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Distinct Receptor Domains Determine Subtype-Specific Coupling and Desensitization Phenotypes for Human β_1- and β_2- Adrenergic Receptors

GUY ROUSSEAU, FRANÇOIS NANTEL, and MICHEL BOUVIER

Département de Biochimie et Groupe de Recherche sur le Système Nerveux Autonome, Université de Montréal, Montréal, Québec, H3C 3J7 Canada

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SUMMARY

Human β_1- and β_2-adrenergic receptor (β_1AR and β_2AR) coupling and desensitization characteristics were compared in defined heterologous expression systems. Significant differences in the coupling efficacies of the two subtypes were found in both Chinese hamster fibroblasts and murine Ltk$^-$ fibroblasts, which were used as surrogate cell lines. At the maximal level of stimulation with the nonselective β-adrenergic agonist isoproterenol, β_1AR-mediated adenylyl cyclase activation represented 70% and 20% of that mediated by β_2AR in Chinese hamster and murine Ltk$^-$ fibroblasts, respectively. Sustained (15 min) stimulation with subsaturating concentration of isoproterenol (<10% of receptor occupancy) led to identical desensitization of the β-adrenergic-stimulated adenylyl cyclase activity for both β_1AR- and β_2AR-expressing cells. In contrast, when a nearly saturating concentration of isoproterenol (>90% of receptor occupancy) was used to promote desensitization, the extent of desensitization observed for β_2AR-expressing cells was 1.7–2-fold higher than that of the β_1AR. The carboxyl domain of several G protein-coupled receptors has been shown to play important roles in both coupling efficacy and agonist-promoted desensitization. Therefore, we examined the contribution of this receptor domain in the subtype-selective phenotypes described above. A chimeric receptor composed of the first six transmembrane domains of the β_1AR and of the seventh transmembrane domain and carboxyl tail of the β_2AR maintained a coupling efficacy characteristic of the β_1AR, whereas the extent of desensitization resulting from high receptor occupancy was identical to that of the β_2AR. These results therefore suggest that the carboxyl portion of the β_1AR and β_2AR determines their subtype-selective desensitization patterns but not their respective coupling efficacies.

Sustained stimulation of βARs with agonist leads to a rapid attenuation of responsiveness, a phenomenon referred to as agonist-induced desensitization. The molecular processes involved in this adaptive mechanism have been particularly well studied for the β_2AR. Phosphorylation of the receptor is believed to play a crucial role in the rapid uncoupling of the receptor from G_s, which leads to the early phases of desensitization (for reviews, see Refs. 1–3). This functional uncoupling of the β_2AR has been correlated with its phosphorylation by at least two protein kinases, PKA and βARK.

Target sites have been identified that may be phosphorylated by these kinases in the third intracytoplasmic loop and in the carboxyl-terminal tail of the β_2AR. All of the βARK sites have been proposed to reside in the distal portion of the carboxyl tail, whereas two distinct domains harbor PKA sites: one located in the third cytoplasmic loop and the other located in the proximal portion of the carboxyl tail. Although the role of the first site in the cAMP-mediated desensitization has been well characterized, the accessibility and contribution of the second site have been questioned (4, 5). Interestingly, this site is missing in the β_1AR structure. Indeed, the β_1AR possesses only one PKA consensus site, which is located in the third intracytoplasmic loop in a conserved position with the equivalent site in the β_2AR. Overall, the carboxyl tail sequences of the two receptor subtypes are not well conserved. Nevertheless, as is the case for the β_2AR, several serine and threonine residues that may be target sites for βARK are found in the β_1AR carboxyl tail. Whether differences in the phosphorylation site distribution between the two receptor subtypes, particularly in the carboxyl tail, may result in distinctive patterns of desensitization remains unknown. Recently, Zhou *et al.* (6) proposed that the β_1AR may

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ABBREVIATIONS: βAR, β-adrenergic receptor; βARK, β-adrenergic receptor kinase; CYP, iodocyanopindolol; CHW, Chinese hamster fibroblasts; DMEM, Dulbecco's modified Eagle's medium; LTK$^-$, murine Ltk$^-$ fibroblasts; PKA, cAMP-dependent protein kinase; Chβ_1/β_2AR, chimeric β_1/β_2-adrenergic receptor.

be more resistant to rapid agonist-promoted desensitization than the β_2AR. However, no such difference could be detected in another study (7).

Studies have also suggested that the β_1AR and β_2AR may have different coupling efficacies to G_s (8, 9). In a recent report, Green and Liggett (10) suggested that a proline-rich region of the third cytoplasmic loop of the β_1AR may be responsible for this difference. However, deletion of this domain in the β_1AR or its insertion in the β_2AR sequence only partially attenuated the difference in the coupling ability between the two receptor subtypes. Other receptor domains have been implicated in β_2AR/G_s coupling. In particular, the proximal portion of the carboxyl tail has been shown to play an important role (11, 12). However, whether the sequence dissimilarities in these regions of the two receptor subtypes could also contribute to their different coupling abilities has not been investigated.

The present study was undertaken to investigate whether sequence differences in the carboxyl-terminal domain of the two receptor subtypes might contribute to distinct coupling and desensitization phenotypes. To address this question, the coupling and agonist-promoted desensitization patterns of the human β_1AR and β_2AR were assessed in CHW and LTK. We also generated a Chβ_1/β_2AR in which the seventh transmembrane domain and the carboxyl-terminal tail of the β_1AR were replaced with the corresponding region of the β_2AR. This substitution imparted a desensitization profile characteristic of the β_2AR but maintained coupling properties typical of the β_1AR. These data demonstrate that the carboxyl-terminal domain of the βAR is an important determinant of the subtype-specific desensitization patterns but not of their distinct basal coupling abilities.

Experimental Procedures

Materials. [^{125}I]CYP, [α-^{32}P]ATP, and [^{3}H]cAMP were obtained from New England Nuclear. Isoproterenol, isobutylmethylxanthine, ATP, GTP, cAMP, phosphoenolpyruvate, and myokinase were purchased from Sigma Chemical Co. Pyruvate kinase was obtained from Calbiochem, and geneticin, DMEM, fetal calf serum, penicillin, streptomycin, amphotericin B, and trypsin were purchased from GIBCO-BRL.

DNA constructions, cell transfection, and culture. Human β_1AR and β_2AR cDNAs were subcloned into the pBC12BI expression vector as previously described (13). A chimeric receptor consisting of the first six transmembrane domains of the β_1AR with the seventh transmembrane domain and the carboxyl-terminal tail of the β_2AR was constructed as follows. Silent mutations were created in the β_2AR cDNA by site-directed mutagenesis according to the method of Kunkel *et al.* (14) to generate a unique restriction site *Xmn*1 at position 943. A *Xmn*1 restriction site already exists in the β_1AR sequence (position 1128) and allowed for the insertion of a *Xmn*1/*Sal*1 fragment (encoding the seventh transmembrane and the carboxyl-terminal tail) of the β_2AR into the corresponding region of the β_1AR. The coding sequence of the chimeric receptor corresponded to the β_1AR up to Lys^{347}, where the interface with the β_2AR sequence started with Ile^{298}. This strategy had the advantage of not adding new amino acids. Site-directed mutagenesis and subcloning were carried out in pTZβ_1AR (13) and pTZβ_2AR (15), respectively. A human β_2AR cDNA clone encoding alanine substitutions of Ser^{345,346} (Ala^{345,346}/β_2AR) was constructed through digestion of pSP65 plasmids containing wild-type β_2AR (15) and Ala^{345,346}/β_2AR cDNAs (5), respectively, with *Avi*II. The appropriate restriction fragments were isolated and ligated, and a resulting pSP65 plasmid containing a cDNA for Ala^{345,346}/β_2AR was obtained. The identity of this mutant was confirmed with dideoxynucleotide sequencing. The Chβ_1/β_2AR cDNA fragment and the Ala^{345,346}/β_2AR cDNA were then subcloned into the pBC12BI vector.

The constructs were cotransfected with the neomycin-resistant plasmid pSV2-Neo (Pharmacia) into LTK$^-$ and CHW according to the calcium phosphate precipitation procedure (16). Neomycin-resistant cells were selected through culturing in DMEM plus 10% fetal calf serum and G418 (450 μg/ml for LTK$^-$ and 150 μg/ml for CHW). Individual G418-resistant clonal colonies were carefully selected and transferred onto 24-well plates. When the cells reached confluency, they were harvested and split into two 25-cm^2 flasks. One flask was used to screen for βAR expression by radioligand binding assays with the use of [^{125}I]CYP, whereas the other flask was kept to maintain the clonal line. The transfected cells were then grown as monolayers in 75-cm^2 flasks containing DMEM supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin, 1 mM glutamine, and G418 (450 μg/ml for LTK$^-$ and 150 μg/ml for CHW) in an atmosphere of 95% air/5% CO_2 at 37°. At 18–20 hr before experiments, the medium was changed to a medium without fetal bovine serum.

Membrane preparation. Cells were washed three times with 10 ml of phosphate-buffered saline at 4° and mechanically detached in 10 ml of ice-cold buffer containing 5 mM Tris·HCl, pH 7.4, 2 mM EDTA, 5 μg/ml leupeptin, 5 μg/ml soybean trypsin inhibitor, and 10 μg/ml benzamidine. Cells were then lysed with a polytron homogenizer (1 burst for 7 sec at maximum speed), and the lysates were centrifuged at 45,000 × *g* for 20 min at 4°. The pelleted membranes were washed twice in the same buffer; resuspended in 0.5 ml of a buffer containing 75 mM Tris·HCl, pH 7.4, 5 mM $MgCl_2$, 2 mM EDTA, 5 μg/ml leupeptin, 5 μg/ml soybean trypsin inhibitor, and 10 μg/ml benzamidine; and used immediately for adenylyl cyclase activity determination and radioligand binding assays as described below. Protein content was determined according to the method of Bradford (Bio-Rad).

Adenylyl cyclase assay. Adenylyl cyclase activity was measured according to the method of Salomon *et al.* (17). Briefly, ~5 μg of membrane proteins was added in a total volume of 50 μl and incubated for 30 min at 37°. The incubation mixture included 120 μM ATP, 1 μCi [α-^{32}P]ATP, 100 μM cAMP, 53 μM GTP, 2.8 mM phosphoenolpyruvate, 0.2 unit pyruvate kinase, 1 unit myokinase, 30 mM Tris·HCl, pH 7.4, 2 mM $MgCl_2$, 0.8 mM EDTA, and 0.1 mM isobutylmethylxanthine. Enzyme activity was determined in duplicate in the absence (basal activity) or in the presence of activators (1×10^{-9}–1×10^{-4} M isoproterenol or 10 mM NaF). The concentration of free magnesium in the assay calculated according to the method of Iyengar and Birnbaumer (18) was 1.1 mM. Under the conditions studied, both basal and isoproterenol-stimulated activities were linear for an incubation period ranging from 5 to 30 min.

Radioligand binding assay. Radioligand binding assays were conducted essentially as described previously (13) with ~5 μg of membrane proteins in a total volume of 0.5 ml containing 250 pM [^{125}I]CYP in the presence or absence of 10 μM alprenolol to define nonspecific binding. The binding reactions were incubated at room temperature for 90 min and terminated by rapid filtration with ice-cold 25 mM Tris·HCl, pH 7.4, over Whatman GF/C glass fiber filters preincubated for ≥30 min in a buffer containing 25 mM Tris·HCl, pH 7.4, 0.1% bovine serum albumin, and 0.3% polyethylenimine.

Statistical analysis. All results are expressed as mean ± standard error. When two cell lines or two conditions were compared, we used an unpaired Student's *t* test to assess the statistical significance of the differences. When three or more conditions or cell lines were compared, an analysis of variance was performed; if statistically significant differences were indicated, we used Bonferroni's *t* test to specify which comparison resulted in a significant difference. Data from adenylyl cyclase activity were analyzed with the use of a nonlinear least-squares regression (Scatfit; Ref. 19) with a slope factor fixed at 1. Basal and maximal stimulation values presented in tables

were estimated with regression analysis. Differences were considered statistically significant when $p < 0.05$.

Results

Expression of β_1AR, β_2AR, and chimera in LTK$^-$ or CHW. CHW and LTK$^-$ with no detectable βAR-stimulated adenylyl cyclase activity were transfected with cDNAs encoding human β_1AR, human β_2AR, or Chβ_1/β_2AR. Cellular clones were selected by virtue of cotransfection with a G418 resistance marker (150 μg/ml for the CHW and 450 μg/liter for the LTK$^-$), and βAR expression was assessed in the resistant clones with the use of [^{125}I]CYP binding assays. Cell lines expressing similar levels of receptors (900–1300 fmol/mg of membrane protein for CHW and 100–250 fmol/mg of membrane protein for LTK$^-$) were chosen for further study.

Adenylyl cyclase stimulation. In the two cell types studied, the stimulation of β_1AR- or β_2AR-containing cells with isoproterenol yielded different efficacies of cAMP production. Indeed, when expressed in CHW, the β_1AR is significantly less efficacious than the β_2AR in stimulating adenylyl cyclase activity (Fig. 1 and Table 1, $p < 0.05$). Maximal β_1AR-mediated stimulation of adenylyl cyclase activity represented only 70% of the stimulation obtained in cells expressing a comparable number of β_2AR (1191 ± 140 fmol of β_1/mg of membrane proteins versus 942 ± 100 fmol of β_2/mg of membrane proteins). A significant difference ($p = 0.006$) in the potency of isoproterenol to stimulate the adenylyl cyclase activity was also found between the β_1AR- and β_2AR-expressing cells (Table 1). No difference was found when the nonstimulated adenylyl cyclase activities of these two cell lines were compared.

The difference between isoproterenol-stimulated adenylyl cyclase activity mediated by the β_1AR and β_2AR was even more dramatic when the receptors were expressed in LTK$^-$. The maximal response elicited by the β_1 subtype barely reached 20% of the β_2AR-mediated response (Fig. 1 and Table 1). The small difference in receptor number (133 ± 15 fmol of β_1/mg of membrane proteins versus 225 ± 25 fmol of β_2/mg of membrane proteins) could not account for the dramatic difference in stimulation because in several experiments the number of β_1ARs were equal or even greater than the number of β_2ARs yet the level of stimulation obtained was always significantly less. No difference in the EC_{50} for the isoproterenol-stimulated adenylyl cyclase activity was observed between β_1AR- and β_2AR-expressing cells. Interestingly, a modest but significantly elevated basal adenylyl cyclase activity was observed in the LTK$^-$ expressing the β_2AR compared with the β_1AR-expressing cells. This may suggest a higher level of spontaneous activity for the β_2AR in this cell line. The lower ability of the β_1AR to stimulate adenylyl cyclase activity does not result from a greater propensity of this receptor subtype to be proteolysed during the assay because the differences in adenylyl cyclase activity were

TABLE 1

Adenylyl cyclase activity in cells expression the human β_1-, β_2, or Chβ_1/β_2 AR.

	Basal	NaF (10 mM)	Isoproterenol-stimulated	
			Maximal stimulation	EC_{50}
	pmol/min/mg of proteins		*pmol/min/mg of proteins*	*nM*
CHW-β_1	3.0 ± 0.3	10.1 ± 1.6	12.4 ± 1.4	529 ± 124
CHW-β_2	4.0 ± 0.7	12.7 ± 2.6	17.7 ± 1.1[a]	131 ± 17[a]
LTK-β_1	1.7 ± 0.2	11.2 ± 0.9	5.2 ± 0.4	468 ± 169
LTK-β_2	3.4 ± 0.4[a,b]	13.8 ± 1.3	25.1 ± 2.0[a,b]	292 ± 41
LTK-Chβ_1/β_2	1.6 ± 0.2	15.2 ± 1.9	5.9 ± 0.7	459 ± 85

Data represent the mean ± standard error of 12–23 experiments.
[a] $p < 0.05$ versus β_1.
[b] $p < 0.05$ versus Chβ_1/β_2.

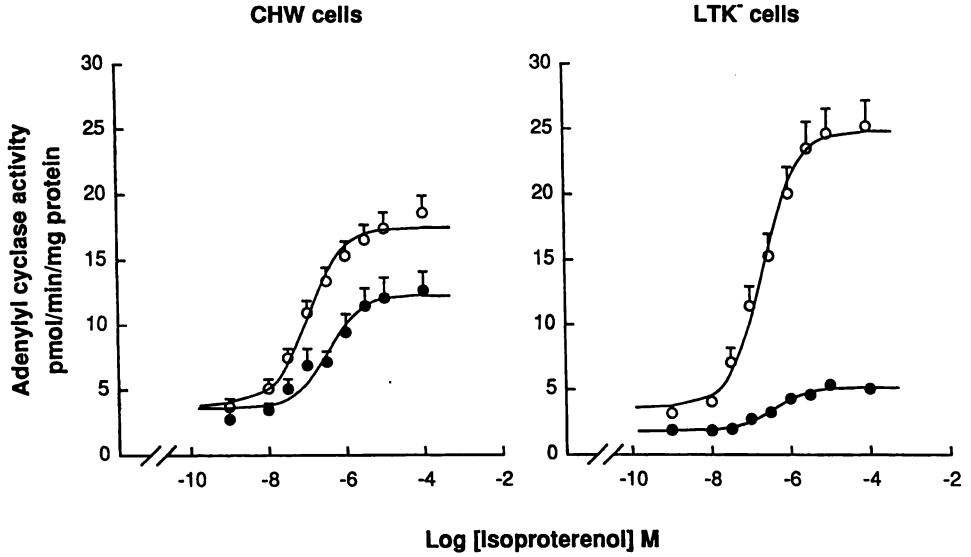

Fig. 1. Isoproterenol-stimulated adenylyl cyclase activity in membranes derived from CHW (*left*) and LTK$^-$ (*right*) expressing human β_1AR (●) or β_2AR (○). The adenylyl cyclase activity is expressed as pmol of cAMP produced/min/mg of protein. Data points represent the mean ± standard error of 13–23 independent experiments performed in duplicate (CHW β_1AR, 15 experiments; CHW β_2AR, 13 experiments; LTK$^-$ β_1AR, 15 experiments; and LTK$^-$ β_2AR, 23 experiments). The absence of error bars indicates that they are smaller than the symbol.

TABLE 2

Adenylyl cyclase activity: effects of desensitization induced by subsaturating concentration of agonist.

	Condition	Basal	NaF (10 mM)	Isoproterenol-stimulated	
				Maximal stimulation	EC_{50}
		pmol/min/mg of proteins		*pmol/min/mg of protein*	*nM*
CHW-β_1	CTL	3.1 ± 0.6	9.0 ± 2.0	11.8 ± 1.9	578 ± 261
	DES	1.6 ± 0.4	9.7 ± 3.0	9.3 ± 1.4	543 ± 420
CHW-β_2	CTL	5.6 ± 1.3	12.5 ± 4.4	20.0 ± 1.3	128 ± 13
	DES	2.9 ± 0.3	7.8 ± 2.1	15.1 ± 1.1[a]	561 ± 118[a]
LTK-β_1	CTL	1.5 ± 0.3	11.1 ± 1.4	5.0 ± 0.4	577 ± 344
	DES	1.6 ± 0.3	10.5 ± 1.4	4.0 ± 0.5	305 ± 83
LTK-β_2	CTL	4.0 ± 0.7	10.5 ± 0.8	23.7 ± 2.1	331 ± 81
	DES	3.1 ± 0.7	10.2 ± 1.0	19.1 ± 2.1	697 ± 55[a]
LTK-Chβ_1/β_2	CTL	1.6 ± 0.4	11.0 ± 1.8	5.0 ± 1.2	385 ± 188
	DES	0.9 ± 0.3	13.0 ± 1.9	4.4 ± 0.9	332 ± 42

Data represent the mean ± standard error of 5–13 experiments.
[a] $p < 0.05$ versus control condition.
DES, pretreatment with 10 nM isoproterenol for 15 min; CTL, control condition.

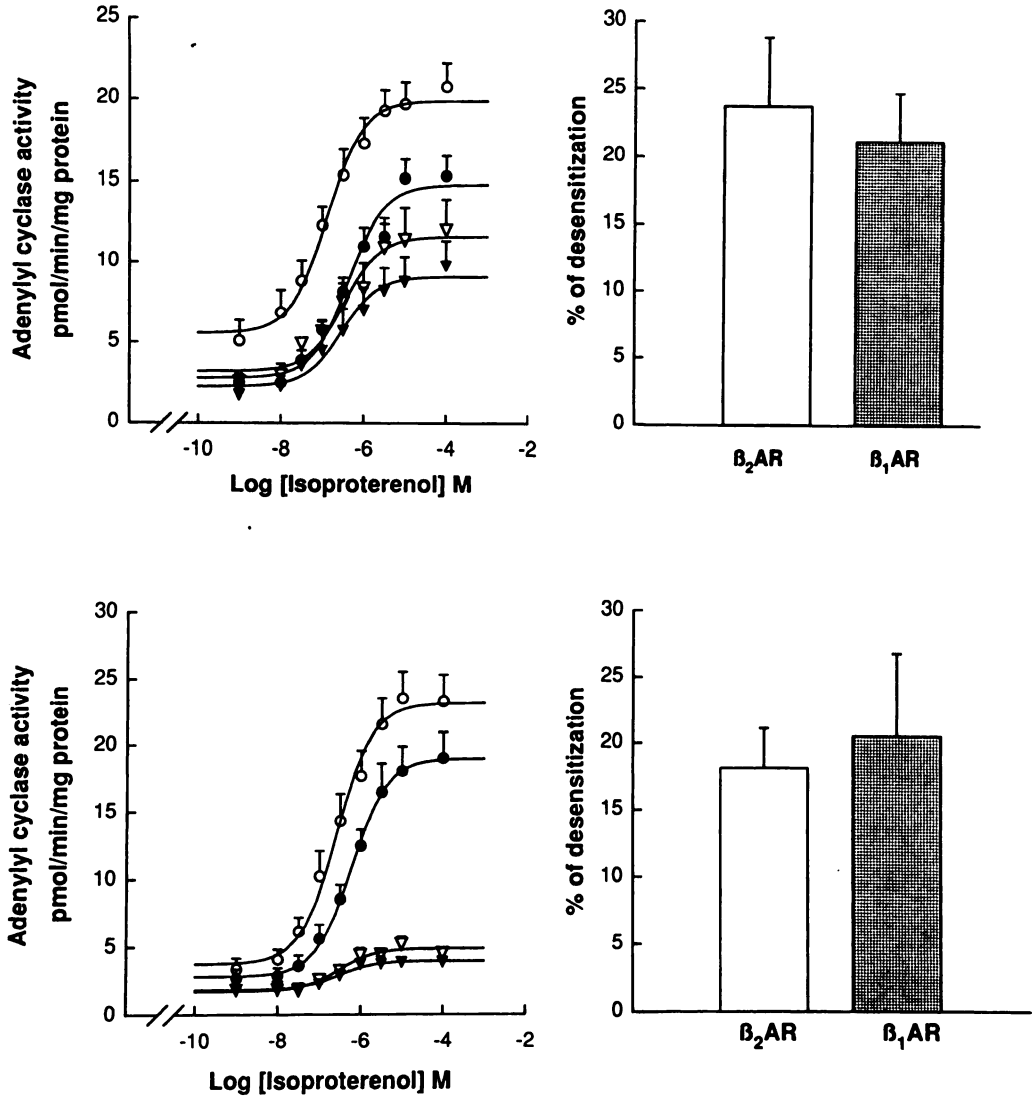

Fig. 2. *Left*, in a subset of the experiments described in the legend to Fig. 1, human β_1AR (▽, ▼) and β_2AR (○, ●) expressed in CHW (*top*) or LTK$^-$ (*bottom*) were preincubated with 10 nM isoproterenol (▼, ●) or with the vehicle (▽, ○) for 15 min at 37°. The isoproterenol-stimulated adenylyl cyclase activity was then measured in membranes derived from these cells. Results are expressed as pmol of cAMP produced/min/mg of protein. Data points represent the mean ± standard error of 5–10 independent experiments performed in duplicate (CHW β_1AR, five experiments; CHW β_2AR, five experiments; LTK$^-$ β_1AR, seven experiments; and LTK$^-$ β_2AR, 10 experiments). *Right*, the reduction in the maximally stimulated adenylyl cyclase activity induced by the pretreatment (desensitization) is expressed as percentage of the stimulation obtained in membranes derived from the vehicle-treated cells. Maximally stimulated activities were defined as the asymptote generated by nonlinear least-squares regressions with the use of the computer program Scatfit (19). The absence of error bars indicates that they are smaller than the symbol.

identical regardless of whether the membranes were assayed for 5, 10, 20, or 30 min (data not shown).

In both cell lines, stimulation with 10 mM NaF led to a similar augmentation of cAMP production, and this was independent of the receptor subtype expressed (Table 1). These results suggest that the differences observed above are receptor specific and do not result from interclonal differences in other components of the adenylyl cyclase stimulatory pathway.

Desensitization with 10 nM isoproterenol. Sustained stimulation of receptor with a low agonist concentration (10

TABLE 3

Adenylyl cyclase activity: effects of desensitization induced by saturating concentration of agonist.

	Condition	Basal	NaF (10 mM)	Isoproterenol-stimulated	
				Maximal stimulation	EC_{50}
		pmol/min/mg of proteins		*pmol/min/mg of proteins*	*nM*
CHW-β_1	CTL	3.1 ± 0.3	10.7 ± 2.3	12.7 ± 1.8	508 ± 128
	DES	2.5 ± 0.5	10.9 ± 2.1	10.0 ± 1.5	162 ± 58[a]
CHW-β_2	CTL	3.8 ± 0.9	12.8 ± 3.5	16.5 ± 1.5	98 ± 21
	DES	2.1 ± 0.3	13.9 ± 3.5	10.7 ± 1.0[a]	414 ± 129[a]
LTK-β_1	CTL	1.9 ± 0.3	11.3 ± 1.4	5.3 ± 0.6	532 ± 287
	DES	1.5 ± 0.2	10.4 ± 1.1	4.3 ± 0.4	613 ± 455
LTK-β_2	CTL	3.0 ± 0.4	16.0 ± 1.9	26.1 ± 3.1	201 ± 17
	DES	3.4 ± 0.7	15.6 ± 2.3	14.4 ± 2.4[a]	752 ± 132[a]
LTK-Chβ_1/β_2	CTL	1.5 ± 0.3	17.3 ± 2.4	6.4 ± 0.9	572 ± 229
	DES	1.1 ± 0.2	16.3 ± 2.2	3.6 ± 0.5[a]	383 ± 298

Data represent the mean ± standard error of 8–13 experiments.
[a] $p < 0.05$ versus control condition.
DES, pretreatment with 10 μM isoproterenol for 15 min; CTL, control condition.

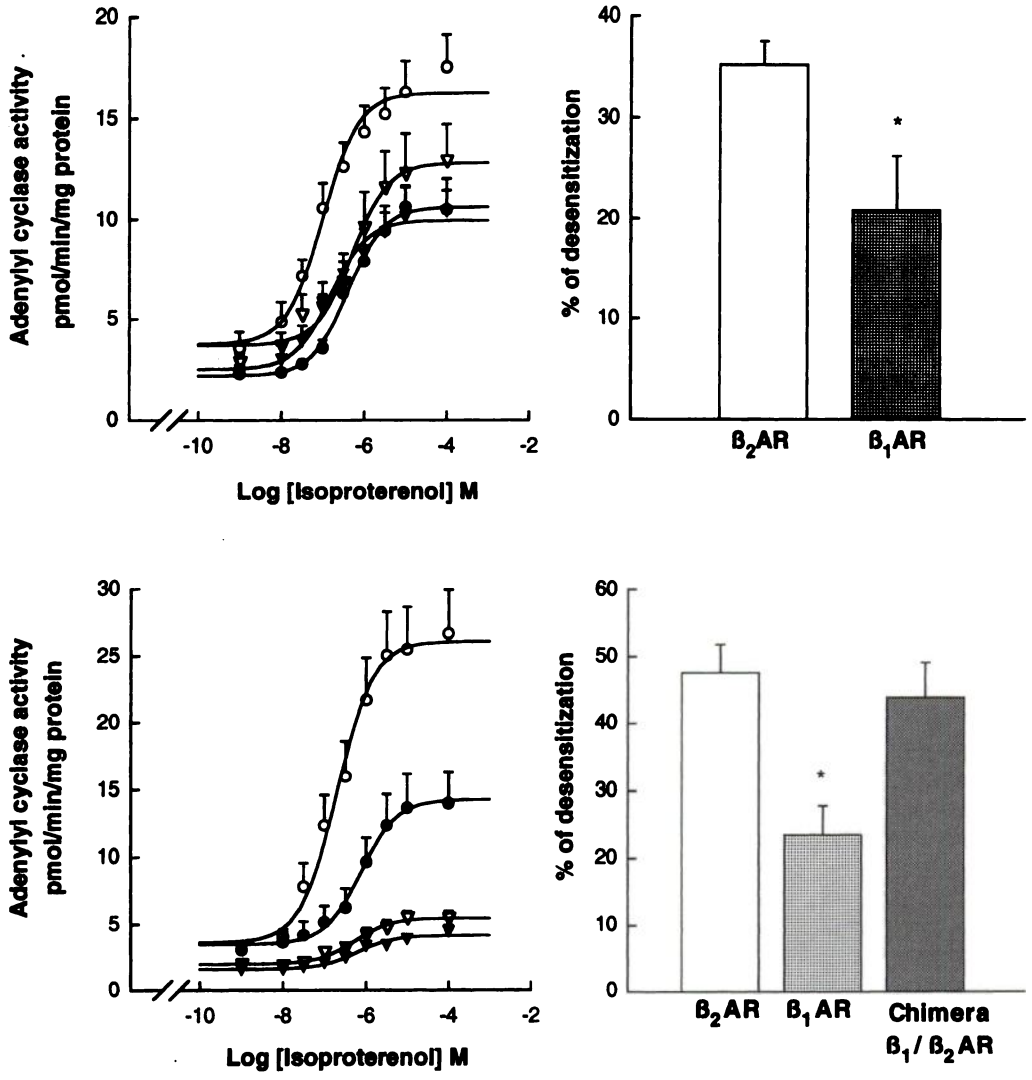

Fig. 3. *Left*, in a subset of the experiments described in the legend to Fig. 1, human β_1AR (▽, ▼) and β_2AR (○, ●) expressed in CHW (*top*) or LTK$^-$ (*bottom*) were preincubated with 1 μM isoproterenol (▼, ●) or with the vehicle (▽, ○) for 15 min at 37°. The isoproterenol-stimulated adenylyl cyclase activity was then measured in membranes derived from these cells. Results are expressed as pmol of cAMP produced/min/mg of protein. Data points represent the mean ± standard error of 9–13 independent experiments performed in duplicate (CHW β_1AR, 11 experiments; CHW β_2AR, nine experiments; LTK$^-$ β_1AR, nine experiments; and LTK$^-$ β_2AR, 13 experiments). *Right*, Isoproterenol-induced desensitization of the βAR-stimulated adenylyl cyclase activity measured in membranes derived from cells expressing β_1AR, β_2AR, or Chβ_1/β_2AR (*Chimera* β_1/β_2*AR*). Results are expressed as percentage of the stimulation obtained in membranes derived from the vehicle-treated cells. Maximally stimulated activities were defined as the asymptote generated by nonlinear least-squares regressions with the use of the computer program Scatfit. *, $p <$ 0.05 compared with β_2AR and Chβ_1/β_2AR. The absence of error bars indicates that they are smaller than the symbol.

nM isoproterenol for 15 min at 37°) led to lower cAMP production on subsequent isoproterenol stimulation in both β_1AR- and β_2AR-expressing cells. Indeed, in a comparison of the maximal level of stimulation obtained after these desensitizing conditions, the isoproterenol-stimulated adenylyl cyclase activity was reduced by 23.5 ± 5.1% in β_2AR-expressing CHW and 21.2 ± 3.5% in β_1AR-expressing cells (Fig. 2 and Table 2). This reduction in maximal stimulation was accompanied by a significant rightward shift of the isoproterenol dose-response curve in the β_2AR- but not the β_1AR-expressing cells. Similar profiles of desensitization were also observed for both receptor subtypes when expressed in LTK$^-$.

As can be seen in Fig. 2, the desensitization observed in β_1AR-expressing LTK$^-$ was very modest in absolute terms. However, when expressed as a percentage, this desensitization was not different than that observed in the β_2AR-expressing cells. Indeed, the agonist pretreatment led to a desensitization of 19.7 ± 3.4% for β_2AR and 20.8 ± 7.4% for β_1AR. Although agonist treatment led to an apparent reduction in basal adenylyl cyclase activity in the CHW expressing the β_2AR (Fig. 2), no statistically significant decrease in either basal or NaF-stimulated adenylyl cyclase activity was detected in any of the cell lines studied (Table 2).

Desensitization with 1 μM isoproterenol. Sustained stimulation of the β_1AR and β_2AR with a high concentration of agonist (1 μM isoproterenol for 15 min at 37°) led to desensitization patterns that are different for the two receptor subtypes. The β_2AR CHW cell line showed a 35.2 ± 2.3% desensitization in comparison with a desensitization of only 20.8 ± 5.4% in cells expressing the β_1 subtype ($p = 0.036$; Fig. 3 and Table 3). A similar difference in the extent of desensitization was observed when the receptors were expressed in LTK$^-$ (Fig. 3). In these cells, the β_2AR-stimulated adenylyl cyclase activity was desensitized by 47.5 ± 4.3% on sustained stimulation with 1 μM isoproterenol, whereas a desensitization of only 23.5 ± 4.4% was observed for the β_1AR-stimulated activity under the same conditions ($p = 0.001$). In this desensitization paradigm, a reduction in the potency of isoproterenol in stimulating adenylyl cyclase was observed in both cell lines but only for the β_2AR (Table 3). As was found for desensitization induced by lower agonist concentration, neither basal nor NaF-activated adenylyl activity was affected in any of the cell lines (Table 3).

Desensitization profiles of the Chβ_1/β_2AR. To determine the potential contribution of the carboxyl domains in the distinct coupling properties and desensitization profiles of the β_1AR and β_2AR, a Chβ_1/β_2AR was constructed. This chimera consisted of a β_1AR core with the seventh transmembrane domain and the carboxyl tail of the β_2AR. Because the most dramatic differences between the β_1AR and β_2AR were observed in the LTK$^-$, the responsiveness of the chimeric receptor was assessed in this cell type under basal conditions and after sustained stimulation with either low or high agonist concentrations. Both the stimulation and desensitization profiles of the chimeric receptor were compared with those observed for the wild-type β_2AR and β_1AR. To eliminate any interclonal variability, three different cellular clones expressing similar levels of the Chβ_1/β_2AR were used, and identical results were obtained. The maximal level of isoproterenol-stimulated adenylyl cyclase activity obtained for the Chβ_1/β_2AR was similar to that observed for the β_1AR (V_{max}, 5.9 ± 0.7 pmol/min/mg of proteins) and thus was significantly less than that observed for the β_2AR (Fig. 4 and Table 1; $p < 0.005$). Stimulation of cells expressing Chβ_1/β_2AR with NaF yielded a level of adenylyl cyclase activity similar to those observed in cells expressing either the β_1AR or the β_2AR (Table 1). Treatment of Chβ_1/β_2AR cells with a low concentration of isoproterenol (10 nM) for 15 min led to a desensitization pattern similar to the one observed for both β_1AR and β_2AR (data not shown). However, pretreatment of the cells with the higher concentration of the agonist for 15 min (1 μM isoproterenol) led to a 43.9 ± 5.2% (eight experiments) desensitization, which is similar to that obtained for the β_2AR but significantly higher than that observed for the wild-type β_1AR (Fig. 3). As previously observed for both wild-type receptors, neither of the two desensitization protocols led to a change in basal or NaF-stimulated adenylyl cyclase activity (Tables 2 and 3). Thus, the substitution of the seventh transmembrane domain and carboxyl tail of the β_1AR for that of the β_2AR conferred a desensitization profile characteristic of the β_2AR without changing the coupling efficacy characteristic of the β_1AR. These results therefore suggest that the different patterns of desensitization observed for the β_1AR and β_2AR are related to molecular determinants present in their carboxyl domains. A noticeable difference within these domains is the presence of a PKA phosphorylation site in the proximal portion of the β_2AR tail but not the β_1AR. To assess the potential role of this PKA site in the distinct desensitization profile of the β_2AR, it was mutated by substituting alanine residues for Ser^{345,346} (Ala$^{345,346}/\beta_2$AR). As shown in Fig. 5, this mutation reduced the extent of desensitization obtained after stimulation with isoproterenol (1 μM) for 15 min by 31%, thus supporting a role for this PKA phosphorylation site in the desensitization process.

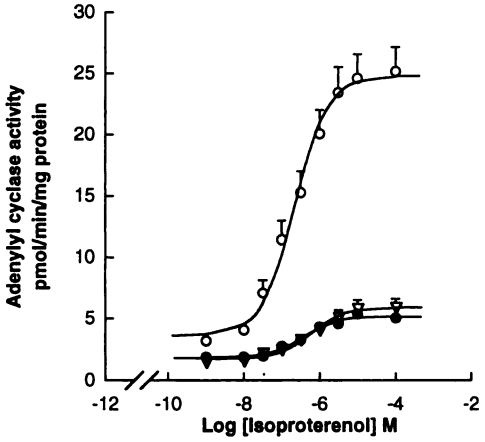

Fig. 4. Isoproterenol-stimulated adenylyl cyclase activity measured in membranes derived from LTK$^-$ expressing Chβ_1/β_2AR (▽). Stimulation curves for the β_1AR and β_2AR (Fig. 1) are reproduced here for comparison purposes [β_1AR (●) or β_2AR (○)]. Results are expressed as pmol of cAMP produced/min/mg of protein. Data points for Chβ_1/β_2AR represent the mean ± standard error of 12 experiments performed in duplicate. The absence of error bars indicates that they are smaller than the symbol.

Discussion

The results of the current study demonstrate that the human β_1AR has a lower coupling efficacy with the adenylyl cyclase system than does the β_2AR. This seems to be an intrinsic property of β_1AR as similar results are observed in two different surrogate cell lines. These results are consistent with previous studies from different laboratories that suggest a weaker coupling of the β_1AR subtype in cells or tissue expressing both receptors (9, 20, 21). In human heart, several groups observed that although the number of β_1ARs is greater in this tissue, the percentage of adenylyl cyclase stimulation attributable to this receptor is significantly less than that resulting from β_2AR stimulation (8, 22). However, the identity of the cells expressing each of the two receptor subtypes and whether the degree of pathology could selectively influence the extent of coupling of a given subtype are

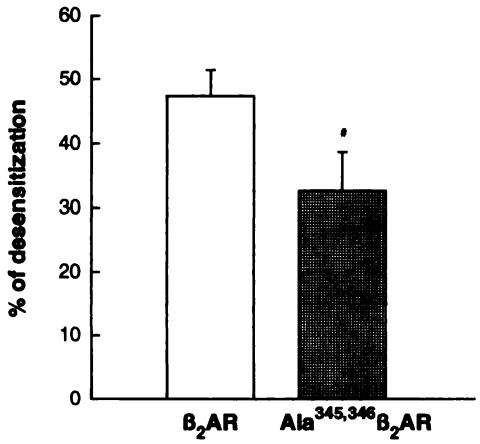

Fig. 5. Isoproterenol-induced desensitization of the βAR-stimulated adenylyl cyclase activity measured in membranes derived from LTK$^-$ expressing β_2AR and Ala^{345,346}/β_2AR. Desensitization was induced by pretreatment of the cells with 1 μM isoproterenol for 15 min at 37°. Results are expressed as percentage desensitization as described in legend to Fig. 3 (#, $p = 0.059$ compared with β_2AR; nine experiments). Each desensitization experiment with the LTK$^-$ Ala^{345,346}/β_2AR was carried out in parallel with a desensitization performed with the LTK$^-$ β_2AR.

still unknown. A study by Levy *et al.* (9) has shown that in LTK$^-$ coexpressing both human β_1AR and β_2AR subtypes, the contribution of the β_1AR to isoproterenol-stimulated adenylyl cyclase is significantly less than that of the β_2AR. However, in that study, the two receptors were present at different levels of expression. To overcome the difficulty in interpreting results in cells coexpressing both subtypes, we chose to study each receptor separately in the same surrogate cell lines. In CHW, the difference in adenylyl cyclase stimulation between receptor subtypes is less than that observed in LTK$^-$. This difference may be due to the higher number of receptors expressed in CHW. Indeed, the results of Levy *et al.* (9) suggest that the level of stimulation of adenylyl cyclase reaches a plateau for the β_2AR at a level of expression approaching 1000 fmol/mg of protein, whereas the stimulation by the β_1AR continues to increase with higher numbers of receptor. In the current study, the CHW used expressed between 900 and 1300 fmol of receptors/mg of protein, whereas in the LTK$^-$, receptor expression varied between 100 and 250 fmol/mg protein. This may explain the smaller difference observed in CHW. We must also consider that the difference might be due to the presence of specific adenylyl cyclase isoforms, βγ subunits, or splice variants of G_s, which might influence the degree of stimulation observed in the two cell lines considered. A modest difference in the signaling efficacies of the two receptors when expressed in CHW was also observed in other studies (6, 7) but could not be detected by others using the same cell line (13) or Chinese hamster ovary cells (6). These apparent contradictions emphasize the fact that the difference in signaling efficacy is relatively modest when the receptors are expressed at high levels in CHW, that special care must be taken when choosing clonal lines expressing similar numbers of receptors, and that a sufficient number of determinations must be carried out to detect the difference.

Green *et al.* (21) also observed different coupling abilities between the two βAR subtypes when expressed in CHW. However, in their study, the difference was reflected by a lower potency of isoproterenol to stimulate adenylyl cyclase activity via β_1AR without any apparent difference in the efficacy of the two receptor subtypes to maximally stimulate the enzyme. The current study, in agreement with the results of Levy *et al.* (9), suggests that the difference between the two receptor subtype does not only lie in a different affinity of the agonist for the receptor/G_s complex but rather reflects a difference in the extent to which the two subtypes may maximally activate adenylyl cyclase. This is particularly clear when the receptors are expressed in LTK$^-$. In an attempt to determine the molecular determinants dictating the distinct abilities of βAR subtypes to stimulate adenylyl cyclase, Green and Liggett (10) deleted a proline-rich 24-amino acid sequence from the third intracytoplasmic loop of the β_1AR that is absent from the β_2AR. Although this deletion improved the ability of the β_1AR to stimulate adenylyl cyclase while the insertion of the proline-rich sequence into the β_2AR impaired its coupling, neither of these mutations fully restored the coupling pattern characteristic of the other subtype (10). This may suggest that other regions could be important in determining the subtype-selective coupling ability of the two βARs. The observation in the current study that substitution of the seventh transmembrane domain and carboxyl tail of the β_1AR for that of the β_2AR did not improve coupling to G_s rules out a possible contribution of these domains in the subtype-selective coupling.

Desensitization patterns observed on stimulation with a low concentration of isoproterenol (10 nM) were similar for the two receptor subtypes, suggesting that molecular determinants involved in this desensitization are present in both receptors. Previous studies have shown that for the β_2AR, PKA-mediated phosphorylation of serines located in the third intracytoplasmic loop plays a dominant role in the desensitization initiated by low levels of stimulation (4, 23). The observation that the level of desensitization induced by a low concentration of isoproterenol is identical for the two receptor subtypes is consistent with these findings. Indeed, a consensus sequence for phosphorylation by PKA is also found in an identical position in the third cytoplasmic loop of the β_1AR. Also, the desensitization induced in the Chβ_1/β_2AR at low agonist levels is similar to that observed in the wild-type β_1AR receptor, suggesting that the β_2-carboxyl tail has no influence on this pattern of desensitization, as might be expected. If this domain contributes to the desensitization elicited by low levels of stimulation, it does so to a similar extent for both receptor subtypes.

The major difference observed in the desensitization patterns of the β_1AR and β_2AR was manifested at high concentrations of agonist. Previous reports have shown that in addition to PKA-mediated phosphorylation, phosphorylation of the agonist-occupied β_2AR by βARK contributes to the desensitization elicited on stimulation with high concentrations of agonists (e.g., 1 μM) (23, 24). For the β_2AR, the principal phosphorylation targets for βARK are believed to be serine and threonine residues located in the distal portion of the carboxyl tail of the receptor (23, 25), possibly in the vicinity of acidic amino acids (26). Although the exact residues have not been identified yet for the β_2AR, a similar number of serine and threonine residues are found in the carboxyl tail of the β_1AR (10) and the β_2AR (11). If only serines and threonines located one or two residues downstream of acidic residues are considered (as they are believed

to represent favored sites for βARK; Ref. 26), four potential sites exist in each receptor. Therefore, the differences between the two receptor subtype-specific desensitization patterns most likely do not result simply from a different number of βARK phosphorylation sites. However, using a protein kinase A inhibitor (PKI) and heparin to inhibit βARK, Zhou *et al.* (6, 27) suggested a greater resistance of the β_1AR to βARK-mediated desensitization. If this greater resistance is responsible for the difference in the desensitization patterns between the two receptor subtypes, one would have to suggest that the β_1AR is a poorer substrate for βARK due to divergence in the primary sequence between the two receptor subtypes or that β-arrestin may have a lower affinity for the phosphorylated β_1AR than for the phosphorylated β_2AR. In a recent report, Freedman *et al.* (7) showed that the β_1AR is a good substrate for βARK-1 and suggested that βARK-mediated phosphorylation plays an important role in the agonist-promoted desensitization of the β_1AR. In any case, our results clearly show that substituting the last 130 residues of the β_1AR with those of the β_2AR restored the desensitization pattern observed for wild-type β_2AR, suggesting that the determinants present in this receptor domain are responsible for the receptor-selective pattern of desensitization evoked by 1 μM isoproterenol. A noticeable difference between the two receptors, in this domain, is the presence of a second consensus phosphorylation site for PKA (^{343}RRSSLK) in the proximal portion of the β_2AR carboxyl tail, which is absent from the β_1AR sequence. Although this site has not been shown to be phosphorylated on direct activation of PKA by cAMP analogues (4, 5), recent evidence suggests that it may become accessible to the kinase once the receptor is agonist bound (28). Phosphorylation of this site could therefore be important in the desensitization patterns observed for the β_2AR and the Chβ_1/β_2AR on high stimulation levels, which lead to full occupation of the receptor. Our observation that mutation of $Ser^{345,346}$ in the carboxyl tail of the β_2AR significantly reduces the desensitization induced by the high concentration of isoproterenol is consistent with an important role of these residues in desensitization of the β_2AR. However, the contribution of other sites to the subtype-specific desensitization patterns cannot be excluded. In particular and as discussed above, differences in βARK phosphorylation may also contribute to distinct desensitization patterns. For some receptors, phosphorylation of specific sites has been shown to facilitate phosphorylation of other residues (29, 30). Whether phosphorylation of $Ser^{345,346}$ facilitates subsequent phosphorylation by βARK is an intriguing possibility that remains to be tested.

The relative resistance of the β_1AR to agonist-promoted desensitization compared with that of the β_2AR has also been observed in a recent studies with CHW and Chinese hamster ovary cells as surrogate cell lines (6). In contrast, no difference between the two receptor desensitization patterns could be detected in another study with CHW (7). It is not clear why the difference was not detected in this latter study; it could be argued that the lower desensitization observed for the β_1AR results from its reduced efficient coupling to G_s and, therefore, to lower cAMP production. This is highly unlikely because the level of cAMP production obtained for the Chβ_1/β_2AR was comparable to that observed for the β_1AR, whereas the level of desensitization was identical to that observed for the β_2AR.

The results presented here therefore suggest that distinct receptor domains are responsible for the βAR subtype-selective coupling and desensitization phenotypes. Indeed, although the carboxyl domain determines the extent of desensitization observed for a given subtype, this portion of the receptor does not influence their maximal coupling efficacy. Further studies are required to identify regions that confer subtype-selective coupling efficacy.

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Send reprint requests to: Dr. Michel Bouvier, Département de Biochimie, Faculté de Médecine, Université de Montréal, P.O. Box 6128, Succursale Centre-ville, Montréal, Québec, H3C 3J7, Canada. E-mail: bouvier@bch.umontreal.ca

Erratum

In the February 1996 issue, the article by Leeder et al. [Leeder, S. J., A. Gaedijk, X. Lu, and V. A. Cook. Epitope mapping studies with human anti-cytochrome P450 3A antibodies. *Mol. Pharmacol.* **49**:234–243 (1996)] was incorrectly designated as an *Accelerated Communication*. It was, in fact, a standard *Article*.

We regret this error and apologize for any inconvenience it may have caused.