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Genetics/Genomics

Mutation E169K in Junctophilin-2 Causes Atrial Fibrillation Due to Impaired RyR2 Stabilization

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Objectives This study sought to study the role of junctophilin-2 (JPH2) in atrial fibrillation (AF).

Background JPH2 is believed to have an important role in sarcoplasmic reticulum (SR) Ca^{2+} handling and modulation of ryanodine receptor Ca^{2+} channels (RyR2). Whereas defective RyR2-mediated Ca^{2+} release contributes to the pathogenesis of AF, nothing is known about the potential role of JPH2 in atrial arrhythmias.

Methods Screening 203 unrelated hypertrophic cardiomyopathy patients uncovered a novel JPH2 missense mutation (E169K) in 2 patients with juvenile-onset paroxysmal AF (pAF). Pseudoknock-in (PKI) mouse models were generated to determine the molecular defects underlying the development of AF caused by this JPH2 mutation.

Results PKI mice expressing E169K mutant JPH2 exhibited a higher incidence of inducible AF than wild type (WT)-PKI mice, whereas A399S-PKI mice expressing a hypertrophic cardiomyopathy-linked JPH2 mutation not associated with atrial arrhythmias were not significantly different from WT-PKI. E169K-PKI but not A399A-PKI atrial cardiomyocytes showed an increased incidence of abnormal SR Ca^{2+} release events. These changes were attributed to reduced binding of E169K-JPH2 to RyR2. Atrial JPH2 levels in WT-JPH2 transgenic, nontransgenic, and JPH2 knockdown mice correlated negatively with the incidence of pacing-induced AF. Ca^{2+} spark frequency in atrial myocytes and the open probability of single RyR2 channels from JPH2 knockdown mice was significantly reduced by a small JPH2-mimicking oligopeptide. Moreover, patients with pAF had reduced atrial JPH2 levels per RyR2 channel compared to sinus rhythm patients and an increased frequency of spontaneous Ca^{2+} release events.

Conclusions Our data suggest a novel mechanism by which reduced JPH2-mediated stabilization of RyR2 due to loss-of-function mutation or reduced JPH2/RyR2 ratios can promote SR Ca^{2+} leak and atrial arrhythmias, representing a potential novel therapeutic target for AF. (J Am Coll Cardiol 2013;62:2010–9) © 2013 by the American College of Cardiology Foundation

Atrial fibrillation (AF) is the most common sustained cardiac arrhythmia and causes significant morbidity and mortality (1). Although the mechanisms behind AF pathogenesis are complex, it is believed that AF is induced and maintained by a combination of re-entry and triggered activity that includes early afteredepolarizations (EADs)

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and delayed afterdepolarizations (DADs) (2,3). Spontaneous diastolic Ca^{2+} release from the sarcoplasmic reticulum (SR) generates a depolarizing Na^{+}/Ca^{2+} exchanger (NCX) current, which may induce DADs and triggered activity (4). The major SR Ca^{2+} release channel responsible for arrhythmogenic Ca^{2+} release is the type 2 ryanodine receptor channel (RyR2) located within junctional membrane complexes (JMCs) (5–7). Defective regulation and activity of RyR2 has been linked to AF in humans and various animal models (4,8,9). In addition, structural changes in the JMC may precipitate destabilization of RyR2 and diastolic Ca^{2+} leakage (10). However, the molecular mechanisms underlying abnormal RyR2 Ca^{2+} leakage from JMCs remain poorly understood, especially in the atrial myocardium.

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Junctophilin-2 (JPH2) plays a critical structural role within JMCs (10,11). In mouse studies, knockdown of JPH2 was associated with loss of JMC numbers, reduced Ca^{2+}-induced Ca^{2+} release, and development of acute heart failure (10). Several mutations in JPH2 were previously identified in patients with hypertrophic cardiomyopathy (HCM) (12,13). Here, we report two novel *JPH2* mutations, one of which, E169K, was identified in a proband with the unusual clinical presentation of juvenile-onset paroxysmal AF (pAF). The proband's father, carrying the same mutation, also exhibited supraventricular tachycardia in addition to HCM. The patient with the other HCM-associated JPH2 mutation, A405S, did not exhibit atrial arrhythmias, similar to all other previously reported HCM patients with JPH2 mutations (12,13).

To determine how the E169K mutation in *JPH2* might cause atrial arrhythmias, we generated genetically modified mouse lines carrying the E169K or A399S (A405S in humans) mutation in JPH2. Subsequently, pseudoknock-in (PKI) mice with total cardiac JPH2 levels similar to those of nontransgenic (NTg) mice were generated by crossing JPH2 transgenic (Tg) mice with inducible, cardiac-specific JPH2 knockdown mice. The E169K-PKI but not A399S-PKI mice exhibited an enhanced susceptibility to pacing-induced AF. Atrial myocytes from E169K-PKI but not A399S-PKI mice showed an increased frequency of spontaneous SR Ca^{2+} release events. Interestingly, the JPH2 E169K mutation but not the A399S mutation decreased the binding affinity of JPH2 to RyR2. To further examine the importance of JPH2 loss-of-function in AF, atrial JPH2 levels in mice were shown to have an inverse correlation with AF inducibility.

Addition of a small peptide containing the E169 region of JPH2 was able to ameliorate the Ca^{2+} spark frequency in atrial cardiomyocytes isolated from JPH2 knockdown mice, and to normalize the open probability (Po) of RyR2 channels from JPH2 knockdown mice. Finally, to assess the importance of JPH2 in clinical AF, JPH2 protein levels were assessed in atrial samples from non-HCM patients with pAF. JPH2:RyR2 ratios were reduced significantly in pAF patients compared with those in sinus rhythm, and isolated atrial cardiomyocytes showed an increased frequency of potential proarrhythmic SR Ca^{2+} release events, consistent with enhanced RyR2 activity.

Together, these data suggest that reduced JPH2-mediated stabilization of RyR2 (either because of the unique inherited loss-of-function E169K mutation in JPH2 or because of the reduced JPH2 expression levels per RyR2 channel) can promote abnormal RyR2-mediated SR Ca^{2+} release events associated with AF. These findings may open new avenues for the development of anti-AF drugs that target either JPH2 or RyR2 abnormalities, to reduce spontaneous proarrhythmic diastolic SR Ca^{2+} release events.

Abbreviations and Acronyms

- **AF** = atrial fibrillation
- **Ca^{2+}** = calcium
- **DAD** = delayed afterdepolarizations
- **EAD** = early afterdepolarizations
- **HCM** = hypertrophic cardiomyopathy
- **JMC** = junctional membrane complex
- **JPH2** = junctophilin-2
- **NCX** = Na^{+}/Ca^{2+} exchanger
- **NTg** = nontransgenic
- **PKI** = pseudoknock-in
- **RyR2** = ryanodine receptor type 2
- **SR** = sarcoplasmic reticulum
- **Tg** = transgenic
- **WT** = wild type

Methods

Expanded methods can be found in the Online Appendix.

Genetic analysis. Comprehensive encoding region genetic analysis of *JPH2* was accomplished by polymerase chain reaction, denaturing high performance liquid chromatography, and direct DNA sequencing as described previously (12).

Generation of animals. All animal studies were performed according to protocols approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine, conforming to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH publication 85-23, revised 1996). Generation of Tg mice, including the WT-Tg and E169K and A399S JPH2 mutant Tg and short hairpin RNA (shRNA) knockdown mice, is described in detail in the Online Appendix. Pseudoknock-in mice were generated by crossing the JPH2 Tg mice (WT and E169K and A399S mutants) with an inducible cardiac-specific JPH2 knockdown mouse line. The offspring were given doses of tamoxifen to induce shRNA-mediated knockdown of total JPH2 levels to protein levels similar to those in NTg mouse hearts. Experiments were performed between 2 and 3 weeks post-knockdown when JPH2 levels were stable.

Programmed electrical stimulation. Atrial and ventricular intracardiac electrocardiography (ECG) traces were recorded using a 1.1F octapolar electrode catheter (EPR-800, Millar Instruments, Houston, Texas) inserted into the right ventricle via the right jugular vein, as described previously (14). Atrial

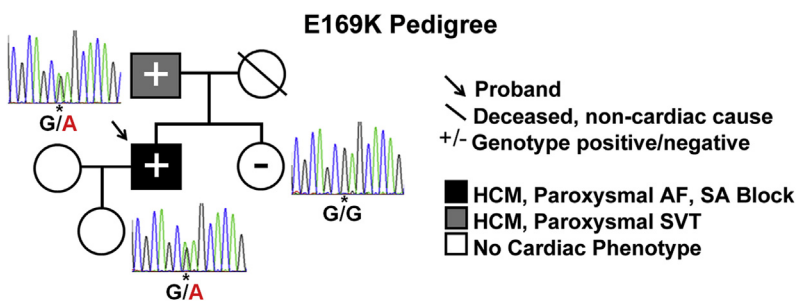

Figure 1 **E169K-JPH2 Is Associated With HCM and pAF**

Pedigree shows presence of HCM and supraventricular arrhythmias (pAF, SA block, SVT) in proband **(arrow)** and his father **(gray square)**, both of whom were mutation positive (heterozygous G > A), and his sister, who was genotype negative **(white circle)**, as indicated by the asterisk. HCM = hypertrophic cardiomyopathy; pAF = paroxysmal atrial fibrillation; SA = sinoatrial; SVT = supraventricular tachycardia.

fibrillation inducibility was determined using rapid atrial pacing; AF was considered positive only if it lasted for ≥1 s.

Mouse myocyte isolation and confocal Ca^{2+} imaging. Mouse atrial myocytes were isolated and loaded with the Ca^{2+}-sensitive dye Fluo-4 AM (Invitrogen, Eugene, Oregon) as described previously (9). Once steady-state Ca^{2+} transients were observed, pacing was paused for 30 s to record spontaneous Ca^{2+} release events (Ca^{2+} waves and Ca^{2+} sparks). Steady-state SR Ca^{2+} was estimated by rapid application of 10 mM of caffeine after pacing.

Simultaneous intracellular Ca^{2+} and membrane current measurements in human atrial myocytes. Right atrial biopsy samples were dissected from appendages of patients with pAF or those in sinus rhythm who underwent coronary artery bypass or valve replacement surgery. Protocols were approved by the medical ethics committees of the Dresden University of Technology and the Medical Faculty Mannheim, University of Heidelberg, Germany.

Statistical analyses. Results are expressed as mean ± standard error of the mean. Normality was examined using the Shapiro-Wilk test. Continuous variables with normal distribution were compared using analysis of variance with a post-hoc Tukey test for equal variance and Dunnett's T3 test for unequal variance, and paired samples were analyzed using a paired Student *t* test. Continuous variables that failed the test of normality were compared using the Kruskal-Wallis test, and pairs of data were analyzed using the Mann-Whitney test. Ordinal variables were compared using the Kendall tau B coefficient. Categorical variables were compared using the Pearson chi-square test, and pairs of data were examined with the Fisher exact test. A p value <0.05 was considered statistically significant.

Results

Identification of the JPH2 E169K mutation in patients with HCM and AF. Previous studies have demonstrated the presence of rare, functionally disruptive *JPH2* mutations in patients with HCM (12,13). Analyzing *JPH2* for mutations in an independent cohort of 203 unrelated patients with HCM revealed a new missense mutation annotated as E169K in a male index case with unusually early onset paroxysmal AF in the context of HCM (Fig. 1). This *JPH2* genotype-positive patient was negative for mutations in the 9 canonical sarcomeric HCM-associated genes. Diagnosed with HCM at 5 months of age, this patient went on to develop profound septal hypertrophy (32 mm) with marked diastolic dysfunction and labile left ventricular outflow obstruction (Online Fig. 1). The onset of pAF was documented at the young age of 22 prior to significant atrial remodeling. At age 24, he required pacemaker implantation due to paroxysmal sinoatrial (SA) blocks and pre-syncope. Clinical and genetic evaluation of family members revealed the E169K mutation in the proband's father, who had HCM with 15 mm maximal left ventricular thickness and paroxysmal supraventricular tachycardia during clinical evaluation blinded to genotype (Fig. 1). In contrast, the proband's sister was mutation-negative and had a normal cardiac work-up. Both the proband and his father had prolonged QTc intervals, whereas the sister's QTc was normal (Online Fig. 1). No other family members were available for genetic testing. In the same cohort, another JPH2 variant (A405S) was identified in a Caucasian male whose HCM was diagnosed at age 16 years. This proband presented with dyspnea, chest pain, and dizziness. He later developed basal septal morphology hypertrophy (23 mm) and was diagnosed with HCM. He had no family history of HCM or sudden cardiac death; both parents were genotype negative and never developed QTc prolongation or supraventricular arrhythmias.

E169K-PKI mice are more susceptible to AF induction. The unusual association of atrial arrhythmias with HCM in the 2 patients with the E169K mutation in JPH2 prompted further mechanistic analysis in animal models. In order to create a model resembling the heterozygous disease state of patients, we generated PKI mice that expressed hemagglutinin-tagged E169K mutant JPH2 (E169K-PKI),

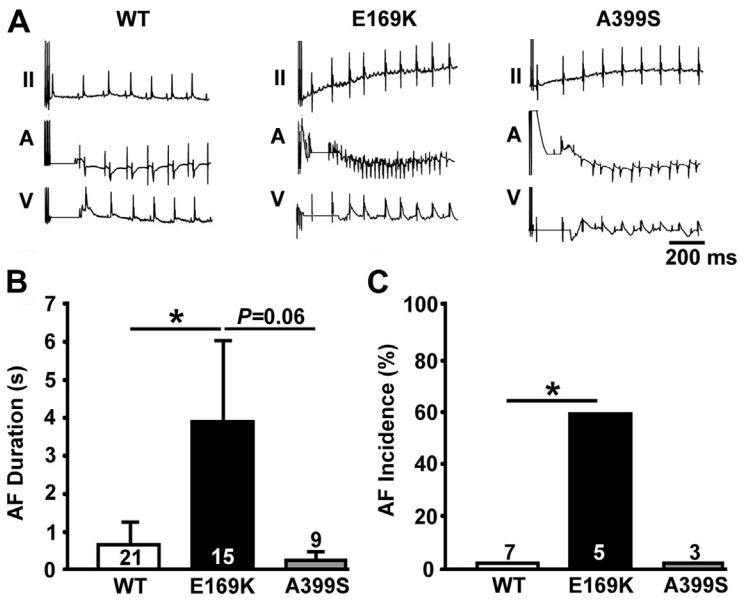

Figure 2 **Higher Susceptibility to Pacing-Induced AF in E169K-PKI But Not A399S Mice**

(A) Representative ECG traces after rapid atrial burst pacing in E169K-PKI mouse reveal AF compared with sinus rhythm in WT-PKI and A399S-PKI mice. **(B)** Graph shows average duration of AF episodes following rapid atrial pacing bursts. Number of occurrences are shown inside bars. **(C)** Graph shows increased incidence of pacing-induced AF (reproducible and lasting >1 s) in E169K-PKI mice compared with WT-PKI and A399S-PKI mice. Numbers of mice per group are shown inside bars. *$p < 0.05$. AF = atrial fibrillation; ECG = electrocardiography.

wild-type JPH2 (WT-PKI), or A399S (A405S in humans) mutant JPH2 (A399S-PKI). The total (i.e., combined endogenous and transgenic) cardiac JPH2 levels in these WT and mutant PKI mouse lines were similar to those in NTg littermates (Online Fig. 2), whereas approximately half of the JPH2 in PKI mice was mutant/transgenic and the other half endogenous JPH2.

To determine whether the E169K mutation in JPH2 increased susceptibility to AF, we subjected E169K-PKI mice to programmed electrical stimulation at an age prior to the onset of cardiac hypertrophy or dysfunction (Online Fig. 3) (8). Following a rapid atrial burst pacing sequence, E169K-PKI mice exhibited a higher incidence of AF than WT-PKI and A399S-PKI mice (Fig. 2A). The average duration of AF episodes in E169K-PKI mice was 3.9 ± 2.1 s, whereas the duration of (nonreproducible) AF episodes in WT-PKI mice was only 0.6 ± 0.6 s ($p < 0.05$ vs. E169K-PKI) and 0.2 ± 0.2 s for A399S-PKI ($p = 0.06$ vs. E169K-PKI) (Fig. 2B). The longest episode of pacing-induced AF recorded in an E169K-PKI mouse was 29 s. The incidence of reproducible AF (defined as ≥2 episodes of AF per 3 attempts) was 60% in E169K-PKI mice, whereas it was 0% in WT-PKI mice ($p < 0.05$ vs. E169K-PKI) and 0% in A399S-PKI mice ($p = 0.19$ vs. E169K-PKI) (Fig. 2C).

Mutation E169K in JPH2 increases spontaneous SR Ca^{2+} leakage in atrial myocytes. To determine whether the E169K mutation in JPH2 increased susceptibility to AF by altering intracellular Ca^{2+} handling, we assessed the incidence of spontaneous SR Ca^{2+} release events in isolated atrial myocytes. Atrial myocytes isolated from E169K-PKI mice exhibited a significantly increased incidence of spontaneous Ca^{2+} waves following a 1-Hz pacing train (3.67 ± 1.47/min) compared with myocytes from WT-PKI and A399S-PKI mice (0.33 ± 0.33/min and 0.11 ± 0.11/min, respectively; $p < 0.05$) (Figs. 3A and 3C). These cell-wide spontaneous Ca^{2+} waves can directly activate NCX, trigger DADs, and cause triggered activity associated with AF (4).

To gain more insight into the mechanisms of altered SR Ca^{2+} release, we quantified the incidence of spontaneous subcellular Ca^{2+} sparks (Fig. 3B). The Ca^{2+} spark frequency was significantly increased in atrial myocytes from E169K-PKI mice (5.86 ± 0.88/min) compared with those from WT-PKI mice and A399S-PKI mice (3.74 ± 0.45 and 3.48 ± 0.87 respectively; $p < 0.05$) (Figs. 3B and 3D). This abnormal diastolic Ca^{2+} leakage produced reduced SR Ca^{2+} load in E169K-PKI mice compared with WT-PKI mice (Online Fig. 4). Thus, our data show that the E169K mutation in JPH2 leads to abnormal SR Ca^{2+} release via RyR2, suggesting that the Ca^{2+} release channel is less stable in mice with the E169K but not the A399S JPH2 mutant variant.

E169K mutation reduces JPH2-RyR2 binding. To gain more insights into the mechanisms by which E169K-JPH2

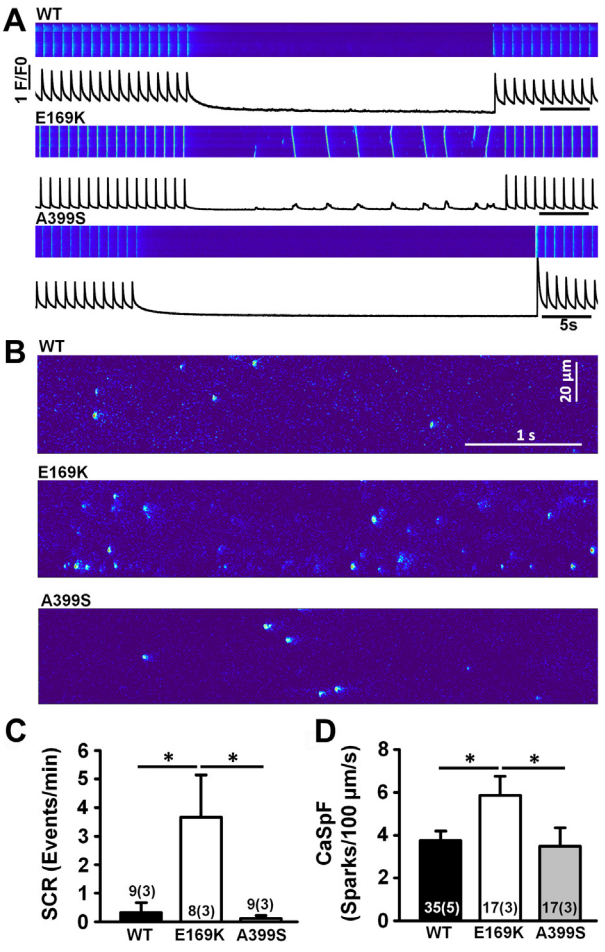

Figure 3 Enhanced SR Ca^{2+} Leak in E169K-PKI But Not A399S-PKI Mouse Myocytes

(A) Representative confocal line scan images and profiles show spontaneous cell-wide Ca^{2+} waves in atrial myocytes from E169K-PKI mice following 1-Hz field stimulation. **(B)** Representative confocal line scan images show increased incidence of Ca^{2+} sparks in atrial myocytes from E169K-PKI mice following 1-Hz field stimulation. **(C, D)** Graphs show quantification of SCR and CaSpF. *$p < 0.05$. CaSpF = Ca^{2+} spark frequencies; SCR = spontaneous Ca^{2+} release; SR = sarcoplasmic reticulum.

potentially affects RyR2 activity, we first assessed the amount of JPH2 binding to RyR2. Immunoprecipitation using anti-RyR2 antibody was performed using cardiac lysates from NTg, WT-PKI, E169K-PKI, and A399S-PKI mice (Fig. 4A). Subsequent Western blotting for RyR2 and JPH2 revealed significantly reduced co-immunoprecipitation of E169K-mutant JPH2 with RyR2 compared with WT-JPH2 and A399S mutant JPH2 (Fig. 4B). These results suggest that the E169K mutation in JPH2 specifically disrupts its binding domain with RyR2.

JPH2 levels inversely correlate with atrial arrhythmogenesis in mice. To determine whether there is an association between reduced atrial JPH2 protein levels and AF susceptibility, we studied 3 genetically modified mouse models expressing different levels of JPH2 in the heart. Tg mice overexpressing WT JPH2 (WT-Tg) had ~2.9-fold higher JPH2 protein levels than NTg mice (Fig. 4C). Compared with NTg mice, cardiac-specific knockdown mice (JPH2-KD) (10) exhibited ~1.9-fold lower JPH2 levels in the heart. When subjected to rapid atrial burst pacing, WT-Tg mice were less susceptible to pacing-induced AF compared with NTg mice, whereas JPH2-KD mice were more susceptible ($p = 0.04$ across the groups) (Fig. 4D). Thus, these experiments demonstrated that susceptibility to AF in mouse models is inversely correlated with atrial JPH2 protein levels. These data suggest that reduced JPH2 levels might be the culprit mechanism for RyR2 dysfunction and the increased AF susceptibility identified in E169K-PKI mice.

JPH2-derived peptide stabilizes RyR2-mediated SR Ca^{2+} release. To assess whether JPH2 binding to RyR2 modulates SR Ca^{2+} release, we synthesized a peptide corresponding to the 25 amino acids of the JPH2 region that flanks residue E169. Next, the JPH2 peptide was added to permeabilized atrial myocytes from JPH2-KD mice, in which JPH2 expression was reduced. Baseline Ca^{2+} spark frequency in vehicle-treated JPH2 knockdown was relatively high (8.2 ± 0.6 sparks/100 µm/s) (Figs. 5A and 5B). Addition of the JPH2-derived peptide significantly decreased the Ca^{2+} spark frequency in a dose-dependent manner (1 µM peptide: 5.2 ± 0.4; $p < 0.01$; and 10 µM peptide: 0.48 ± 0.27; $p < 0.001$ vs. vehicle). A control peptide (10 µM) in which the same 25 amino acids were scrambled did not affect the Ca^{2+} spark frequency (9.4 ± 1.5; p = NS vs. vehicle).

Furthermore, single-channel measurements in planar lipid bilayers revealed a high average Po of RyR2 channels isolated from JPH2-KD mice (0.12 ± 0.048), which was significantly reduced after the addition of JPH2 peptide (0.048 ± 0.024; $p < 0.05$) (Figs. 5C and 5D). In contrast, the addition of JPH2 peptide did not change the average Po of RyR2 isolated from alpha myosin heavy chain (αMHC)-mER-Cre-mER (MCM) control mice (0.032 ± 0.02 vs. 0.024 ± 0.01; p = NS). The specificity of the peptide effects was confirmed by the demonstration that the scrambled peptide did not have a significant effect on the Po of RyR2 isolated from both MCM control mice and JPH2-KD mice (Online Fig. 5). Taken together, these data strongly suggest that JPH2, in particular the domain containing the E169 residue, are critical for stabilization of RyR2 channel activity.

Decreased JPH2/RyR2 ratio in patients with paroxysmal AF correlates with enhanced SR Ca^{2+} leak. In view of the inverse correlation between atrial JPH2 levels and AF susceptibility in Tg mice, we set out to determine whether changes in JPH2 and RyR2 expression levels might occur in more common AF patients without either JPH2 mutations or HCM. Overall, the pAF and sinus rhythm patient cohorts included mostly elderly patients with significant

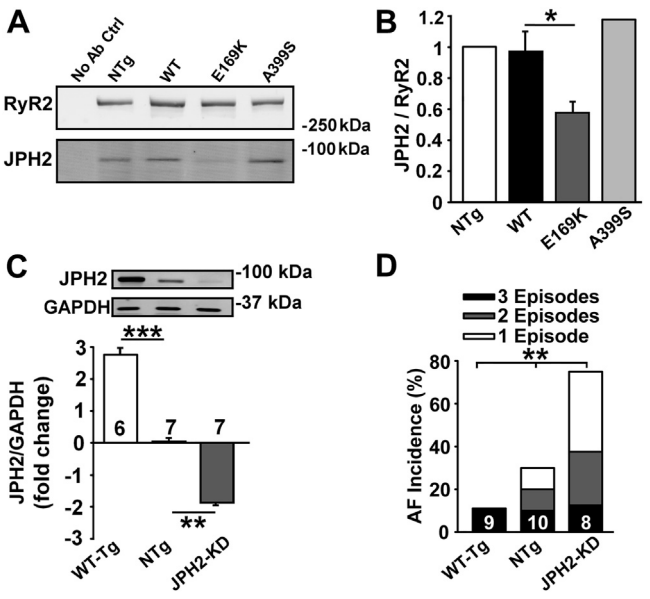

Figure 4 **JPH2 E169K Mutant But Not A399S Mutant Reduces JPH2-RyR2 Binding, and JPH2 Levels Inversely Correlate With AF Inducibility**

(A) Western blots show reduced JPH2 co-immunoprecipitation following RyR2 pull-down from cardiac lysates of E169K-PKI mouse heart compared with those of WT-PKI, A399S-PKI, and NTg mice. **(B)** Graph shows quantification of JPH2 co-immunoprecipitated with RyR2 pull-down. **(C)** Western blots show total JPH2 levels compared with GAPDH in atria from mice that overexpressed higher cardiac JPH2 levels (WT-Tg), control non-Tg Mer-Cre-Mer (MCM) mice with normal JPH2 levels (NTg), or MCM-shJPH2 knockdown mice with reduced cardiac JPH2 levels (JPH2-KD). Graphs show JPH2 levels normalized to GAPDH in these three groups on a logarithmic scale. **(D)** Stacked bar graph shows AF incidence after atrial burst pacing in the 3 groups of mice. Numbers of mice per group are shown inside bars. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. AF = atrial fibrillation.

co-morbidities including ischemic and valvular heart disease, diabetes mellitus, hypertension, and hyperlipidemia (Online Table 1). There were no significant differences between patient characteristics, except for a slightly higher average age in the pAF group. However, there was no significant correlation between age and atrial JPH2 protein levels (Online Fig. 6). Western blotting revealed increased RyR2 but unaltered JPH2 levels in atrial tissues from patients with pAF compared with those in sinus rhythm, resulting in a significantly lower JPH2/RyR2 ratio (Figs. 6A to 6D). In contrast, the protein levels of SERCA2a and phospholamban (PLN), Ca^{2+} cycling proteins involved in Ca^{2+} resequestration into the SR, were unchanged (Figs. 6E and 6G).

Whereas several recent studies have documented profound changes in SR Ca^{2+} handling in atrial myocytes from patients with chronic AF, much less is known about Ca^{2+} handling in paroxysmal AF. Therefore, atrial myocytes were isolated from right atrial samples from pAF patients and control individuals in sinus rhythm. In the presence of high intracellular Ca^{2+} concentrations, atrial myocytes from pAF patients were significantly more likely to exhibit spontaneous SR Ca^{2+} release (SCR) events compared with myocytes from sinus rhythm patients (Figs. 7A and 7B). In addition, the frequency of SCR was increased in atrial myocytes from patients in pAF, and there was a trend toward a shorter interval between the final regularly triggered Ca^{2+} transient and first SCR (Fig. 7C). There was also a nonsignificant increase in the amplitude of the SCR in patients with pAF compared to that in sinus rhythm patients. Finally, the amplitude of the NCX current induced by SCR was approximately 2-fold higher in atrial myocytes from patients with pAF than in those in sinus rhythm (Fig. 7D). Thus, reduced atrial JPH2 levels per RyR2 channel correlated with an increased incidence of spontaneous SR Ca^{2+} release events and enhanced proarrhythmogenic NCX currents in atrial myocytes from patients with pAF.

Discussion

This study describes the first example of a defect in a structural protein (i.e., junctophilin-2) that causes atrial arrhythmias due to perturbed Ca^{2+} handling. Specifically, the inherited mutation E169K in *JPH2* affects RyR2-mediated SR Ca^{2+} release from the SR, causing triggered activity and supraventricular arrhythmias in both patients and mice with the JPH2 E169K mutation in the heart. Biochemical studies revealed that the E169K mutation impairs binding of JPH2 to RyR2. This molecular defect was not observed for the JPH2 mutation A405S identified in HCM patients without atrial arrhythmias. Our studies identified the residue E169 as being part of a critical domain within JPH2 that inhibits RyR2 channel activity.

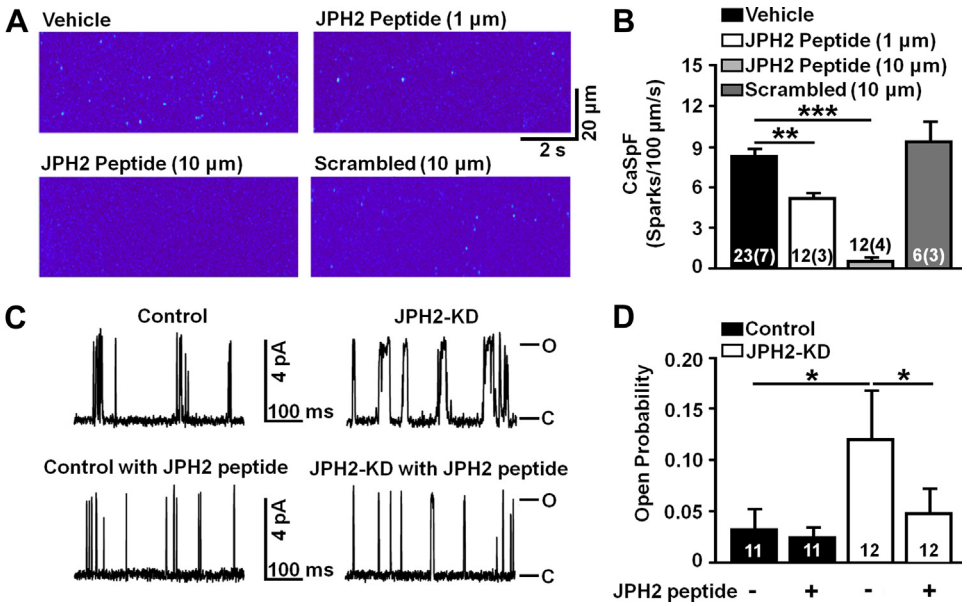

Figure 5 **JPH2-Derived Small Peptide Stabilizes RyR2-Mediated SR Ca^{2+} Leak in Atrial Myocytes From JPH2 Knockdown Mice**

(A) Representative confocal line scans show spontaneous Ca^{2+} sparks recorded from permeabilized atrial myocytes treated with vehicle only **(upper left)**, JPH2 peptide at 1 μM **(upper right)**, JPH2 peptide at 10 μM **(bottom left)**, and scrambled control peptide (10 μM; **bottom right**). **(B)** Graph shows CaSpF, which was significantly decreased by JPH2-derived peptide but not by scrambled control peptides. *$p < 0.05$,**$p < 0.01$, ***$p < 0.001$. Numbers indicate number of cells (number of mice). **(C)** Representative planar lipid bilayer RyR2 single-channel tracings from Mer-Cre-Mer (control) and JPH2 knockdown (JPH2-KD) mice at baseline **(top)** and with the addition of JPH2 peptide **(bottom)**. **(D)** Graph shows significant increase in RyR2 Po in JPH2-KD versus control and significant reduction in RyR2 Po with the addition of JPH2 peptide in JPH2-KD mice. $p < 0.05$. CaSpF = Ca^{2+} spark frequency; Po = open probability; SR = sarcoplasmic reticulum.

Furthermore, reduced amounts of functionally intact (WT) JPH2 per RyR2 channel, resulting in a loss of JPH2-mediated stabilization of RyR2 may also contribute to arrhythmogenesis in patients with paroxysmal AF (15).

Recent studies have shown that genetic mutations in *JPH2* are a rare cause of HCM (12,13,16). The first *JPH2* variant specifically linked to cardiac arrhythmias, E169K, was identified in an HCM index patient with atypical disease features, most notably juvenile-onset paroxysmal AF, supraventricular arrhythmias, and paroxysmal SA blocks, which are rarely seen in patients with HCM in the absence of significant atrial remodeling (17). In contrast, AF is more commonly observed in HCM with increasing age and/or atrial dilatation (17). Thus, these clinical findings suggested that the E169K mutation in JPH2 promoted atrial arrhythmias independently of the mechanism related to HCM formation caused by other JPH2 variants, including A405S (A399S in mice). Indeed, our findings revealed that E169K-PKI mice were susceptible to AF without evidence of structural cardiomyopathy at the age of 2 months. Moreover, A399S-PKI mice did not develop a susceptibility to AF, similarly to WT-PKI mice. Therefore, it is likely that the proarrhythmogenic effects of the E169K mutation are due to the reduced binding of E169K-JPH2 to RyR2, whereas HCM development likely involves other cellular mechanisms that remain to be explored in future studies.

The E169K mutation in JPH2 is located in a flexible "joining domain" connecting 2 sets of membrane-binding domains that attach JPH2 to the sarcolemma (16). Because the RyR2 channel is large and predicted to occupy most of the dyadic space between the sarcolemma and SR, the joining domain is a prime candidate to mediate the physical interaction of JPH2 with RyR2 (18). The region around residue E169 is highly conserved in evolution, highlighting the functional importance of this domain (16). Indeed, our data using a small JPH2-derived peptide containing the E169 residue was able to alter RyR2 functional activity, indicating direct channel modulation. Furthermore, co-immunoprecipitation experiments revealed that mutation E169K greatly reduced JPH2 binding to RyR2, whereas another HCM-associated mutation A399S (in mice) did not affect RyR2 binding. The effects of the peptide centered around the E169 residue were very specific, as a scrambled peptide containing the same amino acids did not inhibit RyR2 in single-channel experiments, nor did it suppress Ca^{2+} sparks in atrial myocytes.

JPH2 was first identified as a structural protein within the Ca^{2+}-release unit (CRU) that anchors the SR membrane to the plasma membrane (11). The current study uncovered an important additional cellular role of JPH2 in cardiomyocytes, namely regulation of RyR2-mediated SR Ca^{2+} release. Mutation E169K in JPH2, which interferes with RyR2 modulation, caused increased SR Ca^{2+} leakage

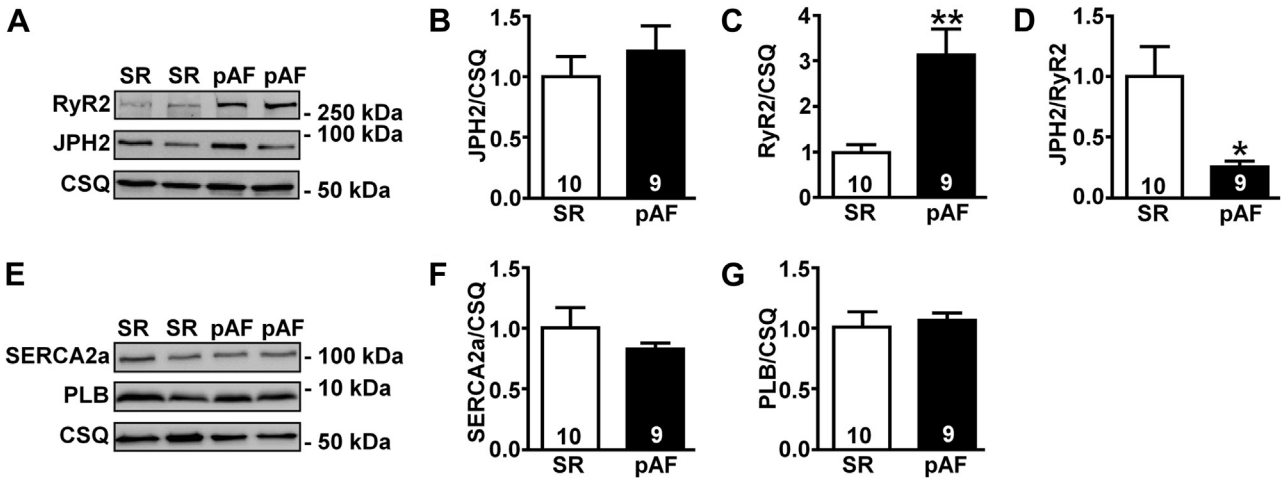

Figure 6 Reduced JPH2/RyR2 Ratios in Patients With Paroxysmal AF

Representative Western blots **(A)** and graphs **(B, C)** show RyR2 and JPH2 protein levels normalized to CSQ in atria from patients in sinus rhythm (SR) and paroxysmal AF (pAF). **(D)** Ratio of JPH2-to-RyR2 levels in human atria. Representative Western blots **(E)** and bar graphs **(F, G)** showing SERCA2a and PLB levels normalized to CSQ. AF = atrial fibrillation; CSQ = calsequestrin.

(Fig. 3), which is similar to what we recently demonstrated with knockdown of JPH2 in ventricular myocytes (10). It is theoretically possible that the JPH2 mutation also affects regulation of other known JPH2-binding partners within the CRU such as the L-type Ca^{2+} channel (LTCC) and the transient-receptor potential channel, subfamily C, member 3 (TRPC3) (15,19,20). However, it is unlikely that the E169K mutation affects the LTCC function, as I_{Ca} was not altered in atrial myocytes isolated from E169K-PKI mice (data not shown). Interestingly, it has been shown that in skeletal muscle, mutation S165F in JPH2 can block a potential PKC phosphorylation site, which in turn might reduce binding of JPH2 with TRPC3 (15). However, at this time it is unknown whether the JPH2 mutation E169K affects gating of the TRPC3 Ca^{2+} channel. Our peptide data suggest that JPH2 strongly regulates RyR2-mediated Ca^{2+} release, and that the E169K mutation decreases binding to RyR2. Thus, it is very likely that the cellular mechanisms underlying arrhythmogenesis caused by the E169K mutation involve reduced JPH2 binding to RyR2, diastolic SR Ca^{2+} release, and arrhythmogenic after-depolarizations leading to atrial arrhythmias.

A growing body of literature suggests that altered intracellular Ca^{2+} handling is a key contributor to AF, in particular ectopic activity associated with AF induction (21,22). Ectopic activity is caused by abnormal spontaneous action potentials that can result from DADs induced by diastolic SR Ca^{2+} release (4,9,22,23). However, the mechanisms by which RyR2 exhibit increased sensitivity to Ca^{2+}-mediated activation have been only partially understood and likely involve hyperphosphorylation of RyR2 in patients with chronic AF (4,9). The present study extends these previous findings by showing that another cellular defect, namely reduced JPH2 levels per RyR2 channel could also contribute to RyR2 dysfunction, especially in patients with pAF.

Our current data suggest that a lower JPH2/RyR2 ratio may promote increased RyR2-mediated SR Ca^{2+} release events that are likely to cause proarrhythmic DADs in pAF patients. Similar findings have been reported for another accessory subunit that stabilizes RyR2, namely FK506-binding protein 12.6 (FKBP12.6). Patients with chronic AF exhibit a reduced FKBP12.6:RyR2 ratio (23). In mice, reduced FKBP12.6 levels predispose to both ventricular and atrial arrhythmias due to an increased sensitivity to Ca^{2+}-mediated activation of RyR2 resulting in spontaneous Ca^{2+} release events and triggered activity (9,24,25).

Finally, studies in atrial cardiomyocytes from both mouse models and patients with chronic AF have established that aberrant RyR2-mediated SR Ca^{2+} release events activate NCX, which causes DADs, providing the trigger for AF (4,8,9). Future studies using JPH2 peptides might prove whether loss of JPH2-mediated RyR2 stabilization is causally linked to arrhythmogenic SR Ca^{2+} release events in patients with AF.

Study limitations. The link between the E169K mutation in JPH2 and atrial arrhythmias is based on 2 patients with the unusual presentation of atrial arrhythmias and HCM. Unfortunately, there were no additional family members available for genotyping. On the other hand, the association of HCM and supraventricular arrhythmias at a young age and prior to significant atrial remodeling is rather unique and not seen in carriers of the A405S mutation or other previously published JPH2 mutations linked to HCM (12,13).

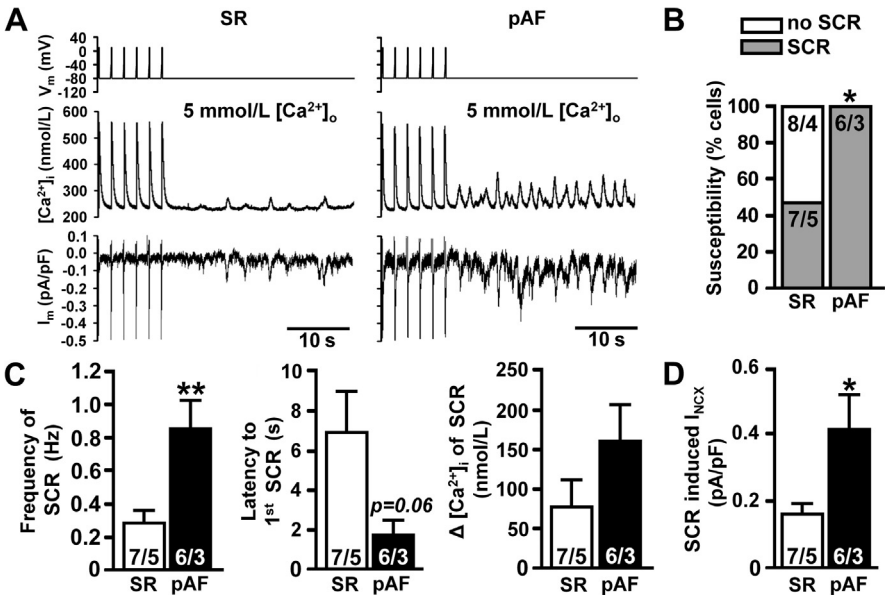

Figure 7 Enhanced Proarrhythmic Ca^{2+} Release Events in Patients With Paroxysmal AF

(A) Representative Ca^{2+} transient and membrane current (I_m) tracings from isolated atrial myocytes from patients in sinus rhythm and those in pAF following 1 min of pacing at 0.5 Hz. **(B)** Graph shows incidence of SCR events. **(C)** Frequency **(left)**, latency **(middle)**, amplitude **(right)** of SCR. **(D)** Amplitude of SCR-mediated NCX current. *$p < 0.05$, **$p < 0.01$. Numbers represent number of cells/number of patients. AF = atrial fibrillation; NCX = Na^+/Ca^{2+} exchanger; pAF = paroxysmal atrial fibrillation; SCR = spontaneous Ca^{2+} release.

Although knock-in mice can be used to study the effects of point mutations on protein function, our repeated efforts to generate E169K knock-in mice failed thus far. However, we were able to establish E169K mutant mice with physiological JPH2 levels in the heart by crossing E169K-Tg mice with MCM-shJPH2 knockdown mice (10). Thus, our pseudoknock-in mouse models serve as an adequate model when more traditional knock-in mice are not available or feasible.

The E169K-PKI mice did not develop spontaneous atrial arrhythmias, unlike the human carriers of the E169K mutation in JPH2. This is not surprising, as it is well established that mice are relatively resistant to developing AF unless there is gross atrial remodeling or dilation. In other mouse models of genetic variants that cause spontaneous AF in humans, we did not observe spontaneous AF although such mutant mice were vulnerable to pacing-induced arrhythmias (8,26). Although it is well accepted that an enhanced susceptibility to AF in mice is an approximate validation of a genetic variant that causes spontaneous AF in humans, using mice as an experimental model is a clear general limitation.

Finally, we used only right atrial tissue samples collected from the right atrial appendage of humans with paroxysmal AF. Although it is well established that ectopic activity often arises from the pulmonary vein region, there is also evidence for highly frequent focal sources in the right atrium in up to 33% of patients (27). Our finding of reduced JPH2 levels per RyR2 channel might not apply to other atrial regions or the left atrium. On the other hand, analysis of right atrial tissue samples and myocytes has proven to be useful and confirmed the presence of increased Ca^{2+} sparks and remodeling of Ca^{2+} handling proteins in patients with paroxysmal and chronic AF (4,28,29).

Conclusions

We describe a novel mechanism by which the JPH2 E169K mutation can cause atrial arrhythmias as a result of defective RyR2-mediated SR Ca^{2+} release events. The inherited mutation E169K in JPH2 disrupts the binding of mutant JPH2 to RyR2 and destabilizes RyR2, thereby increasing SR Ca^{2+} leak and the inducibility of AF in mice. These mechanisms are only observed in E169K mutant mice and not in another HCM-associated (A399S) mutant mouse, suggesting that the mechanisms underlying HCM-related ventricular remodeling are distinct and unrelated to RyR2 destabilization. Interestingly, patients with paroxysmal AF (nongenetic and not associated with HCM) exhibit decreased atrial JPH2 levels per RyR2 channel, suggesting that a similar mechanism of RyR2 destabilization due to a relative depletion of JPH2 could be involved in a much larger population of patients with paroxysmal AF. Our work suggests that future studies are warranted to explore whether JPH2-mediated stabilization of RyR2 might represent a novel therapeutic target for atrial arrhythmias. Specifically,

with the emergence of cell-penetrating peptides and small peptide therapies, it may be possible to develop a targeted therapy to reduce atrial arrhythmias (30).

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REFERENCES

1. Lloyd-Jones DM, Wang TJ, Leip EP, et al. Lifetime risk for development of atrial fibrillation: the Framingham Heart Study. Circulation 2004;110:1042–6.
2. Burashnikov A, Antzelevitch C. Late-phase 3 EAD. A unique mechanism contributing to initiation of atrial fibrillation. Pacing Clin Electrophysiol 2006;29:290–5.
3. Yeh YH, Wakili R, Qi XY, et al. Calcium-handling abnormalities underlying atrial arrhythmogenesis and contractile dysfunction in dogs with congestive heart failure. Circ Arrhythm Electrophysiol 2008;1:93–102.
4. Voigt N, Li N, Wang Q, et al. Enhanced sarcoplasmic reticulum Ca2$^+$ leak and increased Na^+-Ca^{2+} exchanger function underlie delayed afterdepolarizations in patients with chronic atrial fibrillation. Circulation 2012;125:2059–70.
5. Greiser M, Neuberger HR, Harks E, et al. Distinct contractile and molecular differences between two goat models of atrial dysfunction: AV block-induced atrial dilatation and atrial fibrillation. J Mol Cell Cardiol 2009;46:385–94.
6. Wehrens XH, Ather S, Dobrev D. Role of abnormal sarcoplasmic reticulum function in the management of atrial fibrillation. Therapy 2010;7:147–58.
7. Jayasinghe ID, Baddeley D, Kong CH, Wehrens XH, Cannell MB, Soeller C. Nanoscale organization of junctophilin-2 and ryanodine receptors within peripheral couplings of rat ventricular cardiomyocytes. Biophys J 2012;102:L19–21.
8. Chelu MG, Sarma S, Sood S, et al. Calmodulin kinase II-mediated sarcoplasmic reticulum Ca2+ leak promotes atrial fibrillation in mice. J Clin Invest 2009;119:1940–51.
9. Li N, Wang T, Wang W, et al. Inhibition of CaMKII phosphorylation of RyR2 prevents induction of atrial fibrillation in FKBP12.6 knockout mice. Circ Res 2012;110:465–70.
10. van Oort RJ, Garbino A, Wang W, et al. Disrupted junctional membrane complexes and hyperactive ryanodine receptors after acute junctophilin knockdown in mice. Circulation 2011;123:979–88.
11. Takeshima H, Komazaki S, Nishi M, Iino M, Kangawa K. Junctophilins: a novel family of junctional membrane complex proteins. Mol Cell 2000;6:11–22.
12. Landstrom AP, Weisleder N, Batalden KB, et al. Mutations in JPH2-encoded junctophilin-2 associated with hypertrophic cardiomyopathy in humans. J Mol Cell Cardiol 2007;42:1026–35.
13. Matsushita Y, Furukawa T, Kasanuki H, et al. Mutation of junctophilin type 2 associated with hypertrophic cardiomyopathy. J Hum Genet 2007;52:543–8.
14. Li N, Wehrens XH. Programmed electrical stimulation in mice. J Vis Exp 2010;39:1730.
15. Woo JS, Hwang JH, Ko JK, et al. S165F mutation of junctophilin 2 affects Ca^{2+} signalling in skeletal muscle. Biochem J 2010;427:125–34.
16. Garbino A, van Oort RJ, Dixit SS, Landstrom AP, Ackerman MJ, Wehrens XH. Molecular evolution of the junctophilin gene family. Physiol Genomics 2009;37:175–86.
17. Olivotto I, Cecchi F, Casey SA, Dolara A, Traverse JH, Maron BJ. Impact of atrial fibrillation on the clinical course of hypertrophic cardiomyopathy. Circulation 2001;104:2517–24.
18. Inui M, Saito A, Fleischer S. Isolation of the ryanodine receptor from cardiac sarcoplasmic reticulum and identity with the feet structures. J Biol Chem 1987;262:15637–42.
19. Golini L, Chouabe C, Berthier C, et al. Junctophilin 1 and 2 proteins interact with the L-type Ca^{2+} channel dihydropyridine receptors (DHPRs) in skeletal muscle. J Biol Chem 2011;286:43717–25.
20. Yamazaki D, Yamazaki T, Takeshima H. New molecular components supporting ryanodine receptor-mediated Ca(2+) release: roles of junctophilin and TRIC channel in embryonic cardiomyocytes. Pharmacol Ther 2009;121:265–72.
21. Nattel S, Burstein B, Dobrev D. Molecular remodeling and chronic atrial fibrillation. In: Zipes DP, Jalife J, editors. Cardiac Electrophysiology: From Cell to Bedside. 5th ed. Philadelphia: WB Saunders, 2009:453–65.
22. Dobrev D, Voigt N, Wehrens XH. The ryanodine receptor channel as a molecular motif in atrial fibrillation: pathophysiological and therapeutic implications. Cardiovasc Res 2011;89:734–43.
23. Vest JA, Wehrens XH, Reiken SR, et al. Defective cardiac ryanodine receptor regulation during atrial fibrillation. Circulation 2005;111:2025–32.
24. Wehrens XH, Lehnart SE, Reiken SR, et al. Protection from cardiac arrhythmia through ryanodine receptor-stabilizing protein calstabin2. Science 2004;304:292–6.
25. Wehrens XH, Lehnart SE, Huang F, et al. FKBP12.6 deficiency and defective calcium release channel (ryanodine receptor) function linked to exercise-induced sudden cardiac death. Cell 2003;113:829–40.
26. Wang J, Klysik E, Sood S, Johnson RL, Wehrens XH, Martin JF. Pitx2 prevents susceptibility to atrial arrhythmias by inhibiting left-sided pacemaker specification. Proc Natl Acad Sci U S A 2010;107:9753–8.
27. Narayan SM, Krummen DE, Clopton P, Shivkumar K, Miller JM. Direct or coincidental elimination of stable rotors or focal sources may explain successful atrial fibrillation ablation: on-treatment analysis of the CONFIRM (CONventional ablation for AF with or without Focal Impulse and Rotor Modulation) trial. J Am Coll Cardiol 2013;62:138–47.
28. Hove-Madsen L, Llach A, Bayes-Genis A, et al. Atrial fibrillation is associated with increased spontaneous calcium release from the sarcoplasmic reticulum in human atrial myocytes. Circulation 2004;110:1358–63.
29. Neef S, Dybkova N, Sossalla S, et al. CaMKII-dependent diastolic SR Ca^{2+} leak and elevated diastolic Ca^{2+} levels in right atrial myocardium of patients with atrial fibrillation. Circ Res 2010;106:1134–44.
30. Heitz F, Morris MC, Divita G. Twenty years of cell-penetrating peptides: from molecular mechanisms to therapeutics. Br J Pharmacol 2009;157:195–206.

Key Words: atrial fibrillation ■ calcium ■ junctophilin ■ ryanodine receptor ■ sarcoplasmic reticulum.

APPENDIX

For and expanded methods section, and supplemental tables and figures, please see the online version of this article.