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Expression of a Human cDNA Encoding the β_2-Adrenergic Receptor in Chinese Hamster Fibroblasts (CHW): Functionality and Regulation of the Expressed Receptors

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SUMMARY

A human β-adrenergic receptor cDNA was transfected and expressed in transformed Chinese hamster fibroblasts (CHW). The expressed receptor exhibited a typical β_2-adrenergic selectivity for agonists and antagonists as assessed by radioligand binding and adenylate cyclase activation. Guanine nucleotide-sensitive high affinity binding of the agonist, isoproterenol, indicated effective coupling of the expressed receptor to a guanine nucleotide-regulatory protein. The level of expression of β_2-AR in various cell clones varied over 200-fold and was positively correlated with the levels of β_2-AR mRNA. In cells expressing between 0.04 and 3.0 pmol of β_2-AR/mg of membrane protein, the efficacy of isoproterenol for stimulating adenylate cyclase increased with increasing numbers of expressed receptors but reached a plateau and started to decrease in clones with higher β_2-AR density (3.0–8.0 pmol/mg of membrane protein). Preincubation of β_2-AR-expressing cells with isoproterenol for 15 min led to significant reduction in the level of isoproterenol-sensitive adenylate cyclase activity. This agonist-induced desensitization was also accompanied by phosphorylation of the β_2-AR. These data indicate that the expressed human β_2-AR displays typical functional characteristics of adenylate cyclase-coupled receptors including agonist-induced desensitization. Moreover, the availability of this series of cellular clones, which differ markedly in their density of β_2-AR, provides a unique set of biological reagents for future studies of β_2-AR function and regulation.

The β_2-AR is a member of a family of integral membrane proteins that selectively bind hormones, neurotransmitters, and drugs. Agonist occupancy of the receptor initiates a series of interactions which ultimately lead to activation of adenylate cyclase via interaction with the stimulatory guanine nucleotide-regulatory protein, G_s (1). The interactive dynamics of this system indicate that its signaling efficiency is dictated by the relative affinities of the three principal components for each other, as well as their relative concentrations within the cell membrane (2). In fact, the molecular basis of agonist-induced desensitization of the β_2-AR-coupled adenylate cyclase is believed to comprise modulation of β_2-AR coupling to G_s via phosphorylation events as well as sequestration and down-regulation of the receptor (3). Thus, the relationship between the number of receptors expressed in a given cell and the adenylate cyclase responsiveness of the cell to hormonal stimulation is an important feature of the regulation of the adenylate cyclase-coupled signaling pathways. In this study, we document the expression of the recently cloned human β_2-AR cDNA (4) in CHW cells and characterize the ability of this receptor, expressed at widely different levels, to interact with the adenylate cyclase signaling pathway. Furthermore, we demonstrate the ability of the expressed receptor to mediate agonist-induced desensitization and to undergo phosphorylation.

Experimental Procedures

Materials. Carrier-free $^{32}P_i$, [^{125}I]CYP, [^{125}I]iodopindolol, [^{125}I]CYP diazarine, [α-^{32}P]ATP, [^{3}H]cAMP, and Endoglycosidase F were obtained from New England Nuclear. [^{3}H]CGP 12177 was obtained from Amersham. Isoproterenol, norepinephrine, epinephrine, sotalol, alprenolol, ATP, GTP, Gpp(NH)p, cAMP, phosphoenolpyruvate, myokinase, and collagenase were purchased from Sigma. Pyruvate kinase and isobutylmethylxanthine were from Calbiochem. Digitonin was

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ABBREVIATIONS: β_2-AR, β_2-adrenergic receptor; G_s, stimulatory guanine nucleotide-regulatory protein; CHW cell, Chinese hamster fibroblast; [^{125}I]CYP, [^{125}I]iodocyanopindolol; DMEM, Dulbecco's modified Eagle's medium; SV40, simian virus 40; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetate; SDS, sodium dodecyl sulfate; β-AR, β-adrenergic receptor; G_i, inhibitory guanine nucleotide-regulatory protein; Endo-F, Endo-β-*N*-acetylglucosaminidase.

purchased from Gallard-Schlessinger. The expression vector pKSV-10 was obtained from Pharmacia. Geneticin (G418), DMEM, and fetal calf serum were purchased from Gibco. ICI 118551 and betaxolol were generously provided by Imperial Chemical Industries and Synthelab, respectively.

Expression vector and cell transfection. The human β_2-AR cDNA clone pTF (4) containing 190 base pairs of 5′-untranslated sequence (UTS) was cloned into the Bgl II site of the eukaryotic expression vector pKSV-10 containing the SV40 early promoter, the intron from the SV40 large T antigen, and the polyadenylation signal from the SV40 early region. The resulting plasmid, pKSVTF (190bp5′UTS), was cotransfected with pSV Neo into CHW cells by coprecipitating the DNA with calcium phosphate (5). Cells were selected in DMEM + 10% fetal calf serum containing 150 μg/ml Geneticin. Clones were then screened for β_2-AR expression in a [^{125} I]CYP radioligand binding assay.

Cell culture. The transfected CHW cells were grown as monolayers in 75-cm^2 flasks containing DMEM supplemented with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and amphotericin B (0.02 μg/ml) until apparent confluency in an atmosphere of 95% air, 5% CO_2 at 37°.

Whole cell radioligand binding assays. The cells were detached, washed twice with PBS, and resuspended at a concentration of 2×10^6 cells/ml. Fifty and 200 μl of cell suspension were used for [^{125} I] iodopindolol and [^{3}H]CGP 12177 binding assays, respectively. The binding assays were performed as previously described (6) in DMEM at 37° for 1 hr in a final volume of 1 ml. The binding reactions were terminated by the addition of 10 ml of ice-cold PBS and rapid filtration through Whatman GF/C glass fiber filters. Specific binding was defined as the amount of radioligand binding inhibited by 1 μM (−)-propranolol.

Desensitization experiments. Nearly confluent cells were washed twice in the flask with 5 ml of fresh DMEM. DMEM containing isoproterenol (100 μM) and ascorbic acid (1 mM) or ascorbic acid alone was added to experimental and control flasks, respectively, and the cells were incubated at 37° for 15 or 120 min. Reactions were terminated by addition of 10 ml of ice-cold PBS and membranes were prepared as described below.

Membrane preparations. Cells were washed twice with 5 ml of ice-cold PBS, scraped in an additional 5 ml of ice-cold PBS and pelleted by centrifugation at 200 × *g*. Cells were then lysed in ice-cold 5 mM Tris-HCl, pH 7.4, 2 mM EDTA with a Polytron homogenizer (four bursts for 5 sec at maximum setting). The lysate was centrifuged at 200 × *g* to pellet unbroken cells and large particles and the supernatant was centrifuged at 40,000 × *g* for 30 min at 4°. The pelleted membranes were washed once and resuspended in 0.6 ml of a buffer containing 75 mM Tris, pH 7.4, 25 mM $MgCl_2$, 2 mM EDTA. The membrane suspension was assayed immediately.

Membrane radioligand binding assays. Membranes were incubated with various concentrations of [^{125} I]CYP in a buffer containing 75 mM Tris-HCl, pH 7.4, 25 mM $MgCl_2$, 2 mM EDTA at 25° for 120 min. The binding reactions were terminated by filtration over Whatman GF/C glass fiber filters. Specific binding was defined as the amount of [^{125} I]CYP binding inhibited by 10 μM (−)-alprenolol. Competition assays with agonists and antagonists were conducted using 80–100 pM [^{125} I]CYP as radioligand. Determinations were performed in triplicate. Data analysis was carried out using nonlinear least squares regression as previously described (7).

β_2-AR mRNA levels. Total cellular RNA was prepared from individual cell lines by the cesium chloride gradient method of Chirgwin *et al.* (8). For slot blot analysis, RNA was denatured in 6.15 M formaldehyde in 10× saline sodium citrate (1× = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) and applied to nitrocellulose membranes using a slot-blotting apparatus (Schleicher & Schuell) according to the manufacturer's instructions. For Northern blotting, RNA was denatured by the glyoxal procedure (9), electrophoresed through agarose gels (1.2% in 10 mM sodium phosphate buffer, pH 7.2), and blotted onto Biodyne nylon membrane. The blots were hybridized to cDNA probes of human β_2-AR (4) or chicken β-actin (10) under previously described conditions (11). The probes were labeled with [α-^{32} P]dCTP by nick translation (12). Specific activities of the probes ranged from 2 to 5×10^8 cpm/μg of DNA.

Adenylate cyclase assay. Adenylate cyclase activities were measured by the method of Salomon *et al.* (13). Assay mixtures contained 0.02 ml of membrane suspension, 30 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 0.8 mM EDTA, 0.12 mM ATP, 1 μCi of [α-^{32} P[ATP, 0.1 mM cAMP, 0.06 mM GTP, 2.8 mM phosphoenolpyruvate, 5.2 μg/ml pyruvate kinase, and 10 μg/ml myokinase in a final volume of 0.05 ml. Enzyme activities were determined in the absence of activators or with 100 μM (−)-isoproterenol, 10 mM NaF, or 100 μM forskolin. Reactions were initiated by the addition of membranes and the assay mixtures incubated for 20 min at 37°. Reactions were terminated by the addition of 1 ml of 0.4 mM ATP, 0.3 mM cAMP, and [^{3}H]cAMP (~20,000 cpm), and cAMP was isolated by sequential chromatography on Dowex cation exchange resin and aluminum oxide. Determinations were performed in triplicate.

Whole cell phosphorylation experiments. Nearly confluent cells were detached from the flasks by treatment with collagenase (1 mg/ml) containing soybean trypsin inhibitor (0.05 mg/ml) for 60 min at 37°. The cells were then washed twice with fresh phosphate-free DMEM and preincubated with carrier-free ^{32} P_i (30 mCi) in 60 ml of phosphate-free DMEM at 37° for 60 min to allow ^{32} P_i incorporation into the cells. (−)-Isoproterenol (100 μM) and ascorbic acid (5 mM) were added to the cells to induce desensitization as described above. Reactions were terminated by centrifugation at 200 × *g*, and the cells were washed twice with ice-cold, phosphate-free DMEM and disrupted in ice-cold 20 mM Tris-HCl, pH 7.4, 5 mM EDTA, 10 mM Na_2HPO_4 (buffer A) by sonication (six bursts for 10 sec). The lysates were centrifuged at 40,000 × *g* and the pellets were washed twice with ice-cold buffer A. The resulting membranes were solubilized in 100 mM NaCl, 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 2% digitonin, and the β_2-AR purified by alprenolol-Sepharose affinity chromatography as described (14).

Endoglycosidase F treatment. Phosphorylated β_2-AR (~0.1 pmol) purified by alprenolol-Sepharose affinity chromatography was incubated with Endoglycosidase F (3 units) in a final volume of 0.1 ml containing 10 mM ammonium bicarbonate, pH 6.5, 50 mM EDTA, 0.05% digitonin, 100 μM alprenolol at 25° for 12 hr. The reactions were stopped by the addition of SDS sample buffer. Control incubations were conducted in the absence of Endoglycosidase F.

Photoaffinity labeling. Membranes prepared as described above were incubated in the dark with [^{125} I]iodocyanopindolol diazarine (25 pM) in PBS containing 5 mM EDTA for 3 hr at 25°. Membranes were then washed once with ice cold PBS/EDTA containing bovine serum albumin (0.5%), twice with ice-cold PBS/EDTA without bovine serum albumin, and resuspended in 1 ml of PBS/EDTA. The suspensions were UV-irradiated with an Hanovia 450-W medium pressure mercury lamp filtered with 5 mm of pyrex glass for 5 min, and the pelleted membranes were dissolved in SDS-polyacrylamide gel electrophoresis sample buffer (see below).

SDS-polyacrylamide gel electrophoresis. Gel electrophoresis was performed according to the method of Laemmli (15) with 12% slab gels. Sample buffer consisted of 8% SDS, 10% glycerol, 5% β-mercaptoethanol, 25 mM Tris-HCl, pH 6.5, and 0.003% bromophenol blue. After electrophoresis, the gels were dried and autoradiographed at −90° with Kodak XAR-5 film and intensifying screens.

Statistical analysis. Statistical significance of the differences between groups was measured using a paired Student's *t* test. A probability of $p < 0.05$ was considered to represent a significant difference.

Results

Different levels of β-adrenergic receptor expression. Membranes from 50 cellular clones, selected by virtue of their cotransfection with the neomycin resistance marker, were pre-

pared and assayed for [^{125}I]CYP binding activity. Of 34 clones (designated CTF-##) exhibiting significantly greater specific [^{125}I]CYP binding than the nontransfected CHW cells, 9 were selected for further characterization. As shown in Table 1, the number of [^{125}I]CYP-binding sites in transfected cells varied between 2- and 200-fold of that observed in nontransfected CHW cells. No signficant difference in the affinity for [^{125}I]CYP was observed among the clones (data not shown).

Radioligand binding assays performed on whole cells using the hydrophilic radioligand [^{3}H]CGP 12177, which detects only cell surface receptors (16), and the lipophilic compound [^{125}I]iodopindolol, which detects total cellular receptors, indicated that >95% of the total β_2-AR was present at the cell surface in three cellular clones (CTF-33, CTF-23, and CTF-21) expressing different levels of β_2-AR (data not shown). Thus, the differences in β-AR number among the various clones appear not to reflect differences in cellular distribution.

As shown in Fig. 1, the level of β-AR mRNA assessed in CHW, four different CTF clones, and A431 epidermoid carcinoma cells is highly correlated (r = 0.97) with the number of [^{125}I]CYP-binding sites determined in these cells. Thus, the number of membrane-binding sites expressed at the cell surface appears to be a direct function of the level of β-AR mRNA present in any given clonal cell. Northern blot analysis of RNA from three of these clones also indicates that β-AR mRNA levels are coincident with receptor levels. The size of these transcripts (~4.2 kilobases) is significantly larger than the 2-kilobase β-AR messages observed in A431 cells (4). However, this is the expected length for the β-AR cDNA in the expression construct used here, which contains additional SV40 coding sequences and polyadenylation signal.

Pharmacological characteristics of the expressed β-AR. One of the clones, CTF-23, was selected for more complete pharmacological characterization of the [^{125}I]CYP-binding sites. A K_d of 116 pM was calculated, in good agreement with that obtained for [^{125}I]CYP binding in A431 cells (134 pM). In competition experiments, the order of potency of agonists to inhibit [^{125}I]CYP binding was: (−)-isoproterenol > (−)-epinephrine > (−)-norepinephrine > (+)-isoproterenol, whereas for antagonists, the order was: alprenolol > ICI 118551 > betaxolol (Table 2). These results indicate that a β-AR of the β_2-subtype was expressed in these cells (17).

The expression of the human β-AR cDNA in CTF-23 cells confers isoproterenol sensitivity to adenylate cyclase which is lacking in the nontransfected CHW cells. The EC_{50} values of various adrenergic agonists to stimulate adenylate cyclase, as well as the IC_{50} values of antagonists to inhibit isoproterenol-stimulated adenylate cyclase activity shown in Table 2 are consistent with a β_2-adrenergic stimulation of the adenylate

TABLE 1

[^{125}I]CYP binding activity in nontransfected and transfected CHW cells

The data are expressed as the mean ± standard error.

Cell line	n	B_{max}
		pmol/mg
CHW	3	0.042 ± 0.007
CTF-40	4	0.084 ± 0.023
CTF-17	5	0.43 ± 0.09
CTF-33	4	0.97 ± 0.08
CTF-39	5	2.93 ± 0.64
CTF-31	13	3.18 ± 0.34
CTF-23	14	3.55 ± 0.43
CTF-21	7	5.08 ± 0.76
CTF-37	3	8.39 ± 1.45
CTF-36	14	8.42 ± 1.28
A431	6	1.25 ± 0.20

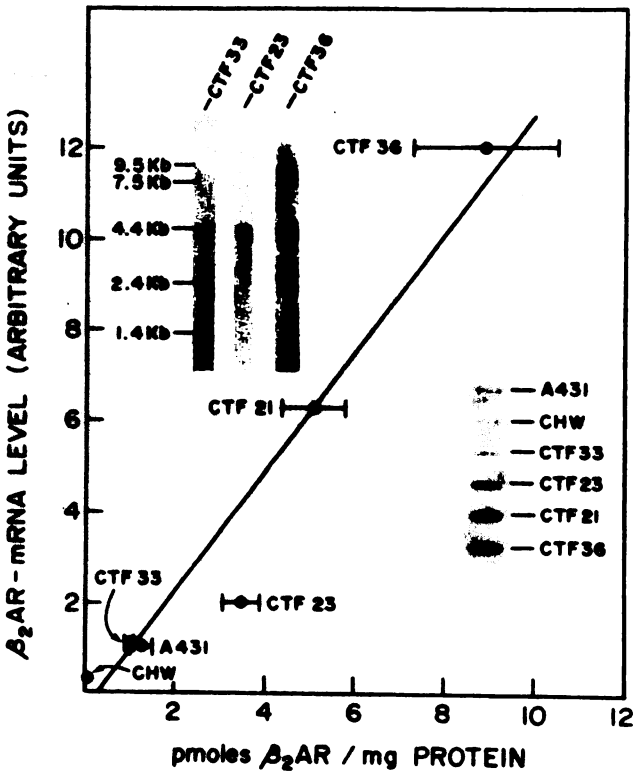

Fig. 1. Correlation between the level of β_2-AR mRNA and the β_2-AR membrane density in several CTF cell lines, CHW cells, and A431 cells. The mRNA levels were quantitated by slot blot analysis as described under Experimental Procedures and a representative slot blot is shown in the (*lower right*) *inset*. The density of membrane β_2-AR was assessed by [^{125}I]CYP binding and the values shown are means ± standard errors of 3 to 14 independent determinations. The linear regression is described by: $y = 1.33x + 0.68$ and $r = 0.97$. A representative Northern blot of three of the clones (20 μg total RNA/lane) is shown in the *upper left inset*.

TABLE 2

Stimulation of adenylate cyclase activity by various agonists (EC_{50}) and inhibition of adenylate cyclase activity by various antagonists (IC_{50})

The data are representative of at least three experiments. The binding competition curves were obtained using 81 pM [^{125}I]CYP. The dose response curves for the inhibition of stimulated adenylate cyclase by various antagonists were obtained using 0.2 μM isoproterenol to stimulate the adenylate cyclase.

	[^{125}I]CYP binding competition	Adenylate cyclase activation
	K_i	EC_{50}
	μM	μM
Agonists		
(−)-Isoproterenol	0.80	0.13
(−)-Epinephrine	2.0	0.72
(−)-Norepinephrine	35	7.1
(+)-Isoproterenol	59	17
	K_i	IC_{50}
	nM	nM
Alprenolol	0.78	8.8
ICI 118551	2.3	19
Betaxolol	600	3600

cyclase system (17). The effective coupling of the transfected human β_2-AR with the hamster adenylate cyclase through endogenous G_s is further evidenced by a guanine nucleotide-sensitive high affinity state of the receptor for agonists observed in competition binding experiments (data not shown).

Role of receptor number in determining signaling efficacy. As seen in Fig. 2, the level of expression of the receptors directly influences the maximum obtainable stimulation of adenylate cyclase by isoproterenol. Maximum isoproterenol-stimulated adenylate cyclase activity increases sharply with the number of β_2-ARs expressed in different CTF clones until receptor concentrations of ~3.0 pmol/mg of membrane protein are reached. Above this receptor concentration, maximal isoproterenol stimulation of adenylate cyclase plateaus and then decreases at receptor densities above ~8.0 pmol/mg of membrane protein. Reduction of maximal isoproterenol-stimulated adenylate cyclase activity was statistically significant ($p < 0.05$) in comparison with the maximal activity observed in cells expressing between 3 and 5 pmol of β-AR/mg of membrane protein. These results suggest that signal transduction through the adenylate cyclase system is optimal in these cells at a receptor concentration of 3.0–5.0 pmol/mg of membrane protein. Above this receptor concentration, however, the efficacy of isoproterenol is apparently reduced. Such a pattern was observed in all the experiments performed. Interestingly, maximal isoproterenol-stimulated adenylate cyclase activity in A431 cells falls reasonably well on the curve relating β_2-AR number to enzyme stimulation in the CTF clones (Fig. 2, *open circle*). It should be noted that A431 cells express the highest number of β-ARs of any naturally occurring cell.

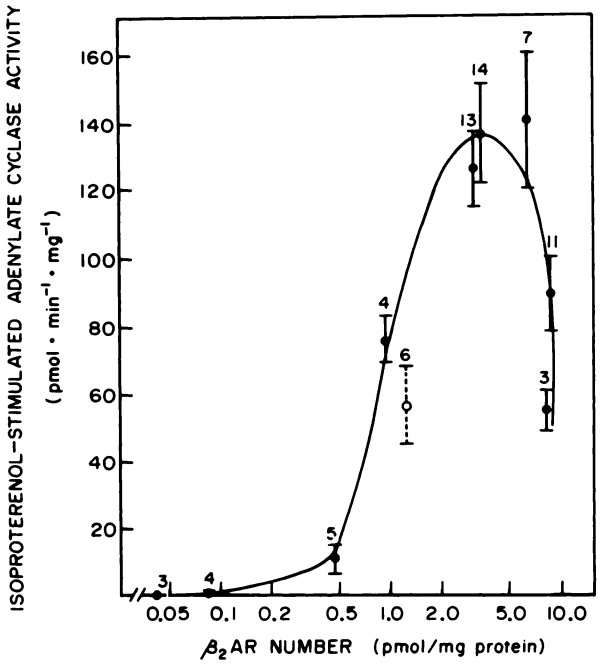

Fig. 2. Relationship between expressed β_2-AR density and the extent of agonist stimulation of adenylate cyclase. Each data point represents the mean ± standard error of adenylate cyclase activity obtained in 3 to 14 separate determinations for individual CTF cell lines. O, the value obtained for the human epidermoid carcinoma A431 cell line. The β_2-AR concentration was determined by [^{125}I]CYP binding to membranes as described under Experimental Procedures and expressed on a logarithmic scale. The *numbers* above the standard error *bars* indicate the number of experiments.

In contrast with the marked (i.e., ~200-fold) variations in the level of isoproterenol stimulation observed with variations in β_2-AR number, only modest (~2-fold) variations in other parameters of adenylate cyclase function such as basal, sodium fluoride- and forskolin-stimulated activities were observed. Interestingly, these variations followed the same pattern as that observed for isoproterenol-stimulated adenylate cyclase activity (data not shown).

Although isoproterenol had reduced efficacy in stimulating adenylate cyclase activity in cells expressing large numbers of β_2-ARs (e.g., CTF-36 and CTF-37), the apparent affinity of this agonist was actually greater in these cells (Fig. 3). The EC_{50} values for isoproterenol stimulation of adenylate cyclase were shifted to the left in the "high expressors" as compared to the values obtained with cells expressing ~3.5 (CTF-23) or ~1.0 (CTF-33) pmol/mg of membrane protein [52 ± 15 nM ($n = 4$) for the "high expressors," 120 ± 20 nM ($n = 8$) for CTF-23, 170 ± 20 nM ($n = 9$) for CTF-33]. This finding is consistent with the presence of a receptor contingent in excess of that required for maximal stimulation of the adenylate cyclase or "spare receptors."

Several hypotheses were considered that might account for the apparent reduction in maximal agonist-stimulated adenylate cyclase activity in cells expressing the greatest numbers of β_2-ARs. First, catecholamines that might be present in the culture media could induce tonic desensitization in the cells. However, the reduced efficacy of isoproterenol to stimulate adenylate cyclase in "high expressors" was not significantly altered by growing these cells in the presence of the β-adrenergic antagonist sotalol, at 1.0 μM, for a period of up to 6 days (data not shown). Second, to test the possibility that expression of the high number of β_2-ARs might result in an increased interaction with the inhibitory guanine nucleotide-regulatory protein (G_i), thus reducing the maximum adenylate cyclase stimulation, the effects of pertussis toxin were studied. A 24-hr incubation with the toxin (16 μg/flask) led to an increase in the maximum level of isoproterenol-stimulated adenylate cyclase in every clone studied (CTF-33, CTF-23, CTF-36). However, the treatment did not restore the full efficacy of isoproterenol to stimulate the enzyme in "high expressors" (data not

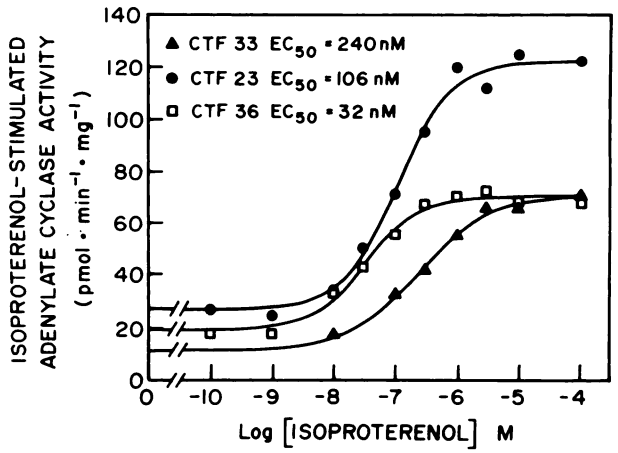

Fig. 3. Dose response curves for the ability of (−)-isoproterenol to stimulate adenylate cyclase activity in membranes from three CTF clonal cells, CTF-23, CTF-33, and CTF-36. The adenylate cyclase activity was measured in the membranes as described under Experimental Procedures and expressed as pmol of cAMP produced/min × mg of protein. The data were analyzed using nonlinear least squares regression (7).

shown). Thus, increased coupling of β_2-AR to G_i does not appear to adequately account for the decreased maximum stimulation of adenylate cyclase by isoproterenol in these clones.

Expressed β_2-AR mediates regulation of the adenylate cyclase signal-transducing system. As shown in Fig. 4A, the expressed human β_2-AR can mediate agonist-induced desensitization of adenylate cyclase. Preincubation of cells expressing between 2.9 and 3.6 pmol of β_2-ARs/mg of membrane protein (CTF-23, CTF-31, CTF-39) with isoproterenol (100 μM) for either 15 or 120 min induced significant ($p < 0.05$) desensitization to further stimulation by isoproterenol. In addition, the ability of sodium fluoride to stimulate adenylate cyclase activity was reduced following a 15-min preincubation, becoming significant ($p < 0.05$) following a 120-min preincubation with isoproterenol. Slight reductions in forskolin-stimulated adenylate cyclase activity were also observed but did not reach statistical significance following isoproterenol treatment.

The concentration of membrane-bound β_2-AR, as assessed by [^{125}I]CYP binding, was significantly reduced ($p < 0.05$) following 15 or 120 min preincubation with isoproterenol (Fig. 4B). The number of receptors present at the cell surface, as defined by the binding of the hydrophilic ligand [^{3}H]CGP 12177 to whole cells, was also reduced by ~30% following agonist pretreatment (data not shown). Similar desensitization patterns were observed when CTF cell lines expressing lower or higher numbers of receptors than those described above were preincubated with isoproterenol.

Phosphorylation of the expressed β_2-AR. As shown in Fig. 5A, incubation of CTF-23 cells with 100 μM isoproterenol for 15 min leads to phosphorylation of the expressed human β_2-AR, as previously shown for the normally expressed mammalian β_2-AR (18). However, the affinity-purified phosphorylated receptor migrates on SDS-polyacrylamide gels with a different mobility than the β_2-AR purified from hamster lung and phosphorylated with the β-AR kinase (Fig. 5, lane 2-3 versus lane 1). The human receptor that underwent phosphorylation in the transfected (CTF-23) cells migrated as a broader band with an M_r of ~80,000. Accordingly, photolabeling experiments were performed with CTF-23 cell membranes to confirm that the human β-AR expressed in these cells, in fact, migrates during SDS-PAGE as a broad band with an M_r of ~80,000. These results are shown in Fig. 5B. A specifically labeled band of M_r ~80,000 is observed following binding and photoincorporation of [^{125}I]CYP diazarine into a CTF-23-derived membrane preparation. To assess whether the mobility of the expressed human β_2-AR during electrophoresis could result from a different glycosylation state of the protein, the phosphorylated receptor was subjected to Endo-B-N-acetylglucosaminidase F (Endo F) treatment. As shown in Fig. 5C, incubation of the receptor, phosphorylated *in vivo* under conditions of agonist-induced desensitization, with Endo F generates an M_r ~45,000 form of the receptor as well as an intermediary form of M_r ~64,000. Similarly, treatment of the phosphorylated β-AR, purified from hamster lung, with Endo F also gives rise to an M_r ~45,000 form of the receptor believed to represent the deglycosylated hamster β_2-AR (19, 29). Cleavage of the N-linked sugar moieties of both the human β_2-AR expressed in CHW cells and the hamster lung β_2-AR by Endo F treatment therefore generates a peptide core of identical relative mobility (~45,000) on SDS-polyacrylamide gel. Thus, the different apparent molecular weights of the human β_2-ARs expressed in CTF-23 appear to be the consequence of different levels of glycosylation of the receptor.

Discussion

A human cDNA encoding the β_2-AR has been transfected and expressed at varying levels in CHW cells. In agreement with Strader and co-workers (21), who have quite recently reported the expression of this cDNA in COS-7 cells, we document the β_2 nature of the receptor encoded by this cDNA and its ability to stimulate the adenylate cyclase. The major emphasis of the present work has been to assess the relationship between the concentration of β_2-ARs expressed in the cells and their functional properties. Thus, the different levels of expression of the β_2-ARs in these CHW cells has provided a unique way to directly address the relationship between the number of membrane-bound β_2-ARs and isoproterenol-sensitive adenylate cyclase activity in a particular cell line. Previously, indirect approaches have been used to surmise the relationship between β_2-AR expression and hormonal responsiveness. These have included irreversible blockade of receptors (22, 23) or studies of the cell cycle dependence of receptor density (23, 24) and adenylate cyclase hormonal sensitivity. The present study in CHW cells directly demonstrates that the adrenergic-sensitive adenylate cyclase activity is proportional to the β_2-AR expression up to a very high membrane concentration of β_2-AR (e.g.,

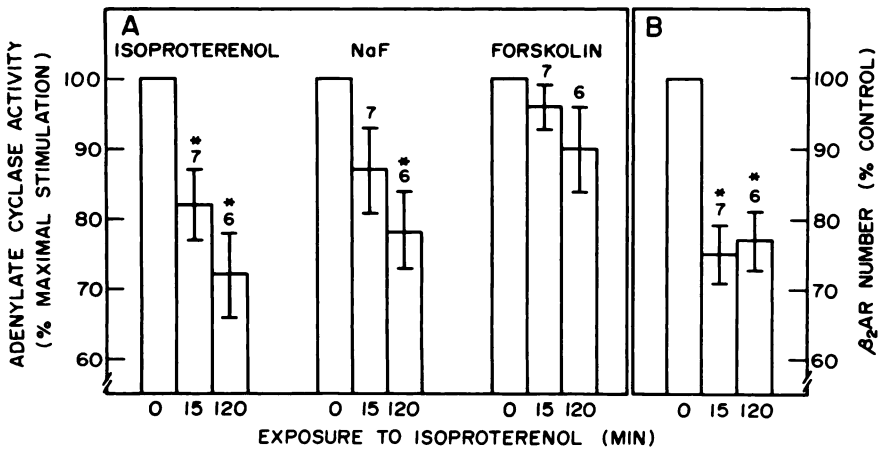

Fig. 4. Effect of exposure to isoproterenol on CTF-23 membrane adenylate cyclase activity and β_2-AR number. A. Cells were incubated for the time period shown. Isoproterenol-, NaF-, and forskolin-stimulated adenylate cyclase activities were measured as described under Experimental Procedures in membranes derived from control CTF-23 cells (0 min) as well as from cells exposed to isoproterenol (100 μM) for 15 or 120 min. The data are expressed as the mean ± standard error in percentage of the maximal stimulation observed in membranes derived from control cells. B. β_2-AR concentrations in membranes derived from CTF-23 cells exposed to isoproterenol for various periods of time were assessed by [^{125}I]CYP binding to membranes, as described under Experimental Procedures. The data are expressed as the mean ± standard error in percentage of the β_2-AR concentration observed in the membranes derived from control cells. *, $p < 0.05$. The *numbers* above the standard error *bars* indicate the number of experiments.

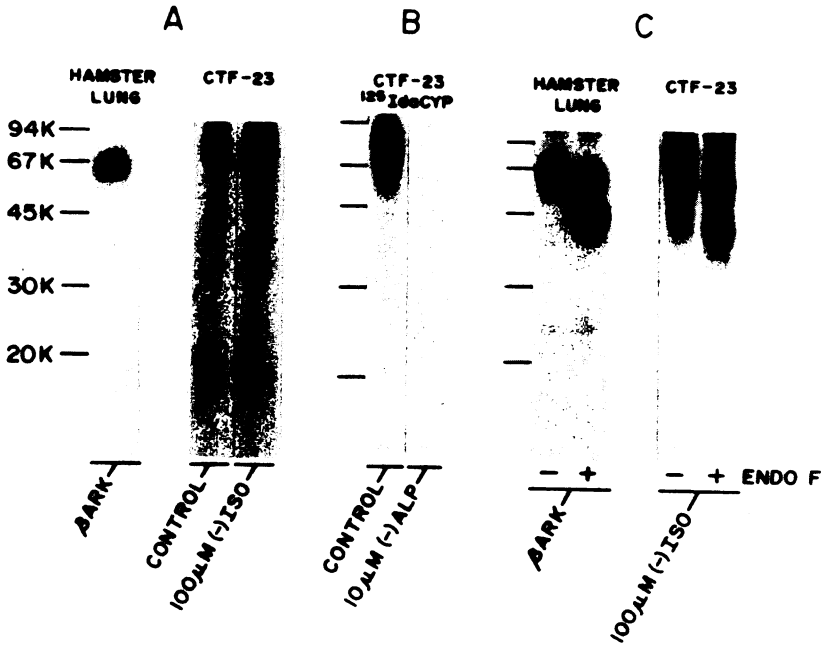

Fig. 5. Whole cell phosphorylation of the β_2-AR during agonist-induced desensitization in CTF-23 cells and photoaffinity labeling of the β_2-AR in CTF-23-derived membranes. A. The first lane is an autoradiogram of the β-AR purified from hamster lung and phosphorylated *in vitro* by the β_2-AR kinase (βARK) in the presence of [γ-^{32} P]ATP. The second and third lanes are autoradiograms of affinity-purified β_2-AR from CTF-23 cells prelabeled with carrier-free ^{32} P_i and exposed (third lane) or not to isoproterenol [(−)ISO] (100 μM) for 15 min (second lane), as described under Experimental Procedures. B. Photoaffinity labeling of membranes prepared from CTF-23 cells using [^{125} I]CYP diazerine in the absence (first lane) or presence (second lane) of 10 μM alprenolol [(−)ALP] as described under Experimental Procedures. C. Endo F treatment of phosphorylated β_2-AR. In the first and second lanes, the hamster lung affinity-purified β_2-AR phosphorylated by the β-AR kinase was treated (+) or not (−) with Endo F as described under Experimental Procedures. In the third and fourth lanes the β_2-AR phosphorylated during desensitization in CTF-23 cells and purified by affinity chromatography was treated (+) or not (−) with Endo F.

3.0 pmol/mg of protein). It should be noted that 3.0 pmol/mg already represents a β_2-AR concentration considerably higher (~3-fold) than the concentration of β_2-ARs found in A431 cells, which contain the highest density of β_2-ARs in a cultured cell line. It therefore appears that until a very high concentration of receptor is reached, the isoproterenol-sensitive adenylate cyclase activity of a cellular system is limited by the number of β_2-ARs expressed. Previous studies have suggested that no "spare" β_2-ARs are present in turkey erythrocytes, (25) S49 murine lymphoma cells (23), or C_6 rat glioma cells (22, 26). More directly, Johnson *et al.* (27), by selecting β_2-AR-deficient variants of the S49 lymphoma cell line, showed that a diminished β_2-AR number is associated in these cells with a proportional reduction of the isoproterenol-sensitive adenylate cyclase activity. However, at β_2-AR levels between 3 and 5 pmol/mg, it is likely that other components of the systems, such as G_s or the cyclase moiety itself, become the limiting factor and, thus, the isoproterenol-sensitive adenylate cyclase activity reaches a plateau.

In cells expressing very high numbers of β_2-ARs (~8 pmol/mg), a significant decrease in adenylate cyclase stimulation was observed. This blunting of the responsiveness of the system appears to be unrelated to serum catecholamine-induced desensitization, as has been shown to occur in S49 (28) and C_6 cells (29), since growing the cells in the presence of a β-adrenergic blocker did not prevent the phenomenon. Coupling of the "excess" receptors through G_i also appears unlikely since treatment with pertussis toxin did not restore the maximal isoproterenol-sensitive adenylate cyclase activity.

The present study also shows that, with the cDNA construct used, the number of receptors functionally expressed in the membranes of the CTF cells is directly proportional to the level of β_2-AR mRNA found in these cells. Thus, it appears that post-translational modifications do not limit the full expression of the receptor in this system.

Exposure of the CHW cells expressing the human β_2-AR to the agonist isoproterenol leads to desensitization of the isoproterenol- and sodium fluoride-sensitive adenylate cyclase activity. The observation that sodium fluoride sensitivity was also affected by preincubation with isoproterenol for 120 min suggests a broad pattern of desensitization of the system, similar to that previously observed in the heterologous form of desensitization (3). As observed in cells normally expressing β_2-AR (3, 30), the desensitization process is accompanied by an increase in the phosphate content of the β_2-AR. Phosphorylation of the receptor by cAMP-dependent protein kinase, as well as by β-AR kinase (30), has been associated with the heterologous and homologous forms of desensitization, respectively. The nature of the kinase(s) responsible for phosphorylation of β_2-AR during desensitization in these cells remains unknown. However, it is noteworthy that β-AR kinase activity was demonstrated in these cells (data not shown).

Whether the decrease in cell surface β_2-AR observed in desensitized cells reflects sequestration of the receptor, as is classically associated with the homologous form of desensitization, or down-regulation of the receptor, which is generally associated with longer exposure of the cells to agonist (3), remains to be determined. The observation that this decrease in cell surface β_2-AR is also seen in the membranes prepared from desensitized cells favors the latter possibility. This cellular transfection system therefore offers a powerful approach to study the molecular basis of β_2-AR regulation using site-directed mutagensis of the human β_2-AR cDNA.

The apparent molecular weight of the human β_2-AR expressed in the CHW cells is different from that generally reported for mammalian β_2-AR. The receptor either photolabeled in membranes or affinity-purified following phosphorylation was found to migrate as a broad band of M_r ~80,000. This is significantly larger than is observed for the β_2-AR purified from hamster lung membranes (M_r ~64,000). The observation that Endo F treatment of both the hamster lung β_2-AR and the human β_2-AR-expressed CTF-23 cells generates a protein with M_r ~45,000 suggests that a difference in the carbohydrate chains linked to the receptor is responsible for the altered

electrophoretic mobility. Indeed, an M_r ~45,000 has been previously observed for the deglycosylated mammalian β_2-AR (19). Moreover, heterogeneous patterns of glycosylation have been previously shown to account for differences in electrophoretic mobility of the rat erythrocyte β_2-AR (20).

In conclusion, we have described a cellular expression system for the human β_2-AR and characterized the relationship between the receptor number expressed and β-adrenergic agonist-sensitive adenylate cyclase in this system. Although "spare" receptors were undetectable up to a concentration of 3 pmol of β_2-AR/mg of protein, very high densities of β_2-AR (e.g., ~8.0 pmol/mg of protein) are associated with a nonspecific desensitization of the isoproterenol-sensitive adenylate cyclase. In addition, agonist-induced desensitization of β_2-AR-coupled adenylate cyclase could be demonstrated in this expression system and was associated with phosphorylation of the β_2-AR.

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