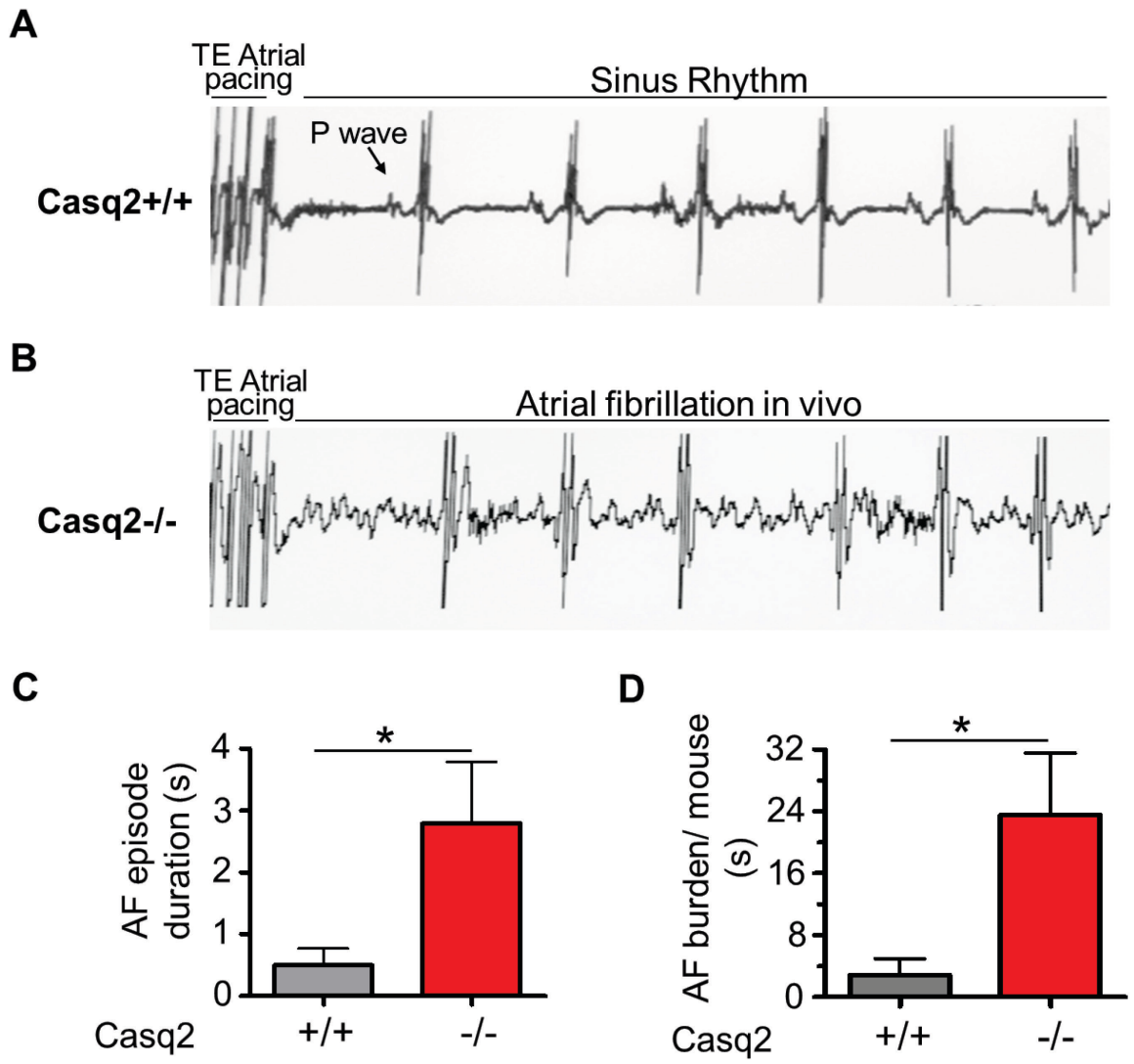
SUPPLEMENTAL MATERIAL

Supplemental Figure 1



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-aminoethylesther)-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.[2] These LS were analyzed as previously described.[2]

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 analysing 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 catecholammines 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

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 us[4]. 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 where 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

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