

Increased Expression of G_i-Coupled Muscarinic Acetylcholine Receptor and G_i in Atrium of Elderly Diabetic Subjects

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In an ongoing investigation of the effects of age on G protein-coupled receptor signaling in human atrial tissue, we have found that the density of atrial muscarinic acetylcholine receptor (mAChR) increases with age but reaches statistical significance only in patients with diabetes. Moreover, we find that in elderly subjects of similar ages, those with diabetes have 1.7-fold higher levels of G α_{12} and twofold higher levels of G β_1 . Diabetes does not affect other atrial G proteins, including G α_{13} , G α_s , G α_o , and G β_2 . These data represent the first demonstration of an increase in a G_i-coupled receptor, G α_{12} , and G β_1 , in atrium of patients with diabetes. These findings suggest a molecular explanation for the increased risk of cardiac disease in patients with diabetes, because increased signaling through G_i has been shown to lead to the development of dilated cardiomyopathy. *Diabetes* 53:2392–2396, 2004

Several large studies have established an association between age and declining heart function. For example, the Baltimore Longitudinal Study on Aging found that even in the absence of disease, there is still a significant loss of cardiac reserve characterized by a reduction in maximum achievable heart rate during stress (1). Another longitudinal aging study, the Honolulu Heart Program, found that in the absence of hypertension the risk of developing coronary heart disease (CHD) is significantly higher in elderly men, with incidence of CHD increasing from 1.8% in younger (45–54 years) adults to 8.1% in elderly (75–93 years) adults ($P = 0.001$) (2). In a retrospective study, Tsang et al. (3) reported age as an independent risk factor ($P < 0.0001$) for the development of both atrial fibrillation (AF) and congestive heart failure (CHF). The molecular mechanisms underlying the effects of age on the heart are not well

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AC, adenylyl cyclase; AF, atrial fibrillation; AR, adrenergic receptor; CHD, coronary heart disease; CHF, congestive heart failure; DCM, dilated cardiomyopathy; GPCR, G protein-coupled receptor; I_{KACH}, inwardly rectifying potassium channel; mAChR, muscarinic acetylcholine receptor; OD, optical density unit; PTX, pertussis toxin.

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understood; however, G protein-coupled receptors (GPCRs) are likely to play a key role given their importance in heart function. Many GPCRs are known to influence heart activity, including β -adrenergic receptor (β -AR), a major regulator of cardiac inotropy (4), and α_1 -adrenergic and endothelin receptors, which both have roles in cardiac hypertrophy (5,6).

To better understand the age-dependent decline in heart function at a molecular level, our laboratory has been studying the effects of age on cardiac GPCR signaling. We have recently reported that levels of cardiac G_i increase with age in both rats (7) and humans (8). Moreover, the elevation of G_i levels increases activation of G_i through multiple GPCRs (7,8). Increased G_i activity is likely to have an adverse effect on heart function since G_i-coupled signaling pathways in the heart reduce both the rate and force of contraction (4). Some recent studies have used cardiac-specific expression of a modified G_i-coupled opioid receptor called RO1, which has low affinity for endogenous ligands but high affinity for synthetic ligands (9). Consistent with a role for G_i in heart dysfunction, these studies demonstrated adverse effects that were simply due to the elevation of basal G_i activity caused by expression of RO1 in the heart. It was found that expression of RO1 in mice, even without stimulation by the synthetic ligand, resulted in the development of dilated cardiomyopathy (DCM) and a decrease in contractile force (10). These effects were reversed when expression of RO1 was blocked for an additional 8 weeks, indicating that increased G_i activity leads to the development of cardiac pathophysiologies (10). A more recent study showed that increased expression of G α_{12} in neonatal cardiomyocytes attenuates β -adrenergic stimulation of adenylyl cyclase (AC) by up to 39% (11). Moreover, increased expression of G α_{12} in adult cardiomyocytes reduces the maximum shortening amplitude of beating cells in the presence of isoprenaline and decreases the potency of isoprenaline, shifting the half-maximal effective concentration (EC₅₀) from 4 to 28 nmol/l (11).

Given the ability of elevated G_i signaling to cause adverse cardiac effects, we have begun to examine whether age affects G_i-coupled GPCRs in human heart. To this end, we examined cardiac muscarinic acetylcholine receptor (mAChR), which acts through G_i in heart (4) to mediate parasympathetic regulation of heart function (12). We find that cardiac mAChR density increases with age, but that the increase is significant only in those subjects with diabetes. Moreover, we find that atrial membranes

TABLE 1
Characteristics of study subjects ($n = 51$)

Male sex	61
CHF	22
Diabetes*	39
Hypercholesterolemia	47
Hypertension	71
β -Blockers	47
Calcium inotropes	8

Data are percent. *Type of diabetes was not available.

from subjects with diabetes have 1.7-fold higher levels of $G\alpha_{i2}$ and twofold higher levels of $G\beta_1$ than age-matched subjects without diabetes. This is the first demonstration of a diabetes-induced increase in both G_i and a G_i -coupled GPCR in human heart. These findings are likely to improve our understanding of the molecular mechanisms underlying those heart pathophysiologies that are common to diabetes.

RESEARCH DESIGN AND METHODS

Quinuclidinyl benzilate, L-[benzilic-4,4'- ^3H]-([^3H]-QNB), AF-DX 384, [2,3-dipropylamino- ^3H] ([^3H]-AF-DX 384), [^{32}P]-NAD, and [α - ^{32}P]-ATP and antibodies directed against $G\alpha_{i1}/G\alpha_{i2}$ (AS/7), $G\alpha_{i1}/G\alpha_{i3}$ (EC/2), $G\alpha_s$ (RM/1), and $G\alpha_o$ were purchased from PerkinElmer Life Sciences (Boston, MA). Forskolin, atropine, and carbamylcholine chloride (carbachol) were purchased from Sigma (St. Louis, MO). Goat anti-rabbit IgG conjugated to horseradish peroxidase and antibodies against $G\beta_1$ and $G\beta_2$ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). SuperSignal West Pico ECL reagent was purchased from Pierce (Rockford, IL). Other reagents were of the highest commercial grade.

Atrial tissue. Right atrial appendages were obtained during cardiopulmonary bypass, under an institutional review board-approved protocol with informed consent of the patients. The tissues were immediately frozen in liquid nitrogen and stored at -80°C . Subjects ($n = 51$) ranged in age from 41 to 85 years; the mean age \pm SE was 64.9 ± 11.1 years. Medical conditions, sex, and preoperative medications are indicated in Table 1; none of the subjects had stroke, acute renal failure, or dialysis. Atrial membranes were prepared as previously described and stored at -80°C (8).

Radioligand binding. mAChR density was measured using saturating concentrations (2–3 nmol/l) of the radioligand [^3H]-QNB. Atrial membranes (12–15 μg protein) and [^3H]-QNB were incubated together for 2 h at 24°C in a buffer containing 10 mmol/l $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 7.4, 1 mmol/l EDTA, and 10 mmol/l MgCl_2 . The total volume was 0.25 ml. Nonspecific binding of [^3H]-QNB was determined in the presence of 10 $\mu\text{mol/l}$ atropine. m2-mAChR density was measured at a saturating concentration (10 nmol/l) of the m2-selective radioligand, [^3H]-AF-DX 384. [^3H]-AF-DX 384 and atrial membranes (50–60 μg protein) were incubated together for 30 min at 24°C in a buffer consisting of 25 mmol/l Tris-HCl, pH 7.4, 1 mmol/l EDTA, and 2 mmol/l MgCl_2 at a total volume of 0.25 ml. Nonspecific binding of [^3H]-AF-DX 384 was determined in the presence of 10 $\mu\text{mol/l}$ atropine. Because the binding of [^3H]-AF-DX 384 has not been previously characterized in human atrial membranes, we determined that binding equilibrium was attained within 30 min at 24°C and that the K_d was 4 nmol/l (data not shown). Receptor-bound radioligand was isolated by vacuum filtration over Whatman GF/C paper presoaked in 0.3% polyethyleneimine; the filters were then washed with ice-cold 20 mmol/l Tris, pH 7.4, buffer and counted in a liquid scintillation counter (13).

Quantitation of atrial G proteins by immunoblotting and pertussis toxin-catalyzed ADP-ribosylation. Detection of G_i by immunoblotting and pertussis toxin-catalyzed [^{32}P]-ADP-ribosylation was performed as previously described (8). Total protein staining by Coomassie blue was used to confirm equal protein loading on gels. Mean pixel intensities expressed in optical density units (ODs) were obtained using a GS-700 scanning densitometer and Quantity One software (BioRad, Hercules, CA). After subtracting background, relative expression levels of atrial G proteins in diabetic and nondiabetic subjects were quantitated as mean OD \pm SE.

Inhibition of AC activity. AC activity was measured as previously described (8). Briefly, atrial membranes (15 μg protein) were incubated with [α - ^{32}P]-ATP in triplicate for 10 min at 30°C (total volume 50 μl) in an incubation buffer that included 10 $\mu\text{mol/l}$ GTP. Atrial mAChR activity was assessed by the ability of 100 $\mu\text{mol/l}$ carbachol to inhibit AC activity following stimulation by 10 $\mu\text{mol/l}$ forskolin.

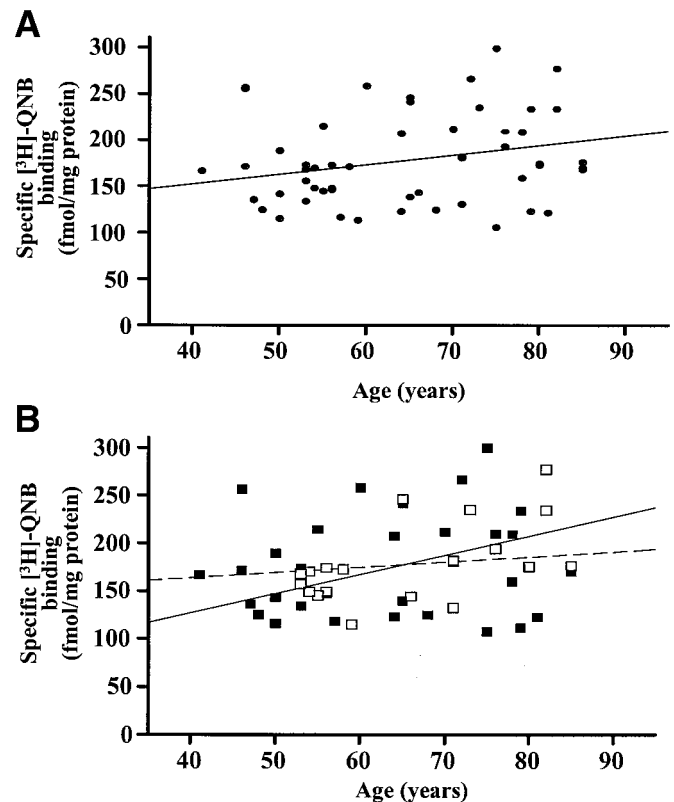


FIG. 1. Higher mAChR density in right atrial membranes from elderly diabetic subjects. Membranes were prepared from human right atrial appendages as described in RESEARCH DESIGN AND METHODS. Binding of [^3H]-QNB (2–3 nmol/l) to atrial membranes (12–15 μg of protein each) was determined in triplicate. A: Specific [^3H]-QNB binding was measured as described in RESEARCH DESIGN AND METHODS, and mAChR density (fmol/mg protein) is shown as a function of age for all 51 subjects. Linear regression analysis performed on these data yields a positive slope that is not significant ($P = 0.062$). B: Specific [^3H]-QNB binding data from all 51 subjects are shown according to whether the subjects are diabetic (\square , solid line) or nondiabetic (\blacksquare , dashed line). Linear regression analysis yields a significant positive slope for diabetic subjects ($P = 0.013$) but not for nondiabetic subjects ($P = 0.442$).

Statistical methods. Interaction of all covariables with age and mAChR density was assessed by multivariable regression analysis using SAS statistical software (SAS Institute, Cary, NC). Serial univariate regression indicated that the distribution of covariables listed in Table 1 is not affected by age ($P < 0.05$). Mean densities of [^3H]-AF-DX 384 binding for old and young diabetic subjects were compared by a two-tailed Student's t test ($P < 0.05$).

RESULTS

Diabetes increases the density and function of atrial mAChR in elderly subjects. To study the effect of age on cardiac mAChR, we first determined whether age affects the density of mAChR expression in human atrium, which is readily available as discarded tissue from patients undergoing cardiopulmonary bypass surgery. Atrial membranes were prepared from subjects ranging in age from 41 to 85 years ($n = 51$), then mAChR density was measured by specific binding of the muscarinic radioligand, [^3H]-QNB. Linear regression analysis of the receptor binding data showed that the relationship between mAChR density and age had a positive slope, suggesting an upward trend in mAChR density with advancing age. However, the slope was not statistically significant ($P = 0.062$, Fig. 1A). To determine whether preexisting medical conditions (listed in Table 1) may influence the relationship between mAChR density and age, we analyzed each covariable by multivar-

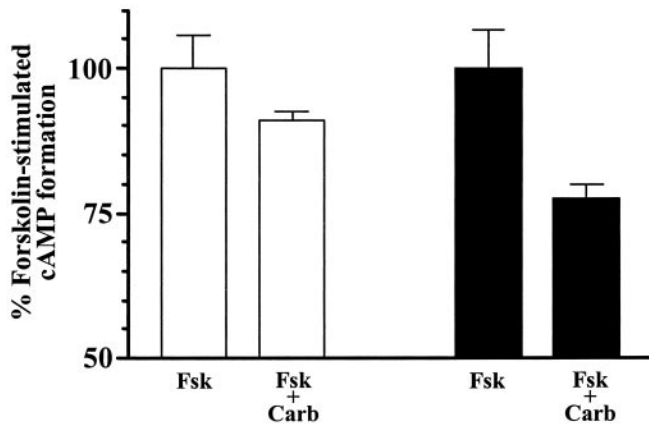


FIG. 2. Age increases mAChR-mediated inhibition of atrial AC activity in diabetic adults. AC activity was stimulated with 10 $\mu\text{mol/l}$ forskolin (FSK) in the absence or presence of 100 $\mu\text{mol/l}$ carbachol (FSK+Carb). Inhibition of AC activity by carbachol is expressed as a percentage of the maximal stimulation by forskolin. Open bars (\square) represent younger diabetic adults (41–55 years, $n = 6$) and filled bars (\blacksquare) represent elderly diabetic adults (70–85 years, $n = 6$). Carbachol produced 9 \pm 1% inhibition in younger diabetic adults and 22 \pm 2% inhibition in elderly diabetic adults.

iate regression. Figure 1B shows that with diabetes as a covariable, mAChR density increases significantly with age (*solid line*, $P = 0.013$). To quantitate the increase in atrial mAChR density, we calculated mean values for specific [^3H]-QNB binding in younger (41–55 years, $n = 11$) and elderly (70–85 years, $n = 5$) diabetic subjects. The values were 158 \pm 10 fmol/mg and 191 \pm 45 fmol/mg for younger and elderly subjects, respectively, yielding an increase of 21 \pm 5%. We conclude that atrial mAChR density increases significantly with advanced age in subjects with diabetes.

There are five subtypes of mAChR, called m1 through m5. The predominant mAChR subtype in human heart is

m2, although m1 and m3 may also be present (12). To determine whether the increase in mAChR density is due to m2, we measured atrial mAChR density using saturation binding of the m2-selective radioligand, [^3H]-AF-DX 384 (14). The density of m2-mAChR in elderly diabetic subjects (70–85 years, $n = 6$) was 188 \pm 23 fmol/mg, whereas in younger diabetic subjects (41–55 years, $n = 6$) the density was 149 \pm 17 fmol/mg, yielding an increase of 26 \pm 4%. We conclude that the increase in m2-mAChR accounts for the entire increase in atrial mAChR density in elderly diabetic subjects.

We next determined whether the increase in mAChR density with age is accompanied by an increase in mAChR activity by measuring the ability of carbachol, a muscarinic agonist, to inhibit forskolin-stimulated AC activity. mAChR-mediated AC inhibition was measured in atrial membranes from diabetic subjects divided into younger (41–55 years, $n = 6$) and elderly (70–85 years, $n = 6$) age-groups. As Fig. 2 shows, maximal inhibition of AC activity by carbachol was doubled in elderly diabetic subjects, increasing from 9 \pm 1% in the younger diabetic subjects to 22 \pm 2% in the elderly group. We conclude that higher atrial mAChR density in elderly diabetic subjects is accompanied by higher mAChR function.

Diabetes increases atrial G α_{i2} and G β_1 . The linkage observed between diabetes and increased atrial mAChR density with age led us to examine whether diabetes also affects expression of atrial G α_{i2} , the main G $_i$ subtype in atria (15). To this end, we used immunoblotting in atrial membranes from age-matched subjects with diabetes (mean age = 62 \pm 6 years, $n = 14$) and without diabetes (mean age = 61 \pm 11 years, $n = 16$). Representative immunoblots are shown in Fig. 3A. Scanning densitometry performed on these blots produced mean OD values of 5.7 \pm 1.3 for nondiabetic subjects and 9.7 \pm 1.6 for diabetic subjects, yielding an increase in G α_{i2} of 1.7 \pm

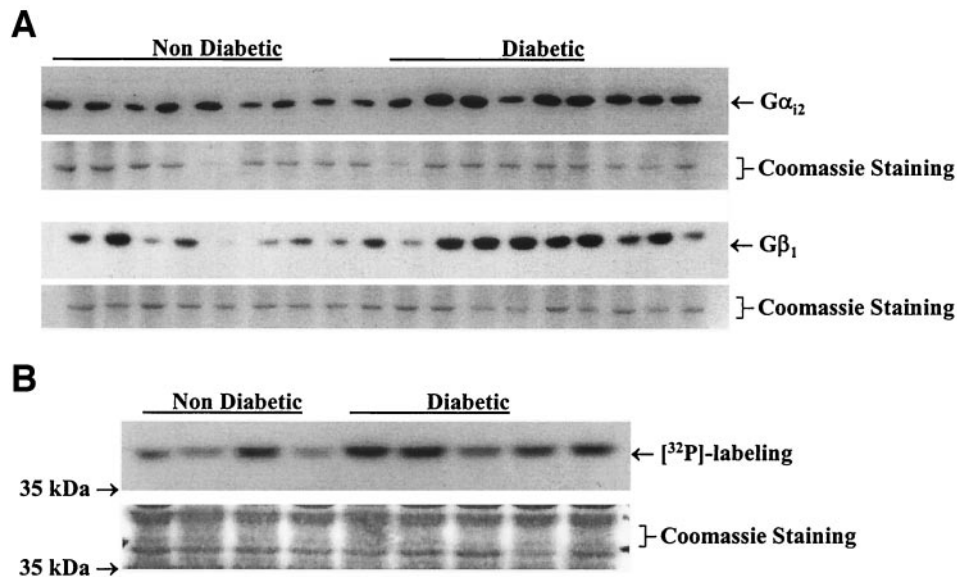


FIG. 3. Diabetes increases atrial G α_{i2} and G β_1 . **A:** Atrial membrane proteins (25 $\mu\text{g/lane}$) from subjects without (*lanes 1–9*) or with (*lanes 10–18*) diabetes were subjected to immunoblotting using antibodies directed against G α_{i2} (*top panel*) or G β_1 (*third panel from top*). Equal protein loading on gels was assessed by total protein staining with Coomassie blue (*second and fourth panels from top*), and G protein expression levels were quantitated by scanning densitometry following enhanced chemiluminescence. The immunoblots shown are representative of three similar experiments. **B:** Atrial G β_1 was labeled with [^{32}P] using PTX-catalyzed [^{32}P]-ADP-ribosylation by incubating [^{32}P]NAD and preactivated PTX with atrial membranes from diabetic ($n = 5$) and nondiabetic ($n = 4$) subjects, as described in RESEARCH DESIGN AND METHODS. Autoradiograms were obtained following SDS-PAGE, and G $_i$ /G $_o$ labeling was quantitated by scanning densitometry. The autoradiogram shown is representative of three similar results.

0.5-fold (Fig. 3A, *top panel*). Examination of other atrial G proteins showed that $G\beta_1$ was twofold higher in diabetic subjects (Fig. 3A, *third panel from top*), while no significant difference was found in $G\alpha_{i3}$, $G\alpha_s$, $G\alpha_o$, or $G\beta_2$ (data not shown). We conclude that diabetes increases atrial expression of $G\alpha_{i2}$ and $G\beta_1$. To assess the functionality of the elevated G_i levels in diabetic subjects we used pertussis toxin (PTX)-catalyzed [32 P]-ADP-ribosylation, which labels active G_i and G_o subunits (16). Autoradiograms were obtained following [32 P]-ADP-ribosylation of atrial membranes, and G_i/G_o labeling was quantitated by scanning densitometry. A representative autoradiogram demonstrating increased G_i/G_o in diabetic subjects is shown in Fig. 3B (*upper panel*). The level of active G_i in diabetic subjects was 1.7-fold higher than that found in nondiabetic subjects.

DISCUSSION

A key finding of the present study is that atrial mAChR density and function increase with age in subjects with diabetes. This finding is consistent with animal studies reporting that induction of diabetes enhances mAChR-mediated effects on heart function (17–21), indicating increased mAChR activity. However, atrial mAChR density in diabetic animals has been variably reported to increase (18), decrease (22), or remain unchanged (20,23). Although there are presently no human data available on the relationship between diabetes and mAChR density, a recent study using live human atrial slices showed that stimulation-induced outflow of acetylcholine is reduced in subjects over the age of 70 years and in subjects with late diabetic complications (24). This observation suggests an increase in atrial mAChR with both diabetes and old age, because reduced acetylcholine release is consistent with increased presynaptic muscarinic autoreceptors since activation of autoreceptors by acetylcholine inhibits further acetylcholine release (24).

Increased mAChR density can have adverse effects on heart function, as suggested by a recent study using positron emission tomography that correlates increased incidence of DCM with increased cardiac mAChR density in human subjects (25). Another negative consequence of increased atrial mAChR and $G\beta_1$ may be increased incidence of AF. This notion is based on two recent studies examining a mAChR-sensitive inwardly rectifying potassium channel (I_{KACH}), which is activated by $G\beta\gamma$ in the heart (26). One study reported that AF could not be experimentally induced in transgenic mice having decreased expression of I_{KACH} but was readily inducible in control mice having normal I_{KACH} expression, establishing I_{KACH} activity as a prerequisite for AF (26). A second study using transgenic mice showed that animals having reduced cardiac expression of functional $G\beta\gamma$ displayed a substantially weaker mAChR-mediated slowing of the heart rate, indicating that the effects of cardiac mAChR depend on mAChR-stimulated release of cardiac $G\beta\gamma$ (27). The same study also showed that AF was inducible in 73% of control mice but in only 23% of the mice having reduced cardiac $G\beta\gamma$ expression, suggesting that an increase in mAChR-coupled $G\beta\gamma$ release in the heart may increase the incidence of AF (27). A link between AF and diabetes in humans is found in a study showing that although diabetic

patients with AF can be converted back to a normal sinus rhythm like nondiabetic patients, their risk of arrhythmia recurrence is 4.6-fold higher than that of nondiabetic patients (28). It is well known that incidence of AF in humans increases with both diabetes and old age (29,30). Thus, if our observations of increased $G\beta_1$ with diabetes and increased mAChR in older subjects with diabetes are considered in light of the studies cited above, there emerges a strong likelihood that increased mAChR signaling underlies increased AF in older diabetic patients.

A second key finding of this study is that atrial G_i levels are elevated in diabetic subjects. This finding is significant because increased G_i signaling has long been associated with heart failure and increased incidence of DCM (15,31,32), an association recently reinforced by a study documenting the development of DCM in transgenic mice having elevated basal G_i activity (10). Furthermore, increased expression of $G\alpha_{i2}$ in neonatal cardiomyocytes attenuates β -adrenergic stimulation of AC (11), while in adult cardiomyocytes it reduces the maximum shortening amplitude of beating cells in the presence of isoprenaline and shifts the EC_{50} for isoprenaline to the right. These results demonstrate that increased expression of G_i decreases cardiac function. Further, increased cardiac G_i signaling is linked to the development of DCM, which leads to CHF. CHF is known to increase with both diabetes (3) and old age, surpassing 10% in people 75 years or older (33).

Finally, the present study also establishes that mAChR density increases in advanced age rather than decreasing, as concluded by Brodde et al. (34). A probable reason for this discrepancy is that Brodde et al. measured atrial mAChR density in subjects ranging in age from 5 days to 76 years, whereas our study examined only adult subjects between 41 and 85 years of age. To support this idea, we examined the data of Brodde et al. in their own Fig. 2 and found that for patients >50 years of age, mAChR density exhibits an upward trend, similar to our findings. We conclude that after 50 years of age, mAChR density exhibits an upward trend with age. An upward trend in mAChR density is certainly consistent with a number of animal studies that report that mAChR-mediated effects on heart activity increase with age (35–37). However, animal studies examining atrial mAChR density have been less conclusive. Receptor binding studies in young (6 month) and old (24 month) rat atria have reported a 24% increase in mAChR density with age (38), as well as smaller increases that fail to reach statistical significance (39,40), while studies of mAChR mRNA content report both increased (39) and decreased (41) mAChR expression with age.

In summary, our results indicate an increase in atrial G_i signaling and an increase in atrial mAChR density in elderly diabetic adults. Further investigation will determine whether inhibition of mAChR and G_i activity improves cardiac function in the elderly diabetic patients.

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