Suppression of Spontaneous Ca Elevations Prevents Atrial Fibrillation in Calsequestrin 2-Null Hearts

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Background—Atrial fibrillation (AF) risk has been associated with leaky ryanodine receptor 2 (RyR2) Ca release channels. Patients with mutations in RyR2 or in the sarcoplasmic reticulum Ca-binding protein calsequestrin 2 (Casq2) display an increased risk for AF. Here, we examine the underlying mechanisms of AF associated with loss of Casq2 and test mechanism-based drug therapy.

Methods and Results—Compared with wild-type Casq2+/+ mice, atrial burst pacing consistently induced atrial flutter or AF in Casq2−/− mice and in isolated Casq2−/− hearts. Atrial optical voltage maps obtained from isolated hearts revealed multiple independent activation sites arising predominantly from the pulmonary vein region. Ca and voltage mapping demonstrated diastolic subthreshold spontaneous Ca elevations (SCaEs) and delayed afterdepolarizations whenever the pacing train failed to induce AF. The dual RyR2 and Na channel inhibitor R-propafenone (3 μmol/L) significantly reduced frequency and amplitude of SCaEs and delayed afterdepolarizations in atrial myocytes and intact atria and prevented induction of AF. In contrast, the S-enantiomer of propafenone, an equipotent Na channel blocker but much weaker RyR2 inhibitor, did not reduce SCaEs and delayed afterdepolarizations and failed to prevent AF.

Conclusions—Loss of Casq2 increases risk of AF by promoting regional SCaEs and delayed afterdepolarizations in atrial tissue, which can be prevented by RyR2 inhibition with R-propafenone. Targeting AF caused by leaky RyR2 Ca channels with R-propafenone may be a more mechanism-based approach to treating this common arrhythmia. (Circ Arrhythm Electrophysiol. 2014;7:313-320.)

Key Words: atrial fibrillation • calsequestrin 2 • delayed afterdepolarization • propafenone • ryanodine receptor calcium release channel • tachycardia, ventricular
spontaneous Ca release with the R-enantiomer of propafenone, which is a much more potent RyR2 Ca release channel inhibitor than S-propafenone.9

Methods
Detailed methods are provided in the Data Supplement.

Animal Use
All studies were performed according to National Institutes of Health guidelines and were approved by the institutional animal care and use committees at Vanderbilt University.

AF Induction
Casq2+/+ and Casq2−/− hearts were harvested and perfused in Langendorff mode as previously described.10 AF was induced by delivering repeated trains of atrial burst pacing (50 Hz, 2 s). Volume-conducted ECG was recorded continuously: AF was defined as rapid and fragmented atrial electrograms present for ≥150 ms. Incidence and duration of AF episodes were quantified in both Casq2+/+ and Casq2−/− hearts.

Optical Mapping
Isolated perfused hearts from Casq2+/+ and Casq2−/− mice were stained with either di-4-ANEPPS or Rhod-2 dye. Both voltage and Ca maps were acquired with a RedShirt charge-coupled device camera (14 bit, 80x80 pixels, 1000 fps, CardioCCD-SMQ; RedShirt Imaging), during the postpacing interval. All maps were analyzed by 1 operator in blinded fashion with MATLAB (Mathworks, Natick, MA) using custom algorithms. Voltage maps were used to study atrial activation during both sinus rhythm and AF and to quantify the incidence of DADs in Casq2+/+ and Casq2−/− atria. In addition, activation maps of the atria during epicardial pacing at constant cycle lengths were obtained to measure atrial conduction velocity (CV) and action potential duration at 90% of repolarization levels. Incidence of distolic spontaneous Ca elevations (SCaEs) in the atria of perfused hearts was investigated by generating Ca fluorescent maps in the postpacing interval (pacing bursts at 50 Hz, 2 s). The amplitude of every SCaE recorded was quantified as a percentage of the Ca transient amplitude during pacing. Only elevations ≥10% of the preceding atrial Ca transient during pacing were considered for the analysis.

To test the effect of class I antiarrhythmic drugs on SCaEs and AF, Casq2−/− isolated perfused hearts stained with Rhod-2 AM underwent the pacing protocol twice: first during vehicle infusion and then in the presence of R-propafenone (3 μmol/L), S-propafenone (3 μmol/L), or lidocaine (20 μmol/L), respectively. The lidocaine concentration was chosen to achieve a similar degree of Na channel block as 3 μmol/L of R-propafenone and S-propafenone as evidenced by a comparable increase in the QRS interval (≥25%). Drugs were continuously infused for 15 minutes before pacing was resumed. AF episodes as well as number and amplitude of SCaEs after R-propafenone, S-propafenone, or lidocaine (20 μmol/L), respectively. The lidocaine concentration

Isolated Myocyte Studies
Atrial myocytes were isolated from Casq2+/+ and Casq2−/− atria. First, isolated hearts were perfused for 7 to 8 minutes with tyrode buffer containing collagenase type II (25 mg) and protease (2 mg) to obtain a primary digestion. Then, atria were removed and exposed to a secondary enzyme digestion for an additional 15 to 30 minutes. For permeabilized myocyte studies, cells were placed in laminin-coated chambers, washed with relaxing solution for 30 s, and chemically permeabilized by exposure to internal solution containing saponin (40 μg/mL). After 1 minute, the saponin solution was replaced by internal solution containing Fluor 4 pentapotassium salt (0.02 mmol/L). The permeabilized cells were imaged in the line-scan mode after 10 minutes incubation with either vehicle, S- or R-propafenone (10 μmol/L) and analyzed as described.11 For intact myocyte studies, cells were loaded with Fura-2 AM, and spontaneous Ca waves and trigger beats were measured as described.12

Statistical Analysis
Data are presented as mean±SEM. Number and duration of AF episodes, Ca sparks and Ca wave frequency and amplitude in isolated myocytes, and DADs and SCaE frequency and amplitude were compared by means of the Mann–Whitney U test. Incidence of AF was compared by Fisher exact test. A 2-tailed P value <0.05 was considered statistically significant. For statistical comparison of ≥2 groups, the Mann–Whitney U test and Fisher exact test were used for post hoc analysis whenever the nonparametric ANOVA (Kruskal–Wallis ANOVA) showed an overall statistically significant difference.

AF Susceptibility of Casq2−/− Mice and Isolated Casq2−/− Hearts
We first determined AF susceptibility in vivo by transesophageal atrial burst pacing. Consistent with other mouse models with increased Ca leak,13 atrial burst pacing resulted in a significantly higher AF burden and AF duration in Casq2−/− mice compared with wild-type Casq2+/+ littermates (Figure I in the Data Supplement). To investigate the underlying mechanism for the AF susceptibility, volume-conducted ECGs and optical voltage maps were recorded simultaneously in Casq2−/− and Casq2+/+ isolated perfused hearts. Neither group displayed spontaneous episodes of AF. AF susceptibility was tested with repeated trains of atrial burst pacing (Figure 1). Atrial burst pacing did not induce significant episodes (>150-ms duration) of atrial tachyarhythmia in wild-type hearts (0/10 hearts, Figure 1). In Casq2−/− hearts, burst pacing induced frequent runs of atrial tachyarhythmias resembling atrial flutter (Figure 1B) and more disorganized forms resembling AF (Figure 1C). For data analysis, both atrial flutter and more disorganized forms were grouped together and labeled as AF. A total of 10 Casq2+/+ and 12 Casq2−/− hearts were analyzed as described.12

Figure 1. Calsequestrin 2-null (Casq2−/−) hearts are susceptible to pacing-induced atrial fibrillation (AF). AF susceptibility was evaluated by atrial burst pacing (50 Hz, 2 s). Representative ECG records from Casq2−/− (A) and Casq2+/+ (B and C) hearts. Atrial tachyarhythmias resembling atrial flutter (B and AF) were observed in Casq2−/− hearts. For statistical analysis, episodes of AF and atrial flutter were grouped together and labeled as AF. Average rate (D) and duration (E) of AF in each group. N=10 Casq2+/+ and 12 Casq2−/−: **P<0.01, ***P<0.001.
111 AF episodes were recorded in 11 of 12 Casq2−/− hearts (9.3±2.6 episodes per heart). Approximately 14% of all pacing trains triggered AF. Once induced, AF lasted on average for 21.9±13.1 s (Figure 1D).

Next, we compared electric activation in Casq2−/− atria during sinus rhythm and AF (Figure 2). During sinus rhythm, atrial activation started from 1 single activation site consistent with the sinoatrial node region near the crista terminalis in the posterior right atrium (Figure 2A). The activation wavefronts then spread simultaneously in 2 directions: to the right atrial appendage and across the pulmonary vein region of the left atrium toward the left atrial appendage. Atrial activation was completed within 12 ms (Figure 2A). During AF, atrial activation occurred quasi-simultaneously from multiple independent sites (usually 2 or 3) from both atria (Figure 2B), with independent foci observed as close as 1 mm. The ectopic activation was preceded by electric silence. A re-entrant pattern of atrial activation was not observed.

We quantified the ectopic activation sites observed during AF by anatomic region: right atrium, pulmonary vein region, and left atrial appendage (Figure 2C). Most of the AF activation sites were found in the pulmonary vein area (48%), especially from the lower portion of the pulmonary vein region. The remaining activation sites were equally distributed among both atrial appendages, with 25% of activation sites in the left atrium and 27% in the right atrium (Figure 2C). Interestingly, the sinoatrial node region was spared. Taken together, the spatial and temporal activation pattern and the regional distribution suggest that focal activity (ie, by DADs)14 was responsible for triggering AF in Casq2−/− hearts.

To test this hypothesis, we examined voltage maps of pacing trains that failed to induce AF for evidence of DADs. Because DADs are best observed in the pause following a pacing train, for this analysis we only examined maps with a postpacing pause of ≥100 ms before atrial or ventricular activation resumed. DADs were observed in 43 of the 95 voltage maps analyzed (45%; Figure 3A). Consistent with the anatomic sites of atrial activation during AF (Figure 2C), the majority (50%) of DADs were detected in the pulmonary vein region (Figure 3B). Pacing-induced DADs were never observed in Casq2+/+ hearts (n=4).

**Casq2+/− Atria Display Unchanged Atrial Action Potential and CV**

Although no clear re-entrant activation was observed in our optical maps, we next investigated whether changes in atrial action potential or CV might contribute to AF susceptibility in Casq2+/− mice. Action potentials were measured optically (Figure 4A) in the right and left atrial appendage of Casq2+/− and Casq2−/− hearts during atrial pacing at a cycle length of 100 ms. Mean action potential duration was not statistically different (Figure 4B). Atrial CV was determined for each heart from right atrial appendage activation maps as illustrated in Figure 4C. Mean maximum CV was not statistically different in the 2 groups (Figure 4D).

**Spontaneous Ca Release in Isolated Casq2+/− Atrial Myocytes and Intact Casq2−/− Atria**

Altered Ca homeostasis with increased frequency and magnitude of Ca waves and sparks has been proposed as a cellular mechanism that can contribute to AF inducibility and maintenance. Therefore, we quantified Ca sparks and Ca waves in isolated permeabilized atrial cells. Compared with Casq2+/+, Casq2−/− atrial cells displayed a significantly higher rate of spontaneous Ca sparks (Figure 5A). Furthermore, the frequency of propagated Ca waves was also significantly increased in Casq2−/− cells (Figure 5B).

To investigate Ca handling in intact atria, we obtained Ca maps from Casq2−/− and Casq2+/− intact hearts during the postpacing pause. A 5×5 pixel selection was used to sample different areas of both atria to obtain the corresponding Ca fluorescent trace. SCaEs were present in 60% of pacing atrial activation sites during AF episodes in 7 Casq2−/− hearts. Size of the circles is proportional to the number of events arising from that specific area. **Right**, Distribution of atrial activation sites by region: RA, PV region, and LA appendage.

![Figure 2](image-url)
trains, with 100% (11/11) Casq2−/− hearts exhibiting SCaEs (Figure 5C and 5D). SCaEs were never observed in Casq2+/+ hearts. Shape, amplitude, and timing of the SCaEs varied greatly in the different areas of the atria consistent with the local nature of these events. The anatomic origin of the SCaE was defined as the atrial site where the deflection in the Ca transient amplitude was most prominent. Only SCaEs with ≥10% of the pacing-induced Ca transient amplitude were considered for analysis. SCaEs arose most frequently from the pulmonary vein area (58%) followed by the left atrial appendage (22%) and the right atrium (20%; Figure 5E). The anatomic distribution of SCaEs matched that of DADs (Figure 3B) and focal activation sites during AF (Figure 2C). These results suggest that Casq2−/− atria display increased susceptibility to SCaEs in the pulmonary vein region that can lead to DADs and ectopic triggered activity in the same area.

Figure 3. Anatomic origin of delayed afterdepolarizations (DADs) in intact calsequestrin 2-null (Casq2−/−) atria. A, Representative example of a DAD. The red optical voltage signal originating from the atria shows a deflection in the postspacing pause consistent with a DAD. Only deflections ≥10% of the atrial action potential amplitude during pacing were considered as DADs. No influence from ventricular fluorescence (black signal) is present during the atrial DAD. B, Anatomic distribution of DADs in Casq2−/− atria: the majority of the DADs occurred in the pulmonary vein (PV) region. LA indicates left atrium; and RA, right atrium.

Figure 4. Action potential duration (APD) and conduction velocity (CV) of Casq2+/+ and calsequestrin 2-null (Casq2−/−) atria. A, Representative example of action potentials recorded optically from the right atrial (RA) appendage in Casq2+/+ and Casq2−/− hearts. B, Average APD at 90% repolarization (APD90) measured during constant pacing at 100-ms pacing cycle length was not statistically different. C, Optical activation maps of the RA appendage during pacing. CV was calculated by defining isochrones where the time of maximum upstroke velocity of the fluorescent signal was the same. D, Average CV values were not statistically different between Casq2+/+ and Casq2−/− atria. N=4 to 5 hearts per group. LA indicates left atrium.

RyR2 Block by R-Propafenone Suppresses SCaEs in Isolated Atrial Myocytes and Intact Atria and Prevents AF

R-propafenone is a class I antiarrhythmic drug with potent RyR2 blocking properties. Incubating Casq2−/− permeabilized atrial myocytes with R-propafenone for 10 minutes significantly reduced frequency, amplitude, and propagation speed of Ca waves (Figure 6A and 6B). In contrast, S-propafenone, which is a much weaker RyR2 channel inhibitor than R-propafenone, had no significant effect on Ca waves (Figure 6A and 6B). We next tested R- and S-propafenone in intact atrial myocytes using burst pacing to induce spontaneous Ca waves, DADs, and triggered beats (Figure 6C). R-propafenone significantly reduced the incidence of spontaneous Ca waves and completely prevented triggered beats, whereas S-propafenone did not (Figure 6D).

Given its efficacy in isolated myocytes, we tested R-propafenone in intact hearts. Atrial pacing induced only 4 SCaEs overall in 3 of 8 Casq2−/− hearts pretreated with R-propafenone, resulting in a significant reduction in SCaE incidence (Figure 7A and 7B). The amplitude of the few remaining SCaEs recorded in the presence of R-propafenone was also reduced compared with vehicle (Figure 7C). The inhibition of spontaneous Ca waves and triggered beats by R-propafenone but not S-propafenone in Casq2−/− atrial myocytes (Figure 6) strongly suggested that propafenone’s suppression of SCaEs in the intact atria is mainly related to its inhibitory effect on RyR2 rather than Na channel block. To test this hypothesis directly, we used S-propafenone and lidocaine on intact atria. S-propafenone is a much weaker RyR2 inhibitor than R-propafenone but blocks Na channels with equal potency. Lidocaine is a class I antiarrhythmic drug with no RyR2 blocking properties. All 3 drugs were administered at concentrations that produced a 25% wider QRS complex, indicating a comparable degree of Na channel block (Figure 7D). In contrast to R-propafenone, S-propafenone, and lidocaine had no effect on frequency or amplitude of SCaEs in intact atria (Figure 7B and 7C). Furthermore, R-propafenone completely prevented pacing-induced AF (Figure 7E), whereas both S-propafenone and lidocaine were ineffective (Figure 7E). Collectively, these results suggest that R-propafenone’s antiarrhythmic efficacy in the Casq2−/− AF model is attributable to its suppression of Ca waves and SCaEs by inhibition of RyR2 channels.

Discussion

Major New Findings

Our experiments demonstrate a mechanistic link between enhanced frequency of Ca waves in single atrial myocytes, SCaEs and DADs in intact atria, and AF risk. An increased frequency of Ca waves has been observed in atrial cells from patients with AF as well as from several AF animal models. Ca handling dysfunction may contribute both to the initiation and maintenance of chronic AF in patients. Although it has been speculated that increased frequency of Ca waves in atrial myocytes could be responsible for DADs and triggered AF, it has not been shown that these cellular events observed in isolated myocytes are sufficient to cause any changes in intact atria. Here, we identify and localize pacing-induced SCaEs...
in the intact atria of Casq2−/− hearts. These local events likely result from the synchronization of Ca waves in several cells and generate DADs and triggered AF as suggested by the colocalization of SCaEs, DADs, and ectopic activation sites close to the pulmonary vein region. This mechanism is further supported by our finding that R-propafenone, a class I antiarrhythmic drug clinically used for the treatment of AF, not only decreases Ca wave rate in single atrial myocytes through inhibition of RyR2 Ca release channels but also significantly reduces SCaEs in intact atria and completely suppresses AF in Casq2−/− hearts. Importantly, S-propafenone and lidocaine, two Na channel inhibitors with either less or no RyR2 blocking properties, fail to prevent SCaEs and AF in intact Casq2−/− atria.

**Regional SCaEs in Intact Atria Increase the Risk of AF in Casq2−/− Hearts**

Our results show that lack of Casq2 increases susceptibility to AF in mice. Both re-entrant and triggered activation patterns have been previously described during AF in RyR2-linked catecholamine-induced polymorphic ventricular tachycardia models. Our optical mapping of AF events in Casq2−/− hearts consistently revealed 2 to 3 sites of near-simultaneous activation that then propagated to the surrounding areas. This pattern is most consistent with focal triggered activation. Although our mouse model does not show any sign of macroscopic structural abnormality or fibrosis in atria or ventricles, we cannot completely exclude the possibility that micro-re-entrant circuits are responsible for sustaining AF. However, when we measured action potential duration at 90% of repolarization levels and CV, 2 main parameters implicated in the risk of functional re-entry, we found them unchanged compared with Casq2+/+ controls. Consistent with SCaEs triggering focal activation in Casq2−/− atria, spontaneous SR Ca releases in the form of both Ca sparks (local Ca releases) and Ca waves (propagated subcellular Ca releases) were significantly more frequent in Casq2−/− isolated atrial myocytes than in controls. Our results support the hypothesis that when Ca waves occur in a sufficient number of adjacent cells at the same time, the phenomenon can be observed at the tissue level and can cause local DADs and triggered activity, as suggested by our findings.
Sinus rhythm
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Abnormalities are present in these cells, thus making it possible that acquired Casq2 reduction might promote AF in some chronic acquired heart diseases that are associated with increased risk of AF, such as congestive heart failure. It is possible that acquired Casq2 reduction might promote AF in these patients as a complication of a pre-existing chronic cardiac disease. In this context, acquired Ca handling dysfunction could provide local triggers to initiate paroxysmal re-entrant AF sustained by anatomic substrates. Similarly, weakness of the SR could contribute to postoperative AF where the β-adrenergic stimulation after surgery may increase heart rate by rapid atrial pacing, which may not be relevant to persistent or chronic forms of AF in humans. However, in the clinical electrophysiology laboratory, atrial pacing is often performed to induce paroxysmal AF in patients. Because >70%

Figure 6. R-propafenone (R-Prop) but not S-propafenone (S-Prop) reduces the frequency of spontaneous Ca waves (SCWs) in permeabilized calsequestrin-null (Casq2−/−) atrial myocytes and suppresses pacing-induced SCWs and triggered beats (TBs) in intact atrial Casq2−/− myocytes. A, Representative line scans recorded from permeabilized Casq2−/− atrial myocytes in the presence of vehicle (VEH, dimethyl sulfoxide), R-Prop (10 μmol/L), or S-Prop. B, Averaged data. N=5 to 8 cells per group; *P<0.05, **P<0.001. C, Representative Ca fluorescence records from Fura-2 AM loaded, intact atrial myocytes after 15 minutes exposure to VEH, R-Prop (3 μmol/L), or S-Prop (3 μmol/L). Both TBs (arrow) and SCWs (arrowhead) were induced by a 20-s pacing train (3 Hz). D, Incidence of TBs and SCWs. ***P<0.01 vs VEH, #P<0.05 vs S-Prop; N=9 to 13 per group.

Figure 7. Ryanodine receptor channel block is necessary for prevention of pacing-induced diastolic spontaneous Ca elevations (SCaEs) and atrial fibrillation (AF) in calsequestrin 2-null (Casq2−/−) atria. A. Representative examples of fluorescent Ca signals obtained from Ca maps of intact Casq2−/− hearts. As opposed to R-propafenone (R-Prop), administration of 3 μmol/L of S-propafenone (S-Prop) or 20 μmol/L of lidocaine (Lido) did not significantly reduce the rate (B) and amplitude (C) of SCaEs in intact atria compared with vehicle (VEH). Despite a similar level of Na channel block evidenced by comparable QRS widening (D), S-Prop and Lido failed to prevent AF in Casq2−/− hearts (E). N=5 to 6 Casq2−/− hearts; **P<0.01.
of paroxysmal AF is thought to be driven by enhanced spontaneous activity originating in the pulmonary veins, rapid atrial pacing may be most relevant to unmasking increased susceptibility to paroxysmal AF in humans.

**RyR2 Block by R-Propafenone Prevents AF in Casq2−/− Isolated Hearts**

Currently, the drug management of AF in clinical practice varies considerably, in part that is attributable to the lack of reliably effective therapies for maintaining sinus rhythm and the toxicities associated with antiarrhythmic drugs. Drug efficacy could be improved by targeting the underlying pathophysiology of AF. For example, increased protein kinase A-mediated phosphorylation of RyR2 as well as altered phosphatase and phosphodiesterase-4 activity have all been proposed as mechanisms contributing to Ca-triggered AF. The experimental compound S107, a RyR2 channel modulator, has been successfully used to prevent AF in a variety of RyR2 mutant mouse models. Calcium/calmodulin-dependent protein kinase-II has also been identified as a potential therapeutic target because it seems to contribute to the risk of chronic AF by altering RyR2 permeability to Ca; experimentally, calcium/calmodulin-dependent protein kinase-II inhibition reduces SCaEs in isolated atrial myocytes from patients with chronic AF. However, these compounds are not yet ready for patient treatment and will require further testing and clinical trials. Hence, we tested the R-enantiomer of propafenone (the racemic mixture of propafenone is routinely used in clinical practice) and investigated its mode of action in our model of Ca-triggered AF. We previously reported the efficacy of R-propafenone in suppressing ventricular arrhythmias in catecholamine-induced polymorphic ventricular tachycardia because of its dual action as both Na and RyR2 channel blockers. Our new results reported here show that R-propafenone can also completely prevent AF in Casq2−/− mice, likely through a significant reduction of diastolic SCaEs in intact atria. Importantly, Na channel block in the absence of RyR2 inhibition, as obtained with S-propafenone or lidocaine, was not sufficient to prevent SCaEs and AF in our model of Ca-triggered AF (Figure 7). However, we cannot exclude that block of transient outward K currents or L-type Ca currents by propafenone contributed to suppression of SCaEs and AF in Casq2 knockout mice.

Propafenone is clinically used as a racemic mixture of R- and S-enantiomers to treat recurrent symptomatic AF as well as pill-in-the-pocket treatment for patients with sporadic paroxysmal AF. However, ≤20% of patients are not responsive to propafenone. Incomplete efficacy of the drug could be attributable to heterogeneity of the underlying AF pathophysiological mechanisms. We speculate that in patients with atrial remodeling and fixed re-entry circuits, propafenone may not be as effective because in that case propafenone RyR2 blocking properties are not exploited. Another possible explanation for the lack of propafenone efficacy in a subset of patients with AF is the use of racemic propafenone. R-propafenone inhibits the metabolism and elimination of S-propafenone, leading to accumulation of S-propafenone during chronic administration. Because S-propafenone was not effective for preventing Ca-triggered AF (our results) and because leaky RyR2s have been observed in many forms of AF, we speculate that R-propafenone might be a more effective mechanism-based treatment than the propafenone racemate currently used in clinical practice. R-propafenone could be considered for clinical drug development.

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**Disclosures**

None.

**References**

Recent studies have demonstrated that increased Ca leak from intracellular Ca stores can be associated with both chronic and paroxysmal forms of atrial fibrillation (AF). Questions remain whether this abnormal Ca leakiness found in cardiomyocytes isolated from patients with AF is a consequence of AF-induced cellular remodeling or if it is a primary defect sufficient to induce AF. This study uses a genetic mouse model to demonstrate that enhanced diastolic Ca releases caused by loss of the Ca-binding protein calsequestrin can cause AF by promoting regional Ca elevations and delayed afterdepolarizations. Moreover, inhibition of spontaneous Ca release prevented AF by significantly reducing the rate of delayed afterdepolarizations and triggered atrial activity: The R-enantiomer of propafenone, which has potent ryanodine receptor channel blocking properties, completely prevented atrial arrhythmias in this mouse model, whereas other class I Na channel inhibitors that lack ryanodine receptor blocking properties were not effective. Taken together, the results of the study provide proof of principle that AF can be triggered by spontaneous Ca release in mouse atria. Propafenone, which is used clinically as a racemic mixture of R- and S-enantiomers, may be the drug of choice for treating patients with AF in whom spontaneous Ca release is suspected to contribute to AF pathophysiology.
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Supplemental Figure 1. Casq2-/- mice are susceptible to pacing induced AF. AF susceptibility was evaluated in anesthetized Casq2+/+ and Casq2-/- by transesophageal (TE) atrial burst pacing. Representative ECG records from Casq2+/+ (A) and Casq2-/- (B) hearts. Average AF duration (C) and total AF burden/mouse (D) in each group. n = 12 Casq2+/+ and 7 Casq2-/- mice; *P<0.05 by Mann-Whitney Test.
SUPPLEMENTAL METHODS

Animal use

All studies were carried out according to National Institutes of Health guidelines and were approved by the institutional animal care and use committees at Vanderbilt University. Casq2−/− mice generation as well as in vitro and in vivo characterization have been previously described.1

In-vivo studies – transesophageal (TE) pacing.

Mice were anesthetized with inhaled isoflurane (3% for induction, 1% for maintenance) while breathing spontaneously and placed supine on a heating pad. A surface ECG (lead I) was obtained by placement of subcutaneous 27-guage needles in each arm. The surface ECG was monitored continuously and stored on an optical disk. An octapolar 2F electrode catheter (CiBer cath; NuMED, Inc) was placed in the esophagus via the mouth guided by electrogram tracings to verify position. Bipolar electrogram recordings were obtained from the atrium and ventricle with the esophageal catheter. Bipolar pacing was performed using a programmable stimulator to deliver coupling intervals as short as 10 ms. Atrial pacing threshold (in milliamperes) was determined and stimulation is performed for 1.0 to 2.0-ms pulse width at twice the diastolic capture threshold. Electrophysiologic intervals (RR, PR, QRS, and AV) were measured in standard fashion. To test for inducibility of atrial fibrillation, burst atrial pacing is performed at 50, 40, 30, 25, 20, and 15 ms for 15 seconds each. Episodes of atrial fibrillation (defined as rapid atrial depolarizations with irregular ventricular rate lasting at least 1 second) were noted. The duration of atrial fibrillation was measured from the end of the pacing train to the first P-wave with regular atrioventricular conduction after termination of atrial fibrillation. The number of induced episodes, total duration of AF, and mean duration of induced episodes are calculated for each animal. At the end of the procedure, the esophageal catheter and subcutaneous needles are removed and isoflurane discontinued. The animal is placed in a cage with food and water and observed until recovery.
Atrial myocyte isolation and confocal imaging of saponin-permeabilized atrial myocytes

Atrial myocytes were isolated from Casq2-/- and Casq2+/+ hearts. Hearts were perfused for 5 min with Tyrode’s buffer (in mmol/L: NaCl 137, KCl 5.4, MgCl2 0.5, HEPES 10, and glucose 10, pH 7.4, 36.5 °C) and for 7 to 9 min with 50 ml Tyrode’s buffer containing 25 mg collagenase type II (Worthington) and 2 mg protease (type IV, Sigma). After primary digestion, the atria were cut and minced in 2ml of enzyme solution and gently pipetted for 1 minute. The tissue was then collected into 2ml tubes and further digested at 37°C for 15-30 min. When a large population of atrial cells was available digestion was interrupted adding 0.2mM Ca solution containing albumin (1mg/ml). This procedure was repeated 3 times (3 cycles of 20 min each) in order to wash out the remaining enzyme and stop the digestion. Aliquots of the solution containing the myocytes were placed in laminin-coated chambers and allowed to settle for 5-6 minutes. After the cells were washed out with a relaxing solution (ethylene glycol-bis (2-aminoethyl)ether)-N, N, N’, N’-tetraacetic acid (EGTA) 0.1 mM, HEPES 10 mM, K-aspartate 150 mM, MgCl2 0.25 mM, and Adenosine TriPhosphate di-Na+ (di-Na+ ATP) 5 mM), the supernatant was removed and replaced with internal solution containing saponin (40 μg/ml). After one minute the saponin solution was removed and replaced by control internal solution (in mM): K-aspartate 100, KCl 15, KH2PO4 5, CaCl2 0.04 - 0.06, MgCl2 0.75, Dextran (40,000) 8 %, HEPES 10, MgATP 5, phosphocreatine DiNa+ 10, Creatine phosphokinase 10 U/ml, Glutathione (reduced) 10 and Fluo 4 pentapotassium salt 0.02. The permeabilized cells were imaged with an LSM 510 Zeiss inverted microscope in the line-scan (LS) mode and a (40x oil immersion objective lens (Nikon, Tokyo, Japan) after 10 minutes incubation of either vehicle or R-Propafenone (10 μM). This same basic procedure, but with some changes in the buffer concentration in the internal solution (high EGTA for sparks (0.4 mM) and low EGTA for calcium waves (0.05 mM)), was used to acquire the LS for the Ca release events. These LS were analyzed as previously described.
Intact myocyte studies

Atrial myocytes were isolated from Casq2+/+ and Casq2-/- atria as described above. In intact myocytes, after myocyte-loaded Fura-2 AM, spontaneous Ca waves and trigger beats were measured as previously described.19 Briefly, in the presence of 1 µM of Isoprotenerol, myocytes were field-stimulated at 3 Hz for 20 seconds and continuously recorded for 30 seconds without stimulation after 15 minutes incubation of vehicle (DMSO), R-propafenone (3µM), or S-propafenone (3µM). The ratiometric fluorescent records were analyzing using commercially available data analysis software (IonWizard, IonOptix, Milton, MA).

R-propafenone, S-propafenone and Lidocaine preparation

Lidocaine hydrochloride and Propafenone hydrochloride were obtained from Sigma (St. Louis, MO). Racemic propafenone was separated into two enantiomers S- and R-propafenone as described.3

Atrial pacing protocol to quantify AF inducibility in isolated hearts

Casq2-/- mice and wild-type littermates (Casq2+/+) were anaesthetized with 5% isofluorane inhalation. After harvesting, hearts were retrogradely perfused through the aorta with Tyrode buffer (130 mM NaCl, 4 mM KCl, 23 mM NaHCO3, 1.5 mM NaH2PO4, 1 mM MgCl2, 2mM CaCl2, 10 mM Glucose) at a temperature of 36°C, bubbled with 95% O2 and 5% CO2. Propranonol (0.2 μM) was present in all perfusate solutions to avoid any confounding effects due to beta adrenergic stimulation by tissue catecholamines potentially mobilized by the fast pacing. Hearts were allowed 10 minutes to equilibrate. Volume conducted ECG was recorded continuously using AD Instrument bioamplifiers and Labchart 7 software. After an equilibration period of 10 min, a bipolar platinum stimulation electrode was placed on the right atrial appendage, and the pacing threshold determined at 12 Hz pacing rate. To induce AF, each heart underwent 5 sets of 10 pacing bursts (50 Hz, 2 s duration, 5 s apart to allow evaluation of atrial rhythm) at twice pacing threshold. AF was defined as rapid and fragmented atrial electrograms present for at least 150ms. The AF burden for each heart was
determined by the ratio between the number of AF episodes recorded and the number of pacing bursts delivered during the entire protocol.

Atrial mapping studies

Optical voltage maps were obtained from Casq2-/- and wild-type hearts at rest and under pacing stimulation. Hearts were perfused at constant pressure as described above. All solutions contained the contractile uncoupling agent (-/-) blebbistatin (3 µM) to prevent motion artefacts. For voltage mapping, hearts were stained with 10-15 µl of di-4-ANEPPS stock solution (0.5 mg/ml dimethyl sulfoxide). Volume conducted ECG was recorded continuously. AF was induced with trains of atrial burst pacing (50Hz, 2 s). Optical maps were recorded starting from the last 20 pacing pulses and during the post pacing pause for up to 4 seconds. Hearts were illuminated with a coherent diode-pumped, solid-state Coherent Verdi laser (532 nm). The fluorescence emitted from the hearts was collected with a RedShirt charge-coupled device camera (14-bit, 80 × 80 pixels, 1,000 fps, CardioCCD-SMQ; RedShirt Imaging), equipped with a 52-mm standard lens in combination with a magnifying lens (+4; Tiffen), and passed through a cut-off filter (No. 25 Red, 607 nm; Tiffen). Optical data were recorded at frame rates of 1000/s for periods of 4s. Voltage maps were obtained from the posterior view of the atria. This view had the advantage that both posterior atrial appendages, pulmonary veins, superior and inferior vena cava and sinoatrial node could be obtained on one image from the intact heart. Additional maps from the anterior and lateral views of the atria were also recorded for action potential and conduction velocity measurements.

Optical Data Analysis

Voltage maps were used to study the atrial activation during both sinus rhythm and AF. When AF was identified on the ECG, the optical maps relative to the episode were analysed to generate activation maps and identify the atrial activation pattern. In addition, after filtering the signal with a 3x3 filter, voltage maps were analysed for evidence of DADs. A small 5x5
A pixel probe was used to select different areas of both atria. When a regional deflection consistent with a DAD was observed, its amplitude was quantified as a percentage of the atrial action potential amplitude during pacing. DAD amplitudes of at least 10% were considered for analysis. Quantification of DADs was done by dividing the number of DADs observed overall and the number of pacing trains delivered to the heart. DADs were analysed during the pause following the last pacing stimulus.

For CV measurements, the right atrium was paced from an epicardial stimulation site at the anterior aspect of the right atrial appendage for at least 90 seconds at constant cycle lengths prior to the optical recordings. The activation time and therefore the isochrones were defined as the time of maximum upstroke velocity of the filtered, averaged and inverted fluorescence signal relative to the time of the stimulus. Activation maps were generated and CV calculated as described by us⁴. Due to the small measurement area, only the maximum CV was considered for each heart. Optical action potential duration (APDs) were computed from inverted fluorescence data corresponding to a 3x3 pixel window. The window was located at the centre of the RA or LA appendage. APDs were calculated at 90% repolarization levels.

**Ca transient measurements in intact hearts**

The incidence of spontaneous diastolic Ca elevations in the atria of perfused hearts was investigated by generating Ca fluorescent maps. Isolated perfused hearts were stained with a dye solution made by sonicating for 10 minutes 50 μg Rhod-2AM dissolved in 22.5μl DMSO, 7.5μl 20% pluronic and 500μl bicarbonate buffer. The dye was recirculated through the hearts for 15 minutes with an external rolling pump. An additional 10-15 min of buffer perfusion were used to wash out the extracellular dye.

Hearts were subjected to trains of S1 atrial burst pacing 50Hz for 2 s; maps were acquired during the post pacing pause. All maps were analysed by one operator in blinded fashion with MATLAB (Mathworks, Natick, MA) using custom algorithms. Recordings were initially processed with a temporal 3-frame moving average filter. Then, a 5x5 pixel window covering only a small portion of the atria was used to probe different areas to identify regional
spontaneous Ca elevations (SCaE) in the fluorescence trace. The number of SCaE was determined as the ratio between SCaE episodes recorded and number of burst pacing trains delivered. The amplitude of every SCaE recorded was quantified as a percentage of the Ca transient amplitude during pacing. Only elevations of at least 10% of the preceding atrial Ca transient during pacing were considered for the analysis. As described for the DADs quantification, SCaE were observed during the pause following the last pacing stimulus. To test the effect of class I antiarrhythmic drugs on SCaE and AF, Casq2-/- isolated perfused hearts stained with Rhod2 AM underwent the pacing protocol twice: first during vehicle infusion and then in the presence of R-Propafenone (3µM), S-Propafenone (3µM) or Lidocaine (20 µM), respectively. The lidocaine concentration was chosen to achieve a similar degree of Na channel block as 3 µM of R- or S-Propafenone, as evidenced by a comparable increase in the QRS interval (around 25%). Drugs were continuously infused for 15 min before pacing was resumed. AF episodes as well as number and amplitude of SCaE after R-Propafenone, S-propafenone or Lidocaine infusion were quantified and compared to the same parameters obtained with vehicle.

References
2 Galimberti ES, Knollmann BC. Efficacy and potency of class I antiarrhythmic drugs for suppression of Ca2+ waves in permeabilized myocytes lacking calsequestrin. J Mol Cell Cardiol Nov 2011;51:760-768.