Autonomic and surgical substrate modulation of atrial fibrillation
Krul, Sébastien

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Chapter 5

Acetylcholine induced conduction block in atrial tissue of patients with atrial fibrillation

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Submitted
ABSTRACT

Introduction
Vagal stimulation facilitates the onset and maintenance of atrial fibrillation (AF). Besides repolarization changes, vagal stimulation may affect atrial conduction. We investigated the effects of acetylcholine (ACH) on conduction properties in the left atrial appendage (LAA) from AF patients.

Methods
Activation maps (1cm²) were constructed using optical mapping. Preparations were epicardially stimulated at 600ms basic cycle length. Effective refractory period (ERP) was determined with short-coupled stimuli. Eight LAAs were superfused with 100uM ACH and six without ACH (control). Mean activation time (AT) of an area of 10x11 pixels was determined. Changes in normalized AT, local dispersion (LD), longitudinal and transverse conduction velocity (CL, CV) were calculated to determine differences between groups. The number and length of lines of conduction block were quantified.

Results
At short coupled extra stimuli, ACH increased the number of lines of block from 0.6±0.7 to 2.3±1.4 (p=0.016) while no changes occurred in the control group (1.2±1.2 vs 1.5±1.0, p=NS). Length of lines of activation block increased after ACH from short/intermediate ±<2mm to long ±>2mm (p=0.004), but not in control, short/intermediate ±<2mm in both series (p=NS). At basic cycle length, mean normalized AT, LD, CVL and CVT were similar between ACH and control groups (p=NS). However, ACH significantly increased LD at ERP (p=0.028).

Conclusions
ACH application increased the number and area of lines of conduction block in human atrial tissue. ACH increased dispersion of conduction times, especially at short coupled premature activations. ACH thus facilitates conduction block and thus the occurrence of reentry.
INTRODUCTION

The parasympathetic nervous system modulates the electrophysiology of atrial and pulmonary vein myocardium and may facilitate the onset and maintenance of atrial fibrillation (AF).\(^1,2\) This effect is mainly attributed to the effect of acetylcholine, which shortens atrial action potential duration, and thus promotes the occurrence of reentry\(^3\). However, in clinical studies the parasympathetic nervous system appears not only to shorten repolarization, but also to slow conduction.\(^4,5\) The acetylcholine dependent repolarizing K\(^+\) current may indeed interfere with the upstroke of the atrial action potential.\(^6\) During AF, areas of slow discontinuous conduction can be identified by fractionated electrograms, the size of which seems to be influenced by local parasympathetic stimulation.\(^7,8\) In fibrotic myocardium a small reduction in excitability likely results more easily in conduction block than in normal myocardium.\(^7\) We hypothesize that the effects of the parasympathetic system may therefore involve conduction properties, especially in remodeled human atria.\(^4\) A conduction dependent mechanism may impact pharmacological treatment in patients with vagal induced AF. In this study we therefore investigated the effects of acetylcholine on conduction properties in the excised left atrial appendage (LAA) from patients with AF.

METHODS

Left atrial tissue

Left LAAs were excised during thoracoscopic surgery from patients with AF.\(^9\) The amputated LAA tissue was immersed in cooled modified Tyrode’s solution and transported to an optical mapping setup, as described earlier.\(^10\) The patient characteristics are shown in Table 1. The study was in accordance with the declaration of Helsinki and approved by the institutional review board. All patients gave written informed consent.

Data acquisition

Left atrial tissue was equilibrated for at least thirty minutes in a tissue bath with a temperature of 36.5°C-37.5°C.\(^10\) A MiCAM Ultima camera (SciMedia USA Ltd, Costa Mesa, CA, USA) was used to record epicardial images of an area of 1cm\(^2\) with a resolution of 100x100 pixels and a sample time of 0.5 ms. Di-4-ANEPPS (Tebu Bio, Le-Perray-en-Yvelines, France) was used as a membrane potential-sensitive fluorescent dye. Motion artefacts prohibited recording of fluorescent action potentials in 2 LAAs and in those a contraction uncoupler 2-10 mM 2-3-butanedione monoxime (DAM, Sigma-Aldrich, B0753) was added. A custom-made analysis program based on MATLAB R2006b (The MathWorks, Inc, Natick, Massachusetts, USA) was used to construct epicardial activation maps.\(^11\)
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Table 1. – Patient Characteristics

<table>
<thead>
<tr>
<th></th>
<th>All Patients (n=14)</th>
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</thead>
<tbody>
<tr>
<td>Age, mean ± SD (range), years</td>
<td>60±10 (43-78)</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>9 (64)</td>
</tr>
<tr>
<td>Years of AF, mean ± SD (range), years</td>
<td>4±3 (1-11)</td>
</tr>
<tr>
<td>Type AF</td>
<td></td>
</tr>
<tr>
<td>Paroxysmal, n (%)</td>
<td>5 (36)</td>
</tr>
<tr>
<td>Persistent, n (%)</td>
<td>9 (64)</td>
</tr>
<tr>
<td>Previous PVI, n (%)</td>
<td>3 (21)</td>
</tr>
<tr>
<td>CHADSVASc, median, range</td>
<td></td>
</tr>
<tr>
<td>0-1</td>
<td>1 (0-7)</td>
</tr>
<tr>
<td>&gt;2</td>
<td>8 (57)</td>
</tr>
<tr>
<td>Left atrial size</td>
<td></td>
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<tr>
<td>Atrial volume index, mean ± SD (range), m²/ml</td>
<td>41±10 (26-58)</td>
</tr>
</tbody>
</table>

Anti-arrhythmic medication

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<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>Flecainide, n (%)</td>
<td>7 (50)</td>
</tr>
<tr>
<td>Beta-blocker, n (%)</td>
<td>10 (71)</td>
</tr>
<tr>
<td>Sotalol, n (%)</td>
<td>2 (14)</td>
</tr>
<tr>
<td>Amiodarone, n (%)</td>
<td>2 (14)</td>
</tr>
<tr>
<td>Verapamil, n (%)</td>
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</tr>
</tbody>
</table>


Stimulation protocol

Left atrial tissue was stimulated with a basic cycle length of 600 ms at twice diastolic threshold and a pulse width of 2 ms using an bipolar epicardial electrode. Premature stimuli were applied with 10 ms decremental coupling intervals, starting at 400 ms down to the shortest S1-S2 interval resulting in capture. Capture of premature stimuli was determined by the presence of a local bipolar electrogram recorded from another epicardial recording electrode. The shortest S1-S2 interval that resulted in capture defined the effective refractory period (ERP). After baseline recordings at which conduction parameters and the ERP were determined (first experiment series) to provide reference values of the LAA, 10 minutes stimulation at basic cycle length was performed. Thereafter, in the second experiment series the same parameters were determined either without (group I, n=6) or with acetylcholine (A2261, Sigma), in a concentration of 100 uM (group II, N=8) added to the superfusate (Figure 1). A concentration of 100uM was used to reflect earlier studies that applied acetylcholine to the atrium.12,13
Conduction parameters

Activation times were determined from the time of \( \frac{df}{dt_{\text{max}}} \) of the optical action potential at each pixel and activation maps were constructed with 5x5 pixel spatial temporal averaging. Longitudinal conduction velocity (CV\(_L\)) and transverse CV (CV\(_T\)) were calculated from the slope of the linear portion of the relation between distance to the stimulation site and activation times along which activation spread most rapidly starting at the point of earliest activation, and the line perpendicular to that respectively, as reported previously (Figure 2).\(^{10,14}\) Additionally, to assess the heterogeneity in activation times in a larger area of the activation map an area of 10x11 pixels was selected that had the same activation pattern and stable electrograms throughout all recordings in both experiment series to calculate mean activation time of this area (mean AT) (Figure 2). Local dispersion (LD) of activation times within this area was calculated. To correct for variability between the different experiments, mean AT, LD and CV of the second experiment series were normalized with the first experiments series to assess the effect of the intervention between experiment series. Restitution curves were created from the normalized differences of

**Figure 1.** – Experiment stimulation protocol.
*ERP: effective refractory period, LAA: left atrial appendage*

**Figure 2.** – LAA activation map measurements (1 cm\(^2\)). The isochronal lines are 2 ms apart and color scale is in ms; red represents the earliest and purple the latest activation. The CV\(_L\) and CV\(_T\) are calculated from the slope of the linear portion of the relation between distance and AT. The mean AT is calculated of an area of 10x11 pixels of the activation map.
*AT: activation time, CV\(_L\): longitudinal conduction velocity, CV\(_T\): transversal conduction velocity, LAA: left atrial appendage*
mean AT and LD to observe changes in conduction between experiments without (group I) and with acetylcholine (group II).

**Assessment of activation maps**

Activation maps were constructed from local activation times at baseline and each 10ms decremental interval until the ERP. These maps were analyzed by two blinded observers (SK, NB). The number of lines of conduction block were counted and the length of these lines were graded (overall short ± <1 mm, intermediate ± 1-2 mm or long lines of block ± > 2 mm). The mean number or the mean length of lines was the defined for each LAA per stimulation protocol based on the average number or length of lines in every single activation maps in a stimulation protocol. Additionally, changes in activation direction were assessed and defined as a complete change in activation pattern and direction during the short-coupled extrastimulation, with absence of these activation changes during baseline stimulation.

**Statistics**

Data are presented as mean ± standard deviation or median and interquartile range for parametric and non-parametric variables respectively. Categorical variables are presented in numbers with percentages. An independent Student’s T-test was used to determine differences for parametric and a Mann-Whitney U-test for non-parametric distributed data. To assess correlation in normally distributed data, the Pearson test was used and in case of non-parametric data Spearman’s test was used. A p-value of p<0.05 was considered significant. Statistical analyses were performed using IBM SPSS Statistics version 23.

**RESULTS**

**Mean activation time**

There were no differences in mean AT at basic cycle length between group I and II during the first series of measurements (identical conditions) 17.4±6.7 and 14.7±5.5 ms, respectively (p=0.49). In the first experiments series mean AT increased significantly at the ERP to 35.2±9.0 ms (p=0.028) and 40.5±23.7 ms (p=0.012) compared to stimulation at basic cycle length in both groups, group I and II respectively, no significant differences between the groups (p=0.95). Acetylcholine superfusion did not change AT, as in the second experiment series, there was no significant change in the mean normalized AT between groups (p=0.49) at basic cycle length and ERP (p=0.76, Figure 3).
Local dispersion of activation

Dispersion of activation was not significantly different between group I and II (7.0±2.9 and 4.1±3.1 ms respectively) in the first experiment series (p=0.06). At coupling intervals close to the ERP in the first experiment series (identical conditions), there was an increase in LD compared to basic cycle length in both groups I (10.9±4.8 ms, p=0.028) and II (7.1±4.0 ms, p=0.042). LD at basic cycle length did not differ between groups (p=0.57). However, a significant change in LD was observed after superfusion of acetylcholine compared to group I, due to increased LD at short-coupled stimuli (p=0.028, Figure 3).

Conduction block and wave front direction

Acetylcholine superfusion in Group II caused the number of lines of conduction block to increase from 0.6±0.7 in the first experiment series to 2.3±1.4 in the second experiment series (p=0.016). Additionally, the length of the lines of conduction block were classified as predominantly short and intermediate lines of block ± <2 mm (none n=3, short n=3, intermediate n=2) before, to more long lines of block ± >2 mm (short n=1, intermediate n=2, long n=5) after superfusion with acetylcholine (p=0.004)[Figure 3]. In group I the mean number of lines of block remained the same between the first and second experiment series (1.2±1.2 vs 1.5±1.0 (p=0.157), and the mean length of lines of conduction block remained predominantly small and intermediate ± <2 mm (none n=2, short n=2, intermediate n=2) vs none n=2, short n=1, intermediate n=2, p=1.00). Furthermore,
changes in the activation wavefront direction were observed in 4 acetylcholine experiments (50%) compared to none control experiments (Figure 4).

**Conduction velocity**

In the first series of experiments, at basic cycle length no significant differences were observed in CV between group I and group II. At basic cycle length, mean CV\(_L\) was 0.55±0.32 and 0.42±0.15 m/s (p=0.44), mean CV\(_T\) was 0.20±0.10 and 0.22±0.14 m/s (p=0.94) in group I and II respectively. Areas of block and motion artifacts precluded CV calculations at ERP in 5 LAAs (1 in group I, 4 in group II), therefore reliable comparisons in conduction at ERP could not be made. In the second experiment series, normalized conduction velocity remained unchanged between groups at basic cycle length in CV\(_L\) (p=0.76) and CV\(_T\) (p=0.88).

**Effective refractory period**

The mean ERP in group I was 230±40 ms in the first experiment series and 250±40 ms in the second series (p=0.06). In group II the mean ERP was 240±50 ms in the first experiment series and 240±60 ms in the second series after administration of acetylcholine (p=0.91). ERP did not differ between group I and group II (p=0.23). ERP was shorter in patients with persistent AF (215±40 ms), compared to patients with paroxysmal AF (265±15 ms, p=0.029) (Figure 5).
In this study, application of acetylcholine to superfused left atrial tissue of patients with AF, resulted in increased dispersion of local activation times after short-coupled premature stimuli compared to control experiments. Additionally, an increased number of lines of conduction block and changes in activation direction.
of conduction block and increase in the area of conduction block was observed at short coupling intervals. Furthermore, changes in activation direction occurred exclusively in LAAs exposed to acetylcholine. The occurrence of conduction block was not directly related to the changes in transversal and longitudinal CV. Mean AT increased as the coupling interval of the premature stimulus shortened, but were not different between acetylcholine and control experiments. Acetylcholine superfusion did not affect ERP.

**Acetylcholine as arrhythmogenic facilitator of reentry**

Acetylcholine is the main parasympathetic neurotransmitter in the heart. Acetylcholine activates the inward rectifier K⁺ current $I_{K_{ach}}$. Activation of $I_{K_{ach}}$ results in action potential duration shortening and hyperpolarization of the resting membrane of the cardiomyocyte.\(^\text{15}\) Although the exact electrophysiological mechanism of AF is disputed and probably multifactorial, reentry plays an important role in the perpetuation of AF.\(^\text{16–18}\) Maintenance of reentry depends on the wavelength, the mathematical product of refractory period and conduction velocity.\(^\text{3}\) Therefore, shortening of the action potential duration and consequently of the ERP by activation of $I_{K_{ach}}$ may increase the vulnerability for AF. We did not observe a significant decrease in ERP after acetylcholine application. Interestingly, changes in conduction after vagal stimulation have been observed in human models.\(^\text{4,5}\) We recently demonstrated that ganglion plexus stimulation affected conduction times and homogeneity of conduction during sinus rhythm in patients with paroxysmal or persistent AF undergoing thoracoscopic surgery. Stimulation of the ganglion plexus results in both a sympathetic and parasympathetic effect. In tissue obtained from patients using beta-blockers (hence in whom only vagal stimulation remained), increases in AT were observed.\(^\text{4}\) Likewise, in a study in patients with paroxysmal AF, carotid sinus massage prolonged left atrial appendage and inter-atrial conduction times.\(^\text{5}\) Interestingly, and consistent with the findings reported here, acetylcholine appears to increase electrogram fractionation during sinus rhythm. In 30 patients with paroxysmal AF application of adenosine, mimicking acetylcholine signaling, increased fractionation in atrial electrograms during sinus rhythm.\(^\text{19}\) These high amplitude fractionated electrograms could be reproduced in a computer model by simulating the effects of acetylcholine on atrial tissue.\(^\text{19}\) Additionally, both vagal stimulation and application of acetylcholine during AF increase complex fractionated electrograms in animals.\(^\text{12}\) These studies, as well as the observations in our study, strongly suggest that the effects of acetylcholine are not limited to changes in action potential duration, but also affect conduction properties of the atrial myocardium beyond the sinus and AV node. We speculate that the acetylcholine dependent potassium current interferes with the upstroke of the atrial action potential.\(^\text{6}\)
Mechanism of dispersion in conduction time and block

The autonomic nervous system is highly regionally heterogeneous and acetylcholine receptors are regionally distributed.\textsuperscript{20,21} We observed that the effects of superfusion with acetylcholine on conduction velocity are also heterogeneous within the atrium. This may be point to a heterogeneous distribution of the acetylcholine receptors or a regionally distributed sensitivity to the effects of acetylcholine (e.g. resulting from fibrosis, see below). Alternatively, the density of $I_{\text{ACh}}$ may show regional differences, which might result in a heterogeneous effect of parasympathetic stimulation.\textsuperscript{22,23} Besides shortening of the action potential duration, acetylcholine induces hyperpolarization due to an inward $K^+$ rectifier current.\textsuperscript{6,24} Hyperpolarization may reduce excitability due to the larger amount of current necessary to reach the activation threshold.\textsuperscript{25,26} Otherwise, an increased inward $K^+$ current via $I_{\text{ACh}}$ results in a more rapid repolarization after phase 0 of the action potential, thereby decreasing the plateau phase and repolarization duration. These changes decrease the net potential gradient between cardiomyocytes and subsequently reduce the safety of conduction. Another hypothesis is that decreasing intercellular coupling might be an indirect effect of acetylcholine that results in increased dispersion of conduction velocity and conduction block. Acetylcholine has indeed been described to reduce phosphorylation which could change the function or expression of connexin 40 and 43 in the atrium.\textsuperscript{27-30} For instance, in rat atrial trabeculae superfusion of acetylcholine increased intracellular resistance.\textsuperscript{31} However, little is known of the direct effects of acetylcholine on the more prevalent connexin 40 in the atrium.\textsuperscript{32} In a substrate with discontinuous bundles of cardiomyocytes with interspersed with a high degree of interstitial fibrosis, as is observed in the human atria from patients with atrial fibrillation, the electrophysiological changes to the atrial myocardium caused by acetylcholine facilitate conduction delay and block. Therefore, the arrhythmogenic effects of acetylcholine, reducing the safety of conduction, might be more prominent in structurally remodeled atria than in normal healthy atria.\textsuperscript{10,33}

Clinical implications

Based on our results interventions aimed at the decrease of the effects of the parasympathetic nervous system may potentially form an anti-arrhythmic strategy for the treatment of AF.\textsuperscript{34} Ablation of the ganglionic plexuses decreases the parasympathetic effects on the sinus node and AV node and has been described to lower the re-occurrence of AF in patient with paroxysmal AF.\textsuperscript{35,36} Some Class IC anti-arrhythmic drugs such as flecainide and dysopyramide are effective in patients with vagal AF.\textsuperscript{37} Part of this effectiveness can be explained by the ability of these drugs to modulate the effects of parasympathetic stimulation.\textsuperscript{38} Drugs that selectively target $I_{\text{ACh}}$ current are not effective against paroxysmal AF.\textsuperscript{39} Although this may indicate that the conduction slowing effect of acetylcholine is not mediated by this channel, it may also point to the importance of the arrhythmio-
genic substrate. In patients with paroxysmal AF the atrial fibrotic changes are less than in patients with persistent AF. This is supported by our own observation that the conduction changes occurred in the absence of a change in ERP.\textsuperscript{10} The influence on cellular coupling might be just as important for the arrhythmic properties of acetylcholine.

**Limitations**

LAA contraction creates motion artefacts that precludes reliable determination of repolarization characteristics from the optical action potential, hence we were not able to determine the effects of acetylcholine on action potential duration. However, the ERP was not decreased after acetylcholine application. In this study we obtained LAAs from patients with AF who underwent surgery for AF. No left atrial samples or tissue from healthy control patients could be obtained. The patients remained on anti-arrhythmic drugs throughout the procedure, which could have influenced the electrophysiological properties of the LAA. On the other hand it shows that even in the presence of an optimal antiarrhythmic regimen, the conduction slowing effects of autonomic stimulation can be observed. This emphasizes the need for an additional conduction centered therapy in patients with vagally induced AF.

**CONCLUSIONS**

Our observations show that acetylcholine induces conduction dispersion and conduction block particularly after a short-coupled extra stimuli in atrial tissue from patients with paroxysmal and persistent AF. These factors are a prerequisite for the induction of reentry.

**ACKNOWLEDGEMENTS**

We would like to thank Jan W.T. Fiolet, PhD, Antoni C.G. van Ginneken, PhD and B. Boukens, PhD for their help in maintaining and assisting with the optical mapping setup and experiments. We would like to thank Rebecca Holman, PhD for her help with the statistical analysis of our data.
REFERENCES

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