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Calcium Leak Through Ryanodine Receptors Leads To Atrial Fibrillation In Three Mouse Models of Catecholaminergic Polymorphic Ventricular Tachycardia

Jian Shan[1], **Wenjun Xie**[1], **Matthew Betzenhauser**[1], **Steven Reiken**[1], **Bi-Xing Chen**[1], **Anetta Wronska**[1], and **Andrew R. Marks**[1,2]

[1]Department of Physiology and Cellular Biophysics, Clyde and Helen Wu Center for Molecular Cardiology, College of Physicians and Surgeons of Columbia University, New York, NY 10032, USA

[2]Department of Medicine, College of Physicians and Surgeons of Columbia University, New York, NY 10032, USA

Abstract

Rationale—Atrial fibrillation (AF) is the most common cardiac arrhythmia, however the mechanism(s) causing AF remain poorly understood and therapy is suboptimal. The ryanodine receptor (RyR2) is the major calcium (Ca^{2+}) release channel on the sarcoplasmic reticulum (SR) required for excitation-contraction coupling in cardiac muscle.

Objective—In the present study we sought to determine whether intracellular diastolic SR Ca^{2+} leak via RyR2 plays a role in triggering AF and whether inhibiting this leak can prevent AF.

Methods and Results—We generated three knock-in mice with mutations introduced into RyR2 that result in leaky channels and cause exercise induced polymorphic ventricular tachycardia in humans [catecholaminergic polymorphic ventricular tachycardia (CPVT)]. We examined AF susceptibility in these three CPVT mouse models harboring RyR2 mutations to explore the role of diastolic SR Ca^{2+} leak in AF. AF was stimulated with an intra-esophageal burst pacing protocol in the three CPVT mouse models (RyR2-R2474S$^{+/-}$, 70%; RyR2-N2386I$^{+/-}$, 60%; RyR2-L433P$^{+/-}$, 35.71%), but not in wild type (WT) mice ($P<0.05$). Consistent with these *in vivo* results, there was a significant diastolic SR Ca^{2+} leak in atrial myocytes isolated from the CPVT mouse models. Calstabin2 (FKBP12.6) is an RyR2 subunit that stabilizes the closed state of RyR2 and prevents a Ca^{2+} leak through the channel. Atrial RyR2 from RyR2-R2474S$^{+/-}$ mice were oxidized and the RyR2 macromolecular complex was depleted of calstabin2. The Rycal drug S107 stabilizes the closed state of RyR2 by inhibiting the oxidation/phosphorylation induced dissociation of calstabin2 from the channel. S107 reduced the diastolic SR Ca^{2+} leak in atrial myocytes and decreased burst pacing-induced AF *in vivo*. S107 did not reduce the increased prevalence of burst pacing-induced AF in calstabin2-deficient mice, confirming that calstabin2 is required for the mechanism of action of the drug.

Address correspondence to: Dr. Andrew R. Marks, Medicine, College of Physicians and Surgeons, Columbia University, New York, NY 10032, USA, Phone: (212) 851-5340, Fax (212) 851-5345, arm42@columbia.edu.

DISCLOSURES

ARM is a consultant for and owns shares in ARMGO Pharma, Inc. a biotech company targeting RyR2 treatment for prevention of CPVT.

Conclusions—The present study demonstrates that RyR2-mediated diastolic SR Ca^{2+} leak in atrial myocytes is associated with AF in CPVT mice. Moreover, the Rycal S107 inhibited diastolic SR Ca^{2+} leak through RyR2 and pacing-induced AF associated with CPVT mutations.

Keywords

CPVT; atrial fibrillation; SR Ca^{2+} leak; Ca^{2+} sparks; ryanodine receptor

INTRODUCTION

Atrial fibrillation (AF) is the most common arrhythmia and is especially prevalent in the elderly [1]. AF accounts for more than one-third of all arrhythmia-related hospitalizations [2]. Although AF itself is not typically lethal, complications related to AF including thromboembolism, hemodynamic compromise and arrhythmogenesis make it one of the leading causes of cardiovascular morbidity and mortality. Current clinical management of AF is focused on rate control and chronic anticoagulation with concomitant potential bleeding risk and impaired cardiac function, especially in patients with congestive heart failure (CHF).

The mechanism of AF is not well understood despite more than 100 years of study. Most hypotheses regarding the induction of AF are based on observational studies in patients with chronic AF or studies of chronic AF animal models. Based on these studies, structural remodeling including atrial enlargement and fibrosis are proposed to play important roles in both triggering and maintaining AF [3]. However, it has proven difficult to distinguish whether these factors are the cause or the consequence of AF. Structural changes may directly or indirectly induce atrial electrical abnormalities leading to atrial ectopic events and AF. More recently, the role of Ca^{2+} in AF has been explored as a possible contributing factor to the well-known reentry mechanism and in 'triggered activity' models [4].

Our laboratory has reported that SR Ca^{2+} leak via PKA hyperphosphroylated and/or oxidized RyR2 channels contributes to heart failure (HF) progression [5] and triggers ventricular arrhythmias [6–8]. Furthermore, we showed that CPVT-linked RyR2 mutations cause a diastolic SR Ca^{2+} leak, delayed after depolarizations (DADs) and lethal ventricular arrhythmias in mice [6]. Recent reports demonstrate that patients with CPVT mutations have AF [9–13]. Mouse models harboring CPVT mutations recapitulate the ventricular human phenotype manifesting exercise-induced polymorphic VT and sudden death and studies with knock-in mice have helped to establish the role of diastolic SR Ca^{2+} leak through mutant RyR2 in ventricular arrhythmias [6, 14–16]. The normal cardiac structure and function of CPVT mouse models makes them ideal tools to study the role of diastolic SR Ca^{2+} leak via RyR2 in triggering AF in the absence of structural cardiac defects.

In the present study, AF was inducible by burst atrial pacing in mice harboring CPVT mutations *in vivo* but not in WT littermates. Moreover, we examined the difference in diastolic SR Ca^{2+} leak between atrial and ventricular myocytes isolated from both WT and CPVT mice. Consistent with *in vivo* study results, there was an increase in diastolic SR Ca^{2+} leak in atrial myocytes isolated from RyR2-R2474S$^{+/-}$, RyR2-R2386I$^{+/-}$, and RyR2-L433P$^{+/-}$ mice compared to atrial myocytes from WT mice. Increased diastolic SR Ca^{2+} leak, associated with depletion of calstabin2 (FKBP12.6) from the RyR2 channel complex, was observed in atrial myocytes from RyR2-R2474S$^{+/-}$ mice, but not in WT mice. The small molecule Rycal S107, which stabilizes RyR2-calstabin2 interactions, significantly decreased the diastolic SR Ca^{2+} leak in RyR2-R2474S$^{+/-}$ mice at the cellular level and prevented burst pacing-induced AF *in vivo*. These data suggest a role for diastolic SR Ca^{2+}

leak in initiating AF. Furthermore, inhibiting diastolic SR Ca^{2+} leak with a Rycal could be a potential therapeutic approach for preventing AF.

METHODS

Detailed methods are provided in the Online Supplement including generation of RyR2-knock-in mice, murine atrial myocytes isolation, intracellular Ca^{2+} measurements, measurement of total SR Ca^{2+} leak, intra-esophageal burst pacing, intra-cardiac burst pacing ECG recording, immunoprecipitation and immunoblot analyses.

S107 and Metoprolol treatment

S107 and metoprolol were diluted in drinking water at concentrations of 0.25 mg/ml and 0.1 mg/ml respectively. The drinking water was changed every week and the water consumption was recorded. There were no differences in water consumption between vehicle, S107 or metoprolol treated groups.

Statistical analysis

Data are reported as mean ± s.e.m unless otherwise indicated. *In vivo* AF stimulation studies were analyzed with chi-square. $P < 0.05$ was accepted as statistically significant. All experiments with animals were approved by Columbia University's Institutional Animal Care and Use Committee.

RESULTS

Atrial fibrillation (AF) in CPVT mouse models

Diastolic SR Ca^{2+} leak via mutant RyR2 triggers lethal ventricular arrhythmias during stress in CPVT patients [6–8, 14]. However, whether these mutant RyR2 in atria lead to atrial arrhythmias is not clear, although there are several clinical case reports demonstrating atrial premature complexes which may trigger atrial tachycardia, atrial flutter or AF in CPVT patients [9–13, 17]. As we have shown previously, RyR2-R2474S$^{+/-}$ mice developed typical bidirectional ventricular tachycardia (VT) and polymorphic VT during stress, which mimics the human CPVT phenotype [6]. To further study the molecular mechanism of CPVT, we generated two new mice harboring human CPVT mutations: RyR2-N2386I$^{+/-}$ and RyR2-L433P$^{+/-}$. Using stress protocols we confirmed the phenotype of these two new CPVT mouse models (supplemental figure I).

An intra-esophageal pacing method was developed to verify the role of diastolic SR Ca^{2+} leak via RyR2 in AF using three CPVT knock-in mouse models. In these experiments, the left atrium was paced via an intra-esophageal pacing catheter using a previously reported atrial burst pacing protocol [18]. Compared to WT mice which exhibited no AF under these conditions, mice harboring CPVT mutations could be stimulated into AF by atrial burst pacing (RyR2-R2474S$^{+/-}$; 7/10, RyR2-N2386I$^{+/-}$; 9/15, RyR2-L433P$^{+/-}$; 5/14 (Figures 1 A and B). The duration of AF and heart rate during atrial burst pacing-induced AF were similar among the groups (data not shown). To further verify the AF induced by intra-esophageal burst pacing, an octapolar catheter was introduced into the right ventricle via the jugular vein to record intra-cardiac ECGs during intra-esophageal pacing in three RyR2-N2386I$^{+/-}$ mice. AF events recognized on surface ECGs in these mice were confirmed by the presence of typical irregular atrial waves recorded from the intra-cardiac atrial leads (Figure 1C).

Sarcoplasmic reticulum Ca^{2+} leak in atrial myocytes from CPVT mice

To further explore the mechanism of increased prevalence of atrial burst pacing-induced AF in CPVT mice, we characterized the SR Ca^{2+} release in atrial myocytes isolated from CPVT and WT mice. Ca^{2+} spark frequencies were significantly increased in atrial myocytes from RyR2-R2474S$^{+/-}$ (5.59±0.49 /100 μm/s), RyR2-N2386I$^{+/-}$ (4.63±0.63 /100 μm/s) and RyR2-L433P$^{+/-}$ (4.14±0.45 /100 μm/s) compared to WT myocytes (2.60±0.39 /100 μm/s, $P<0.05$, Figure 2B). As the morphologies of Ca^{2+} sparks showed profound variations between these groups (Figure 2A), we developed a new parameter (methods described in supplemental data) to quantitatively assess the total SR Ca^{2+} leak between different groups. The results of total SR Ca^{2+} leak were similar to but more profound than that measured by Ca^{2+} spark frequencies. In agreement with the results of diastolic SR Ca^{2+} leak, the SR Ca^{2+} content in atrial myocytes isolated from RyR2-R2474S$^{+/-}$, RyR2-N2386I$^{+/-}$, and RyR2-L433P$^{+/-}$ mice were decreased compared to WT atrial myocytes ($\Delta F/F_0$: 6.93±0.38, 7.16±0.30, and 7.28±0.32 vs 8.47±0.42 in WT, $P<0.05$, Figure 2C).

S107 prevents SR Ca^{2+} leak in atrial myocytes from RyR2-R2474S$^{+/-}$ mice

Calstabin2 binding to the RyR2 channel complex stabilizes the closed state of the channel thereby preventing pathological diastolic SR Ca^{2+} leak [7, 8, 19]. Calstabin2 knock-out mice exhibit a higher prevalence of AF induced by endocardial burst pacing and diastolic SR Ca^{2+} leak in isolated atrial myocytes [20]. To test whether calstabin2 plays a role in diastolic SR Ca^{2+} leak in atrial myocytes of RyR2-R2474S$^{+/-}$ mice we treated isolated atrial myocytes with the Rycal S107, a 1,4-benzothiazepine, which is known to inhibit diastolic SR Ca^{2+} leak via RyR2 by inhibiting dissociation of calstabin2 from RyR2 [21]. Pre-incubation with S107 (10 μM) for 2 hrs significantly reduced the total diastolic SR Ca^{2+} leak in RyR2-R2474S$^{+/-}$ group by 46% (Figure 2B). Consistent with the reduction in diastolic SR Ca^{2+} leak, SR Ca^{2+} content was returned to normal by S107 ($\Delta F/F_0$: 8.47±0.42 in WT, 6.93±0.38 in RyR2-R2474S$^{+/-}$, $P<0.05$, and 8.21±0.35 in S107 treated RyR2-R2474S$^{+/-}$, P=NS, Figure 2C).

Decreased calstabin2 binding to RyR2 in atria but not ventricles isolated from RyR2-R2474S$^{+/-}$ mice

As we previously reported, PKA phosphorylation of RyR2 and castabin2 binding to RyR2 were unchanged in ventriclular cardiomyocytes from RyR2-R2474S$^{+/-}$ mice compared to WT littermates [6]. Stress-induced PKA phosphorylation of the channel depletes calstabin2 from RyR2 causing diastolic SR Ca^{2+} leak and ventricular arrhythmias [6]. In the present study atrial burst pacing did not cause PKA phosphorylation of RyR2 (Figure 3A and 3B); instead, it caused CaMKII phosphorylation of RyR2 as previously reported [22, 23] (supplemental figure XII). However, compared to WT mice, the calstabin2 level was significantly decreased in atrial RyR2 immunoprecipitated from RyR2-R2474S$^{+/-}$ mice. This was not the case in ventricular immunoprecipitates as we previously reported [6].

To further explore the cause of this difference between atrial and ventricular RyR2, we examined the PKA phosphorylation, oxidation and nitrosylation of RyR2 in atrial and ventricular tissues from WT and RyR2-R2474S$^{+/-}$ mice. We previous reported that RyR2 are oxidized and nitrosylated in cardiomyocytes from a knock-in mouse harboring RyR2 that mimic constitutively PKA hyperphosphorylated channels, RyR2-S2808D, as well as in human, rat and mouse HF [21]. Interestingly, the RyR2 from atrial tissue in RyR2-R2474S$^{+/-}$ mice exhibited increased oxidation but no detectable PKA phosphorylation or nitrosylation. In contrast there was no oxidation, PKA phosphorylation, or nitrosylation of atrial RyR2 from WT mice (Figure 3A and 3B). The cause of atrial RyR2-R2474S$^{+/-}$ channel oxidation is unknown, but provides an explanation for the depletion of calstabin2 from the RyR2 channel complex in atrial tissue of RyR2-R2474S$^{+/-}$ mice.

In order to explore the mechanism underlying S107 treatment in prevention of both burst pacing-induced AF and diastolic R Ca^{2+} leak in RyR2-R2474S$^{+/-}$ mice, we examined castabin2 binding and oxidation of atrial RyR2 in atrial samples from WT and RyR2-R2474S$^{+/-}$ mice. S107 inhibited depletion of calstabin2 from the RyR2 channel complex without reducing RyR2 oxidation (Figure 3C).

Since oxidation of the RyR2 channel complex was observed in RyR2-R2474S$^{+/-}$ atria, to test whether RyR2 oxidation can cause calstabin2 depletion from the channel complex, we applied the anti-oxidant DTT to atria from RyR2-R2474S$^{+/-}$ mice. Incubation with DTT reversed the oxidation of RyR2 and restored calstabin2 binding to RyR2 to levels comparable to those observed in RyR2 from WT atria (Figure 3D).

Atrial burst pacing-induced AF in PLN-KO and PLN-DM mice

Atrial burst pacing did not induce AF in phospholamban (PLN)-DM mice in which SR Ca^{2+} content is not increased by adrenergic stimulation due to mutations in the PKA and CaMKII phosphorylation sites in PLN [24] (Table 1). However, in PLN knockout mice, in which SR Ca^{2+} is maximally loaded, the prevalence of atrial burst pacing-induced AF was 37.5% (3/8) (Table 1). Thus, atrial burst pacing-induced AF in mice can be triggered by leaky RyR2 and/or SR Ca^{2+} overload and preventing adrenergic or rate related increases in SR Ca^{2+} content can inhibit AF triggered by leaky RyR2 channels.

S107 prevents atrial burst pacing-induced AF in RyR2-R2474S$^{+/-}$ and RyR2-N2386I$^{+/-}$ mice

RyR2-R2474S$^{+/-}$ and RyR2-N2386I$^{+/-}$ mice were treated with S107 in the drinking water (20 mg/kg/day) for two weeks. Compared to control groups, S107 treatment significantly decreased the susceptibility to AF in both RyR2-R2474S$^{+/-}$ (from 70% to 0%, Figure 4A) and RyR2-N2386I$^{+/-}$ (from 60% to 9.1%, Figure 4B) mice, indicating that diastolic SR Ca^{2+} leak plays a major role in atrial burst pacing-induced AF in these CPVT mice.

Increased SR Ca^{2+} leak in atrial vs. ventricular myocytes from RyR2-R2474S$^{+/-}$ mice

The difference in calstabin2 binding to RyR2 and increased oxidation in atrial RyR2 but not in ventricular RyR2 in RyR2-R2474S$^{+/-}$ mice could result in differences in diastolic SR Ca^{2+} leak between atria or ventricular cardiomyocytes. We isolated atrial and ventricular cardiomyocytes from WT and RyR2-R2474S$^{+/-}$ mice and compared diastolic SR Ca^{2+} leaks. As reported previously [25], ventricular myocytes isolated from RyR2-R2474S$^{+/-}$ mice exhibited higher Ca^{2+} spark frequencies compared to ventricular myocytes isolated from WT mice. Interestingly, the Ca^{2+} spark frequencies of atrial myocytes were higher than their ventricular counterparts in both WT and RyR2-R2474S$^{+/-}$ mice (Figure 5A), indicating an intrinsic difference in Ca^{2+} cycling between atria and ventricles. These findings are consistent with *in vivo* programmed electrical stimulation results in which the same burst pacing protocol introduced via both atrial (intra-esophageal) and ventricular (endocardial) routes in RyR2-R2474S$^{+/-}$ mice resulted in only atrial but not ventricular burst pacing-induced arrthythmias (Figure 5B). In addition, the Ca^{2+} spark frequencies in atrial myocytes isolated from WT mice and ventricular myocytes from RyR2-R2474S$^{+/-}$ mice were similar indicating a higher resting diastolic SR Ca^{2+} leak rate in atrial myocytes vs. ventricular myocytes. These data are consistent with *in vivo* data showing that without stress (exercise + epinephrine), none of RyR2-R2474S$^{+/-}$ or WT mice can be stimulated into ventricular arrhythmias and AF, respectively.

Calstabin2 knockout mice have increased atrial burst pacing-induced AF which was not prevented by S107 treatment

Although our previous work showed that both the 1,4-benzothiazepine JTV519 and the Rycal S107 prevent diastolic SR Ca^{2+} leak in murine models of heart failure and ventricular arrhythmias by preventing the stress induced depletion of calstabin2 from the RyR2 macromolecular complex [5, 6, 8, 19, 26–29], the mechanism of these drugs continues to be questioned [8]. In order to clarify the molecular mechanism of S107 we tested the effect of S107 in calstabin2 knockout mice. Consistent with a previous report [20], compared to WT, calstabin2 knockout mice showed significantly increased prevalence of AF with intra-esophageal burst pacing (Figure 6A and 6B). After 2-weeks of oral S107 (20 mg/kg/day delivered in the drinking water), the incidence of AF by atrial burst pacing was not changed in calstabin2 knockout mice, indicating that the mechanism of action of S107 depends on the presence of calstabin2. Consistent with these *in vivo* experiments, atrial myocytes isolated from calstabin2 knockout mice showed significantly increased Ca^{2+} spark frequencies that were not reduced by incubation with S107 (Figure 6C).

The role of CaMKII phosphorylation of RyR2 in atrial burst pacing-induced AF in mice with CHF

It has been reported that CaMKII phosphorylation of RyR2 is a major cause of SR Ca^{2+} leak in atrial myocytes with RyR2 mutations and leads to AF [22, 23, 30]. To test the role of CaMKII phosphorylation of RyR2 in triggering AF, we examined AF in a knock-in mouse, RyR2-S2814A, harboring an RyR2 that cannot be CaMKII phosphorylated. In agreement with a previous report by Chelu et al. [22] there was no AF stimulated in RyR2-S2814A mice by our intra-esophageal burst pacing protocol. To further confirm the activation of CaMKII during our intra-esophageal burst pacing procedure, we showed that RyR2 CaMKII phosphorylation at Ser2814 in our WT and CPVT mice was increased[22, 23, 31] (supplemental figure XII). However, as we previously reported, RyR2-S2814A knock-in mice were not protected against heart failure after myocardial infarction [32]. Moreover, there was no difference in pacing-induced AF between WT and RyR2-S2814A mice (50.0% vs. 63.6%), indicating that in mice with post-MI heart failure, the CaMKII phosphorylation of RyR2 does not play a pivotal role in atrial burst pacing-induced AF (supplemental figure V).

Catecholamines do not play an important role in atrial burst pacing-induced AF in 3 CPVT mouse models

CPVT is characterized by stress-induced polymorphic ventricular arrhythmias. To further explore the role of sympathetic activation in atrial burst pacing-induced AF in CPVT mouse models, we treated CPVT mice with metoprolol, a β-blocker, via drinking water for 4 weeks in a previously reported dose (30 mg/kg/day) [21]. Compared to vehicle groups, there was no significant therapeutic effect of metoprolol in the CPVT mice (supplemental figure VI), suggesting that activation of sympathetic system likely does not play an important role in triggering AF in our CPVT mouse models.

DISCUSSION

SR Ca^{2+} leak triggers AF

Although SR Ca^{2+} leak has been observed in cardiomyocytes from chronic AF patients[33], it is still not clear whether SR Ca^{2+} leak is the cause of or results from AF. Chronic sustained AF leads to atrial remodeling of both heart structure (increased fibrosis and atrial dilatation) and ion channel function [34]. We previously showed that there is RyR2 PKA hyperphosphorylation and calstabin2 dissociation from RyR2 in atrial samples from humans with AF and in an animal model with chronic AF [35]. These findings suggested that dynamic

molecular changes to RyR2 channels occur during chronic AF and that these changes may relate to the maintenance of AF. In the current study, using knock-in mouse models harboring human CPVT mutations which have diastolic SR Ca^{2+} leak without structural or functional abnormalities in the heart (supplemental figure IV), we explored the molecular basis of pacing-induced AF in detail. The CPVT mice had a significantly higher prevalence of pacing-induced AF compared to WT littermates. In isolated atrial myocytes from these mice, diastolic SR Ca^{2+} leak was significantly increased compared to WT atrial myocytes. This increased diastolic SR Ca^{2+} leak in myocytes isolated from all three CPVT mouse models was associated with decreased SR Ca^{2+} content compared to WT, indicating that a sustained diastolic SR Ca^{2+} leak via RyR2 occurs in atrial myocytes.

Calstabin2 dissociation leads to SR Ca^{2+} leak and burst pacing-induced AF

Both congestive heart failure (CHF) and CPVT are characterized by calstabin2 dissociation from the RyR2 macromolecular complex resulting in “leaky” RyR2 channels. Our previous work showed that RyR2-castabin2 binding stabilizes the RyR2 channel complex and prevents diastolic SR Ca^{2+} leak in CHF or CPVT [6, 19, 21, 36, 37]. The role of castabin2 binding to RyR2 in AF was first reported in myocytes from human chronic AF patients [35] and Sood et al. showed endocardial right atrial burst pacing could induce AF in calstabin2 knockout mice [20]. Our intra-esophageal pacing protocol showed a significant increase in AF prevalence in calstabin2 knockout mice and S107 treatment failed to inhibit the burst pacing-induced AF *in vivo* and diastolic SR Ca^{2+} leak *in vitro* in calstabin2 deficient mice. However, in the CPVT models, in contrast to ventricular arrhythmias that are induced by both exercise and epinephrine, AF was induced by fast atrial pacing without any catecholamine treatment. Inhibition of sympathetic activity by the β-blocker metoprolol did not suppress AF in our CPVT mouse models (supplemental figure VI), further indicating that catecholamines may not be important in triggering AF in CPVT. This discrepancy between atrial and ventricular arrhythmias in the CPVT mouse models is likely explained by the depletion of calstabin2 from the RyR2 molecular complex in atrial but not ventricular tissues from resting RyR-R2474S$^{+/-}$ mice (Figure 3). We previously reported that the dissociation of calstabin2 from RyR2 is caused by remodeling of the RyR2 channel complex including PKA phosphorylation, nitrosylation, and oxidation of RyR2 [21]. We now show that RyR2 from RyR2-R2474S$^{+/-}$ atrial tissue are oxidized at baseline indicating chronic remodeling of the channel in this CPVT mouse. Indeed, clinical reports have suggested a close link between elevated levels of derivatives of reactive oxygen metabolites and persistent AF and AF recurrence after radio frequency catheter ablation in paroxysmal AF patients [38]. We previously showed that oxidation and PKA phosphorylation cause calstabin2 depletion from RyR2 [21]. We now show that calstabin2 dissociation from RyR2 can occur when the channel is oxidized. Using the reducing reagent DTT to inhibit RyR2 oxidation in atrial tissue isolated from RyR2-R2474S$^{+/-}$ mice we further examined the role of RyR2 oxidation in depletion of calstabin2 from the RyR2 channel complex in our CPVT mouse model. It is possible that the CPVT mutations may alter RyR2 conformation rendering it more accessible to oxidation and more sensitive to calstabin2 depletion.

Restoring calstabin2 binding to RyR2 stabilizes the channel and prevents burst pacing-induced AF

As we previously reported [6, 21, 26, 27], S107 stabilizes RyR channels and prevents Ca^{2+} leak by enhancing RyR-calstabin interactions. Here we show that incubation of atrial myocytes isolated from RyR2-R2474S$^{+/-}$ mice with S107 inhibited diastolic SR Ca^{2+} leak. S107 treatment of mice also prevented intra-esophageal burst pacing-induced AF in RyR2-R2474S$^{+/-}$, RyR2-N2386I$^{+/-}$ mice but had no effect in mice lacking calstabin2. The effect of S107 in stabilizing leaky RyR2 channels in the atria indicates that dissociation of calstabin2 from RyR2 channel complex likely plays an important role in AF in these CPVT

mouse models. Furthermore, according to the Framingham Study, CHF is the strongest predictor for the development of AF [39]. CHF results in RyR2 PKA hyperphosphorylation, oxidation, nitrosylation and calstabin2 dissociation from RyR2 molecular complex. Dissociation of calstabin2 from the RyR2 channel complex could be one of the causes of AF in patients with CHF. Therefore, the RyR2 stabilizing Rycal S107 has potential as a possible therapeutic for the prevention and treatment of AF related to CHF or CPVT.

The role of CaMKII in triggering AF in CPVT

Chelu et al. reported that AF stimulated by intracardiac pacing is associated with CaMKII phosphorylation of RyR2 [22]. However, the finding that the CaMKII inhibitor KN93 suppressed burst pacing-induced AF does not prove that CaMKII phosphorylation of RyR2 plays a pivotal role in AF since CaMKII phosphorylates other Ca^{2+} cycling proteins including the L-type Ca^{2+} channel and PLN which modulates SERCA2a to regulate SR Ca^{2+} uptake. Consistent with previous reports [22, 23, 31], our experiment using freshly isolated atria from WT and RyR2-R2474S$^{+/-}$ mice with or without atrial burst pacing showed that atrial burst pacing led to RyR2 CaMKII phosphorylation at Ser2814 in both groups (supplemental figure XII), suggesting a role for CaMKII phosphorylation of RyR2 during atrial burst pacing induced AF. To further clarify the role of CaMKII phosphorylation of RyR2 in triggering AF, we used a clinically relevant acute myocardial infarction induced HF model as AF occurs in 15% to 30% of patients with HF[40]. According to our previously published results, RyR2-S2814A mice showed similar progression of heart failure after myocardial infarction compared to WT littermates [32]. In these HF mice, the atrial burst pacing protocol induced similar incidences of AF in both RyR2-S2814A and WT groups, arguing that CaMKII phosphorylation of RyR2 does not play a major role in triggering AF in HF. However, due to the multiple substrates of CaMKII in Ca^{2+} cycling proteins, the role of pacing-induced activation of CaMKII in modulating Ca^{2+} cycling requires further study.

The different characteristics of arrhythmias between atria and ventricles

Increased diastolic SR Ca^{2+} leak in atrial myocytes isolated from RyR2-R2474S$^{+/-}$ mice is consistent with *in vivo* intra-esophageal and intra-cardiac burst pacing-induced AF and ventricular arrhythmias respectively in RyR2-R2474S$^{+/-}$ mice (Figure 6). A recent report showed that rat atrial myocytes have higher SR mediated Ca^{2+} uptake and a ~3-fold higher SR Ca^{2+} load compared to ventricular myocytes [41]. Higher SR Ca^{2+} load and increased SR Ca^{2+} uptake may explain the increased Ca^{2+} spark frequencies in both WT and RyR2-R2474S$^{+/-}$ atrial myocytes compared to their ventricular counterparts and may lower the threshold for induction of atrial arrhythmias induced by burst atrial pacing. The baseline Ca^{2+} spark frequencies of WT atrial myocytes and RyR2-R2474S$^{+/-}$ ventricular myocytes were comparable (Figure 5, first and fourth bar), indicating comparable diastolic SR Ca^{2+} leak. This leak by itself is not sufficient to induce AF during *in vivo* burst pacing stimulation in WT mice, or ventricular arrhythmias in RyR2-R2474S$^{+/-}$ mice. Clinically, VT is observed during exercise in patients with the RyR2-R2474S mutation indicating the importance of sympathetic activation of the SR Ca^{2+} uptake pathway and loading of the SR to increase the amplitude of the leak. The exact reasons for these differences are still not well understood. However, it is well known that unlike ventricular fibrillation which leads to sudden cardiac death, AF is typically not lethal in the absence of a by-pass tract. Therefore there might be less evolutionary pressure to maintain a higher threshold for arrhythmias in the atria.

Mechanism of AF

Although re-entry and multiple wavelets are observed in AF, the molecular events initiating AF remain uncertain. In the current study using CPVT mouse models with known RyR2 mutations and normal cardiac structure and function (supplemental figure IV), we explored

the role of diastolic SR Ca^{2+} leak via mutant RyR2 in the atria. In agreement with previous reports implicating abnormal Ca^{2+} handling in AF, we did observe Ca^{2+} waves (supplemental figure IX) and indirect evidence of Ca^{2+}-activated inward current (DAD, supplemental figure VIII) in our studies. Together with the normal cardiac structure and function of the CPVT mouse models, our data indicate that the diastolic SR Ca^{2+} leak via RyR2 leads to Ca^{2+} waves and DADs which form multiple wavelets and possible re-entry loops that trigger atrial tachycardia (AT) and AF.

Limitations and disadvantages

The CPVT RyR2 mutations in our study were originally discovered in CPVT patients. However, due to the low prevalence of CPVT in population and extremely low number of patients with each specific RyR2 mutation there are no reports of AF in patients with RyR2-R2474S, RyR2-N2386I, or RyR2-L433P mutations. Due to technical limitations, it is impossible to pace a single cardiomyocyte at physiologic frequencies (for mice) e.g. 9–10Hz. Therefore, the Ca^{2+} spark measurements may not reflect physiological conditions in intact atria. Also, the burst pacing protocol needed to elicit AF in mice may not represent maintained AF in humans. In addition, abnormal function of pacemaker cells in the CPVT mouse models is potentially a factor in triggering AF.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations

AF	atrial fibrillation
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CHF	congestive heart failure
CPVT	catecholaminergic polymorphic ventricular tachycardia
DAD	delayed after depolarizations
DNP	2, 4-dinitrophenyl
DTT	dithiothreitol
HF	heart failure
PKA	protein kinase A
PLN	phospholamban
PLN-DM	mutant PLN in which both phosphorylation residues (Ser16 and Thr17) were replaced by Ala
RyR2	ryanodine receptor type 2
SERCA	sarco/endoplasmic reticulum Ca^{2+}-ATPase

SR	sarcoplasmic reticulum
VT	ventricular tachycardia
WT	wild-type

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Novelty and Significance

What Is Known?

- Chronic atrial fibrillation (AF) is associated with increased diastolic sarcoplasmic reticulum (SR) Ca^{2+} leak in atrial cardiac myocytes.
- Catecholaminergic polymorphic ventricular tachycardia (CPVT)-linked ryanodine receptor 2 (RyR2) mutations cause diastolic SR Ca^{2+} leak in ventricular cardiac myocytes.
- Recent reports show that CPVT patients have increased prevalence of AF.

What New Information Does This Article Contribute?

- Diastolic SR Ca^{2+} leak likely plays a critical role in initiating AF in murine models of human CPVT-linked RyR2 mutations.
- S107 (Rycal) significantly inhibits diastolic SR Ca^{2+} leak in atrial myocytes and prevents pacing-induced AF in models of human CPVT-linked RyR2 mutations.

Atrial fibrillation (AF) is the most common cardiac arrhythmia. However, the mechanisms underlying AF are not well understood despite more than 100 years of study. This has impaired the development of therapeutics for AF. Intracellular Ca^{2+} plays a central role in the action potential and contraction of atrial myocytes. While the role of diastolic SR Ca^{2+} leak in the generation of ventricular arrhythmias has been demonstrated, the impact of diastolic SR Ca^{2+} leak in the genesis of AF remains to be established. It has been shown that CPVT-linked RyR2 mutations cause diastolic SR Ca^{2+} leak, delayed after depolarizations and lethal ventricular arrhythmias. Recent reports demonstrate that patients with CPVT-linked RyR2 mutations have a higher prevalence of AF as well. Murine models harboring CPVT-linked RyR2 mutatoins exhibit increased burst pacing-induced AF. Atrial myocytes isolated from these mice showed increased diastolic SR Ca^{2+} leak. The rycal drug S107 that inhibits loss of the stabilizing subunit calstabin2 from the RyR2 macromolecular complex inhibited diastolic SR Ca^{2+} SR leak and prevented burst pacing-induced AF in murine models of CPVT-linked RyR2 mutations. These findings indicate that inhibition of diastolic SR Ca^{2+} leak with a rycal drug could be a potential therapeutic approach for preventing AF.

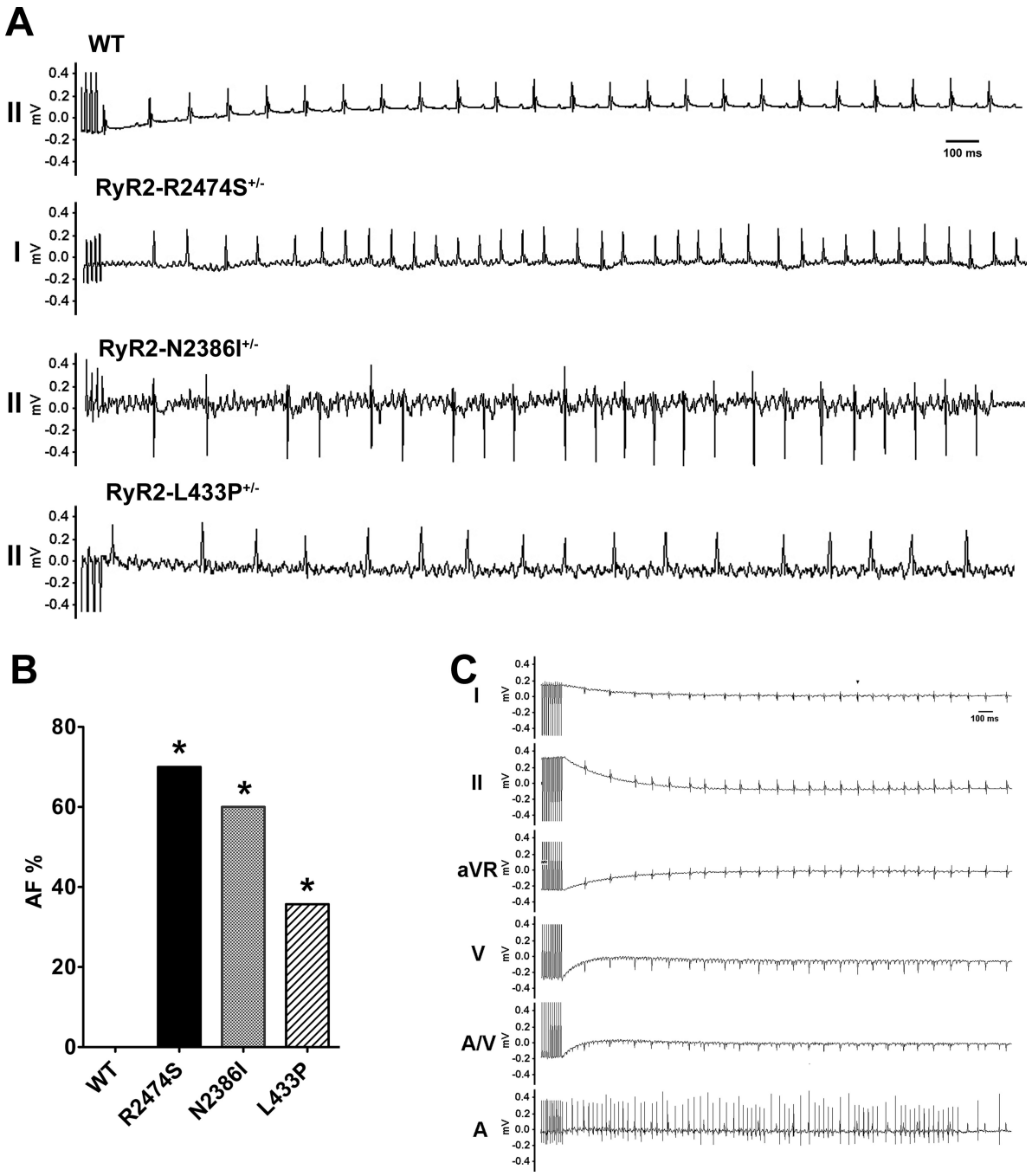

Figure 1. Intra-esophageal burst pacing induces AF in three murine CPVT models

A) Representative surface ECG traces from WT, RyR2-R2474S$^{+/-}$, RyR2-N2386I$^{+/-}$, and RyR2-L433P$^{+/-}$ mice during intra-esophageal burst pacing. B) Prevalence of AF in WT (n=33), RyR2-R2474S$^{+/-}$ (n=10), RyR2-N2386I$^{+/-}$ (n=15), and RyR2-L433P$^{+/-}$ (n=14) mice during intra-esophageal burst pacing. *, P<0.05 vs. WT. C) Representative AF in surface and intra-cardiac ECG trace from a RyR2-N2386I$^{+/-}$ mouse after intra-esophageal burst pacing stimulation (I, II, and aVR, surface leads; V, ventricular leads; A/V, atrioventricular node leads; A, atrial leads).

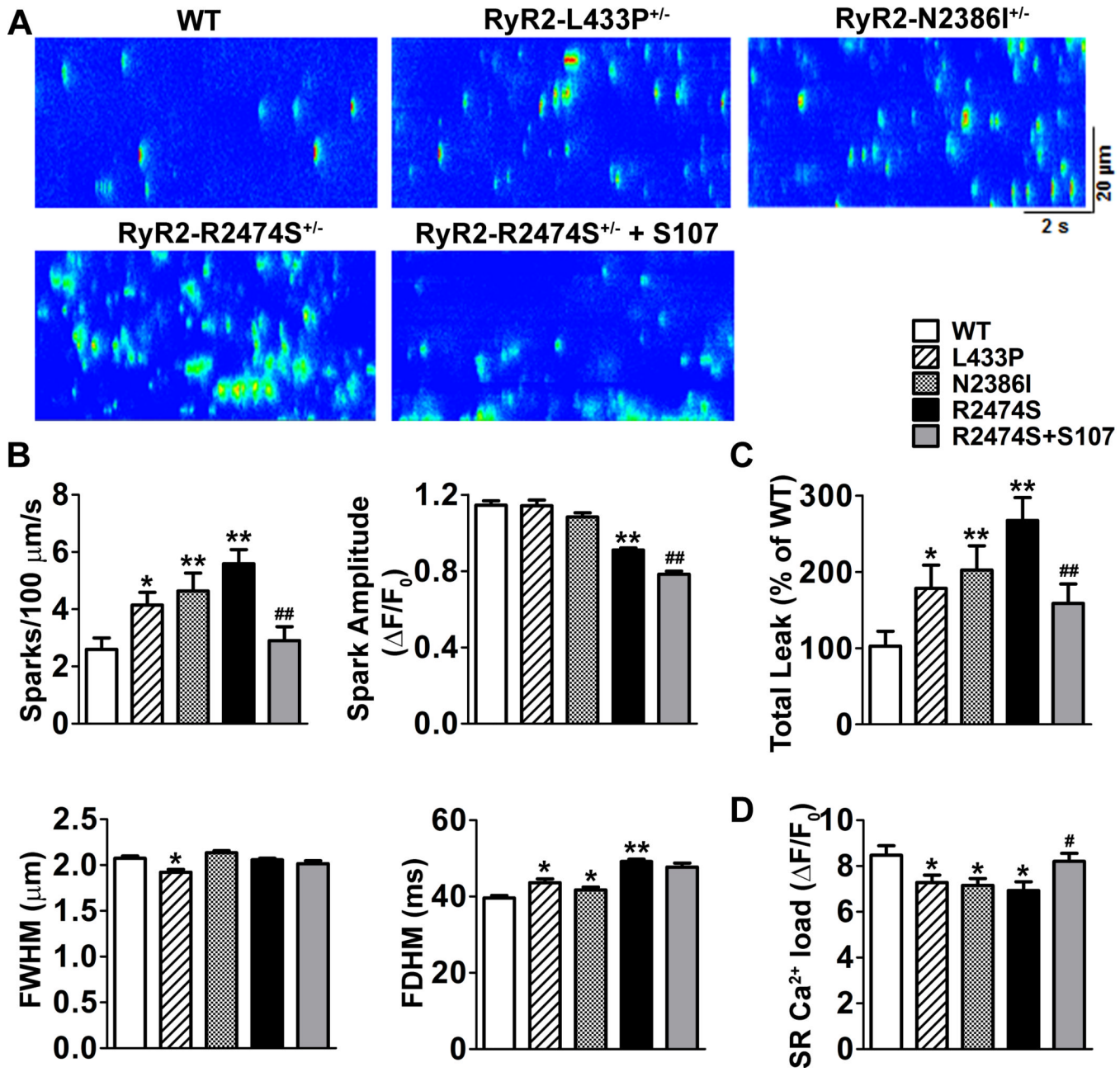

Figure 2. SR Ca^{2+} leak in atrial myocytes isolated from CVPT mice

A) Representative raw line scan of WT, RyR2-L433P$^{+/-}$, RyR2-N2386I$^{+/-}$, RyR2-R2474S$^{+/-}$, and S107 treated RyR2-R2474S$^{+/-}$ atrial myocytes. B) Ca^{2+} leak parameters for different groups, n=20~25 cells in each group. C), SR Ca^{2+} contents, n=7~14 cells in each group. * P<0.05 and ** P<0.01 compared to WT; # P<0.05 and ##, P<0.01 compared to RyR2-R2474S$^{+/-}$.

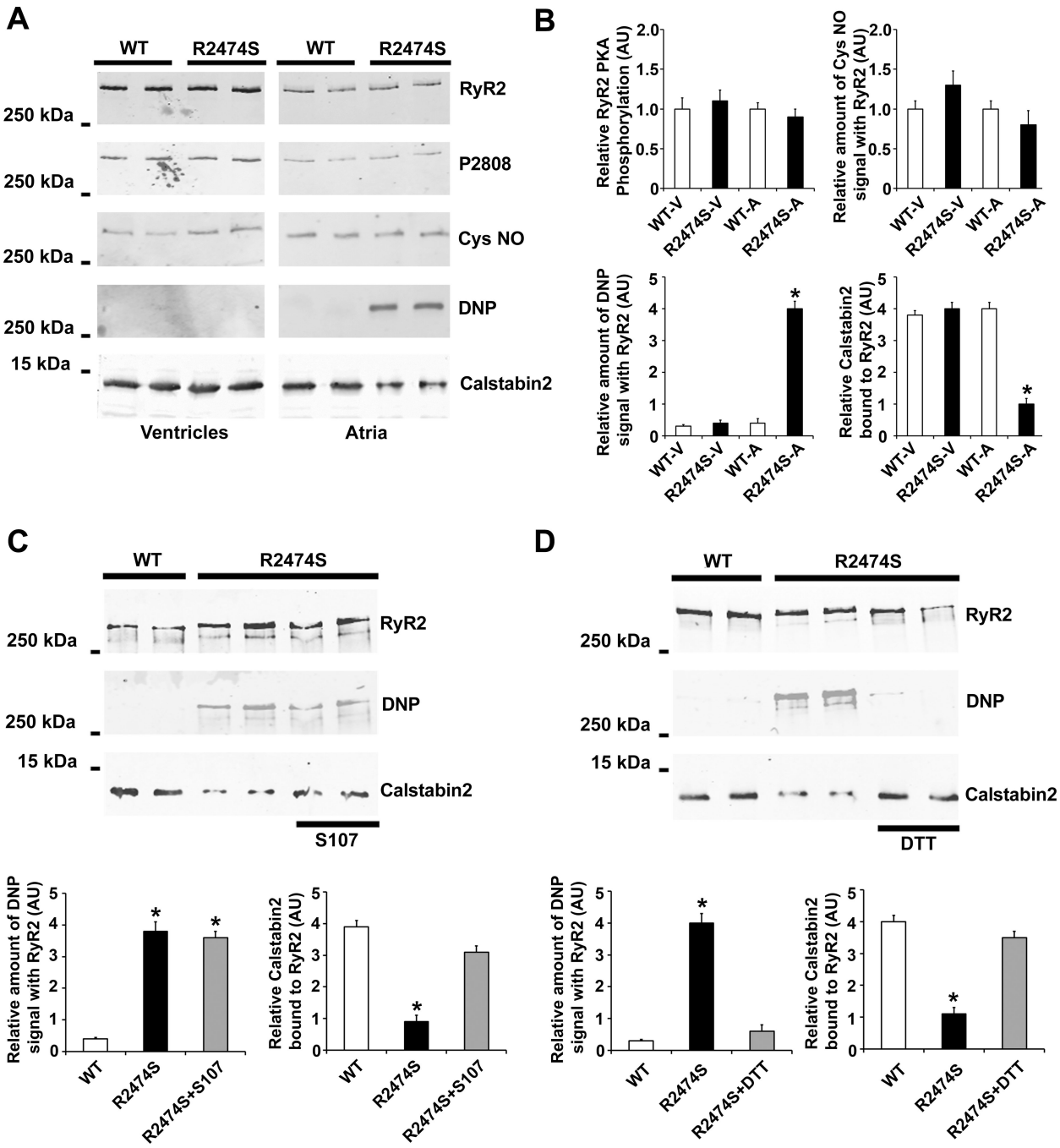

Figure 3. Atrial RyR2 channel complex remodeling in RyR2-R2474S$^{+/-}$ mice

A) Representitive immunoprecipitation of RyR2 from ventricular and atrial tissues from WT and RyR2-R2474S$^{+/-}$ mice showing oxidation (DNP) and calstabin2 dissociation from RyR2 channel complex only in atria of RyR2-R2474S$^{+/-}$ mice. B) Pooled data from 4 separate immunoblots. V, ventricles; A, atria; *, P<0.05 vs. WT. C) Representitive immunoprecipitation of RyR2 from atrial tissues from WT and RyR2-R2474S$^{+/-}$ treated vehicle or S107. Bottom, pooled data from 3 separate immunoblots, *, P<0.05 vs. WT. D) Representitive immunoprecipitation of RyR2 from atrial tissues from WT, RyR2-R2474S$^{+/-}$ and RyR2-R2474S$^{+/-}$ following *in vitro* treatment with DTT. Bottom, pooled data from 3 separate immunoblots, *, P<0.05 vs. WT.

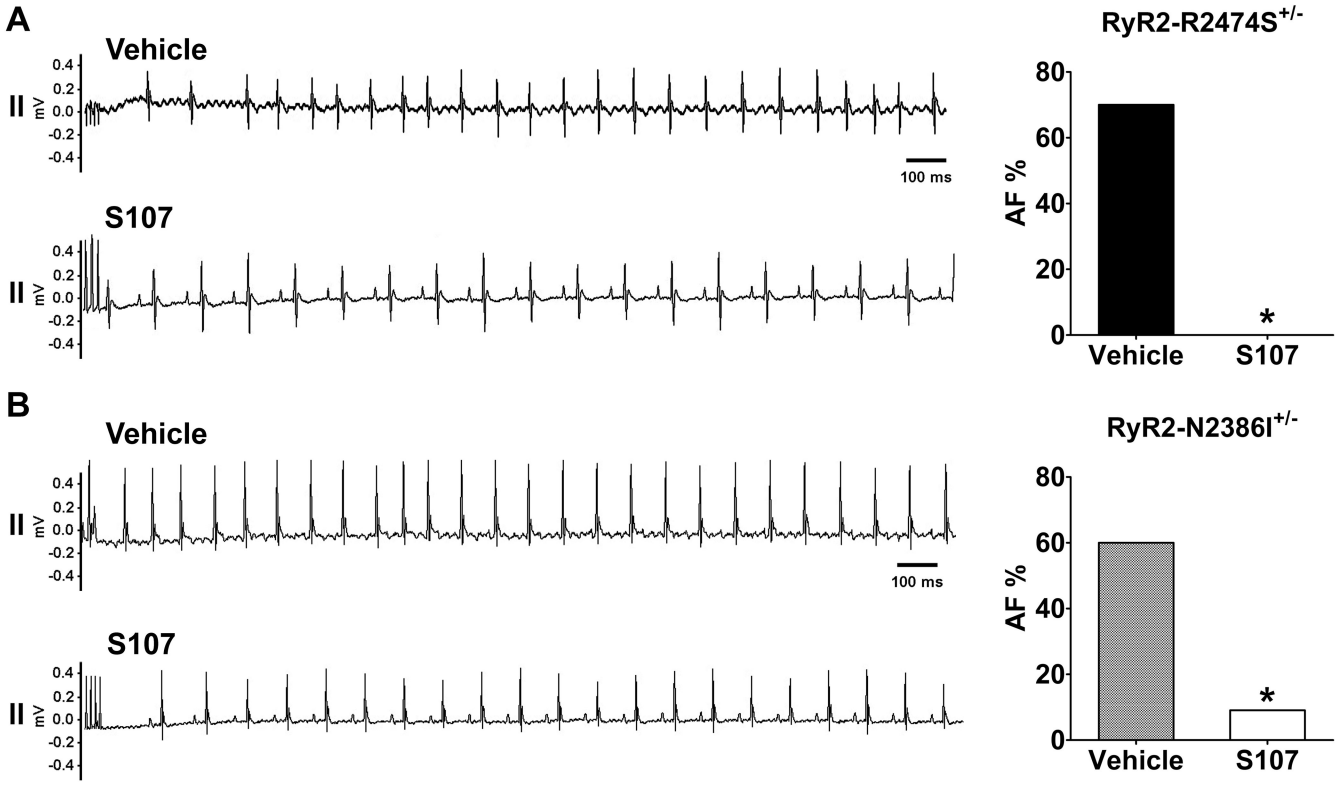

Figure 4. S107 prevents atrial burst pacing-induced AF in RyR2-R2474S$^{+/-}$ and RyR2-N2386I$^{+/-}$ mice

A) Left panel: representative ECG traces from RyR2-R2474S$^{+/-}$ mice during intra-esophageal burst pacing in vehicle or S107 treatment groups. Right panel: prevalence of AF in RyR2-R2474S$^{+/-}$ mice treated with vehicle (n=10) or S107 (n=10). B) Left panel: representative ECG traces of RyR2-N2386I$^{+/-}$ mice during intra-esophageal burst pacing in vehicle or S107 treatment groups. Right panel: prevalence of AF in RyR2-N2386I$^{+/-}$ mice treated with vehicle (n=15) or S107 (n=11). *, P<0.05 vs. vehicle treatment group.

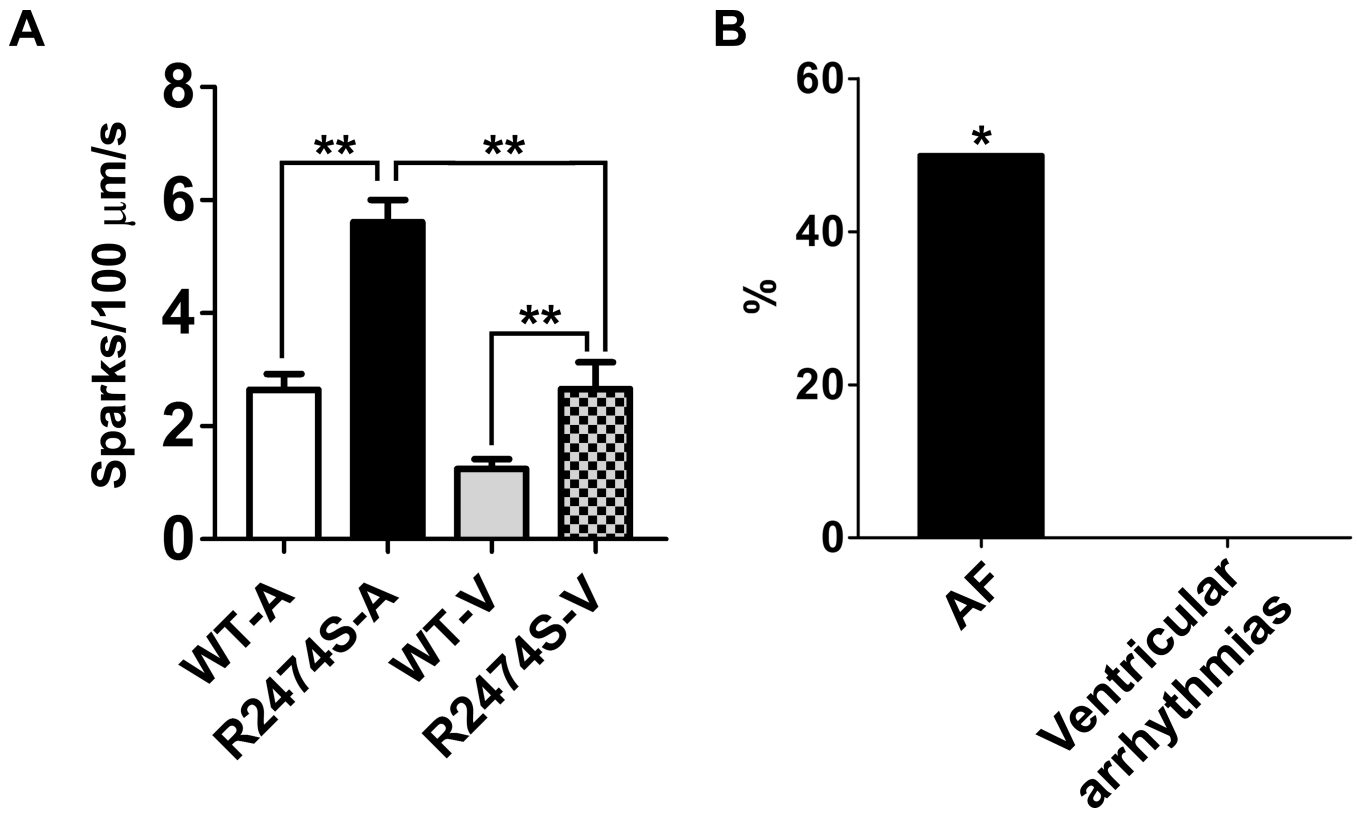

Figure 5. Comparison between atrial and ventricular Ca^{2+} sparks and arrhythmias in WT and RyR2-R2474S$^{+/-}$ mice

A) Differences in diastolic SR Ca^{2+} leak measured as Ca^{2+} spark frequency in atrial and ventricular cardiomyocytes isolated from WT and RyR2-R2474S$^{+/-}$ mice. For ventricular myocytes, we use the same solutions as those used for atrial cardiomyocytes (see methods) and 1~3 Hz pacing to induce Ca^{2+} sparks. n=42 atrial and ventricular myocytes from WT mice, n=31 atrial and ventricular myocytes from RyR2-R2474S$^{+/-}$ mice. **, p<0.01. B) Prevalence of burst pacing-induced AF and ventricular arrhythmias in RyR2-R2474S$^{+/-}$ mice. AF was stimulated by a intra-esophageal pacing protocol, ventricular arrhythmias were stiumlated by an intra-cardiac pacing protocol (n=10 in both groups). *, P<0.05 vs. ventricular arrhythmias.

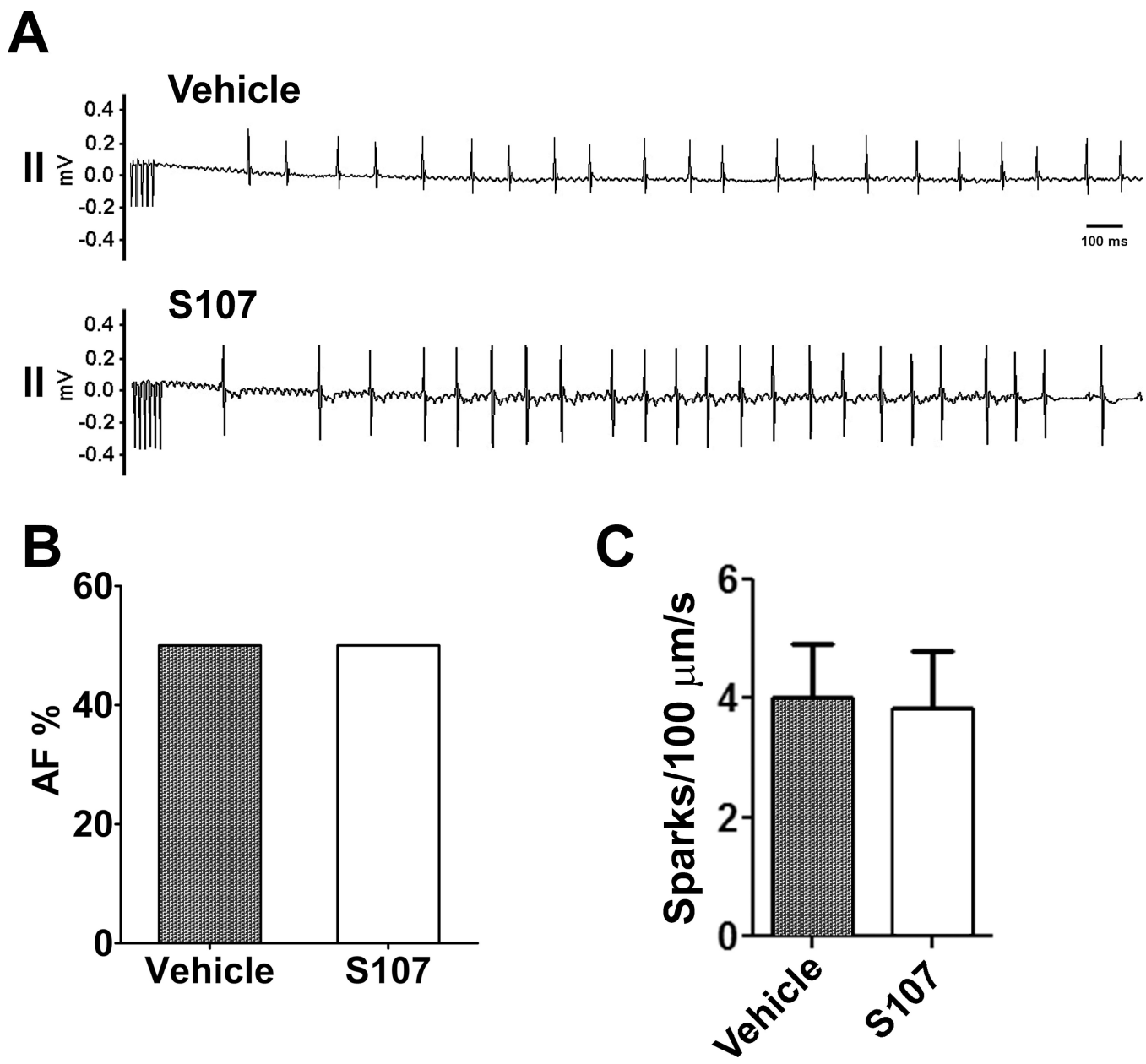

Figure 6. S107 has no effect on atrial burst pacing-induced AF in castabin2 KO mice

A) Representative surface ECG traces from a calstabin2 KO mouse treated with vehicle or S107. B) Prevalence of AF in calstabin2 KO mice in vehicle (n=10) and S107 treatment (n=12) groups during intra-esophageal burst pacing. C) Atrial myocytes isolated from calstabin2 KO mice were incubated with 10 μM S107 for 2 hrs before sampling for Ca^{2+} spark frequencies (n=13 cells in both groups).

Table 1

Atrial burst pacing-induced AF in WT, PLN KO and PLN-DM mice before and after caffeine treatment

	WT (n=33)	PLN KO (n=8)	PLN-DM (n=8)
Vehicle (%)	0	37.5*	0
Caffeine (120 mg/kg) (%)	45.5	62.5	50

*P<0.05 vs. WT