Online Appendix

Methods

Canine Model of Pacing-Induced AF

All animal study protocols were approved by the Institutional Animal Care and Use Committee of Indiana University School of Medicine and the Methodist Research Institute. The pacing-induced AF was produced in 7 adult mongrel dogs weighing 25 to 30 kg with the use of previously described techniques.[1] In short, after anesthesia with isoflurane (1.5% to 2%), active fixation leads were implanted in the RA appendage and right ventricular septum through the external jugular vein. Atrial and ventricular leads were connected to an EnPulse DR pacemaker (Medtronic, Inc., MN, USA) and an Identity XL DR pacemaker (St. Jude Medical, Inc., MN, USA), respectively. Both pacemakers were implanted in a submuscular pocket in the right lateral part of the neck. The ventricular pacemaker was programmed at a rate of 80-100 bpm. Complete atrioventricular block was produced with catheter radiofrequency ablation. After 1 week of recovery, intermittent atrial pacing was instituted at a rate of 640 bpm from the modified EnPulse DR pacemaker and maintained for more than 6 weeks to induce both paroxysmal and persistent (>24 hr) AF (Figure 1). Atrial EnPulse DR pacemaker was programmed for high rate atrial pacing with a modified Medtronic CareLink® Programmer (Medtronic, Inc., MN, USA), and was interrogated to confirm the generation of AF during every monitoring period.

Ambulatory Holter recordings were obtained for 48 hours in dogs numbered 3 through 7. All recordings were made with two-channel tape recorders (Marquette Electronics, WI, USA) and two bipolar leads. The data were manually analyzed. Afterwards the dogs were

continuously paced at an atrial rate of 640 bpm for at least an additional 16 days (mean 21 ± 4 days, range 16-26 days) before sacrifice.

Langendorff-Perfused Canine Isolated RA Preparation

We studied isolated RA from 7 dogs with pacing-induced AF. The results were compared with that of 19 normal dogs. The data of 11 of the 19 normal dogs were included in a manuscript published elsewhere.[2] These studies (normal and AF dogs) were performed over 6 months during which an average 1-2 AF dogs were available per month. The heart was rapidly excised under general anesthesia and the right coronary artery was perfused with 37°C Tyrode's solution equilibrated with 95% O_2 and 5% CO_2 to maintain a pH of 7.4. The composition of Tyrode's solution was (in mmol/L): 125 NaCl, 4.5 KCl, 0.25 $MgCl_2$, 24 $NaHCO_3$, 1.8 NaH_2PO_4, 1.8 $CaCl_2$, and 5.5 glucose). The coronary perfusion pressure was regulated between 50 mmHg and 60 mmHg. To ensure adequate atrial perfusion, all ventricular coronary branches were tied off. Both ventricles and left atrium were removed. During optical recordings, contractility was inhibited by 10-17 µmol/L of blebbistatin, and the motion artifact was negligible even after isoproterenol infusion.[2] Pseudo-ECG was recorded with widely spaced bipolar RA electrodes using ISO-DAM8A (World precision instruments, FL, USA).

Assessment of SAN Function

SNRT was determined by bipolar pacing with a programmable stimulator (Bloom Associates, Ltd., Reading, PA, USA) for 30 seconds at progressively shorter pacing cycle lengths (400, 350, and 300 ms) with the two electrodes placed near the SAN. The longest time interval from the last paced atrial depolarization to the first spontaneous sinus beat was recorded as the SNRT. The corrected SNRT (cSNRT) was determined by subtracting an average of 3 sinus cycle lengths prior to the commencement of atrial pacing from the SNRT.

The SAN conduction time (SACT) was measured by the method described by Narula: the SACT = (return cycle length - basic cycle length)/2.[3]

Dual V_m and Ca_i Recordings

The simultaneous V_m and Ca_i mapping was performed with previously described techniques.[2] Briefly, the RAs were stained with Rhod-2 AM and RH237 (Molecular Probes) and excited with laser light. Fluorescence was collected using 2 cameras (MiCAM Ultima, BrainVision, Tokyo, Japan) at 1 ms/frame and 100 X 100 pixels with spatial resolution of 0.35 x 0.35 mm^2/pixel. After dual V_m and Ca_i optical mapping of baseline spontaneous heart beats, pharmacologic intervention was performed. These interventions included isoproterenol infusion of 1 µmol/L in all RAs, and a bolus injection of 2 ml caffeine (20 mmol/L) in 7 normal and 5 AF dog RAs. The heart rate response to isoproterenol (0.01, 0.03, 0.1, 0.3, 1, 3, and 10 µmol/L) was tested in 7 normal and all 7 AF RAs. SNRT and corrected SNRT (cSNRT) were measured in RAs from 15 normal and all 7 AF dogs.

Assay of Ca^{2+} Handling Proteins

To evaluate the expression of type 2 ryanodine receptor (RyR2) and sarcoplasmic reticulum Ca^{2+}-ATPase 2a (SERCA2a), 70 µg of membrane homogenates of superior SAN of normal and AF dogs were loaded on a 5% polyacrylamide SDS-PAGE and transferred to a nitrocellulose membrane. The SAN was identified both by the anatomical location (near upper end of the crista terminalis) and by the presence of diastolic depolarization on the Vm signals. The tissues were then stained with Masson's Trichrome and HCN4. The slides were examined with light microscopy to confirm the correct identification of the SAN. The superior SAN was defined as the superior third of the SAN. That area consistently showed LDCAE during isoproterenol infusion in all 19 normal SAN studied.[2] For protein analyses, we removed the

endocardial half of the SAN tissue and used the remaining tissue for analyses. The amount of RyR2 and SERCA2a proteins on the blot was determined by signals of the anti-RyR monoclonal antibody, 2C11, and the anti-SERCA2a monoclonal antibody, 2A7A1, respectively. To evaluate the expression level of SERCA2a and phospholamban, 10 μg and 20 μg of membrane homogenates were used on an 8% polyacrylamide SDS-PAGE and transferred to an immobilon. The amount of phospholamban proteins on the blot was determined by signals of the anti-phospholamban monoclonal antibody, 1F1.[4] Antibody-binding protein bands are visualized by ^{125}I-protein A and quantified with a Bio-Rad Personal Fx phosphorimager. All results represent at least 3 experiments for 4 normal and 3 AF dogs.

Data analysis

The Ca_i and V_m traces were normalized to their respective peak-to-peak amplitude for comparison of timing and morphology. The slopes of LDCAE and 90% Ca_i relaxation time were measured by the previously described method.[2] The onset of LDCAE was defined by the time of the transition between negative to positive values in dCa_i/dt curve. The slope of Ca_i upstroke was measured according to Ca_i tracing from the onset (phase 0) of V_m to the time when maximum Ca_i was reached. The "90% Ca_i relaxation time" was measured from the maximum systolic Ca_i to 90% reduction of Ca_i. The isochronal map of the RA was generated by the traditional criteria for detecting activation times (50% of the optical AP amplitude). We applied a 3x3x3 spatiotemporal filter before generation of the isochronal map. The method averages 3 pixels in X-axis, 3 pixels in Y axis over 3 frames of time.

Data were expressed as the mean ± SEM. Student's *t* tests with Bonferroni's correction were used to compare the means of two numeric values. The Pearson's chi-square tests were used to compare two categorical variables. The repeated-measure ANOVA model was used to

compare isoproterenol-induced heart rate response between AF and control dogs. A *p* value of < 0.05 was considered significant.

References

1. Elvan A, Wylie K, Zipes DP. Pacing-induced chronic atrial fibrillation impairs sinus node function in dogs--electrophysiological remodeling. *Circulation*. 1996;94:2953-2960.
2. Joung B, Tang L, Maruyama M et al. Intracellular calcium dynamics and the acceleration of sinus rhythm by {beta}-adrenergic stimulation. *Circulation*. 2009;119:788-796.
3. Narula OS, Shantha N, Vasquez M et al. A new method for measurement of sinoatrial conduction time. *Circulation*. 1978;58:706-714.
4. Chen Z, Akin BL, Jones LR. Mechanism of reversal of phospholamban inhibition of the cardiac Ca2+-ATPase by protein kinase A and by anti-phospholamban monoclonal antibody 2D12. *J Biol Chem*. 2007;282:20968-20976.

Supplementary Figures

Figure S1. A, The SNRT (left panel) and cSNRT (right panel) of normal (n=15, square) and AF isolated RA preparations (n=7, circle). After termination of AF, the RAs from AF dogs had longer SNRT and cSNRT than those from normal dogs at the all pacing cycle length. B, The action potential duration measured to 90% repolarization (APD_{90}) from the isolated RA preparations of normal and AF dogs. The APD_{90} in AF dogs were significantly shorter than normal dogs at all pacing cycle lengths.

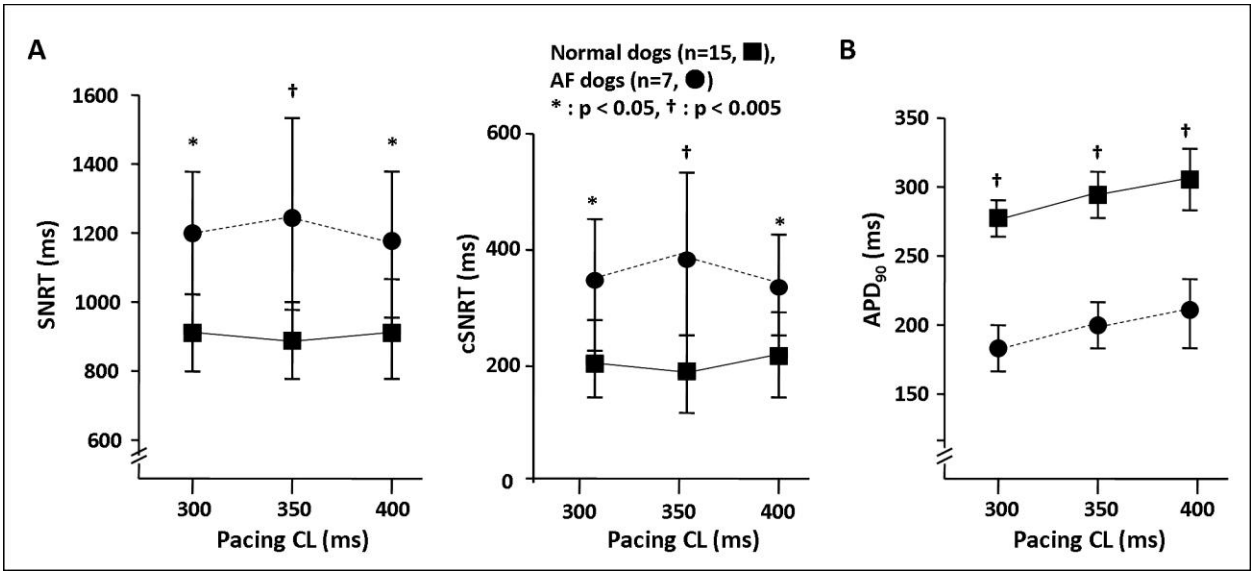

Figure S2. The LDCAE (upper panels), slope of Ca_i upstroke (middle panels) and 90% Ca_i relaxation time (lower panels) measured at middle SAN (A), inferior SAN (B) and RA (C) of intact RA preparations from normal (n=15, square) and AF (n=7, circle) dogs. The response after ISO infusion was grouped according to the heart rate.