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

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Abstract

Rationale—Abnormal calcium release from sarcoplasmic reticulum (SR) is considered an important trigger of atrial fibrillation (AF). Whereas increased 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 to wildtype mice. Atrial myocytes from FKBP12.6−/− mice exhibited spontaneous Ca^{2+} waves (SCaWs) leading to Na^{+}/Ca^{2+}-exchanger (NCX) 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, NCX 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 that suppression of RyR2-S2814 phosphorylation as a potential anti-AF therapeutic target.

Keywords

Atrial fibrillation; CaMKII; delayed afterdepolarization; FKBP12.6; ryanodine receptor

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Disclosures. None

Introduction

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 (PKA),[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) via RyR2 generate Na^{+}/Ca^{2+}-exchanger (NCX) current sufficiently large to cause delayed afterdepolarizations (DADs) and triggered action potentials (APs). Here, 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[6], and assessed the underlying mechanisms at the cellular, organ and whole-animal level.

Material and Methods

An expanded Methods section is available in Supplemental Materials and Methods. All animal studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. 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[4]. *In vivo* electrophysiology was performed as previously described [4, 6]. Ca^{2+} imaging and patch clamp studies were conducted in isolated atrial myocytes[4, 6].

Results

FKBP12.6 deficiency promotes triggered activity in mouse atria

To determine whether FKBP12.6 deficiency can cause atrial arrhythmias at tissue level, optical mapping of isolated atria was performed. Figure 1 shows representative optical APs recorded at 3 sites in a 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 (CL) = 80 ms) compared to areas of variable activation (Site 2 – variable voltage amplitude) and 2:1 activation (Site 3 – CL = 160 ms) (Fig. 1A). The incidence of spontaneous AF and atrial flutter was higher in FKBP12.6−/− mice compared to wildtype (WT) mice ($P<0.05$, Fig. 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 Ca^{2+} release may produce DADs and triggered beats in intact tissue[7]. Therefore, we next measured SCaW-induced DADs and membrane depolarizing currents (I_{NCX}) in atrial myocytes. Simultaneous recordings of Ca^{2+} transients and membrane voltage revealed an increased incidence of SCaWs-induced DADs (Fig. 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 (Fig. 1C), directly linking SR Ca^{2+} 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}.

Ca^{2+}-spark frequency in FKBP12.6−/− mice depends on RyR2-S2814 phosphorylation

Previous studies have demonstrated that FKBP12.6 deficiency promotes atrial arrhythmias owing to a destabilizing effect on RyR2.[6] Frequency of spontaneous Ca^{2+} sparks (CaSF) in atrial myocytes from FKBP12.6−/− mice was higher than those from WT mice (7.9±0.9 vs. 2.9±0.51 sparks/100μm/s, $P<0.001$). Compared to FKBP12.6−/− mice, CaSF was significantly reduced in myocytes from FKBP12.6−/−:S2814A mice (7.9±0.90 vs. 4.0±0.42 sparks/100μm/s, $P<0.001$) (Fig. 2A). There was no difference in full-duration of half-maximum of Ca^{2+} sparks among the three genotypes (Fig. 2B), whereas there was a small decrease in full-width of half-maximum in FKBP12.6−/−:S2814A mice (Fig. 2C). Amplitude of the SR Ca^{2+} transient as an index of SR Ca^{2+} content was reduced in FKBP12.6−/− mice compared to WT mice (4.6±0.5 vs. 7.5±1.1 F/F0; $P<0.05$), and was partially restored in FKBP12.6−/−:S2814A mice (5.9±0.9 F/F0; $P=0.31$ vs. FKBP12.6−/−) (Fig. 2D).

Recent studies suggest that a fraction of SR Ca^{2+} leak is not detectable as Ca^{2+} sparks [8]. Therefore, total SR Ca^{2+} leak was also measured using the tetracaine protocol (Online Figure I). The ratio of SR Ca^{2+} leak to SR Ca^{2+} load was significantly larger in FKBP12.6−/− mice compared with WT (11.8±0.8 vs. 5.8±0.8 %; $P<0.001$; Online Figure I).[6] Conversely, SR Ca^{2+} 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 Ca^{2+} leak in FKBP12.6−/− myocytes.

Delayed afterdepolarizations 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 (Fig. 3). Mean AP durations at 30% and 50% repolarization (APD_{30} and APD_{50}, respectively) were similar in the three genotypes (APD_{30} and APD_{50}: 3.7±0.9; 9.9±1.9ms in WT; 4.5±0.9; 11.9±2.3ms in FKBP12.6−/−; 4.7±0.5; 10.2±0.9ms in FKBP12.6−/−:S2814A). Whereas WT myocytes typically showed pacing-induced APs only, we observed frequent non-paced (spontaneous) APs (Fig. 3A) and/or DADs (Fig. 3B) in FKBP12.6−/− myocytes. In fact, 73% (8 out of 11) cells from FKBP12.6−/− mice developed DADs, whereas only 16.7% (1 out of 6) of WT myocytes showed this behavior ($P<0.05$). In contrast, incidence of DADs was significantly reduced to 25% (4 out of 16) in FKBP12.6−/−:S2814A myocytes ($P<0.05$ vs. FKBP12.6−/−; Fig. 3C). Finally, simultaneous recordings of Ca^{2+} transients and membrane voltage, or Ca^{2+} transients and membrane current in FKBP12.6:S2814A myocytes under the same conditions as in Fig. 1B–C revealed significantly reduced incidence of SCaW-induced DADs and SCaW-induced I_{NCX} in FKBP12.6−/−:S2814A compared to FKBP12.6−/− myocytes (Online Figure II). Thus, inhibition of S2814 phosphorylation on RyR2 prevents triggered activity in FKBP12.6−/− mice 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 out of 19) of FKBP12.6−/− mice developed AF/atrial flutter following atrial-burst pacing, compared to 13% (2 out of 15) of WT mice (Fig. 4A). Incidence of AF decreased to 15% (3 out of 22) in S2814-ablated FKBP12.6−/−:S2814A mice ($P<0.05$ vs. FKBP12.6−/−), but was not decreased in FKBP12.6−/−:S2808A mice with S2808 ablation (43%; 10 out of 23; P=N.S. vs. FKBP12.6−/−). The number of burst-pacing trials leading to AF/atrial flutter induction (out of 3 attempts) was 1.63±0.27 in FKBP12.6−/− compared to 0.53±0.19 in WT mice ($P<0.01$; Fig. 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 towards AF as a result of heterozygosity for mutation R176Q in RyR2 (R176Q/+ mice)[4] (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 following 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 auto-phosphorylation at baseline among the different genotypes. Following atrial-burst pacing, activation of CaMKII (evidenced by increased CaMKII-T287 auto-phosphorylation) resulted in enhanced phosphorylation of both RyR2 at S2814 and phospholamban (PLN) at T17 (Online Figure IV). There were no differences in S2808 phosphorylation on RyR2 and S16 on PLN before and after pacing (Online Figure V). Finally, protein expression levels of sarco/endoplasmic reticulum Ca^{2+}-ATPase-2a (SERCA2a) and NCX were similar among all four 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_{NCX} current, which occurs in response to the removal of excess Ca^{2+} release from the cytosol.[9,10] Here 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 mice examined in this study. This 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 PLN phosphorylation at T17, which might help preserve SR Ca^{2+} content in AF,[11] by increasing SR Ca^{2+} uptake through SERCA2a disinhibition. We demonstrated that (partial) inhibition of S2814 phosphorylation on RyR2 is sufficient to suppress inducibility of AF following atrial-burst pacing in R176Q/+:S2814A/+ mice. Whereas this suggests that CaMKII phosphorylation of RyR2 contributes to SR Ca^{2+} leak associated with arrhythmogenesis, this did not exclude the possibility that CaMKII phosphorylation of PLN 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 contributes to AF initiation in FKBP12.6−/− mice by amplifying SR Ca^{2+} leak and inducing 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.

Novelty and Significance

What Is Known?

- Atrial fibrillation (AF) is the most prevalent sustained cardiac arrhythmia.
- Increased open probability of ryanodine receptors (RyR2) contributes to defective intracellular Ca^{2+} handling in AF.
- Ca^{2+}/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 Ca^{2+} waves, activation of inward Na^{+}/Ca^{2+} exchange current, and delayed after-depolarizations in atrial myocytes from FKBP12.6 deficient mice.

Previous studies demonstrated higher open probability of RyR2 in patients with chronic atrial fibrillation. Biochemical studies revealed increased phosphorylation levels of serine 2808 and serine 2814 on RyR2, as well as increased CaMKII activity. Here, we present evidence that inhibition of serine 2814 but not serine 2808 prevents induction of atrial fibrillation in the FKBP12.6-deficient mouse model of AF. Atrial myocytes from FKBP12.6-deficient mice exhibited spontaneous Ca^{2+} waves (SCaWs) leading to Na^{+}/Ca^{2+}-exchange current activation, and delayed afterdepolarizations (DADs). We therefore conclude that serine 2814 is an important downstream target of CaMKII in atrial fibrillation. We propose that defective sarcoplasmic reticulum calcium release via 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Non-standard Abbreviations and Acronyms

AF	Atrial Fibrillation
AP	Action potential
CaMKII	Ca^{2+}/Calmodulin-Dependent Protein Kinase II
CaSF	Ca^{2+} sparks frequency
CL	Cycle length

DADs	Delayed Afterdepolarizations
FDHM	full duration of half maximum
FKBP12.6	FK506-Binding Protein 12.6
FWHM	full width of half maximum
NCX	Na^+/Ca^{2+} Exchanger
PKA	Protein Kinase A
PLN	Phospholamban
RyR2	Type 2 Ryanodine Receptor
SCaWs	spontaneous Ca^{2+} waves
SERCA2a	Sarco/Endoplasmic Reticulum Ca^{2+}-ATPase 2a
SR	Sarcoplasmic Reticulum

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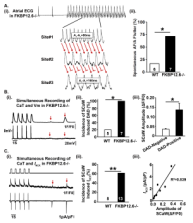

Figure 1. Triggered activity in FKBP12.6−/− mouse atria

A. i. Representative optical action potentials (Aps) recorded at 3-sites in FKBP12.6−/− mouse during spontaneous atrial tachyarrhythmia. **ii**. Incidence of spontaneous AF/atrial flutter. Numbers in bars indicate numbers of mice. **B–C. i.** Representative simultaneous recording in FKBP12.6−/− myocytes of Ca^{2+} transients and APs (**B**) or membrane currents (**C**) **ii.** Incidence of spontaneous Ca^{2+} wave (SCaW)-induced delayed afterdepolarizations (DADs) (**B**) or I_{NCX} (**C**). **iii.** Amplitude of DAD-negative and DAD-positive SCaWs (**B**) and correlation of SCaW and I_{NCX} amplitudes (**C**). Numbers in bars indicate numbers of cells from 3 animals. **P*<0.05, ***P*<0.01

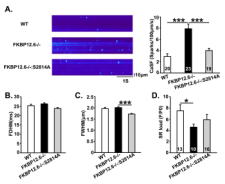

Figure 2. Increased Ca^{2+}-spark frequency in FKBP12.6−/− mice depends on RyR2-S2814 phosphorylation level

A. Confocal line-scan images of Ca^{2+} sparks in intact myocytes from WT, FKBP12.6−/− and FKBP12.6−/−:S2814A mice. **B–C.** Full-duration at half-maximum (FDHM) and full-width at half-maximum (FWHM) of Ca^{2+} sparks. **D**. SR Ca^{2+} load assessed with rapid caffeine application. Numbers in bars indicate numbers of cells from 3–4 mice in each group. **P*<0.05, ****P*<0.001

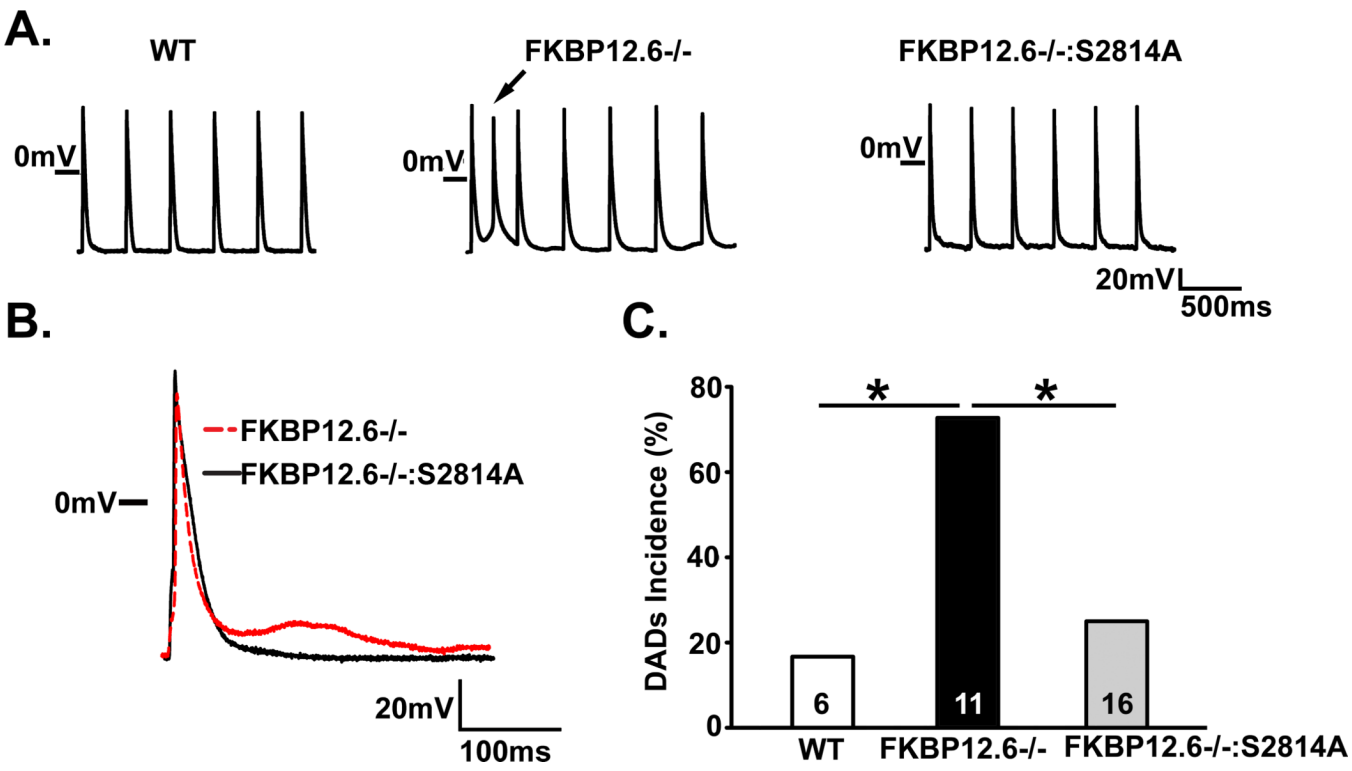

Figure 3. Delayed afterdepolarizations (DADs) in FKBP12.6−/− mice depend on CaMKII phosphorylation of RyR2

A–B. Representative AP recordings at 3Hz and overlay of single APs with DAD in FKBP12.6−/− and FKBP12.6−/−:S2814A mouse. **C**. Incidence of DADs. Numbers in bars indicate numbers of cells from 3–4 mice in each group. **P*<0.05, ****P*<0.001.

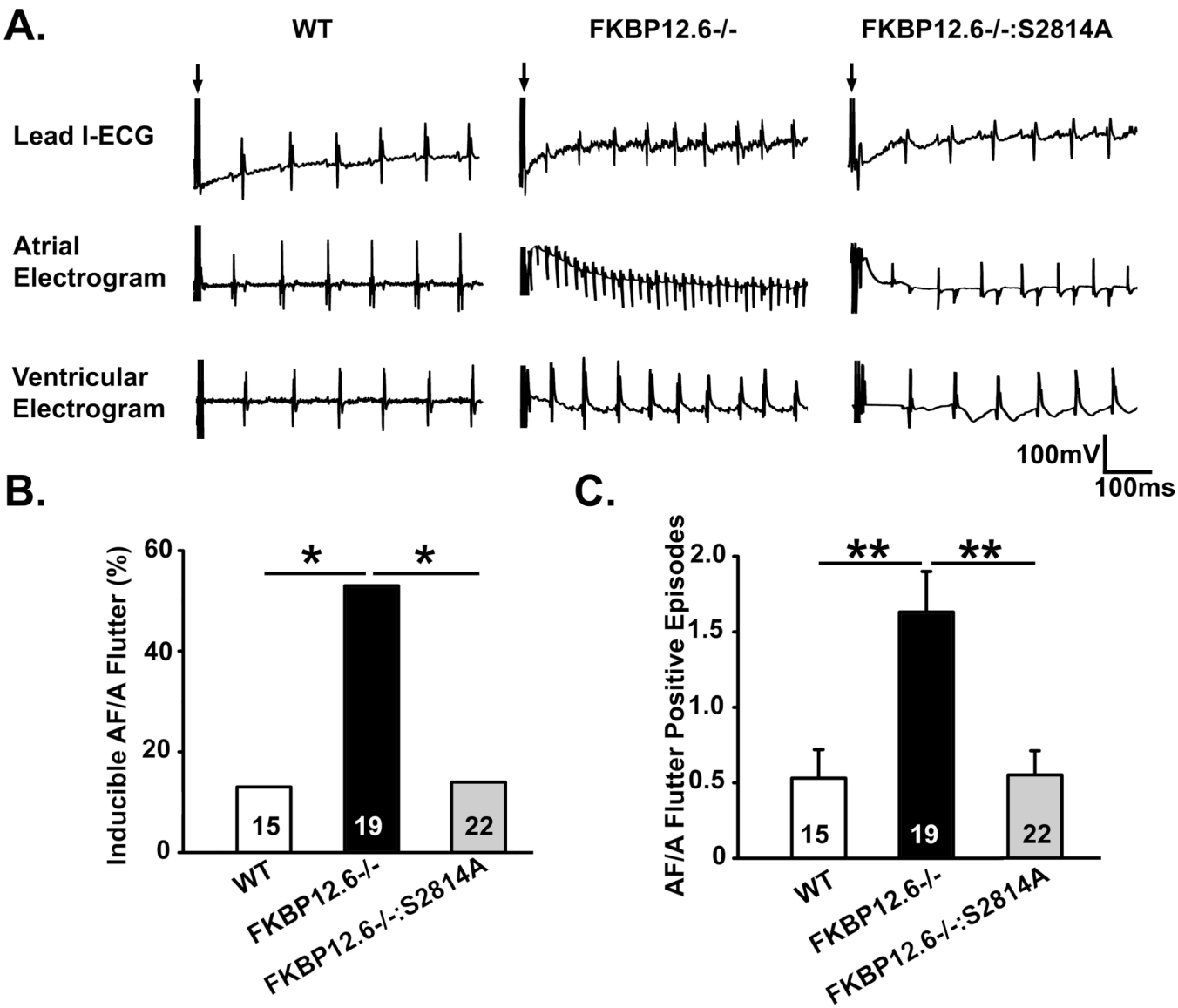

Figure 4. Inhibition of CaMKII phosphorylation of RyR2 prevents AF induction in FKBP12.6−/−:S2814A mice

A. Simultaneous surface ECG (lead 1), intracardiac atrial and ventricular electrograms with absent P-waves and irregular RR intervals suggestive of AF in FKBP12.6−/− mice after burst pacing. The WT and FKBP12.6−/−:S2814A mice show sinus rhythm following rapid pacing. **B–C.** Bar graph summarizing the incidence (**B**) and number of episodes (**C**) of reproducible AF/atrial flutter. Numbers in bars indicate numbers of mice. **P*<0.05, ***P*<0.01