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

Jian Shan¹, Wenjun Xie¹, Matthew Betzenhauser¹, Steven Reiken¹, Bi-Xing Chen¹, Anetta Wronska¹, Andrew R. Marks¹,²

¹Departments 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 and ²Medicine, College of Physicians and Surgeons of Columbia University, New York, NY 10032, USA.

Running title: Ca²⁺ Leak Through RyR Leads to AF in CPVT Mice

Subject codes:
[130] Animal models of human disease
[132] Arrhythmias-basic studies

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
E-mail: arm42@columbia.edu

In June 2012, the average time from submission to first decision for all original research papers submitted to Circulation Research was 13.35 days.

DOI: 10.1161/CIRCRESAHA.112.273342
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.

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

Non-standard Abbreviations:

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

DOI: 10.1161/CIRCRESAHA.112.273342
INTRODUCTION

Atrial fibrillation (AF) is the most common arrhythmia and is especially prevalent in the elderly. AF accounts for more than one-third of all arrhythmia-related hospitalizations. 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. 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.

Our laboratory has reported that SR Ca\(^{2+}\) leak via PKA hyperphosphorylated and/or oxidized RyR2 channels contributes to heart failure (HF) progression and triggers ventricular arrhythmias. 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. Recent reports demonstrate that patients with CPVT mutations have AF. 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. 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.

**DOI:** 10.1161/CIRCRESAHA.112.273342
METHODS

Detailed methods are provided in the Online Supplement including generation of RyR2- knock-in mice, murine atrial myocytes isolation, intracellular Ca\textsuperscript{2+} measurements, measurement of total SR Ca\textsuperscript{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\textsuperscript{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\textsuperscript{+/-} 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\textsuperscript{+/-} and RyR2-L433P\textsuperscript{+/-}. 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\textsuperscript{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\textsuperscript{+/-}; 7/10, RyR2-N2386I\textsuperscript{+/-}; 9/15, RyR2-L433P\textsuperscript{+/-}; 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\textsuperscript{+/-} 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\textsuperscript{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\textsuperscript{2+} release in atrial myocytes isolated from CPVT and WT mice. Ca\textsuperscript{2+} spark frequencies were significantly increased in atrial myocytes from RyR2-R2474S\textsuperscript{+/-} (5.59±0.49

**DOI:** 10.1161/CIRCRESAHA.112.273342 4
/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 Ca²⁺ 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 Ca²⁺ leak between different groups. The results of total SR Ca²⁺ leak were similar to but more profound than that measured by Ca²⁺ spark frequencies. In agreement with the results of diastolic SR Ca²⁺ leak, the SR Ca²⁺ content in atrial myocytes isolated from RyR2-R2474S+/−, RyR2-N2386I+/−, and RyR2-L433P+/− mice were decreased compared to WT atrial myocytes (ΔF/F₀: 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 Ca²⁺ 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 Ca²⁺ leak 7, 8, 19. Calstabin2 knock-out mice exhibit a higher prevalence of AF induced by endocardial burst pacing and diastolic SR Ca²⁺ leak in isolated atrial myocytes 20. To test whether calstabin2 plays a role in diastolic SR Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ leak in RyR2-R2474S+/− group by 46% (Figure 2B). Consistent with the reduction in diastolic SR Ca²⁺ leak, SR Ca²⁺ content was returned to normal by S107 (ΔF/F₀: 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 calstabin2 binding to RyR2 were unchanged in ventricular cardiomyocytes from RyR2-R2474S+/− mice compared to WT littermates 6. Stress-induced PKA phosphorylation of the channel depletes calstabin2 from RyR2 causing diastolic SR Ca²⁺ 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 Ca²⁺ leak in RyR2-R2474S+/− mice, we examined calstabin2 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 antioxidant 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 Ca2+ content is not increased by adrenergic stimulation due to mutations in the PKA and CaMKII phosphorylation sites in PLN (Table 1). However, in PLN knockout mice, in which SR Ca2+ 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 Ca2+ overload and preventing adrenergic or rate related increases in SR Ca2+ 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 Ca2+ leak plays a major role in atrial burst pacing-induced AF in these CPVT mice.

**Increased SR Ca2+ 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 Ca2+ leak between atria or ventricular cardiomyocytes. We isolated atrial and ventricular cardiomyocytes from WT and RyR2-R2474S/−/+ mice and compared diastolic SR Ca2+ leaks. As reported previously, ventricular myocytes isolated from RyR2-R2474S/−/+ mice exhibited higher Ca2+ spark frequencies compared to ventricular myocytes isolated from WT mice. Interestingly, the Ca2+ 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 Ca2+ 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 arrhythmias (Figure 5B). In addition, the Ca2+ spark frequencies in atrial myocytes isolated from WT mice and ventricular myocytes from RyR2-R2474S/−/+ mice were similar indicating a higher resting diastolic SR Ca2+ 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 Ca2+ leak in murine models of heart failure and ventricular arrhythmias by preventing the stress induced depletion of calstabin2 from the RyR2 macromolecular complex, the mechanism of these drugs continues to be questioned. In order to clarify the molecular mechanism of S107 we tested the effect of S107 in calstabin2 knockout mice. Consistent with a previous report,
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 \(\beta\)-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

DOI: 10.1161/CIRCRESAHA.112.273342
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-calstabin2 binding stabilizes the RyR2 channel complex and prevents diastolic SR Ca\(^{2+}\) leak in CHF or CPVT. The role of calstabin2 binding to RyR2 in AF was first reported in myocytes from human chronic AF patients and Sood et al. showed endocardial right atrial burst pacing could induce AF in calstabin2 knockout mice. 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 \(\beta\)-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. 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. We previously showed that oxidation and PKA phosphorylation cause calstabin2 depletion from RyR2. 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, 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. 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 Ryca 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. 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, 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. According to our previously published results, RyR2-S2814A mice showed similar progression of heart failure after myocardial infarction compared to WT littermates. 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. 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.

DOI: 10.1161/CIRCRESAHA.112.273342
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.

ACKNOWLEDGEMENTS
We thank Dr. John Vest for help in analyses of in vivo AF stimulation data.

SOURCES OF FUNDING
This work was supported a grant from the NHLBI to ARM (1R01HL102040-01A1). M.J.B. was supported by a Postdoctoral Fellowship (F32-HL107029) from the NIH.

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

REFERENCES

DOI: 10.1161/CIRCRESAHA.112.273342


20. Sood S, Chelu MG, van Oort RJ, Skapura D, Santonastasi M, Dobrev D, Wehrens XHT. Intracellular calcium leak due to fkbp12.6 deficiency in mice facilitates the inducibility of atrial fibrillation. _Heart Rhythm_. 2008;5:1047-1054


DOI: 10.1161/CIRCRESAHA.112.273342


DOI: 10.1161/CIRCRESAHA.112.273342


FIGURE LEGENDS

**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 Ca2+ 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) Ca2+ leak parameters for different groups, n=20~25 cells in each group. C), SR Ca2+ 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) Representative 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) Represenative 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+/− and RyR2-N2386I+/− 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 Ca2+ sparks and arrhythmias in WT and RyR2-R2474S+/− mice. A) Differences in diastolic SR Ca2+ leak measured as Ca2+ 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 Ca2+ sparks. n=42 atrial and ventricular myocytes from WT mice, n=31 atrial and

DOI: 10.1161/CIRCRESHA.112.273342
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 an intra-esophageal pacing protocol, ventricular arrhythmias were stimulated 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 Ca2+ 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

<table>
<thead>
<tr>
<th></th>
<th>WT (n=33)</th>
<th>PLN KO (n=8)</th>
<th>PLN-DM (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vehicle (%)</strong></td>
<td>0</td>
<td>37.5*</td>
<td>0</td>
</tr>
<tr>
<td><strong>Caffeine (120 mg/kg) (%)</strong></td>
<td>45.5</td>
<td>62.5</td>
<td>50</td>
</tr>
</tbody>
</table>

*, P<0.05 vs. WT
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. Intraacellular 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 mutations 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\(^{2+}\) 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.
Calcium Leak Through Ryanodine Receptors Leads to Atrial Fibrillation in Three Mouse Models of Catecholaminergic Polymorphic Ventricular Tachycardia
Jian Shan, Wenjun Xie, Matthew Betzenhauser, Steven Reiken, Bixing Chen, Anetta Wronska and Andrew R. Marks

_Circ Res._ published online July 24, 2012;
_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/early/2012/07/24/CIRCRESAHA.112.273342

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2012/07/24/CIRCRESAHA.112.273342.DC1

**Permissions:** Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

**Reprints:** Information about reprints can be found online at:
http://www.lww.com/reprints

**Subscriptions:** Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org//subscriptions/
**Supplemental materials**

**Stress induced ventricular arrhythmias in three CPVT mouse models**

Mice harboring CPVT mutations (RyR2-R2474S+/−, RyR2-N2386I+/−, and RyR2-L433P+/−) were studied using two conventional stress protocols to demonstrate CPVT: 1, treadmill exercise plus epinephrine (EPI) injection1 and 2, isoproterenol (Iso) plus caffeine injection2. Following treadmill exercise plus EPI (2 mg/kg i.p.) injection, all (6/6) RyR2-R2474S+/− mice displayed typical bidirectional VT, while the RyR2-N2386I+/− and RyR2-L433P+/− mice failed to exhibit any VT (Supplemental Figure IA). We further examined arrhythmogenesis by treating the mice with Iso (0.5 mg/kg i.p.) plus caffeine (120 mg/kg i.p). Under these conditions, mice with CPVT mutations exhibited ventricular bigeminy and again, all RyR2-R2474S+/− mice developed bidirectional VT (Supplemental Figure IB). Thus, the 3 CPVT mouse models recapitulated the CPVT phenotype.

**Generation of RyR2-N2386I knock-in mouse**

The targeting vector for homologous recombination consisted of an 8.9-kb genomic DNA fragment including exons 44-51 of the RyR2 genomic sequence. The 5’ and 3’ flanking regions were amplified from 129S mouse genomic DNA using PCR with the following 2 sets of primers: 5’-ATGCAGGCGGAGAATCTACTACATCCCCCTTG-3’ and 5’-TCAATCGATGCACCTTATGAGATTTCTTCAA-3’ (5’ flanking region) and 5’- TCAACAGCACTGAAAGGTGCCACC-3’ with 5’-TCCTGCCACAGCATAGGCACAGAGACC-3’ (3’ flanking region). The resulting PCR fragments were subcloned into the pBlueScript SK− plasmid (Stratagene). Mutagenesis was performed in the 5’ flanking region using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer’s instructions to introduce the codon change leading to the N2366I variant in exon 47.
(Supplemental Figure IIA). To facilitate screening for the mutation a BspH1 site was deleted. The two arms were subsequently cloned into Sall sites of pACN. The resulting plasmid constitutes the TVN2386I targeting vector consisting of 2 arms separated by the pACN cassette. TVN2386I was linearized with SacII and transfected by electroporation into ES cells. Transfected ES cells underwent positive selection with G418 (200 μM) for 10 days. Genomic DNA from resistant clones was analyzed for occurrence of homologous recombination by Southern blotting. Recombinant ES cells were injected into C57BL/6 blastocyst-stage mouse embryos. Chimeric male mice were bred to C57BL/6 female mice to establish a hybrid line. Germine transmission generated RyR2+RyR2-N2368I (RyR2-N2386I+/−) mice. The genotypes from the F1 generations were determined by Southern blotting and PCR on DNA using tail biopsy specimens. Genomic DNA extracted from the tail was digested with EcoRV and analyzed by hybridization with an external 5’probe. The 9.1-kb band corresponds to the WT allele, whereas the 6.0-kb band corresponds to the mutant allele (Supplemental Figure IIB). Mouse genotyping was performed using the following primers: 5’-GGAATCAATTATTGGTGAAACA-3’ and 5’-ATCCTAACATGCCCTTGCAG-3. A PCR product of 688 bp was digested with BspH1 which results in 2 specific bands at 409 bp and 279 bp from the WT but not the mutant allele (Supplemental Figure IIC).

**Generation of RyR2-L433P knock-in mouse**

A targeting construct for homologous recombination was constructed using the BAC modification system. A 200 kb fragment of C57BL/6 BAC clone containing RyR2 genomic sequence was purchased from Cori.org. It was designed to introduce a leucine to proline mutation in E15 by galk selection, along with a loxp-neo-loxp cassette and DTA cassette insertion in upstream of E15 by Red system (Supplemental Figure IIIA). This targeting construct containing BAC was linearized and electroporated into C57BL/6 129 sv hybrid embryonic stem cells. Targeted clones were identified by PCR using primer: 5’-TGGTTTCCCTTGCCCTGGTTT
located in the region where the RyR2 sequences were replaced by DTA cassette, and 3’ primer: 5’-CTACCCGGTGGATGTAAG located in the neo cassette (Supplemental Figure IIIB). Two of these targeted clones were injected into blastocytes. Heterozygous mice carrying the targeted allele were obtained. They were mated with Ell2a Cre transgenic mice to remove the neo cassette flanked by a pair of loxp sites. Mice carrying the L433P knock-in allele were identified by a PCR genotyping using the following primer: 5’- AGCAATGCAGTGCTTGGAAGA and 3’- CCTGAATCAGCAAAACACTTC (Supplemental Figure IIIC).

Generation of RyR2-R2474S knock-in mouse was as previously described 1.

**Murine atrial myocytes isolation**

Atrial myocytes were isolated from WT and CPVT mice according to a modified version of AfiCS Procedure Protocol PP00000125. Briefly, the heart was perfused with AfiCS perfusion buffer, includes (mM): NaCl 113, KCl 4.7, KH₂PO₄ 0.6, Na₂HPO₄ 0.6, MgSO₄ 1.2, NaHCO₃ 12, KHCO₃ 10, Hepes 10, taurine 30, glucose 1.5 and BDM 10, for 5 min at a speed of 3 ml/min after rapidly excised and canulated. The isolated heart was then perfused with digestion buffer (0.65 mg/mL Collagenase type 2 and 50 µM CaCl₂ in AfiCS) for 10–15 min. After enzyme digestion of the heart was complete (heart appeared swollen, pale and flaccid), the atria were excised and teased into small pieces in stop 1 buffer (0.65 mg/mL Collagenase type 2, 0.065 mg/mL Protease XIV, 15 mg/mL BSA and 50 µM CaCl₂ in AfiCS) and bath at 37°C for 10 min. Pipets were used to dissociate the heart tissue gently until all large pieces were dispersed. After separation from the enzyme by centrifugation for 4 min at 200 rpm, cells were resuspended in stop 2 buffer (15 mg/mL BSA and 50 µM CaCl₂ in AfiCS) and recovered [Ca²⁺] to 1.2 mM. The cells were maintained in stop 2 buffer until use.

**Intracellular calcium measurements**
Atrial myocytes were loaded with 5 µM fluo-4 AM for 15 min at room temperature, then washed and maintained in K-H solution (mM): NaCl 125, KCl 4.75, MgSO$_4$ 1.2, KH$_2$PO$_4$ 1.2, HEPES 30, glucose 10, taurine 50, CaCl$_2$ 1.2, titrated to pH 7.4. A Leica TCS SP2 confocal microscopy with 63x, 1.4 NA oil immersion objectives was used for confocal linescan imaging. The scan zoom was adjusted to fit the cells, and the scan line was along the long axis of cells. The excitation for Fluo-4 is 488 nm, and emission was collected at 505-530 nm. For Ca$^{2+}$ sparks measurement, cells were scanned at 400 Hz for 20 s immediately after pacing at 0.5 Hz > 20 beats. For SR Ca$^{2+}$ content measurement, cells were exposed to 10 mM caffeine immediately after pacing at 0.5 Hz for 1 min, and sampling started 10 s before caffeine treatment. For S107 treatment, cells were pre-treated with 10 µM S107 for 2 hrs before loading and imaging. Ca$^{2+}$ sparks detection and analyses used algorithms described previously.

**Total SR Ca$^{2+}$ leak**

The background noise of a confocal laser scan images follows a Poisson distribution. For a normalized linescan image, the background noise can be fit into a modified Gaussian distribution:

$$N = N_0 \times e^{-\frac{(x-1)^2}{b_i}}$$  \hspace{1cm} (S1)

where, x is normalized fluorescence. $b_i$, i=1 for $x<1$ and i=2 for $x>1$ ($b_2$ is usually a bit greater than $b_1$). As shown in Supplemental Fig. IX, for a linescan fluo-4 fluorescence image (A) without Ca$^{2+}$ sparks (which means rare Ca$^{2+}$ release events), the histogram can be fit according to equation S1 (B), while for images with Ca$^{2+}$ sparks, only the background can be fitted into Gaussian curves (C). The differences between the real curves and fitted background noises curves represent all the Ca$^{2+}$ release signals pixels. Thus, we define a parameter for total Ca$^{2+}$ release flux (total SR Ca$^{2+}$ leak):
\[ P_{\text{leak}} = \frac{\sum (\Delta N \times x)}{\sum N} \]  

(S2)

where, \( x \) is normalized fluorescence and \( \Delta N \) is the difference between real and fitted curves.

**Intra-esophageal burst pacing in mouse**

Intra-esophageal pacing was performed using either a 1.1-Fr octapolar catheter (EPR-800, Millar Instruments, Houston, Texas) or 1-Fr bipolar pacing catheter (model EP118-2, NuMED; Hopkinton, NY) placed in the esophagus close to the left atrium using an external stimulator (STG-3008, MultiChannel Systems, Reutlingen, Germany). A computerized data acquisition system (EMKA Technologies, Falls Church, VA) was used to record a 3-lead body surface ECG, and up to 4 intra-esophageal bipolar electrograms. Inducibility of atrial arrhythmias was tested by applying a series of 2-second bursts. The first 2-second burst had a cycle length (CL) of 40 ms, and CL was decreased in each successive 2-ms decrements until reaching a CL of 10 ms. These series of bursts were repeated once. AF was defined as a period of rapid irregular atrial rhythm lasting at least 1 sec. If 1 or more bursts in the 2 series of bursts evoked an AF episode, AF was considered to be inducible in that animal; otherwise, AF was considered to be non-inducible. All data were analyzed by two independent board certified electrophysiologists blinded to the genotype and treatment groups of the animals.

**Intra-cardiac burst pacing and ECG recording in mouse**

Mice were anesthetized with isoflurane (1.5-2%) in 100% oxygen. Animals were placed in supine position on a temperature-controlled surgical table to maintain rectal temperature at 37°C and were allowed to breathe spontaneously. Under a dissecting microscope (model SZ61, Olympus; Tokyo, Japan), the right jugular vein was isolated, and a 1.1-Fr octapolar catheter was
inserted (EPR-800, Millar Instruments, Houston, Texas). Intra-cardiac ECG was recorded showing typical ventricular, atrioventricular node, and atrial ECG. Atrial and ventricular pacing was established using a stimulator (STG-3008, MultiChannel Systems, Reutlingen, Germany).

**Immunoprecipitation and immunoblot analyses**

RyR2 was immunoprecipitated from cardiac homogenates (100 µg) using anti-RyR antibody \(^8\) (2 µl 5029 Ab) in 0.5 ml of a modified RIPA buffer (50 mM Tris-HCl pH 7.4, 0.9% NaCl, 5.0 mM NaF, 1.0 mM Na\(_3\)VO\(_4\), 0.5% Triton-X100, and protease inhibitors) for 2 hrs at 4°C. The samples were incubated with protein A sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ) at 4°C for 1 hr and washed five times with 1.0 ml RIPA. Samples were heated to 95°C and size fractionated by PAGE (6% for RyR2, 15% for calstabin2). Proteins were transferred to nitrocellulose membranes and immunoblots were developed using the following antibodies: anti-calstabin (1:1,000) \(^8\), anti-RyR (5029; 1:3,000) \(^8\), anti-phospho-RyR2-pSer\(^{2808}\) (1:5,000) \(^9\). Levels of RyR2 bound proteins were normalized to the total RyR2 immunoprecipitated (arbitrary units). All immunoblots were developed using the Odyssey system (LI-COR, Inc., Lincoln, NE) with IR labeled anti-mouse and anti-Rabbit IgG (1:10,000 dilution) secondary antibodies.

**RyR2 Oxidation**

To detect RyR2 protein oxidation, SR membrane samples (50 ug) were immunoprecipitated as described above. Immunoprecipitate was treated with 2, 4-dinitrophenyl hydrazine (DNPH) and the derivatized carbonyls were detected using an OxyBlot™ Protein Oxidation Detection Kit (Cat # S7150, Chemicon International, Inc., Temecula, CA). Proteins were size fractionated on 6% SDS-PAGE gels and transferred onto nitrocellulose membranes and immunoblots were developed with an anti-RyR antibody (Affinity Bioreagents, Bolder, CO
1:2,000). The DNP signal associated with RyR was determined using an anti-DNP antibody (1:2000).
Supplemental figure 1. Ventricular arrhythmias induced by exercise plus epinephrine and epinephrine plus caffeine in 3 CPVT mice. A) Upper panel: representative ECG traces recorded by telemetry transducers from WT (n=10), RyR2-R2474S+/− (n=16), RyR2-N2386I+/− (n=11), and RyR2-L433P+/− (n=5) after maximal treadmill exercise and epinephrine (2 mg/kg) injection. Lower panel: incidence of PVCs, ventricular bigeminy and bidirectional ventricular tachycardia (BVT) in WT and mice with RyR2 mutations. B) Upper panel: representative ECG traces recorded by surface ECG from WT (n=11), RyR2-R2474S+/− (n=11), RyR2-N2386I+/− (n=8), RyR2-L433P+/− (n=11) mice after ISO (0.5 mg/kg) plus caffeine 120 (mg/kg) injection. Lower panel: incidences of PVC, ventricular bigeminy and bidirectional ventricular tachycardia (BVT) in WT and mice with RyR2 mutations. *, P<0.05 vs. WT.
Supplemental figure II. Generation of RyR2-N2386I knock-in mouse model. A) Top line: the wild-type locus of the murine RyR2 gene containing Exon 44 -50. Second line: The targeting construct containing of 5' homologous arm (4.1 kb) and 4.8 kb of 3' homologous arm. The N2386I mutation was engineered in Exon 47. Third line: the homologous recombinant mutant allele containing the RyR2-N2386I mutation and ACN selection marker cassette. Bottom line: final RyR2-N2386I allele after excision of ACN selection marker. B) Southern blot result in screening RyR2-N2386I knock-in mice: genomic DNA extracted from tail of F1 mice digested with EcoRV and analyzed by hybridization with an external 5-probe. The 9.1 kb band corresponds to the WT allele whereas the 6kb band corresponds to the Mutant allele. C) PCR result in screening RyR2-N2386I knock-in mice: mouse genotyping was performed using the following primers: 5'-TCCTAACATGCCCTTGCAG-3' and 5'-GGAATCAATTATTGGTGGAAAC-3'. PCR product of 688bp was digested with BspHI and resulted in 2 specific band at 409bp and 279bp from WT but not the mutant.
Supplemental figure IV. Echocardiography and histology studies showed no difference between WT and 3 CPVT mouse models. A) Echocardiography results in 6-8 months old WT (n=12), RyR2-R2474S+/− (n=8), RyR2-N2386I+/− (n=9), RyR2-L433P+/− (n=8). B) Representative histology sections using Masson’s trichrome stain for atrium, left and right ventricles from WT, RyR2-R2474S+/−, RyR2-N2386I+/−, and RyR2-L433P+/− mice. Bar = 10μm.
Supplemental figure V. Preventing CaMKII phosphorylation of RyR2 does not impact the prevalence of burst pacing-induced AF in heart failure. Prevalence of burst pacing-induced AF in WT (n=10) and RyR2-S2814A mice (n=11) before (Pre-MI) and 4 weeks post myocardial infarction (HF). The heart function data was published previously 10.
Supplemental figure VI. Metoprolol treatment for 4 weeks does not decrease the prevalence of burst pacing-induced AF in all 3 CPVT mouse models. Prevalence of burst pacing-induced AF in RyR2-R2474S+/− (n=10), RyR2-N2386I mice (n=14), and RyR2-L433P (n=9) treated by vehicle or metoprolol (30 mg/kg/d) for 4 weeks.
Supplemental figure VII. DTT treatment decreased Ca\(^{2+}\) leak in atrial myocytes from RyR2-R2474S\(^{+/-}\) mice. Atrial myocytes were bathed in imaging buffer with 5 mM DTT for 3 min before sampling. (DTT is membrane-permeable, and the experiment are done with intact cells). n = 20-25 cells in each group. **, p<0.01 vs WT group; # and ##, p<0.05 and 0.01 vs R2474S group.
Supplemental figure VIII. Line scan of atrial myocytes isolated from RyR2-R2474S+/− mice showed increased DAD/EADs comparing to WT and S107 treated animals. Atrial myocytes were bathed in 2mM [Ca$^{2+}$] imaging buffers. n = 20~24 cells from 3 hearts in each group. **, p<0.01 vs WT group; ##, p<0.01 vs R2474S group.
Supplemental figure IX. Line scan of atrial myocytes isolated from RyR2-R2474S+/− mice showed spontaneous Ca\(^{2+}\) waves comparing to WT and S107 treated animals. Atrial myocytes were bathed in 2mM [Ca\(^{2+}\)] imaging buffers and pacing at 3 Hz for at least 1 minute. After stop pacing, 8 out of 21 atrial myocytes in R2474S group occurred Ca\(^{2+}\) wave, which is obviously higher than WT (0/9) and R2474S+S107 (2/14) groups.
Supplemental figure X. Total SR Ca\textsuperscript{2+} leak measurement. A) A confocal line scan image for cardiac myocyte loading with fluo-4, which shown rare Ca\textsuperscript{2+} release. B) Histogram of normalized images in A (blue) can be well fitted into Gaussian distribution (red). C) Histogram curves (blue) and fitted curves (blue) for Ca\textsuperscript{2+} images of atrial myocytes from WT (upper) and RyR-R2474S\textsuperscript{+-} (lower) mice. The differences between real and fitted curves show the total leak.
Supplemental figure XI. Amplitudes and Spatio-temporal characters of Ca$^{2+}$ sparks in atrial myocytes isolated from different mice models.
Supplemental figure XII. CaMKII phosphorylation of RyR2 in atrial tissues freshly isolated from both WT and RyR2-R2474S+/− mice immediately after atrial burst pacing.
References

1. Lehnart SE, Mongillo M, Bellinger A, Lindegger N, Chen BX, Hsueh W, Reiken S, Wronska A,
   channel/ryanodine receptor 2 causes seizures and sudden cardiac death in mice. J Clin Invest.
   2008;118:2230-2245

   Bidirectional ventricular tachycardia and fibrillation elicited in a knock-in mouse model carrier
   of a mutation in the cardiac ryanodine receptor. Circ Res. 2005;96:e77-82

3. Bunting M, Bernstein KE, Greer JM, Capecci MR, Thomas KR. Targeting genes for self-excision

4. Copeland NG, Jenkins NA, Court DL. Recombineering: A powerful new tool for mouse

   NG. A highly efficient escherichia coli-based chromosome engineering system adapted for
   recombinogenic targeting and subcloning of bac DNA. Genomics. 2001;73:56-65

6. Warming S, Costantino N, Court DL, Jenkins NA, Copeland NG. Simple and highly efficient bac

   distribution of calcium sparks in confocal images: Theory and studies with an automatic
   detection method. Biophys J. 1999;76:606-617

8. Jayaraman T, Brillantes AM, Timerman AP, Fleischer S, Erdjument-Bromage H, Tempst P,
   Marks AR. Fk506 binding protein associated with the calcium release channel (ryanodine

9. Wehrens XH, Lehnart SE, Reiken SR, Marks AR. Ca2+/calmodulin-dependent protein kinase ii
   phosphorylation regulates the cardiac ryanodine receptor. Circ Res. 2004;94:e61-70

    of the cardiac ryanodine receptor in the force frequency relationship and heart failure. Proc
    Natl Acad Sci U S A. 2010;107:10274-10279