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Selective β_1-adrenoceptor blockade enhances the activity of the stimulatory G-protein in human atrial myocardium

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1 Chronic selective β_1-adrenoceptor (β_1AR) blocker treatment enhances the sensitivity of β_2-adrenoceptor (β_2AR) in human heart (Hall *et al*., 1990; 1991). To clarify the mechanism of the cross-sensitization between β_1AR and β_2AR, we determined whether the stimulatory G-protein ($G_s\alpha$) function is increased in atria from β_1AR-blocker treated patients compared with non-β-blocked patients, and investigated whether this change is caused by an alteration of post-translational modification of $G_s\alpha$ protein.

2 $G_s\alpha$ function was determined by reconstitution of human atrial $G_s\alpha$ into S49 cyc$^-$ cell membranes. In the reconstitution system, GTP$_\gamma$S stimulated cyclic AMP generation in a dose-dependent manner. Upon 10^{-4} M GTP$_\gamma$S stimulation, $G_s\alpha$ activity in the β_1AR-blocker, atenolol, treated group (78.2 ± 10.3 pmol cyclic AMP mg^{-1} min^{-1} 10^{-3}) was 65% higher than that in non-β-blocked patients (47.3 ± 6.3 pmol cyclic AMP mg^{-1} min^{-1} 10^{-3}, $n=15$, $P=0.02$).

3 Isoelectric point (pI) values of $G_s\alpha$ were measured by two dimensional gel electrophoresis (2D-E) and the amount of each isoform quantified by image analysis of a Western blot of the gel using specific antibody. Multiple isoforms of $G_s\alpha$ were detected by 2D-E with different pI values. There were no significant differences between the groups of patients in either pI values or the proportions of the acidic isoforms of $G_s\alpha$ to the main basic form ($n=12$, $P>0.05$).

4 The results suggest that chronic β_1AR-blockade enhances $G_s\alpha$ function in human atrium, and this may account in part for the hypersensitivity of β_2AR and other G_s-coupled receptors during β_1AR-blockade. The increased $G_s\alpha$ function is unlikely to be caused directly by blockade of protein kinase A phosphorylation of $G_s\alpha$ protein.

Keywords: β_1-adrenoceptor blockade; G-protein; S49 cyc$^-$ reconstitution; two-dimensional gel electrophoresis; receptor cross-regulation; human

Abbreviations: β_1AR, β_1-adrenoceptor; β_2AR, β_2-adrenoceptor; βARK, β-adrenergic receptor kinase; cyclic AMP, adenosine 3′:5′-cyclic monophosphate; CPK, carbamylated creatine phosphokinase; 2D-E, two-dimensional gel electrophoresis; ECL, enhanced chemiluminescence; Gβ, β-subunit of G-protein; Giα, α-subunit of inhibitory G-protein; G_s, stimulatory heterotrimeric G-protein; $G_s\alpha$, α-subunit of stimulatory G-protein; $G_s\alpha$L, the long isoform of the stimulatory G-protein α-subunit; $G_s\alpha$S, the shot isoform of the stimulatory G-protein α-subunit; GTP, guanosine triphosphate; GTP$_\gamma$S, guanosin-5′-3-*O*-(thio)triphosphate; IEF, isoelectric focusing; PKA, protein kinase A; pI, isoelectric point; RAA, right atrial appendage; SDS–PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Introduction

β-Adrenergic receptor (βAR) antagonists are widely used for the treatment of ischaemic heart disease, hypertension and heart failure. To minimize the adverse effects of β_2AR-blockade, so-called cardio-selective (β_1AR selective) βAR-blockers were introduced. However, subsequent evidence has shown that human atrium and ventricle contain an appreciable proportion of functional β_2AR (Kaumann & Lemoine, 1987; Del Monte *et al*., 1993), and that, moreover, chronic treatment of patients with a β_1AR-selective blocker causes marked enhancement of β_2AR mediated atrial inotropic responses (Hall *et al*., 1990). The β_2AR hypersensitization by β_1AR blockade has been confirmed *in vivo*, where there is a 6 fold increase of the potency of salbutamol during intra-coronary infusion of this drug in patients receiving a β_1AR-blocker (Hall *et al*., 1991). This phenomenon may benefit the patients with chronic heart failure by improving the pumping function during β_1AR-blockade, but on the other hand, in patients with myocardial infarction the increased sensitivity to β_2AR stimulation is likely to increase heart rate and myocardial oxygen consumption, thereby increasing the risks of arrhythmia and the extent of infarction.

When the βAR is occupied by an agonist, the resulting conformational change of the receptor activates the stimulatory heterotrimeric G-protein (G_s) to bind guanosine triphosphate (GTP) to its α-subunit. The $G_s\alpha$-GTP complex then stimulates adenylyl cyclase (AC) to generate the second messenger cyclic AMP. Cyclic AMP subsequently gives rises to the activation of protein kinase A (PKA) which can phosphorylate a series of proteins. Elements involved in G_s-mediated signal transduction cascade are obvious targets for clarifying the mechanism of cross regulation between receptors coupled to adenylyl cyclase. In support of this suggestion is the evidence that two other G_s-coupled receptors, the 5-HT$_4$-receptor and histamine H$_2$-receptor, mediate inotropic responses in human atria which are potentiated by chronic β_1AR-blockade (Sanders *et al*., 1995; 1996).

Indirect evidence for the involvement of the coupling mechanism in the receptor 'cross-talk' comes also from the findings that there is no increase in β_2AR density or affinity for ligands in β_1AR blocked right atria, nor any change in the

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cellular sensitivity to exogenously applied cyclic AMP analogues (Hall *et al*., 1990). However no quantitative change has been found following β_1AR-blocker treatment in the level of G-protein subunits ($G_s\alpha$, $G_i\alpha$, $G\beta$) (Ferro *et al*., 1993) or the mRNA of the four splice variants of $G_s\alpha$ and $G_i2\alpha$ (the principle cardiac G_i protein) (Jia *et al*., 1995; Monteith *et al*., 1995). A variety of post-translational modifications of $G_s\alpha$ have been reported, such as phosphorylation and ribosylation, some occurring in response to cyclic AMP stimulation (Yamane *et al*., 1993; Degtyarev *et al*., 1993; Linder *et al*., 1993; Pyne *et al*., 1993). These modifications therefore raise the possibility of a functional feedback regulation of G_s function without changes in its transcription.

The present study was designed to measure G_s protein function by reconstitution of $G_s\alpha$ protein from human atrium into S49 cyc$^-$ lymphoma cell membranes. In this system, S49 cyc$^-$ cells are genetically deficient in $G_s\alpha$ and therefore lack G_s-mediated adenylyl cyclase activity. The addition of solubilized $G_s\alpha$ to cyc$^-$ cell membranes can restore G_s-mediated adenylyl cyclase activity. The rate of cyclic AMP generation theoretically reflects pure G_s function.

As a consequence of the activation of the G_s-mediated signal transduction pathway, the phosphorylation status and possibly function of numerous proteins may be increased by PKA. $G_s\alpha$ can be phosphorylated by PKA *in vitro* (Pyne *et al*., 1992). It is not clear whether this occurs *in vivo*. However we have reported that the serine$^+$ variants of $G_s\alpha$, which might be more prone to PKA phosphorylation, have a more acidic isoelectric point (pI) – consistent with *in vivo* modification by phosphorylation (Monteith *et al*., 1995). In the present study, two dimensional gel electrophoresis was employed to determine whether there is any change in the pI value of $G_s\alpha$ protein from human atria after chronic treatment with a β_1AR-blocker.

Methods

Patients

Right atrial appendage (RAA) was obtained immediately following its routine removal from patients undergoing coronary artery bypass grafting, at the time of institution of cardiopulmonary bypass. Premedication was with papaveretum and hyoscine; anaesthesia was induced with midazolam, fentanyl and propofol, with pancuronium as muscle relaxant. Propofol infusion was used for maintenance of anaesthesia. RAA were received from 54 patients; 30 samples were used for G_s functional assay, and the other 24 were used for two-dimensional gel electrophoresis. Half the samples for each assay were from patients receiving chronic β_1AR-blocker atenolol treatment (50 mg day^{-1}, over 1 month) at the time of surgery, and half from non-β-blocked patients. Although the choice was not randomized, it tended to reflect individual referring physicians' preference for β-blockade or Ca^{2+}-blockade as secondary treatment in patients already receiving nitrates. Patients were matched as far as possible in terms of age, sex and diagnosis in both sets of studies (Table 1). Other drug therapy included aspirin, calcium channel antagonists, diuretics and nitrates. Tissue samples were immediately snap-frozen in liquid nitrogen as soon as they were removed during the heart operation and stored at -70°C until use. The use of tissue routinely removed and discarded during surgery was approved by the local research ethics committee without patients written consent being required.

Cell culture and preparation of S49 cyc$^-$ cell membranes

S49 cyc$^-$ lymphoma cells were grown in suspension culture in HEPES buffered Dulbecco's modified Eagle's medium that had been supplemented with antibiotics (100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin), antimycotic (0.25 μg ml^{-1} amphotericin B) and 5% foetal calf serum. The population density was maintained at about 10^6 cells ml^{-1}. 2.4×10^9 cyc$^-$ cells were harvested, the cells pelleted by centrifuging at $1000 \times g$ for 20 min at 4°C and washed twice with HEPES buffer (HEPES 50 mM, EDTA (pH 8.0) 5 mM). The cell pellet was suspended in HEPES buffer and homogenized in a ground glass homogenizer. The homogenate was centrifuged at $1000 \times g$ for 10 min at 4°C and the supernatant collected as supernatant 1. The pellet was re-homogenized in HEPES buffer and the supernatant collected as supernatant 2. Supernatants 1 and 2 were pooled, filtered through four layers of gauze and centrifuged at $48{,}000 \times g$ for 30 min at 4°C. The pellet was resuspended in HEPES buffer. Protein concentration was measured in 96-well plates using the Bio-Rad protein assay with bovine serum albumin as standard. The cyc$^-$ cell membrane preparation with a final concentration of $\sim$2 mg ml^{-1} was stored in separate aliquots at -70°C.

Cholate extraction of G_s protein from human right atrium

Right atrial appendage tissues (40–200 mg) were debrided of fat and connective tissue and homogenized in buffer A (170 μl 100 mg^{-1} tissue, HEPES (pH 8.0) 10 mM, EDTA 1 mM, benzamidine 3 mM and 1 μg ml^{-1} aprotinin). Sodium cholate and β-mercaptoethanol were added to the homogenate to give a final concentration of 1% and 20 mM respectively. After vortexing on ice for 60 min the homogenate was centrifuged at $100{,}000 \times g$ for 60 min at 4°C. Protein concentration of the supernatant was measured as above to be 10 μg μl^{-1} on average, and the remainder aliquoted and stored at -70°C until use.

Table 1 Characteristics of patients

	Reconstitution assay		*2D-gel electrophoresis*	
	β_1-blockade (n = 15)	*Non-β_1-blockade* (n = 15)	*β_1-blockade* (n = 12)	*Non-β_1-blockade* (n = 12)
Age*	65.3 ± 2.1	65.4 ± 2.2	57 ± 2.7	63.6 ± 2.5
Sex	All male	All male	10M/2F	8M/4F
Diagnosis†				
Coronary artery disease	15	15	12	12
Valve disease	0	0	0	1
Diabetics	0	0	0	1
Hypertension	1	1	1	0
Drug therapy†				
β_1-blocker	15	0	15	0
Ca^{2+} antagonist	11	11	8	8
Nitrates	14	11	10	9
Aspirin	15	11	9	8
ACE inhibitor	3	2	4	7
Diuretic	4	4	2	4
Cardiac function†$^+$				
Good	4	6	7	6
Mild to moderate impairment	11	9	5	6
Severely impaired	0	0	0	0

*Values are mean ± s.e.mean. †Number of patients. $^+$Cardiac function was assessed by angiography.

Reconstitution of $G_s\alpha$ activity in S49 cyc$^-$ lymphoma membrane

Cholate extracts were diluted in buffer A. Frozen cyc$^-$ membranes were resuspended in fresh HEPES buffer. Cyc$^-$ membranes (40 μg) and RAA cholate extracts were incubated together in a 100 μl reconstitution mix containing cyclic AMP 1 mM, ATP 0.1 mM, creatine phosphate 20 mM, 40 U creatine phosphokinase, 40 U myosin kinase, GTP 0.05 mM HEPES 50 mM, EGTA 0.1 mM, $MgCl_2$ 10 mM, 0.1 mg ml^{-1} BSA and 5 μCi [α-^{32}P]-ATP. After 40 min incubation in a water bath, the reaction was terminated by adding 100 μl of 40 mM ATP and heating at 90°C for 5 min. The cyclic AMP formed was isolated by sequential chromatography using a Dowex 50 cation exchange and neutral alumina column. Recovery of cyclic AMP was monitored by the addition of [^{3}H]-cAMP to each tube. The [^{32}P]- and [^{3}H]-cAMP eluted from the alumina were quantified by double-isotope liquid-scintillation spectroscopy. A concentration curve of Guanosine-5′-3-*O*-(thio)triphosphate ($GTP_\gamma S$) stimulated adenylyl cyclase activities was examined in an atrial sample with triplicate determinations of each concentration of $GTP_\gamma S$, and 10^{-4} M $GTP_\gamma S$ stimulated cyclic AMP generation with different amount of membrane protein (12–70 μg) was tested. Results were expressed as the amount of cyclic AMP formed per minute per milligram of protein.

Membrane preparation from human right atrium

Samples of right atrial appendage were debrided of fat and connective tissue. The remaining material was placed in ice cold buffer B (β-glycerophosphate 10 mM, HEPES 10 mM, benzamidine 2 mM, PMSF 1 mM, 5 μl mg^{-1} tissue), minced with scissors and homogenized using a Polytron at maximum speed (setting 10) for 5 × 5 s. The homogenate was centrifuged at 2000 × *g* for 10 min at 4°C. The resulting supernatant was centrifuged at 48,000 × *g* for 15 min at 4°C to obtain a crude plasma membrane fraction that was resuspended in buffer B and stored in separate aliquots at −70°C. Protein concentration was measured as above.

Isoelectric focusing (IEF)

Two dimensional electrophoresis was performed according to the method of O'Farrel (1975) using the MINI-PROTEAN II 2-D CELL system. IEF gel monomer solution was made containing 9.2 M urea, 2% NP40, 0.018% ammonium persulphate, 0.025% TEMED, 2% pH 3–10 ampholyte, 1% pH 5–7 ampholyte, 1% pH 3.5–5 ampholyte and 1% pH 7–9 ampholyte. IEF tube gels were cast in glass capillary tubes of 10 cm long and 1 mm in diameter. Samples of crude plasma membrane were mixed with an equal volume of first dimensional sample buffer (9.5 M urea, 4% NP40, 5% β-mercaptoethanol, 0.4% pH 3.5–10 ampholyte, 1.6% pH 5–7 ampholyte) and incubated at room temperature for 10–15 min. The tube gel was pre-run at 200 V for 10 min, 300 V for 15 min and 400 V for 15 min with the upper chamber running buffer NaOH 20 mM (degassed) and lower chamber running buffer H_3PO_4 10 mM. About 10 μg membrane proteins (25 μl) were loaded onto the tube gel. On the top of the sample, 30 μl sample overlay buffer (9 M urea, 0.8% pH 5–7 ampholyte, 0.2% pH 3–10 ampholyte) was loaded. The gel was run at 500 V for 10 min, 750 V for 3.5 h and 2 KV for 30 min. After IEF was completed, the tube gels were ejected into equilibration buffer (Tris-HCl (pH 6.8) 0.0625 M, 2.3% (w v^{-1}) SDS, 2-mercaptoethanol 10 mM, 10% (w v^{-1}) glycerol) and frozen at −70°C until the second-dimension was run.

For measurement of the pH gradient along the tube gel, Pharmacia 2-D Electrophoresis Calibration Kit was used. Carbamylated creatine phosphokinase (CPK) protein was included with each atrial sample prior to iso-electric focusing and SDS–PAGE. The series of spots (approximately 34 spots) between pI values of 4.9–7.1 were visualized by Ponsau S staining of the nitro-cellulose membrane after Western blotting. The pI value of $G_s\alpha$ was calculated by comparison with these standard protein spots.

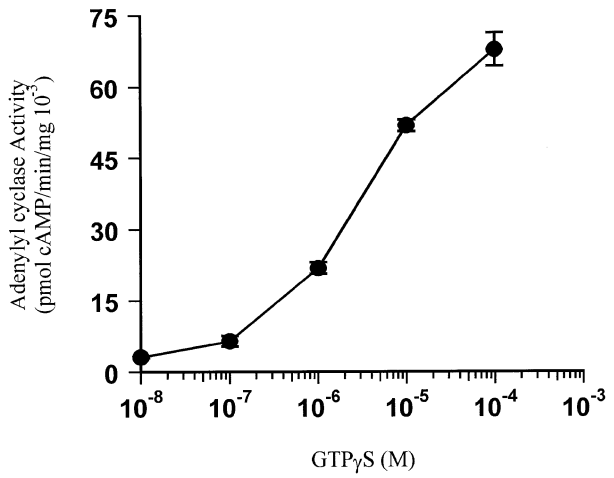

Figure 1 Dose-dependent increase of $G_s\alpha$ activity by $GTP_\gamma S$ stimulation in the $G_s\alpha$ reconstitution assay. Data are mean ± s.e.mean of triplicate determinations from one patient's right atrial appendage.

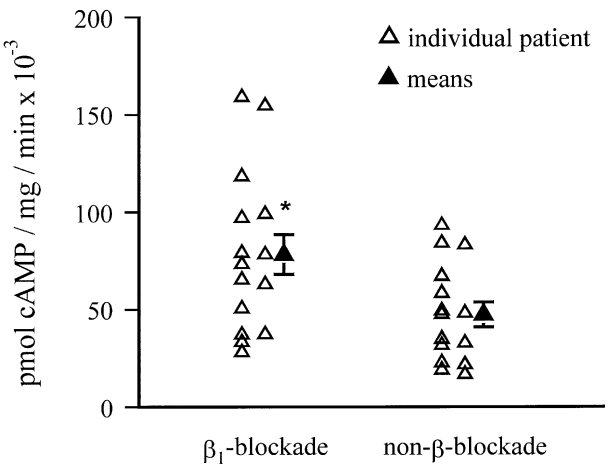

Figure 2 Comparison of atrial $G_s\alpha$ activity between β_1AR-blocked and non-β-blocked patients. $G_s\alpha$ activity was measured as the increase in adenylyl cyclase activity in S49 cyc$^-$ cell membranes in the presence of 10^{-4} M $GTP_\gamma S$. Data are represented as means of triplicate determinations of each patients' sample (open triangles) and means of data from 15 patients' right atrial appendage (solid triangle, mean ± s.e.mean). *$P = 0.02$.

SDS–PAGE and immunoblotting

IEF gel strips were loaded directly on the top of the slab gel, excluding any air bubbles. The second-dimension separation was carried out at room temperature with a mini stacking gel ($1 \times 8 \times 0.05$ cm, 4%T) and separating gel ($5 \times 8 \times 0.05$ cm, 10%T) system. Voltage was set at 200 V and current at maximum.

Following electrophoresis, immunoblotting of $G_s\alpha$ protein was performed as described previously (Monteith *et al*., 1995). Briefly, proteins were electro-blotted onto a nitro-cellulose membrane for 1 h at 0.8 mA cm^{-2} with LKB117-250 Novablot apparatus. After blocking the non-specific binding sites by immersing in 5% dried milk (Marvel) for 30 min, the membrane was incubated at room temperature with anti-$G_s\alpha$ antiserum (1 : 750) for 2 h. This antiserum was generated against a synthetic $G_S\alpha$ C-terminal decapeptide (RMHLRQYELL) and its characterization has been described previously (Miligan & Unson, 1989; Ohisalo *et al*., 1989). After washing with 0.1% Tween-20 (v v^{-1}) made in Tris-buffer saline (TBS, NaCl 200 mM, Tris-HCl, (pH 7.4) 50 mM), the membrane was then incubated for 1 h with horseradish peroxidase-labelled goat anti-rabbit immunoglobulin (1 : 500 dilution). After the membrane was washed again, G-protein dots were visualized by incubating with enhanced chemiluminescence (ECL) detection reagents.

Quantification of $G_s\alpha$ protein

There was inadequate RAA protein to permit combined $G_s\alpha$ function and mass quantification except in four β-blocked and seven non β-blocked patients. Quantification was performed by 1-D Western blot, as previously described (Ferro *et al*., 1993), and results expressed in arbitrary optical density units. This permitted an assessment of $G_s\alpha$ specific activity in the 11 samples.

Reagents

Dulbecco's modified Eagle's medium was from Life Technologies (Paisley, Scotland, U.K.). Foetal calf serum was from Globepharm. [α-^{32}P]-ATP, [^{3}H]-cAMP and enhanced chemiluminescence (ECL) detection reagents were from Amersham (Amersham International plc., U.K.). Anti-$G_s\alpha$ antiserum was kindly donated by Professor G. Milligan (Molecular Pharmacology Group, Department of Biochemistry, University of Glasgow, Scotland, U.K.). Horseradish Peroxidase-labelled goat anti-rabbit immunoglobulin was from DAKO (Denmark). Protein assay kit was from Bio-Rad (Bio-Rad Laboratories Ltd, Herculaes, CA, U.S.A.). 2-D Electrophoresis Calibration Kit was from Pharmacia (Pharmacia Inc, U.S.A.). Other chemicals were from Sigma (St. Louis, MO, U.S.A.).

Statistics

All data were expressed as mean $\pm$ s.e.mean. Statistical comparison of the reconstituted $G_s\alpha$ activity and the pI value between β_1AR-blocked and non-β-blocked samples were by means of unpaired Student's *t*-test. The changes in the proportions of the acidic isoforms of $G_s\alpha$ to the main basic form after β_1AR-blockade was assessed by a multiple regression analysis incorporating patients' ages and estimated left ventricular function. A value of $P < 0.05$ was considered significant.

Results

$G_s\alpha$ activity in cyc$^-$ reconstitution assay

$GTP_\gamma S$ stimulation of adenylyl cyclase activity in cyc$^-$ cell membranes was restored after combination with atrial extracts containing human $G_s\alpha$. Cyclic AMP generation was dose-dependent up to the maximal concentration used of 10^{-4} M

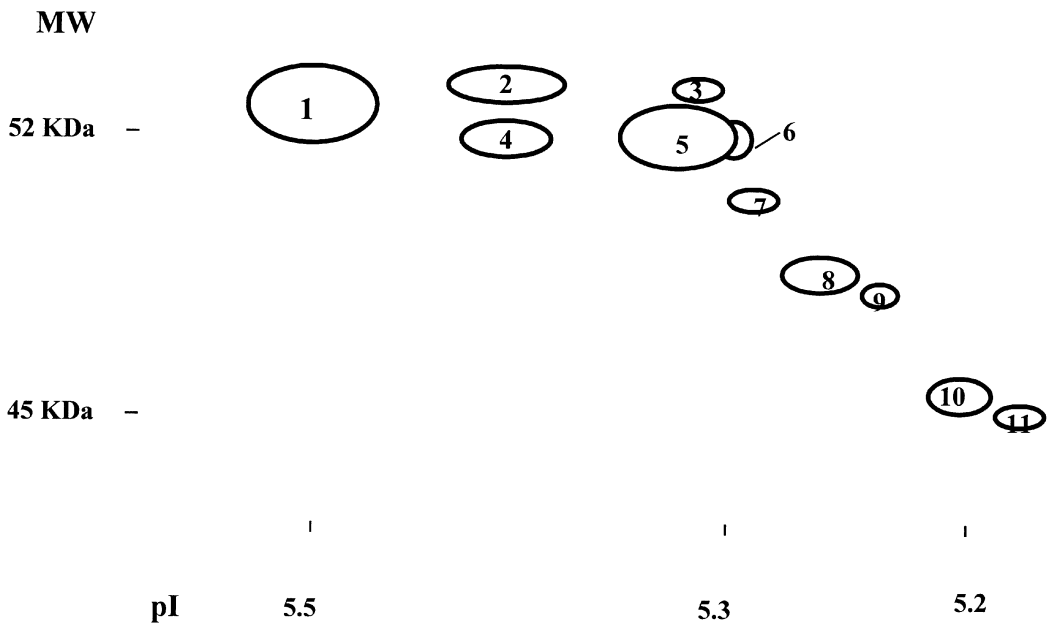

Figure 3 Sketchgraph shows the 2D-pattern of the $G_s\alpha$ protein from human atria. Membrane proteins were separated horizontally by iso-electric point and vertically by mass. $G_s\alpha$ was detected by immunoblotting with specific antibody. The dots were numbered from 1–11.

$GTP_\gamma S$ (Figure 1). 10^{-4} M $GTP_\gamma S$-stimulated cyclic AMP generation varied linearly with the amount of membrane protein used up to 28 μg. Fifteen micrograms membrane protein was available from all RAA, and was used for the comparison of $G_s\alpha$ activity between β_1AR-blocked and non-β-blocked human atria. Cholate extracts were diluted before addition to the reconstitution mix, and in the absence of S49 cell membranes, restored negligible $G_s\alpha$ mediated adenylyl cyclase activity upon $GTP_\gamma S$ stimulation (data not shown). Reconstituted $G_s\alpha$ activity in the presence of 10^{-4} M $GTP_\gamma S$ was 78.2 ± 10.3 pmol cyclic AMP mg^{-1} min^{-1} 10^{-3} in 15 atenolol treated patients, which was 65% greater than in 15 non-β-blocked patients, 47.3 ± 6.3 pmol cyclic AMP mg^{-1} min^{-1} 10^{-3} ($P = 0.02$, Figure 2).

In the 11 patients where the $G_s\alpha$ protein could be quantified, there was a trend for higher specific activity in the β_1-blocked atria (13.0 ± 2.1 arbitrary units) than that in the non-β-blocked atria (7.3 ± 2.3), although this was not a significant difference in the small number of samples.

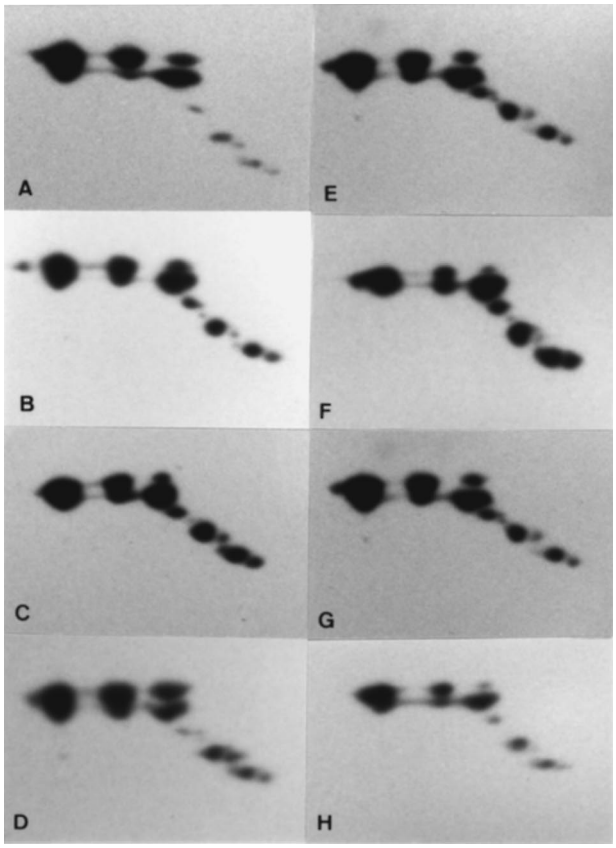

Figure 4 Comparison of two-dimensional pattern of $G_s\alpha$ in right atrial appendage between patients receiving β_1AR-blocker and non-β-blocker treatment. Four typical 2D-patterns of $G_s\alpha$ from each group are shown here. (A, B, C and D) β_1AR-blockade; (E, F, G and H) non-β-blockade.

Table 2 Quantification of $G_s\alpha$ isoforms in atria from β_1AR-blocked and non-β-blocked patients

	β_1AR-blockade (%)	*Non-β-blockade* (%)
Long		
Dot No.2/dot No.1	61.8 ± 3.7	53.1 ± 9.6
Dot No.3/dot No.1	25.0 ± 2.3	24.9 ± 5.4
Dot No.4/dot No.1	52.4 ± 7.2	46.0 ± 9.5
Not No.5+6/dot No.1	101.2 ± 11.5	112.1 ± 35
Short		
Dot No.10/dot No.1	31.4 ± 5.3	35.4 ± 14.1
Dot No.11/dot No.1	9.7 ± 2.0	8.4 ± 2.7
Undetermined		
Dot No.7/dot No.1	16.2 ± 2.0	22.0 ± 8.6
Dot No.8/dot No.1	39.0 ± 5.4	48.0 ± 18.6
Dot No.9/dot No.1	9.4 ± 1.2	8.7 ± 2.5

2D-gel electrophoresis and immunoblotting was used to quantify each of 11 $G_s\alpha$ residues. The results obtained by image densitometry are shown for dots 2–11 (see Figure 3) as a proportion of the principle dot (No.1) Dots 1–6 have the MW of $G_s\alpha$ Long, and dots 10–11 have the MW of $G_s\alpha$ Short, with dots 7–9 lying in between.

2D-gel electrophoresis and immunoblotting

Typical 2D-patterns of $G_s\alpha$ from human atrium are shown in Figures 3 and 4. A total of 11 forms of $G_s\alpha$ were detected. Those at MW 52 KDa correspond to $G_s\alpha$ long isoform ($G_s\alpha$L) and those at 45 KDa to $G_s\alpha$ short isoform ($G_s\alpha$S). The pI for the $G_s\alpha$L residues (pI 5.53 ± 0.01) were less acidic than $G_s\alpha$S (pI 5.19 ± 0.01, $P < 0.001$), which confirms the previous finding. The densest and most basic spot was taken as the reference point and assumed to be the unmodified $G_s\alpha$.

The 2D-pattern of $G_s\alpha$ from the atria of 12 atenolol treated and 12 matched non-β-blocked patients were compared. The patterns were very reproducible. The pI value of the major isoforms of $G_s\alpha$L and $G_s\alpha$S were 5.53 ± 0.01 and 5.19 ± 0.01 for the β_1AR-blocked group, compared to 5.53 ± 0.01 and 5.17 ± 0.01 for the non-β-blocked group, ($P > 0.05$, Figure 4). In order to determine whether the relative amount of these isoforms changed after β_1AR-blockade, the isoforms on the 2D-pattern from each individual were numbered from 1–11 (Figure 3) and were quantified by laser-densitometry, and the ratio of each dot to the main basic dot (number 1) calculated. The results are shown in Table 2. No significant differences were observed in the proportions of the acidic residues of $G_s\alpha$ relative to the main basic residue between these two groups of patients ($P > 0.05$).

Discussion

Previous studies showed that chronic β_1-blocker treatment causes a several fold increase in the potency of β_2AR-agonists and antagonists at adenylyl cyclase coupled receptors (Hall *et al*., 1990; 1991; 1993; Sanders *et al*., 1995; 1996). These observations have been made both *in vitro* and *in vivo*, the most striking being a 6 fold increase in potency of sulbutamol when infused into the right coronary artery of atenolol treated patients. This sensitization occurs with no increase in receptor number or occupation, or of cyclic AMP responsiveness (Hall *et al*., 1990; Brodde, 1991), pointing to enhanced coupling of the receptors to adenylyl cyclase through G_s. However in several studies of $G_s\alpha$ and β subunit mRNA and protein levels, no overall differences in levels were found (Ferro *et al*., 1993; Jia *et al*., 1995; Monteith *et al*., 1995). The present study shows that there is indeed an increase in G_s function in the atria of β_1AR-blocked patients, but fails to elucidate the mechanism.

There was reason to expect that β_1AR-blockade might influence post-translational modification of G-proteins. In the heart, the βAR–G-protein–adenylyl cyclase signal transduction pathway is normally under a high level of adrenergic stimulation, which results in tonic PKA activity. There is evidence that $G_s\alpha$ can be phosphorylated through PKA *in vitro*

(Pyne *et al*., 1992), and it is plausible therefore that the PKA activation could cause $G_s\alpha$ to be phosphorylated *in vivo*. Chronic blockade of β_1AR interrupts the activation cascade and would therefore reverse any PKA stimulated phosphorylation of $G_s\alpha$. Other kinds of post-translational modification of $G_s\alpha$, such as palmitoylation and ADP-ribosylation, could also be affected in the same way, especially the latter which has been shown to be activated by cyclic AMP (Yamane *et al*., 1993; Degtyarev *et al*., 1993; Linder *et al*., 1993; Pyne *et al*., 1992). We have ourselves found indirect evidence that sympathetic tone *in vivo* does influence the post-translational modifications of $G_s\alpha$ identified by 2D-pattern changes. The finding was that the proportion of some of the acidic isoforms of $G_s\alpha$L is much lower in saphenous vein than in the heart (Wang & Brown, 1996), consistent with the much lower sympathetic tone in the former. The lack of difference in the present study of pI values in atria of $G_s\alpha$ between β_1AR-blocked and non-β-blocked patients implies that $G_s\alpha$ is unlikely to be modified directly by cyclic AMP-dependent protein kinase A *in vivo*. It is conceivable that such phosphorylation has still occurred through the β_2AR, but in a small number of atria from patients receiving non-selective β-blockade, a similar 2D-pattern is still observed.

The precise mechanisms that result in the increase of $G_s\alpha$ activity remain intriguing. The reproducibility of the 2D-gel electrophoresis means that we could have readily detected changes in modification as great as the increase in G_s function present. The possibility of changes of some post-translational modifications that do not alter the pI values of the $G_s\alpha$ protein could not however be excluded.

There was substantial overlapping of the $G_s\alpha$ function data between the two groups of patients, even though the means were significantly different. This overlapping, together with the modest mean increase in $G_s\alpha$ function raises the question whether this difference is sufficient to explain the 5–10 fold increase of the inotropic response of the atria from β_1AR-blocked patients (Hall *et al*., 1990; 1991). Since the signal transduction cascade is an amplification process, we would not expect 1 : 1 equivalence between G-protein activity and the final physiological response, but we have not attempted to measure the gearing. The increased $G_s\alpha$ function following β_1AR-blockade shows that receptor coupling to adenylyl cyclase is one site of receptor cross talk, but it is likely this is not the unique change which contributes to the increased β_2AR sensitivity. At the receptor level, chronic administration of bisoprolol, a β_1AR-selective blocker, to pigs has been reported to cause a reduction in left ventricular β-adrenergic receptor kinase (βARK) activity (Ping *et al*., 1995). The reduction of βARK expression was also observed in mouse model with long-term administration of atenolol (Iaccarino *et al*., 1998). Beyond $G_s\alpha$ in the transduction pathway, there are up to nine types of adenylyl cyclase that have been identified so far (Krupinski *et al*., 1989; Gao & Gilman, 1991; Ishikawa *et al*., 1992; Premont *et al*., 1992; Katsushika *et al*., 1992; Watson *et al*., 1994). Of these at least four types are present in the heart (AC IV, V, VI and VII) (Ishikawa & Homcy, 1997; Iyengar, 1993). Are these cardiac adenylyl cyclases coupled equally to different receptors or are some of the adenylyl cyclases switched on or off upon β_1AR-blockade? Determination of the gene expression of each subtype of the adenylyl cyclases upon β_1AR-blockade is ongoing. The multiple isoforms of the adenylyl cyclase present in the heart, and evidence of compartmentalization of cyclic AMP activation (Zhou *et al*., 1997) limit the value of comparison of the overall adenylyl cyclase activity between the two groups of patients. Cross-talk between the β_2AR and other receptors or cell signalling systems has been described in the organ bath. We believe these observations are not relevant to the β_2AR cross-talk we observe, which develops over several hours or days.

$G_s\alpha$ reconstitution study is a classic method to test $G_s\alpha$ function with the advantage of the availability of S49 cyc^- cell line genetically deficient in $G_s\alpha$. The early experiments with the method by Ross and Gilman led to the discovery of $G_s\alpha$ (Haga *et al*., 1977; Ross & Gilman, 1977). However, when used to test $G_s\alpha$ function, the disadvantage is that cholate extraction causes subunit dissociation of G-protein. Therefore the method would under-estimate changes related to subunit interaction, for instance if reduced G_s activation during β_1AR-blockade results in a higher proportion of intact trimeric G_s-protein in the atrial membrane.

In summary, chronic β_1AR-blockade can enhance $G_s\alpha$ function in human atrium, explaining at least partially the sensitization of β_2AR mediated responses by β_1AR-blockade. The lack of difference in pI values of $G_s\alpha$ between β_1AR-blocked and non-β-blocked atria suggests that the increased $G_s\alpha$ activity is not due to a change in the phosphorylation status of $G_s\alpha$ protein consequent on the reduced activity of cyclic AMP-dependent protein kinase A.

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