP12.6 is co-immunoprecipitated with RyR2 from HEK293 cells co-transfected with FKBP12.6 (21, 22). Fig. 1A shows the channel activity of a single RyR2 channel from HEK293 cells co-transfected with RyR2 and FKBP12.6 before (Fig. 1Aa) and after (Fig. 1Ab) the addition of 5 µM FK506, an immunosuppressant known to dissociate FKBP12.6 from RyR2. As can be seen, the conductance and gating of the channel remained largely unchanged after the FK506 treatment. The average open probability (Po) after the FK506 treatment was 100 ± 8.4% (n=7) that before the treatment. These data indicate that dissociation of FKBP12.6 from RyR2 does not induce long-lasting subconductance states.

Fig. 1B shows single channel current traces of an RyR2 channel from HEK293 cells transfected with RyR2 alone without FKBP12.6. We have previously shown that RyR2 expressed alone in HEK293 cells is devoid of FKBP12.6 (21, 22). As seen in Fig. 1B, single RyR2 channels devoid of FKBP12.6 displayed a single channel conductance identical to that of RyR2 co-expressed with FKBP12.6 (Fig. 1A) and native canine RyR2 (Fig. 1C). Importantly, single RyR2 channels without FKBP12.6 exhibited no long-lived subconductance states. As with the FKBP12.6/RyR2 channels, FK506 (5 µM) had no significant effect on the conductance or gating of the FKBP12.6-devoid single RyR2 channels (Fig.1Bb). The average Po after FK506 treatment was 112 ± 5.2% (n=10, P < 0.236) that before the treatment. These observations demonstrate that the loss of FKBP12.6 does not affect the conductance of the RyR2 channel.

It is clear that removal of FKBP12.6 does not affect the conductance or gating of the recombinant RyR2 channels. To determine whether the dissociation of FKBP12.6 alters the properties of native RyR2 channels, we tested the effect of FK506 on native canine RyR2 channels incorporated into lipid bilayers. As shown in Fig. 1C, treating single native canine RyR2 channels with 5 µM FK506 did not produce
subconductance states or change the gating of the channel. The average P₀ after the FK506 treatment was 100 ± 4.6% (n=8) that before the treatment. We have previously shown that FKBP12.6 was co-immunoprecipitated with the recombinant or canine RyR2 and dissociated from RyR2 by rapamycin (19, 21, 22), an analogue of FK506. Similarly, we found that FKBP12.6 was dissociated from the recombinant or native canine RyR2 channels by 5 µM FK506 (Fig. 1D). Hence, taken together, our data indicate that the dissociation of FKBP12.6 does not induce subconductance states in either recombinant or native RyR2 channels.

The removal of FKBP12.6 does not alter the Ca²⁺ dependence or caffeine response of [³H]ryanodine binding to RyR2 — To determine whether dissociation of FKBP12.6 affects the sensitivity of RyR2 to activation by stimuli, we assessed the effect of FK506 on [³H]ryanodine binding to cell lysates from HEK293 cells transfected with RyR2 alone or co-transfected with RyR2 and FKBP12.6 at various concentrations of Ca²⁺ or caffeine. As shown in Fig. 2A, the Ca²⁺ dependence of [³H]ryanodine binding to RyR2 co-expressed with FKBP12.6 (EC₅₀ = 3.65 ± 0.22 µM, n=4) was identical. Treatment with 5 µM FK506 did not change the Ca²⁺ dependence of [³H]ryanodine binding to RyR2 co-expressed with FKBP12.6 (Fig. 2B). In the presence of FK506, the EC₅₀ value for [³H]ryanodine binding to RyR2/FKBP12.6 was 0.17 ± 0.02 µM (n=3), which is similar to that of [³H]ryanodine binding to RyR2/FKBP12.6 in the absence of FK506 (0.16 ± 0.01 µM, n=5).

Fig.3 shows the caffeine-response of [³H]ryanodine binding to RyR2 expressed alone or co-expressed with FKBP12.6 in the absence or presence of 5 µM FK506. The caffeine responses of [³H]ryanodine binding to RyR2 co-expressed with FKBP12.6 (EC₅₀ = 3.65 ± 0.22 µM, n = 4) or without FKBP12.6 (EC₅₀ = 4.25 ± 0.46 µM, n = 4) were similar (Fig. 3A). FK506-treatment did not alter the sensitivity of RyR2 to activation by caffeine. The EC₅₀ values for caffeine activation of [³H]ryanodine binding to RyR2 co-expressed with FKBP12.6 in the presence and absence of FK506 were 3.93 ± 0.69 mM (n = 3) and 3.65 ± 0.22 mM (n = 4), respectively. Collectively, these observations indicate that the removal of FKBP12.6 does not affect the sensitivity of RyR2 to activation by Ca²⁺ or caffeine.

Effect of FK506 on SOICR in HEK293 cells expressing RyR2 and in rat cardiac myocytes — Given the importance of spontaneous Ca²⁺ release or store-overload-induced Ca²⁺ release (SOICR) in arrhythmogenesis and the potential role of FKBP12.6 in cardiac arrhythmias, we examined whether the removal of FKBP12.6 by FK506 alters the SOICR properties of HEK293 cells expressing RyR2 and cardiac myocytes. To this end, we generated a stable, inducible HEK293 cell line expressing both RyR2 and FKBP12.6. To confirm that RyR2 forms a complex with FKBP12.6 in these cells, we performed immunoprecipitation and immunoblotting experiments. As shown in Fig. 4F, FKBP12.6 was co-immunoprecipitated with RyR2 by an anti-RyR antibody (34C). The levels of co-immunoprecipitated FKBP12.6 were comparable after 24-48 hours of induction. Accordingly, the RyR2/FKBP12.6-expressing cells were induced for 24 hours. The cells were then loaded with the Ca²⁺ fluorescent indicator fura-2-AM and perfused with 2 mM external Ca²⁺ to produce SOICR. After a stable SOICR was achieved, FK506 (5 µM) was added to the perfusate. The addition of FK506 did not increase SOICR activity, as would be expected given the common belief that the dissociation of FKBP12.6 enhances the activity of the RyR2 channel. Interestingly and on the contrary, we found that FK506 slightly increased the amplitude to 104.4 ± 0.4% (P < 0.0001) and reduced the frequency of SOICR to 80.5 ± 2.0% (P < 0.002) (n=77) (Fig. 4A,D,E).

The effect of FK506 on SOICR was also assessed in rat cardiac myocytes. Freshly isolated rat cardiac myocytes were perfused with 6 mM external Ca²⁺ to induce SOICR. The cells were then switched to perfusate containing 6 mM Ca²⁺ and 5 µM FK506. As seen in Fig. 4B, perfusion with FK506 did not considerably affect the SOICR activity. A small increase in the amplitude of SOICR was observed (103.0 ± 0.8%, P < 0.0001), but no significant change in the frequency was detected (96.9 ± 6.4%, P < 0.323) (n=74) (Fig. 4B,D,E).
To further investigate whether the modest impact of FK506 on SOICR is mediated by FKBP12.6, we examined the effect of FK506 on SOICR in HEK293 cells expressing RyR2 alone without FKBP12.6. As shown in Fig. 4C, the addition of FK506 (5 µM) was still able to increase the amplitude (104.9 ± 1.6%, P < 0.02) and reduce the frequency (80.5 ± 1.1%, P < 0.0002) (n = 180) of SOICR in these FKBP12.6-deficient cells (Fig. 4D,E). Taken together, these observations demonstrate that the removal of FKBP12.6 does not enhance spontaneous Ca\(^{2+}\) release in HEK293 cells or in cardiac cells. They also indicate that the effect of FK506 on SOICR is FKBP12.6-independent.

**Effect of FKBP12.6 on SOICR in HEK293 cells expressing RyR2** -- To examine the role of FKBP12.6 in SOICR without the interference of FK506, we compared the SOICR activity of HEK293 cells expressing RyR2 alone and HEK293 cells expressing both RyR2 and FKBP12.6. To ensure a comparable level of expression of RyR2 in these cell lines, we examined the time course of expression of RyR2. As shown in Fig. 5A, the expression level of RyR2 and FKBP12.6 increased in both cell lines with increasing induction times. The expression level of RyR2 in RyR2-expressing HEK293 cells after 30 h induction was found to be comparable with that in RyR2/FKBP12.6-expressing HEK293 cells after 24 h induction. Hence, SOICR was compared between cells expressing RyR2 after 30 h induction and cells expressing both RyR2 and FKBP12.6 after 24 h induction. It should be noted that FKBP12.6 is absent in HEK293 cells expressing only RyR2, and that the expression levels of the endogenous FKBP12.0 were not changed under these conditions. Analyzing a number of oscillating cells revealed that HEK293 cells expressing RyR2 with (Fig. 5B, n=508 cells) or without (Fig. 5C, n=403 cells) FKBP12.6 exhibited the same propensity for SOICR. No significant differences in the level of intracellular Ca\(^{2+}\) store or the frequency of SOICR were detected (Fig. 5E). These data indicate that the loss of FKBP12.6 does not alter the propensity for SOICR.

**Single channel properties of RyR2 from FKBP12.6-null mice** -- The role of FKBP12.6 in RyR2 function was further investigated using FKBP12.6-null mice. Cardiac microsomes from WT and FKBP12.6-null mice were isolated and reconstituted into planar lipid bilayers. As shown in Figs. 6A-D, single RyR2 channels from FKBP12.6-null mice displayed properties indistinguishable from those of the WT mice. Their current voltage relationships overlapped (Fig. 6A). Channels openings to various subconductance states, such as those shown in Fig. 6B, were rare. The probability of the subconductance states (the time of the channel in substate/the total time of the channel in full-open state) was 0.0005 ± 0.0002 (n=4) for the WT channels, and 0.0004 ± 0.0002 (n=4) for the FKBP12.6-deficient channels. The Ca\(^{2+}\) dependent activation (Fig. 6C) and Mg\(^{2+}\) dependent inhibition (Fig. 6D) of RyR2 from WT and FKBP12.6-null mice were identical. The FKBP12.6-deficient RyR2 channels also responded to ATP, caffeine (Fig. 6E), ruthenium red, and ryanodine in a manner similar to RyR2 WT (not shown). In addition, the gating properties of RyR2 from FKBP12.6-null mice were not affected by the addition of the exogenous FKBP12.6 protein (Figs. 6E,F). These observations indicate that the loss of FKBP12.6 does not alter the single channel properties of native mouse RyR2 channels. These findings are consistent with our previous observations that FKBP12.6 removal/association does not have any noticeable effect on the activity of RyR2 (29, 30, 33).

**Impact of FKBP12.6-deficiency on stress-induced ventricular arrhythmias** -- It has been shown that one model of FKBP12.6-null mice is highly susceptible to stress-induced ventricular tachycardia (VT) and sudden death (11). As shown above, we found no apparent differences between RyR2 from another FKBP12.6-null mice and WT. Moreover, all our studies suggest that FKBP12.6 removal does not affect single RyR2 channel properties, the sensitivity of RyR2 to activation, or SOICR, a well-known trigger of cardiac arrhythmias. Our FKBP12.6-null mice only display some mild cardiac abnormalities (24). Still, the susceptibility of these animals to stress-induced VT was never tested. Accordingly, we decided to address whether VT and sudden
death could be readily induced by stress in our FKBP12.6-null mice.

Telemetric electrocardiogram (ECG) devices were implanted into the FKBP12.6-null and WT mice and their ECG was monitored before and after the injection of a mixture of epinephrine and caffeine, a combination that has been shown to induce ventricular arrhythmias in mice harboring a RyR2 mutation linked to VT and sudden death (34, 35). Using this approach, we observed that the injection of a combination of epinephrine (2 mg/kg) and caffeine (120 mg/kg) induced no ventricular arrhythmias in either WT (n=9) or FKBP12.6-null mice (n=9) (Fig. 7). These observations indicate that FKBP12.6-deficiency does not lead to stress-induced ventricular arrhythmias and sudden death in our FKBP12.6-null mice.

**DISCUSSION**

FKBP12.6, a small RyR2-associated protein, has recently been proposed as a promising therapeutic target for the treatment of HF and cardiac arrhythmias (16). This proposal is largely based on the observations that (1) the level of FKBP12.6 bound to RyR2 was reduced in HF as a result of PKA-dependent hyperphosphorylation of RyR2 (9), (2) the dissociation of FKBP12.6 from RyR2 induced subconductance states and increased the sensitivity of the channel to Ca\(^{2+}\) activation, consequently leading to SR Ca\(^{2+}\) leak (9), (3) an experimental drug, K201(JTV519) which is known to have cardioprotective and anti-arrhythmic properties, inhibited SR Ca\(^{2+}\) leak and cardiac arrhythmias in an FKBP12.6-dependent manner (16), and (4) FKBP12.6-null mice were susceptible to stress-induced ventricular arrhythmias (11). Accordingly, restoring or stabilizing the binding of FKBP12.6 to RyR2 is thought to be an effective strategy for suppressing SR Ca\(^{2+}\) leak and cardiac arrhythmias (16). However, the phosphorylation status of RyR2 by PKA in HF, the impact of PKA-dependent phosphorylation on the FKBP12.6-RyR2 interaction, and the role of FKBP12.6 in the inhibitory action of K201(JTV519) have recently been questioned (19-24, 30, 35). In the present study, we have focused on the questions of whether the dissociation of FKBP12.6 alters the conductance and activation of the RyR2 channel, and whether the removal of FKBP12.6 enhances the propensity for spontaneous Ca\(^{2+}\) release (SOICR) and the susceptibility to stress-induced ventricular arrhythmias. We found that the removal of FKBP12.6 has little effect on RyR2 function and spontaneous Ca\(^{2+}\) release. Importantly, the ablation of FKBP12.6 does not render mice susceptible to stress-induced ventricular arrhythmias. Taken together, the results of the present study and those of previous studies do not support the notion that impaired RyR2-FKBP12.6 interaction is a major mechanism underlying cardiac dysfunction in HF.

The role of FKBP12.6 in the conduction and activation of the RyR2 channel -- FK506 and rapamycin are two immunosuppressants capable of binding to and dissociating FKBP12.6 from the RyR2 channel. Because of their unique properties, they have been widely used as probes for assessing the role of FKBP12.6 in RyR2 function. It has been shown that treating single RyR2 channels incorporated into planar lipid bilayers with FK506 or rapamycin induced the appearance of various subconductance states and increased the Po of the channel (36, 37). These FK506- or rapamycin-induced changes in single channel behavior were thought to result from the dissociation of FKBP12.6. However, SR membrane vesicles pre-treated with FK590, an analogue of FK506, to remove FKBP12.6 before their fusion into planar lipid bilayers displayed single RyR2 channel activities without subconductance states (30). Consistent with this observation, we have demonstrated that single recombinant RyR2 channels expressed in HEK293 cells without FKBP12.6 and single native mouse RyR2 channels from FKBP12.6-null mice exhibited no subconductance states (Figs. 1 and 6). These observations indicate that the removal of FKBP12.6 in and of itself does not induce subconductance states. The exact mechanism by which FK506 or rapamycin induces subconductance states is not clear. The occurrence of subconductance states may be influenced by the concentrations, types, and sources of the experimental drugs used (FK590, FK506, or rapamycin), the preparation, forms (SR membrane vesicles or purified channels), and species of the RyR2 channels, and the recording conditions (bilayer compositions or charge...
carriers). In any event, it is clear that the drug-induced subconductance states are not due to a lack of FKBP12.6, but may result from a direct effect of the drug or the drug-FKBP12.6 complex on RyR2.

The dissociation of FKBP12.6 is generally believed to increase the sensitivity of RyR2 to Ca\(^{2+}\) activation. However, there is no direct evidence to support this belief. Despite an increase in the Po of the channel as a result of the appearance of long-lived subconductance states, rapamycin did not affect the overall Ca\(^{2+}\) dependence of activation of the RyR2 channel (36). The Ca\(^{2+}\) dependence of \([\text{H}]\)ryanodine binding to SR membrane vesicles was also found to remain unchanged after the removal of FKBP12.6 (30). In this study, we found that the EC50 values for Ca\(^{2+}\) and caffeine activation of \([\text{H}]\)ryanodine binding to RyR2 co-expressed with or without FKBP12.6 or treated with or without FK506 were indistinguishable (Figs. 2 and 3). We also found that the Ca\(^{2+}\) response of single RyR2 channels from FKBP12.6 null mice is virtually identical to that of single wt channels. Therefore, the removal of FKBP12.6 does not alter the sensitivity of the RyR2 channel to activation by Ca\(^{2+}\) or caffeine.

**The significance of FKBP12.6 in SR Ca\(^{2+}\) release** -- The significance of FKBP12.6 in SR Ca\(^{2+}\) release has been extensively studied, but its exact role remains largely undefined and controversial. Fleischer and colleagues found that FKBP12.0-stripped or FK590-treated canine skeletal muscle SR vesicles displayed a decreased rate of SR Ca\(^{2+}\) loading, suggesting an increased SR Ca\(^{2+}\) release (38, 39). Surprisingly, the removal of FKBP12.6 or FK590-treatment had no effect on the rate of SR Ca\(^{2+}\) loading in canine cardiac microsomes (29, 30). Similarly, Prestle et al. showed that rapamycin has no significant effect on the rate of SR Ca\(^{2+}\) uptake in rabbit cardiac myocytes (40). In contrast, Yano et al. showed that the addition of FK506 to canine cardiac microsomes led to an increase in SR Ca\(^{2+}\) release (12). Controversies also exist with respect to the impact of FK506 on Ca\(^{2+}\) sparks. It has been shown that FK506 increased, decreased, or had no effect on the amplitude, frequency, or duration of Ca\(^{2+}\) sparks (37, 41-43). The reasons for these different results are unclear, but they could be cause by factors other than FKBP12.6. In this regard, Guo et al. recently showed that rapamycin rapidly dissociated a fluorescent FKBP12 from RyR2, but did not alter the frequency of Ca\(^{2+}\) sparks in permeabilized rat ventricular myocytes, suggesting that the dissociation of FKBP12.6 from RyR2 is unlikely to affect the activity of RyR2 (44). The effect of FK506 on stimulated Ca\(^{2+}\) transients is also inconsistent, and may be species-dependent. FK506 increased the amplitude of stimulated Ca\(^{2+}\) transients in rat cardiac myocytes, but decreased the amplitude of Ca\(^{2+}\) transients in rabbit cardiac myocytes (45). This difference is thought to be attributable to the different Na/Ca exchange activities between rat and rabbit cardiac cells (45). On the other hand, Dubell et al. showed no change in Ca\(^{2+}\) transients after FK506 treatment in voltage-clamped rat cardiac cells (46). Instead, FK506 inhibited outward K\(^{+}\) currents and prolonged the action potential duration (46). Furthermore, Milting et al. found that FK506 has no effect on the contractility of human and rabbit myocardium (47).

It should be noted that the FK506-induced impact on stimulated Ca\(^{2+}\) transients in both rat and rabbit cardiac cells is sustained, suggesting that FK506 is unlikely to alter only the activity of RyR2. This is because modest modulation of RyR2, for example, by caffeine or tetracaine, has only a transient effect on stimulated Ca\(^{2+}\) transients due to the auto-regulation of Ca\(^{2+}\) release by the SR Ca\(^{2+}\) content ((48, 49). In line with this view, FK506 has been shown to act on a number of targets in cardiac cells. It inhibits the Na/Ca exchange and the outward K\(^{+}\) currents (41, 46), the sarcolemmal Na\(^{+}/K\(^{+}\) pump (50), and the SR Ca\(^{2+}\) pump (51), in addition to the inhibition of the phosphatase calcineurin (52). Inhibition of the Na/Ca exchange current by FK506 is thought to partially account for the enhanced stimulated Ca\(^{2+}\) transients in rat cardiac myocytes by reducing Ca\(^{2+}\) extrusion and thus increasing the SR Ca\(^{2+}\) content (41). It is not clear whether this effect is mediated by FKBP12.6 or a direct effect of the drug FK506. Hence, FK506 (or rapamycin) affects multiple targets in cells.

The significance of FKBP12.6 in SR Ca\(^{2+}\) release has also been assessed by knocking out the FKBP12.6 gene in mice or overexpressing it in rabbit cardiac myocytes. The ablation of FKBP12.6 slightly increased the amplitude and
prolonged the duration of Ca\textsuperscript{2+} sparks, and enhanced the Ca\textsuperscript{2+}-induced and Ca\textsuperscript{2+} release (CICR) gain in mouse cardiac myocytes (24). Hence, the lack of FKBP12.6 is unlikely to be associated with HF, which exhibits a decreased CICR gain (53). On the other hand, the overexpression of FKBP12.6 in rabbit cardiac myocytes decreased the amplitude, frequency, and duration of Ca\textsuperscript{2+} sparks, but augmented stimulated Ca\textsuperscript{2+} transients and the magnitude of spontaneous Ca\textsuperscript{2+} waves (40, 42, 54). It is unclear why FKBP12.6-ablation and FKBP12.6-overexpression can both enhance stimulated Ca\textsuperscript{2+} transients. It should be also noted that FKBP12.6 KO and overexpression both resulted in a maintained impact on Ca\textsuperscript{2+} transients.

Although modulation of RyR2 has only a transient impact on stimulated Ca\textsuperscript{2+} transients, it has a maintained effect on spontaneous Ca\textsuperscript{2+} release (SOICR). Accordingly, in the present study, we determined the impact of FK506 or FKBP12.6 on SOICR. We found that co-expression with or without FKBP12.6 did not affect the propensity for SOICR in HEK293 cells. We further demonstrated that FK506 treatment did not reduce the amplitude of SOICR either in HEK293 cells co-expressing RyR2 and FKBP12.6 or in rat cardiac myocytes, as would be expected if FK506 enhances the activity of RyR2. On the contrary, we found that FK506 slightly increased the amplitude and decreased the frequency of SOICR in HEK293 cells and in cardiac myocytes. Interestingly, this effect of FK506 on SOICR is similar to that of tetracaine (55), indicating that FK506 inhibits, rather than enhances the activity of RyR2. Importantly, this effect of FK506 was also observed in HEK293 cells expressing RyR2 alone without FKBP12.6, indicating that the effect of FK506 on SOICR is independent of FKBP12.6. These data and those from the FKBP12.6 KO mouse studies indicate that the removal of FKBP12.6 does not increase the propensity for spontaneous Ca\textsuperscript{2+} release or SOICR, which is consistent with the observation that the dissociation of FKBP12.6 does not alter the conductance and activation of the RyR2 channels.

**REFERENCES**

1. Bers, D. M. (2002) *Nature* **415**, 198-205.

The role of FKBP12.6 in ventricular arrhythmias -- Since defective RyR2 function as a result of naturally occurring mutations is linked to ventricular arrhythmias, altered RyR2 regulation is potentially arrhythmogenic. Based on the observation that the removal of FKBP12.6 from RyR2 dramatically increases the sensitivity of the channel to Ca\textsuperscript{2+} activation and enhances SR Ca\textsuperscript{2+} leakage (9), which can lead to DADs and triggered arrhythmias, the dissociation of FKBP12.6 itself is believed to trigger or to render the heart susceptible to cardiac arrhythmias (9). However, based on our observation that the removal of FKBP12.6 does not alter RyR2 function or the propensity for SOICR, which is a common defect of disease-linked RyR2 mutations, we expected that, unlike RyR2 mutations, the loss of FKBP12.6 would not result in ventricular arrhythmias. Consistent with this view, we found that mice lacking FKBP12.6 responded to epinephrine- and caffeine-challenge in a manner indistinguishable from the wt mice. Neither FKBP12.6-null nor wt mice developed ventricular arrhythmias after the injection of caffeine and epinephrine, although, the same doses of epinephrine and caffeine induced ventricular tachycardia in mice harboring a CPVT RyR2 mutation, R4496C. Marks and colleagues also generated an FKBP12.6-null mouse line and found no structural and ECG abnormalities or arrhythmias in these mice at rest, but they exhibited exercise-induced ventricular arrhythmias in these mice at rest, but they exhibited exercise-induced ventricular arrhythmias in these mice at rest, but they exhibited exercise-induced ventricular arrhythmias in these mice at rest, but they exhibited exercise-induced ventricular arrhythmias in these mice at rest, but they exhibited exercise-induced ventricular arrhythmias in these mice at rest, but they exhibited exercise-induced ventricular arrhythmias in these mice at rest, but they exhibited exercise-induced ventricular arrhythmias in these mice at rest, but they exhibited exercise-induced ventricular arrhythmias (11). On the other hand, our FKBP12.6-null mice showed mild, sex-dependent cardiac hypertrophy, but exhibited no stress-induced VT (24). The reasons for this discrepancy are unknown. Differences in the genetic background may, in part, account for the different phenotypes. Further investigations, particularly a detailed functional characterization of FKBP12.6-null mice, will be required to understand the exact physiological role of FKBP12.6 in RyR2 and cardiac function.
2. Ter Keurs, H. E., and Boyden, P. A. (2007) *Physiol. Rev.* 87, 457-506
3. George, C. H., Jundi, H., Thomas, N. L., Fry, D. L., and Lai, F. A. (2007) *J. Mol. Cell. Cardiol.* 42, 34-50
4. Mohamed, U., Napolitano, C., and Priori, S. G. (2007) *J. Cardiovasc. Electrophysiol.* 18, 791-797
5. Hove-Madsen, L., Llach, A., Bayes-Genis, A., Roura, S., Rodriguez Font, E., Aris, A., and Cinca, J. (2004) *Circulation* 110, 1358-1363
6. Vest, J. A., Wehrens, X. H. T., Reiken, S. R., Lehnart, S. E., Dobrev, D., Chandra, P., Danilo, P., Ravens, U., Rosen, M. R., and Marks, A. R. (2005) *Circulation* 111, 2025-2032
7. Yano, M., Yamamoto, T., Ikeda, Y., and Matsuzaki, M. (2006) *Nat. Clin. Pract. Cardiovasc. Med.* 3, 43-52
8. Lam, E., Martin, M. M.,Timerman, A. P., Sabers, C., Fleischer, S., Lukas, T., Abraham, R. T., O'Keefe, S. J., O'Neill, E. A., and Wiederrecht, G. J. (1995) *J. Biol. Chem.* 270, 26511-26522
9. Marx, S. O., Reiken, S., Hisamatsu, Y., Jayaraman, T., Burkhoff, D., Rosembliit, N., and Marks, A. R. (2000) *Cell* 101, 365-376
10. Witcher, D. R., Kovacs, R. J., Schulman, H., Cefali, D. C., and Jones, L. R. (1991) *J. Biol. Chem.* 266, 11144-11152
11. Wehrens, X., Lehnart, S., Huang, F. et al. (2003) *Cell* 113, 829-840
12. Yano, M., Ono, K., Ohkusa, T. et al. (2000) *Circulation* 102, 2131-2136
13. Lehnart, S. E., Terrenoire, C., Reiken, S. et al. (2006) *Circulation* 103, 7906-7910
14. Kaneko, N. (1994) *Drug Dev. Res.* 33, 429-438
15. Kumagai, K., Nakashima, H., Gondo, N., and Saku, K. (2003) *J. Cardiovasc. Electrophysiol.* 14, 880-884
16. Wehrens, X. H. T., Lehnart, S. E., Reiken, S. R., Deng, S.-., Vest, J. A., Cervantes, D., Coromilas, J., Landry, D. W., and Marks, A. R. (2004) *Science* 304, 292-296
17. Ono, K., Yano, M., Ohkusa, T., Kohno, M., Hisaoka, T., Tanigawa, T., Kobayashi, S., and Matsuzaki, M. (2000) *Cardiovasc. Res.* 48, 323-331
18. Yano, M., Kobayashi, S., Kohno, M. et al. (2003) *Circulation* 107, 477-484
19. Hunt, D. J., Jones, P. P., Wang, R., Chen, W., Bolstad, J., Chen, K., Shimoni, Y., and Chen, S. R. (2007) *Biochem. J.* 404, 431-438
20. Jiang, M. T., Lokuta, A. J., Farrell, E. F., Wolff, M. R., Haworth, R. A., and Valdivia, H. H. (2002) *Circ. Res.* 91, 1015-1022
21. Xiao, B., Jiang, M. T., Zhao, M., Yang, D., Sutherland, C., Lai, F. A., Walsh, M. P., Warltier, D. C., Cheng, H., and Chen, S. R. W. (2005) *Circ. Res.* 96, 847-855
22. Xiao, B., Sutherland, C., Walsh, M. P., and Chen, S. R. W. (2004) *Circ. Res.* 94, 487-495
23. Stange, M., Xu, L., Balshaw, D., Yamaguchi, N., and Meissner, G. (2003) *J. Biol. Chem.* 278, 51693-51702
24. Xin, H. B., Senbonmatsu, T., Cheng, D. S. et al. (2002) *Nature* 416, 334-38.
25. Li, P., and Chen, S. R. (2001) *J. Gen. Physiol.* 118, 33-44.
26. Shimoni, Y., Chuang, M., Abel, E. D., and Severson, D. L. (2004) *Proc. Natl. Acad. Sci. U. S. A.* 94, 345-354
27. Jiang, D., Wang, R., Xiao, B., Kong, H., Hunt, D. J., Choi, P., Zhang, L., and Chen, S. R. W. (2005) *Circ. Res.* 97, 1173-1181
28. Fabiato, A., and Fabiato, F. (1979) *J. Physiol. (Paris)* 75, 463-505
29. Barg, S., Copello, J. A., and Fleischer, S. (1997) *Am. J. Physiol.* 272, C1726-C1726
30. Timerman, A. P., Onoue, H., Xin, H. B., Barg, S., Copello, J., Wiederrecht, G., and Fleischer, S. (1996) *J. Biol. Chem.* 271, 20385-20391
31. Laemmli, U. K. (1970) *Nature* 227, 680-685.
32. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* 76, 4350-434.
33. Copello, J. A., Qi, Y., Jeyakumar, L. H., Ogubunmu, E., and Fleischer, S. (2001) *Cell Calcium* 30, 269-284
34. Cerrone, M., Colombi, B., Santoro, M., di Barletta, M. R., Scelsi, M., Villani, L., Napolitano, C., and Priori, S. G. (2005) *Circ. Res.* 96, e77-82
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**FIGURE LEGENDS**

**Fig.1.** FKBP12.6-removal does not affect the conductance of single RyR2 channels. Single channel activities of the recombinant RyR2 wt co-expressed with FKBP12.6 (A), recombinant RyR2 wt expressed alone (B), and the native canine RyR2 (C) were recorded in a symmetrical recording solution containing 250 mM KCl and 25 mM Hepes (pH 7.4) at a holding potential of -20 mV. EGTA was added to either the cis or trans chamber to determine the orientation of the incorporated channel. The side of the channel to
which the addition of EGTA inhibited the activity of the incorporated channel presumably corresponds to the cytosolic face of the channel. Panel a shows control single channel activities in the presence of ~100-200 nM cytosolic Ca$^{2+}$ and 45 nM luminal Ca$^{2+}$. Single channel activities ~10 min after the addition of 5 µM FK506 are shown in panel b. A total of 7 RyR2/FKBP12.6, 10 RyR2 without FKBP12.6, and 8 canine RyR2 channels were tested, and none showed FK506-induced long-lived subconductance states. Openings are downward. Open probability (Po), arithmetic mean open time (To), and arithmetic mean closed time (Tc) are indicated at the top of the traces. A short line to the right of each current trace indicates the baseline. (D) Western blots of RyR2 and FKBP12.6 immunoprecipitated with an anti-RyR antibody (34c) from either HEK293 cell lysate expressing RyR2 or co-expressing RyR2 and FKBP12.6, or solubilized canine cardiac microsomes treated with or without 5 µM FK506.

Fig.2. FKBP12.6-removal does not alter the Ca$^{2+}$ dependence of $[^{3}H]$ryanodine binding. $[^{3}H]$ryanodine binding to cell lysate prepared from HEK293 cells expressing RyR2 or expressing both RyR2 and FKBP12.6 was carried out at various Ca$^{2+}$ concentrations (~0.2 nM to 0.1 mM), 5 mM $[^{3}H]$ryanodine, and 500 mM KCl. Panel A shows $[^{3}H]$ryanodine to RyR2 (filled circles) and RyR2/FKBP12.6 (open circles). Pane B shows $[^{3}H]$ryanodine binding to RyR2/FKBP12.6 in the presence (open squares) or absence (open circles) of 5 µM FK506. Data points shown are mean ± SEM from 3-5 separate experiments.

Fig.3. FKBP12.6-removal does not alter the sensitivity of RyR2 to activation by caffeine. $[^{3}H]$ryanodine binding to cell lysate prepared from HEK293 cells expressing RyR2 or expressing both RyR2 and FKBP12.6 was carried out at various caffeine concentrations (0.01 to 20 mM), 5 mM $[^{3}H]$ryanodine, and 500 mM KCl. Panel A shows $[^{3}H]$ryanodine to RyR2 (filled circles) and RyR2/FKBP12.6 (open circles). Pane B shows $[^{3}H]$ryanodine binding to RyR2/FKBP12.6 in the presence (open squares) or absence (open circles) of 5 µM FK506. Data points shown are mean ± SEM from 4 separate experiments.

Fig.4. Effect of FK506 on SOICR in HEK293 cells and in rat cardiac cells. HEK293 cells expressing both RyR2 and FKBP12.6 (panel A) or expressing RyR2 alone (Panel C) were grown on glass coverslips. The cells were loaded with 5 µM fura-2-AM in KRH buffer for 20 minutes at room temperature (22 °C). Fura-2 ratios of representative cells in the presence of 2 mM external Ca$^{2+}$ before and after the addition of 5 µM FK506 are shown. Panel B, freshly isolated rat ventricular myocytes were loaded with Fluo-4-AM in KRH buffer for 20 minutes at room temperature (22 °C). Fluo-4 intensities of a representative cell in the presence of 6 mM external Ca$^{2+}$ before and after the addition of FK506 (5 µM) are shown. Panels D and E show the impact of FK506 on the amplitude and frequency of Ca$^{2+}$ oscillations in HEK293 cells expressing both RyR2 and FKBP12.6 or RyR2 alone in rat cardiac myocytes. The amplitude and frequency were determined during the last 5-min of FK506 treatment and normalized to those during the control recordings before FK506 treatment (100%). A number of RyR2/FKBP12.6 (n=172), RyR2 (n=180), and rat cardiac (n=74) cells from 4-5 separate experiments were analyzed. Values shown are mean ± SEM (*, P < 0.02-0.0001). Panel F, Western blot showing the interaction between RyR2 and FKBP12.6 at various induction times with tetracycline. The FKBP12.6/RyR2 complex was immunoprecipitated using the anti-RyR antibody, followed by immunoblotting with the anti-RyR (top panel) and anti-FKBP12/12.6 (bottom panel) antibodies. Loadings to the SDS-PAGE gel were adjusted in an attempt to achieve similar loadings of RyR2 in each lane for easy comparison of the levels of associated FKBP12.6 between different induction times. Data shown are representative of 3 separate experiments.

Fig.5. The lack of FKBP12.6 does not alter the SOICR properties. A, HEK293 cells expressing RyR2 alone (left) or both RyR2 and FKBP12.6 (right) were induced by tetracycline for different lengths of time (12-48 h). RyR2 and FKBP12.6 proteins from the same amount of cell lysate were immunoblotted with the anti-RyR antibody (top) or anti-FKBP12.0/12.6 antibody (bottom). Note that FKBP12.6 is
absence in HEK293 cells expressing RyR2 alone. Fura-2 ratios of single RyR2/FKBP12.6 cells induced for 24 h (B) and of single RyR2 cells induced for 30 h (C) at elevated [Ca$^{2+}$]$_o$ (0.1-2.0 mM) are shown. (D) The fraction (%, mean ± SEM) of RyR2 cells (open circles) and RyR2/FKBP12.6 cells (filled circles) that displayed Ca$^{2+}$ oscillations at various [Ca$^{2+}$]$_o$. The total numbers of cells analyzed for Ca$^{2+}$ oscillations were 403 for RyR2 and 508 for RyR2/FKBP12.6. (E) Store Ca$^{2+}$ contents in HEK293 cells expressing RyR2 alone or expressing both RyR2 and FKBP12.6 were determined by measuring the amplitude of caffeine (10 mM)-induced Ca$^{2+}$ release, while the frequency of Ca$^{2+}$ oscillations was estimated from the number of peaks in the presence of 2 mM [Ca$^{2+}$]$_o$. Data shown are mean ± SEM from 4 separate experiments.

**Fig.6. Single channel properties of RyR2 from FKBP12.6$^{+/+}$ and FKBP12.6$^{-/-}$ mice.** (A) The I-V relationship of single RyR2 channels activated by 100 µM Ca$^{2+}$ from FKBP12.6$^{+/+}$ (open circles) and FKBP12.6$^{-/-}$ mice (open triangles) is shown. The slope conductances of FKBP12.6$^{+/+}$ and FKBP12.6$^{-/-}$ RyR2 channels were similar (~90 pS at 0 mV). Panel B shows examples of subconductance states, which were rarely observed in either WT or KO channels (<0.05% of channels openings, see text). Openings to full conductance state (o) and to subconductance states (s) and the closed state (c) are indicated. Panel C shows the sensitivity of RyR2 from FKBP12.6$^{+/+}$ (open circles) and FKBP12.6$^{-/-}$ mice (open triangles) to activation by Ca$^{2+}$. Data points shown are mean ± SEM (n=4, each). The Mg$^{2+}$ dependent inhibition of single RyR2 channels is shown in panel D. Single FKBP12.6$^{+/+}$ and FKBP12.6$^{-/-}$ RyR2 channels were activated by 5 µM cytosolic Ca$^{2+}$ and were inhibited by various Mg$^{2+}$ concentrations in a similar manner. Values are mean ± SEM (n=4, each). (E) Single FKBP12.6$^{-/-}$ RyR2 channel exhibited low Po at cytosolic [Ca$^{2+}$] of 2 µM in the presence of 2 mM Mg$^{2+}$ and 1 mM ATP (free [Mg$^{2+}$] ~1 mM). Addition of 5 mM caffeine activated the channel (Po ~ 0.9). Subsequent addition of 1 µM FKBP12.6 did not significantly change the Po of the channel (Po ~ 0.86) (n=4). Panel F shows the diary plot of single channel activities shown in pane E.

**Fig.7. FKBP12.6-null mice display no stress-induced ventricular arrhythmias.** WT and FKBP12.6-null mice implanted with ECG telemetric transmitters were injected with epinephrine (2 mg/kg) and caffeine (120 mg/kg). ECG was continuously monitored for 10 min before and 60-90 min after injection. Representative ECG traces from a WT (A) and a FKBP12.6-null mouse (B) before and after the injection are shown. The number of mice tested was 9 WT and 9 FKBP12.6 KO.
Figure 1

A) RyR2 (wt)/FKBP12.6
   a) Control
      Po = 0.147, To = 1.72 ms, Tc = 9.89 ms
      100 ms
      20 pA

   b) +5 μM FK506
      Po = 0.148, To = 1.68 ms, Tc = 9.39 ms
      -20 mV (cyto)

B) RyR2 (wt) (with no FKBP12.6)
   a) Control
      Po = 0.270, To = 2.04 ms, Tc = 5.55 ms
      100 ms
      20 pA

   b) +5 μM FK506
      Po = 0.273, To = 1.78 ms, Tc = 5.32 ms
      -20 mV (cyto)

C) Canine RyR2
   a) Control
      Po = 0.177, To = 1.92 ms, Tc = 8.75 ms
      100 ms
      20 pA

   b) +5 μM FK506
      Po = 0.209, To = 2.02 ms, Tc = 7.16 ms
      -20 mV (cyto)

D) WB
   RyR2
   FKBP12.6
   FK506
   Cardiac Microsomes
Figure 2

A

[\text{\[^3H\]Ryanodine Bound (\%)}]

\[ \text{pCa} \]

- RyR2 (wt)
- RyR2 (wt)/FKBP12.6

B

[\text{\[^3H\]Ryanodine Bound (\%)}]

\[ \text{pCa} \]

- RyR2 (wt)/FKBP12.6
  - -FK506
  - +FK506
Figure 3

A

$[^3]H$Ryanodine Bound (%)

RyR2 (wt)

RyR2 (wt)/FKBP12.6

Caffeine (mM)

0.01 0.1 1.0 10 100

0 20 40 60 80 100

B

RyR2 (wt)/FKBP12.6

$[^3]H$Ryanodine Bound (%)

-FK506

+FK506

Caffeine (mM)

0.01 0.1 1.0 10 100

0 20 40 60 80 100
Figure 5

A

RyR2 expressing cells

RyR2/FKBP12.6 co-expressing cells

Anti-RyR2

Anti-FKBP

12 18 24 30 36 42 48hr

12 18 24 30 36 42 48hr

B

HEK293 cells (RyR2/FKBP12.6)

[calf] 10 mM

0.1 0.2 0.3 0.5 1.0 2.0

[C\textsuperscript{2+}]\textsubscript{o} (mM)

Fura-2 Ratio

Time (second)

0 200 400 600 800 1000 1200 1400 1600 1800 2000 2200

C

HEK293 cells (RyR2)

[calf] 10 mM

0.1 0.2 0.3 0.5 1.0 2.0

[C\textsuperscript{2+}]\textsubscript{o} (mM)

Fura-2 Ratio

Time (second)

0 200 400 600 800 1000 1200 1400 1600 1800 2000 2200

D

Oscillating cells (%)

\textbullet RyR2

\textbullet RyR2/ FKBP12.6

\[\text{Ca}^{2+}\textsubscript{o} (\text{mM})\]

0 0.4 0.8 1.2 1.6 2.0

E

Store level (%)

Freq (%)

RyR2

RyR2/ FKBP

RyR2/ FKBP
Figure 6

A

B

C

D

E

F

(i) FKBP12.6\(^{-/-}\); Mg/ATP

(ii) + caffeine

(iii) + FKBP12.6

(i) 

(ii) 

(iii)
Figure 7

A  WT mice

Before

After

B  FKBP12.6 KO mice

Before

After
Removal of FKBP12.6 does not alter the conductance and activation of cardiac ryanodine receptor and the susceptibility to stress-induced ventricular arrhythmias

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