Acetylcholine Release in Human Heart Atrium
Influence of Muscarinic Autoreceptors, Diabetes, and Age

Vitus Oberhauser, PhD; Eckhard Schwertfeger, MD; Tobias Rutz; Friedhelm Beyersdorf, MD; Lars Christian Rump, MD

Background—An imbalance of sympathetic and parasympathetic drive to the heart is an important risk factor for cardiac death in patients with coronary heart disease, diabetes, and renal insufficiency. The amount of neurotransmitter released from peripheral autonomic nerves is modulated by presynaptic receptor systems. In analogy to α-autoreceptors on sympathetic nerves, muscarinic autoreceptors activated by endogenous acetylcholine may exist on parasympathetic nerves in the human heart.

Methods and Results—We developed a technique to study acetylcholine release from human atria and investigated muscarinic autoreceptor function. A pharmacological and molecular approach was used to characterize the subtype involved. Of the 5 muscarinic receptor subtypes cloned, only mRNA encoding for M1- and M2-receptors were detected. Potencies of several muscarinic antagonists against the release-inhibiting effect of the nonselective muscarinic agonist carbachol at the cardiac autoreceptor were correlated with published data for human cloned M1- through M5-receptors.

Conclusions—This analysis clearly indicates that acetylcholine release in human atria is controlled by muscarinic M2-receptors. Blockade of these receptors by atropine doubles the amount of acetylcholine released at a stimulation frequency of 5 Hz. In atria of patients >70 years of age and patients with late diabetic complications, acetylcholine release is reduced. Locally impaired cardiac acetylcholine release may therefore represent a pathophysiological link to sudden cardiac death in elderly and diabetic patients. (Circulation. 2001;103:1638-1643.)

Key Words: coronary disease ■ nervous system, sympathetic ■ acetylcholine ■ receptors ■ diabetes mellitus

Sympathetic overactivity plays a dominant prognostic role in myocardial infarction and heart failure of various causes, and the role of enhanced cardiac norepinephrine release is well described. Surprisingly, little is known about the role of the cholinergic nervous system, which is considered to be a functional counterpart of sympathetic influences to the heart.1 Parasympathetic activity is suppressed in heart failure,2 diabetes,3 and old age,4 and an imbalance of sympathetic and parasympathetic drive may contribute to the high incidence of sudden cardiac death in these patients. This view is mainly based on indirect measures of cardiac parasympathetic dysfunction such as altered heart rate variability5 and baroreflex sensitivity6; however, nothing precise is known about presynaptic mechanisms controlling cardiac acetylcholine release in humans. Parasympathetic nerve endings may have presynaptic receptors, which when activated inhibit acetylcholine release.7–9 The most important presynaptic receptor systems are those activated by endogenous ligands. In analogy to presynaptic α-autoreceptors on sympathetic nerve terminals,10 muscarinic autoreceptors activated by neuronally released acetylcholine may exist on parasympathetic nerves in the human heart. The existence of presynaptic muscarinic autoreceptors may explain the clinical observation of why low doses of the M1-selective antagonist pirenzepine decrease heart rate in humans.11 Five human muscarinic receptors (M1 through M5) have been cloned,12 and one could hypothesize that presynaptic and postsynaptic receptor subtypes differ. Therefore, we determined the muscarinic autoreceptor subtype of parasympathetic nerves regulating acetylcholine release in the human heart and investigated whether diabetes or old age modifies acetylcholine release.

Methods

Patients and Tissue Sources
The study was approved by the Ethics Committee of the University of Freiburg. Atrial tissues were taken from patients undergoing open heart surgery for coronary bypass grafting (78 patients) or aortic valve replacement (18 patients). Specimens of ~100 to 200 mg were harvested from the tip of the right auricle during venous cannulation for extracorporal circulation. The age of the patients (21 women; 75 men) averaged 67 ± 1 years (range, 41 to 82). Patients with diabetes were separated into groups according to the presence (10 patients) or absence (8 patients) of diabetic complications (nephropathy, polyneuropathy, and retinopathy). Diabetic nephropathy was assumed in diabetic patients with a serum creatinine of ≥1.2 mg/dL and pathological microalbuminuria (>30 mg/24 h) or overt proteinuria (>300 mg/24 h). Diagnosis of retinopathy and polyneuropathy was based on diagnosed typical findings of funduscropy and neurological examination, respectively.
Release Experiments

Connective tissue was removed and multiple segments were prepared. Segments were incubated with [3H]-choline (25 μCi, 0.31 nmol) between platinum electrodes in Krebs-Henseleit solution gassed with 95% O₂/5% CO₂. During incubation (75 minutes), field stimulation (40 V, 20 mA, 2 ms) was applied every 5 seconds to increase uptake of radioactivity.¹³ The segments were then placed into superfusion chambers (volume, 250 μL). Eight segments were superfused (2 mL/min) in parallel at 37°C. Hemicholine (3 μmol/L) was added after 65 minutes to prevent reuptake of [3H]-choline. After washout, twenty 3-minute fractions of the superfusion solution were collected. Two stimulations (40 mA, 5 Hz, 2 minutes) were applied (S1 at 7 minutes, S2 at 45 minutes after start of collections). S1 served as reference stimulation. The effect of drugs was tested by adding them 15 minutes before S2. In those experiments in which a drug was present in both stimulation periods (throughout), it was added 15 minutes before S1.

Estimation of Radioactivity

The radioactivity of the 3-minute samples was detected by liquid scintillation counting. Total tissue radioactivity was determined at the end of each experiment after the tissue was dissolved in Soluene (Packard).

Calculation of Results

The spontaneous outflow of radioactivity was determined as the mean of radioactivity of 2 samples, collected before and after electrical stimulation. The stimulation-induced (S-I) outflow of radioactivity was calculated by subtracting the spontaneous outflow of radioactivity from the radioactivity present in the three 3-minute samples collected immediately after onset of stimulation (Figure 1). S-I outflow of radioactivity is subsequently expressed as a percentage of the total tissue content of radioactivity at the time of stimulation (fractional S-I outflow in S1 and S2; FR1, FR2). When effects of drugs were tested, results were calculated as FR2 to FR1 ratios and expressed as percentages of the corresponding controls. Antagonist potencies (pKᵦ values) were estimated according to Equation 4 of Furchgott.¹⁴

Statistics

All data are expressed as mean±SEM. Data points were always determined in duplicate; n gives the number of atria used in each group. Data were analyzed by unpaired Student’s t test or ANOVA where appropriate. Values of P<0.05 were considered statistically significant.

RNA Extraction and Reverse Transcription–Polymerase Chain Reaction

Atrial tissue was homogenized and total RNA was isolated from the supernatant with the RNeasy Kit (Quiagen). DNA was isolated by the same method with RNAase instead of DNAse. cDNA synthesis and reverse transcription–polymerase chain reaction (RT-PCR) were performed at 57°C and 35 cycles with the SuperScript preamplification system (GIBCO BRL). Homologous primers for human M1- through M₄-receptors were used (Table 1). RT-PCR products were cloned in the pCR2.1-TOPO plasmid with the TOPO-TA cloning kit (Invitrogen) and sequenced at the Human Genetic Institute (Freiburg, Germany). Sequences were verified by BLAST search at the National Center for Biotechnology Information.

Drugs and Vehicles

Krebs-Henseleit solution (mmol/L) contained NaCl 118, KCl 4.7, CaCl₂ 2.5, MgCl₂ 0.8, NaHCO₃ 25, KH₂PO₄ 1.03, D(+)-glucose 11.1, disodium-EDTA 0.067, and ascorbic acid 0.07. The following drugs were used: [3H]-choline (Amersham); atropine, pirenzepine, himbacine, TTX, EGTA, and carbachol (all Sigma); tropicamide and AFDX-116 (Toceis); and hemicholinum (ICI Chemicals). Drugs were dissolved with either distilled water, DMSO, or ethanol.

Results

[3H]-Acetylcholine Release From Human Atria

Human atrial segments incubated with [3H]-choline accumulated 33 140±1170 dpm per milligram of tissue wet weight. Electrical field stimulation with 600 pulses at 5 Hz (Figure 1) induced an increase of the fractional outflow of radioactivity of 0.27±0.02% (n=49). Fractional outflow in the two stimulation periods (S1, S2) was stable (Figure 1), with a ratio (FR2/FR1) of 89±2.1% (n=49). Tetrodotoxin (1 μmol/L) and omission of Ca²⁺ abolished the release of S-I radioactivity (Figure 1). Thus, the S-I release of radioactivity from human atrial tissue could be taken as an index of [3H]-acetylcholine release, as shown previously for other species.⁹

Modulation of Acetylcholine Release by Muscarinic Autoreceptors in Human Atria

The nonselective muscarinic agonist carbachol (0.1 to 10 μmol/L) inhibited the release of radioactivity concentration dependently (EC₅₀ of 0.9 μmol/L) and at a maximum of 78% (Figure 2). Atropine (0.1 μmol/L), a nonselective muscarinic antagonist, when present throughout superfusion, shifted the dose-response curve of carbachol to the right (Figure 2). Atropine by itself enhanced the release of radioactivity dose dependently. To further characterize the receptor subtype involved, dissociation constants (pKᵦ values) of 5 muscarinic antagonists were determined (Table 2) against carbachol and plotted against affinity estimates of cloned human muscarinic receptors. Whenever possible, pKᵦ values¹⁵,¹⁶ were taken for correlation. In the case that pKᵦ values were not available, pKᵦ values were used instead.¹⁷,¹⁸ There was a significant correlation for the M₂- and M₃-receptors, with the best correlation coefficient calculated for the M₃-receptor (Figure 3). This view is supported by the rank order of potencies: atropine>himbacine>AFDX-116>tropicamide>pirenzepine, compatible with the involvement of an M₂ receptor.

RT-PCR of Muscarinic Receptors in Human Atria

Under the conditions applied, only significant mRNA levels of M₂- and M₃-receptor subtypes were found (Figure 4). The

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**TABLE 1. Primer Settings for RT-PCR/PCR for Human Muscarinic Receptors**

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Primer</th>
<th>Location</th>
<th>Product Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>M₁</td>
<td>UP: 5' CAGAAGCCCCCGTGAAAGAG 3'</td>
<td>1121...1141</td>
<td>401 bp</td>
</tr>
<tr>
<td></td>
<td>LP: 5' CGGGGACCTGATGGTGAAG 3'</td>
<td>1521...1504</td>
<td></td>
</tr>
<tr>
<td>M₂</td>
<td>UP: 5' GTTACGGCTTGGGACCTGT 3'</td>
<td>360...381</td>
<td>491 bp</td>
</tr>
<tr>
<td></td>
<td>LP: 5' GCTGGGATGTGGTATGTTG 3'</td>
<td>850...828</td>
<td></td>
</tr>
<tr>
<td>M₃</td>
<td>UP: 5' ATOGCTTGATGGGGTATGCTCTC 3'</td>
<td>665...687</td>
<td>367 bp</td>
</tr>
<tr>
<td></td>
<td>LP: 5' ACGGGGATCATCTCCCTGTTG 3'</td>
<td>1031...1009</td>
<td></td>
</tr>
<tr>
<td>M₄</td>
<td>UP: 5' CACTGGAGCTGGCTTCTTCA 3'</td>
<td>395...414</td>
<td>403 bp</td>
</tr>
<tr>
<td></td>
<td>LP: 5' CTCGGGCGCCGTGCCTGGA 3'</td>
<td>797...777</td>
<td></td>
</tr>
<tr>
<td>h₁</td>
<td>UP: 5' ATCTCTCCGGCTTTCCTTAG 3'</td>
<td>836...858</td>
<td>422 bp</td>
</tr>
<tr>
<td></td>
<td>LP: 5' ATCTCTCCGGCTTTCCTTAG 3'</td>
<td>1257...1234</td>
<td></td>
</tr>
</tbody>
</table>
M2-receptor band was more prominent than the M3-receptor band. RT-PCR without reverse transcriptase (−RT; Figure 4) showed that the RT-PCR products obtained originated from mRNA. PCR of genomic DNA revealed bands for 5 muscarinic receptors (data not shown), proving the specificity of the primers.

Influence of Age and Late Diabetic Complications on Acetylcholine Release From Human Atria

Uptake of radioactivity after incubation with [3H]-choline was similar in all age groups (Figure 5). However, S-I release of [3H]-acetylcholine decreased with age. Segments of patients >70 years of age showed a lower release of [3H]-acetylcholine than atria of patients ≤70 years of age. Uptake of radioactivity after incubation with [3H]-choline was similar in patients with or without diabetes but lower in diabetic patients with late complications (Figure 6). Atrial segments of patients with late diabetic complications also had a significant lower fractional release of [3H]-acetylcholine than control subjects and diabetic patients without late complications (Figure 6). The average age of the 3 groups was not different (63±3, 68±2, and 66±1 years).

Discussion

A suppressed parasympathetic nervous system of the heart may play an important role for prognosis in diseases such as heart failure, myocardial infarction, and diabetes. In this study, we established a method to investigate acetylcholine release and its modulation by inhibitory muscarinic autoreceptors from human cardiac tissue. Because there was indirect evidence to suggest an impaired release of acetylcholine release in old age11 and diabetes,19 we took advantage of our technique to look into this matter.

Acetylcholine Release From Human Atria

Labeling of the cholinergic transmitter stores with [3H]-choline taken up by cholinergic nerves and synthesized to [3H]-acetylcholine by choline acetyltransferase has been used in atrial tissue of guinea pigs, rats, and mice20–22 but not in atrial tissue of

TABLE 2. pKb Values of [3H]-Acetylcholine Release From Human Atria

<table>
<thead>
<tr>
<th>Muscarinic Antagonist</th>
<th>pKb Value</th>
</tr>
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<tbody>
<tr>
<td>Atropine</td>
<td>8.40±0.09</td>
</tr>
<tr>
<td>Himbacine</td>
<td>7.26±0.04</td>
</tr>
<tr>
<td>AFDX-116</td>
<td>6.37±0.04</td>
</tr>
<tr>
<td>Tropicamide</td>
<td>6.20±0.05</td>
</tr>
<tr>
<td>Pirenzepine</td>
<td>5.95±0.06</td>
</tr>
</tbody>
</table>

Antagonist potencies of muscarinic antagonists (pKb values) against carbachol estimated according to Reference 14. Data are mean±SEM of 8 to 10 segments for each antagonist used.
humans. We modified this technique for human atria and showed a significant outflow of [3 H] activity in response to electrical field stimulation. Within 1 hour after obtaining the tissue in the operation theater, experiments were started, which then lasted for '3 hours. This short time interval makes it unlikely that effects of denervation may have altered uptake and release functions of parasympathetic nerve endings. The S-I release of radioactivity was completely tetrodotoxin sensitive and dependent on extracellular Ca\textsuperscript{2+}, fulfilling the criteria of exocytotic release processes. Therefore, the S-I outflow of radioactivity from human atria preincubated with [3 H]-choline was taken as an index of endogenous acetylcholine release.

Characterization of Cardiac Inhibitory Autoreceptor

The amount of acetylcholine released at parasympathetic neuroeffector junctions in the heart is primarily regulated by the central nervous system integrating afferent inputs from the baroreceptor reflex and cardiopulmonary mechanoreceptors and chemoreceptors. However, postganglionic cholinergic nerve endings have presynaptic receptors, which, when activated by endogenous acetylcholine, inhibit the release of cardiac acetylcholine. In several animal species, such muscarinic autoreceptors have been demonstrated in cardiac tissue. These receptors involved in autoinhibition of acetylcholine release are of the M\textsubscript{1}-subtype in chicken heart\textsuperscript{7} and of the M\textsubscript{2}-subtype in guinea pig, rat, and rabbit heart.\textsuperscript{7–9} Functional studies suggest the existence of presynaptic M\textsubscript{1}-receptors in humans. It was postulated that the decrease of heart rate by low concentrations of the preferential M\textsubscript{1}-receptor antagonist pirenepine is due to blockade of presynaptic M\textsubscript{1}-receptors enhancing acetylcholine release.\textsuperscript{11} However, binding studies revealed only the existence of M\textsubscript{2}-receptors in the human heart.\textsuperscript{23} To clarify this situation, we used two different approaches: pharmacological characterization with Equation 4 of Furchgott\textsuperscript{14} and molecular characterization with RT-PCR. First, potencies (pK\textsubscript{B} values) of 5 muscarinic receptor antagonists against the release-inhibiting effect of the nonselective agonist carbachol were determined and compared with pK\textsubscript{B}/pK\textsubscript{i} values of cloned human muscarinic receptors.\textsuperscript{15–18} The highest correlation coefficient of 0.98 was determined for the M\textsubscript{2}-subtype, and the slope of the regression was close to unity. pK\textsubscript{B} values obtained for human autoreceptors were 10-fold lower than those for the cloned receptors. This
difference is expected because in our study, the antagonists used had to compete with carbachol and endogenous acetylcholine for the presynaptic autoreceptor. The role of endogenous acetylcholine activating presynaptic autoreceptors is clearly demonstrated by the marked enhancement of acetylcholine release induced by atropine. Taken together, the pharmacological characterization suggests the involvement of presynaptic autoreceptors of the M2- but not the M1-subtype in human atria. This is in accordance with a previous study in human trachea. In contrast, at parasympathetic ganglia, the M1-subtype, in urinary bladder the M3-subtype and in detrusor the M2-subtype, has been described to modulate acetylcholine release.

Pharmacological characterization may lead to a false interpretation because of imperfect specificity of muscarinic receptor antagonists available. However, the operation of cardiac muscarinic M2-autoreceptors is supported by results with RT-PCR. With the use of homologous primers, RT-PCR amplified only cDNA encoding for M2- and M3-receptor subtypes. The detection of RT-PCR products for M2-receptors fits well with our pharmacological characterization. However, it is likely that the RT-PCR signal for M2-receptors comes to some extent from mRNA encoding for postsynaptic M1-receptors. M1-receptors have as yet not been found in human heart, but there were some indications from in vitro and in vivo studies that an additional muscarinic receptor, different from the M2-subtype, may exist in human heart.

M3-receptors have already been demonstrated in chicken and dog heart. However, our pharmacological analysis excludes presynaptic M3-receptors in human atria. M4- and M5-receptors were excluded by RT-PCR. With the pharmacological and molecular evidence taken together, it is feasible that in the human heart, acetylcholine release is modulated exclusively by M2-autoreceptors.

**Influence of Age and Late Diabetic Complications**

The physiological process of aging as well as advanced stages of diabetes have been associated with profound changes in autonomic function. In older age, circulating plasma norepinephrine levels are increased. More recently, an imbalance between sympathetic and parasympathetic influences to the heart in diabetes and old age have been suggested on the basis of heart rate variability studies. This dysregulation may be due to defective afferent and efferent signaling processes; however, in addition, alterations of local presynaptic release mechanisms of acetylcholine may be involved. It has already been shown that muscarinic receptor density and function are decreased in old age. The concept of a locally altered parasympathetic nervous function is now supported by the present in vitro study, which demonstrates that cardiac acetylcholine release from parasympathetic nerves is decreased in diabetes and old age. As for diabetes, this impairment was observed only in patients with advanced stages of diabetes.
the disease (nephropathy, retinopathy, neuropathy). This fits well with a study in diabetic impotent men, which showed that synthesis and release of acetylcholine in corporeal tissue was reduced and worsened with the duration of diabetes. Furthermore, animal studies investigating acetylcholine release from parasympathetic nerves to atrial myocardium have demonstrated that the uptake of tritiated choline was reduced in atria of patients treated with streptozotocin-induced diabetes in rats support the concept of autonomic neuropathy being a primary cause of cardiac dysfunction in diabetes. In the present study, the uptake of tritiated choline was reduced in atria of diabetic patients with late complications. This may reflect a reduced density of parasympathetic innervation or reduced ability of choline-acetyltransferase generation. However, fractional release calculated as a percentage of the total accumulated tissue radioactivity was also diminished, suggesting an additional functional component. This appears to be true also for atria of patients >70 years of age. Uptake of tritiated choline was similar to atria of younger patients; however, S-I release of acetylcholine was reduced. One possible explanation is that presynaptic M2-autoreceptor function is upregulated, leading to reduced acetylcholine release, as shown for rat diabetic lungs. Taken together, this is the first evidence to show impaired cardiac acetylcholine release in diabetics and elderly people. This impaired local parasympathetic activity may be an important risk factor for sudden cardiac death in these groups of patients. Furthermore, alterations of presynaptic modulation of acetylcholine release must be taken into account when interpreting results of heart rate variability studies, in which pharmacological manipulations are used to assess vagal activity.

Acknowledgments

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