Functional β_3-Adrenoceptor in the Human Heart

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Abstract

β_3-adrenoceptors are involved in metabolism, gut relaxation, and vascular vasodilation. However, their existence and role in the human heart have not been documented. We investigated the effects of several β-adrenoceptor agonists and antagonists on the mechanical properties of ventricular endomyocardial biopsies. In the presence of nadolol, a β_1- and β_2-adrenoceptor antagonist, isoprenaline produced consistent negative inotropic effects. Similar negative inotropic effects also resulted from the action of β_3-adrenoceptor agonists with an order of potency: BRL 37344 > SR 58611 ≈ CL 316243 > CGP 12177. The dose-response curve to BRL 37344–decreasing myocardial contractility was not modified by pretreatment with nadolol, but was shifted to the right by bupranolol, a nonselective β-adrenoceptor antagonist. β_3-adrenoceptor agonists also induced a reduction in the amplitude and an acceleration in the repolarization phase of the human action potential. β_3-adrenoceptor transcripts were detected in human ventricle by a polymerase chain reaction assay. These results indicate that: (*a*) β_3-adrenoceptors are present and functional in the human heart; and (*b*) these receptors are responsible for the unexpected negative inotropic effects of catecholamines and may be involved in pathophysiological mechanisms leading to heart failure. (*J. Clin. Invest.* 1996. 98:556–562.) Key words: β-adrenoceptors • negative inotropy • human action potential • β_3-adrenoceptor transcripts • human myocardium

Introduction

β_3-Adrenoceptor differs from classical β_1- and β_2-adrenoceptor subtypes by its molecular structure and pharmacological profile (for review, see reference 1). β_3-adrenoceptor shares only 40–50% amino acid sequence identity with β_1- and β_2-adrenoceptors, possesses an intron whereas β_1- and β_2-adrenoceptors are intronless (2), and lacks recognition sites for the cAMP-dependent and β-adrenoceptor kinases implicated in the desensitization of β_2-adrenoceptors (3). β_3-adrenoceptor is activated by preferential pharmacological agonists (e.g., BRL 37344 and SR 58611), which have little effect on β_1- and β_2-adrenoceptors. The biological effects mediated by β_3-adrenoceptor stimulation have been identified in a variety of tissues. As β_3-adrenoceptors mediate lipolysis in white adipose tissues and thermogenesis in brown adipose tissues (4–7), they constitute a target for antiobesity and antidiabetic drugs (8, 9). They also inhibit the contractile activity of ileum and colon (10–12). β_3-adrenoceptors modulate neural bronchomotor control, inducing relaxation of airway smooth muscle (13) and producing sustained peripheral vasodilation that is predominant in skin and fat (14, 15).

Although considerable information is available on β_3-adrenoceptor physiology in fat and the gastrointestinal tract, it is still unclear whether or not β_3-adrenoceptors exist in the human heart. In animal models, the use of partial agonists inducing chronotropic and inotropic effects resistant to blockade by conventional β_1- and β_2-adrenoceptor antagonists has suggested the existence of a third cardiac β-adrenoceptor, designated as atypical β-adrenoceptor (16). However, these partial agonists have no effect on inotropy in the human heart (17). In vivo studies have recently demonstrated that positive β_3-adrenoceptor–related chronotropic effects were prevented by β_1- or β_2-adrenoceptor antagonists and are likely due to baroreflex activation in response to β_3-adrenoceptor agonist–induced vasodilation (18–21). Thus, the existence of a putative β_3-adrenoceptor in the heart has not been clearly demonstrated. The purpose of the present study was to investigate a combination of preferential β_3-adrenoceptor agonists and β-adrenoceptor antagonists in human myocardial fragments obtained from endomyocardial biopsies. We found that β_3-adrenoceptor stimulation of the human cardiac muscle, in stark contrast with β_1- and β_2-adrenoceptor stimulation, resulted in a profound dose-dependent negative inotropic effect. This unexpected finding suggests that β_3-adrenoceptors may participate in the pathogenesis of cardiac failure, during which modification of β_1- and β_2-adrenoceptor expression occurs (22).

Methods

Human ventricular biopsies. All protocols were approved by the Ethics Committee of the Centre National de la Recherche Scientifique (France). 45 human endomyocardial biopsies were obtained from the right interventricular septum of cardiac transplant patients (40 men and 5 women, mean age 55.9±1.3 years) during right jugular vein catheterization performed routinely to detect possible rejection. None of the patients had evidence of cardiac rejection. All received immunosuppressive therapy (azathioprine, prednisolone and cyclosporine). In addition, 11 were given a calcium antagonist, 2 were given an α-adrenoceptor antagonist, and 6 were given a diuretic. 27 had no treatment known to possess cardiovascular effects. The effects of β-agonists obtained in biopsies from patients treated with calcium antagonists were similar to those obtained in biopsies from patients not receiving these drugs. Biopsies were also performed in two pa-

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tients undergoing open-heart surgery and receiving no cardioactive or immunosuppressive drugs. In these two patients, the effects of β_3-adrenoceptor agonists were similar to those obtained in biopsies from transplanted patients.

Experimental protocol. Tissues were placed in a transport solution containing (in mM): 120 NaCl, 5 KCl, 1 $CaCl_2$, 1.1 $MgCl_2$, 0.33 NaH_2PO_4, 5 glucose, and 10 Hepes (pH adjusted to 7.4 with NaOH) and transported to the laboratory in less than 5 min. Preparations were then placed in an experimental chamber and superfused at a flow rate of 5 ml/min with oxygenated (95% O_2, 5% CO_2) Tyrode's solution (37±0.5°C) containing (in mM): 120 NaCl, 5 KCl, 2.7 $CaCl_2$, 1.1 $MgCl_2$, 0.33 NaH_2PO_4, 5 glucose, and 20 $NaHCO_3$. Tissues were equilibrated for 60 min and then subjected to field stimulation at a pacing cycle length of 1,700 ms. Stimulus pulse width was 1 to 2 ms and amplitude was twice the diastolic threshold.

Tension and action potentials were measured as previously described (23). Tension was recorded using a mechanoelectric force transducer (Akers, AE 801; SensoNor, Norway). Endomyocardial biopsies were stretched stepwise (10-μm increments) to a length at which contraction force was maximal. Studies were then performed at 90% of maximal tension. Action potentials were recorded using conventional 3 M KCl-filled microelectrodes (resistance, 10 to 25 MΩ). Electrodes were coupled to an Ag–AgCl electrode connected to an amplifier (VF 102; Biologic, Claix, France). The tissue chamber was grounded through an Ag-AgCl electrode.

After equilibration, the cumulative dose-response curves of β-adrenoceptor agonists were determined by superfusion with successively increasing concentrations of drugs. For all concentrations, tension and action potentials were recorded at steady state.

Data analysis. Tension and action potentials were recorded on a digital storage oscilloscope (400; Gould Inc., Les Ullis, France), a strip chart recorder (8188; Gould Inc.) and a digital tape recorder (DTR-1200; Biologic). Twitch and action potential parameters were analyzed using DATAPAC software (Caen University, France).

The results are expressed as mean±SEM of n number of experiments. The statistical significance of the drug effect was assessed using one-way analysis of variance, followed by a Bonferonni test. To determine agonist potencies from the dose-response curves, the concentrations producing 50% of maximum effect (EC_{50}) were determined by fitting curves with the Bolzmann equation. pD_2 values were then calculated according to the equation $pD_2 = -\log(EC_{50})$ and compared using Student's t test ($P < 0.05$ being considered significant). Apparent pA_2 values were calculated according to $pA_2 = -\log$ ([antagonist]/DR-1), where DR was the dose ratio between the EC_{50} value for an agonist in the presence of a given antagonist concentration and the EC_{50} value in the absence of the antagonist.

mRNA preparation. Tissue samples weighing between 4 and 10 mg were homogenized in liquid nitrogen in 1.5-ml Eppendorf tubes. The samples were selected visually for absence of fat. The powder was resuspended in 0.8 ml RNA-PLUS™ extraction solution (Bioprobe Systems, Montreuil, France), to which 0.1 ml chloroform was added. The suspension was then shaken vigorously and kept on ice for 5 min before being centrifuged at 13,000 rpm (4°C) for 15 min. The aqueous phase was transferred to a fresh tube and an equal volume of isopropanol was added. The sample was then kept on ice for 45 min and centrifuged at 13,000 rpm (4°C) for 15 min. The supernatant was removed and the pellet washed once with 0.8 ml 75% ethanol. The RNA pellet was then dried and resuspended with an appropriate volume of diethylpyrocarbonate-treated water.

To avoid contamination with genomic DNA, total RNA was treated with amplification-grade DNase I (GIBCO BRL, Cergy Pontoise, France). Digestion was carried out at room temperature for 15 min and stopped by addition of 20 mM EDTA, pH 8.0, and incubation at 65°C for 10 min.

$PolyA^+$ RNA was directly prepared from DNase I–treated RNA, using the Dynabeads mRNA purification kit (Biosys, Compiègne, France). Briefly, total RNA was heated at 65°C for 5 min, and then hybridized to oligo (dT)25-linked magnetic beads at room temperature for 10 min, cleaned twice with washing solution (10 mM Tris-HCl, pH 7.5, 0.15 M LiCl, and 1 mM EDTA) and eluted with 24 μl of 2 mM EDTA, pH 8.0, at 65°C. $PolyA^+$ RNA was used immediately or stored at −20°C for subsequent analysis.

PCR analysis. $PolyA^+$ RNA was treated with 200 U Superscript TM II RNase H^- reverse transcriptase (GIBCO BRL) in 20 μl reverse transcriptase buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, and 3 mM $MgCl_2$) containing 5 mM DTT and 0.5 mM each dNTP, 100 ng oligo (dT)12-18 and 2 U/ml RNase Inhibitor (Pharmacia, St. Quentin en Yvelines, France). A control without reverse transcriptase was performed to verify that amplification did not proceed from residual genomic DNA. cDNA was heated 5 min at 92°C, and then amplified by 30 cycles (92°C, 1 min; 55°C, 1.5 min; 72°C, 1.5 min), followed by a 5-min extension at 72°C in a temperature cycler (Biometra Biomed. Analytik GmbH, Göttingen, Germany) in 100 μl of PCR buffer containing 2.5 U of Thermus aquaticus polymerase (Promega Corp., Madison, WI), 2.5 mM $MgCl_2$, 50 μM each dNTP, 125 nM each sense and antisense oligonucleotide primer, 2.5% (vol/vol) formamide, and 10% (vol/vol) DMSO. For β_3-adrenoceptor gene, the sequences of sense and antisense oligonucleotide primers were 5′-GCATGCTC-CGTGGCCTCACGAGAA-3′ and 5′-CTGGCTCATGATGGG-CGC-3′, respectively. The expected fragment length was 525 bp. PCR products were visualized by electrophoresis through 2% agarose ethidium bromide–stained gels. Gels were blotted onto nylon membranes (Hybond N; Amersham International, Little Chalfont, UK) that were hybridized at 65°C to the human β_3-adrenoceptor cDNA (24). Final washing conditions were 15 mM NaCl, 1.5 mM sodium citrate, and 0.1% SDS at 65°C. Membranes were then subjected to autoradiography.

Drugs. (−)-Isoprenaline, norepinephrine, nadolol, adenosine, and pertussis toxin were obtained from Sigma Chemical Co. (St. Louis, MO). Metoprolol and CGP 12177 (4-[3-*t*-butylamino-2-hydroxypropoxy]benzimidazol-2-1) were gifts from Ciba Geigy (Basel, Switzerland), bupranolol from Schwarz Pharma (Monheim, Germany), BRL 37344 (4-[-[2-hydroxy-(3-chlorophenyl)ethyl-amino]propyl] phenoxyacetate) from Smith Kline-Beecham Pharmaceuticals (Surrey, UK) and CL 316243 (5-(2-{[2-(3-chlorophenyl)-2-hydroxyethyl]-amino}propyl)-1,3-benz odioxole-2,2-dicarboxylate) from American Cyanamid (Pearl River, NY) and SR58611 ((RS)-*N*-[(25)-7-ethoxycarbonylmethoxy-1,2,3,4-tetrahydronapth-2-yl]-(2R)-2-(3-chlorophenyl)-2-hydroxyethanamine hydrochloride) from Sanofi Recherche (Montpellier, France).

Results

Effects of isoprenaline on mechanical responses. We explored the effects of cumulative concentrations of isoprenaline on the mechanical properties of human endomyocardial biopsies (Fig. 1, *A* and *B*). As expected, isoprenaline alone ($n = 5$) induced a dose-dependent increase in peak tension and produced an acceleration of twitch, the maximal effect being obtained with a 1-μM concentration. At this concentration, isoprenaline increased peak tension by 268±74% ($P < 0.05$) and accelerated twitch, decreasing total duration from 496±9 to 429±16 ms ($P < 0.05$), time-to-peak from 186±5 to 145±4 ms ($P < 0.05$), half-contraction time from 82±4 to 65±2 ms ($P < 0.05$) and half-relaxation time from 142±9 to 110±9 ms ($P < 0.05$). In another set of experiments ($n = 4$), we explored the effects of isoprenaline in the presence of 10 μM nadolol, a potent β_1- and β_2-adrenoceptor antagonist (25). Under these conditions, isoprenaline induced a dose-dependent decrease in peak tension at concentrations ranging from 0.7 to 10 μM. 10 μM isoprenaline decreased peak tension by 27.2±4.5% ($P < 0.05$). Total twitch duration was also significantly decreased from 530±35 to 513±33 ms ($P < 0.05$), but other twitch parameters were not significantly modified. At higher concentrations (e.g.,

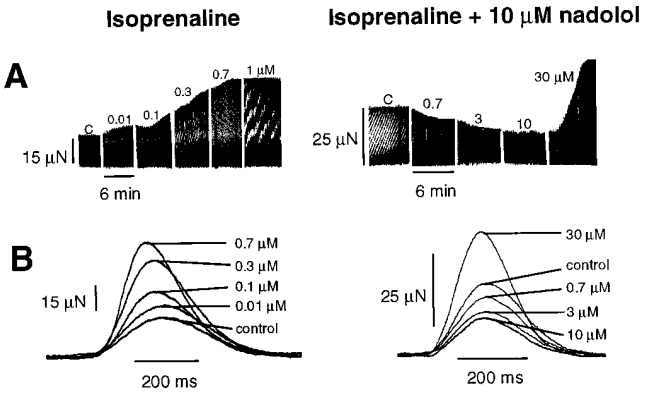

Figure 1. Effects of isoprenaline on the twitch tension of human endomyocardial biopsies alone and in the presence of nadolol. (*A*) Time course of the effects of cumulative concentrations of isoprenaline alone (*left*) and in the presence of 10 μM nadolol, a β_1- and β_2-adrenoceptor antagonist (*right*), on the twitch tension. In both cases, each concentration of isoprenaline was perfused for 6 min. When nadolol was used, it was first perfused alone and defined as control (*C*) when steady state was reached (15–20 min). Then, cumulative concentrations of isoprenaline were also perfused in the presence of nadolol. This figure is representative of data obtained in four other experiments. (*B*) Superimposed twitches obtained from the experiments illustrated in *A*.

30–100 μM), isoprenaline induced a positive inotropic response. In the presence of 10 μM nadolol, norepinephrine also decreased peak tension for concentrations ranging from 0.7 to 10 μM. At a 10-μM concentration, peak tension was decreased by 21.3±15.3% ($n = 5$, $P < 0.05$). Thus, depending on the presence of a competitive β_1- and β_2-adrenoceptor antagonist, catecholamines exerted opposite effects on the mechanical properties of the human ventricle.

Effects of β_3-adrenoceptor agonists on mechanical and electrical responses. The first set of experiments suggested that the stimulation of a third cardiac β-adrenoceptor, possibly β_3, was responsible for the unexpected negative inotropic effect produced by isoprenaline. To further investigate this possibility, a series of preferential β_3-adrenoceptor agonists was used on human endomyocardial biopsies. As illustrated in Fig. 2, BRL 37344 induced a dose-dependent negative inotropic effect at concentrations ranging from 0.1 nM to 1 mM. pD_2 values for this effect are presented in Table I. The maximum effect, obtained at a concentration of 1 μM, decreased peak tension by 59.4±2.8% ($P < 0.05$) as compared to the control. BRL 37344 accelerated twitch at the highest concentrations tested (0.7 to 3 μM). At 1 μM, it decreased total duration from 512±47 to 452±42 ms ($P < 0.05$), time-to-peak from 186±19 to 169±19 ms ($P < 0.05$), half-contraction time from 79±7 to 72±8 ms ($P < 0.05$) and half-relaxation time from 145±12 to 119±12 ms ($P < 0.05$). Since BRL 37344 possesses low affinity for β_1- and β_2-adrenoceptors (26), dose-response curves for this agonist were also plotted in the presence of several β-adrenoceptor antagonists. The dose-response curve to BRL 37344 was not modified by pretreatment with 1 μM metoprolol, a β_1-adrenoceptor antagonist (Fig. 3 *A*) or with 10 μM nadolol, a β_1- and β_2-adrenoceptor antagonist (Fig. 3 *B*). We also used bupranolol, which combines β_1-, β_2- and β_3-adrenoceptor antagonist properties (6, 16, 27). In the presence of 1 μM bupranolol, the dose-response curve to BRL 37344 on peak tension was shifted about tenfold to the right (apparent pA_2 value for bupranolol was 6.88; Fig. 3 *C*). Acceleration in the twitch, as observed at the highest concentrations of BRL 37344, was prevented in the presence of 1 μM bupranolol (data not shown).

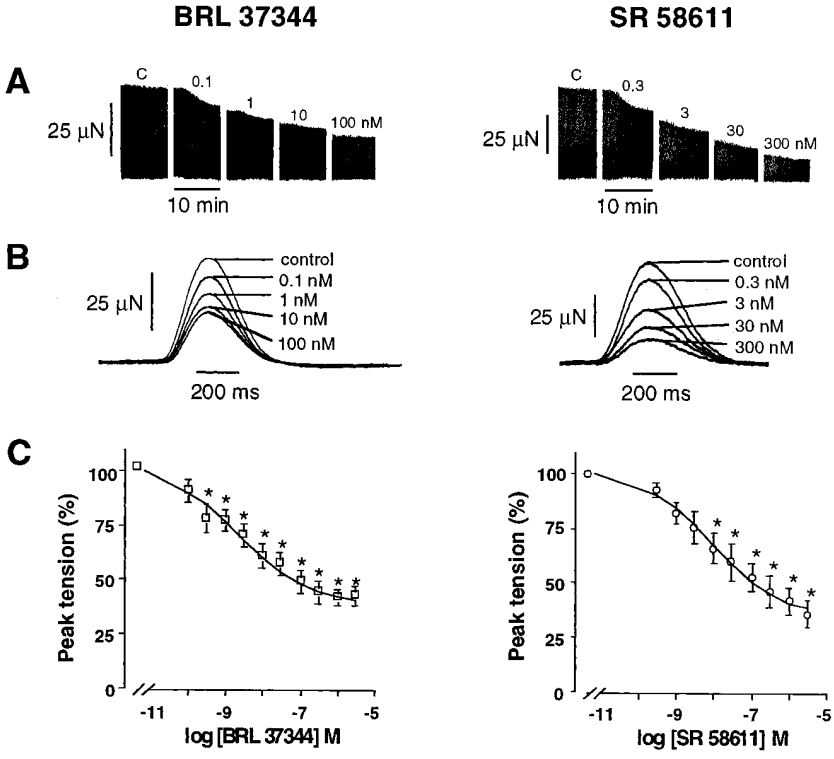

Figure 2. Effects of BRL 37344 and SR 58611 on the twitch tension of human endomyocardial biopsies. (*A*) Time course of the effects of cumulative concentrations of BRL 37344 (*left*) and SR 58611 (*right*) on twitch tension. After control (*C*), the β_3-adrenoceptor agonists were perfused for 10 min for each concentration to obtain a steady state effect. This figure is representative of the data obtained in six other experiments for BRL 37344 and five other experiments for SR 58611. (*B*) Superimposed twitches obtained from the experiments illustrated in *A*. For the clarity of *A* and *B*, data obtained at 1 and 3 μM are not shown. (*C*) Dose-response curves for the negative inotropic effect of BRL 37344 (*left*) and SR 58611 (*right*) on peak tension. Values are the means±SEM of seven experiments for BRL 37344 (□) and six experiments for SR 58611 (○). The response is expressed as a percentage of decrease in peak tension compared to the control. The continuous line was obtained by curve fitting using the Bolzmann equation. *Significant statistical difference ($P < 0.05$) from basal peak tension.

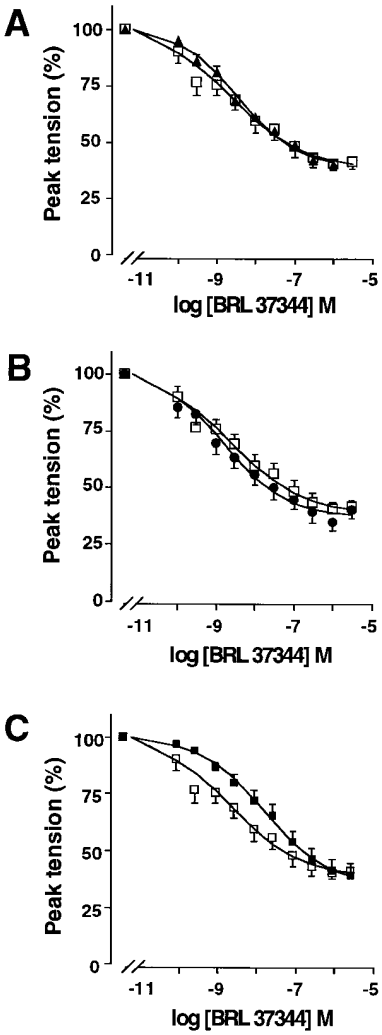

Figure 3. Dose-response curves for the negative inotropic effect of BRL 37344 in the presence of several β-adrenoceptor antagonists. When the antagonist was used, it was perfused alone until a steady state was reached (15–20 min). This was defined as control. Then, cumulative concentrations of BRL 37344 were perfused in presence of the antagonist. (*A*) Dose-response curves for the effect of BRL 37344 alone (□, $n = 7$) and in the presence of 1 μM metoprolol (▲, $n = 5$). (*B*) Dose-response curves for the effect of BRL 37344 alone (□, $n = 7$) and in the presence of 10 μM nadolol (●, $n = 7$). (*C*) Dose-response curves for the effect of BRL 37344 alone (□, $n = 7$) and in the presence of 1 μM bupranolol (■, $n = 5$). For all curves, values are the means±SEM of n experiments. The continuous lines were obtained by curve fitting using the Bolzmann equation.

Table I. Negative Inotropic Effects of Several β_3-Adrenoceptor Agonists Alone or in the Presence of Antagonists on Human Endomyocardial Biopsies

Agonists	Antagonists	pD_2	Maximal decrease	n
	μM		*% (μM)*	
BRL 37344		8.74±0.22	59.4±2.8 (1)	7
BRL 37344	10 Nadolol	8.65±0.20	65.7±3.5 (1)	7
BRL 37344	1 Metoprolol	8.34±0.08	60.0±2.5 (1)	5
BRL 37344	1 Bupranolol	7.64±0.14*	60.2±5.3 (3)	5
SR 58611		8.16±0.18	64.3±6.1 (3)	6
CL 316 243		8.24±0.21	49.9±4.8 (10)	5
CGP 12177		5.15±0.78	34.8±5.7 (100)	5

The pD_2 values of the agonists (- log EC_{50}) were estimated from each concentration–response curve. The pD_2 values are means±SEM of n experiments. The percentage of maximal decrease of peak tension is reported for each agonist. The results are mean±SEM of n experiments. The value of concentration in brackets is the concentration giving the maximal effect. *$P < 0.05$ for BRL 37344 + bupranolol vs BRL 37344 alone.

Dose-dependent negative inotropic effects were also observed with SR 58611 at concentrations ranging from 0.3 nM to 3 μM (Fig. 2). The maximum effect was observed with 3 μM, which decreased peak tension by 64.3±6.1% ($P < 0.05$). As observed with BRL 37344, an acceleration in the twitch occurred at the highest doses: at 3 μM, total duration decreased from 558±7 to 479±17 ms ($P < 0.05$), time-to-peak from 202±4 to 173±11 ms ($P < 0.05$) and half-relaxation time from 164±4 to 128±10 ms ($P < 0.05$). Two other β_3-adroneceptor agonists also induced dose-dependent negative inotropic effects: CL 316243 reduced peak tension for concentrations ranging from 1 nM to 10 μM (at 10 μM, peak tension decreased by 49.9±4.8%, $P < 0.05$) and CGP 12177 decreased peak tension for concentrations ranging from 0.01 to 100 μM (at 100 μM, peak tension decreased by 34.8±5.7%, $P < 0.05$). pD_2 values for SR 58611, CL 316243, and CGP 12177 are shown in Table I.

The effects of β_3-adrenoceptor stimulation were also studied on the electrophysiological characteristics of human endomyocardial biopsies (Fig. 4). BRL 37344 modified action potential parameters in the same range of concentrations in which it altered mechanical response (Fig. 4, *A* and *B*). At a 1-μM concentration ($n = 4$), BRL 37344 induced a reduction in the action potential duration measured at 30% of repolarization (APD_{30})[1] from 203±15 to 164±9 ms ($P < 0.05$), APD_{50} from 256±17 to 218±17 ms ($P < 0.05$) and APD_{90} from 336±30 to 294±31 ms ($P < 0.05$). Action potential amplitude was also reduced from 111.3±2.1 to 107.5±1.6 mV ($P < 0.05$). Comparable effects were produced by SR 58611 (Fig. 4 *C*). It is noteworthy that a similar reduction in action potential duration was observed with isoproterenol in the presence of 10 μM nadolol (data not shown).

Effects of β_3-adroneceptor stimulation in the presence of pertussis toxin. In an attempt to define the involvement of G_i proteins in the negative inotropic effects produced by β_3-adrenoceptor agonists, the effects of BRL 37344 were tested on human endomyocardial biopsies pretreated with pertussis toxin (PTX, Fig. 5). To determine PTX concentrations that would block G_i proteins, adenosine stimulation was used as an assay because adenosine is generally thought to negatively modulate β-adrenoceptor stimulation by activating a PTX-sensitive G-protein pathway. Norepinephrine was used to stimulate adenylate cyclase in the presence of 1 μM prazosin to block α-adrenoceptors. In non-PTX–pretreated tissues, 1 μM norepinephrine alone produced a positive inotropic effect (peak tension increased by 61.7±19.3%, $n = 6$). When a steady state was reached in the presence of norepinephrine, 10 μM adenosine was added. Under these conditions, adenosine blunted the positive inotropic response to norepinephrine (peak tension decreased by 31.44±5.24%, $n = 6$). The same experiments were repeated in endomyocardial biopsies pretreated with 0.5 μg/ml PTX for 2 h at 30°C. In the presence of PTX, 1 μM norepinephrine increased peak tension by 42.1±13.2% ($n = 5$). But in this case, adenosine did not induce negative inotropic effects, demonstrating that activation of G_i was effectively blocked. The effects of 10 nM BRL 37344 were then studied in control and PTX-pretreated endomyocardial biopsies. In con-

1. *Abbreviations used in this paper:* APD, action potential duration; PTX, pertussis toxin.

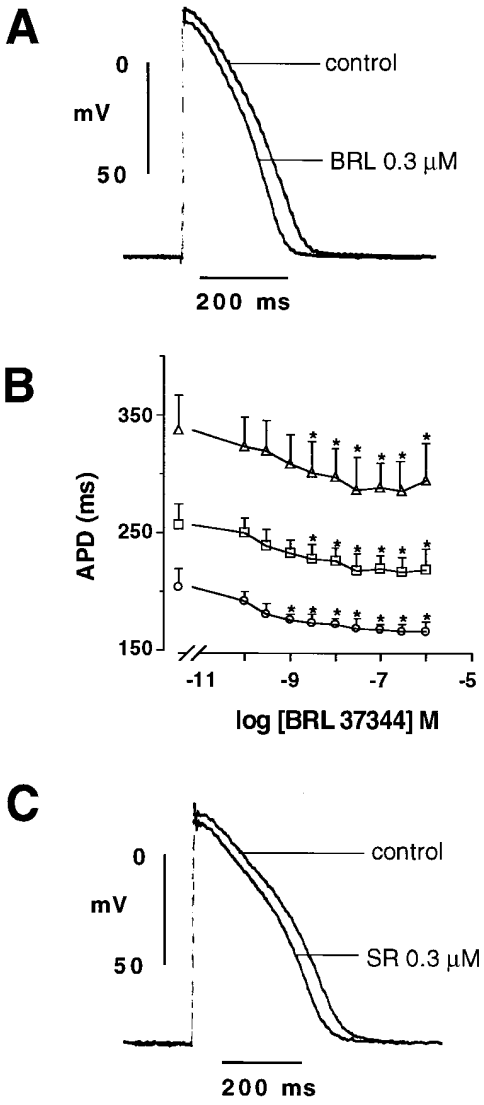

Figure 4. Effects of BRL 37344 and SR 58611 on action potentials of human endomyocardial biopsies. (*A*) Superimposed action potentials recorded from an endomyocardial biopsy before (*control*) and after perfusion of 0.3 μM BRL 37344. BRL 37344 decreased the amplitude of action potential from 117 (*control*) to 112 mV and reduced APD_{30} from 190 to 146 ms, APD_{50} from 231 to 191 ms, and APD_{90} from 290 to 250 ms. (*B*) Dose-response curves for the effects of BRL 37344 on the action potential duration at 30% (○), 50% (□) and 90% (△) of repolarization (APD). Values are the means±SEM of four experiments. *Significant statistical difference ($P < 0.05$) from the control value. (*C*) Superimposed action potentials recorded from an endomyocardial biopsy before (*control*) and after perfusion of 0.3 μM of SR 58611. SR 58611 decreased the amplitude of action potential from 109 (*control*) to 104 mV and reduced APD_{30} from 210 to 178 ms, APD_{50} from 290 to 254 ms, and APD_{90} from 300 to 256 ms.

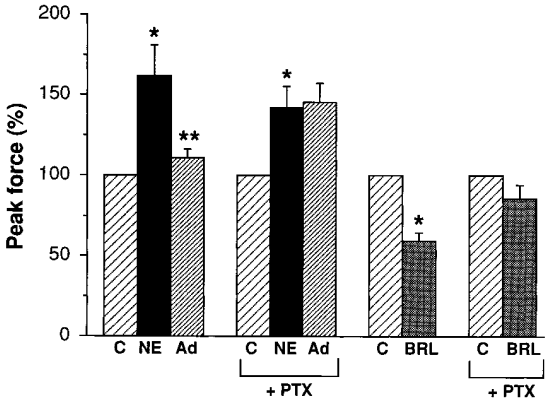

Figure 5. The effects of PTX (0.5 μg/ml for 2 h) on the negative inotropic responses of adenosine (*Ad*) and BRL 37344 (*BRL*) in human endomyocardial biopsies. To demonstrate adenosine inhibition, 1 μM norepinephrine (*NE*), in the presence of 1 μM prazosin, was used to stimulate β_1-adrenoceptors increasing contractility. For adenosine, the bars from left to right represent control peak force (*C*), peak force in the presence of NE (*NE*), and the effect of 10 μM adenosine on NE-stimulated peak force (*Ad*). Results before ($n = 6$) and after ($n = 5$) PTX treatment are illustrated. For BRL 37344, the bars illustrate control peak force and the inhibitory effect of BRL 37344 before ($n = 7$) and after ($n = 3$) PTX treatment. Values are the means±SEM of n experiments. *$P < 0.05$ vs C; **$P < 0.05$ vs NE.

trol tissues, BRL 37344 induced a decrease in peak tension of 40.5±5.1% ($n = 7$). In PTX-pretreated tissues, the negative inotropic effects of BRL 37344 were markedly reduced (peak tension only decreased by 14.1±8.3%, $n = 3$).

β_3-adrenoceptor mRNA in human myocardium. To confirm the identity of the receptor involved in β_3-adrenoceptor agonist effects, mRNA expression of β_3-adrenoceptors was studied in human endomyocardial biopsies. A reverse transcription PCR protocol on $PolyA^+$ RNA was developed to avoid contamination by genomic DNA (Fig. 6). When human β_3-adrenoceptor gene primers were used, the length of PCR-amplified product corresponded to the gene structure. Hybridization to human β_3-adrenoceptor cDNA confirmed the identity of the fragment. With this technique, β_3-adrenoceptor mRNA expression was detected in the myocardium of 5/5 subjects. We checked for possible contamination of the biopsies with fat cells expressing β_3-adrenoceptors, using hormone-sensitive lipase as a marker of brown and white fat cells. When primers specific for the human hormone-sensitive lipase gene were used (28), no specific products were amplified from human myocardium; whereas, strong signals were obtained from human white adipocytes (data not shown).

Discussion

Various studies have shown that β_1- and β_2-adrenoceptors coexist in the human heart and that their stimulation produces positive inotropic effects in in vitro human atrial and ventricular preparations (29–33). To our knowledge, there have been no previous reports as to a direct cardiac effect through β_3-adrenoceptor stimulation. Our study conclusively demonstrates the presence of functional β_3-adrenoceptors in the human heart ventricle and indicates that their stimulation induces surprisingly negative inotropic effects.

In stark contrast with the effects of isoprenaline alone, we observed that isoprenaline induced a negative inotropic effect when applied in the presence of nadolol, a potent β_1- and β_2-adrenoceptor antagonist possessing no β_3-adrenoceptor antagonist properties (27, 34). From this result, the existence of another adrenoceptor distinct from β_1 and β_2 was suspected. The existence of β_3-adrenoceptors in the human myocardium was further assumed when we observed that β_3-adrenoceptor ago-

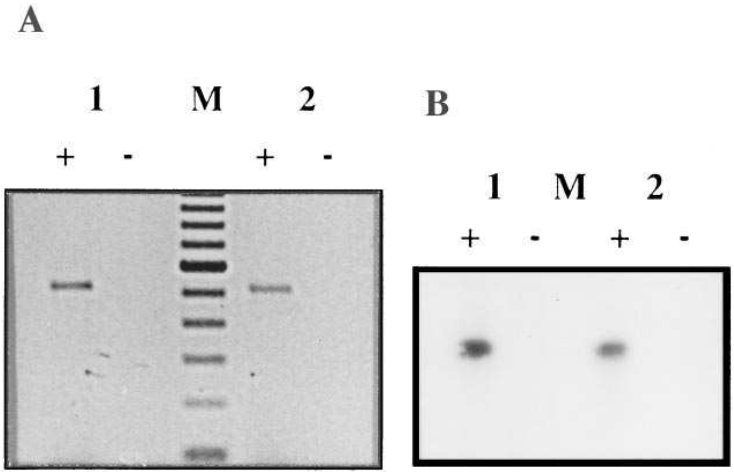

Figure 6. β_3-adrenoceptor mRNA expression in human myocardium. (*A*) The negative of an agarose gel electrophoresis photo of β_3-adrenoceptor cDNA amplified by PCR. PolyA$^+$ RNAs were subjected (+) or not (−) to reverse transcription. Size markers (*M*) are the 100-bp DNA ladder. (*B*) Southern blot hybridized to a human β_3-adrenoceptor cDNA probe. The membrane was autoradiographed for 14 h.

nists also induced negative inotropic effects with an order of potency: BRL 37344 > SR 58611 ≈ CL 316243 > CGP 12177 similar to that observed in chinese hamster ovary cells transfected with human β_3-adrenoceptors (8, 26). The most convincing pharmacologic evidence for the presence of β_3-adrenoceptors in the human heart was supported by the use of β-adrenoceptor antagonists. The mechanical effects of BRL 37344 were not modified by pretreatment with metoprolol (a β_1-adrenoceptor antagonist) or nadolol indicating that this effect was not mediated by β_1- or β_2-adrenoceptors. By contrast, bupranolol, which possesses β_3-adrenoceptor antagonist properties (6, 27, 35), antagonized the negative inotropic effects of BRL 37344 with a pA_2 value similar to that determined in adipocytes (6, 27). Finally, pharmacological evidence for myocardial β_3-adrenoceptor was strengthened by detection of β_3-adrenoceptor transcripts in the human ventricle. β_3-adrenoceptor mRNA expression has already been reported in human heart biopsies, particularly at the atrial level (36, 37). However, these reports did not exclude an adipose origin for β_3-adrenoceptor transcripts since a significant amount of adipose tissue was found in the samples (36). As adipocytes are rare in endomyocardial biopsies, it is not surprising that no specific products corresponding to the human hormone-sensitive lipase gene were detected in our own samples. Yet this would indicate that expression of β_3-adrenoceptors in the human ventricle was not due to the presence of adipocytes.

Previous in vivo studies have evaluated whether β_3-adrenoceptors exist in the cardiovascular system. In dogs, intravenously administered β_3-adrenoceptor agonists induced a positive chronotropic effect (18). Because positive chronotropic effects were not observed in denervated animals, it was concluded that tachycardia resulted from a baroreceptor-mediated reflex in response to a drop in blood pressure caused by the vasodilating action of β_3-adrenoceptor agonists (14, 15). Positive chronotropic and inotropic effects were also reported in isolated dog atrium perfused with blood from a donor dog injected with β_3-adrenoceptor agonists. These effects were attributed to β_1-adrenoceptor stimulation (19) and could be interpreted as resulting from baroreflex activation in the donor dog. In the clinical setting, β_3-adrenoceptor agonists were shown to increase heart rate and blood pressure in man, an effect that was prevented by β_2-adrenoceptor antagonists (21). In vitro studies are more suitable to analyzing the cardiac effects of β_3-adrenoceptors. A typical example of the masking effects of baroreflex activation lies with 1,4-dihydropyridines, which induce a negative inotropic effect in vitro, but a positive chronotropic and inotropic effect in vivo as a consequence of vasodilation (38). In mammalian hearts, purported "atypical β-adrenoceptors" were suggested to be responsible for the positive inotropic and chronotropic effects of partial β-adrenoceptor agonists such as pindolol and alprenolol (16). However, human β_3-adrenoceptors are distinct from atypical β-adrenoceptors of the mammalian heart inasmuch as the positive inotropic and chronotropic effects of partial agonists are not observed in isolated human tissues (17), and β_3-adrenoceptor agonists induced a negative inotropic effect opposite the reported effects of "atypical β-adrenoceptors" stimulation in animals.

As the β_3-adrenoceptor stimulation induced a marked negative inotropic effect, it could be hypothesized that this receptor is not coupled to G_s protein. It has been previously shown that in adipocytes β_3-adrenoceptors could be linked to G_i proteins (39, 40). Furthemore, the β_2-adrenoceptor has recently been shown to activate both G_s and G_i proteins (41). In our study, in PTX-pretreated tissues, the negative inotropic effects of BRL 37344 were markedly reduced, suggesting the involvement of G_i proteins in the β_3-adrenoceptor signaling pathway. However, the negative inotropic effects of BRL 37344 are not fully suppressed, either because G_i proteins are not completely blocked by PTX or because another mechanism coexists with the G_i pathway. Therefore, a more complete study will be necessary to determine the receptor-effector pathways.

The present study raises the question as to the role of β_3-adrenoceptors in cardiac diseases. In heart failure, increased activity of the sympathetic nervous system leads to downregulation of cardiac β_1- and β_2-adrenoceptors (22) resulting from their phosphorylation by cAMP-dependent protein kinase or β-adrenoceptor kinase. Reduced β_1- and β_2-adrenoceptors lead to a decrease in the contractile response to β-adrenoceptor agonists. Contrary to β_1- and β_2-adrenoceptors, β_3-adrenoceptors lack phosphorylation sites for cAMP-dependent protein kinase or β-adrenoceptor kinase (3), and thus may not be downregulated in heart failure. According to this hypothesis, the high adrenoceptor tone during heart failure may alter the cardiac contractile activity as a result of unmasked β_3-adrenoceptor stimulation in the presence of reduced β_1- and β_2-adrenoceptors. Finally, the present study also has implication in clinical pharmacology. Indeed, the development of β_3-adrenoceptor agonists have led to the elaboration of prom-

ising new drugs. The predicted therapeutic indications for these drugs are obesity and obesity-linked diabetes. However, we have demonstrated that β_3-adrenoceptors are also involved in regulation of the mechanical and electrophysiological activities of the human heart and thus may produce cardiac side effects. Clearly, further studies are needed to address the precise role of cardiac β_3-adrenoceptor in human cardiac physiology and physiopathology.

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References

1. Bylund, D.V., D.C. Einkenberg, J.P. Hieble, S.Z. Langer, R.J. Lefkowitz, K.P. Minneman, P.B. Molinoff, R.R. Ruffolo, and U. Trendelenburg. 1994. International union of pharmacology nomenclature of adrenoceptors. *Pharmacol. Rev.* 46:121–136.

2. Granneman, J.G., K.N. Lahners, and A. Chaudhry. 1993. Characterization of the human β_3-adrenoceptor receptor gene. *Mol. Pharmacol.* 44:264–270.

3. Strosberg, A.D. 1993. Structure, function, and regulation of adrenoceptor receptors. *Protein Sci.* 2:1198–1209.

4. Arch, J.R.S., A.T. Ainsworth, M.A. Cawthorne, V. Piercy, M.V. Sennit, V.E. Thody, C. Wilson, and S. Wilson. 1984. Atypical β-adrenoceptor on brown adipocytes as target for anti-obesity drugs. *Nature (Lond.).* 309:163–165.

5. Zaagsma, J., and S.R. Nahorski. 1990. Is the adipocyte β-adrenoceptor a prototype for the recently cloned atypical *beta* 3-adrenoceptor? *Trends Pharmacol. Sci.* 11:3–7.

6. Langin, D., M.P. Portillo, J.S. Saulnier-Blache, and M. Lafontan. 1991. Coexistence of three β-adrenoceptor subtypes in white fat cells of various mammalian species. *Eur. J. Pharmacol.* 199:291–301.

7. Lönnqvist, F., S. Krief, A.D. Strosberg, B. Nyberg, L.J. Emorine, and P. Arner. 1993. Evidence for a functional β_3-adrenoceptor in man. *Br. J. Pharmacol.* 110:929–936.

8. Pietri-Rouxel, F., and A.D. Strosberg. 1995. Pharmacological characteristics and species-related variations of β_3-adroneceptor receptors. *Fundam. & Clin. Pharmacol.* 9:211–218.

9. Lowell, B.B., and J.S. Flier. 1995. The potential significance of β_3-adrenoceptor receptors. *J. Clin. Invest.* 95:923.

10. Bond, R.A., and D.E. Clarke. 1988. Agonist and antagonist characterization of a putative adrenoceptor with distinct pharmacological properties from the α- and β-subtypes. *Br. J. Pharmacol.* 95:723–734.

11. Manara, L., and A. Bianchetti. 1990. The phenylethanoaminotetralines: new selective agonists for atypical β-adrenoceptors. *Trends Pharmacol. Sci.* 11: 229–230.

12. Koike, K., I. Takayanagi, M. Muramatsu, S.I. Ohki, and T. Horinouchi. 1994. Involvement of β_3-adrenoceptor in the relaxation response in guinea pig taenia caecum. *Jpn. J. Pharmacol.* 66:213–220.

13. Martin, C.A.E., and C. Advenier. 1995. $Beta_3$-adrenoceptors and airways. *Fundam. & Clin. Pharmacol.* 9:114–118.

14. Berlan, M., J. Galitzky, A. Bousquet-Melou, M. Lafontan, and J.L. Montastruc. 1994. *beta-3* adrenoceptor-mediated increase in cutaneous blood flow in the dog. *J. Pharmacol. Exp. Ther.* 268:1444–1451.

15. Shen, Y.T., H. Zhang, and F. Vatner. 1994. Peripheral vascular effects of *beta*-3 adrenoceptor receptor stimulation in conscious dogs. *J. Pharmacol. Exp. Ther.* 268:466–473.

16. Kaumann, A.J. 1989. Is there a third heart β-adrenoceptor? *Trends Pharmacol. Sci.* 10:316–320.

17. Kaumann, A.J., and B.M. Lobnig. 1986. Mode of action of (−)-pindolol on feline and human myocardium. *Br. J. Pharmacol.* 89:207–218.

18. Tavernier, G., J. Galitzky, A. Bousquet-Melou, J.L. Montastruc, and M. Berlan. 1992. The positive chronotropic effect induced by BRL 37344 and CGP 12177, two *beta*-3 adrenoceptor agonists, does not involve cardiac *beta* adrenoceptors but baroreflex mechanisms. *J. Pharmacol. Exp. Ther.* 263:1083–1090.

19. Takayama, S., Y. Furukawa, L.M. Ren, Y. Inoue, S. Sawaki, and S. Chiba. 1993. Positive chronotropic and inotropic responses to BRL 37344, a β_3-adrenoceptor agonist in isolated, blood-perfused dog atria. *Eur. J. Pharmacol.* 231:315–321.

20. Wheeldon, N.M., D.G. McDevitt, and B.J. Lipworth. 1993. Investigation of putative cardiac β_3-adrenoceptors in man. *Q. J. Med.* 86:255–261.

21. Wheeldon, N.M., D.G. McDevitt, and B.J. Lipworth.1994. Cardiac effects of the β_3-adrenoceptor agonist BRL 35135 in man. *Br. J. Clin. Pharmacol.* 37:363–369.

22. Brodde, O.E. 1993. β-adrenoceptors in cardiac disease. *Pharmacol. & Ther.* 60:405–430.

23. Gauthier, C., K. Laurent, F. Charpentier, E. Drouin, J.C. Chevallier, and H. Le Marec. 1994. Endomyocardial biopsies: a new approach for studying the electrical and mechanical properties of human ventricular myocardium. *J. Mol. Cell. Cardiol.* 26:1267–1271.

24. Lelias, J.M., M. Kaghad, M. Rodriguez, P. Chalon, J. Bonnin, I. Dupre, B. Delpech, M. Bensaid, G. LeFur, P. Ferrara, and D. Caput. Molecular cloning of a human beta 3-adrenoceptor receptor cDNA. *FEBS Lett.* 324:127–130.

25. Lee, R.J., D.B. Evans, S.H. Baky, and R.J. Laffan. 1975. Pharmacology of nadolol (SQ 11725), a beta-adrenoceptor antagonist lacking direct myocardial depression. *Eur. J. Pharmacol.* 33:371–382.

26. Dolan, J.A., H.A. Muenkel, M.G. Burns, S.M. Pellegrino, C.M. Fraser, F. Pietri, A.D. Strosberg, E.E. Largis, M.D. Dutia, J.D. Bloom et al. 1994. *Beta*-3 adrenoceptor selectivity of the dioxolane dicarboxylate phenethanolamines. *J. Pharmacol. Exp. Ther.* 269:1000–1006.

27. Galitzky, J., M. Reverte, C. Carpene, M. Lafontan, and M. Berlan. 1993. Beta 3-adrenoceptors in dog adipose tissue: studies on their involvement in the lipomobilizing effect of catecholamines. *J. Pharmacol. Exp. Ther.* 266:358–366.

28. Langin, D., H. Laurell, L. Stenson Holst, P. Belfrage, and C. Holm. 1993. Gene organization and primary structure of human hormone-sensitive lipase: possible significance of a sequence of homology with a lipase of Moraxella TA144, an antartic bacterium. *Proc. Natl. Acad. Sci. USA.* 90:4897–4901.

29. Jones, C.R., P. Molenaar, and R.J. Summers. 1989. New views of human cardiac β-adrenoceptors. *J. Mol. Cell. Cardiol.* 21:519–535.

30. Kaumann, A.J., J.A. Hall, K.J. Murray, F.C. Wells, and M.J. Brown. 1989. A comparison of the effects of adrenaline and noradrenaline on human heart: the role of β_1- and β_2-adrenoceptors in the stimulation of adenylate cyclase and contractile force. *Eur. Heart J.* 10(Suppl. B):29–37.

31. Bristow, M.R., R.E. Hershberger, J.D. Port, E.M. Gilbert, A. Sandoval, R. Rasmussen, A.E. Cates, and A.M. Felman. 1990. β-adrenoceptor pathways in nonfailing and failing human ventricular myocardium. *Circulation.* 82(Suppl. I):12–25.

32. Brodde, O.E. 1991. β_1- and β_2-adrenoceptors in the human heart: properties, function, and alterations in chronic heart failure. *Pharmacol. Rev.* 43: 203–242.

33. Del Monte, F., A.J. Kaumann, P.A. Poole-Wilson, D.G. Wynne, J. Pepper, and S.E. Harding.1993. Coexistence of functioning β_1- and β_2-adrenoceptors in single myocytes from human ventricle. *Circulation.* 88:854–863.

34. Emorine, L.J., S. Marullo, M.M. Briend-Sutren, G. Patey, K. Tate, C. Delavier-Klutchko, and A.D. Strosberg. 1989. Molecular characterization of the human β_3-adrenoceptor receptor. *Science (Wash. DC).* 245:1118–1121.

35. Sugasawa, T., M. Matsuzaki, S. Morooka, N. Foignant, N. Blin, and A.D. Strosberg. 1992. *In vitro* study of a novel atypical beta-adrenoceptor agonist, SM-1044. *Eur. J. Pharmacol.* 216:207–215.

36. Krief, S., F. Lönnqvist, S. Raimbault, B. Baude, A. Van Spronsen, P. Arner, A.D. Strosberg, D. Ricquier, and L.J. Emorine. 1993. Tissue distribution of β_3-adrenoceptor receptor mRNA in man. *J. Clin. Invest.* 91:344–349.

37. Berkowitz, D.E., N.A. Nardone, R.M. Smiley, D.T. Price, D.K. Kreutter, R.T. Fremeau, and D.A. Schwinn. 1995. Distribution of β_3-adrenoceptor mRNA in human tissues. *Eur. J. Pharmacol.* 289:223–228.

38. Piepho, R.W. 1991. Heterogeneity of calcium channel blockers. *Hosp. Pharm.* 26:856–864.

39. Chaudhry, A., R.G. MacKenzie, L.M. Georgic, and J.G. Granneman. 1994. Differential interaction of β_1- and β_3-adrenoceptor receptors with G_i in rat adipocytes. *Cell Signalling.* 6:457–465.

40. Begin-Heick, N. 1995. β_3-adrenoceptor activation of adenylyl cyclase in mouse white adipocytes: modulation by GTP and effect of obesity. *J. Cell. Biochem.* 58:464–473.

41. Xiao, R.P., X. Ji, and E.G., Lakatta. 1995. Functional coupling of the β_2-adrenoceptor to a pertussis toxin-sensitive G protein in cardiac myocytes. *Mol. Pharmacol.* 47:322–329.