High sensitivity of the sheep pulmonary vein antrum to acetylcholine stimulation

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Atrial fibrillation is the most common arrhythmia encountered in clinical practice. It can cause complications such as congestive heart failure and stroke (7, 24). Catheter ablation is thought to be a breakthrough in the history of the treatment of the arrhythmia (30). Although the technique has been modified, its critical point is isolation of the pulmonary vein antrum (4, 8, 25–27). Association of cholinergic nerves with atrial fibrillation has been well documented in, for example, humans, canine, and sheep (17, 19, 27, 32). In human heart, cholinergic nerve fibers distribute mainly in the left atrial posterior wall (LAPW), especially in the pulmonary vein antrum or the vicinity (5, 29). Cholinergic denervation achieved by catheter ablation can effectively reduce the recurrence of the arrhythmia (27). We hypothesize that cholinergic nerves may alter the electrophysiological activity of the pulmonary vein antrum via muscarinic type 2 receptors (M2R). The activation of the receptor produces an electrophysiological substrate involved in the initiation or maintenance of the arrhythmia, whereas isolation of the pulmonary vein antrum may block the interaction of the electrophysiological substrate with other parts of the atria. In this study, we investigated the electrophysiological response of the sheep pulmonary vein antrum to cholinergic agonists and found that its effective refractory period (ERP) was reduced more than in other locations of the atrium. This heterogeneity in electrophysiological response is correlated with the distribution of the M2R. These results are consistent with our hypothesis.

MATERIALS AND METHODS

All procedures for animals were approved by the Animal Ethics and Experimentation Committee of the Tongji University, Shanghai, China, and were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (publication no. 85-23, revised 1996). The research protocol for humans was reviewed and approved by the Ethical Committee at the Tongji University School of Medicine, Shanghai, China, and conformed to the guidelines of the World Medical Assembly (the Declaration of Helsinki).

Tissue preparation. Young sheep weighing 13–17 kg were anesthetized with pentobarbital sodium. Mechanical ventilation was applied with an inducted endotracheal tube. Thoracotomy was performed on all animals via the sternum. For the immunofluorescent staining of M2R, sheep hearts were continuously perfused with a Langendorff apparatus at 36.5°C Tyrode solution (in mM: 145 NaCl, 4.5 KCl, 2.5 CaCl2, 1.0 MgCl2, 20 HEPES, 11.1 glucose, pH 7.35, 95% O2-5% CO2) through the coronary artery for 5–10 min. For the intracellular microelectrode recording, sheep hearts were quickly excised and stored in oxygenated (95% O2-5% CO2) cold above 4°C for 15 min.

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The research also included the pulmonary vein antrum, LAPW, roof, LAFW, LAA, and RAFW of human hearts of both sexes (4 women, 2 men) from 10 to 40 years of age, which were taken from cadavers without basal heart disease.

Intracellular microelectrode recordings. Standard intracellular microelectrode recording techniques were used (16). The voltage signal was amplified with a DC preamplifier and head stage (NL102; Digitimer, Welwyn Garden City, UK) and analyzed by a PowerLab/8SP data-acquisition system (Chart software, version 5.0; AD Instruments, Colorado Springs, CO). All samples were stimulated at 1 Hz with square-wave pulses of 1 ms in duration and with the amplitude at two times the threshold. Glass capillary microelectrodes with tip resistances of 10–20 MΩ and filled with 3 M KCl were used to impale into endocardial surface. Experiments were started after 2 h of perfusion in 36.5°C Tyrode solution as mentioned above. After the sample was perfused with 150 μM acetylcholine for 30 min, resting membrane potential, action potential amplitude, action potential duration (APD) at 50% and 90% of repolarization (APD50 and APD90, respectively), and ERP were acquired. One cell was recorded at each of the following sites: the pulmonary vein antrum, LAPW, roof, LAFW, LAA, and RAFW (one cell each site from one sheep). The ERP was measured with 15 basic (S1) stimuli followed by a premature (S2) stimulus at an S1S2 interval that was decreased by 1- to 10-ms decrements from the basic cycle length, with the ERP defined as the longest S1S2 interval failing to produce a new, propagated action potential. The ERP was determined twice at each basic cycle length, and the mean of the ERP values was used for data analysis.

Pharmacological studies. To examine the EC50, tissues from pulmonary vein antrum and other atrial regions were discretely perfused with different doses of acetylcholine for ~10 min and then washed out for 5 min. The dose-response curves were acquired via logistic equation.

Immunofluorescent staining for M4-R in the atrium. We analyzed the distribution of the M4-R in the pulmonary vein antrum, LAPW, roof, LAFW, LAA, and RAFW in sheep (n = 6) and humans (n = 6) by immunohistochemistry and confocal laser scanning biological microscopy, following modifications to previous methods (6). After the pericardium and fatty tissue were eliminated from the surface of the atrium, the atrium specimens were fixed by 4% paraformaldehyde in PBS for 24 h and embedded in paraffin. Five-micrometer-thick sections were then sliced from each block. After samples were immersed in dimethylbenzene and graded ethanol successively, slides of the pulmonary vein antrum, LAPW, roof, LAFW, LAA, and RAFW were processed for immunohistochemistry.
RAFW were sequentially treated as follows: 1) exposure to 0.01 mol/l citrate buffer (pH 6.0) at 95°C for 15 min, 2) blocking nonspecific antigen by 10% normal rabbit serum for 10 min, 3) incubation overnight at 4°C with M2R (Sigma-Aldrich), muscarinic type 3 receptors (M2R; MBL), muscarinic type 4 receptors (M4R; MBL), GIRQ3.1 (US Biological), GIRQ3.4 polyclonal antibody (1:100; Santa Cruz Biotechnology), and distilled water (negative control). Rat hippocampus was used as a positive control. Slides of different sites were treated with the same antibody at the same time. Thereafter, slides were reacted with goat anti-rabbit IgG-FITC (1:100; Santa Cruz Biotechnology) for 45 min at room temperature. Signals were visualized at 500–550 nm by FV1000 confocal laser-scanning biological microscope (Olympus). Immunofluorescence was analyzed from 50 cardiomyocytes of each slide in five slides of each atrial site. Moreover, the fluorescence of slides on the same sample was measured three times over 3 days. The cellular fluorescent intensity was evaluated by ImageJ 1.34 software (National Institutes of Health) (1, 20).

Statistics. Data are expressed as means ± SE. Statistical analysis of data was performed by applying one-way ANOVA followed by Student-Newman-Keuls post hoc test. A P value of <0.05 was considered statistically significant.

RESULTS

Electrophysiological response of the pulmonary vein antrum to M₃R agonist. We examined the effect of acetylcholine, an M₃R agonist, on the parameters of action potential and ERP of the sheep atrial myocardium using the standard intracellular microelectrode recording technique. Figure 1 displays the backsides of the atrium and the pulmonary vein antrum in the sheep. Figure 2 shows a dose-response curve and a time dependence of the effect of acetylcholine on the ERP in cardiomyocytes of sheep pulmonary vein antrum, LAPW, roof, LAFW, LAA, and RAFW. In the present study, we used the data from the left superior pulmonary vein antrum to represent the different pulmonary vein antrums. The ERP was measured at 500–550 nm by FV1000 confocal laser-scanning biological microscope (Olympus). Immunofluorescence was analyzed from 50 cardiomyocytes of each slide in five slides of each atrial site. Moreover, the fluorescence of slides on the same sample was measured three times over 3 days. The cellular fluorescent intensity was evaluated by ImageJ 1.34 software (National Institutes of Health) (1, 20).

Statistics. Data are expressed as means ± SE. Statistical analysis of data was performed by applying one-way ANOVA followed by Student-Newman-Keuls post hoc test. A P value of <0.05 was considered statistically significant.

Table 1. Electrophysiological changes of atrial cardiomyocytes before and after acetylcholine application

<table>
<thead>
<tr>
<th>Before Acetylcholine Application (n = 20)</th>
<th>After 15 μM Acetylcholine Application (n = 20)</th>
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</thead>
<tbody>
<tr>
<td>Antrum</td>
<td>RP, mV</td>
</tr>
<tr>
<td>Antrum</td>
<td>−79.7 ± 0.9*</td>
</tr>
<tr>
<td>LAPW</td>
<td>−77.8 ± 0.7</td>
</tr>
<tr>
<td>Roof</td>
<td>−76.6 ± 0.7†</td>
</tr>
<tr>
<td>LAFW</td>
<td>−76.1 ± 0.6†</td>
</tr>
<tr>
<td>LAA</td>
<td>−78.9 ± 0.7</td>
</tr>
<tr>
<td>RAFW</td>
<td>−77.3 ± 0.7†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of sheep. Antrum, left superior pulmonary vein antrum (data from antrum were similar to those from the other pulmonary vein antrums); LAPW, left atrial posterior wall; roof, left atrial roof; LAFW and RAFW, left and right atrial free wall; LAA, left atrial appendage; RP, resting potential; APA, action potential amplitude; APD₀ and APD₂₀₀, action potential duration and 90% and 50% repolarization; ERP, effective refractory period. *P < 0.05 vs. RAFW; †P < 0.05 vs. antrum.

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We also noted that, after acetylcholine was washed out, action potential parameters and ERP of sheep atrial cardiomyocytes returned to original levels before treatment with acetylcholine. Another M2R agonist, carbachol (1 μM), also produced an effect (Table 2) similar to acetylcholine, whereas the M3R blocker methoctramine (1 μM) and the selective blocker of the acetylcholine-activated potassium current (I\(_{K\text{-}Ach}\)) terttiapin-Q (0.15 μM) abolished the effect of acetylcholine (15 μM) (data not shown).

To further test which type of muscarinic receptors is responsible for the effects of acetylcholine, we used the M2R, M3R, and M4R blockers to inhibit the acetylcholine-induced ERP shortening. The results showed that IC\(_{50}\) values for methoctramine, an M3R blocker, 4-diphenylacetoxy-N-methylpiperidine, an M3R blocker, and tropicamide, an M2R blocker, were 0.151, 1.063, and 2.497 μM (Fig. 4), respectively. These results indicate that M2R plays a major role in the response of atrial myocytes to acetylcholine stimulation.

**M2R distribution in the atrial myocardium.** Our results revealed that the M2R widely distributed within the atrial myocardium, but the distribution was not homogenous. The relative fluorescence intensities of the receptors in the pulmonary vein antrum, LAPW, roof, LAFW, LAA, and RAFW were 62.64 ± 0.9 2.56, 53.12 ± 2.76, 51.83 ± 2.45, 47.90 ± 2.32, 55.27 ± 2.08, and 45.53 ± 2.02, respectively. The pulmonary vein antrum displayed the highest M2R density (Fig. 5, A–G). A similar distribution of the M2R was also found in human hearts (Fig. 5, H–N). The coefficient of variation of the fluorescence was 5.07–12.31% for the same sample. In contrast, the expression of M1R, M2R, GIRK3.1, and GIRK3.4 showed no significant difference among different sites (data not shown).

**DISCUSSION**

In the present study, we for the first time demonstrated that atrial ERP was heterogeneously shortened by cholinergic agonist administration, with the shortest ERP occurring in the pulmonary vein antrum. In conformity with the heterogeneity of ERP distribution, M2R also heterogeneously distributed in the atrial myocardium, and the highest density of M2R was found in the pulmonary vein antrum, indicating that acetylcholine-induced heterogeneous ERP distribution may be a consequence of heterogeneous distribution of M2R. However, other muscarinic receptors, M3R and M4R, and I\(_{K\text{-}Ach}\) channel subunits, GIRK3.1 and GIRK3.4, demonstrated no significant differences of spatial distribution in the atrium.

The heterogeneous shortening of ERP by acetylcholine as a consequence of the heterogeneity of M2R distribution is also supported by the following results. First, the M2R agonist carbachol could produce an effect similar to that of acetylcholine. Second, the effect of acetylcholine was inhibited by the M2R blocker methoctramine with a much higher sensitivity than by the M3R or M4R blockers 4-diphenylethacetoxy-N-methylpiperidine or tropicamide. Third, the inhibition of I\(_{K\text{-}Ach}\) channel, which is activated by the M2R signaling pathway, by terttiapin-Q also effectively abolished the effect of acetylcholine. These results were supported by the present findings on muscarinic expression, left atrial refractory period, and I\(_{K\text{-}Ach}\) channel blockade (2, 9, 10).

Recently, Mansour et al. (22) identified sequential wave fronts with similar spatial patterns of propagation and temporal periodicity during atrial fibrillation in the isolated sheep heart by combining high-resolution video imaging, electrocardiographic recordings, and spectral analyses. These results showed a high degree of spatiotemporal periodicity accompanying atrial fibrillation, which thereby led to the hypothesis that atrial fibrillation maintenance depends on the uninterrupted periodic activity of a single or small number of reentrant sources to give rise to fibrillatory conduction through interactions with anatomic and/or functional obstacles (24, 15). Increasing studies of animals and humans have demonstrated that the pulmonary vein antrum or junction of pulmonary vein left atrium is the leading reentrant source (7, 12, 17, 21). Most of

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**Table 2. Electrophysiological changes of atrial cardiomyocytes before and after carbachol application**

<table>
<thead>
<tr>
<th></th>
<th>Before Carbachol Application (n = 8)</th>
<th>After 1 μM Carbachol Application (n = 8)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>RP, mV</td>
<td>APA, mV</td>
</tr>
<tr>
<td>Antrum</td>
<td>−80.1±0.9</td>
<td>105.8±2.29*</td>
</tr>
<tr>
<td>LAPW</td>
<td>−78.5±1.3</td>
<td>105.3±1.0*</td>
</tr>
<tr>
<td>Roof</td>
<td>−75.9±0.8†</td>
<td>102.3±1.3</td>
</tr>
<tr>
<td>LAFW</td>
<td>−76.3±2.1†</td>
<td>96.2±1.5*</td>
</tr>
<tr>
<td>LAA</td>
<td>−78.0±1.0</td>
<td>99.7±1.9*</td>
</tr>
<tr>
<td>RAFW</td>
<td>−78.0±1.2</td>
<td>98.0±2.1†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of sheep. Data from the left superior pulmonary vein antrum was similar to those from the other pulmonary vein antrums.

*P < 0.05 vs. RAFW; †P < 0.05 vs. antrum.
the clinical catheter ablation practices, such as circumferential pulmonary vein ablation and electrogram-guided ablation, also target substructures in the pulmonary vein antrum or junction of pulmonary vein and left atrium (3, 4, 8, 14, 18, 23, 26, 31). The pulmonary vein antrum apparently plays an important role in atrial fibrillation. Shortening of ERP has long been regarded as an important substrate for atrial fibrillation (24). On the other hand, the refractory heterogeneity has also become a subject of attention, which may be associated with fractionation of wave fronts and fibrillatory conduction (17, 19). The present study identified a marked ERP shortening in the pulmonary vein antrum (104.0 ± 2.1 ms before acetylcholine application vs. 52.0 ± 1.6 ms after acetylcholine application) and a distinct refractory heterogeneity between the pulmonary vein antrum (ERP in the pulmonary vein antrum: 52.0 ± 1.6 ms) and its vicinity (ERP in the LAPW, roof, LAFW, LAA, and RA FW: 75.1 ± 2.0, 77.2 ± 1.7, 85.6 ± 1.7, 64.3 ± 2.1, and 90.5 ± 1.3 ms, respectively) as induced by acetylcholine. Thus the pulmonary vein antrum may be a site with high response to acetylcholine. Remarkable ERP shortening induced by acetylcholine (from 104.0 ± 2.1 to 52.0 ± 1.6 ms), which is consistent with the clinical findings (28), together with the ERP heterogeneity, may be inclined to promote reentry and ultimately atrial fibrillation. The therapeutic effect of the pulmonary vein antrum isolation is most likely attributed to the isolation of the atrial fibrillation substrates within the pulmonary vein antrum (cholinergic stimulation-induced ERP shortening) and the abolition of the electrophysiological basis for fractionation of wave fronts and fibrillatory conduction (cholinergic stimulation-induced refractory heterogeneity).

In summary, the pulmonary vein antrum is a region of unique electrophysiological importance with high sensitivity to acetylcholine stimulation. The dense M2R distribution in the pulmonary vein antrum is most likely to be responsible for such high sensitivity to acetylcholine. Cholinergic stimulation induced dramatic ERP heterogeneity in the atrium, which may be a possible substrate for atrial fibrillation. Our work also presents the potential value of cholinergic antagonists in the treatment of certain types of atrial fibrillation.

Limitations

The relationship between ERP and atrial fibrillation has been identified previously, and our study suggested that cholinergic stimulation induced dramatic ERP heterogeneity in the atrium. However, the direct relationship between muscarinic receptor distribution heterogeneity and atrial fibrillation was not established in the present study.

Arora et al. (2) showed that M2R is predominantly located in the LAPW, whereas Huang et al. and Zhao et al. demonstrate that M2R is more abundant in atrial appendage than in other sites (11, 32). However, the present study showed that M2R density in the pulmonary vein antrum was the highest. It is possible that this discrepancy is attributed to species differences.

GRANTS

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REFERENCES


