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FKBP12.6 Deficiency and Defective Calcium Release Channel (Ryanodine Receptor) Function Linked to Exercise-Induced Sudden Cardiac Death

Xander H.T. Wehrens,[1,5] Stephan E. Lehnart,[1,5] Fannie Huang,[1] John A. Vest,[1] Steven R. Reiken,[1] Peter J. Mohler,[2] Jie Sun,[1] Silvia Guatimosim,[3] Long-Sheng Song,[3] Nora Rosemblit,[1] Jeanine M. D'Armiento,[1] Carlo Napolitano,[4] Mirella Memmi,[4] Silvia G. Priori,[4] W.J. Lederer,[3] and Andrew R. Marks[1,*]

[1]Department of Physiology and Cellular Biophysics
Center for Molecular Cardiology
Department of Medicine
Columbia University College of Physicians and Surgeons
New York, New York, 10032
[2]Howard Hughes Medical Institute
Department of Cell Biology
Duke University Medical Center
Durham, North Carolina 27710
[3]Medical Biotechnology Center
University of Maryland Biotechnology Institute
Department of Physiology
School of Medicine
University of Maryland
Baltimore, Maryland 21201
[4]Molecular Cardiology
IRCCS Fondazione S. Maugeri
University of Pavia
Via Ferrata 8
27100 Pavia
Italy

Summary

Arrhythmias, a common cause of sudden cardiac death, can occur in structurally normal hearts, although the mechanism is not known. In cardiac muscle, the ryanodine receptor (RyR2) on the sarcoplasmic reticulum releases the calcium required for muscle contraction. The FK506 binding protein (FKBP12.6) stabilizes RyR2, preventing aberrant activation of the channel during the resting phase of the cardiac cycle. We show that during exercise, RyR2 phosphorylation by cAMP-dependent protein kinase A (PKA) partially dissociates FKBP12.6 from the channel, increasing intracellular Ca^{2+} release and cardiac contractility. *FKBP12.6*$^{-/-}$ mice consistently exhibited exercise-induced cardiac ventricular arrhythmias that cause sudden cardiac death. Mutations in RyR2 linked to exercise-induced arrhythmias (in patients with catecholaminergic polymorphic ventricular tachycardia [CPVT]) reduced the affinity of FKBP12.6 for RyR2 and increased single-channel activity under conditions that simulate exercise. These data suggest that "leaky" RyR2 channels can trigger fatal cardiac arrhythmias, providing a possible explanation for CPVT.

*Correspondence: arm42@columbia.edu
[5]These authors contributed equally to this work.

Introduction

Ventricular arrhythmias in the heart can be rapidly fatal, a phenomenon referred to as sudden cardiac death. Sudden cardiac death is associated with common cardiac diseases, most notably heart failure, in which approximately 50% of patients die from fatal cardiac arrhythmias. However, these fatal arrhythmias can also occur in young, otherwise healthy individuals without known structural heart disease. In structurally normal hearts, the most common mechanism for induction and maintenance of ventricular tachycardia is abnormal automaticity. One form of abnormal automaticity, known as "triggered arrhythmias," is associated with aberrant release of sarcoplasmic reticulum (SR) Ca^{2+} that initiates delayed after depolarizations (DADs) (Fozzard, 1992; Wit and Rosen, 1983). DADs, which can trigger fatal ventricular arrhythmias, are abnormal depolarizations in cardiomyocytes that occur after repolarization of a cardiac action potential. The molecular basis for abnormal SR Ca^{2+} release that causes DADs has not been fully elucidated. DADs are, however, known to be blocked by ryanodine, providing evidence that RyR2 may play a key role in the pathogenesis of this aberrant Ca^{2+} release (Marban et al., 1986; Song and Belardinelli, 1994).

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is an arrhythmogenic disorder of the heart characterized by stress-induced bidirectional or polymorphic ventricular tachycardia that leads to sudden cardiac death in the absence of structural heart disease (Laitinen et al., 2001; Leenhardt et al., 1995; Priori et al., 2002, 2001; Swan et al., 1999). CPVT is predominantly inherited in an autosomal-dominant fashion. Affected individuals present during childhood or adolescence with repetitive exercise-induced syncopal events with 30%–50% mortality by age 30 (Fisher et al., 1999; Swan et al., 1999). Linkage studies and direct sequencing have identified mutations in the human cardiac ryanodine receptor gene (*hRyR2*) on chromosome 1q42-q43 in individuals with CPVT (Laitinen et al., 2001; Priori et al., 2001; Swan et al., 1999). Importantly, individuals with CPVT have ventricular arrhythmias when subjected to exercise testing, but they do not have these arrhythmias at rest.

RyR2 is the major Ca^{2+} release channel required for excitation-contraction coupling in cardiac muscle. During excitation-contraction coupling, depolarization of the cardiac muscle cell membrane in phase zero of the action potential (during the upstroke) activates voltage-gated Ca^{2+} channels. Ca^{2+} influx through these channels in turn initiates Ca^{2+} release via RyR2 from the sarcoplasmic reticulum. This process is known as Ca^{2+}-induced Ca^{2+} release (Fabiato, 1983; Nabauer et al., 1989). RyR2-mediated Ca^{2+}-induced Ca^{2+} release activates the contractile proteins, which are responsible for cardiac muscle contraction.

During exercise, sympathetic nervous system stimulation leads to catecholaminergic activation of β-adrenergic receptors (β-AR) in the heart. β-ARs are coupled via G proteins to the activation of adenylyl cyclase that

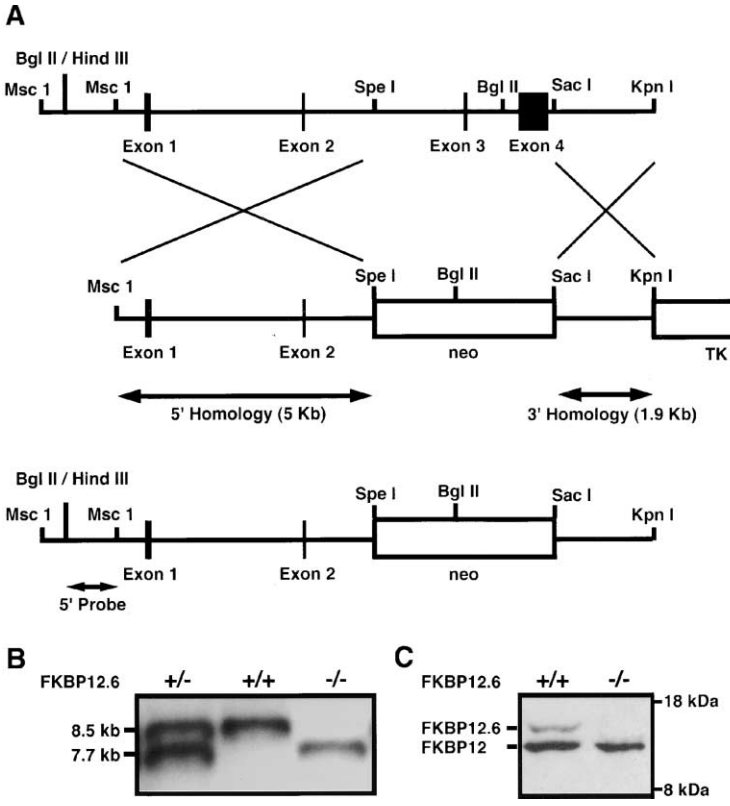

Figure 1. Generation of *FKBP12.6*$^{-/-}$ Mice

(A) The mouse FKBP12.6 gene (top), targeting vector (middle), and homologous recombinant mutant allele (bottom). A 3.5 kb Spe I-Sac I segment that includes exons 3 and 4 was replaced with a neomycin-resistance (neo) expression cassette.

(B) Southern blot analyses of FKBP12.6 heterozygous (+/−) or homozygous (−/−) and wild-type mouse (+/+).

(C) FKBP12/12.6 immunoblot of heart homogenate from *FKBP12.6*$^{+/+}$ and *FKBP12.6*$^{-/-}$ mice.

increases intracellular cAMP concentration that in turn activates cAMP-dependent protein kinase A (PKA). PKA phosphorylation of RyR2 causes the release of the FK506 binding protein (FKBP12.6) from the channel complex. RyR2 is a tetrameric channel, and each RyR2 monomer binds one FKBP12.6. Phosphorylation of RyR2 (Valdivia et al., 1995) and depletion of FKBP12.6 from the RyR2 macromolecular complex result in an increase in RyR2 channel open probability (Marx et al., 2000).

In the present study, we show that FKBP12.6-deficient (*FKBP12.6*$^{-/-}$) mice consistently exhibited exercise-induced ventricular arrhythmias that are similar to those observed in CPVT patients. Moreover, termination of SR Ca^{2+} release was defective in cardiomyocytes from *FKBP12.6*$^{-/-}$ mice (Xin et al., 2002), and cardiomyocytes from *FKBP12.6*$^{-/-}$ mice exhibited DADs following catecholamine application that can trigger extrasystoles and cardiac arrhythmias. RyR2 channels from *FKBP12.6*$^{-/-}$ mice exhibited increased open probability during exercise compared to channels from *FKBP12.6*$^{+/+}$ mice.

We also report the biophysical properties of three distinct RyR2 mutations linked to CPVT in individuals who manifest exercise-induced ventricular arrhythmias and have clinical features consistent with triggered arrhythmias known to be associated with DADs. All three mutant RyR2s exhibited reduced affinity for FKBP12.6 and increased open probability compared to wild-type (wt) channels. Importantly, the increased channel activity compared to wt was only observed after PKA phosphorylation of the channels (conditions that mimic the effect of exercise on the channel). This is in agreement with the clinical phenotype as individuals with these mutant RyR2 only have arrhythmias when they exercise.

Together, these studies provide a link between altered RyR2 channel function and ventricular arrhythmias and suggest that defective RyR2 channel function due to depletion of FKBP12.6 from the channel macromolecular complex may be one molecular mechanism underlying the aberrant SR Ca^{2+} release that can cause DADs and triggered ventricular arrhythmias.

Results

Exercise-Induced Arrhythmias in *FKBP12.6*$^{-/-}$ Mice

FKBP12.6$^{-/-}$ mice were generated by homologous recombination, resulting in the absence of FKBP12.6 protein in the heart (Figure 1). The hearts of male and female *FKBP12.6*$^{-/-}$ mice were structurally normal based on echocardiography and histology (including absence of fibrosis, see Supplemental Figure S1 online at http://www.cell.com/cgi/content/full/113/7/829/DC1). Examination of ECG parameters of conscious *FKBP12.6*$^{+/+}$ and *FKBP12.6*$^{-/-}$ mice revealed no significant differences in RR (+/+, 97.3 ± 5 ms; −/−, 92.1 ± 4 ms), PR (+/+, 36.2 ± 2.1 ms; −/−, 35.4 ± 2.0 ms), QRS (+/+, 17.4 ± 1.8 ms; −/−, 16.4 ± 1.4 ms), or rate corrected QT intervals (QTc; +/+, 56.7 ± 3.0 ms; −/−, 55.1 ± 4.0 ms; n = 10 [+/+] and n = 6 [−/−], not significant). Thus, *FKBP12.6*$^{-/-}$ mice show no structural cardiac abnormalities and no ECG abnormalities or arrhythmias at rest (Figures 2A and 2B).

To test for cardiac arrhythmias, *FKBP12.6*$^{+/+}$ and *FKBP12.6*$^{-/-}$ mice were subjected to an exercise proto-

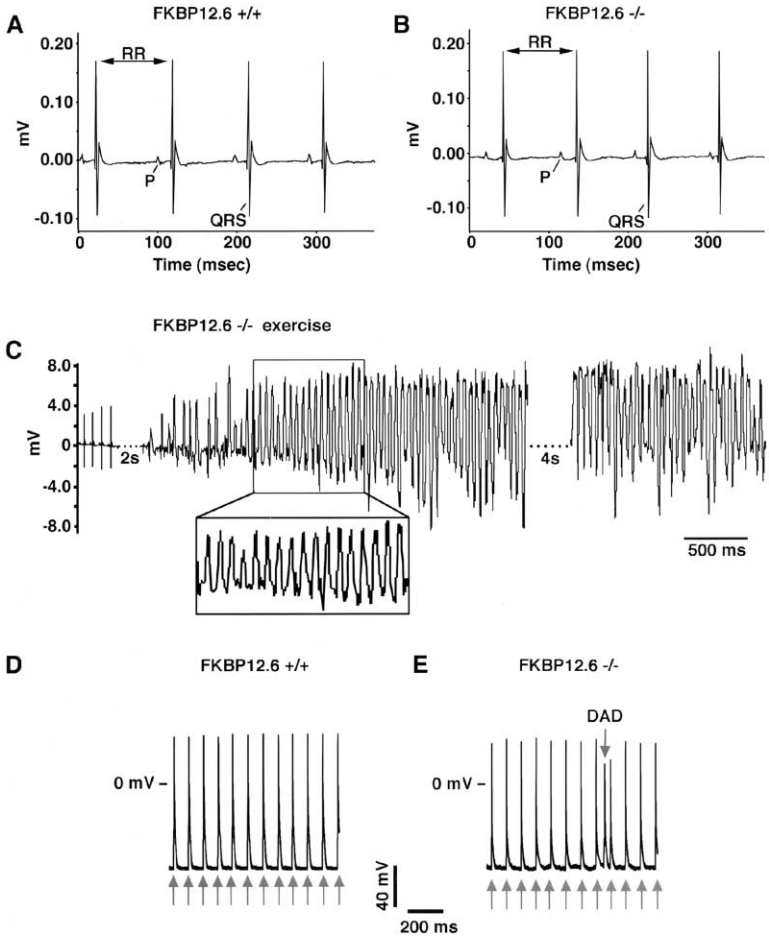

Figure 2. Exercise-Induced Fatal Ventricular Arrhythmias and DADs in *FKBP12.6*$^{-/-}$ Mice

(A and B) There were no significant ECG abnormalities comparing conscious *FKBP12.6*$^{+/+}$ mice to *FKBP12.6*$^{-/-}$ mice.

(C) ECG following exercise and epinephrine. Following strenuous exercise and injection with epinephrine (~8 min before the trace), FKBP12.6$^{-/-}$ mouse displayed polymorphic ventricular tachycardia followed by death 4 min following this trace. This stress test resulted in ventricular arrhythmias in 8/8 (100%) of male (n = 3) and female (n = 5) *FKBP12.6*$^{-/-}$ mice, compared to 0/6 (0%) of the *FKBP12.6*$^{+/+}$ mice.

(D and E) Action potentials were recorded in (D) *FKBP12.6*$^{+/+}$ (n = 19) and (E) *FKBP12.6*$^{-/-}$ (n = 38) cardiomyocytes during rapid pacing. Action potentials were produced in patch-clamped single cardiomyocytes stimulated by brief current injections at 12 Hz in current clamp mode. Upward arrows at the bottom of (D) and (E) indicate the timing of the induced action potentials. After depolarizations, as the one shown in (E), were observed in *FKBP12.6*$^{-/-}$ cardiomyocytes (n = 6) from four *FKBP12.6*$^{-/-}$ mice. The downward arrow shows a DAD that produced an extrasystole in a FKBP12.6$^{-/-}$ cardiomyocyte.

col that has been used previously (Mohler et al., 2003). While no *FKBP12.6*$^{+/+}$ mice (0 of 10) displayed arrhythmias or syncopic events during the protocol, two of eight *FKBP12.6*$^{-/-}$ mice became unresponsive for 2–5 s after strenuous exercise, and all of the *FKBP12.6*$^{-/-}$ mice (8/8) died following exercise plus epinephrine injection. Following exercise, when epinephrine was administered, no wt mice had arrhythmias with this stress protocol, whereas 100% (8/8) of the *FKBP12.6*$^{-/-}$ mice had fatal arrhythmias consisting of a progression from a sinus rhythm (heart rate ~700–850 bpm) with episodes of polymorphic ventricular arrhythmias (heart rate >1200 bpm) to sustained ventricular arrhythmias (Figure 2C). Before death, two of the mice displayed significant prolonged polymorphic arrhythmias (>6 s), four displayed multiple short (0.5–2 s) runs of ventricular arrhythmia, and two mice died without ECG monitoring. A previous report using a different *FKBP12.6*$^{-/-}$ mouse model showed that male mice had a cardiomyopathy, and in agreement with our findings, no cardiac arrhythmias were observed in nonexercised animals (Xin et al., 2002). The effects of exercise were not reported. Moreover, male mice in the Xin et al. (2002) study had significant hypertension that likely contributed to cardiac hypertrophy. There was no evidence of hypertension or cardiac hypertrophy in our *FKBP12.6*$^{-/-}$ mice, nor any differences between males and females (differences between the Xin et al. mice and ours could be due to distinct genetic backgrounds, 129SvEv versus DBA in the present study).

Delayed after Depolarizations in *FKBP12.6*$^{-/-}$ Cardiomyocytes

To determine whether or not FKBP12.6 deficiency is associated with increased risk of delayed after depolarizations (DADs) that can trigger arrhythmias, we examined cardiomyocytes isolated from *FKBP12.6*$^{-/-}$ and control *FKBP12.6*$^{+/+}$ mice with patch clamp. There were no significant changes of the single-cell action potential (AP) in myocytes from the hearts of both genotypes. However, after depolarizations were observed in *FKBP12.6*$^{-/-}$ cardiomyocytes from four *FKBP12.6*$^{-/-}$ mice examined under conditions that simulate exercise (i.e., following the application of isoproterenol [1 μM] or epinephrine [1 μM], and stimulation with action potentials at 12 Hz to correspond to the heart rate of ~700–750 bpm, at which fatal exercise-induced cardiac arrhythmias occurred in *FKBP12.6*$^{-/-}$ mice) (Figures 2D–2E). Since both *FKBP12.6*$^{+/+}$ and *FKBP12.6*$^{-/-}$ hearts were subjected to increased SERCA2 activity (due to PKA-dependent phosphorylation of phospholamban, data not shown) and increased L type Ca^{2+} channel activity (due to PKA-dependent phosphorylation), and both genotypes were subjected to RyR2 phosphorylation by PKA, the remaining difference was the absence of FKBP12.6 in the *FKBP12.6*$^{-/-}$ mouse heart cells.

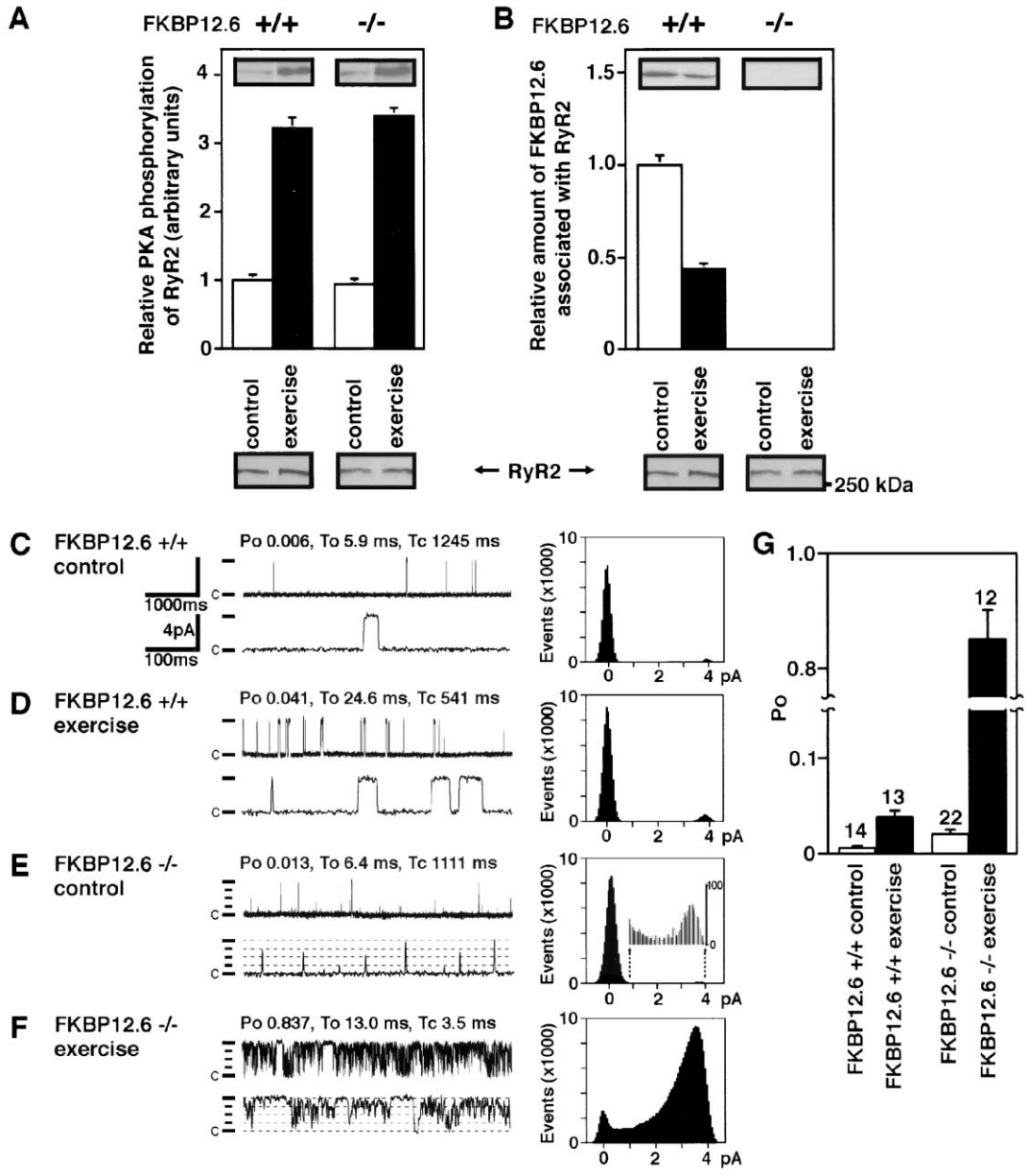

Figure 3. Exercise-Induced Increased Open Probability of RyR2 from *FKBP12.6*$^{-/-}$ Mice

(A) PKA phosphorylation of RyR2 from *FKBP12.6*$^{+/+}$ and *FKBP12.6*$^{-/-}$ mice was increased during exercise. Mice were exercised as described in Experimental Procedures; at the termination of exercise, the heart was rapidly removed and flash frozen. RyR2 was immunoprecipitated from cardiac homogenates and PKA phosphorylation of RyR2 was assessed using a phosphoepitope-specific antibody (anti-RyR2-P2809) that specifically recognizes RyR2 PKA phosphorylated at RyR2-Ser^{2809}. Immunoblots of RyR2 with anti-RyR2-5029 (Jayaraman et al., 1992), an antibody that recognizes the carboxy terminus of RyR2, were used to show that equal amounts of RyR2 protein were in each sample.

(B) FKBP12.6 bound to RyR2 after exercise was decreased (due to PKA phosphorylation of RyR2, which dissociates FKBP12.6 from the channel) in *FKBP12.6*$^{+/+}$ mice. No FKBP12.6 was detected in the RyR2 macromolecular complex in *FKBP12.6*$^{-/-}$ mice.

(C–F) Single-channel studies showing increased open probability of RyR2 from *FKBP12.6*$^{-/-}$ mice compared to *FKBP12.6*$^{+/+}$ after exercise. Channel openings are upward, the dash indicates the level of full openings (4 pA), and the letter "c" indicates the closed state. Channels are shown at compressed (5 s, upper tracing) and expanded (500 ms, lower tracing) time scales, and recordings are at 0mV. Amplitude histograms of *FKBP12.6*$^{+/+}$ and *FKBP12.6*$^{-/-}$ channels revealed increased activity and subconductance openings in resting (see [E] tracing and insert in amplitude histogram) and exercised *FKBP12.6*$^{-/-}$ channels.

(G) Open probabilities (Po) of control and exercised *FKBP12.6*$^{+/+}$ and *FKBP12.6*$^{-/-}$ mice, revealing increased Po in *FKBP12.6*$^{-/-}$ channels after exercise (please note interruption in *y* axis) under conditions (low cytosolic [Ca^{2+}] = 150 nM) that simulate diastole (the resting phase) in the heart. Numbers above each bar indicate how many single channels were measured.

RyR2 Channels from *FKBP12.6*$^{-/-}$ Mice Exhibit Defective Gating during Exercise

Native RyR2 are tetramers comprised of four RyR2 monomers, each of which binds a single FKBP12.6 molecule. FKBP12.6 stabilizes the RyR2 channel in the closed state and reduces its activity (Brillantes et al., 1994; Kaftan et al., 1996). Stimulation of the sympathetic nervous system during exercise causes the release of catecholamines that activate β-AR, which raises cAMP levels and activates protein kinase A in cardiac muscle. PKA phosphorylation of serine 2809 on RyR2 (RyR2-Ser^{2809}) dissociates FKBP12.6 from the channel complex (Marx et al., 2000) and increases the sensitivity of RyR2 to be activated by transient increases in $[Ca^{2+}]_i$ (Valdivia et al., 1995). Therefore, PKA phosphorylation-induced dissociation of FKBP12.6 from RyR2 and the increased sensitivity of RyR2 to $[Ca^{2+}]_i$ (see below) are physiological mechanisms involved in upregulating RyR2 activity (Marx et al., 2000), which increase SR Ca^{2+} release. This occurs as part of a signaling system (the "fight-or-flight" stress response) that increases cardiac output to meet the metabolic demands of exercise.

To determine the effects of exercise on RyR2, PKA phosphorylation of the channel was examined in *FKBP12.6*$^{+/+}$ and *FKBP12.6*$^{-/-}$ mice after exercise. We found that during exercise, RyR2s are PKA phosphorylated (Figure 3A) and, in the case of the *FKBP12.6*$^{+/+}$ mice, partially depleted of FKBP12.6 (Figure 3B). RyR2s from both *FKBP12.6*$^{+/+}$ and *FKBP12.6*$^{-/-}$ were PKA phosphorylated to a similar extent during exercise (Figure 3A). During exercise, the open probabilities (Po) of RyR2 channels were increased, compared to channels from nonexercised animals (Figures 3C–3F). However, *FKBP12.6*$^{-/-}$ RyR2 channels from exercised mice exhibited ~10 fold greater increase in open probability (compare Figures 3D and 3F) compared to age- and sex-matched *FKBP12.6*$^{+/+}$ control animals subjected to the same degree of exercise (Figure 3G). These channels were studied under conditions of low *cis* (cytosolic) $[Ca^{2+}]$ of 150 nM to approximate the conditions in the heart during diastole when the RyR2 channel should have very low open probability to prevent diastolic SR Ca^{2+} leak that can trigger cardiac arrhythmias. Thus, the significant increase in RyR2 open probability under these conditions in the *FKBP12.6*$^{-/-}$ channels suggests that these channels could be leaky during diastole when the mice are exercised.

Exercise-Induced Sudden Cardiac Death Is Linked to Defective RyR2 Gating

The clinical phenotype of CPVT consists of ventricular arrhythmias inducible with exercise stress testing. During exercise, patients may display a typical progression from isolated premature ventricular contractions to polymorphic ventricular tachycardia that may degenerate into ventricular fibrillation and cause sudden cardiac death (Leenhardt et al., 1995; Priori et al., 2002).

To determine whether the exercise-induced arrhythmias in CPVT patients are associated with defects in SR Ca^{2+} release channel function, we expressed three mutant forms of RyR2 corresponding to known CPVT missense mutations (RyR2-S2246L, RyR2-R2474S, and RyR2-R4497C) (Figure 4A). Experiments were performed at a low *cis* (cytosolic) $[Ca^{2+}]$ of 150 nM to approximate the conditions of cardiac muscle during diastole when the heart is relaxed, and RyR2 channels have very low open probability. In the absence of PKA phosphorylation, wt and all three mutant RyR2 had extremely low activity (see upper tracings in Figure 4B). There were no differences in single-channel properties of wt-RyR2 and mutant RyR2 examined over a wide range of *cis* (cytosolic) $[Ca^{2+}]$ from 50 nM to 5 mM (Figure 4B, top right graph).

The findings—that under basal conditions all three CPVT-associated mutant RyR2 exhibited normal single-channel properties, indistinguishable from those of RyR2-wt channels—were not surprising, given that patients with CPVT do not have arrhythmias at rest (Leenhardt et al., 1995; Priori et al., 2002). However, patients with CPVT have exercise-induced arrhythmias (Leenhardt et al., 1995; Priori et al., 2002). To approximate the effects of exercise, which activates PKA through β-AR signaling pathways in cardiomyocytes, we compared the single-channel properties of PKA phosphorylated wt and mutant RyR2 channels in planar lipid bilayers.

PKA phosphorylation significantly increased the activities (open probability; Po) of the wt-RyR2 and CPVT mutant channels (bottom tracings in Figure 4B). The open probabilities of the mutant RyR2 (RyR2-S2246L, RyR2-R2474S, RyR2-R4497C) were, however, significantly higher than those for wt-RyR2 channels (Figure 4C, $n = 9$, $p < 0.05$). Similarly, gating frequencies were significantly higher in the mutant channels ($n = 9$, $p < 0.05$, data not shown). The increased open probabilities and gating frequencies exhibited by the mutant channels indicate that they are more sensitive to activation by PKA. After PKA phosphorylation, both wt and CPVT-associated mutant RyR2 were more sensitive to Ca^{2+}-induced activation at moderate $[Ca^{2+}]_i$ (Figure 4B, bottom right panel), and this effect was exaggerated in the mutant channels, suggesting that they are more active than the wt channels during exercise because they exhibit increased Ca^{2+}-dependent activation at low $[Ca^{2+}]_i$.

To determine the effects of the CPVT mutations on FKBP12.6 binding to RyR2, microsomes were prepared from HEK293 cells expressing wt-RyR2 and three CVPT mutations (RyR2-S2246L, RyR2-R2474S, and RyR2-R4497C). The amount of RyR2 in these microsomes was determined by [^{3}H] ryanodine binding. FKBP12.6 binding curves were obtained by incubating the microsomes with ^{35}S-labeled FKBP12.6. The dissociation constants (K_d) for FKBP12.6 binding to the channels ($n = 3$) was determined from Scatchard analyses (Figure 4D): 108.3 ± 9.1 nM for wt-RyR2, 182.7 ± 8.1 nM for RyR2-S2246L, 215.7 ± 6.0 nM for RyR2-R2474S, and 202.2 ± 11.2 nM for RyR2-R4497C. The significant increase ($p < 0.001$) in K_d indicates that the CVPT mutants have decreased affinity for FKBP12.6 compared to wt channels.

Because patients with CPVT are heterozygous for the mutant RyR2 allele, we also examined the function of heterotetrameric channels by expressing equal amounts of wt-RyR2 and CPVT mutant RyR2 in HEK293 cells. Because the precise composition of any given channel studied in a bilayer experiment cannot be determined, we pooled data from multiple heterotetrameric channels. As with the homotetrameric CPVT-associated mu-

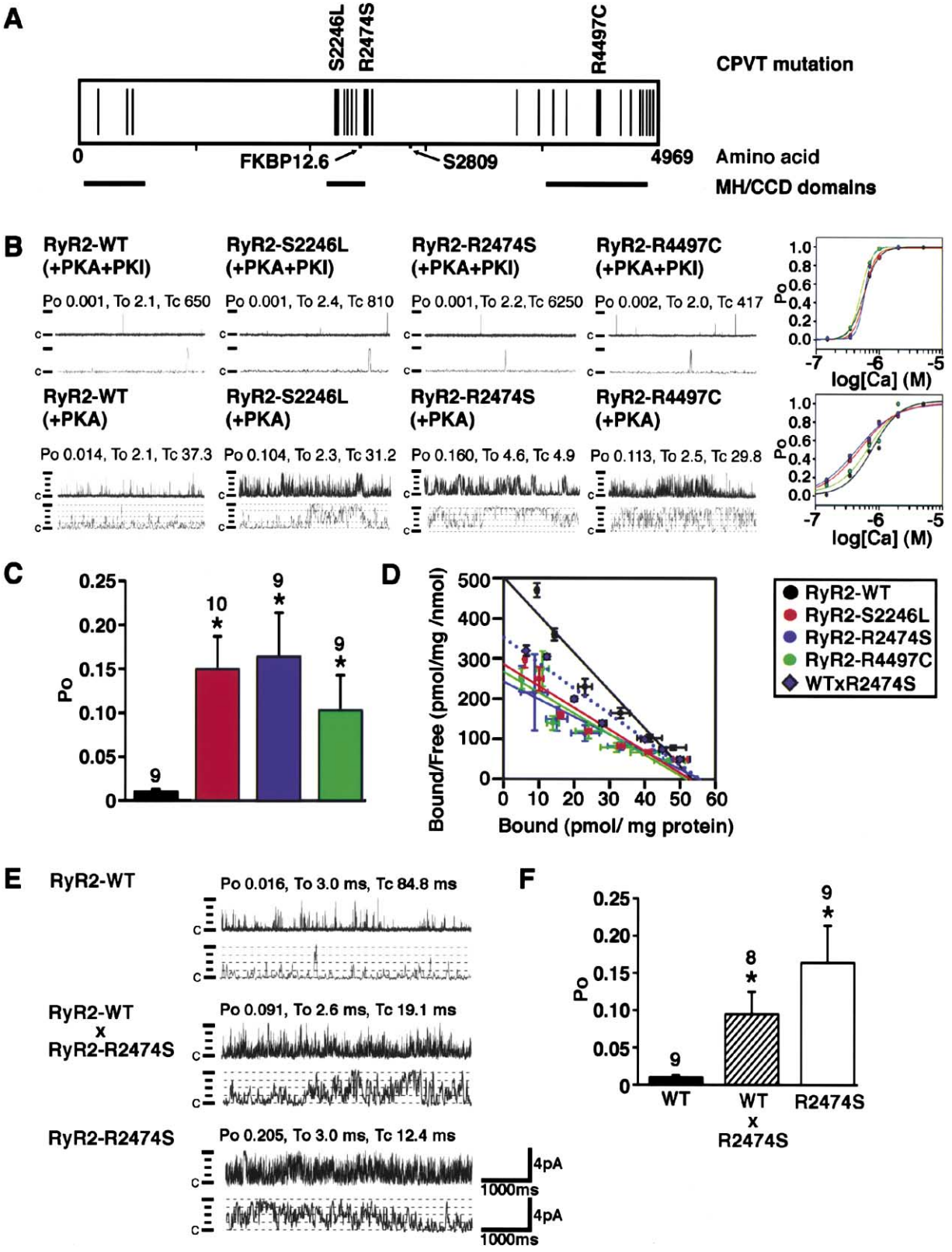

Figure 4. RyR2 Mutations Linked to Exercise-Induced Sudden Cardiac Death Reduce FKBP12.6 Affinity and Increase RyR2 Activation by PKA Phosphorylation.

(A) Location of CPVT-associated RyR2 mutations. CPVT mutations in RyR2 are indicated with thin vertical lines; the three CPVT mutations studied in this paper are indicated with thick vertical lines. FKBP12.6, binding site in RyR2; S2809, PKA phosphorylation site on RyR2. Three regions containing skeletal muscle RyR1 mutations linked to malignant hyperthermia (MH) and central core disease (CCD) are indicated with

tant RyR2 channels, heterotetrameric RyR2 channels also exhibited the same altered single-channel properties, including an increase in open probability after PKA phosphorylation (Figures 4E–4F). In addition, the affinity of FKBP12.6 for the heterotetrameric RyR2-wt × RyR2-R2474S was also reduced (158.3 ± 3.1 nM), compared to RyR2-wt (108.3 ± 9.1 nM for wt, $p < 0.01$). Taken together, these data show that CPVT-associated mutant RyR2 channels exhibit significantly altered single-channel properties compared to wt-RyR2 channels, but only after PKA phosphorylation.

FKBP12.6 Restores Normal Gating to Defective RyR2 Channels

We have previously shown that FKBP12.6 cannot bind to PKA phosphorylated RyR2 (Marx et al., 2001, 2000). We now show that FKBP12.6 also cannot bind to a mutant RyR2-S2809D that mimics constitutively PKA phosphorylated RyR2 (Figure 5A). We wanted to determine whether or not the defective gating in the mutant CPVT-associated RyR2 was due to reduced FKBP12.6 binding. However, since wild-type FKBP12.6 cannot bind to PKA phosphorylated RyR2, we generated a mutant form of FKBP12.6 with serine residue 37 substituted for aspartate acid (FKBP12.6-D37S) that is capable of binding to PKA phosphorylated RyR2 or to the RyR2-S2809D mutant (Figure 5A). We chose to modify the negative charge on Asp37 because this residue is near the hydrophobic binding pocket when FKBP12 is bound to the TGFβ receptor I (Huse et al., 1999) and might be involved in PKA phosphorylation-induced dissociation of FKBP12 or FKBP12.6 from RyR1 or RyR2, respectively, when an additional negative charge is added to the channel after phosphorylation. Indeed, addition of the mutant FKBP12.6-D37S restored normal (low activity) channel function to RyR2-S2809D channels (Figure 5B).

Moreover, in contrast to wild-type FKBP12.6, FKBP12.6-D37S was capable of binding to RyR2 channels isolated from exercised *FKBP12.6*$^{-/-}$ mouse hearts (Figure 5C) and restored normal channel function (Figure 5D). Finally, FKBP12.6-D37S, but not wild-type FKBP12.6, also bound to PKA-phosphorylated CPVT-associated RyR2 mutant channels (Figure 5E) and restored normal channel function (Figure 5F). Thus, we have generated a mutant form of FKBP12.6 that, unlike wild-type FKBP12.6, binds to PKA phosphorylated RyR2 and restores normal channel function. Taken together, these results suggest that partial depletion of FKBP12.6 from the RyR2 macromolecular complex, which occurs physiologically during exercise, is associated with increased RyR2 open probability, but more severe deficiency of FKBP12.6 in the RyR2 complex (such as in the FKBP12.6$^{-/-}$ mouse or patients with the CPVT mutations) can result in channels with significantly increased open probability during diastole that is not observed with wild-type channels from normal hearts.

Discussion

In the present study, we show that both RyR2 from *FKBP12.6*$^{-/-}$ mice that exhibit exercise-induced sudden cardiac death and CPVT-associated mutant RyR2s that are linked to exercise-induced arrhythmias in patients exhibit significantly increased open probabilities under conditions that correspond to diastole in the heart during exercise. These data suggest that in vivo, SR Ca^{2+} leak through PKA phosphorylated, FKBP12.6-depleted RyR2 can occur. Indeed, DADs were observed in cardiomyocytes from *FKBP12.6*$^{-/-}$ mice during simulated exercise. Importantly, RyR2 from *FKBP12.6*$^{-/-}$ mice and CPVT-RyR2 exhibit normal channel function under basal conditions and only show defective function (i.e., increased open probability) when they are examined under conditions that correspond to exercise-induced stimulation. The increased open probabilities of the channels from *FKBP12.6*$^{-/-}$ mice during exercise and the CPVT-associated mutant RyR2, combined with DADs observed in cardiomyocytes from the *FKBP12.6*$^{-/-}$ mice, suggest that SR Ca^{2+} leak via RyR2 can initiate DAD-triggered arrhythmias (Figure 6).

FKBP12.6 Deficiency Causes Exercise-Induced Sudden Cardiac Death

Cardiac ventricular arrhythmias are a major cause of mortality, but the molecular basis for the triggers that initiate arrhythmias is not well understood. In the present study, we have found that RyR2 channels from exercised *FKBP12.6*$^{-/-}$ mice display dramatically increased open probabilities that were not observed in exercised *FKBP12.6*$^{+/+}$ mice. These data suggest that the remaining FKBP12.6 in the RyR2 channel complexes from exercised *FKBP12.6*$^{+/+}$ mice (Figure 5B) suffice to keep

thick gray lines. CPVT and MH/CCD mutations occur in highly homologous regions of RyR2 and RyR1, respectively, suggesting that common defects in SR Ca^{2+} release may play a role in these disorders.

(B) CPVT mutant RyR2 channels exhibit altered channel activity only under conditions that simulate exercise. Single-channel recordings of wt and CPVT mutant RyR2 expressed in HEK293 cells treated with PKA plus the PKA inhibitor PKI_{5-24}. RyR2-wt and CPVT-associated mutant channels exhibit low activity under basal conditions with *cis* (cytosolic) [Ca^{2+}] of 150 nM. PKA phosphorylated CPVT mutant RyR2 exhibit increased activity compared to RyR2-wt channels. A minimum of 8–12 channels from at least two separate preparations were recorded for the RyR2-wt and each mutant RyR2 channel for each condition shown. Right-hand panels show normalized Po plotted as a function of cytosolic [Ca^{2+}].

(C) Po of RyR2-wt and CPVT-associated mutant RyR2 channels after PKA phosphorylation; the asterisk indicates $p < 0.05$; numbers above each bar indicate how many single channels were measured.

(D) ^{35}S-labeled FKBP12.6 binding to microsomes containing RyR2-wt and CPVT-associated mutant RyR2 channels, indicating decreased FKBP12.6 affinity for the CPVT-associated mutant RyR2 channels.

(E) RyR2-wt and one of the CPVT-associated mutant RyR2 (RyR2-R2474S) were expressed as homo- or heterotetrameric channels. Representative single-channel traces of RyR2-wt, RyR2-R2474S, and heterotetrameric wt × R2474S channels are shown, revealing increased activity of wt × R2474S channels after PKA phosphorylation.

(F) Average open probabilities for RyR2-wt, wt × R2474S, and RyR2-R2474S channels after PKA phosphorylation; asterisk indicates $p < 0.05$.

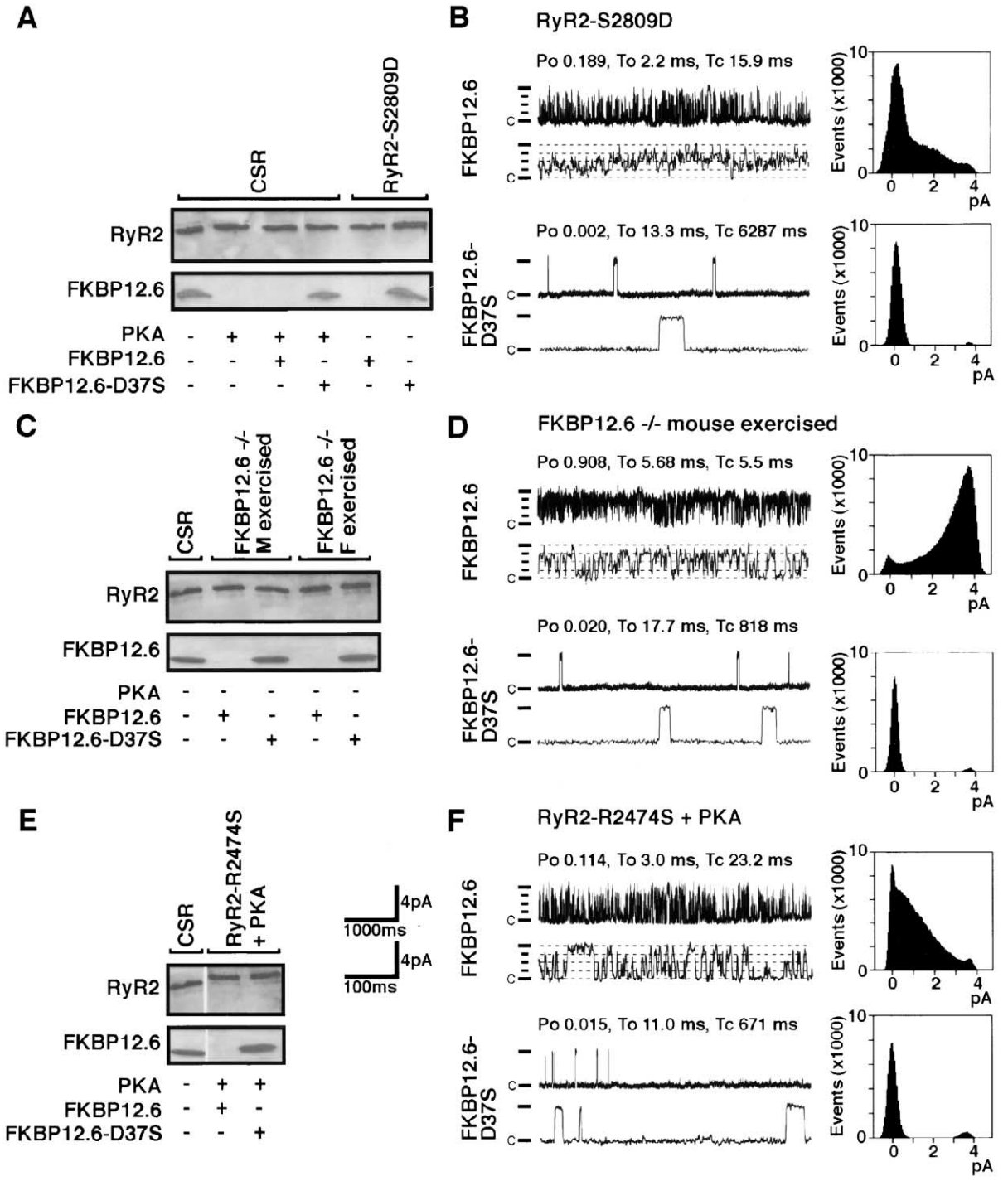

Figure 5. A Mutant FKBP12.6-D37S Restores Normal Single-Channel Function to Channels from Exercised *FKBP12.6*$^{-/-}$ Mice and PKA-Phosphorylated CPVT Mutant Channels

(A) Immunoblot showing equivalent amounts of RyR2 immunoprecipitated from murine cardiac SR (CSR) and recombinant RyR2-S2809D expressed in HEK293 cells, using anti-RyR-5029 antibody that recognizes the carboxy terminus of RyR (Jayaraman et al., 1992). Immunoblot of FKBP12.6 showing dissociation of FKBP12.6 from RyR2 following PKA phosphorylation. Wild-type FKBP12.6 (final concentration ~250 nM) does not bind to PKA phosphorylated RyR2. The mutant FKBP12.6-D37S (~250 nM) binds to PKA phosphorylated RyR2 channels. RyR2-S2809D mimics constitutively PKA-phosphorylated RyR2 channels and, therefore, does not bind FKBP12.6. However, mutant FKBP12.6-D37S binds RyR2-S2809D channels.

(B) Single-channel tracings of RyR2-S2809D channels treated with FKBP12.6 (top; n = 10) and mutant FKBP12.6-D37S (bottom; n = 9), showing increased open probability of RyR2-S2809D channels, similar to PKA-phosphorylated RyR2 channels (Marx et al., 2000). Binding of FKBP12.6-D37S to RyR2-S2809D channels rescued normal channel gating and decreased open probability (bottom). Amplitude histograms are shown on the right, revealing the presence of partial openings or subconductance states in the FKBP12.6-treated channels, but not in the presence of FKBP12.6-D37S.

PKA phosphorylated RyR2 channels closed during diastole. Previous studies examining the phenotype of an independently generated *FKBP12.6*$^{-/-}$ mouse reported defects in cardiomyocyte Ca^{2+} signaling, but exercise-induced arrhythmias were not studied (Xin et al., 2002). Our results indicate that the absence of FKBP12.6 in the RyR2 channel complex predisposes mice to DADs, ventricular arrhythmias, and sudden cardiac death during exercise and stimulation of the β-AR pathway.

We have previously shown that PKA phosphorylation of RyR2 causes dissociation of FKBP12.6 from the channel complex (Marx et al., 2000). In this study, we confirmed this finding using a mutant channel, RyR2-S2809D, that mimics constitutively PKA phosphorylated RyR2 and, therefore, cannot bind FKBP12.6 (Figures 5A and 5B). The finding that mutant FKBP12.6-D37S is able to bind PKA phosphorylated RyR2 or RyR2-S2809D is consistent with the model that charge repulsion secondary to the addition of the negatively charged phosphate group to RyR2-S2809 causes dissociation of FKBP12.6 from the channel complex.

The finding that FKBP12.6-D37S could rescue the (low activity) channel phenotype in RyR2 from exercised *FKBP12.6*$^{-/-}$ mice or CPVT mutant RyR2 channels strongly suggests that the absence of FKBP12.6 following PKA phosphorylation causes RyR2 channel hyperactivity. The absence of FKBP12.6 may trigger arrhythmias in *FKBP12.6*$^{-/-}$ mice and patients with CPVT-associated mutations in RyR2. Moreover, our results suggest that increasing FKBP12.6 binding to phosphorylated RyR2 channels may provide a novel and very specific therapeutic strategy to prevent triggered arrhythmias in CPVT and heart failure.

PKA Phoshorylation of RyR2 Is Part of the Fight-or-Flight Response

PKA phosphorylation of RyR2 occurs as part of an important physiological stress pathway known as the fight-or-flight response (Marks, 2000). This signaling pathway provides a mechanism whereby sympathetic nervous system activation in response to exercise or stress results in enhanced cardiac output required to meet the metabolic demands of the relevant stress. Sympathetic nervous system stimulation during exercise causes the release of catecholamines that activate β-AR, which raises intracellular cAMP levels and, hence, activates PKA in cardiac muscle. PKA phosphorylation of RyR2 at Ser^{2809} dissociates FKBP12.6 from the channel complex and provides a physiological mechanism involved in upregulating RyR2 activity (Marx et al., 2000) to increase SR Ca^{2+} release in response to exercise or stress.

CPVT RyR2 Mutations Linked to Exercise-Induced Sudden Cardiac Death

Although CPVT-associated RyR2 mutations exhibit reduced binding of FKBP12.6 to RyR2, these mutant RyR2 channels were able to bind FKBP12.6 under basal conditions. This finding is consistent with the fact that CPVT patients do not exhibit arrhythmias under resting conditions. Since PKA phosphorylation-induced dissociation of FKBP12.6 is part of the mechanism by which RyR2 channels are activated during exercise, the reduced affinity of FKBP12.6 likely plays a role in the increased sensitivity of mutant channels to activation by PKA. Indeed, following PKA phosphorylation, the CPVT-associated RyR2 mutations examined in this study resulted in channels that have increased activities compared to wt channels examined under the same conditions. Thus, during exercise, PKA phosphorylation of CPVT mutant RyR2 increases the probability of after depolarizations that can trigger arrhythmias (Figure 6).

We found that under basal conditions, CPVT-associated RyR2 channels exhibited normal activity, in contrast to a previous report that the RyR2-R4497C mutant had increased basal activity at very low [Ca^{2+}] (Jiang et al., 2002). We did not observe any difference in channel function even at a [Ca^{2+}] of 50 nM, which is below that of resting cardiomyocytes or during diastole when cardiac muscle is relaxed. The fact that in the present study mutant RyR2 only exhibited defects after PKA phosphorylation, corresponding to the condition of the channels during exercise, supports the relevance of these specific alterations in channel function to the exercise-induced arrhythmias in CPVT patients. Moreover, it is difficult to understand how any defect found in the CPVT-associated RyR2 channels under nonexercise conditions can be linked to SCD in these patients because they never have arrhythmias at rest.

Delayed after Depolarizations Associated with Leaky Ryanodine Receptors

DADs are oscillations in the plasma membrane potential occurring after completion of the cardiac action potential that are caused by aberrant SR Ca^{2+} release, resulting in a Ca^{2+}-activated transient inward (depolarizing) current (I_{ti}) (Fozzard, 1992; Wit and Rosen, 1983). Previous pharmacological studies, showing that ryanodine specifically inhibits DADs, suggest that they may

(C) Immunoblots of RyR2 and FKBP12.6 of CSR from two exercised male (M) and two female (F) *FKBP12.6*$^{-/-}$ mice. Whereas wild-type FKBP12.6 does not, FKBP12.6-D37S does bind to RyR2 isolated from exercised *FKBP12.6*$^{-/-}$ mouse hearts.

(D) Single-channel tracings of RyR2 channels isolated from exercised *FKBP12.6*$^{-/-}$ hearts treated with wild-type FKBP12.6 (n = 14), showing high open probability and subconductance states (see histogram on the right). Binding of FKBP12.6-D37S completely rescued the channel phenotype (n = 9): open probability is dramatically decreased, and no subconductance openings were observed.

(E) Immunoblots of recombinant RyR2-R2474S expressed in HEK293 cells. FKBP12.6-D37S also binds to PKA-phosphorylated CPVT-associated mutant RyR2-R2474S channels.

(F) Single-channel tracings of CPVT-associated mutant RyR2-R2474S channels after PKA phosphorylation and treatment with wild-type FKBP12.6 (n = 13), revealing high open probability and the presence of subconductance states (see amplitude histogram). Binding of FKBP12.6-D37S completely normalized channel gating and open probability (n = 11). For each tracing, channel openings are upward, the dash indicates the level of full openings (4 pA), and the letter "c" indicates the closed state. Channels are shown at compressed (5 s, upper tracing) and expanded (500 ms, lower tracing) time scales, and recordings are at 0mV.

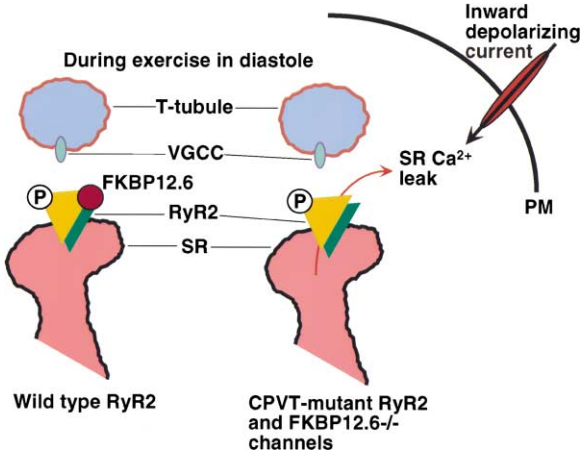

Figure 6. Model of Mechanism by Which SR Ca^{2+} Leak May Initiate Delayed after Depolarizations that Trigger Cardiac Arrhythmias

During EC coupling in cardiac muscle RyR2 on the SR is activated by the influx of Ca^{2+} through the voltage-gated calcium channel (VGCC) on the transverse tubule (T tubule). In response to stress/exercise-induced activation of the sympathetic nervous system, RyR2 is PKA phosphorylated, which causes dissociation of FKBP12.6 from the channel and increases its open probability. This is part of a normal physiological stress response (e.g., fight or flight). In normal hearts, the wild-type RyR2 is able to remain tightly shut, even when it is physiologically PKA phosphorylated, during diastole when the heart muscle is relaxed so that it can refill with blood in preparation for the next contraction (systole). This is necessary to prevent SR Ca^{2+} leak during diastole, which can initiate DADs that can trigger cardiac arrhythmias (Fozzard, 1992; Wit and Rosen, 1983). In the case of the CPVT-associated RyR2 mutant channels, increased activity (increased open probability) of the channels when they are PKA phosphorylated (due to decreased binding affinity of FKBP12.6) increases the probability of aberrant RyR2 opening during diastole, which can activate DADs due to inward depolarizing currents, possibly through the sodium/calcium exchanger, which are triggers for cardiac arrhythmias (Pogwizd et al., 1998). Similar mechanisms may underlie cardiac arrhythmias in heart failure in which structurally normal RyR2 are PKA hyperphosphorylated, resulting in defective channel function (Marx et al., 2000) that may promote SR Ca^{2+} leak that can trigger arrhythmias (Pogwizd et al., 2001). The likelihood of cardiac arrhythmias triggered by activation of inward depolarizing currents via the sodium/calcium exchanger is further enhanced in heart failure due to upregulation of the exchanger (Pogwizd et al., 2001).

be due to defective SR Ca^{2+} release and implicate RyR2 in this process (Marban et al., 1986; Song and Belardinelli, 1994). The present study strengthens this mechanistic link between RyR2 and DADs in the setting of triggered ventricular arrhythmias.

DAD-induced triggered activity has been proposed as the principal mechanism for CPVT-associated exercise-induced arrhythmias (Priori et al., 2002). The biophysical defects in CPVT mutant channels were only observed when the channels were subjected to PKA phosphorylation, which mimics the condition of the channels during exercise. This provides further evidence supporting the relevance of these defects in channel function to the arrhythmias in patients, because the arrhythmias are also exclusively observed during stress and can be elicited with exercise testing.

In support of this mechanism, we found that the onset of premature ventricular contractions (PVCs) in patients with CPVT during exercise testing occurred at a sinus rate >100 beats per minute (107 ± 7 beats per minute, range 100–120, n = 9). Ventricular tachycardia developed at a sinus rate >130 bpm (mean 148 ± 22; range 135–204). Interestingly, the coupling interval of ventricular tachycardias (352 ± 26 ms) was significantly shorter than that of isolated premature beats ($p < 0.0001$), indicating that the rate dependency of arrhythmias in the carriers of the three RyR2 mutations fulfills the criteria for DAD-mediated triggered activity (Fozzard, 1992).

A Potential Molecular Mechanism for Triggered Cardiac Arrhythmias

While CPVT is a rare inherited disorder, our data showing that a defect in RyR2 function is linked to sudden cardiac death may have broader implications. The elucidation of the defect in CPVT-associated RyR2 provides a link between the phenotype of leaky SR Ca^{2+} release channels and cardiac arrhythmias. A transient exercise-induced defect in RyR2 function likely provides a mechanism for the exercise-induced arrhythmias in CPVT (Figure 6).

Heart failure, a leading cause of mortality in the developed world, is associated with altered RyR2 function due to PKA hyperphosphorylation and depletion of FKBP12.6 from the channel complex that may cause aberrant SR Ca^{2+} release (Marx et al., 2000). Indeed, RyR2 from failing hearts exhibits the same defective single-channel properties as the RyR2 from *FKBP12.6*$^{-/-}$ mice and the CPVT-associated RyR2 examined under exercise conditions (Marx et al., 2000). This is likely because heart failure is a chronic hyperadrenergic state, resulting in chronic PKA hyperphosphorylation of RyR2 channels and depletion of FKBP12.6 from the RyR2 macromolecular complex (Marx et al., 2000).

PKA phosphorylated CPVT-associated mutant RyR2 and wild-type RyR2 channels from failing human hearts both exhibit increased activities at low cytosolic [Ca^{2+}], which may promote SR Ca^{2+} leak. The distinction between these two disease states is that in CPVT, the defective channel function is due to inherited mutations in RyR2 that reduce the affinity for FKBP12.6 and impair the ability of the channel to close during diastole, whereas in heart failure, the RyR2 channel complexes are depleted of FKBP12.6 due to chronic PKA hyperphosphorylation (Marx et al., 2000). Taken together, these data suggest that the mechanisms for triggered arrhythmias in heart failure patients may be similar to those in CPVT and in the *FKBP12.6*$^{-/-}$ mouse. It should be noted that cardiac arrhythmias in failing hearts are likely due to multiple causes and those triggered by SR Ca^{2+} leak likely represent a subset of these arrhythmias.

Experimental Procedures

FKBP12.6$^{-/-}$ Mice

Mouse genomic λ-phage clones for the murine ortholog of the human FK506 binding protein 12.6 (FKBP12.6) were isolated from a DBA/1lacJ library using a full-length murine cDNA probe in the laboratory of Dr. John McNeish at Pfizer, Inc. The targeting vector was designed to delete exons 3 and 4, which contain the entire coding sequences for murine FKBP12.6 (Bennett et al., 1998) by replacing 3.5 kb of murine genomic DNA with a PGK-neo selectable marker. A 5.0 kb 5′ fragment and a 1.9 kb 3′ fragment were cloned into pJNS2, a backbone vector with PGK-neo and PGK-TK cassettes.

The DBA/lacJ embryonic stem (ES) cells were grown and transfected using established protocols. Targeted ES cells were first screened by Southern analysis, and five positive ES cell lines were analyzed by PCR to confirm homologous recombination. Targeted ES cells from cell line #97 were injected into C57Bl/6 blastocysts. Male chimeras were bred to DBA/1lacJ females and germline offspring identified by brown coat color. Germline offspring were genotyped using 5′ Southern analysis. Positive *FKBP12.6*$^{+/-}$ males and females were intercrossed and offspring resulted in *FKBP12.6*$^{-/-}$ mice at approximately 25% frequency. *FKBP12.6*$^{-/-}$ mice were fertile. All studies performed with *FKBP12.6*$^{-/-}$ mice used age- and sex-matched *FKBP12.6*$^{+/+}$ mice as controls. No differences were observed between *FKBP12.6*$^{-/-}$ mice raised on the following backgrounds: DBA/C57BL6 mixed, pure DBA, and pure C57BL6.

Southern blot analysis of DNA cut with Bgl II mice yielded an 8.5 kb and 7.7 kb band for wild-type mice and homozygous mutants, respectively. The absence of FKBP12.6 protein was demonstrated by immunoblotting in cardiac tissue (Figure 1C).

Cardiac samples from *FKBP12.6*$^{+/+}$ and *FKBP12.6*$^{-/-}$ mice were homogenized in 1.0 ml buffer (10 mM Tris maleate [pH 6.8], 20 mM NaF, and protease inhibitors), centrifuged at 3000 × g for 10 min, supernatant at 12,000 × g for 20 min, then 120,000 × g for 30 min, resuspended in 50 μl buffer (10 mM Tris maleate [pH 6.8], 0.9% NaCl, and 300 mM sucrose), aliquoted, and stored at −80°C.

Telemetry Recording and Exercise Testing in Mice

FKBP12.6$^{+/+}$ and *FKBP12.6*$^{-/-}$ mice were maintained and studied according to protocols approved by the Institutional Animal Care and Use Committee of Columbia University. Mice were anaesthetized using an intraperitoneal injection of ketamine (50 μg/kg) and xylazine (10 μg/kg). ECG radiotelemetry recordings of ambulatory animals were obtained >48 hr following intraperitoneal implantation (Data Sciences International, St. Paul, MN) (Mitchell et al., 1998). Standard criteria were used to measure ECG parameters (Mitchell et al., 1998). For stress tests, mice were exercised on an inclined treadmill until exhaustion, and then intraperitoneally injected with epinephrine (0.1–0.5 mg/kg) (Mohler et al., 2003). Resting heart rates of ambulatory animals were averaged over 4 hr.

CPVT-Associated RyR2 and FKBP12.6 Mutants

Mutagenesis of CPVT-associated RyR2 and FKBP12.6 mutants is described in the Supplemental Experimental Procedures section online at http://www.cell.com/cgi/content/full/113/7/829/DC1.

HEK293 cells were cotransfected with 20 μg of RyR2-wt or mutant cDNA and with 10 μg FKBP12.6 cDNA using Ca^{2+} phosphate precipitation; vesicles containing RyR2 channels were prepared as previously described (Gaburjakova et al., 2001).

RyR2 PKA Phosphorylation and FKBP12.6 Binding

Cardiac SR membranes were prepared as described (Kaftan et al., 1996; Marx et al., 2000). ^{35}S-labeled FKBP12.6 was generated using the TNT Quick Coupled Transcription/Translation system from Promega (Madison, WI). [^{3}H] ryanodine binding was used to quantify RyR2 levels. 100 μg of microsomes were diluted in 100 μl of 10 mM imidazole buffer (pH 6.8) and incubated with 20 to 1000 nM (final concentration) [^{35}S]-FKBP12.6 at 37°C for 60 min, then quenched with 500 μl ice-cold imidazole buffer. Samples were centrifuged at 100,000 × g for 10 min, washed three times in imidazole buffer, and the amount of bound [^{35}S]-FKBP12.6 was determined by liquid scintillation counting of the pellet.

Immunoblots

Immunoblotting of microsomes (50 μg) was as described with anti-FKBP12/12.6 (1:1,000), anti-RyR (5029, 1:3,000) (Jayaraman et al., 1992), or anti-phosphoRyR2 (P2809, 1:5,000) for 1 hr at room temperature. The P2809 phosphoepitope-specific anti-RyR2 antibody is an affinity-purified polyclonal rabbit antibody custom-made by Zymed Laboratories (San Francisco, CA) using the peptide CRTRRI-(pS)-QTSQ corresponding to RyR2 PKA phosphorylated at Ser2809. After incubation with HRP-labeled anti-rabbit IgG (1:5,000 dilution, Transduction Laboratories; Lexington, KY), the blots were developed using ECL (Amersham Pharmacia, Piscataway, NJ).

Single-Channel Recordings

Single-channel recordings of native RyR2 from mouse hearts or recombinant RyR2 were acquired under voltage-clamp conditions at 0mV, as described (Marx et al., 2000). Symmetric solutions used for channel recordings were *trans* compartment: 250 mM HEPES, 53 mM $Ba(OH)_2$ (in some experiments, $Ba[OH]_2$ was replaced by $Ca[OH]_2$) (pH 7.35), and *cis* compartment: 250 mM HEPES, 125 mM Tris-base, 1.0 mM EGTA, and 0.5 mM $CaCl_2$ (pH 7.35). Unless otherwise indicated, single-channels recordings were made in the presence of 150 nM [Ca^{2+}] and 1.0 mM [Mg^{2+}] in the *cis* compartment. Ryanodine (5 mM) was applied to the *cis* compartment to confirm identity of all channels. Data were analyzed from digitized current recordings using Fetchan software (Axon Instruments, Union City, CA). All data are expressed as mean ± SEM. The unpaired Student's t test was used for statistical comparison of mean values between experiments. A value of $p < 0.05$ was considered statistically significant.

Action Potential Recordings

Cardiomyocytes were isolated from *FKBP12.6*$^{+/+}$ or *FKBP12.6*$^{-/-}$ mouse hearts using the Langendorff method (Reiken et al., 2003) and superfused with 140 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 0.33 mM NaH_2PO_4, 5.5 mM glucose, 0.5 mM NaF, and 5 mM HEPES (pH 7.40) at 35°C–37°C, ± 1 μM isoproterenol or 1 μM epinephrine. Pipette (1–3 MΩ) solution was 10 mM NaCl, 130 mM KCl, 1.0 mM $MgCl_2$, 5 mM MgATP, 10 mM HEPES, and 20 mM TEA-Cl (pH 7.20 with KOH). Axopatch 200A was used in current clamp mode to record action potentials (Mohler et al., 2003). Current injections triggered action potentials at a constant rate 12 Hz.

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