Inhibition of CaMKII Phosphorylation of RyR2 Prevents Induction of Atrial Fibrillation in FKBP12.6 Knockout Mice

Na Li, Tiannan Wang, Wei Wang, Michael J. Cutler, Qiongling Wang, Niels Voigt, David S. Rosenbaum, Dobromir Dobrev, Xander H.T. Wehrens

**Rationale:** Abnormal calcium release from sarcoplasmic reticulum (SR) is considered an important trigger of atrial fibrillation (AF). Whereas increased Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) activity has been proposed to contribute to SR leak and AF induction, downstream targets of CaMKII remain controversial.

**Objective:** To test the hypothesis that inhibition of CaMKII-phosphorylated type-2 ryanodine receptors (RyR2) prevents AF initiation in FKBP12.6-deficient (−/−) mice.

**Methods and Results:** Mice lacking RyR2-stabilizing subunit FKBP12.6 had a higher incidence of spontaneous and pacing-induced AF compared with wild-type mice. Atrial myocytes from FKBP12.6−/− mice exhibited spontaneous Ca\(^{2+}\) waves (SCaWs) leading to Na\(^{+}\)/Ca\(^{2+}\)-exchanger activation and delayed afterdepolarizations (DADs). Mutation S2814A in RyR2, which inhibits CaMKII phosphorylation, reduced Ca\(^{2+}\) spark frequency, SR Ca\(^{2+}\) leak, and DADs in atrial myocytes from FKBP12.6−/−:S2814A mice compared with FKBP12.6−/− mice. Moreover, FKBP12.6−/−:S2814A mice exhibited a reduced susceptibility to inducible AF, whereas FKBP12.6−/−:S2808A mice were not protected from AF.

**Conclusions:** FKBP12.6 mice exhibit AF caused by SR Ca\(^{2+}\) leak, Na\(^{+}\)/Ca\(^{2+}\)-exchanger activation, and DADs, which promote triggered activity. Genetic inhibition of RyR2-S2814 phosphorylation prevents AF induction in FKBP12.6−/− mice by suppressing SR Ca\(^{2+}\) leak and DADs. These results suggest suppression of RyR2-S2814 phosphorylation as a potential anti-AF therapeutic target. (Circ Res. 2012;110:465-470.)

**Key Words:** atrial fibrillation ■ CaMKII ■ delayed afterdepolarization ■ FKBP12.6 ■ ryanodine receptor

Atrial fibrillation (AF) is the most prevalent sustained arrhythmia and is associated with extensive morbidity and mortality.1 Defects in intracellular Ca\(^{2+}\) handling may directly contribute to pathogenic mechanisms underlying AF, such as ectopic activity and reentry.2 Previous studies revealed a higher open probability of type-2 ryanodine receptors (RyR2) isolated from dogs with chronic AF.3 This has been attributed to increased S2808 phosphorylation on RyR2 by protein kinase A,3 decreased levels of FK506-binding protein 12.6 (FKBP12.6) binding to RyR2,3 and increased Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) phosphorylation of RyR2 at S2814.4 Whereas CaMKII inhibition might suppress aberrant sarcoplasmic reticulum (SR) Ca\(^{2+}\) release events associated with AF,4,5 it remains unknown whether phosphorylation of S2808 and/or S2814 play a causal role in AF induction. Moreover, it is unknown whether SR Ca\(^{2+}\) leak and spontaneous Ca\(^{2+}\) waves (SCaWs) through RyR2 generate Na\(^{+}\)/Ca\(^{2+}\)-exchanger (NCX) current sufficiently large to cause delayed afterdepolarizations (DADs) and triggered action potentials (APs). We tested the hypothesis that genetic inhibition of RyR2 phosphorylation at S2808 and/or S2814 phosphorylation sites prevents AF induction in FKBP12.6-deficient (FKBP12.6−/−) mouse model of AF and assessed the underlying mechanisms at the cellular, organ, and whole-animal level.

**Methods**

An expanded Methods section is available in the Data Supplement at http://circres.ahajournals.org. All animal studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. To test the consequences of inhibition of RyR2 phosphorylation at S2814 on Ca\(^{2+}\) release and arrhythmogenesis, we intercrossed FKBP12.6−/− mice with S2814A knock-in mice.6 In vivo electrophysiology was performed as previously.
Non-standard Abbreviations and Acronyms

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<tr>
<td>AF</td>
<td>atrial fibrillation</td>
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<tr>
<td>AP</td>
<td>action potential</td>
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<tr>
<td>CaMKII</td>
<td>Ca²⁺/calmodulin-dependent protein kinase II</td>
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<tr>
<td>CaSF</td>
<td>Ca²⁺ sparks frequency</td>
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<td>DADs</td>
<td>delayed afterdepolarizations</td>
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<td>FKBP12.6</td>
<td>FK506-binding protein 12.6</td>
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<tr>
<td>FWHM</td>
<td>full-width at half-maximum amplitude</td>
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<tr>
<td>NCX</td>
<td>Na⁺/Ca²⁺ exchanger</td>
</tr>
<tr>
<td>RyR2</td>
<td>type 2 ryanodine receptor</td>
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<tr>
<td>ScaWs</td>
<td>spontaneous Ca²⁺ waves</td>
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<tr>
<td>SERCA2a</td>
<td>sarco/endoplasmic reticulum Ca²⁺-ATPase 2a</td>
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Described⁴ ⁵ Ca²⁺ imaging and patch-clamp studies were conducted in isolated atrial myocytes⁴ ⁵.

Results

FKBP12.6 Deficiency Promotes Triggered Activity in Mouse Atria

To determine whether FKBP12.6 deficiency can cause atrial arrhythmias at the tissue level, optical mapping of isolated atria was performed. Figure 1 shows representative optical APs recorded at 3 sites in an FKBP12.6⁻/⁻ mouse atrium during a spontaneous atrial tachyarrhythmia. Voltage mapping revealed fragmented activation as evidenced by an area of rapid activation or potential “driver” (Site 1: cycle length=80 ms) compared with areas of variable activation (Site 2: variable voltage amplitude) and 2:1 activation (Site 3: cycle length=160 ms) (Figure 1A). The incidence of spontaneous AF and atrial flutter was higher in FKBP12.6⁻/⁻ mice compared with wild-type (WT) mice (P<0.05, Figure 1A). This suggests that atria from FKBP12.6⁻/⁻ mice are susceptible to spontaneous arrhythmias, which arise from a focal source driving the remaining atrium.

Localized diastolic Ca²⁺ release may produce DADs and triggered beats in intact tissue.⁶ Therefore, we next measured ScaW-induced DADs and membrane depolarizing currents (I_{NCX}) in atrial myocytes. Simultaneous recordings of Ca²⁺ transients and membrane voltage revealed an increased incidence of ScaW-induced DADs (Figure 1B) in FKBP12.6⁻/⁻ myocytes. Additionally, simultaneous recordings of ScaW and I_{NCX} revealed occurrence of ScaWs with corresponding I_{NCX} in FKBP12.6⁻/⁻ myocytes only (Figure 1C), directly linking SR Ca²⁺ leak with I_{NCX} and potentially arrhythmogenic DADs. For those I_{NCX}-positive FKBP12.6⁻/⁻ myocytes, there was a strong correlation between the amplitudes of ScaW and I_{NCX}.

Ca²⁺ Spark Frequency in FKBP12.6⁻/⁻ Mice Depends on RyR2-S2814 Phosphorylation

Previous studies have demonstrated that FKBP12.6 deficiency promotes atrial arrhythmias as the result of a destabilizing effect on RyR2.* Frequency of spontaneous Ca²⁺ sparks (CaSF) in atrial myocytes from FKBP12.6⁻/⁻ mice was higher than those from WT mice (7.9±0.9 versus 2.9±0.51 sparks/100 μm/s, P<0.001). Compared with FKBP12.6⁻/⁻ mice, CaSF was significantly reduced in myocytes from FKBP12.6⁻/⁻:S2814A mice (7.9±0.90 versus 4.0±0.42 sparks/100 μm/s, P<0.001) (Figure 2A). There was no difference in full-duration at half-maximum amplitude of Ca²⁺ sparks among the 3 genotypes (Figure 2B), whereas there was a small decrease in full-width at half-maximum amplitude in FKBP12.6⁻/⁻:S2814A mice (Figure 2C). Amplitude of the SR Ca²⁺ transient as an index of caffeine-induced Ca²⁺ transient content was reduced in FKBP12.6⁻/⁻ mice compared with WT mice (4.6±0.5 versus 7.5±1.1 F/F; P<0.05) and was partially restored in FKBP12.6⁻/⁻:S2814A mice (5.9±0.9 F/F; P=0.31 versus FKBP12.6⁻/⁻) (Figure 2D).

Recent studies suggest that a fraction of SR Ca²⁺ leak is not detectable as Ca²⁺ sparks.⁸ Therefore, total SR Ca²⁺ leak was also measured using the tetracaine protocol (Online Figure I). The ratio of SR Ca²⁺ leak to SR Ca²⁺ load was significantly larger in FKBP12.6⁻/⁻ mice compared with WT (11.8±0.8 versus 5.8±0.8%; P<0.001; Online Figure I). Conversely, SR Ca²⁺ leak was reduced in FKBP12.6⁻/⁻:S2814A myocytes to levels seen in WT mice (5.9±0.4%; P<0.05), suggesting that prevention of RyR2 phosphorylation at S2814 was sufficient to reverse abnormal SR Ca²⁺ leak in FKBP12.6⁻/⁻ myocytes.

DADs in FKBP12.6⁻/⁻ Mice Depend on CaMKII Phosphorylation of RyR2

Next, we measured membrane potentials in myocytes from WT, FKBP12.6⁻/⁻ and FKBP12.6⁻/⁻:S2814A mice at 3 Hz (Figure 3). Mean AP durations at 30% and 50% repolarization (APD₃₀ and APD₅₀, respectively) were similar in the 3 genotypes (APD₃₀ and APD₅₀: 3.7±0.9 versus 4.0±0.5 ms in WT, 4.5±0.9; 11.9±2.3 ms in FKBP12.6⁻/⁻, 4.7±0.5; 10.2±0.9 ms in FKBP12.6⁻/⁻;S2814A). Whereas WT myocytes typically showed pacing-induced APs only, we observed frequent nonpaced (spontaneous) APs (Figure 3A) and/or DADs (Figure 3B) in FKBP12.6⁻/⁻ myocytes. In fact, 73% (8 of 11) cells from FKBP12.6⁻/⁻ mice developed DADs, whereas only 16.7% (1 of 6) of WT myocytes showed this behavior (P<0.05). In contrast, incidence of DADs was significantly reduced to 25% (4 of 16) in FKBP12.6⁻/⁻:S2814A myocytes (P<0.05 versus FKBP12.6⁻/⁻; Figure 3C). Finally, simultaneous recordings of Ca²⁺ transients and membrane voltage, or Ca²⁺ transients and membrane current in FKBP12.6⁻/⁻:S2814A myocytes under the same conditions as in Figure 1B and 1C, revealed significantly reduced incidence of ScaW-induced DADs and ScaW-induced I_{NCX} in FKBP12.6⁻/⁻:S2814A compared with FKBP12.6⁻/⁻ myocytes (Online Figure II). Thus, inhibition of S2814 phosphorylation on RyR2 prevents triggered activity in FKBP12.6⁻/⁻ myocytes.

Inhibition of CaMKII Phosphorylation of RyR2 Prevents AF Induction in FKBP12.6⁻/⁻ Mice

We also determined whether inhibition of CaMKII phosphorylation of RyR2 could prevent AF induction in FKBP12.6⁻/⁻ mice. We found that 53% (10 of 19) of...
FKBP12.6−/− mice developed AF/atrial flutter after atrial burst pacing, compared with 13% (2 of 15) of WT mice (Figure 4A). The incidence of AF decreased to 15% (3 of 22) in S2814-ablated FKBP12.6−/−:S2814A mice (P<0.05 versus FKBP12.6−/−) but was not decreased in FKBP12.6−/−:S2808A mice with S2808 ablation (43%; 10 out of 23; P=NS versus FKBP12.6−/−). The number of burst-pacing trials leading to AF/atrial flutter induction (of 3 attempts) was
1.63±0.27 in FKBP12.6−/− compared with 0.53±0.19 in WT mice (P<0.01; Figure 4C). Whereas inhibition of S2808 phosphorylation did not significantly reduce the number of episodes (1.35±0.24) in FKBP12.6−/−:S2808A mice, ablation of S2814 in FKBP12.6:S2814A significantly decreased the number of inducible AF episodes to 0.55±0.16 (P<0.01). Inactivation of phosphorylation sites on RyR2 did not alter baseline electrophysiological and conduction properties (Online Table I). Finally, the importance of the S2814 phosphorylation site was confirmed in a second mouse model of RyR2 dysfunction previously associated with an elevated propensity toward AF as a result of heterozygosity for mutation R176Q in RyR2 (R176Q+/− mice) (Online Figure III).

**Rapid Atrial Pacing Leads to CaMKII Activation and Phosphorylation of RyR2 and Phospholamban**

To gain more insight into the mechanisms underlying induction of AF after rapid atrial pacing, we determined whether the CaMKII was activated in atria of paced mice (Online Figure IV). There were no differences in CaMKII expression.
or the level of CaMKII-T287 autophosphorylation at baseline among the different genotypes. After atrial burst pacing, activation of CaMKII (evidenced by increased CaMKII-T287 autophosphorylation) resulted in enhanced phosphorylation of both RyR2 at S2814 and phospholamban at T17 (Online Figure IV). There were no differences in S2808 phosphorylation on RyR2 and S16 on phospholamban before and after pacing (Online Figure V). Finally, protein expression levels of sarcoplasmic reticulum Ca\(^{2+}\)/H\(^+\)-ATPase-2a (SERCA2a) and NCX were similar among all 4 genotypes (Online Figure V), suggesting that RyR2 mutations at S2808A and S2814A did not cause compensatory remodeling of SR Ca\(^{2+}\)-handling proteins.

Discussion

Studies over the past decade have demonstrated that SR Ca\(^{2+}\) release is abnormal in patients with chronic AF.\(^2\) Whereas the amplitude of the L-type Ca\(^{2+}\) current is generally decreased in AF, Ca\(^{2+}\) leak through RyR2 is typically elevated despite similar or decreased SR Ca\(^{2+}\) contents.\(^2,5\) It has been proposed that triggered activity due to DADs is caused by an inward depolarizing I\(_{\text{NCX}}\) current, which occurs in response to the removal of excess Ca\(^{2+}\) release from the cytosol.\(^9,10\) We provide direct experimental evidence for this mechanism in the FKBP12.6\(^{−/−}\) mouse model of AF. Our data revealed that FKBP12.6\(^{−/−}\) mice exhibit atrial focal activity and AF caused by SR Ca\(^{2+}\) leak, NCX activation, and DADs generation. Because recent studies revealed that CaMKII phosphorylation of RyR2 at S2814 is elevated in patients with chronic AF,\(^4,5\) we investigated whether inhibition of S2814 phosphorylation of RyR2 affected susceptibility of FKBP12.6\(^{−/−}\) to AF. Our results demonstrate that inhibition of S2814 but not S2808 phosphorylation suppressed pacing-induced AF in FKBP12.6\(^{−/−}\) mice by preventing spontaneous SCAWs and related DADs. Therefore, our studies suggest that elevated CaMKII phosphorylation on RyR2 might be the primary phosphorylation event associated with triggered activity and AF induction,\(^2\) at least in the particular mutant mouse models examined in this study. These data are consistent with evidence showing that expression levels and activity of cytosolic CaMKII are upregulated in patients with chronic AF.\(^4,5\)

Elevated CaMKII activity not only leads to increased RyR2 phosphorylation at S2814 but also causes increased phospholamban phosphorylation at T17, which might help preserve SR Ca\(^{2+}\) content in AF by increasing SR Ca\(^{2+}\) uptake through SERCA2a disinhibition. We demonstrated that (partial) inhibition of S2814 phosphorylation on RyR2 contributes to SR Ca\(^{2+}\) leak associated with arrhythmogenesis, this did not exclude the possibility that CaMKII phosphorylation of phospholamban or other ion channels/transporters also contribute under some circumstances.

In conclusion, our data demonstrate that an increase in CaMKII phosphorylation of RyR2 at S2814 but not S2808 phosphorylation suppressed pacing-induced AF in FKBP12.6\(^{−/−}\) mice by preventing spontaneous SCAWs and related DADs. Conversely, inhibition of CaMKII phosphorylation of RyR2 prevents AF initiation by decreasing aberrant SR Ca\(^{2+}\) release, NCX activation, and DADs generation, whereas inhibition of S2808 phosphorylation of RyR2 failed to prevent AF induction. Together, our findings imply that CaMKII phosphorylation of S2814 on RyR2 might play an important role in enhancing RyR2-mediated SR Ca\(^{2+}\)
leak that promotes DADs and atrial triggered activity associated with AF.

**Sources of Funding**

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**Disclosures**

None.

**References**


**What Is Known?**

- Atrial fibrillation (AF) is the most prevalent sustained cardiac arrhythmia.
- Increased open probability of ryanodine receptors (RyR2) contributes to defective intracellular Ca2+ handling in AF.
- Ca2+/calmodulin-dependent kinase II (CaMKII) is upregulated in patients with chronic AF.

**What New Information Does This Article Contribute?**

- Genetic inhibition of CaMKII phosphorylation of RyR2 prevents induction of AF in FKBP12.6-deficient mice.
- CaMKII phosphorylation of RyR2 promotes spontaneous Ca2+ waves, activation of inward Na+/Ca2+ exchange current, and delayed afterdepolarizations in atrial myocytes from FKBP12.6-deficient mice.

**Novelty and Significance**

Previous studies demonstrated higher open probability of RyR2 in patients with chronic AF. Biochemical studies revealed increased phosphorylation levels of serine 2808 and serine 2814 on RyR2, as well as increased CaMKII activity. We present evidence that inhibition of serine 2814 but not serine 2808 prevents induction of AF in the FKBP12.6-deficient mouse model of AF. Atrial myocytes from FKBP12.6-deficient mice exhibited spontaneous Ca2+ waves (SCaWs) leading to Na+/Ca2+ exchange current activation and delayed afterdepolarizations. We therefore conclude that serine 2814 is an important downstream target of CaMKII in atrial fibrillation. We propose that defective sarcoplasmic reticulum calcium release through hyperphosphorylated RyR2 may cause triggered activity in atria and contribute to the initiation of AF. Our findings suggest that inhibition of CaMKII phosphorylation of RyR2 could be a potential target for AF treatment.
Inhibition of CaMKII Phosphorylation of RyR2 Prevents Induction of Atrial Fibrillation in FKBPI2.6 Knockout Mice

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Supplemental Material

Inhibition of CaMKII Phosphorylation of RyR2 Prevents Induction of Atrial Fibrillation in FKBP12.6 knock-out Mice

Supplemental Methods

Animals. All animal studies were performed according to protocols approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine conforming the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health. FKBP12.6-deficient (-/-) and R176Q/+ mice were generously provided by Dr. Susan Hamilton (Baylor College of Medicine, TX).\(^1\)\(^2\) RyR2-S2814A knock-in mice (S2814A) were generated as previously described.\(^3\) Serine2814 on RyR2 was substituted with alanine, which inhibits CaMKII phosphorylation of S2814 on RyR2. S2808A knock-in mice were generously provided by Dr. Andrew Marks (Columbia University, NY).\(^4\)\(^5\) Serine2808 on RyR2 was substituted with alanine such that PKA phosphorylation of S2808 on RyR2 was inhibited. FKBP12.6 deficient (-/-) and R176Q/+ mice were intercrossed with S2814A and S2808A mice, to obtain FKBP12.6-/-:S2814A or FKBP12.6-/-:S2808A mice, respectively. These mice were used for electrophysiology, biochemistry, Ca\(^{2+}\) imaging and patch clamp studies. S2814A and S2808A mice were also intercrossed with R176Q/+ mice to compound heterozygous R176Q/+:S2814A/+ or R176Q/+:S2808A/+ mice, respectively. All animals were studied at age of 3-4 month.

Optical mapping of intact atria. Whole atria electrophysiological studies were performed in WT (n=6) and FKBP12.6/-/- (n=7) mice. Briefly, isolated hearts were Langendorff perfused with oxygenated Tyrode’s solution and the voltage sensitive dye di-4-ANEPPS (4 mmol/l). After 5-7 min of Langendorff perfusion the L and R atria were carefully dissected away from the ventricles and placed in a microscope imaging chamber for optical mapping. All preparations were
continuously superfused with normal Tyrode’s solution at 37°C. Voltage dye fluorescence intensity was measured using a high-speed CCD camera that is attached to the microscope imaging system. Spontaneous atrial arrhythmias were defined as any atrial tachyarrhythmia lasting >30 s that developed during a 5 min period of electrophysiological monitoring without external pacing. Statistical differences were assessed with the Chi Square test.

**In vivo electrophysiology in mice.** Briefly, atrial and ventricular intracardiac electrograms were recorded using an 1.1F octapolar catheter (EPR-800, Millar Instruments, Houston, Texas) inserted via the right jugular vein. Surface and intracardiac electrophysiology parameters were assessed at baseline. Right atrial pacing was performed using 2-ms current pulses delivered by an external stimulator (STG-3008, Multi Channel Systems, Reutlingen, Germany). Sinus node recovery time (SNRT) was measured after applying a 15-s atrial pacing train at a basic cycle length of 100 ms. SNRT is defined as the interval between the last stimulus of the pacing train and the onset of first spontaneous sinus beat. Atrial effective refractory period (AERP) and the effective refractory period of the atrioventricular effective refractory period (AVERP) were determined by applying a series of atrial pacing trains at a fixed basic cycle length of 100 ms (S1) with a S2 premature stimulus. The S1-S2 interval was progressively reduced by 2-ms in each pacing train from 70ms to 20ms. The AERP is defined as the longest S1-S2 coupling interval for atria that failed to generate a propagated beat with S2. The AVERP is defined as the longest S1-S2 coupling interval at which the premature stimulation delivered to the atrium is followed by a His potential but not by a QRS complex. Atrial fibrillation (AF) inducibility was determined by using the protocol described by Sood *et al.*, and defined as the occurrence of rapid and fragmented atrial electrograms with irregular AV-nodal conduction and ventricular rhythm for at least 1 second. Inducibility of AF was considered positive if at least 2 of 3 pacing trials induced AF.
**Western blot analysis**  Protein extraction and Western blotting was performed as previously described. Briefly, mouse atrial lysates were subjected to electrophoresis on 5% (for RyR2, SERCA and NCX) and 12% (for PLN and CaMKII) acrylamide gels, and transferred onto polyvinyl difluoride membranes. Membranes were probed with monoclonal anti-RyR2 (1:5,000), monoclonal anti-PLN (1:5,000), polyclonal anti-Thr17-phosphorylated PLN (1:5,000, Badrilla), polyclonal anti-Ser16-phosphorylated PLN (1:5,000, Badrilla), monoclonal anti-Thr287-phosphorylated CaMKII (1:1,000) and polyclonal anti-CaMKIIδ (1:1000) antibodies at room temperature. The polyclonal anti-Ser2808-RyR2 (1:1000) and anti-Ser2814-RyR2 (1:1000) phosphoepitope-specific antibody were custom generated using the peptide C-RTRRI-(pS)-QTSQV corresponding to the PKA phosphorylation site region at serine 2808 on RyR2 and peptide CSQTSQV-(pS)-VD corresponding to RyR2 CaMKII phosphorylated at serine 2814, respectively. Membranes were then incubated with secondary anti-mouse and anti-rabbit antibodies conjugated to Alexa-Fluor 680 (Invitrogen Molecular Probes) and IR800Dye (Rockland Immunochemicals), respectively, and bands were quantified using Image J.

**Atrial Myocyte Isolation.** Mouse atrial myocytes were isolated as described previously. Briefly, the heart was removed and the blood was washed out with 0 Ca²⁺ Tyrode solution (137 mM NaCl, 5.4 mM KCl, 1 mM MgCl₂, 5 mM HEPES, 10 mM glucose, 3 mM NaOH, pH 7.4). The heart was cannulated through the aorta and perfused on a Langendorff apparatus with 0 Ca²⁺ Tyrode for 3 to 5 minutes at 37 °C, followed by 0 Ca²⁺ Tyrode containing 20 μg/mL Liberase (Roche, Indianapolis, IN) for 10 to 15 minutes at 37 °C. After digestion, heart was perfused with 5 mL KB solution (90 mM KCl, 30 mM K₂HPO₄, 5 mM MgSO₄, 5 mM pyruvic acid, 5 mM β-hydroxybutyric acid, 5 mM creatine, 20 mM taurine, 10 mM glucose, 0.5 mM EGTA, 5 mM HEPES, pH 7.2). Both left and right atrium were minced in KB solution and gently agitated, then filtered through a 210 mm polyethylene mesh. Atrial myocytes were stored in KB solution at room temperature before use.
**Calcium imaging.** Only rod-shaped myocytes showing clear striation were selected for further experiments. Atrial myocytes were loaded with 2 μM Fluo-4-AM (Invitrogen, Carlsbad, CA) in normal Tyrode solution containing 1.8 mM Ca²⁺ for 30 minutes at room temperature. Cells were washed with Tyrode solution for 15 minutes for de-esterification and transferred to a chamber equipped with parallel platinum electrodes. For Ca²⁺ sparks recordings, the chamber was placed on a LSM510 confocal microscope (Carl Zeiss, Thornwood, NY). Fluorescence images were recorded in line-scan mode with 1024 pixels per line at 500 Hz. Once steady state Ca²⁺ transient induced by 1Hz-pacing (5 ms, 10 V) was observed, pacing was stopped for 45 seconds and Ca²⁺ sparks were counted. Steady state SR Ca²⁺ content was estimated by rapid application of caffeine (10mmol/L) after pacing. SR Ca²⁺ leak in atrial myocytes were measured as described in detail previously. Myocytes were preconditioned in 1.8 mM Ca²⁺ normal Tyrode solution for 15 seconds by pacing delivered by platinum electrodes. Steady state [Ca²⁺]ᵢ was then monitored after perfusate was switched to a 0Na⁺, 0Ca²⁺ Tyrode solution which blocks the Ca²⁺ exchange via the Na⁺/Ca²⁺ exchanger and the L-type Ca²⁺ channel. Acute application of tetracaine (5 µmol/L) was used to rapidly and reversibly block RyR2, followed by application of caffeine (10mmol/L) to estimate steady-state SR Ca²⁺ content. The tetracaine-dependent shift of Ca²⁺ from cytosol to SR is proportional to SR Ca²⁺ leak.

**Patch Clamp.** Membrane currents and potentials were measured in whole-cell configuration using voltage-clamp and current-clamp techniques, with simultaneous Ca²⁺ transients (CaTs) measurement. The resistances of glass microelectrodes were 2-5 MΩ when filled with pipette solution (mmol/L: K-aspartate 120, KCl 20, MgCl₂ 1, Na₂-ATP 5, EGTA 0.02, HEPES 10; pH=7.2). Series resistance and cell capacitance were compensated. For simultaneous recording of CaT and $I_{\text{NCX}}$, myocytes were superfused with a bath solution containing (mmol/L): NaCl 140, KCl 5.4, MgCl₂ 1, CaCl₂ 3, glucose 10, HEPES 10, BaCl₂ 0.5; pH=7.4). CaT was
induced by a 200 ms conditioning pulse from -40 mV to 0mV at 1Hz.
Online Figure I. Increased SR Ca^{2+} leak in FKBP12.6/- mice depends on RyR2-S2814 phosphorylation level. A-C. Representative [Ca^{2+}]i tracings from fluo-4-AM loaded atrial myocytes paced at 1 Hz followed by rapid switch to Tyrode containing 0 Na^+, 0 Ca^{2+}, and 1 mmol/L tetracaine (TTC) to block RyR2. SR Ca^{2+} content was measured by adding 10 mmol/L caffeine. SR Ca^{2+} leak (curve below red baseline) was quantified and normalized to SR Ca^{2+} content (D). Numbers in bars indicate numbers of cells studied from 3-4 mice in each group. *P < 0.05, ***P<0.001.
Online Figure II. A. Left panel: Representative simultaneous recording of Ca\textsuperscript{2+} transients and membrane potential in WT and FKBP12.6-/-:S2814A atrial myocytes. Right panel: Bar graph summarizing the incidence of ScaW induced DADs. B. Left panel: Representative simultaneous recording of Ca\textsuperscript{2+} transients and membrane current in WT and FKBP12.6-/-:S2814A atrial myocytes. Right panel: Bar graph summarizing the incidence of ScaW induced $I_{NCX}$. Numbers in bars indicate numbers of cells tested from 2-3 animals. *$P$<0.05, **$P$<0.01
Online Figure III. S2814A but not S2808A mutation prevents pacing-induced AF in R176Q/+ knockin mice. A. Because the S2814A mutation is located in the same gene (i.e., RYR2) as the R176Q mutation, compound heterozygous mice were generated with mutation R176Q/+ in one RYR2 allele, and S2814A (or S2808A) in the other allele. Simultaneous surface ECG (lead 1), intracardiac atrial and ventricular electrograms revealed atrial fibrillation in R176Q/+ mice after burst pacing. WT and R176Q/+;S2814A/+ mice typically had sinus rhythm following rapid pacing. B-C. Bar graphs summarizing the incidence (B) and number of episodes (C) of reproducible AF in the respective genotypes of mice. Numbers in bars indicate numbers of mice tested in each group. *P < 0.05.
Online Figure IV. Pacing-induced activation of CaMKII leads to phosphorylation of RyR2 and PLN. A. Western blots were used to measure total CaMKII and T286 phosphorylation levels in atria excised at resting heart rates or immediately after rapid burst pacing. Bar graphs showing T286 phosphorylation normalized to total CaMKII levels. B. Western blots were used to measure total RyR2 and S2814 phosphorylation levels in atria excised at resting heart rates or immediately after rapid burst pacing. Bar graphs showing S2814 phosphorylation normalized to total RyR2 levels. C. Western blots were used to measure total PLN and T17 phosphorylation levels in atria excised at resting heart rates or immediately after rapid burst pacing. Bar graphs showing phosphorylation level normalized to total levels. Numbers in bars indicate numbers of mice tested in each group. *P < 0.05, **P<0.01, ***P<0.001.
Online Figure V. PKA phosphorylation levels of RyR2 and PLN and protein expressions of SERCA and NCX were unaltered.  

A-B. Western blots were used to measure PKA phosphorylation levels of RyR2 at S2808 (A) and PLN at S16 (B) in atria excised at resting heart rates or immediately after rapid burst pacing. Bar graphs showing phosphorylation level normalized to total levels.  

C-D. Western blots were used to measure SERCA (C) and NCX (D) levels in atria excised at resting heart rates or after rapid burst pacing. Bar graphs showing protein level normalized to housekeeping protein GAPDH levels. Numbers in bars indicate numbers of mice tested in each group.
## Supplemental Table

### Online Table I. Cardiac electrophysiological parameters

<table>
<thead>
<tr>
<th></th>
<th>WT (n = 15)</th>
<th>FK (n = 19)</th>
<th>FK-S2808A (n = 23)</th>
<th>FK-S2814A (n = 22)</th>
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<tbody>
<tr>
<td>HR (bpm)</td>
<td>519 ± 15</td>
<td>547 ± 13</td>
<td>544 ± 16</td>
<td>521 ± 10</td>
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<tr>
<td>PR (ms)</td>
<td>35.9 ± 0.9</td>
<td>35.7 ± 1.0</td>
<td>37.9 ± 0.8</td>
<td>38.5 ± 1.0</td>
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<tr>
<td>QRS (ms)</td>
<td>10.1 ± 0.3</td>
<td>10.4 ± 0.4</td>
<td>10.8 ± 0.4</td>
<td>10.1 ± 0.3</td>
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<tr>
<td>QTc (ms)</td>
<td>24.3 ± 0.9</td>
<td>25.9 ± 0.9</td>
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<td>26.1 ± 0.7</td>
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<td>SNRT (ms)</td>
<td>141.8 ± 4.3</td>
<td>142.2 ± 9.9</td>
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<td>AERP (ms)</td>
<td>35.7 ± 1.1</td>
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<tr>
<td>AVERP (ms)</td>
<td>46.1 ± 1.2</td>
<td>45.8 ± 1.9</td>
<td>43.9 ± 1.2</td>
<td>47.3 ± 1.2</td>
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</table>
Supplemental References


