SUPPLEMENTAL MATERIAL

Title: Complex interactions between the sinoatrial node and atrium during reentrant arrhythmias in the canine heart.

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SUPPLEMENTAL METHODS

This protocol was approved by the Washington University Animal Care and Use Committee. The isolated, perfused canine right atrium(1) as well as the experimental techniques(2) have been previously described in detail.

In vitro optical mapping studies

Optical mapping studies were conducted on isolated, coronary-perfused preparations of the canine right atrium.(1,3,4) Normal, healthy, young (10-12 month old) mongrel dogs (n=17) weighing between 18 and 25 kg, without any history of disease were anesthetized (IV 7.5 mg/kg propofol), intubated, and placed on a positive pressure respirator with 2% to 3% isoflurane for anesthesia throughout the procedure. A median sternotomy was performed. The heart was cradled in the pericardium and the azygous vein was ligated and divided. The intra-atrial groove was dissected, separating the left (LA) and right atria (RA). The RA was dissected from the rest of the heart and divided through the superior vena cava (SVC) down to the inferior vena cava (IVC). The right coronary artery was cannulated with a 16-gauge catheter. The RA preparations were positioned in a temperature-controlled glass chamber with the epicardium (Figure 1) or endocardium facing the optical apparatus (Online Figure 1). Two bipolar pacing and recording electrodes were placed on the RA free wall and intra-atrial septum (IAS) regions. The RA preparations were superfused (50 mL/min) and coronarily perfused under a constant pressure of 55±5 mmHg with oxygenated (95% O₂-5% CO₂) modified Tyrode’s solution containing (in mM): 128.2 NaCl, 1.3 CaCl₂, 4.7 KCl, 1.05 MgCl₂, 1.19 NaH₂PO₄, 25 NaHCO₃, and 11 glucose. Temperature and pH were continuously maintained at 36±0.5°C and 7.35±0.05, respectively.
Preparations were equilibrated during normal sinus rhythm in the tissue chamber for 60-90 min before the measurements. During this time, the preparations were stained with voltage-sensitive dye, di-4-ANNEPPS or RH237, and Blebbistatin (10-20 µM) was used to suppress motion artifacts in the optical signals caused by muscle contraction.¹ The SAN preparations were restained with the dye during the experiment as needed. No measurements were performed until 5 min after the restaining procedure. Stability of the preparation was periodically verified by measuring sinus cycle length.

1) In the preliminary experiments, a 16×16 photodiode array (Hamamatsu, Japan) with a spatial resolution of 2.25 mm at a rate of 1,500 frames/s recorded optical fluorescent signals from an optical field of view 36×36 mm² (OFV) at the epicardium, endocardium (Online Figure 1), or both (n=5). The OFVs contained part of the SVC, crista terminalis (CT) and IAS regions.

2) During the main experimental series (n=12), a 100x100 Ultima-L CMOS camera (SciMedia, Japan) with a spatial resolution of 300-400 µm/pixel at a rate of 1,000 frames recorded fluorescent signals from the epicardial OFV ranging in size from 30x30 to 40x40 mm² (Figure 1).

**Experimental protocol**

Programmed atrial stimulation was used to measure atrial conduction properties and SAN recovery time at different cycle lengths (CL) from 350 ms to 175 ms. S1S2 protocol (Online Figure 2) or progressive overdrive atrial pacing up to CL=90 was used to induce atrial fibrillation (AF)/atrial flutter (AFL) (Figure 3) before and after perfusion with acetylcholine (ACh) or isoproterenol (Iso).

In the preliminary series of experiments (n=5), we wanted to induce sustained AF and AFL during perfusion with Acetylcholine (ACh, 0.1 - 10 µM) without completely
depressing the SAN as previously described for this model. Depending on the individual preparation, 0.3-3 µM ACh could promote sustained AF/AFl without completely depressing SAN intrinsic pacemaker activity. Thus, for the remaining experiments (n=12), we used ACh concentrations in this range. To block ACh effects, 3 µM Atropine was used in three preparations (Online Figure 5D). In 5 out of 12 preparations, we also perfused with Isoproterenol (Iso, 0.2-1 µM) to investigate the effects of beta-adrenergic stimulation on the SAN activity during spontaneous rhythm or AF/AFL.

**Optical mapping data analysis and interpretation**

The SAN tissue was electrically isolated from the atrial myocardium except through the sinoatrial exit pathways (SACPs). Based on our previous study, the SAN conduction pathways were defined as areas of preferential conduction between SAN and atrial myocardium which correspond to narrow muscular bundles containing both Connexin 43 positive and negative transitional cells. These bundles spread from the SAN into larger muscular bundles found in the atria, such as the CT. SACPs were located between the superior and inferior borders of the SAN and the atria and served as conduction bridges between these two structures.

There were two separate activation patterns: SAN excitation from the leading pacemaker, and atrial excitation from the exit pathways. While both activations were electrically separated, the SAN and atrial excitations were related to each other. This study defined the leading pacemaker as the earliest activation in the SAN region (by the SAN OAP component) and “atrial breakthrough” as the earliest activation in the atria (by the atrial OAP component). Sinoatrial conduction time (SACT) was defined as the time between the earliest excitation in the SAN and the atrial breakthrough.
A custom Matlab computer program was used to analyze the optical signals in the SAN and the atria as previously described.\(^7\) Optical recordings contained signatures of cardiac excitation to a depth of 1-3 mm.\(^8\text{--}^{11}\) Therefore, our optical recordings contained signals from both the SAN and atrial tissue, which we separated as described in our recent study of the canine SAN.\(^7\)

Using this method, we located the conduction block area around the SAN. This area cannot be detected using surface electrogram recordings.\(^12\) The separation of these components allowed for the measurement of the sequential SAN and atrial activation patterns, and conduction velocity in individual tissue layers (Figure 1).

Activation times and corresponding conduction velocities were defined in the SAN layer at 50\% of the SAN OAP component (AP50\%).\(^2\text{--}^7\text{,}^{13}\) Atrial activation times and patterns were defined by traditional \(\frac{dF}{dt}\)\(_{\text{max}}\).

To estimate the changes in action potential duration at 80\% of repolarization APD (APD80) over the atria and SAN, we selected several regions corresponding to notable anatomic features (Figure 1) and computed summary statistics for the APDs within these regions. The three regions used were: 1) the right atrial free wall, the right atrial tissue anterior to the sulcus terminalis, which contained the trabeculated part of the right atrial wall; 2) the IAS, the right atrial tissue between septal border and SAN block zone; 3) SAN, the central part of the SAN.

Optical signals were filtered using low-pass Butterworth filters (10-200 Hz). During normal sinus rhythm, we distinguished the preceding SAN component of the optical signals from the consequent atrial upstroke (Figures 1 and 2). Figure 3 shows that slow atrial pacing paced the SAN 1:1 (S1S1 = 350 msec); but faster atrial pacing (120 ms – 200 ms) may cause up to even 4:1 Wenckebach block-like interaction between the atria and the SAN. Since SAN excitation came after the atrial component
reconstruction of the SAN activation during atrial pacing required a different algorithm.\(^{(14)}\)

Moreover, during fast atrial pacing and atrial reentrant arrhythmias, the atrial activation frequency was much higher (6 Hz – 32 Hz) than the SAN's (0.5 Hz – 3.5 Hz). This significant difference in frequencies and amplitude masked the SAN component. To measure the SAN activation during pacing and atrial reentrant arrhythmias, we used low frequency filters ranging from 10-20 Hz, separating the slow upstroke SAN signals from the fast atria. Online Figure 3 shows the success of the filtering process in separating low-frequency SAN signals (dark blue) from the high frequency atrial signals (green) during AF/AFI.

We also used Fourier transform analyses to produce the Dominant Frequency (DF) maps. Using the custom Matlab program, we analyzed the power spectrum for each recording site and labeled the frequency with the largest amplitude as the dominant frequency, similar to past studies.\(^{(15)}\) These individual DF values from each recording site were then compiled to create DF maps of the entire OFV. Figure 3 shows the DF maps with the 16 Hz low-pass filtering during atrial pacing CL. Using this DF analysis, we were able to associate the region of low DF as the SAN anatomical region. Online Figure 4 shows that decreasing the low–pass frequency from 200Hz to 16 Hz improved the quality of the SAN optical recordings and DF maps.

**SANRT**

Sinus node recovery time (SANRT) was used as a measure of the SAN function before and after perfusion with ACh and Iso. SANRT was defined as the delay between the last paced beat and the first spontaneous beat (Online Figure 5). Direct SANRT, SANRT\(_d\), was measured from the last paced atrial beat to a 50% rise in the SAN optical upstroke. The rate corrected SANRT was then computed as the difference between the
measured SANRTd and the recorded sinus rhythm taken before pacing began. Indirect SANRT, SANRTi, was computed as the difference between the last paced atrial beat and a 50% rise in the first atrial optical upstroke (Online Figure 5B).

**Histological examination and anatomic correlation**

Histology was performed as previously described.\(^2\) After optical mapping experiments, canine SAN preparations (n=8) were perfused with 3.7% formaldehyde for 5 minutes and left in solution overnight. The SAN preparations were then transferred to 20% sucrose for two days before the tissue was frozen. SAN preparations were embedded in Tissue-Tek OCT compound (Histo Prep; Fisher Scientific, Fairlawn, NJ, USA), frozen in isopentane, cryosectioned parallel (n=4) or perpendicular (n=4) to the epicardium, and stored at -80°C until staining and imaging was performed.

**Online Figure 2** shows that slow conduction areas, conduction block, and the core of the re-entrant wave front were correlated with the macroscopic anatomic and histological findings of the SAN structure.

**Statistics**

Statistical analysis was done using SAS 9.2 (Cary, NC). Mixed linear models included treatment as fixed effect and sample as random effect. Least squares estimates of the ACh and Iso treatments were compared to control with Dunnett’s adjustment. For SCL and SACT variables, each treatment had its own control, thus differences between treatments and controls were used as responses. Mixed linear models were modified to compare these differences to zero and included differences as fixed effect and sample as random effect. For comparison of AF and AF/AFL groups, a paired t-test was used.
Study Limitations

We used denervated preparations from young mongrel dogs without any structural diseases. SAN dysfunctions and AF/AFL were very often caused by structural diseases. It is possible that the suppression of mechanical contractions by Blebbistatin prevented the activation of stretch-activated channels, which could play a role in the pacemaker activity of the SAN\(^{16}\) as well as in the cholinergic AF/AFL mechanism.\(^{17}\) The optical recordings conducted from the epicardium or endocardium surface were likely to carry contributions from both the atrial and SAN intramural layers and represented a weighted average of the transmembrane recordings throughout the canine atrial wall.\(^{7}\) However, the ratio of the atria input amplitude to the SAN signals could be unpredictable due to the variability in nodal anatomy and locations of the leading pacemakers. Thus, it was still not possible to clearly distinguish conduction in the SACPs due to their size and intramural nature.\(^{7}\)
### SUPPLEMENTAL TABLES

**Online Table 1. Effects of Acetylcholine and Isoproterenol on the main electrophysiological parameters of canine right atria (n=12)**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>( \text{ACh} )</th>
<th>( \text{Iso} )</th>
<th>( p)-value (( \text{ACh} ) vs. ( \text{Control} ))</th>
<th>( p)-value (( \text{Iso} ) vs. ( \text{Control} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCL (ms)</td>
<td>519 ± 21.9</td>
<td>841.4 ± 88.7</td>
<td>371.17 ± 70.5</td>
<td>0.035</td>
<td>0.027</td>
</tr>
<tr>
<td>SACT (ms)</td>
<td>54.0 ± 20.3</td>
<td>123.2 ± 40.2</td>
<td>36.7 ± 17.8</td>
<td>0.0036</td>
<td>0.023</td>
</tr>
<tr>
<td>SANRT (ms)</td>
<td>628.0 ± 56.6</td>
<td>981.5 ± 181.6</td>
<td>439.0 ± 96.0</td>
<td>0.031</td>
<td>0.031</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>( \text{ACh} )</th>
<th>( \text{Iso} )</th>
<th>( p)-value (( \text{ACh} ) vs. ( \text{Control} ))</th>
<th>( p)-value (( \text{Iso} ) vs. ( \text{Control} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>IAS (ms)</td>
<td>166.9 ± 11.3</td>
<td>95.31 ± 25.3</td>
<td>108.9 ± 32.8</td>
<td>&lt;0.001</td>
<td>0.0015</td>
</tr>
<tr>
<td>RAFW (ms)</td>
<td>163.4 ± 14.4</td>
<td>90.6 ± 21.1</td>
<td>110.3 ± 32.8</td>
<td>&lt;0.001</td>
<td>0.0006</td>
</tr>
<tr>
<td>SAN (ms)</td>
<td>204.1 ± 21</td>
<td>207.7 ± 20.2</td>
<td>170.3 ± 17.4</td>
<td>0.953</td>
<td>0.019</td>
</tr>
</tbody>
</table>

Sinus cycle length (SCL), sinoatrial node conduction time (SACT), sinoatrial node recovery time (SANRT), right atrial free wall (RAFW), intraatrial septum (IAS), and sinoatrial node (SAN). APD80% - action potential duration at 80% repolarization.

**Online Table 2. SAN and atrial frequency during AFL and AF.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Maximal frequency (Hz)</th>
<th>( \text{AF/AFI} ) (( \text{ACh} )) (n=47)</th>
<th>( \text{AF (ACh and Iso)} ) (n=6)</th>
<th>( \text{AF/ AFL washout from ACh} ) (n=10)</th>
<th>AFL (Iso) (n = 5)</th>
<th>AFL (Control) (n = 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAN (Hz)</td>
<td>1.17 ± 0.21</td>
<td>1.64±0.22*</td>
<td>1.63 ± 0.33*</td>
<td>2.42± 0.36</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>Atrial around SAN (Hz)</td>
<td>11.26 ± 5.19</td>
<td>11.44 ± 1.5</td>
<td>9.28 ± 3.72</td>
<td>9.98 ± 0.87</td>
<td>6.8</td>
<td></td>
</tr>
<tr>
<td>DF (Hz)</td>
<td>22.32 ± 5.86</td>
<td>21.38 ± 2.49</td>
<td>17.8± 7.27</td>
<td>9.98 ± 0.87</td>
<td>6.8</td>
<td></td>
</tr>
<tr>
<td>% Spontaneous activation of SAN</td>
<td>48.6 ± 38.9</td>
<td>62.3 ± 24.8</td>
<td>39.5± 34.6</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
</tbody>
</table>

Atrial fibrillation (AF), atrial flutter (AFL), Acetylcholine (ACh), Isoproterenol (Iso). DF –dominant frequency for AF cases.

* p<0.01 vs AF/AFL (ACh) group (paired t-test)
SUPPLEMENTAL MATERIAL

Figure 1_Online
A. Sinus Rhythm (2uM ACh)

LPF 200Hz

SCL = 792ms

B. AFL

LPF 10Hz

AFLCL = 72 ms
SCL = 804ms
AFL/SCL = 11:1

LPF 200Hz

SAN APs

C. AF

LPF 20Hz

AFCL = 44 ms
SCL = 812ms
AF/SCL = 18:1

LPF 200Hz

SAN APs

Atrial APs
Figure 4 online

S1S1 = 200 ms

LPF 200Hz

LPF 64Hz

LPF 16 Hz
Figure 5-Online

A. Control, SCL=579ms

Pacing CL=300ms  SANRT=660ms

B. 0.3μM ACh, SCL=806ms

Pacing CL=300ms  SANRT_T= 989ms  SANRT_I=3027ms

C. 1 μM ACh, SCL=961ms, Full exit block

0 ms  1000  2000  3000  4000  5000  6000  7000  8000

D. 3μM Atropine, SCL=560ms

Pacing CL=300ms  SANRT=633ms
ONLINE FIGURE LEGENDS

Online Figure 1 - Fluorescent optical mapping of the right atrial endocardium during ACh-induced AFl and AF in the canine isolated atria.
Panel A - Photo of the preparation with an epicardial optical field of view (OFV).
Panels B and C - Atrial activation maps and optical action potentials (OAP) during AFL and AF, which were induced by atrial burst pacing and ACh 2 µM.

Online Figure 2 - Fluorescent optical mapping of the canine right atrial epicardium during sinus rhythm and pacing-induced reentry.
Panel A - Photo of the preparation with an epicardial optical field of view (OFV).
Panel B - Parallel histology section close to the epicardial surface corresponding to the dotted rectangle on panel A. This panel shows a single 2D histology section through different layers of tissue, which also includes the SAN (pink oval) and coronary arteries (light blue arrows).
Panels C and D - Optical action potentials from the recording sites 1-5 in Panel A and activation maps during sinus rhythm and atrial pacing S1-S2=300ms-160ms induced reentry. Abbreviations are the same as in Figure 1.

Online Figure 3 - The low-pass filtering (LPF) used to unmask SAN optical signals during atrial arrhythmias.
Panel A – Endocardial optical recording from the central part of SAN shows the double component upstroke (SAN and Atrial) of the OAPs during sinus rhythm (ACh 2 µM).
Panel B – The optical recording from the same location in Panel A during atrial flutter (AFL) used the high (200Hz - green) and low frequency (10 Hz - dark blue) filters to unmask the SAN signal.
Panel C - The optical recording from the same location in Panel A and B during atrial fibrillation (AF) used the high (200Hz - green) and low frequency (20 Hz - dark blue) filters to unmask the SAN signal.
Online Figure 4 - The low-pass filtering (LPF) unmasks SAN signals and improves Dominant Frequency maps.
The left panels show DF maps of OAPs recorded during atrial pacing CL at 200 ms (same preparation as Figure 1). The optical signals were filtered with three different filtering frequencies 200Hz, 64 Hz and 16 Hz. The right panels show OAPs recordings and their Frequency Power spectrums from the SAN center (1-Blue) and right atria free wall (2-Red).

Online Figure 5 - SAN recovery time in control and after ACh and Atropine.
Panels A and B - Examples of the SANRTd and SANRTi measurements in control and after ACh, respectively. Both panels show two optical recordings from the center of the SAN (blue) and the crista terminalis CT (green). Panel B represents an example of the pacing-induced transient SAN exit block.
Panel C - An example of the full SAN exit block during 1 µM ACh perfusion.
Panel D – SAN recovery after 3 µM Atropine.
ONLINE MOVIE LEGEND

Online Movie 1. AFL Suppresses SAN
The color map movies show the spatial and temporal changes in amplitude of normalized OAP (upper left), and their derivative (upper right) during AFL, respectively. The bottom panel shows two OAP recordings (blue – SAN center, green – Crista terminalis) from the corresponding color point in both color map movies. This movie corresponds to Figure 6A. It shows sustained AFL and its termination by atrial pacing (CL = 145 ms), and the corresponding recovery of the first normal sinus beat.

Online Movie 2. SAN activation during ACh-induced AFL/AF
The color map movies show the spatial and temporal changes in amplitude of normalized OAP (upper left), and their derivative (upper right) during ACh-induced AFL/AF, respectively. The bottom panel shows two OAP recordings (blue- SAN center, green – Intratrial septum) from the corresponding color point in both color map movies. The movie shows distinguishable coronary arteries surrounding the SAN. It shows conversions from AFL to AF due to SAN intrinsic activity at approximately Time=2.5 sec. This movie corresponds to Figures 6C and D.
Reference List


