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Research Article

The M1 muscarinic receptor antagonist pirenzepine in low doses decreases resting heart rate; this effect declines with age (Poller, U., G. Nedelka, J. Radke, K. Pönicke, and O.-E. Brodde. 1997. *J. Am. Coll. Cardiol.* 29:187-193). To study possible mechanisms underlying this effect, we assessed (a) in six young (26 yr old) and six older volunteers (61 yr old), pirenzepine effects (0.32 and 0.64 mg intravenous [i.v.] bolus) on isoprenaline-induced heart rate increases; (b) in five heart transplant recipients, pirenzepine effects (0.05-10 mg i.v. bolus) on resting heart rate in the recipient's native and transplanted sinus nodes; and (c) in right atria from 39 patients of different ages (5 d-76 yr) undergoing open heart surgery, M2 muscarinic receptor density (by [³H]N-methyl-scopolamine binding) and adenylyl cyclase activity. (a) Pirenzepine at both doses decreased heart rate in young volunteers significantly more than in older volunteers; (b) pirenzepine (< 1 mg) decreased resting heart rate in the recipient's native but not transplanted sinus node; and (c) M2 receptor density and carbachol-induced inhibition of forskolin-stimulated adenylyl cyclase activity decreased significantly with the age of the patients. We conclude that pirenzepine decreases heart rate via inhibition of presynaptic M1 autoreceptors, thereby releasing endogenous acetylcholine, and that the heart rate-decreasing effect of acetylcholine declines with age because right atrial M2 receptor density and function decrease.

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Cardiac Muscarinic Receptors Decrease with Age

In Vitro and In Vivo Studies

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Abstract

The M₁ muscarinic receptor antagonist pirenzepine in low doses decreases resting heart rate; this effect declines with age (Poller, U., G. Nedelka, J. Radke, K. Pönicke, and O.-E. Brodde. 1997. *J. Am. Coll. Cardiol.* 29:187–193). To study possible mechanisms underlying this effect, we assessed (a) in six young (26 yr old) and six older volunteers (61 yr old), pirenzepine effects (0.32 and 0.64 mg intravenous [i.v.] bolus) on isoprenaline-induced heart rate increases; (b) in five heart transplant recipients, pirenzepine effects (0.05–10 mg i.v. bolus) on resting heart rate in the recipient's native and transplanted sinus nodes; and (c) in right atria from 39 patients of different ages (5 d–76 yr) undergoing open heart surgery, M₂ muscarinic receptor density (by [³H]N-methylscopolamine binding) and adenylyl cyclase activity. (a) Pirenzepine at both doses decreased heart rate in young volunteers significantly more than in older volunteers; (b) pirenzepine (< 1 mg) decreased resting heart rate in the recipient's native but not transplanted sinus node; and (c) M₂ receptor density and carbachol-induced inhibition of forskolin-stimulated adenylyl cyclase activity decreased significantly with the age of the patients. We conclude that pirenzepine decreases heart rate via inhibition of presynaptic M₁ autoreceptors, thereby releasing endogenous acetylcholine, and that the heart rate-decreasing effect of acetylcholine declines with age because right atrial M₂ receptor density and function decrease. (*J. Clin. Invest.* 1998. 101:471–478.) Key words: human cardiac muscarinic receptors • heart rate • aging • adenylyl cyclase • heart transplantation

Introduction

Aging is associated with a decline in the function of many hormone and neurotransmitter receptors (for a review, see reference 1). While such age-dependent changes in human adrenergic receptors have been studied extensively (for reviews, see references 2–4), relatively little is known about possible age-dependent alterations in human cholinergic receptors. In the

human heart, there are muscarinic receptors that are predominantly of the M₂ subtype and that couple to the inhibitory G protein G_i (5, 6). Stimulation of these receptors causes inhibition of adenylyl cyclase activity and a decrease in heart rate as well as in β -adrenoceptor-mediated increases in ventricular contractility (6–9).

We have shown recently that in healthy volunteers, the M₁ muscarinic receptor antagonist pirenzepine in low doses caused parasympathomimetic effects in an age-dependent manner: it decreased resting heart rate in young volunteers to a significantly greater extent than in older volunteers (10). The aim of this study was to investigate possible mechanisms underlying this age-dependent decrease in muscarinic receptor function in the human heart. For this purpose, we assessed (a) in vivo in six young (age 26 yr) and six older volunteers (age 61 yr) whether pirenzepine might also reduce isoprenaline-induced heart rate increases in an age-dependent manner (b) in vivo in five heart transplant recipients whether an intact parasympathetic innervation is essential for the heart rate-decreasing effect of pirenzepine; and (c) in vitro in right atria from 39 patients of different ages (range 5 d–76 yr) whether there are age-dependent changes in the density (assessed by [³H]N-methylscopolamine ([³H]NMS)¹ binding) and function (assessed by carbachol-induced inhibition of forskolin-stimulated adenylyl cyclase activity) of muscarinic receptors.

Methods

In vivo studies

PIRENZEPINE EFFECTS IN HEALTHY VOLUNTEERS

Subjects. The study included six young healthy male volunteers (23–29 yr old, mean age 26.2±0.6 yr) and six older healthy volunteers (53–68 yr old, mean age 60.8±1.4 yr). At baseline, systolic/diastolic blood pressure was 110±2/76±1 vs. 123±3/78±2 mm Hg; heart rate was 61.9±2 vs. 60.5±2 beats per min (bpm); and body weight was 72.5 vs. 66.5 kg in young and older volunteers, respectively. Normal health status was previously established by medical history, physical examination, biochemical and hematologic screening, and electrocardiogram (ECG). All volunteers had undergone this examination to exclude asthma, chronic pulmonary disease, diabetes mellitus, hypertension, cardiac disease, and other symptoms pertaining to the cardiovascular system. They were of average physical fitness, and had not taken any medication during the last 6 wk before entry into the study. All had fasted from 11 p.m. on the evening before the study. Smoking was prohibited on the morning of the study.

The experimental procedure and its purpose were explained thoroughly to all subjects, and written consent was obtained. The study protocol was approved by the Ethical Committee of the University of Halle-Wittenberg.

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1. Abbreviations used in this paper: Ach, acetylcholine; DSN, donor sinus node; HTX, heart transplant; NMS, N-methylscopolamine; NYHA, New York Heart Association; RSN, recipient sinus node; STI, systolic time interval.

Study protocol. All experiments were carried out in a quiet air-conditioned room with the volunteers in supine position. Subjects were studied on three occasions, with at least 1 wk between each treatment regimen. After arrival at the clinical laboratory at 7 a.m. and after placement of the instruments, indwelling polythene catheters were positioned in an antecubital vein of each arm. Blood samples were drawn from the left arm, and drugs were administered into the right arm. After at least 30 min of rest in supine position, the experiments were begun.

Examination of muscarinic receptor function. For the isoprenaline response, continuous systemic intravenous (i.v.) infusion of isoprenaline was administered with a perfusion pump (B. Braun Melsungen AG, Melsungen, Germany). Incremental doses of isoprenaline (sequential doses of 3.5, 7, 17, and 35 ng/kg/min for 10 min each) were infused. These experiments were repeated with the volunteers pretreated with atropine (15 µg/kg/body wt as bolus, followed by i.v. infusion of 0.15 µg/kg/min until the end of the isoprenaline infusion). 30 min after the start of the atropine administration, i.v. isoprenaline dose-response curves were assessed. Blood pressure and systolic time intervals (STIs) were determined immediately before atropine administration, immediately before the start of the isoprenaline infusion, and at the end of each dose step of the isoprenaline infusion as detailed elsewhere (11, 12).

For the pirenzepine experiments, volunteers were infused i.v. with 17 ng/kg/min isoprenaline for 60 min. 20 min after the beginning of the isoprenaline infusion, pirenzepine was injected over 5 min in doses of 0.32 and 0.64 mg, with each dose step requiring 20 min as described recently (10). Blood pressure and STIs were determined at 5, 10, 15, and 20 min of each dose step. All measurements were performed with the subjects in supine position.

Measurements. Systolic and diastolic (phase V) blood pressure were measured with an automatic device (Dinamap; Critikon, Johnson & Johnson Medical, Inc., Norderstedt, Germany). Measurements of STIs were obtained noninvasively from simultaneous recordings of an ECG lead, a phonocardiogram, and a carotid pulse tracing at high paper speed (100 mm/s) using a multichannel recorder (Bioset 8000; Hörmann Medizintechnik, Zwönitz, Germany). The following parameters were measured: 20 RR intervals (ms) of the ECG from which heart rate (bpm) was calculated; duration of the electromechanical systole (ms); duration of the left ventricular ejection time (ms); and duration of preejection period (ms) derived mathematically by subtracting left ventricular ejection time from electromechanical systole (for details, see references 10–12).

PIRENZEPINE EFFECTS IN HEART TRANSPLANT (HXT) RECIPIENTS

Five orthotopic cardiac transplant (HTX) patients were studied during routine surveillance follow-up on the occasion of routine endomyocardial biopsy. The study protocol was approved by the Ethical Committee of the University of Halle-Wittenberg, and written informed consent was obtained from all patients. Patients were included in the study if they showed no evidence of acute infection or clinical signs of acute rejection. Patients were in a stable maintenance immunosuppressed state (the standard regimen at our institution consists of cyclosporine A and azathioprine, exclusively), and showed no evidence of graft atherosclerosis proven by coronary angiography within the previous 6 mo, or evidence of rejection at the time of investigation proven by histology of the biopsies obtained 30 min after the end of the infusion study. All patients showed histology grades of A0–A2 IUC.

The average cyclosporine A trough level was 229.2 ± 39.0 ng/ml. The clinical data for the patients at the time of study are given in Table I.

In addition to cyclosporine A and azathioprine, patients were treated with angiotensin-converting enzyme inhibitors ($n = 2$), diuretics ($n = 3$), nitrates ($n = 5$), acetyl salicylic acid ($n = 5$), and prednisolone ($n = 1$), alone or in combination; none of the patients had received catecholamines and/or cholinergic drugs for at least 6 wk before the study.

Study protocol. After physical examination, a conventional bi-

Table I. Clinical Characteristics of the Five HTX Recipients

Age (yr)	53.6 ± 3.7
Time after transplantation (d)	296.6 ± 74
Baseline hemodynamics (before pirenzepine administration)	
Systolic blood pressure (mm Hg)	142.4 ± 5.5
Diastolic blood pressure (mm Hg)	89.7 ± 3.1
Heart rate (bpm)	
RSN	67.8 ± 6.8 (range 59–94)
DSN	84.0 ± 3.8 (range 78–99)

Mean ± SEM of five experiments.

opsy sheath was placed into the right internal jugular vein. The patients were prepared for ECG and blood pressure monitoring, and rested for 30 min in supine position. Thereafter, an intraatrial multiple bipolar probe was placed such that recipient sinus node (RSN) and donor sinus node (DSN) rates could be recorded simultaneously.

Pirenzepine was injected over 5 min in increasing doses of 0.05, 0.10, 0.25, 0.50, 1.0, and 10 mg bolus, with every dose step requiring 20 min. Systolic and diastolic blood pressure (phase V) were measured with the Dinamap automatic device. Heart rate and surface ECG as well as peripheral capillary oxygen saturation were recorded continuously. The RSN (remaining sinus node of the recipient) and DSN (actual transplanted heart) were measured via the intracardiac electrode catheter (Bard Electrophysiology, Bellerica, MA) and recorded by a 12-channel recorder at a paper speed of 50 mm/s in the last min of each dose step. Endomyocardial biopsies were taken 30 min after the end of the infusion using a commercial right ventricular biptome (9 French, 2.8-mm head diameter; Scholten Surgical Instruments, Redwood City, CA).

In vitro studies

Right atrial appendages were obtained from 14 children (8 female, 6 male) with acyanotic congenital heart disease who underwent open heart surgery because of ventricular septal defect ($n = 5$), atrioventricular septal defect ($n = 3$), atrial septal defect ($n = 3$), or aortic stenosis ($n = 3$). Their parents had given informed written consent. None of the children suffered from acute heart failure or had been treated with sympathomimetic (i.e., catecholamines) or cholinergic drugs for at least 3 wk before surgery.

Anesthesiological premedication usually consisted of 1 mg/kg i.v. diazepam given immediately before surgery in infants with a body weight < 10 kg, 0.3 mg/kg rectally in children with a body weight of 10–25 kg, and 0.4 mg/kg p.o. in children with a body weight > 25 kg. The operation was carried out under balanced anesthesia with midazolam and isoflurane (up to 1.5 vol percent to effects). N₂O was avoided in all cases. Fentanyl was added for analgesia. Controlled ventilation was performed with an inspired oxygen fraction of 50–100%. Right atrial appendages were removed immediately after installation of the cardiopulmonary bypass.

Right atrial appendages were also obtained from 25 adult patients (18 male, 7 female) undergoing elective coronary artery bypass graft without apparent heart failure (New York Heart Association [NYHA] class I–II, $n = 14$) or undergoing open heart surgery because of aortic valve disease ($n = 9$, NYHA class I–II except one patient class II–III) or mitral valve insufficiency ($n = 2$, NYHA class III), having given informed written consent. None of these patients had been treated with β-adrenoceptor agonists or cholinergic drugs for at least 6 wk before the operation. However, patients had received nitrates ($n = 17$), β-adrenoceptor antagonists ($n = 9$), calcium antagonists ($n = 6$), angiotensin-converting enzyme inhibitors ($n = 10$), diuretics ($n = 7$), digitalis glycosides ($n = 4$), heparin ($n = 13$), 3-hydroxy-3-methylglutaryl CoA reductase inhibitors ($n = 8$), acetyl salicylic acid ($n = 5$), and antibiotics ($n = 4$), alone or in combination.

Premedication usually consisted of 0.5 mg lorazepam given orally the evening before and 0.5–1.0 mg given orally the morning of surgery. The operation was carried out under balanced anesthesia consisting of midazolam, fentanyl, and pancuronium bromide as muscle relaxant, as well as isoflurane (0.6–1.0% vol/vol) for narcosis; N₂O was avoided in all cases. Controlled ventilation was performed with an inspired oxygen fraction of 50–100%. In all patients, right atrial appendages were removed during installation of the cardiopulmonary bypass. Immediately after excision, all specimens were quickly frozen in liquid nitrogen.

Patients were arbitrarily divided into three age-groups: children (group A, < 20 yr), adults (group B, 20–50 yr), and old subjects (group C, > 50 yr). The mean ages of the three groups were as follows: group A, 7.3±1.5 yr (range 5 d–15 yr), *n* = 14; group B, 32.6±2.5 yr (range 22–43 yr), *n* = 9; and group C, 66.8±1.8 yr (range 55–76 yr), *n* = 16.

Radioligand binding studies

For radioligand binding studies, tissues were minced with scissors and homogenized in 10 vol of ice-cold 1 mM KHCO₃ with an Ultra Turrax (Janke & Kunkel, Staufen, Germany) twice for 20 s at full speed in 1-min intervals. The homogenate was diluted with 1 mM KHCO₃ to 50 ml, and centrifuged at 700 *g* for 15 min; the supernatant was passed through four layers of cheesecloth and centrifuged at 21,000 *g* for 45 min; the pellets were washed once by resuspension and recentrifugation. The final pellets were resuspended in incubation buffer (10 mM Na₂HPO₄, 10 mM NaH₂PO₄, pH 7.4) to yield a protein concentration of 0.6–0.7 mg/ml. Protein content was determined by the method of Bradford (13) using bovine IgG as the standard.

[³H]NMS and all drugs used in these experiments were prepared in 10 mM Na₂HPO₄, 10 mM NaH₂PO₄ buffer, pH 7.4. If not stated otherwise, an aliquot of the membrane suspension (150 µl) was incubated with [³H]NMS in a final volume of 250 µl. Incubations were carried out for 60 min at 25°C and terminated by adding 10 ml of washing buffer to the entire incubation mixture, followed by rapid filtration over Whatman GF/C glass fiber filters that had been soaked previously in 1 mM unlabeled NMS.

Each filter was washed with an additional 10 ml of washing buffer. The filters were then dried and transferred to vials containing 5 ml of Lumasafe plus scintillator (Lumac-LSC B.V., Groningen, The Netherlands), and radioactivity was determined in a scintillation counter (LS 6000; Beckman Instruments, Inc., Fullerton, CA) at an efficiency of 42%. Nonspecific binding of [³H]NMS was defined as radioactivity bound to membranes that was not displaced by a high concentration (1 µM) of atropine. Specific binding of [³H]NMS was defined as total binding minus nonspecific binding, and was 80–90% (at 0.5–3 nM) and 70% (at 10 nM) of [³H]NMS.

For determination of the density of muscarinic receptors in membranes from right atrial tissues, the amount of specifically bound [³H]NMS was determined at six different concentrations ranging from 0.1 to 10 nM.

Adenylyl cyclase determination

Adenylyl cyclase activity was assessed as described by Salomon et al. (14) with minor modifications as detailed elsewhere (15). Membranes (35–45 µg of protein) were incubated for 10 min at 30°C in a final volume of 100 µl containing 40 mM Hepes buffer, pH 7.4, 5 mM MgCl₂, 1 mM EDTA, 10 µM GTP, 500 µM ATP, ~1,000,000 cpm [α -³²P]-ATP, 100 µM cAMP, and an ATP regenerating system (5 mM phosphocreatine and 50 U/ml creatine phosphokinase) in the presence or absence of isoprenaline (10 µM), forskolin (10 µM), and various concentrations of carbachol (10 nM–100 µM). The reaction was stopped by addition of 100 µl buffer containing 50 mM Tris, 40 mM ATP, 1.4 mM cAMP, 2% SDS, and [³H]cAMP (~10,000 cpm) at pH 7.5; 800 µl water was then added.

The mixture was poured into Dowex AG 50W-X4 anion-exchange columns (200–400 mesh, hydrogen form), and ATP was eluted twice with 1 ml water. The Dowex columns were then placed over neutral

Table II. Adenylyl Cyclase Activity in Right Atrial Membranes from Two Groups of Patients without Apparent Heart Failure Undergoing Open Heart Surgery

	Adenylyl cyclase activity	
	Group A (<i>n</i> = 7)	Group C (<i>n</i> = 9)
	pmol cAMP/mg protein/min	
Basal	62.1±9.2	39.6±4.4*
GTP	81.9±5.0	57.8±4.1‡
ISO	42.6±5.6	26.4±2.7§
FOR	1104.9±60.5	703.1±53.7

**P* = 0.0324, ‡*P* = 0.0021, §*P* = 0.0148, and ||*P* < 0.0003 vs. the corresponding values for group A (nonpaired two-tailed Student's *t* test). Right atrial adenylyl cyclase activity is given in net increase in activity upon stimulation in picomoles of cAMP per milligram of protein per minute (mean±SEM). GTP, 10 µM GTP - basal. ISO, 10 µM isoprenaline - GTP. FOR, 10 µM forskolin - GTP. Group A, < 20 yr; group C, > 50 yr.

alumina columns, and cAMP was eluted from the Dowex columns with 4 ml water. The alumina columns were placed over scintillation vials, and the cAMP was eluted from the alumina columns with 5 ml 0.1 M imidazole (pH 7.3). Lumasafe plus scintillator (15 ml) was added to the eluate and counted at 42% efficiency. The determined amount of [³H]cAMP in each vial was used to calculate the recovery of cAMP for each column, and the amount of [³²P]cAMP collected from each column was corrected for the recovery rate (usually 70–80%).

Statistical evaluations

Data given are mean±SEM of *n* experiments. The equilibrium dissociation constant (*K*_D) and maximal number of binding sites (*B*_{max}) for [³H]NMS were calculated from plots according to Scatchard (16). For calculation of EC₅₀ values of carbachol-induced adenylyl cyclase inhibition, data were fitted to sigmoid curves; in these calculations, the bottom of the curve was fixed at 0% inhibition, and inhibition of adenylyl cyclase by 100 µM carbachol was considered maximal inhibition; the Hill slopes were kept variable. From these curves, EC₅₀ values were obtained that were not considerably different (maximal difference a factor of 2) from those calculated with a Hill slope fixed at 1.0. Experimental data were analyzed by computer-supported iterative nonlinear regression analysis using the InPlot program (GraphPAD Software for Science, San Diego, CA).

Linear regression analysis of the data was performed by the least squares method (model: [³H]NMS binding sites = *a* × age + *b*). Statistical significance of differences was analyzed by one-way ANOVA followed by Bonferroni testing for multiple comparisons and by repeated measures analysis (17), or, if appropriate, by nonpaired, two-tailed Student's *t* test (for specification, see citations to the figures and Table II). A *P* value < 0.05 was considered significant. Statistical calculations were performed with the InStat program (GraphPAD Software for Science) or with the SPSS Advanced Statistics program, version 7.5 (SPSS Inc., Chicago, IL).

Drugs used

For infusion, atropine (Atropin-sulfat solution) was purchased from Fresenius AG (Bad Homburg, Germany), isoprenaline (Aleudrina) from Boehringer Ingelheim (Ingelheim, Germany), and pirenzepine (Gastrozepin) from Thomae (Biberach an der Riss, Germany). Atropine sulfate, (–)-isoprenaline bitartrate, carbachol chloride, and forskolin were obtained from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany), *N*-methyl-scopolamine nitrate from Merck KGaA (Darmstadt, Germany), [³H]NMS (specific activity 85 Ci/mmol), [α -³²P]ATP (specific activity 30 Ci/mmol), and [³H]cAMP (specific

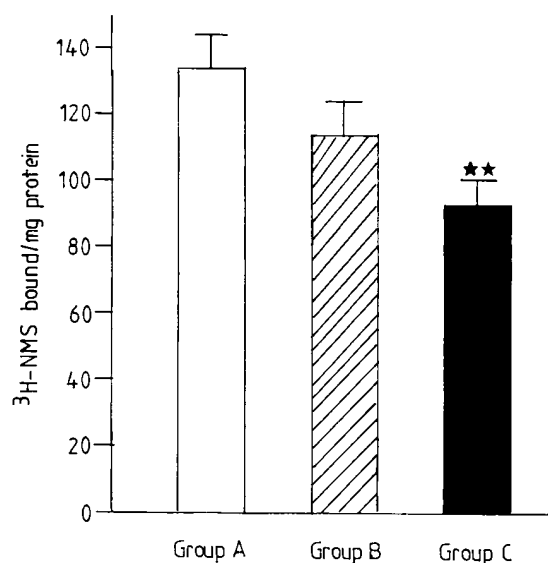


Figure 1. Muscarinic receptor density in right atria from three groups of patients undergoing open heart surgery: group A (< 20 yr old, white bars), group B (20–50 yr old, striated bars), and group C (> 50 yr old, black bars) (see Methods). Right atrial muscarinic receptor density is expressed in femtomoles of [^3H]NMS specifically bound per milligram of protein (Mean \pm SEM). K_D values for [^3H]NMS were 1.43 ± 0.26 nM ($n = 14$) for group A, 1.35 ± 0.27 nM ($n = 9$) for group B, and 1.43 ± 0.34 nM ($n = 16$) for group C. ** $P < 0.01$ vs. group A (one-way ANOVA followed by the Bonferroni test for multiple comparisons).

activity 44.5 Ci/mmol) from New England Nuclear (Dreieich, Germany). All other chemicals were of the purest commercially available grade.

Results

In vitro studies

Right atrial muscarinic receptor density. The mean right atrial muscarinic receptor density decreased across the three groups,

from group A (patients < 20 yr old) via group B (patients 20–50 yr old) to group C (patients > 50 yr old; Fig. 1); the difference between groups A and C was highly significant. Moreover, for all 39 patients there was a significant negative correlation between right atrial muscarinic receptor density and the age of the patients (Fig. 2). K_D values for [^3H]NMS were not significantly different in the three groups (see citation to Fig. 1).

Right atrial adenylyl cyclase activity. Because of limited amount of tissue, we could assess right atrial adenylyl cyclase activity in only seven patients from group A (mean age 10.4 ± 1.8 yr) and nine patients from group C (mean age 67.3 ± 2.7 yr). In agreement with our recently published data (15), basal, 10 μM GTP-, 10 μM isoprenaline-, and 10 μM forskolin-stimulated adenylyl cyclase activity was significantly higher in group A than in group C (Table II).

The muscarinic receptor agonist carbachol (10 nM–100 μM) inhibited 10 μM forskolin-stimulated adenylyl cyclase activity in a concentration-dependent manner (Fig. 3). This inhibitory effect was antagonized by the muscarinic receptor antagonist atropine with a pK_i value of 8.42 (data not shown), indicating that it is mediated by muscarinic receptor stimulation.

However, inhibition of 10 μM forskolin-stimulated adenylyl cyclase activity was significantly more pronounced at each concentration of carbachol in group A than in group C (Fig. 3). In addition, the EC_{50} value for carbachol was significantly lower in group A (0.76 ± 0.22 μM) than in group C (7.2 ± 2.1 μM , $P < 0.05$).

In vivo studies

Pirenzepine effects in young and older volunteers. As mentioned in the Introduction, we have shown recently that pirenzepine in doses of 0.04–1.25 mg decreases basal heart rate in healthy volunteers; maximum effects were obtained at doses of 0.32 and 0.64 mg pirenzepine (10). Therefore, we used these two doses to study whether pirenzepine might also decrease isoprenaline-induced increases in heart rate, and whether this effect is also diminished in older volunteers.

To assess the dose of isoprenaline that increases heart rate by ~ 20 bpm, we first performed dose–response curves for iso-

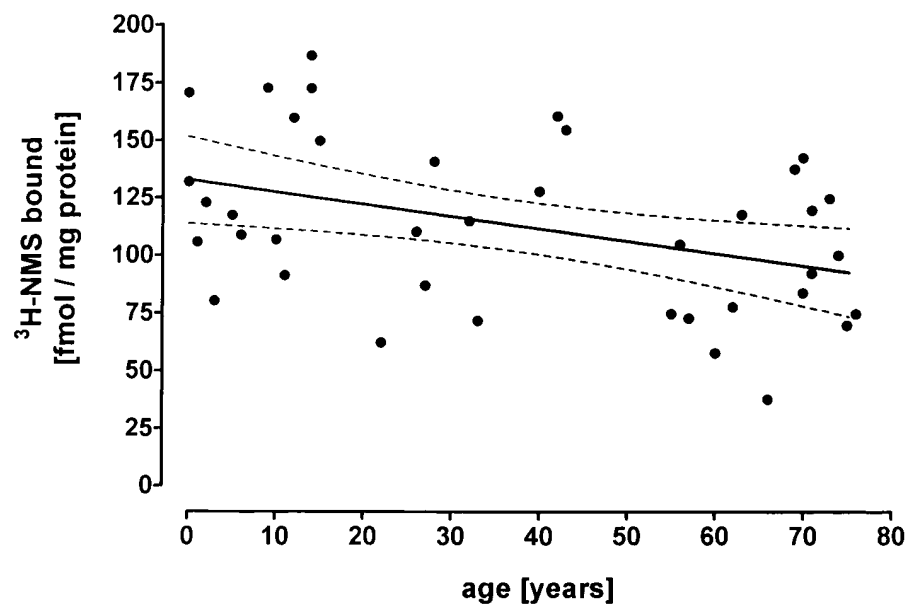


Figure 2. Correlation between right atrial muscarinic receptor density and age of 39 patients undergoing open heart surgery. y axis, right atrial muscarinic receptor density in femtomoles of [^3H]NMS specifically bound per milligram of protein; x axis, age of the patients in years. $y = -0.531x + 132.9$; $n = 39$; r (correlation coefficient) = -0.3946 ; $P = 0.0129$. Dotted curves, 95% confidence limit for the regression line.

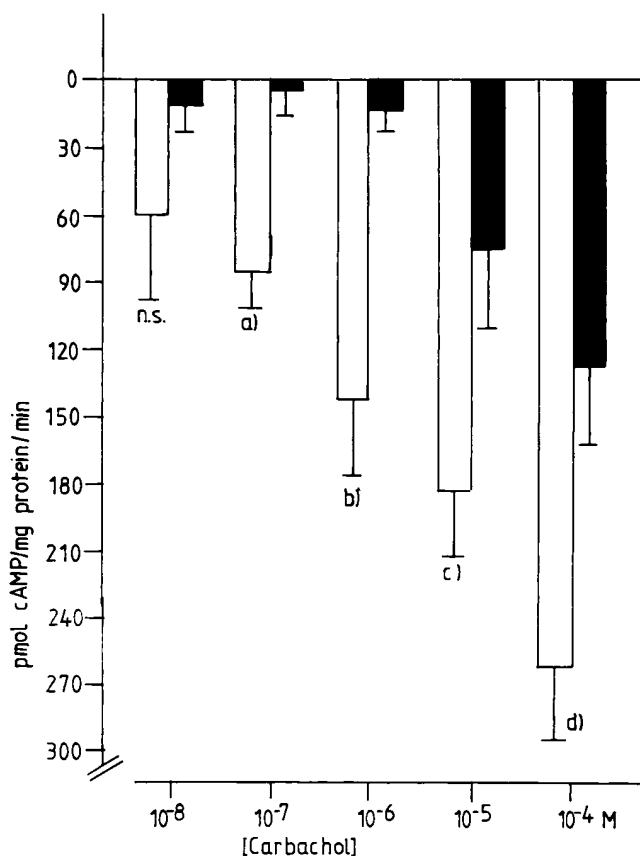


Figure 3. Inhibition of 10 μ M forskolin-stimulated adenylyl cyclase activity by carbachol in right atria from seven patients of group A (< 20 yr old, white bars) and nine patients of group C (> 50 yr old, black bars) (see Results). y axis, net decrease in 10 μ M forskolin-stimulated right atrial adenylyl cyclase activity upon carbachol stimulation in picomoles of cAMP formed per milligram of protein per minute (mean \pm SEM); x axis, molar concentration of carbachol. For

prenaline-induced increases in heart rate in six young and six older volunteers. As shown in Fig. 4, the dose-response curves for isoprenaline-induced increases in heart rate were very similar in both groups; however, when these experiments were repeated in the presence of atropine, thereby inhibiting vagal tone, isoprenaline-induced increases in heart rate were enhanced significantly in the young volunteers, but were affected only marginally and not significantly in the older volunteers. Moreover, atropine increased resting heart rate to a significantly greater extent in the young volunteers ($+ 36.9 \pm 4.3$ bpm) than in the older volunteers ($+ 18.8 \pm 4.0$ bpm, $P < 0.02$). Based on these experiments, we choose a dose of 17 ng/kg/min isoprenaline for the pirenzepine experiments.

At this dose, isoprenaline increased heart rate by 20.7 ± 1.4 bpm in the young and 24.0 ± 4.1 bpm in the older volunteers. Heart rate remained elevated throughout the 1-h infusion. When pirenzepine was added 20 min after the start of isoprenaline infusion, heart rate declined rapidly; however, at both doses of pirenzepine, the decrease in heart rate was markedly more pronounced in the young than in the older volunteers (Fig. 5). Thus, at the 0.64 mg dose of pirenzepine, heart rate in young volunteers returned almost to baseline levels (despite the continuous infusion of isoprenaline), whereas heart rate in the older volunteers was still increased by 13–14 bpm (Fig. 5). On the other hand, pirenzepine in neither dose significantly affected isoprenaline-induced changes in blood pressure and STI (data not shown).

Studies in heart transplant recipients. For this study, pirenzepine was given in doses of 0.05–10 mg. Application of the intracavitary multipolar probe allowed assessment of sinus node

forskolin-stimulated adenylyl cyclase activities, see Table II. n.s., $P = 0.1960$; a), $P = 0.0083$, b), $P = 0.0028$, c), $P = 0.041$, and d), $P = 0.0083$ vs. the corresponding values for group C (nonpaired, two-tailed Student's t test).

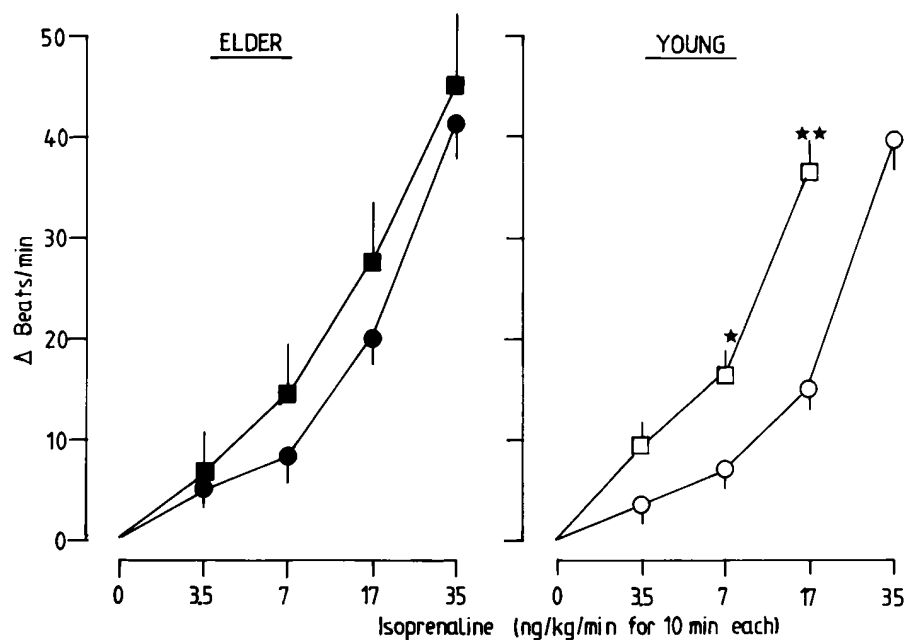


Figure 4. Effects of atropine (15 μ g/kg/body as bolus, followed by i.v. infusion of 0.15 μ g/kg/min until the end of the isoprenaline infusion) on isoprenaline infusion-induced heart rate increases in six older (left, filled symbols) and six young volunteers (right, open symbols) (see Methods). y axis, heart rate increases (Δ Beats/min); x axis, dose of isoprenaline in nanograms per kilogram per minute for 10 min each. Mean \pm SEM of six experiments each. Baseline heart rate in young volunteers was 63.7 ± 3.4 bpm before and 97.3 ± 4.3 bpm after 30 min of atropine infusion; in older volunteers, baseline heart rate was 61.5 ± 2.9 bpm before and 82.4 ± 8.7 bpm after atropine infusion. ** $P < 0.01$, * $P < 0.05$ vs. the corresponding values in the absence of atropine (one-way ANOVA followed by the Bonferroni test for multiple comparisons). When the data were analyzed by repeated measures analysis (17), isoprenaline dose-response curve in the presence of atropine (squares) was significantly different from control (circles) in young ($P = 0.002$) but not in older volunteers ($P = 0.312$).

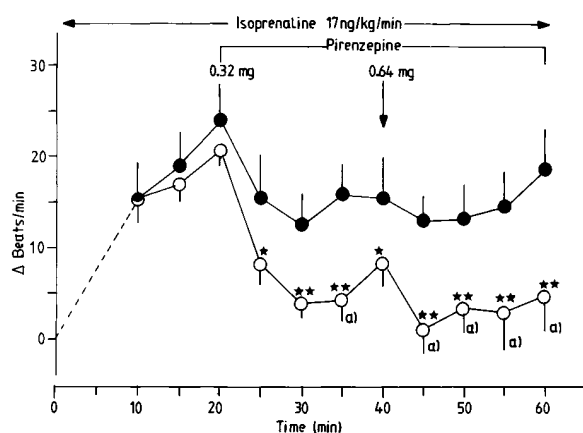


Figure 5. Effects of pirenzepine (0.32 and 0.64 mg i.v. bolus) on isoprenaline infusion (17 ng/kg/min throughout)–induced increases in heart rate in six young (○) and six older volunteers (●). After 20 min of isoprenaline infusion, pirenzepine was added, and heart rate was assessed (see Methods). y axis, heart rate changes (Δ Beats/min); x axis, time in minutes. Mean \pm SEM of six experiments each. ** $P < 0.01$, * $P < 0.05$ vs. the corresponding value after 20 min of isoprenaline infusion (one-way ANOVA followed by the Bonferroni test for multiple comparisons); a, $P < 0.05$ vs. the corresponding values for older volunteers (one-way ANOVA followed by the Bonferroni test for multiple comparisons). When the effects of pirenzepine on isoprenaline-induced heart rate increases were analyzed by repeated measures analysis (17), the difference between young and older volunteers was just under statistical significance ($P = 0.065$).

rates in the native atrial cuffs as well as in the transplanted donor hearts. Pirenzepine in doses of 0.05–0.5 mg caused dose-dependent decreases in sinus node rate in the native atrial cuffs but not in the transplanted donor hearts (Fig. 6); the maximal decrease at 0.5 mg was 4.9 ± 1.1 bpm. At the 10-mg dose, pirenzepine increased heart rate significantly in the RSN, by 10.5 ± 2.4 bpm, and again, did not affect heart rate significantly in the DSN (Fig. 6).

Discussion

In healthy volunteers, atropine and the M_1 -selective muscarinic receptor antagonist pirenzepine cause biphasic effects on resting heart rate: in low (M_1 -selective) doses, they decrease heart rate, whereas in higher doses, they lead to the well-known increase in heart rate (18, 19). We have shown recently that in healthy volunteers, the heart rate–decreasing effects of low doses of atropine and pirenzepine were significantly greater in young than in older volunteers (10). The results of this study confirm and extend these findings: they show that pirenzepine in low doses can also inhibit isoprenaline-induced heart rate increases; again, this effect was markedly more pronounced in young than in older volunteers.

The mechanism of the heart rate–decreasing effects of low doses of pirenzepine is not completely understood. According to its affinity profile for M_1 , M_2 , M_3 , and M_4 muscarinic receptors at the doses used in these experiments, pirenzepine should act selectively at M_1 muscarinic receptors (20). Therefore, it has been suggested that the heart rate–decreasing effect of pirenzepine is due to inhibition of presynaptic M_1 muscarinic autoreceptors; this leads to an increased release of endogenous acetylcholine (ACh) that decreases heart rate via stimulation

of postsynaptic M_2 muscarinic receptors (10, 21). The existence of such presynaptic ACh autoreceptors modulating ACh release has been demonstrated in chicken, rat, rabbit, and guinea pig atria but not yet in human atria: they are species-dependent either of the M_1 (chicken [22, 23]) or the M_2 subtype (guinea pig [22], rat [24], rabbit [25]).

Studies in HTX patients support the idea of an involvement of presynaptic receptors modulating ACh release in the negative chronotropic effects of atropine and pirenzepine. Epstein et al. (26) have shown that in HTX patients, atropine decreases heart rate only in the (innervated) native RSN and not in the (denervated) DSN, and almost identical results were obtained in our study in HTX patients: pirenzepine in low doses decreased heart rate only in the native RSN but not in the DSN. These results strongly indicate that pirenzepine (and atropine) can exert their negative chronotropic effects only when innervation is intact; therefore, it is quite likely that the negative chronotropic effects of low doses of pirenzepine are due to inhibition of presynaptic M_1 muscarinic receptors, thereby releasing endogenous ACh, although the final experimental proof of the existence of such presynaptic M_1 muscarinic receptors in the human right atrium is still lacking. It should be noted that in HTX patients, sympathetic reinnervation can occur long-term after transplantation (27, 28), and

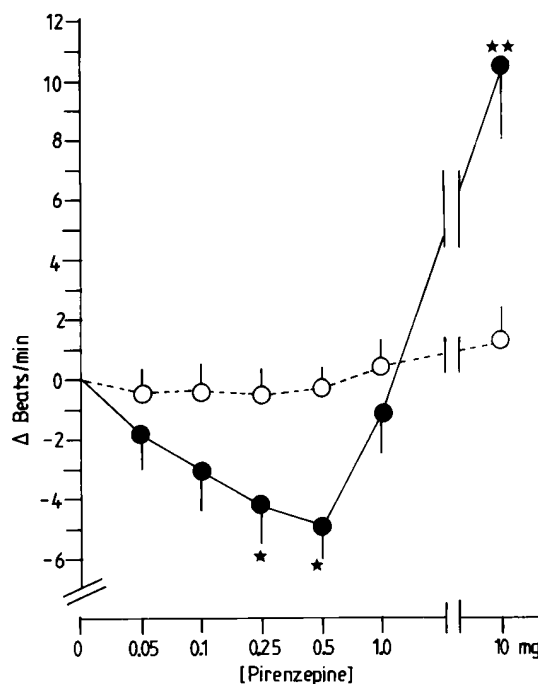


Figure 6. Effects of pirenzepine in five heart transplant recipients on heart rate in the recipient's native (●) and transplanted (○) sinus node (see Methods). Pirenzepine was injected i.v. in six graded doses of 0.05–10 mg bolus each over 5 min. Each dose step took 20 min. Mean \pm SEM of five experiments. y axis, heart rate changes (Δ Beats/min); x axis, dose of pirenzepine in milligrams. ** $P < 0.01$, * $P < 0.05$ vs. the corresponding value before pirenzepine (one-way ANOVA followed by the Bonferroni test for multiple comparisons). When the effects of pirenzepine on heart rate in the recipient's native and transplanted sinus node were analyzed by repeated measures analysis (17), the effects of pirenzepine were significant in the native ($P = 0.004$) but not in the transplanted sinus node ($P = 0.392$).

very recently, preliminary evidence for vagal reinnervation has also been obtained (29). However, in this study, HTX patients were investigated who had undergone transplantation less than a year before (see Table I); therefore, it is very unlikely that any sympathetic or parasympathetic reinnervation occurred in these patients.

The negative chronotropic effect of pirenzepine on resting heart rate (10) as well as on isoprenaline-stimulated heart rate (this study) was markedly reduced in the elderly. Assuming that pirenzepine induces this negative chronotropic effect by inhibition of presynaptic M_1 muscarinic receptors with subsequent release of endogenous ACh, the age-dependent reduction in this effect could be due either to a diminished release of ACh in the elderly or to a diminished response of the postsynaptic M_2 muscarinic receptors to the released ACh. To address this question, in this study, we have measured the density of muscarinic receptors in right atria from patients of ages ranging from 5 d to 76 yr using [3 H]NMS binding studies. By this method, only M_2 muscarinic receptors are identified in the human heart (5, 6). There was a significant decline in receptor number from group A (patients < 20 yr old) via group B (patients 20–50 yr old) to group C (patients > 50 yr old); in addition, right atrial muscarinic receptor density was significantly negatively correlated with patient age. In this study, muscarinic receptor density has been assessed in crude membrane preparations from right atria. It has been shown that the aging heart is associated with a loss of myocytes and with fibrosis, which might contribute considerably to the age-dependent decrease in muscarinic receptors observed in this study. We cannot exclude this possibility, but it seems very unlikely, since several groups have demonstrated that in severely failing human hearts that exhibit a greater loss of cardiomyocytes and fibrosis than the aging human heart, muscarinic receptor density is unchanged compared with nonfailing human hearts (7, 30, 31). The age-dependent decrease in muscarinic receptor density was accompanied by an age-dependent decline in carbachol-induced inhibition of forskolin-stimulated adenylyl cyclase mediated via M_2 muscarinic receptor stimulation; moreover, affinity of carbachol was reduced significantly in aging atrial tissues (Fig. 3). Taken together, these results indicate that an age-dependent decrease in right atrial M_2 muscarinic receptor density and function might contribute considerably to the age-dependence of the negative chronotropic effect of pirenzepine. Whether the decrease in receptor density or the decrease in agonist affinity is more important cannot be determined from this study; however, the fact that correlation between age and receptor density was weak and that many points were outside the 95% confidence limit might favor the idea that decreased agonist affinity is more important for the age-dependent decrease in M_2 receptor function. In addition, since we cannot measure ACh in the intact human heart, we cannot exclude completely the possibility that a diminished release of ACh in the elderly might also contribute to this effect.

It is interesting to note that in this study, the decrease in isoprenaline-increased heart rate induced by pirenzepine was greater in both young and older volunteers than we had recently observed for resting heart rate (Fig. 5, and reference 10). This is obviously due to the very well-known phenomenon, “accentuated antagonism,” where the inhibitory effects of muscarinic receptor stimulation are enhanced when sympathetic activity is increased (32). In contrast, in this study, isoprenaline-induced shortening of STIs (as a measure of positive

inotropism) was not affected significantly by low dose pirenzepine, although it has been shown in vitro and in vivo that β -adrenoceptor agonist-induced increases in left ventricular contractile force can be inhibited in a concentration-dependent manner by carbachol via M_2 muscarinic receptor stimulation (6–9, 33, 34). On the other hand, in vivo and in vitro studies have shown that muscarinic stimulation does not affect basal contractile force of the left ventricular myocardium (6, 8, 9, 33), and muscarinic receptor blockade by atropine augmented only slightly β -adrenoceptor agonist-induced increases in left ventricular contractility (9, 34), if at all (8, 10). These weak parasympathomimetic effects are very likely due to the fact that human ventricular myocardium is only sparsely parasympathetically innervated (35, 36). Such a sparse parasympathetic innervation might also explain the findings of this study that low dose pirenzepine failed to antagonize isoprenaline-induced increases in ventricular contractility: it might well be that the concentration of ACh released under these conditions is too low to lead to a considerable stimulation of the M_2 muscarinic receptor. Another possibility is that in ventricular myocardium, in contrast to the atria, presynaptic M_1 muscarinic receptors modulating ACh release may not exist. Finally, the fact that as in rat, rabbit (37), and chick hearts (38), in human ventricular myocardium the number of M_2 muscarinic receptors is less than in atrial tissue (6), might also contribute to the lack of the negative inotropic effect of low dose pirenzepine.

It is generally accepted that in the human heart, β -adrenoceptor function declines with aging (for reviews, see references 2, 3, and 39). The mechanism underlying this effect is not completely understood, but it might be due to an age-dependent decrease in the catalytic unit of adenylyl cyclase (right atrium [15]) or decrease in β -adrenoceptor number (left ventricle [40]). However, in this study, isoprenaline infusion induced the same increase in heart rate in young as it did in older volunteers. It has been shown that during isoprenaline infusion, vagal tone increases, thus damping isoprenaline effects (41). As discussed above, human right atrial M_2 muscarinic receptor function declines with age. Thus, the depressing effects of increases in vagal tone on isoprenaline infusion-induced heart rate increases are lower in older than in young volunteers; this obviously can mask the age-dependent decrease in cardiac β -adrenoceptor-mediated effects. In fact, after blockade of vagal activity by atropine, isoprenaline showed significantly greater increases in heart rate in the young than in the older volunteers (Fig. 4), thus supporting our recent findings that right atrial β -adrenoceptor function is decreased in the elderly (15). These results are in good agreement with recently published data by White and Leenen (42), who also found a decreased chronotropic response to isoprenaline in older volunteers only after ganglionic blockade by trimethaphan; moreover, these authors demonstrated that an age-dependent decrease in positive inotropic responses to isoprenaline could also be obtained only after trimethaphan treatment. And finally, we have found recently that only in the presence of atropine could differences in the heart rate response to noradrenaline and tyramine infusion be demonstrated between young and older volunteers (12).

In conclusion, in the human heart, the paradoxical parasympathomimetic effect of low doses of the muscarinic receptor antagonist pirenzepine (and atropine) is very likely due to inhibition of presynaptic M_1 muscarinic autoreceptors, thereby enhancing the release of endogenous ACh; the released ACh

decreases heart rate via activation of postsynaptic M₂ muscarinic receptors. The heart rate-decreasing effect of endogenous ACh is diminished with age, because M₂ muscarinic receptor density and function decline. Such an age-dependent decrease in M₂ muscarinic receptor density may well lead to a decrease in cardiac parasympathetic activity, as suggested previously (43), and might contribute significantly to the well-known decrease in baroreflex activity with aging (2–4).

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