0026-895X/93/040548-08$03.00/0

MOLECULAR PHARMACOLOGY, 43:548-555

The Human β_3-Adrenergic Receptor Is Resistant to Short Term Agonist-Promoted Desensitization

FRANÇOIS NANTEL, HÉLÈNE BONIN, LAURENT J. EMORINE, VLADIMIR ZILBERFARB, A. DONNY STROSBERG, MICHEL BOUVIER, and STEFANO MARULLO

Département de Biochimie et Groupe de Recherche sur le Système Nerveux Autonome, Université de Montréal, Montréal, Quebec, H3C 3J7, Canada (F.N., H.B., M.B.), and CNRS-UPR 0415 and Université Paris VII, Institut Cochin de Génétique Moléculaire, F-75014 Paris, France (L.J.E., V.Z., A.D.S., S.M.)

Received September 18, 1992; Accepted February 2, 1993

SUMMARY

The human β_3-adrenergic receptor (β_3AR) lacks most of the structural determinants that, in the β_2AR, contribute to agonist-induced receptor desensitization. To evaluate the effect of these structural differences on the β_3AR desensitization profile, the human β_2- and β_3AR were stably expressed in Chinese hamster fibroblasts (CHW) and murine Ltk$^-$ cells (L cells). Incubation of CHW-β_2 or L-β_2 cells with 10 μM isoproterenol for 30 min induced a decrease in the maximal agonist-stimulated adenylyl cyclase activity and a cAMP-dependent reduction in the potency of isoproterenol to stimulate the receptor. In addition, this pretreatment impaired the formation of the high affinity heterotrimeric agonist-receptor-guanine nucleotide-binding protein complex and induced the sequestration of ~30% of the β_2AR away from the cell surface. In contrast, similar treatment of CHW-β_3 and L-β_3 cells did not affect the maximal receptor-stimulated adenylyl cyclase activity, nor did it induce any significant sequestration of the β_3AR. In fact, only a modest cAMP-independent decrease in the potency of isoproterenol to stimulate the receptor could be observed after isoproterenol treatment. The rapid desensitization pattern of a chimeric β_3AR, in which the third cytoplasmic loop and the carboxyl-terminal tail were exchanged with those of the β_2AR (which include potential phosphorylation sites and other possible molecular determinants of desensitization), was found to be intermediate between those of the two original receptor subtypes. These results demonstrate that (i) the β_3AR is less prone than the β_2AR to undergo rapid agonist-promoted desensitization and, (ii) in addition to the phosphorylation sites located in the third cytoplasmic loop and the carboxyl-terminal tail of the β_2AR, other molecular determinants contribute to short term desensitization.

Sustained stimulation of many hormone receptors leads to a rapid decrease of their responsiveness. This very general regulatory process, known as desensitization, tends to limit the time of activation of the cellular signal and thus prevents overstimulation. The desensitization of the β_2AR has been particularly well studied (1–3). Both a functional uncoupling of the receptor from G_s and a rapid sequestration of the receptor away from the cell surface are believed to contribute to this regulatory process. The functional uncoupling of the β_2AR has been shown to result from its phosphorylation by PKA and βARK (4–8). It was therefore suggested that phosphorylation may represent a general mechanism involved in the desensitization of G protein-coupled receptors (9).

This work was supported, in part, by grants from the Medical Research Council of Canada, the Canadian Heart and Stroke Foundation, the CNRS, the Université de Paris VII, and Bristol Myers Squibb Company. F.N. holds a studentship from the Canadian Heart and Stroke Foundation and the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche du Québec, and M.B. is a Scholar from the Medical Research Council of Canada.

It has been shown that, in the β_2AR, the targets of phosphorylation by PKA and βARK are located in the third cytoplasmic loop and the carboxyl terminus of the receptor (4, 5). Interestingly, most of those phosphorylation sites are absent from the primary sequence of another βAR subtype, the β_3AR (10). This suggests that the β_3AR, which has been implicated in the control of metabolic processes in adipocytes (10–13), may be less prone to agonist-induced desensitization.

To test this hypothesis, the effects of sustained agonist stimulation on β_3AR responsiveness were investigated in CHW and L cells stably expressing the human β_3AR gene. Characterization of the rapid desensitization of this receptor is of considerable interest, because it is considered a potential target for antiobesity drugs that would be selective β_3AR agonists (11, 13).

Here we report that a 30-min exposure to isoproterenol, which leads to a significant desensitization of the β_2AR-stimulated adenylyl cyclase activity, had only a marginal effect on

ABBREVIATIONS: βAR, β-adrenergic receptor(s); Ch$\beta_3\beta_2$AR, chimeric β_3/β_2-adrenergic receptor; PKA, cAMP-dependent protein kinase; βARK, β-adrenergic receptor kinase; CHW, Chinese hamster fibroblasts; L cells, murine Ltk$^-$ cells; ^{125}I-CYP, ^{125}I-cyanopindolol; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; Gpp(NH)p, guanyl-5′-yl-imidodiphosphate; Bt$_2$cAMP, N^6,$O^{2'}$-dibutyryladenosine 3′,5′-cyclic monophosphate; G_s, stimulatory guanine nucleotide-binding regulatory protein; FBS, fetal bovine serum; G protein, guanine nucleotide-binding protein.

β_3AR responsiveness. The addition of the β_2AR phosphorylation sites to the sequence of the β_3AR, by substitution of the third cytoplasmic loop and the carboxyl-terminal tail of the β_3AR with the corresponding regions of the β_2AR, partially restored desensitization. These results, although consistent with the role of phosphorylation sites, suggest that other molecular determinants are also implicated in the development of agonist-promoted desensitization.

Experimental Procedures

Materials. [α-^{32}P]ATP was obtained from New England Nuclear or ICN Biochemicals; [^{3}H]cAMP and ^{125}I-CYP were purchased from New England Nuclear. Isoproterenol, norepinephrine, epinephrine, DL-propranolol, (−)-alprenolol, ATP, GTP, cAMP, phosphoenolpyruvate, myokinase, isobutylmethylxanthine, Bt$_2$cAMP, leupeptin, soybean trypsin inhibitor, and benzamidine were obtained from Sigma. Pyruvate kinase was from Calbiochem. DMEM, PBS, trypsin, FBS, horse serum, geneticin (G418), penicillin, streptomycin, and fungizone were purchased from GIBCO/BRL. CGP-12177 was a generous gift from Ciba Geigy. ICI-118551 and BRL-37344 were kindly provided by Imperial Chemical Industries and SmithKline Beecham, respectively.

DNA construction and cell transfection and culture. The human β_2AR cDNA was cloned into the pBC12BI expression vector (4). A ~2.6-kilobase *Sma*I-*Sac*I fragment of the human β_3AR gene (10), containing the totality of the coding region and part of the 3′ untranslated region, was cloned into the *Hind*III-*Sma*I sites of the pBC12BI plasmid.

A chimeric receptor consisting of the β_3AR with the third cytoplasmic loop and the carboxyl-terminal tail of the β_2AR was constructed as follows. Silent mutations were created in the β_2- and β_3AR genes by site-directed mutagenesis, following the method of Kunkel *et al.* (14), to generate unique restriction sites. These sites were *Avr*II (ccTAGG at position 2085), *Pst*I (CTGCaG at position 2241), and *Sca*I (AGTacT at position 2509) in the β_2AR sequence (15) and *Avr*II (CctaGG at position 1516) and *Pst*I (CTGaGg at position 1674) in the coding region of the β_3AR (10). The *Acc*I-*Avr*II fragment (encoding the third cytoplasmic loop, Val^{218}-Leu^{275}) and the *Pst*I-*Sca*I fragment (encoding the carboxyl-terminal tail, Arg^{328}-Leu^{413}) from the β_2AR were substituted for those of the β_3AR. The Ch$\beta_3\beta_2$AR gene fragment was subcloned in the pBC12BI vector as described above. Identity of the chimeric receptor was confirmed by dideoxynucleotide sequencing.

Each of the constructs described above was cotransfected with the pSVneo plasmid (Pharmacia) into CHW-1102 and/or L cells by calcium phosphate precipitation (16). Neomycin-resistant cells were selected in DMEM supplemented with 10% (v/v) FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml fungizone, 1 mM glutamine, and geneticin at a concentration of 150 μg/ml (for CHW) or 450 μg/ml (for L cells). Individual clones were screened for βAR expression by radioligand binding assays, using ^{125}I-CYP as ligand.

Membrane preparation and radioligand binding assays. Nearly confluent cells, preincubated (when required) for 30 min at 37° with 10 μM isoproterenol in DMEM supplemented as described above, were washed twice with ice-cold PBS. Washed cells were mechanically detached and resuspended in 10 ml of an ice-cold buffer containing 5 mM Tris, 2 mM EDTA, pH 7.4, 5 mg/liter soybean trypsin inhibitor, 5 mg/liter leupeptin, and 10 mg/liter benzamidine (buffer A). The suspensions were homogenized for 5 sec with a Polytron homogenizer (Janke & Undel Ultra-Turrax T25) at maximum setting. The lysates were then centrifuged at 500 × g for 5 min at 4° and the supernatants were centrifuged again at 43,000 × g for 20 min at 4°. The pellets were washed in 10 ml of buffer A and recentrifuged at 43,000 × g for 20 min at 4°. The pelleted membranes were resuspended in buffer B (75 mM Tris, 12.5 mM $MgCl_2$, 2 mM EDTA, pH 7.4, 5 mg/liter soybean trypsin inhibitor, 5 mg/liter leupeptin, 10 mg/liter benzamidine). Protein concentrations were determined by the method of Bradford (17), using the Bio-Rad protein assay system with bovine serum albumin as standard.

Radioligand binding assays were performed as described previously (18), in a total volume of 0.5 ml containing 5–10 μg of protein. Full saturation binding isotherms were conducted using 0–400 pM ^{125}I-CYP for the β_2AR and 0–1400 pM ^{125}I-CYP for the β_3AR and the Ch$\beta_3\beta_2$AR. Specific binding was defined as the binding selectively inhibited by 10 μM (−)-alprenolol. The binding assays were conducted for 90 min at 22° and were terminated by rapid filtration over Whatman GF/C glass fiber filters. To reduce nonspecific binding, filter papers were presoaked in 25 mM Tris, pH 7.4, 0.3% polyethyleneimine, 0.1% bovine serum albumin. K_d values obtained from binding isotherms were not affected by either isoproterenol or Bt$_2$cAMP pretreatments. Therefore, receptor numbers were routinely evaluated using 250 pM ^{125}I-CYP for the β_2AR and 450 pM ^{125}I-CYP for the β_3AR and the Ch$\beta_3\beta_2$AR. It is important to note that 450 pM ^{125}I-CYP does not saturate all β_3AR binding sites. Higher concentrations were not used for economic reasons and because of high nonspecific binding. Estimations obtained using 450 pM ^{125}I-CYP were in good agreement with the numbers obtained in full saturation binding isotherms.

Competition binding assays were performed in duplicate using ~45 pM or ~300 pM ^{125}I-CYP for β_2AR and β_3AR, respectively, and 0–2000 μM concentrations of competitors [isoproterenol, norepinephrine, epinephrine, BRL-37344, ICI-118551, DL-propranolol, and (−)-alprenolol]. Competition assays with agonists were conducted in the presence and absence of 100 μM Gpp(NH)p. Binding data were analyzed by nonlinear least-squares regression using the computer program LIGAND (19).

Adenylyl cyclase assays. The activity of adenylyl cyclase was measured using the method described by Salomon *et al.* (20). Cell membranes were prepared as described above for radioligand binding and were resuspended in buffer B containing 5 mM instead of 12.5 mM $MgCl_2$. The assay mixture contained 0.02 ml of membrane suspension (2–6 μg of protein), 45 mM Tris, pH 7.4, 3 mM $MgCl_2$, 1.2 mM EDTA, 0.12 mM ATP, 0.053 mM GTP, 0.1 mM cAMP, 0.1 mM isobutylmethylxanthine, 1 μCi of [α-^{32}P]ATP, 2.8 mM phosphoenolpyruvate, 0.2 unit of pyruvate kinase, and 1 unit of myokinase, in a final volume of 50 μl. Enzyme activity was determined in the presence of 0–100 μM isoproterenol for 15 min at 37°. Reactions were terminated by the addition of 1 ml of ice-cold stop solution containing 0.4 mM ATP, 0.3 mM cAMP, and [^{3}H]cAMP (~25,000 cpm). The cAMP was then isolated by sequential chromatography on Dowex cation exchange resin and aluminium oxide. The determinations were performed in duplicate and the data were analyzed using nonlinear least-squares regression.

Sequestration assays. Nearly confluent cells grown in 25-cm^2 flasks were washed twice with 5 ml of PBS and were treated for 5 min with 1 ml of trypsin/EDTA. The cells were resuspended in a 15-ml tube in 10 ml of DMEM/10% FBS supplemented with 1 μM ascorbic acid and were equilibrated at 37° for 30 min. They were then incubated in the presence or absence of 10 μM isoproterenol at 37° for the indicated periods of time, with gentle shaking. The cells were then centrifuged at 450 × g for 5 min at 4°, washed twice with ice-cold PBS, and resuspended in 3 ml of ice-cold PBS. Aliquots of 150 μl were used for radioligand binding assays. Whole-cell binding assays were performed at 13° for 3.5 hr in a final volume of 0.5 ml of DMEM supplemented with 10% (v/v) horse serum, 2 μM desipramine (to reduce nonspecific binding), and either ~250 pM or ~450 pM ^{125}I-CYP (for CHW-β_2 and CHW-β_3 cells, respectively). The reactions were terminated by rapid filtration through Whatman GF/C glass fiber filters treated as described above. The total receptor number was defined as the number of ^{125}I-CYP binding sites inhibited by 10 μM DL-propranolol, whereas the number of cell surface receptors was defined as the number of radioligand binding sites inhibited by 3 μM concentrations of the hydrophillic ligand CGP-12177. The cell suspension was homogenized with a Polytron homogenizer for one 5-sec burst before determination of protein concentration.

Agonist-induced sequestration of βAR in L cells was evaluated by

subcellular distribution experiments. Attached cells were incubated or not with 10 μM isoproterenol for the indicated times. After the incubation period, the cells were mechanically detached, collected on ice, and homogenized using a Polytron homogenizer (one 5-sec burst at maximum setting). The lysate was centrifuged at 200 × g for 10 min at 4° and the supernatant was layered on top of a 35% sucrose cushion and centrifuged at 150,000 × g for 90 min. As reported previously (6), the light membrane vesicular fraction was found at the 0–35% interface, whereas the plasma membrane fraction sedimented at the bottom of the sucrose cushion. Each fraction was collected, diluted in buffer A, and centrifuged at 200,000 × g for 60 min. The pelleted membranes were resuspended in 50 mM Tris, pH 7.4, 5 mM EDTA, and used immediately for radioligand binding assays.

Statistical analysis. Differences between data were evaluated using the Bonferroni *t* test with the program PRIMER. Differences were considered statistically significant with $p < 0.05$.

Results

CHW cells, devoid of endogenous βAR binding activity, were transfected with the pBC12BI expression vector containing the β_2AR (CHW-β_2) or the β_3AR (CHW-β_3) coding sequences. Cell lines expressing similar numbers of receptors (~500–1000 fmol/mg of membrane protein, as determined by membrane binding assays) were used throughout this study.

Pharmacology of CHW-β_3 cells. Partial pharmacological characterization of the ^{125}I-CYP binding sites of cells transfected with the β_3AR gene was conducted. A single class of binding sites, with a K_d of 337 ± 109 pM (two experiments), were detected. In competition experiments, the order of potency for the β agonists to displace ^{125}I-CYP binding was BRL-37344 > isoproterenol > norepinephrine > epinephrine (Table 1). The affinity values were in good agreement with those reported previously for the β_3AR (12). Three classical β antagonists inhibited the binding of ^{125}I-CYP to CHW-β_3 membrane preparations with the following order of potency: alprenolol > propranolol > ICI-118551 (Table 1). The K_i calculated for ICI-118551 was close to that determined previously (12). However, the results obtained with (−)-alprenolol and DL-propranolol were in apparent contradiction with a previous report describing the pharmacology of the human β_3AR (10). In that study, (−)-alprenolol and DL-propranolol were shown to be ineffective in blocking isoproterenol-induced cAMP accumulation in Chinese hamster ovary cells expressing the β_3AR. This apparent discrepancy is explained by the partial agonistic properties of these compounds for the human β_3AR.[1]

Isoproterenol- and Bt$_2$cAMP-promoted desensitization. To assess the effects of short term agonist treatment on receptor responsiveness, CHW-β_2 and CHW-β_3 cells were treated with 10 μM isoproterenol for a period of 30 min at 37°. This concentration of isoproterenol was required to ensure >85% saturation of the β_3AR. The isoproterenol-stimulated adenylyl cyclase activities were then determined in membrane preparations derived from these cells. This treatment did not significantly alter the total number of receptors in either cell line. However, in CHW-β_2 cells the agonist exposure caused a 21 ± 4% ($p < 0.05$) decrease in the maximal isoproterenol-stimulated adenylyl cyclase activity (Fig. 1). In contrast, in CHW-β_3 cells the same treatment with isoproterenol caused no change in the maximal agonist stimulation of the enzyme. However, the agonist pretreatment induced a similar decrease (~3-fold, $p < 0.05$) in the potency of isoproterenol to stimulate adenylyl cyclase in the two cell lines. Identical results were

TABLE 1

Competition with ^{125}I-CYP binding in CHW membrane preparatons

Experiments were carried out with membrane preparations derived from CHW-β_3 of -β_2 cells. Competitions with agonists were performed in the presence of 100 μM Gpp(NH)$_p$. The curves were fitted using least-squares regression analysis. Data are expressed as mean ± standard error of three or four experiments performed in duplicate.

	K_i	
	CHW-β_3	CHW-β_2
	μM	
Agonists		
Isoproterenol	3.9 ± 0.7	0.14[a]
Epinephrine	40 ± 14	0.37[a]
Norepinephrine	13 ± 1.5	0.74[a]
BRL-37344	1.4 ± 0.6	ND[b]
Antagonists		
ICI-118551	1.7 ± 0.3	0.002[c]
DL-Propranolol	0.2 ± 0.04	ND
(−)-Alprenolol	0.03 ± 0.006	0.0003[a]

[a] From Ref. 34.
[b] ND, not determined.
[c] From Ref. 18.

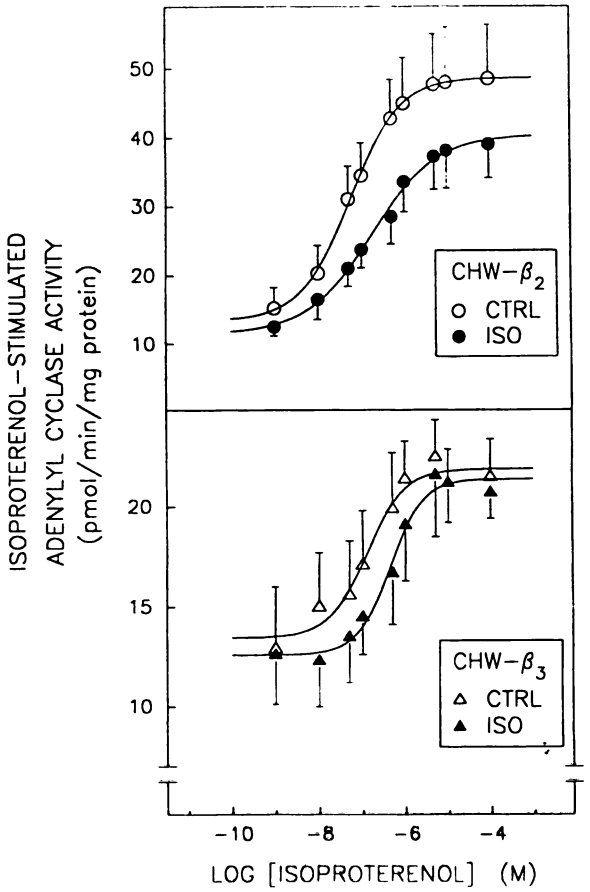


Fig. 1. Isoproterenol-stimulated adenylyl cyclase in membranes from CHW-β_2 (*upper*) and CHW-β_3 (*lower*) cells. CHW-β_2 or CHW-β_3 cells were incubated with 10 μM isoproterenol or with the incubation buffer alone for 30 min at 37°. The adenylyl cyclase activity is expressed as pmol of cAMP produced/min/mg of protein. Data points represent the mean ± standard error of four experiments performed in duplicate. Calculated K_{act} values (in nM) were as follows: CHW-β_2: control, 56.3 ± 6.9; isoproterenol, 178 ± 34; CHW-β_3: control, 126 ± 44; isoproterenol, 435 ± 126.

[1] N. Blin and A. D. Strosberg, unpublished observations.

obtained after pretreatment with 100 μM isoproterenol (data not shown).

Our results also show that the isoproterenol-induced activation of adenylyl cyclase in CHW-β_3 cells is lower than in CHW-β_2 cells, which suggests a lower coupling efficiency for the β_3AR. Whole-cell levels of cAMP, upon agonist stimulation, were also found to be lower in β_3AR-expressing cells (data not shown). To evaluate whether these differences could be responsible for these distinct desensitization patterns, the effect of the permeable cAMP analog Bt_2cAMP on receptor responsiveness was assessed. Treatment of CHW-β_2 cells with 1 mM Bt_2cAMP for 30 min was found to cause a 2.4-fold decrease in the potency of isoproterenol to stimulate the enzyme (Table 2). This decrease was similar to that observed when the cells were pretreated with either isoproterenol alone or isoproterenol plus Bt_2cAMP. This suggests, as reported previously (21–23), that a cAMP-dependent process contributes to the rapid desensitization of the β_2AR. As shown in Fig. 1, pretreatment of CHW-β_3 cells with isoproterenol alone also caused a significant decrease in the potency of isoproterenol to stimulate the enzyme. However, treatment of these cells with Bt_2cAMP had no effect on the potency of the agonist (Table 2). This strongly suggests that the agonist-induced decrease in the potency of isoproterenol to stimulate the β_3AR is independent of intracellular cAMP levels.

Agonist-binding properties. As shown in Fig. 2, isoproterenol competition with ^{125}I-CYP binding in both CHW-β_2 and CHW-β_3 membrane preparations was biphasic. Curves were best fitted by a two-affinity site model, using an iterative least-squares regression analysis of the nontransformed data. In both cases the high affinity component was found to be sensitive to guanyl nucleotides, because the addition of Gpp(NH)p decreased the proportion of receptors in the high affinity state. However, the effect of Gpp(NH)p on the agonist-binding properties was found to be much more dramatic for the β_2AR than for the β_3AR. The guanyl nucleotide-sensitive high affinity state is believed to represent the heterotrimeric hormone-receptor-G protein complex, whereas the low affinity state would represent the hormone-receptor complex uncoupled from G_s (19). Treatment of CHW-β_2 cells with 10 μM isoproterenol caused a 76% decrease ($p < 0.05$) in the number of receptors in the high affinity state. The same treatment led to a smaller reduction of the number of β_3AR in the high affinity state that did not reach statistical significance. These results would be consistent with the notion that the β_3AR is less prone to rapid desensitization than is the β_2AR. However, given the lack of sensitivity of the β_3AR high affinity sites to guanyl nucleotides, these data could reflect a difference in the strengh of receptor/G protein coupling rather than a difference in susceptibility to desensitization.

TABLE 2

Isoproterenol stimulation of adenylyl cyclase in membrane preparations from CHW-β_2 and CHW-β_3 cells

CHW-β_2 or -β_3 cells were incubated for 30 min at 37° with either vehicle alone (control), 10 μM isoproterenol, 1 mM Bt_2cAMP, or 10 μM isoproterenol plus 1 mM Bt_2cAMP. Data are expressed as mean ± standard error; α^E represents the intrinsic activity expressed as a fraction of control cells.

	EC_{50}	α^E	n^a
	nM		
CHW-β_2			
Control	70 ± 18	1.0	9
Isoproterenol	154 ± 18[b]	0.8	6
Bt_2cAMP	171 ± 49[b]	1.2	3
Isoproterenol + Bt_2cAMP	214 ± 66[b]	1.1	3
CHW-β_3			
Control	276 ± 62	1.0	11
Isoproterenol	623 ± 105[b]	1.0	5
Bt_2cAMP	278 ± 98	1.1	3
Isoproterenol + Bt_2cAMP	695 ± 121[b]	1.1	3

[a] *n*, number of experiments.
[b] $p < 0.05$, compared with control.

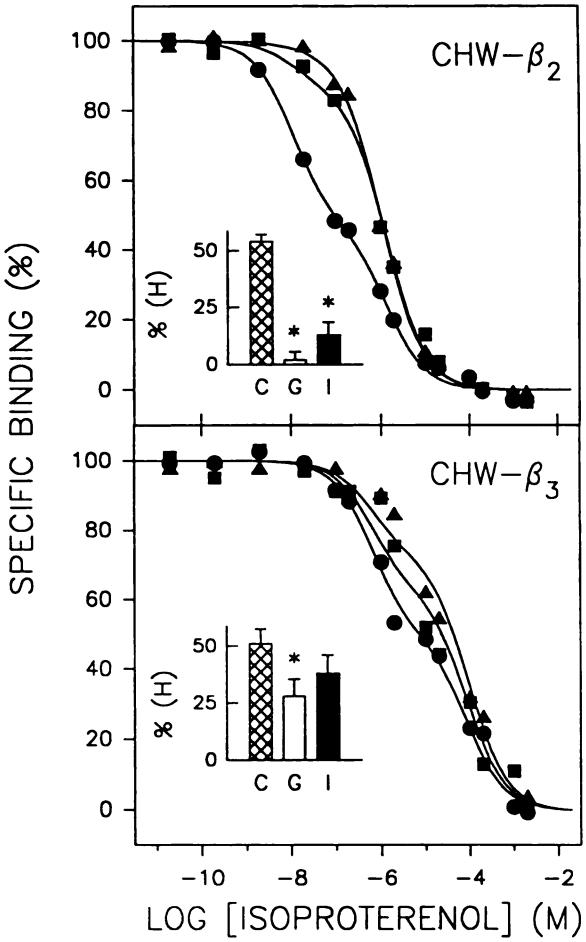


Fig. 2. Competition with ^{125}I-CYP binding in membranes from CHW-β_2 (*upper*) and CHW-β_3 (*lower*) cells. Experiments were carried out with membrane preparations from cells preincubated (■) or not (●, ▲) with 10 μM isoproterenol for 30 min, in the absence (■, ●) or in the presence (▲) of 100 μM Gpp(NH)p. The three *curves* were fitted simultaneously using least-squares regression analysis. K_i values (in μM) were as follows: CHW-β_2: $K_{i(H)}$, 4.3 ± 1.0; $K_{i(L)}$, 500 ± 60; CHW-β_3: $K_{i(H)}$, 380 ± 100; $K_{i(L)}$, 49,000 ± 29,000. Results are expressed as the percentage of maximal ^{125}I-CYP binding and represent the mean of three experiments performed in triplicate. The histograms (*insets*) represent the percentage of high affinity sites [% *(H)*] measured in membrane preparations. *C*, Membranes from untreated cells; *G*, membranes from untreated cells in the presence of 100 μM Gpp(NH)p; *I*, membranes from cells preincubated with 10 μM isoproterenol. *, $p < 0.05$.

Agonist-induced sequestration. Agonist-induced sequestration of the β_2- and β_3AR was evaluated in CHW by comparing the abilities of the hydrophillic ligand CGP-12177 and of the hydrophobic ligand DL-propranolol to inhibit ^{125}I-CYP binding in whole-cell assays (24). Treatment of CHW-β_2 cells with 10 μM isoproterenol caused a rapid decrease in the number of ^{125}I-CYP binding sites accessible to the hydrophillic ligand CGP-12177 (Fig. 3). This sequestration reached a maximum of 30% after 15 min of incubation. However, in CHW-β_3 cells a similar treatment did not cause any significant decrease in the

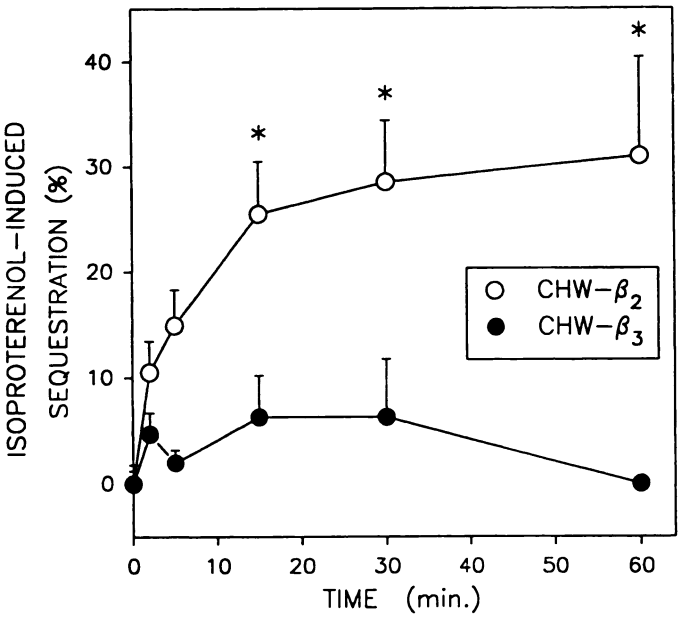


Fig. 3. Isoproterenol-induced sequestration of βAR in CHW-β_2 and CHW-β_3 cells. Sequestration is defined as the difference between the total receptor number, determined as the number of ^{125}I-CYP binding sites accessible to DL-propranolol, and the cell surface receptor number, determined as the number of sites accessible to CGP-12177. The sequestered receptor number is expressed as a percentage of total receptor number. Data represent the mean ± standard error of four experiments performed in triplicate. *, $p < 0.05$.

number of cell surface ^{125}I-CYP binding sites even after 60 min of continuous stimulation with the agonist.

Desensitization profile of Ch$\beta_3\beta_2$AR. To determine whether the presence of phosphorylation sites, or other molecular determinants in the cytoplasmic domains of the β_2AR, could restore a normal β_3AR desensitization profile, a chimeric receptor was constructed. This chimera consisted of a β_3AR backbone in which the third cytoplasmic loop (Val^{223}-Leu^{294}) and the carboxyl-terminal tail (Arg^{348}-Gly^{403}) were replaced with those of the β_2AR (Val^{218}-Leu^{275} and Arg^{328}-Leu^{413}). These segments include potential phosphorylation sites for both PKA and βARK. The chimera was then stably expressed in murine L cells (L-Ch$\beta_3\beta_2$). The effects of sustained agonist stimulation on the chimeric receptor responsiveness were compared with effects observed in L cells expressing either the β_2AR (L-β_2) or the β_3AR (L-β_3). These cell lines expressed between 40,000 and 65,000 receptor molecules/cell, as determined either in radioligand binding assays performed on membrane fractions (300–500 fmol of receptor/mg of membrane protein) or in whole-cell binding assays (40–60 fmol of receptor/mg of cell protein). L cells were chosen because the adenylyl cyclase was more responsive to βAR stimulation and the agonist-induced desensitization was more pronounced than in CHW cells (compare Figs. 1 and 4). Moreover, the two receptor subtypes have identical abilities to stimulate adenylyl cyclase when expressed in this cell line.

A single class of binding sites for ^{125}I-CYP were observed in L-Ch$\beta_3\beta_2$-derived membranes. The K_d of ^{125}I-CYP for the chimeric receptor (315 ± 59 pM; three experiments) was virtually identical to that found for the wild-type β_3AR expressed in either L cells (578 ± 159 pM; two experiments) or CHW (see above). The order of potency of the β agonists, as determined by ^{125}I-CYP competition binding assays, was identical to that for the wild-type β_3AR (Table 3). Although the relative affinities for ICI-118551 and DL-propranolol were unchanged, the affinity for (−)-alprenolol was slightly reduced in the Ch$\beta_3\beta_2$AR chimera (Table 3). Overall, the K_i values of the agonists and antagonists suggest that the chimeric receptor maintained a pharmacological profile characteristic of the β_3AR.

Treatment of L-β_2 cells with isoproterenol induced a pronounced (62 ± 2%, $p < 0.05$) decrease in the maximal isoproterenol-stimulated adenylyl cyclase activity (Fig. 4). However, similar to observations in CHW-β_3 cells, isoproterenol treatment of L-β_3 cells did not significantly affect the maximal agonist-induced stimulation of adenylyl cyclase but significantly reduced ($p < 0.05$) the potency of isoproterenol to stimulate the enzyme. In L-Ch$\beta_3\beta_2$ cells, agonist pretreatment induced a 17 ± 10% ($p < 0.05$) reduction of the maximal isoproterenol-stimulated adenylyl cyclase activity along with a 5-fold ($p < 0.05$) decrease in the potency of isoproterenol. The reason for such a pronounced decrease in potency is not clear but may be related to the relatively modest decrease in maximal response observed.

Isoproterenol competition curves for ^{125}I-CYP binding in L-β_2, L-β_3, and L-Ch$\beta_3\beta_2$ membrane preparations are shown in Fig. 5. The curves were biphasic and were fitted best to a two-site affinity model. The high affinity component was sensitive to guanyl nucleotides; the addition of Gpp(NH)p significantly reduced, by ~70%, the proportion of receptors in the high affinity state for the three receptor subtypes. The effect of agonist pretreatment on high affinity binding was slightly more important for the β_2AR and the Ch$\beta_3\beta_2$AR than for the β_3AR.

The effect of agonist exposure on βAR sequestration was also evaluated in L cells. The isoproterenol-induced sequestration was evaluated by assessing the subcellular distribution of the receptor. The agonist treatment induced a time-dependent redistribution of the β_2AR from the plasma membrane to the light membrane fraction (Fig. 6). This agonist-induced sequestration was identical to that measured in CHW-β_2 cells using the hydrophillic ligand CGP-12177. Also in agreement with what was observed in CHW, no agonist-induced translocation of the β_3AR from the plasma membrane to the sequestered vesicles was observed in L cells (Fig. 6). Similarly, the agonist treatment did not influence the subcellular distribution of the Ch$\beta_3\beta_2$AR expressed in L cells. The latter observation suggests that the molecular determinants for sequestration are located outside of the third cytoplasmic loop and the carboxyl-terminal tail of the β_2AR.

Discussion

In the present study, we have shown that the human β_3AR, expressed in either CHW or L cells, is much less prone to rapid agonist-mediated desensitization than is the human β_2AR. Substituting the third cytoplasmic loop and the carboxyl-terminal tail of the β_3AR with those of the β_2AR (which include phosphorylation sites for PKA and βARK along with other possible molecular determinants of desensitization) enhanced, but did not completely restore, the occurrence of desensitization.

The rapid agonist-mediated desensitization of the β_2AR is reflected by decreased maximal stimulation of adenylyl cyclase and by decreased potency of β agonists in dose-response experiments (5, 25). These phenomena are believed to result, at least in part, from the functional uncoupling of the receptor from G_s. The reduced number of β_2AR in the guanyl nucleotide-

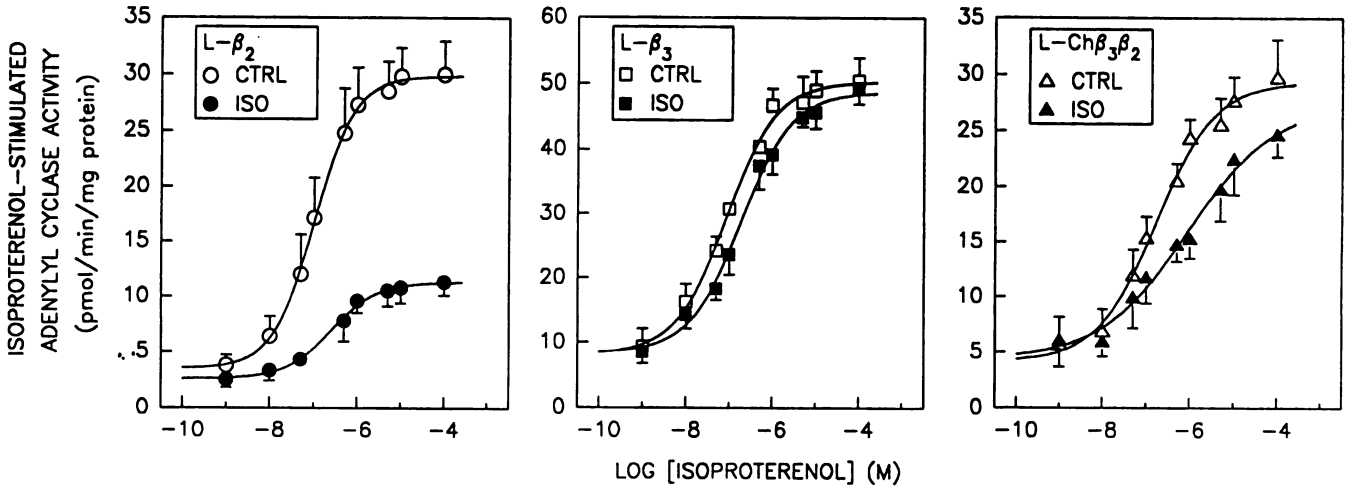


Fig. 4. Isoproterenol-stimulated adenylyl cyclase activity in membranes from L-β_2 (*left*), L-β_3 (*middle*), and L-Ch$\beta_3\beta_2$ (*right*) cells. L-β_2, L-β_3, and L-Ch$\beta_3\beta_2$ cells were incubated with 10 μM isoproterenol (*closed symbols*) or with the vehicle alone (*open symbols*) for 30 min at 37°. The adenylyl cyclase activity is expressed as pmol of cAMP produced/min/mg of protein. Data represent the mean ± standard error of three experiments performed in duplicate. Calculated K_{act} values (in nM) were as follows: L-β_2: control, 101 ± 7; with isoproterenol, 256 ± 69; L-β_3: control, 85 ± 16; with isoproterenol, 183 ± 36; L-Ch$\beta_3\beta_2$: control, 170 ± 44; with isoproterenol, 864 ± 498.

TABLE 3

Competition with ^{125}I-CYP binding in L-Ch$\beta_3\beta_2$ membrane preparations

Experiments were carried out with membrane preparations from L-Ch$\beta_3\beta_2$ cells. Competitions with agonists were performed in the presence of 100 μM Gpp(NH)$_p$. The curves were fitted using least-squares regression analysis. Data are expressed as mean ± standard error of three or four experiments performed in duplicate.

	K_i
	μM
Agonists	
Isoproterenol	2.6 ± 0.4
Epinephrine	17.9 ± 5.5
Norepinephrine	4.1 ± 1.5
Antagonists	
ICI 118-551	0.44 ± 0.13
DL-Propranolol	0.08 ± 0.02
(−)-Alprenolol	0.13 ± 0.03

sensitive high affinity state for agonists has often been used as an index of uncoupling (19). In the present report, agonist stimulation of the β_3AR did not affect the ability of the receptor to maximally stimulate adenylyl cyclase in two different cellular systems and only marginally reduced the potency of isoproterenol. This low level of desensitization cannot be ascribed to the reduced ability of the β_3AR to stimulate cAMP production observed in CHW, because in L cells both receptor subtypes stimulated the enzyme with the same efficacy.

The fact that the β_3AR is less prone to a decrease in its reactivity upon agonist stimulation may have important physiological consequences. Under sympathetic stimulation, this receptor might maintain a minimal β-adrenergic sensitivity, whereas the two other βAR subtypes would be desensitized. Consistent with this hypothesis, Granneman (26) reported that pretreatment of isolated rat adipocytes with isoproterenol did not affect β_3AR-mediated adenylyl cyclase activation. The observation made by us and others (10, 13) that the β_3AR binds the natural catecholamines with much lower affinities than do the β_1AR and the β_2AR is also consistent with this idea. A report by Thomas *et al.* (27) suggested that, in murine 3T3-F442A adipocytes, the β_3AR is also less prone to the down-regulation that occurs after long term exposure to agonist. However, this might not be the case in all species, because in rat adipose tissue β_3AR mRNA levels are decreased after prolonged β-adrenergic stimulation *in vivo* (28). The effects of long term agonist stimulation on the human β_3AR number and mRNA level remain to be studied.

One possible hypothesis explaining the resistance of the β_3AR to rapid desensitization is the absence, in its primary structure, of most of the phosphorylation sites found in the β_2AR. Phosphorylation by βARK and PKA is indeed believed to be the major determinant of the rapid β_2AR desensitization (4–6). The reduced potency of β agonists to stimulate adenylyl cyclase during desensitization has been mainly attributed to PKA-mediated phosphorylation of the receptor (21, 25). The involvement of a cAMP-dependent process in the rapid desensitization of the β_2AR is again shown in the present study, because treatment of the CHW-β_2 cells with the cAMP analog Bt$_2$cAMP reduced the potency of isoproterenol to stimulate adenylyl cyclase. Phosphorylation of the β_3AR by PKA appears very unlikely, because the motif RRXS, which is the canonical phosphorylation site for PKA (29), is absent from the primary sequence of the β_3AR. Moreover, we showed that Bt$_2$cAMP did not decrease β_3AR reactivity. The ability of the β_3AR to act as a substrate for βARK has not yet been determined. In the β_2AR, this enzyme has been shown to phosphorylate serine and threonine residues located in the carboxyl-terminal tail of the receptor (4, 5). Serine residues are indeed present in the carboxyl-terminal tail of the β_3AR. However, none of them are preceded by acidic amino acids (aspartate or glutamate), a context that has been proposed to favor βARK-mediated phosphorylation (30).

The substitution of the β_2AR third cytoplasmic loop and carboxyl-terminal tail, which contain the PKA and βARK potential phosphorylation sites, into the β_3AR sequence partially restored the desensitization profile observed in cells expressing the wild-type β_2AR. In fact, the decrease in maximal receptor-mediated adenylyl cyclase stimulation for the chimeric receptor was found to be intermediate between that observed for the β_2AR and the β_3AR. These results support the notion that sequences, such as phosphorylation sites, located in the

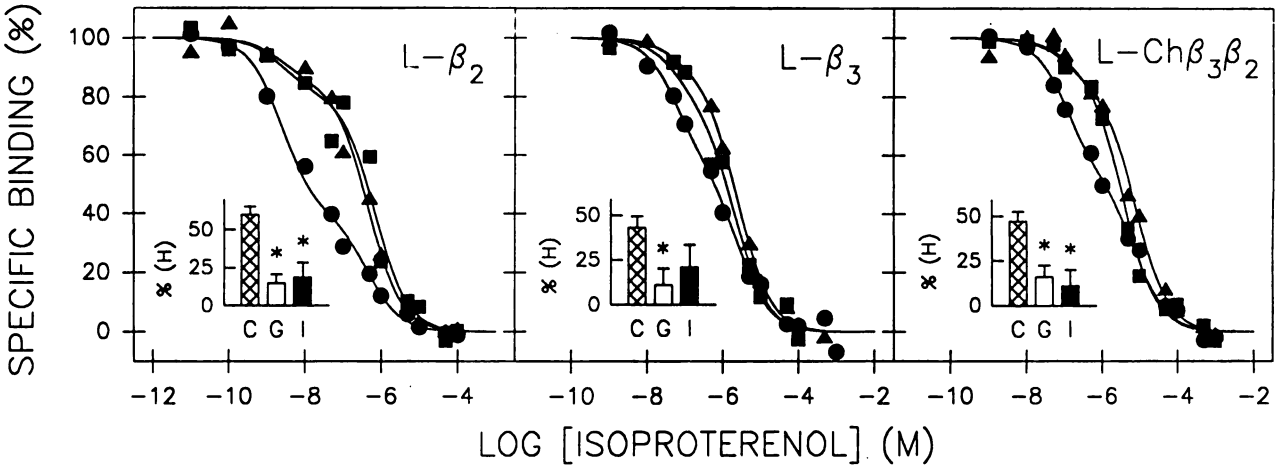
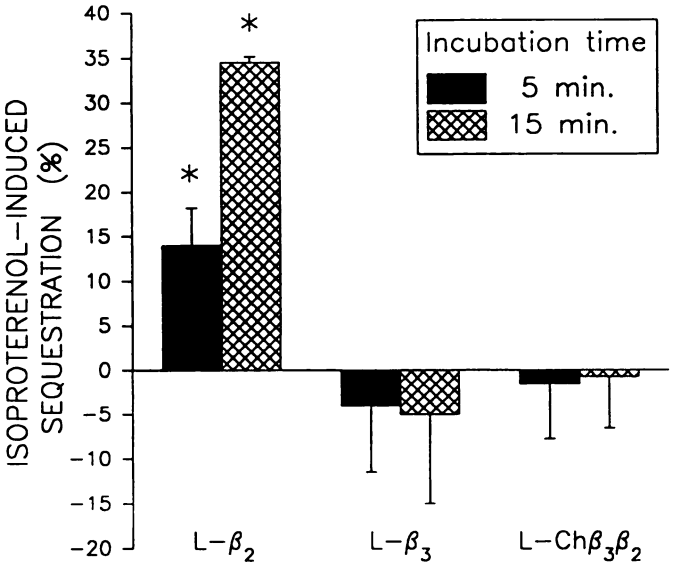


Fig. 5. Competition with ^{125}I-CYP binding in membranes from L-β_2 (*left*), L-β_3 (*middle*), and L-Ch$\beta_3\beta_2$ (*right*) cells. Experiments were carried out with membrane preparations from cells preincubated (■) or not (●, ▲) with 10 μM isoproterenol for 30 min, in the absence (■, ●) or in the presence (▲) of 100 μM Gpp(NH)p. The three *curves* were fitted simultaneously using least-squares regression analysis. K_i values (in nM) were as follows: L-β_2: $K_{i(H)}$, 9.5 ± 3.0; $K_{i(L)}$, 170 ± 40; L-β_3: $K_{i(H)}$, 38 ± 24; $K_{i(L)}$, 1600 ± 300; L-Ch$\beta_3\beta_2$: $K_{i(H)}$, 63 ± 19; $K_{i(L)}$, 4300 ± 700. Results are expressed as the percentage of maximal ^{125}I-CYP binding and represent the mean of three experiments performed in triplicate. The histograms (*insets*) represent the percentage of high affinity sites [% *(H)*] measured in membrane preparations. *C*, Membranes from untreated cells; *G*, membranes from untreated cells in the presence of 100 μM Gpp(NH)p; *I*, membranes from cells preincubated with 10 μM isoproterenol. *, $p < 0.05$.



Nantel et al., Mol. Pharmacol., 1993

fraction. L cells were incubated with 10 μM isoproterenol at 37° for the indicated times. Receptor numbers were measured in the plasma and light membrane fractions, after differential centrifugation, in a ^{125}I-CYP binding assay as described in Experimental Procedures. The data represent the isoproterenol-induced increase in the proportion of receptors located in the light membrane fraction (sequestration) and are expressed as percentage of total receptor number. The data shown are the mean ± standard error of two or three experiments. *, $p < 0.05$.

third cytoplasmic loop and the carboxyl-terminal tail of the β_2AR contribute to the rapid desensitization. However, the fact that only a partial desensitization is restored in the chimera suggests that other molecular determinants, outside of the third cytoplasmic loop and the carboxyl-terminal tail of the β_2AR, are involved in desensitization and that these determinants are lacking in the primary structure of the β_3AR. It was suggested that βARK/β_2AR interaction involves intracellular regions other than the carboxyl-terminal tail. Although no phosphorylation sites are present in the first cytoplasmic loop of the β_2AR, its interaction with βARK might be required for phosphorylation of the receptor at distant sites (31). In the Ch$\beta_3\beta_2$AR, the first cytoplasmic loop is still of β_3AR origin and displays only 56% identity with that of the β_2AR. Improper interaction between the Ch$\beta_3\beta_2$AR and βARK could explain the lack of phosphorylation even though the potential phosphorylation sites are present in the carboxyl-terminal tail of the chimera. To confirm the involvement of phosphorylation, or lack thereof, in the desensitization of the chimera and the β_3AR, purification procedures that allow direct assessement of the phosphorylation level of the receptor will be necessary.

It should be noted that, in our system, the β_3AR is not completely refractory to rapid agonist-promoted desensitization. Indeed, agonist treatment in both CHW and L cells expressing the β_3AR induced a decrease in the potency of isoproterenol to stimulate adenylyl cyclase. Only phosphorylation-dependent mechanisms have been shown to contribute to the rapid uncoupling of the β_2AR. However, Hausdorff *et al.* (5) observed that mutation of both PKA and βARK phosphorylation sites on the β_2AR did not completely abolish desensitization. It should be noted that this mutant receptor had a normal sequestration pattern, which could contribute to the residual desensitization observed in that case. A sequestration of the receptor away from the cell surface cannot account for the small desensitization observed here for the β_3AR because we showed that, in contrast to the β_2AR, this receptor does not undergo agonist-promoted sequestration. The mechanisms leading to the modest β_3AR desensitization therefore remain unknown.

The absence of agonist-mediated sequestration of the β_3AR is also an important finding of the present study. The molecular determinants implicated in the sequestration process are still unknown. Even though phosphorylation of the β_2AR *per se* does not lead to sequestration (5), this process has been proposed as a recycling pathway in which the phosphorylated receptor could be dephosphorylated and recycled back to the plasma membrane (32). Interestingly, the β_2AR, which has the highest number of potential phosphorylation sites, is also the most prone to agonist-promoted sequestration. The β_3AR,

which may not be phosphorylated, does not undergo agonist-induced sequestration, whereas the β_1AR, which has fewer potential phosphorylation sites than does the β_2AR (33), shows a sequestration pattern intermediate between those of the β_2AR and β_3AR subtypes (34). The absence of agonist-promoted sequestration of the Ch$\beta_3\beta_2$AR supports the notion that the molecular determinants of sequestration are not the major phosphorylation sites of the β_2AR. In contrast, Hausdorff *et al.* (35) reported that mutation of a subset of the potential βARK phosphorylation sites in the β_2AR prevented agonist-promoted sequestration. However, the authors concluded that conformationnal changes imposed by the mutation, rather than the removal of the phosphorylation sites *per se*, were responsible for the phenotype. This conclusion is in line with the present data and the previous observations that mutation of all the phosphorylation sites in the β_2AR did not block sequestration (4, 5).

In summary, the human β_3AR displays significant resistance to short term agonist-promoted desensitization. This resistance may not be entirely accounted for by the lack of potential phosphorylation sites in the primary sequence of this receptor.

Note Added in Proof

It has recently been shown that the human β_3AR gene is composed of two exons (36, 37), yielding a protein six amino acids longer than initially thought (10). The β_3AR expression plasmid used here contained both exons. Proper splicing of the β_3AR gene was confirmed by direct sequencing of PCR products and the corresponding mRNA indeed encoded for a full length receptor containing these six supplementary amino acids.

Acknowledgments

The authors are grateful to Nicole Robinson and Yves Villeneuve for their help in preparing the manuscript.

References

1. Benovic, J. L., M. Bouvier, M. G. Caron, and R. J. Lefkowitz. Regulation of adenylyl cyclase-coupled β-adrenergic receptors. *Annu. Rev. Cell Biol.* **4**:405–427 (1988).
2. Dohlman, H. G., J. Thorner, M. G. Caron, and R. J. Lefkowitz. Model systems for the study of seven-transmembrane-segment receptors. *Annu. Rev. Biochem.* **60**:653–688 (1991).
3. Savarese, T. M., and C. M. Fraser. *In vitro* mutagenesis and the search for structure-function relationships among G protein-coupled receptors. *Biochem. J.* **283**:1–19 (1992).
4. Bouvier, M., W. P. Hausdorff, A. De Blasi, B. F. O'Dowd, B. K. Kobilka, M. G. Caron, and R. J. Lefkowitz. Removal of phosphorylation sites from the β_2-adrenergic receptor delays onset of agonist-promoted desensitization. *Nature (Lond.)* **333**:370–373 (1988).
5. Hausdorff, W. P., M. Bouvier, B. F. O'Dowd, G. P. Irons, M. G. Caron, and R. J. Lefkowitz. Phosphorylation sites on two domains of the β_2-adrenergic receptor are involved in distinct pathways of receptor desensitization. *J. Biol. Chem.* **264**:12657–12665 (1989).
6. Lohse, M. J., J. L. Benovic, M. G. Caron, and R. J. Lefkowitz. Multiple pathways of rapid β_2-adrenergic receptor desensitization: delineation with specific inhibitors. *J. Biol. Chem.* **265**:3202–3209 (1990).
7. Benovic, J. L., F. Mayor, Jr., C. Staniszewski, R. J. Lefkowitz, and M. G. Caron. Purification and characterization of the β-adrenergic receptor kinase. *J. Biol. Chem.* **262**:9026–9032 (1987).
8. Bouvier, M., N. Guilbault, and H. Bonin. Phorbol-ester induced phosphorylation of the β_2-adrenergic receptor decreases its coupling to G_s. *FEBS Lett.* **279**:243–248 (1991).
9. Huganir, R. L., and P. Greengard. Regulation of neurotransmitter receptor desensitization by protein phosphorylation. *Neuron* **5**:555–567 (1990).
10. Emorine, L. J., S. Marullo, M.-M. Briend-Sutren, G. Patey, K. M. Tate, C. Delavier-Klutchko, and A. D. Strosberg. Molecular characterization of the human β_3-adrenergic receptor. *Science (Washington D. C.)* **245**:1118–1121 (1989).
11. Arch, J. R. S., A. T. Ainsworth, M. A. Cawthorne, V. Piercy, M. V. Sennitt, V. E. Thody, C. Wilson, and S. Wilson. Atypical β-adrenoceptor on brown adipocytes as target for anti-obesity drugs. *Nature (Lond.)* **309**:163–165 (1984).
12. Feve, B., L. J. Emorine, F. Lasnier, N. Blin, B. Baude, C. Nahmias, A. D. Strosberg, and J. Pairault. Atypical β-adrenergic receptor in 3T3-F442A adipocytes: pharmacological and molecular relationship with the human β_3-adrenergic receptor. *J. Biol. Chem.* **266**:20329–20336 (1991).
13. Muzzin, P., J.-P. Revelli, F. Kuhne, J. D. Gocayne, W. R. McCombie, J. C. Venter, J.-P. Giacobino, and C. M. Fraser. An adipose tissue-specific β-adrenergic receptor: molecular cloning and down-regulation in obesity. *J. Biol. Chem.* **266**:24053–24058 (1991).
14. Kunkel, T. A., J. D. Roberts, and R. A. Zakour. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* **154**:367–382 (1987).
15. Emorine, L. J., S. Marullo, C. Delavier-Klutchko, S. V. Kaveri, O. Durieu-Trautmann, and A. D. Strosberg. Structure of the gene for human β_2-adrenergic receptor: expression and promoter characterization. *Proc. Natl. Acad. Sci. USA* **84**:6995–6999 (1987).
16. Mellon, P. L., V. Parker, Y. Gluzman, and T. Maniatis. Identification of DNA sequences required for transcription of the human α_1-globin gene in a new SV40 host-vector system. *Cell* **27**:279–288 (1981).
17. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
18. Bouvier, M., M. Hnatowich, S. Collins, B. K. Kobilka, A. De Blasi, R. J. Lefkowitz, and M. G. Caron. Expression of a human cDNA encoding the β_2-adrenergic receptor in Chinese hamster fibroblast (CHW): functionality and regulation of the expressed receptors. *Mol. Pharmacol.* **33**:133–139 (1988).
19. De Lean, A., and D. Rodbard. Kinetic analysis of cooperative ligand binding: applications to the insulin receptor. *FASEB J.* **39**:116–120 (1980).
20. Salomon, Y., C. Londos, and M. Rodbell. A highly sensitive adenylate cyclase assay. *Anal. Biochem.* **58**:541–548 (1974).
21. Clark, R. B., J. Friedman, R. A. F. Dixon, and C. D. Strader. Identification of a specific site required for rapid heterologous desensitization of the β-adrenergic receptor by cAMP-dependent protein kinase. *Mol. Pharmacol.* **36**:343–348 (1989).
22. Bouvier, M., L. M. F. Leeb-Lundberg, J. L. Benovic, M. G. Caron, and R. J. Lefkowitz. Regulation of adrenergic receptor function by phosphorylation. II. Effects of agonist occupancy on phosphorylation of α_1- and β_2-adrenergic receptors by protein kinase C and the cyclic AMP-dependent protein kinase. *J. Biol. Chem.* **262**:3106–3113 (1987).
23. Liggett, S. B., M. Bouvier, W. P. Hausdorff, B. F. O'Dowd, M. G. Caron, and R. J. Lefkowitz. Altered patterns of agonist-stimulated cAMP accumulation in cells expressing mutant β_2-adrenergic receptors lacking phosphorylation sites. *Mol. Pharmacol.* **36**:641–646 (1989).
24. Hertel, C., P. Muller, M. Portenier, and M. Staehelin. Determination of the desensitization of β-adrenergic receptors by [^{3}H]CGP-12177. *Biochem. J.* **216**:669–674 (1983).
25. Clark, R. B., J. Friedman, J. A. Johnson, and M. W. Kunkel. β-Adrenergic receptor desensitization of wild-type but not cyc$^-$ lymphoma cells unmasked by submillimolar Mg^{2+}. *FASEB J.* **1**:289–297 (1987).
26. Granneman, J. G. Effects of agonist exposure on the coupling of *beta*-1 and *beta*-3 adrenergic receptors to adenylyl cyclase in isolated adipocytes. *J. Pharmacol. Exp. Ther.* **261**:638–642 (1992).
27. Thomas, R. F., B. D. Holt, D. A. Schwinn, and S. B. Liggett. Long-term agonist exposure induces up-regulation of β_3-adrenergic receptor expression via multiple cAMP response elements. *Proc. Natl. Acad. Sci. USA* **89**:4490–4494 (1992).
28. Granneman, J. G., and K. N. Lahners. Differential adrenergic regulation of β_1- and β_3-adrenoreceptor messenger ribonucleic acids in adipose tissues. *Endocrinology* **130**:109–114 (1992).
29. Kemp, B. E., D. J. Graves, E. Benjamini, and E. G. Krebs. Role of multiple basic residues in determining the substrate specificity of cyclic AMP-dependent protein kinase. *J. Biol. Chem.* **252**:4888–4894 (1977).
30. Onorato, J., K. Palczewski, J. W. Regan, M. G. Caron, R. J. Lefkowitz, and J. L. Benovic. Role of acidic amino acids in peptide substrates of the β-adrenergic receptor kinase and rhodopsin kinase. *Biochemistry* **30**:5118–5125 (1991).
31. Benovic, J. L., J. Onorato, M. J. Lohse, H. G. Dohlman, C. Staniszewski, M. G. Caron, and R. J. Lefkowitz. Synthetic peptides of the hamster β_2-adrenoceptor as substrates and inhibitors of the β-adrenoceptor kinase. *Br. J. Clin. Pharmacol.* **30**:3S-12S (1990).
32. Sibley, D. R., R. H. Strasser, J. L. Benovic, K. Daniel, and R. J. Lefkowitz. Phosphorylation/dephosphorylation of the β-adrenergic receptor regulates its functional coupling to adenylate cyclase and subcellular distribution. *Proc. Natl. Acad. Sci. USA* **83**:9408–9412 (1986).
33. Frielle, T., S. Collins, K. Daniel, M. G. Caron, R. J. Lefkowitz, and B. K. Kobilka. Cloning of the cDNA for the human β_1-adrenergic receptor. *Proc. Natl. Acad. Sci. USA* **84**:7920–7924 (1987).
34. Suzuki, T., C. T. Nguyen, F. Nantel, H. Bonin, M. Valiquette, T. Frielle, and M. Bouvier. Distinct regulation of β_1- and β_2-adrenergic receptors in Chinese hamster fibroblasts. *Mol. Pharmacol.* **41**:542–548 (1992).
35. Hausdorff, W. P., P. T. Campbell, J. Ostrowski, S. S. Yu, M. G. Caron, and R. J. Lefkowitz. A small region of the β-adrenergic receptor is selectively involved in its rapid regulation. *Proc. Natl. Acad. Sci. USA* **88**:2979–2983 (1991).
36. Granneman, J. G., K. N. Lahners, and D. Rao. Rodent and human β_3-adrenergic receptor genes contain an intron within the protein coding block. *Mol. Pharmacol.* **49**:964–970 (1992).
37. Van Spronsen, A., C. Nahmias, S. Krief, M. M. Briend-Sutren, A. D. Strosberg, and L. J. Emorine. The human and mouse β_3-adrenergic receptor genes promoter and intron/exon structure. *Eur. J. Biochem.* In press.

Send reprint requests to: Michel Bouvier, Department of Biochemistry, Université de Montréal, C.P. 6128, succ. A, Montréal (Québec), Canada, H3C 3J7.