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Inducible Expression of β_1- and β_2-Adrenergic Receptors in Rat C_6 Glioma Cells: Functional Interactions between Closely Related Subtypes

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SUMMARY

We examined the role of β_1- and β_2-adrenergic receptor (AR) density and ratio in catecholamine-stimulated cAMP responses in rat C_6 glioma cells. These cells, which normally express both subtypes, were stably transfected with an isopropylthio-β-D-galactoside-inducible vector containing either β_1AR or β_2AR coding sequences, and receptor expression was controlled by the time and concentration of isopropylthio-β-D-galactoside exposure. Induction of the dominant β_1AR subtype increased the potencies of isoproterenol (ISO) and other agonists in stimulating cAMP accumulation by 20–40-fold without changing maximal response. Induction of β_2AR expression caused 7–13-fold increases in the potency of ISO, epinephrine, and zinterol, but not of norepinephrine, and a 20–40% loss in maximal response to all agonists. Selective antagonists showed that both subtypes contributed in a nonadditive manner in the response to ISO under different conditions. After β_2AR induction, the effects of ISO were not blocked by the β_1-selective antagonist CGP 20712A but were shifted 100-fold to the right by the β_2-selective antagonist ICI 118,551. However, in the presence of ICI 118,551, CGP 20712A caused an additional 100-fold decrease in ISO potency, and Schild analysis revealed complex interactions between the two subtypes. Each antagonist alone caused smaller shifts to the right in the dose-response curve to NE and, when present simultaneously, completely abolished the NE response. We conclude that β_1ARs and β_2ARs have different efficiencies in activating cAMP accumulation in C_6 glioma cells. Activation of coexisting subtypes results in complex and sometimes synergistic interactions between the two subtypes, which vary with agonist concentration, selectivity, subtype density, and ratio.

At least nine AR subtypes have been identified through the use of pharmacological and molecular cloning techniques (1–3). They are grouped into three families (α_1, α_2, and β), each containing at least three members. Three closely related subtypes (β_1, β_2, and β_3) have been identified in the βAR family, all of which couple to activation of adenylate cyclase through G_s (4). Relatively little is known about the functional role of multiple receptor subtypes that respond to the same neurotransmitters and couple to the same signaling mechanism. More interestingly, these receptor subtypes often coexist within tissues (5, 6) and on the same cells (7, 8) and can be linked to converging, redundant, or synergistic functional responses (5–9). Their individual actions and interactions have not yet been clearly elucidated.

It is not yet known whether closely related subtypes exhibit different efficiencies in activating the same signaling mechanism. Human β_2ARs have been suggested to couple more efficiently to G_s/adenylate cyclase than do human β_1ARs in transfected (10, 11) and normal (8) cells; however, the contributions of coexisting subtypes to catecholamine stimulation have not been examined. In addition, we have shown that although both coexisting β_1ARs and β_2ARs on rat C_6 glioma cells can contribute to cAMP responses, responses to catecholamines are mediated primarily by the β_1 subtype, even after glucocorticoid treatment, when β_2ARs are numerically dominant (12). Whether such differences are related to species variations, cellular machinery, or other factors is not known.

To help clarify the functional roles and interactions of β_1- and β_2ARs, we used inducible vectors to directly control the expression of each subtype in C_6 cells. This approach allowed us to modify the density of each subtype independent of other changes in cellular physiology, to directly compare the relationship between receptor density and tissue response for each subtype in the same cells, and to identify interactions between subtypes. It also allowed us to begin to define the role of receptor density and subtype ratio in responses to catecholamines.

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ABBREVIATIONS: AR, adrenergic receptor; ISO, isoproterenol; EPI, epinephrine; NE, norepinephrine; ZINT, zinterol; CGP, CGP 20712A; ICI, ICI 118,551; CYP, cyanopindolol; PBS, phosphate-buffered saline; IPTG, isopropylthio-β-D-galactoside.

Experimental Procedures

Materials. Rat C_6 glioma cells were purchased from the American Type Culture Collection (Rockville, MD). Other materials were obtained from the following sources: Dulbecco's modified Eagle's medium, fetal bovine serum, geneticin, and trypsin-EDTA (GIBCO-BRL, Grand Island, NY), hygromycin B (Boehringer Mannheim Biochemicals, Indianapolis, IN), [^{3}H]adenine (~25 Ci/mmol) (DuPont, Boston, MA), [α-^{32}P]dCTP (~10 mCi/ml), (Amersham, Arlington Heights, IL), ICI (Cambridge Research Biochemicals, Macclesfield, UK), (−)NE, (−)ISO, (−)EPI, cAMP, and other chemicals (Sigma Chemical Co., St. Louis, MO), and LacSwitch inducible vector system (Stratagene, La Jolla, CA). ZINT was kindly donated by Mead Johnson Pharmaceuticals (Evansville, IN). CGP was kindly donated by Ciba-Geigy (Summit, NJ). The rat β_1AR cDNA (13) was kindly provided by Dr. Curtis A. Machida (Oregon Regional Primate Research Center, Beaverton, OR). The rat β_2AR cDNA (14) was kindly provided by Dr. Claire M. Fraser (The Institute For Genomic Research, Gaithersburg, MD). A rat genomic library (in Lambda DASH^{II}; Stratagene, La Jolla, CA) was kindly provided by Dr. T. J. Murphy (Department of Pharmacology, Emory University, Atlanta, GA).

Cell culture. C_6 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 20 mg/liter streptomycin, and 20 mg/liter penicillin in a humidified atmosphere containing 7% CO_2 at 37° (12). Cells were grown to confluency, and the medium was changed every 2–3 days. Confluent cells were subcultured at a ratio of 1:4 into Primaria flasks. For cAMP assays, two drops of cells (10^4 cells/ml) were plated onto each well of a 96-well Primaria dish and grown to confluency. For radioligand binding assays, 10^5 cells (in 10 ml of medium) were plated onto 10-cm culture plates and grown to confluency.

cAMP accumulation. cAMP accumulation was measured according to the [^{3}H]adenine prelabeling technique (15). Briefly, confluent cells in 96-well Primaria plates were prelabeled with [^{3}H]adenine (2 μCi/100 μl well) for 2 hr. Culture medium was then removed through aspiration, and cells were washed three times by submerging plates in 500 ml of Krebs-Ringer-bicarbonate buffer at 37°. Drugs were added in 200 μl of Krebs-Ringer-bicarbonate buffer, and cells were incubated at 37° for 10 min. In most experiments, no inhibitors of 3′,5′ cyclic nucleotide phosphodiesterase were included in the assay medium. At the end of the incubation, 20 μl of 77% trichloroacetic acid was added to terminate the reaction, and 10 μl of unlabeled cAMP (5 mM) was added as a carrier. Cells were sonicated for 10 sec. A 10-μl aliquot of the reaction mixture was taken for determination of the incorporation of [^{3}H]adenine, and [^{3}H]cAMP was isolated from the remainder of the supernatant through sequential DOWEX and alumina chromatography (16). Results are expressed as a percentage of the conversion of incorporated tritium to [^{3}H]cAMP. When cells were induced, IPTG was added to the culture medium for the indicated time before cAMP assays were performed as described above.

Radioligand binding assay. Binding of ^{125}I-CYP to C_6 cell membranes was performed as described previously (12). Briefly, confluent cells were washed twice in PBS (20 mM $NaPO_4$, 154 mM NaCl, pH 7.6); harvested; and homogenized in PBS. Homogenates were centrifuged at 30,000 × *g* for 10 min, and pellets were resuspended in PBS (1 ml/confluent plate of untransfected cells). Membranes (0.1 ml) were incubated with ^{125}I-CYP in a final volume of 0.25 ml of PBS at 37° for 1 hr with or without competing drugs. For Scatchard analysis, increasing concentrations (maximal, 300 pM) of ^{125}I-CYP were used. For single-point determinations, 300,000 cpm ^{125}I-CYP (300 pM) was used. At the end of the incubation, the binding was terminated with the addition of 10 ml 10 mM Tris·HCl, pH 7.4, and filtered through glass-fiber filters (#30, Schleicher and Schuell) under a vacuum. Filters were washed with an additional 10 ml buffer, dried, and counted with a γ counter. Total βAR binding was defined as the difference between binding in the absence of drug and in the presence of 50 μM ISO. β_1AR binding was calculated as the difference between binding in the absence of drug and in the presence of CGP (500 nM). β_2AR binding was calculated as the difference between binding in the presence of CGP (500 nM) and in the presence of 50 μM ISO.

Cloning of the rat β_2AR gene and construction of expression vectors. Rat β_1AR cDNA (13) was kindly provided by Dr. Curtis Machida and ligated into the LacSwitch (Stratagene, La Jolla, CA) operator vector (pRSVNot). To study the β_2AR, Dr. Claire Fraser kindly provided the rat β_2AR cDNA (14). Unfortunately, this clone did not express in any of the many vectors or cells we studied, probably due to problems we found in the sequence and restriction map of the 5′ end. Because the rat β_2 genomic clone isolated by Buckland *et al.* (17) had been discarded, we recloned this sequence from a rat genomic library in Lambda DashII by high stringency hybridization to a *Kpn*I fragment of Dr. Fraser's rat β_2AR cDNA. A single *Hin*dIII/*Eco*RI 2.5-kb band was isolated and ligated into pBluescript. Insertion of a *Hin*dIII/*Xba*I 2.1-kb fragment (containing ~100 bp 5′ untranslated region and the entire coding sequence) into the lac operator vector resulted in large increases in β_2AR binding sites in transiently transfected COS-7 and C_6 cells.

Isolation of C_6 clones with low constitutive and highly inducible receptor expression. C_6 cells were cotransfected with 10 μg each of two plasmids, the p3′SS repressor vector and the pRSVNot operator vector, containing β_1AR or β_2AR coding sequences. Transfections were performed through calcium phosphate precipitation. Cells that stably integrated the two vectors were selected by their ability to grow in the presence of 400 μg/ml geneticin and 400 μg/ml hygromycin. C_6 cells were subcloned through limited dilution and expanded in the selection medium. Subclones were tested for low constitutive and high inducible receptor expression by a radioligand binding assay with ^{125}I-CYP. Basal and induced receptor expression remained stable in particular subclones up to 10 passages over a period of several months (data not shown).

Data analysis. Concentration-response curves were analyzed with the use of nonlinear regression analysis, and the best two-site fit was compared with the best one-site fit by minimizing the sum of squares of errors. Data are given as mean ± standard error. Statistical comparisons were made with a Student's *t* test.

Results

Induction of β_1AR expression by IPTG in β_1-transfected cells. Treatment of β_1-transfected C_6 cells with IPTG caused concentration- and time-dependent increases in specific ^{125}I-CYP binding sites (Fig. 1). β_1AR density was determined as the difference between total binding and binding in the presence of the β_1-selective antagonist CGP (500 nM), and β_2AR density was determined as as the difference between binding in the presence of CGP and binding in the presence of the nonselective agonist ISO (50 μM). Fig. 1 shows that IPTG selectively increased β_1AR expression by ~20-fold with an EC_{50} of 10 μM and a $t_{1/2}$ of ~10 hr. No change in K_D for ^{125}I-CYP was observed after IPTG induction (data not shown). Little further rise in receptor expression was observed after 24 hr; however, expression levels remained elevated up to 5 days after IPTG exposure (data not shown). This massive increase in β_1AR expression did not cause a significant change in expression of β_2ARs (Fig. 1), although this was difficult to quantify because the small β_2AR population became almost unmeasurable relative to the overexpressed β_1ARs.

Induction of β_1ARs: Effect on catecholamine responsiveness. cAMP accumulation stimulated by the nonselective βAR agonist ISO was measured at various times after

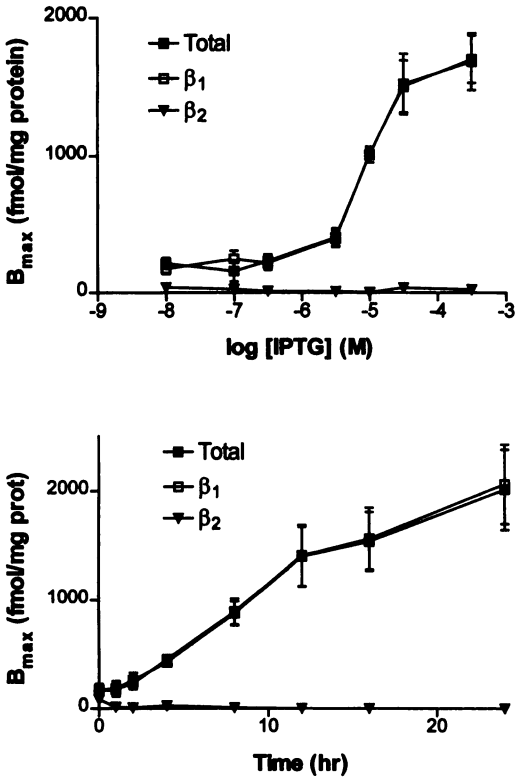

Fig. 1. Concentration and time dependence of IPTG induction of β_1AR expression in β_1-transfected C_6 cells. Total βAR density was determined through Scatchard analysis of specific ^{125}I-CYP binding. β_1AR and β_2AR densities were determined as described in Experimental Procedures. Cells were induced for 24 hr with various concentrations of IPTG (*top*) or with 1 mM IPTG for the indicated time period (*bottom*). Each value is mean ± standard error of data from three experiments performed in duplicate.

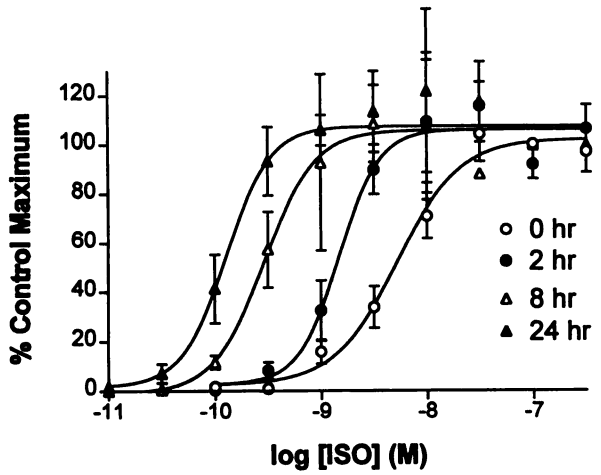

Fig. 2. ISO stimulated cAMP accumulation after various induction times in β_1-transfected C_6 cells. β_1AR-transfected cells were induced with 1 mM IPTG for the indicated time, and ISO-stimulated cAMP accumulation was measured. Data are expressed as a percentage of the maximal response in noninduced (0 hr, *Control*) cells. Each value is mean ± standard error of data from three experiments performed in duplicate. In cells not exposed to IPTG, basal and maximal ISO-stimulated cAMP accumulations were 0.07 ± 0.01% and 6.0 ± 0.58% conversion, respectively. Responses to 300 nM ISO were 106 ± 9.7%, 88 ± 1.6%, and 100 ± 0.4% of the control response at 2-, 8-, and 24-hr exposure to IPTG, respectively.

induction of β_1AR expression. As shown in Fig. 2, time-dependent induction of β_1AR expression caused corresponding increases in the potency of ISO, with no significant effect on the maximal response. A maximal 40-fold increase in potency was observed after 24 hr IPTG exposure.

The rank order of potencies of catecholamines in stimulating cAMP responses in β_1-transfected cells before and after induction with 1 mM IPTG for 24 hr is shown in Table 1. Not surprisingly, a potency order characteristic of β_1ARs (ISO > NE ≥ EPI) was observed both before and after β_1AR induction. Exposure to IPTG (1 mM, 24 hr) caused a similar 20–40-fold increase in the potencies of all three agonists, with no significant changes in maximal response (Table 1).

Induction of β_2AR expression by IPTG in β_2-transfected cells. Treatment of β_2-transfected C_6 cells with 1 mM IPTG for 24 hr caused a 3–4-fold increase in specific ^{125}I-CYP binding sites with no change in apparent K_D (Fig. 3). With CGP (500 nM) to selectively block β_1ARs, IPTG caused a 7–8-fold increase in β_2AR density with no significant change in β_1AR density (Fig. 3). The increase in β_2AR density in β_2-transfected cells was dependent on IPTG concentration and exposure time, with EC_{50} and $t_{1/2}$ values similar to those observed in β_1-transfected cells (Fig. 4). Again, expression levels became essentially maximal after 24 hr and remained elevated for up to five days after IPTG exposure (data not shown).

Induction of β_2ARs: Effect on ISO response. The effect of progressive induction of β_2AR expression on ISO-stimulated cAMP accumulation in C_6 cells is shown in Fig. 5 (*top*). Increases in β_2AR density initially caused little change in the potency of ISO. However, ISO potency increased 7-fold after 24 hr of induction. Surprisingly, this induction also caused a gradual decline in the maximal response to ISO, with a 40% decrease in maximal response 24 hr after induction (Fig. 5 and Table 2). On the other hand, IPTG exposure (1 mM, 24 hr) did not alter the potency or maximal effect of forskolin in stimulating cAMP accumulation in β_2-transfected C_6 cells (Fig. 5, *bottom*). The decline in maximum response to ISO after β_2AR induction occurred at all time points examined (Fig. 6). In addition, inhibition of cAMP breakdown with isobutylmethylxanthine (2 mM) caused large and variable increases in both basal and ISO-stimulated cAMP levels but did not reverse the decrease in maximum response observed after β_2AR induction (data not shown).

Relationship between β_1AR and β_2AR density and the EC_{50} and maximum response to ISO. The relationship between β_1AR and β_2AR density and the potency and maximal response for ISO in stimulating cAMP accumulation in transfected C_6 cells after various induction times are shown in Fig. 7. The log of receptor density (B_{max}) is plotted against the $-\log EC_{50}$ or percentage of maximal uninduced response (18) for each subtype, calculated from the data in Figs. 1, 2, 4, and 5. Linear relationships were observed between log B_{max} and $-\log EC_{50}$ values for both β_1ARs and β_2ARs, although with different slopes. Progressive increases in β_1AR density had no effect on the maximum response to ISO, whereas increases in β_2AR density were associated with a progressive decrease in the maximum ISO response.

Induction of β_2ARs: Effect on responses to subtype-selective agonists. As shown in Fig. 8, induction of β_2AR expression in cells transfected with that subtype (1 mM IPTG, 24 hr) caused 10–13-fold increases in the potencies of EPI and the β_2-selective agonist ZINT (in presence of 500 nM CGP

TABLE 1

Responses to agonists in transfected C_6 cells

Cells were treated with (+IPTG) or without (−IPTG) 1 mM IPTG for 24 hr before agonist concentration-response curves were determined. Curves were analyzed by nonlinear regression to determine EC_{50} values and maximum responses.

	β_1 Transfected					β_2 Transfected				
	−log EC_{50}		-fold shift	Control maximum		−log EC_{50}		-fold shift	Control maximum	
	−IPTG	+IPTG		−IPTG	+IPTG	−IPTG	+IPTG		−IPTG	+IPTG
	M			%		M			%	
ISO	8.28 ± 0.28	9.89 ± 0.11[b]	40	100	117 ± 4.7	8.52 ± 0.03	9.35 ± 0.23[a]	7	100	60 ± 3.6[b]
EPI	7.13 ± 0.09	8.47 ± 0.23[b]	22	101 ± 5.3	96 ± 8.0	7.28 ± 0.07	8.39 ± 0.13[b]	13	100 ± 2.6	65 ± 3.6[b]
NE	7.46 ± 0.03	8.94 ± 0.31[b]	30	101 ± 1.4	112 ± 14	7.04 ± 0.8	7.13 ± 0.09	1.2	105 ± 3.5	71 ± 2.9[b]
ZINT	ND[c]	ND		ND	ND	8.43 ± 0.07	9.41 ± 0.05[b]	10	95 ± 2.7	79 ± 1.7[b]

[a] $p < 0.05$ compared with −IPTG.
[b] $p < 0.01$.
[c] ND, not determined.

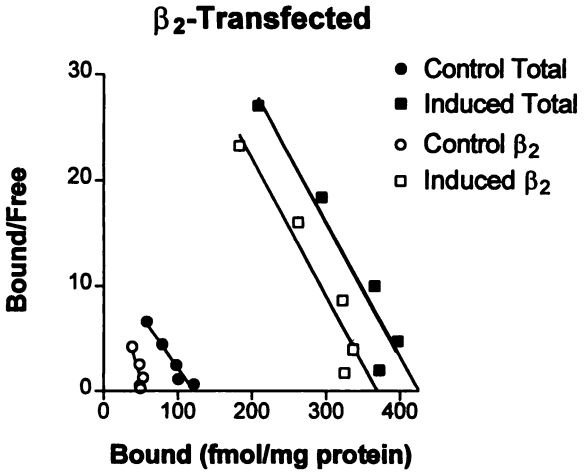

Fig. 3. ^{125}I-CYP binding to membranes from noninduced (*Control*) and induced β_2-transfected C_6 cells. Cells were treated with (*Induced*) or without (*Control*) 1 mM IPTG for 24 hr. Total βAR density (●, ■) was determined through Scatchard analysis of specific ^{125}I-CYP binding, and β_2AR density (○, □) was determined through Scatchard analysis of specific ^{125}I-CYP binding in the presence of the β_1-selective antagonist CGP 20712A (500 nM). Each value is mean of data from three experiments performed in duplicate.

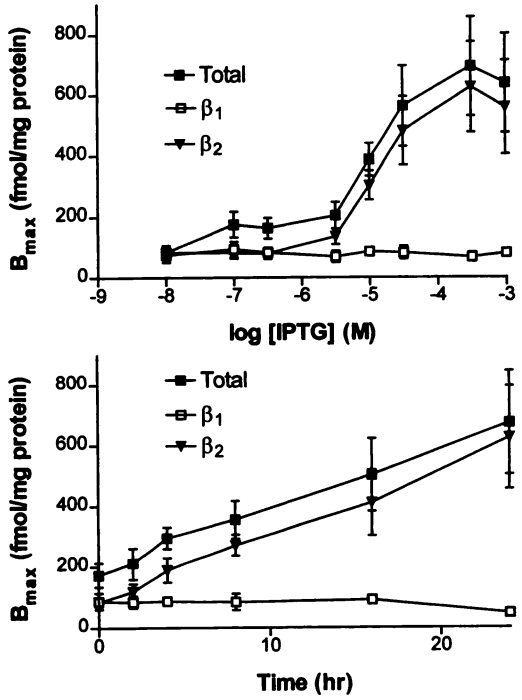

Fig. 4. Concentration and time dependence of IPTG induction of β_2AR expression in β_2-transfected C_6 cells. Total βAR density was determined through Scatchard analysis of specific ^{125}I-CYP binding. β_1AR and β_2AR densities were determined as described in Experimental Procedures. Cells were induced for 24 hr with various concentrations of IPTG (*top*) or with 1 mM IPTG for the indicated time period (*bottom*). Each value is mean ± standard error of data from three experiments performed in duplicate.

to block the β_1 subtype) and decreases in the maximal responses (Table 1). Although the maximal response to NE (β_1 selective) was also decreased, no change in the EC_{50} was observed after β_2AR induction (Fig. 8 and Table 1).

Lack of effect of pertussis toxin on the decreased maximal response caused by β_2AR induction. To examine whether promiscuous coupling to pertussis toxin-sensitive G proteins (19) might account for the decrease in the maximum agonist-induced cAMP response, the effect of pertussis toxin pretreatment on β_2-transfected C_6 cells was examined. Pertussis toxin alone caused a 26–32% decrease in the maximal ISO response in both noninduced and induced cells but did not reverse the decrease in maximal ISO response caused by induction of β_2ARs with IPTG (Table 2). Pertussis toxin pretreatment caused a large, but not complete, reversal of the inhibitory effect of ATP on the response to ISO in these cells, as reported previously (20), which was similar in both noninduced and induced cells (Table 2).

Effects of subtype-selective antagonists on ISO responses in β_2-transfected cells. The β_1-selective antagonist CGP and the β_2-selective antagonist ICI were used to examine the contribution of each subtype to the ISO response in noninduced and induced β_2-transfected cells. Concentrations of ISO resulting in just maximal responses were used, which were different in noninduced (10 nM) and induced (3 nM) cells. Biphasic inhibition curves were observed with both CGP and ICI in noninduced cells (Fig. 9), suggesting a contribution of both subtypes to the ISO response. Inhibition curves seemed to be monophasic in induced cells (ICI > CGP), suggesting only β_2 involvement. Interestingly, the ISO response was reduced to a similar extent (30%) by either CGP or β_2AR induction, and CGP had no further effect after β_2AR induction (Fig. 9, *bottom*).

Synergistic blockade of ISO and NE responses by CGP and ICI after β_2AR induction. To determine

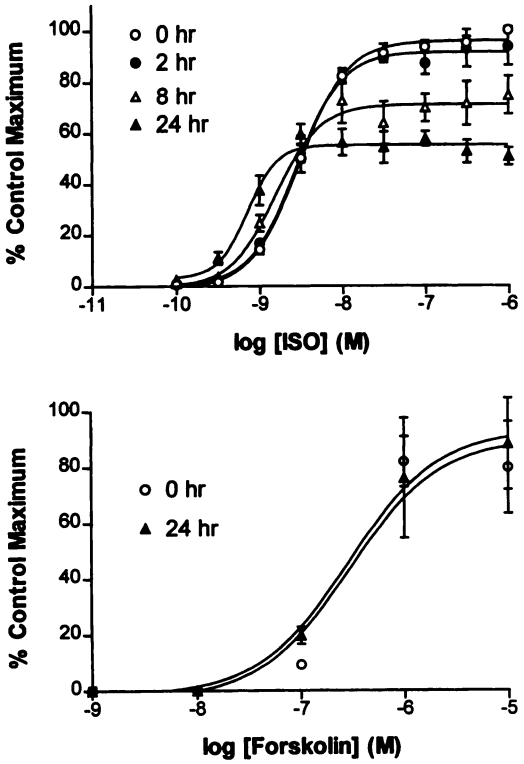

Fig. 5. ISO- and forskolin-stimulated cAMP formation after various induction times in β_2-transfected C_6 cells. Cells transfected with rat β_2AR coding sequences were exposed to 1 mM IPTG for the indicated time. cAMP formation was measured as percentage of conversion of [^{3}H]ATP to [^{3}H]cAMP, and data were normalized to the control maximum as 100%. Each value is mean ± standard error of data from three experiments performed in duplicate.

whether β_1ARs played any role in agonist-induced responses after β_2AR induction, we examined the effects of the selective antagonists CGP and ICI (alone and in combination) on the dose-response curves for ISO and the β_1-selective agonist NE (Fig. 10). Consistent with a primarily β_2-mediated response in induced cells, CGP (500 nM) had no significant effect on the ISO dose-response curve, whereas ICI (500 nM) caused a 100-fold shift to the right. Surprisingly, when CGP was added with ICI, an additional 100-fold shift to the right was observed. CGP and ICI each caused smaller 7–10-fold shifts to the right in the dose-response curve for NE, suggesting complex interactions between the two subtypes in the response to this agonist. Interestingly, a combination of both antagonists again caused a much-more-than-additive shift to the right in NE potency, totally eliminating the response at all concentrations examined (Fig. 10).

Schild analysis reveals complex interactions between the two subtypes after β_2AR induction. The effects of the β_2-selective antagonist ICI on ISO-induced increases in cAMP accumulation after β_2AR induction are shown in Fig. 11. The effects of increasing concentrations of ICI on the concentration response-curve to ISO are shown in the absence of any other drug (*top*), and the effects of increasing concentrations of ICI on the concentration-response curve to ISO are shown in the presence of 500 nM CGP to block the β_1ARs (*bottom*). Although CGP alone had no significant effect on the concentration-response curve to ISO, it greatly increased the ability of ICI to competitively antagonize the response. By plotting these data (Fig. 12) according to the method of Arunlakshana and Schild (21), we show that there is no significant change in the pA_2 value for ICI (8.85 without CGP versus 9.12 with CGP), confirming that the inhibitory effect of ICI is mediated via β_2AR blockade under both conditions. However, a large difference in slope (0.73 without CGP versus 1.29 with CGP) is observed, indicating an interaction between the inhibitory effects of the two antagonists.

Discussion

C_6 glioma cells express both β_1ARs and β_2ARs linked to activation of G_S/adenylate cyclase, making them useful for studying the interactions between closely related subtypes that coexist on the same cell. β_1ARs outnumber β_2ARs by 4:1 in native C_6 cells (7, 12, 22), and catecholamines stimulate cAMP accumulation with a rank order of potency characteristic of β_1ARs (ISO > NE ≥ EPI). This rank order does not change even when β_2ARs become numerically dominant after dexamethasone treatment (12), suggesting that β_1ARs couple more efficiently to cAMP accumulation than do β_2ARs in these cells. Consistent with the higher density, there is a larger receptor reserve for β_1ARs than β_2ARs in C_6 cells (23).

In this study, we stably transfected C_6 cells with an IPTG-inducible vector system containing rat β_1AR or β_2AR coding sequences. This allowed us to control the density of either subtype over an 8–20-fold range by altering the time or concentration of exposure to IPTG. Although we do not know whether receptors are induced equally in all cells in the population, we were able to relate mean receptor expression across the cell population under each condition to agonist responsiveness. Large increases in expression of either β_1ARs or β_2ARs did not cause reciprocal changes in the density of the other subtype, suggesting that synthesis and degradation of each subtype are not affected by the presence or absence of the other receptor type. This system allows direct examination of agonist-induced responses in cells in which the density of one subtype can be varied while the expression of the other subtype remains constant.

Increasing expression of the dominant β_1AR subtype in C_6 cells caused corresponding increases in the potencies of all agonists examined, with no change in maximal response. This suggests that stimulation of the endogenously expressed βARs maximally activates the cAMP signaling system in native C_6 cells, which is consistent with our previous observations of a substantial β_1AR reserve (23). The results seen after induction of the dominant β_1AR subtype are consistent with theoretical predictions in the presence of a receptor reserve. As receptor density increased, we observed a progressive increase in agonist potency with no change in maximal response. As expected, agonists showed a characteristic β_1AR order of potency both before and after β_1AR induction, with IPTG causing similar 20–40-fold increases in potency for ISO, NE, and EPI.

We recently obtained the opposite result when comparing the relationship between α_{1B}AR density and inositol phosphate responses in DDT_1 cells (24). Increasing α_{1B}AR expression in DDT_1 cells caused a linear increase in the maximal response to NE but no change in potency. This suggests the absence of an α_{1B}AR receptor reserve in DDT_1 cells, even with very high receptor expression (2–3 pmol/mg protein). Whether the presence or absence of a receptor reserve at similar receptor densities is related to the AR type (β or α_{1B}),

TABLE 2

Effect of PTX on ISO responses with or without ATP in β_2-transfected cells

C_6 cells were treated with (induced) or without (noninduced) 1 mM IPTG for 24 hr. Cells were also pretreated for 24 hr with (PTX) or without (control) 300 ng/ml pertussis toxin. Data are presented as percent conversion of [^{3}H]ATP to [^{3}H]cAMP; 1 μM ISO and 100 μM ATP were used where indicated.

	Noninduced		Induced	
	Control	PTX	Control	PTX
Basal	0.21 ± 0.05	0.20 ± 0.02	0.19 ± 0.01	0.20 ± 0.001
ISO	4.56 ± 0.31	3.36 ± 0.17[a]	3.10 ± 0.16[b]	2.11 ± 0.10[a, b]
ISO + ATP	0.25 ± 0.03	2.12 ± 0.11[a]	0.21 ± 0.01	1.18 ± 0.04[a, b]

[a] $p < 0.01$ compared with control.
[b] $p < 0.01$ compared with noninduced.

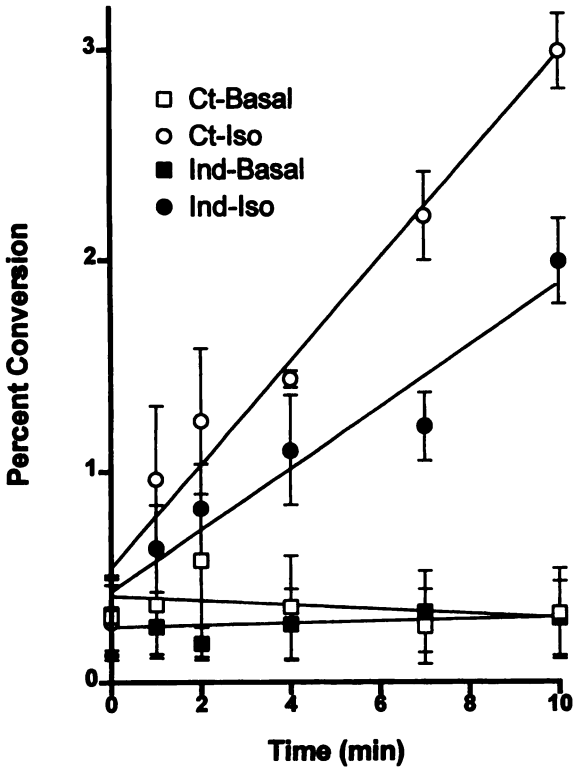

Fig. 6. Time course of cAMP accumulation after induction of β_2AR density in C_6 glioma cells. Cells transfected with rat β_2AR coding sequences were exposed to 0 (*Ct*) or 1 (*Ind*) mM IPTG for 24 hr. cAMP formation was measured in the absence (*Basal*) or presence (*Iso*) of 1 μM ISO after various incubation times. Data are plotted as percentage of conversion of [^{3}H]ATP to [^{3}H]cAMP. Each value is mean ± standard error of data from three experiments performed in duplicate.

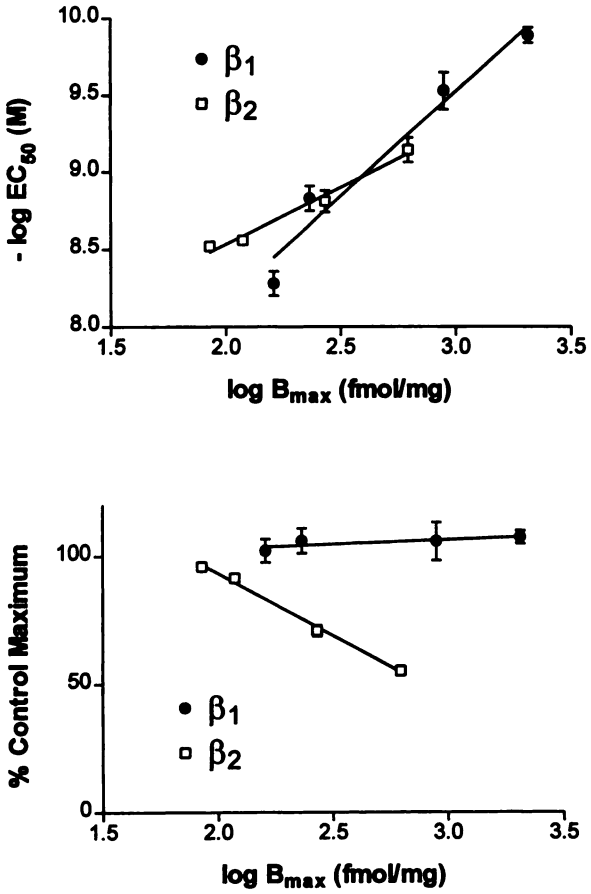

Fig. 7. Relation of receptor density for each subtype to the EC_{50} and the maximum of ISO-stimulated cAMP responses. Receptor densities were increased in β_1- or β_2-transfected cells through induction with 1 mM IPTG for 0, 2, 8, and 24 hr. Data were calculated from Figs. 2 and 5. Receptor densities were determined as described in the legends to Figs. 1 and 4. Each value is mean ± standard error of data from three experiments performed in duplicate.

G protein/signaling mechanisms, or other cell-specific parameters remains to be clarified.

In contrast to the predictable results observed after β_1AR induction, the results obtained after induction of β_2ARs in cells transfected with this subtype were much more complex. β_2ARs compose only a minor proportion of total βARs in native C_6 cells (20%) and normally play little role in catecholamine responsiveness (12). Induction of β_2AR expression caused smaller increases in the potency of ISO than did induction of β_1ARs. This is most clearly shown by the different slopes of the linear relationship between receptor density and agonist potency shown in Fig. 7 (*top*). Interestingly, the lines seem to cross at a receptor density of ~300 fmol/mg protein. Below that number, ISO is more potent at the β_2 than the β_1 subtype for a given receptor density, whereas the situation may reverse when the density is increased (although extrapolation is limited by the maximum density actually achieved in β_2-transfected cells). This may contribute to differences seen between model systems. Sometimes the β_2 subtype seems to be more efficacious than the β_1 subtype (8, 10, 11), whereas in other systems, the reverse seems to be true (12, 23). It will undoubtedly be necessary to take into account the actual receptor densities, as well as other cell-specific parameters such as G protein subunits and densities, when comparing the relative coupling efficiencies of the two subtypes.

Surprisingly, we found a progressive decrease in the maximum response to agonists after β_2AR induction. This decrease was observed at all time points examined and was not

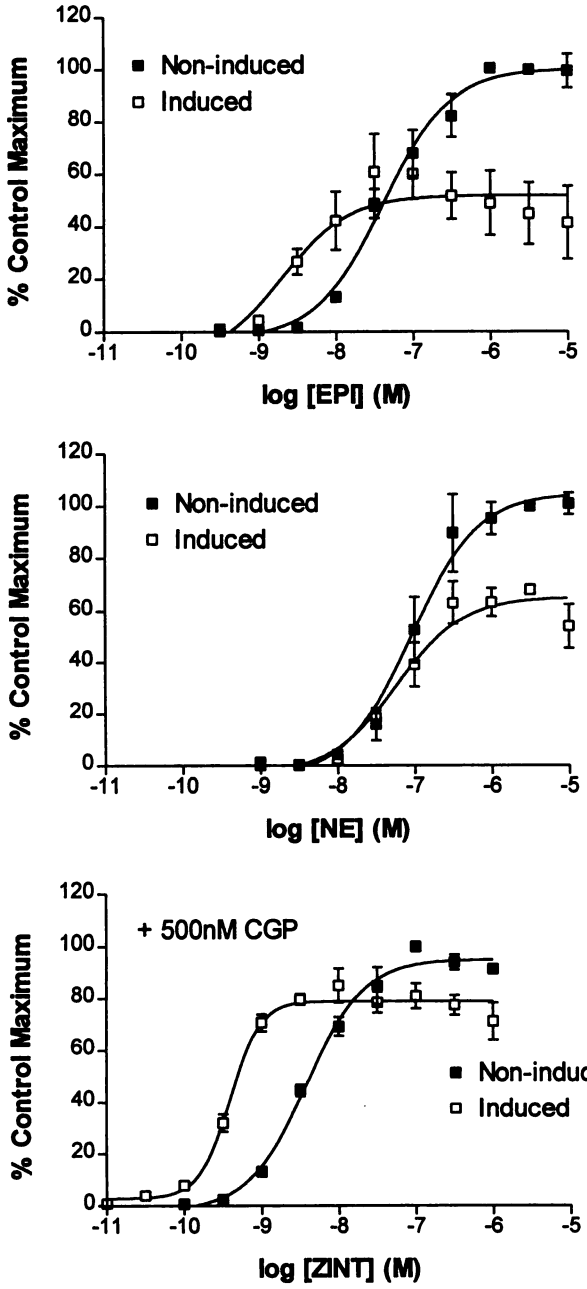

Fig. 8. EPI-, NE-, and ZINT-stimulated cAMP formation in noninduced and induced β_2-transfected C_6 cells. Cells were incubated with (*Induced*) or without (*Non-induced*) 1 mM IPTG for 24 hr. ZINT response was measured in the presence of 500 nM CGP to block β_1ARs. cAMP formation was measured as percentage of conversion of [^{3}H]ATP to [^{3}H]cAMP, and data were normalized to the control maximum for that agonist as 100%. Each value is mean ± standard error of data from three experiments performed in duplicate.

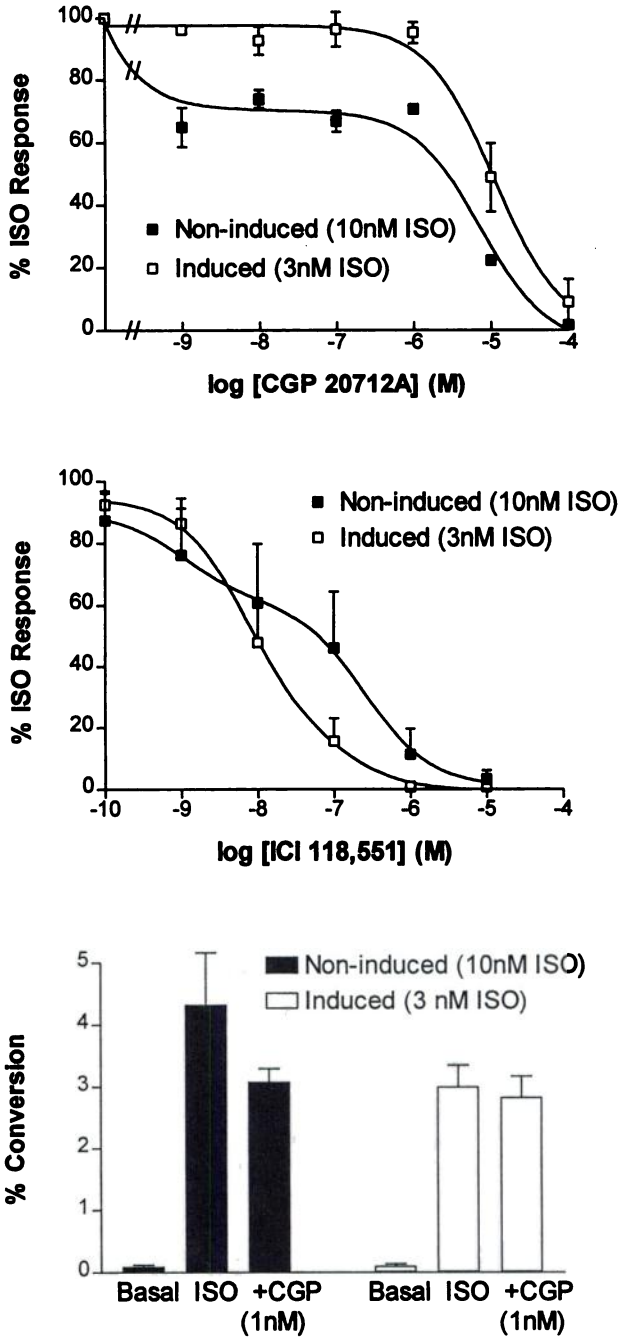

Fig. 9. CGP and ICI inhibition of cAMP responses stimulated by ISO in noninduced and induced β_2-transfected C_6 cells. For noninduced and induced cells, 10 and 3 nM ISO were used, respectively, to give the maximal responses. *Bottom*, magnitude of each response and the effect of CGP. *Top two panels*, data were normalized to the response stimulated by ISO in the absence of antagonist. Noninduced values were normalized to the noninduced ISO response, and induced values were normalized to induced ISO response. Each value is mean ± standard error of data from three experiments performed in duplicate.

blocked by prevention of cAMP breakdown with isobutylmethylxanthine. Several observations suggest that this was a specific response to receptor induction rather than a nonspecific effect; these include the correlation between the decreased maximal response and the time of IPTG exposure, the lack of effect of IPTG on the response to forskolin, and the fact that no such decrease was seen when β_1ARs were induced under similar conditions. Thus, we consider it likely that the decreased response is due to increased β_2AR density; however, the specific mechanism involved is not yet clear.

The decreased maximal response is not due to a promiscuous interaction of these receptors with pertussis toxin sensitive G_i or G_o-like proteins (19) because it was not eliminated with pertussis toxin pretreatment. Interestingly, selective blockade of the β_2AR subtype by ICI also did not reverse the decreased maximal response (Figs. 10 and 11), suggesting that β_2AR induction may be causing additional cellular effects that are not reversed with receptor blockade.

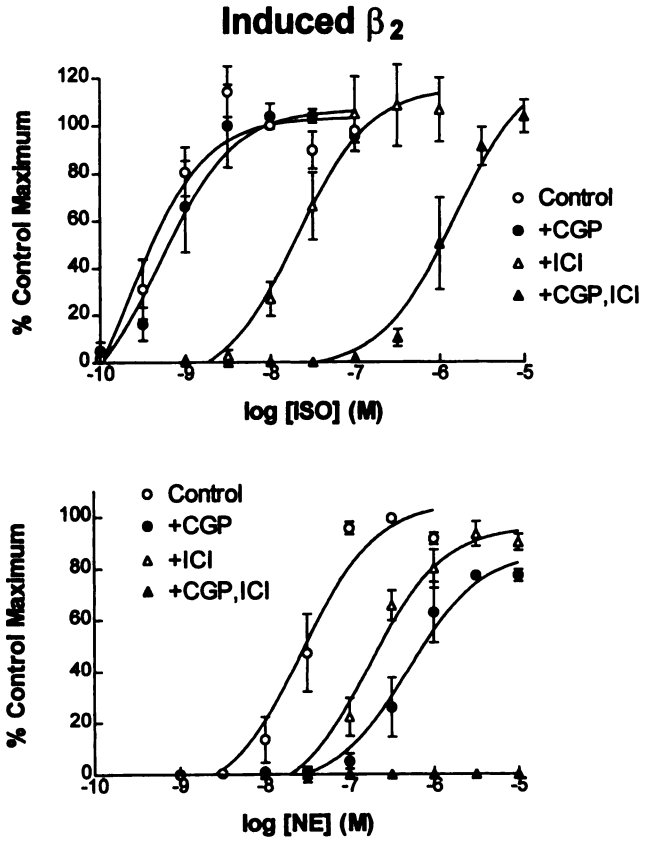

Fig. 10. ISO- and NE-stimulated cAMP accumulation in the presence or absence of CGP and ICI in cells induced for β_2AR expression. Transfected cells were induced for β_2AR expression with 1 mM IPTG for 24 hr. Concentration-response curves to agonists were done in the presence or absence of the indicated antagonists at a concentration of 500 nM. Responses were measured as percentage of conversion of [^{3}H]ATP to [^{3}H]cAMP. Data were normalized to the response stimulated by ISO or NE in the absence of antagonist. Each value is mean ± standard error of data from three experiments performed in duplicate.

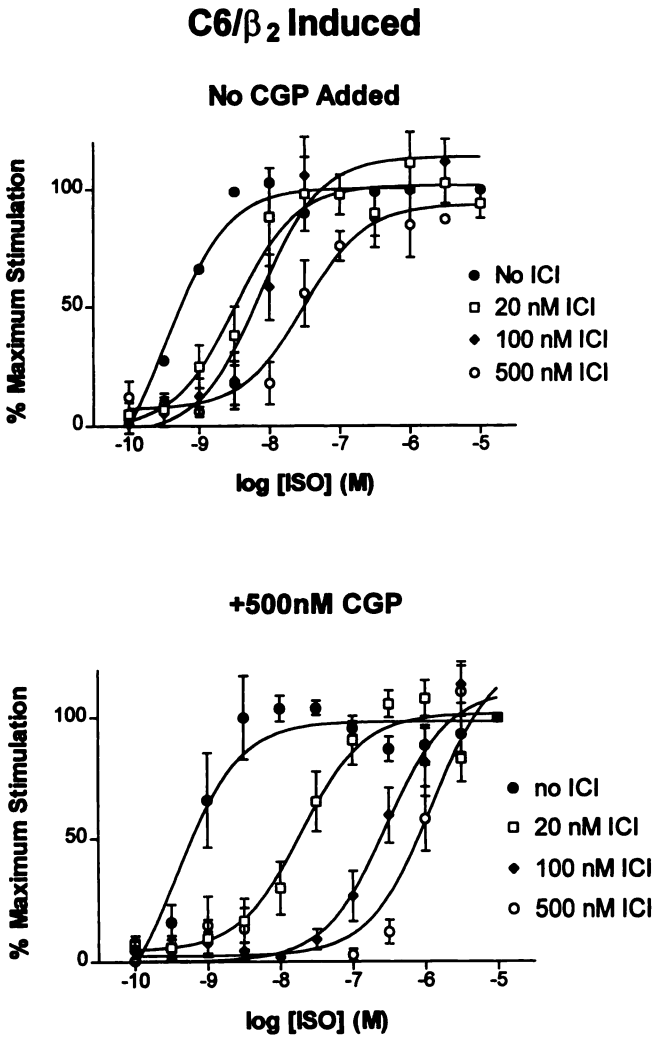

Fig. 11. Competitive inhibition of ISO-induced responses by ICI in the presence or absence of CGP in cells induced for β_2AR expression. Transfected cells were induced for β_2AR expression with 1 mM IPTG for 24 hr. Concentration-response curves for ISO were determined in the presence of the indicated concentrations of ICI in the absence (*top*) or presence (*bottom*) of 500 nM CGP. Responses were measured as percentage of conversion of [^{3}H]ATP to [^{3}H]cAMP. Data were normalized to the response stimulated by ISO in the absence of antagonist. Each value is mean ± standard error of data from four to five experiments performed in duplicate.

One potential explanation for the reduced maximal response to induction of β_2AR expression is a reduction in cellular concentrations of $G_{S\alpha}$ (25, 26) caused by constitutive activity (27) of the newly expressed β_2ARs. However, we did not observe constitutive activity after induction of either subtype in these experiments because no significant increases in whole-cell cAMP accumulation were observed in the absence of agonist. In addition, because forskolin works partially through $G_{S\alpha}$ (28), losses in this G protein might be expected to decrease the forskolin response, which was not observed. At this point, we have no good explanation for this decreased maximum response after β_2AR induction. Bouvier *et al.* (29) also found decreases in maximum response in cell lines with very high β_2AR expression levels; however, such a phenomenon has not been observed in other studies (18, 30). The ability to directly modulate receptor expression in our cell line with inducible vectors should allow us to clarify the mechanisms involved in controlling the maximum response to agonist.

One of the primary goals of our study was to examine the interactions between two closely related subtypes that converge on the same signal transduction system. We are particularly interested in the roles of the different subtypes in response to selective and nonselective agonists under different receptor densities and ratios and their implications for pharmacological analysis. Thus, we used selective antagonists to clarify the roles of the different receptor subtypes in agonist-induced responses when the normally minor β_2AR population was increased.

In β_2-transfected cells that were not exposed to IPTG, some basal constitutive activity of the operator vector resulted in a situation in which the ratio of β_1AR to β_2AR density was ~1. In this case, both subtypes contributed to the response stimulated by the nonselective agonist ISO. After IPTG induction, however, when β_2ARs constituted >80% of the total βAR population, the response to ISO seemed to be mediated exclusively by the β_2 subtype. This suggests that the β_1ARs, although still present as determined with radioligand binding measurements, are not activated by concentrations of ISO maximally activating the β_2AR population or that the β_1ARs might be unable to compete effectively for interaction

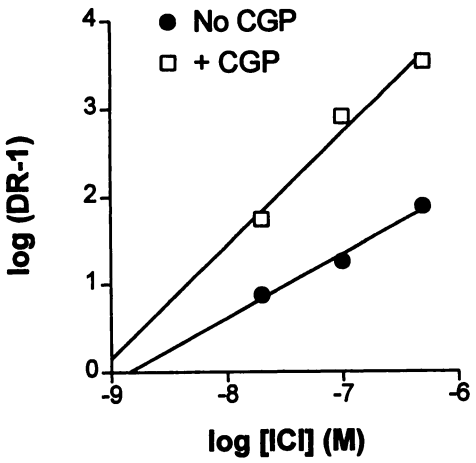

Fig. 12. Schild plots for ICI inhibition of ISO responses in cells induced for β_2AR expression. Data from Fig. 11 were analyzed through nonlinear regression and plotted according to the method of Arunlakshana and Schild (20). *DR*, dose ratio.

with G_S. Clearly, the existence of different receptor reserves for the two subtypes and differences in their relative efficiencies in coupling to the second messenger system will be important in clarifying the roles of the different subtypes. These results suggest that although β_1ARs play an important role in the ISO response in noninduced cells, this contribution becomes overshadowed when β_2AR expression is increased markedly.

Further pharmacological analysis after β_2AR induction suggests some interesting functional interactions between the coexisting subtypes. In β_2-induced C_6 cells, 500 nM CGP had no effect on the concentration-response curve to ISO, whereas 500 nM ICI decreased the potency of ISO by 100-fold, suggesting that the response was mediated primarily through β_2ARs. In the presence of ICI, however, CGP caused an additional 100-fold decrease in ISO potency, suggesting a major role for β_1ARs under these conditions. The further shift by CGP cannot be attributed simply to activation of β_1ARs at higher ISO concentrations. In the presence of CGP, ICI shifts the curve 3000-fold, much greater than the ~100-fold that would be expected from simple competitive antagonism at β_2ARs (assuming a K_i of ~4 nM for ICI; Ref. 12). The response to the β_1-selective agonist NE was shifted to a lesser extent by both CGP and ICI but completely eliminated when both antagonists were present, also suggesting synergistic contributions from the two subtypes. It is interesting that very similar observations have been made in isolated human heart cells, in which β_1ARs and β_2ARs coexist and are both linked to mechanical responses (8), supporting the biological relevance of such interactions.

Competitive antagonism of the response to ISO in β_2-induced cells by the β_2-selective antagonist ICI shows this interaction more clearly. Increasing concentrations of ICI caused a progressive shift to the right in the concentration-response curve for ISO-stimulated cAMP accumulation, which is consistent with a competitive antagonism of the response (Fig. 11). The x-intercept of the resulting Schild plot was 8.85, which is similar to the pA_2 expected for the β_2 subtype and the K_D in competing for β_2AR binding sites in these cells (12). The slope of the Schild plot was low (0.73), suggesting that increasing concentrations of antagonist were not shifting the curve as far as would be expected for a simple competitive antagonist. However, there was no apparent discontinuity (i.e., a large shift at low concentrations of ICI and then no further shift when the ICI concentration was increased), which might be expected if the β_1AR population began to compensate at higher ISO concentrations as the β_2ARs were progressively blocked (31). When the same experiment was performed in the presence of 500 nM CGP to block the β_1 subtype, increasing concentrations of ICI again caused progressive shifts to the right in the ISO-stimulated cAMP accumulation. However, these shifts were now much larger than those observed in the absence of CGP. The Schild plot resulted in a similar pA_2 value (9.1) but a much higher slope (1.29). We do not understand why the slope is higher than unity, although it may be due to a contribution from the β_1 subtype at the high ISO concentrations used. Such ISO concentrations were needed due to the large effects of ICI and might overcome the competitive inhibition of β_1ARs by CGP. In any case, these studies show clearly that both subtypes can each contribute to responses to nonselective agonists but that these interactions are not simply additive. Rather, the presence of one subtype influences the ability of the other subtype to activate a response, and different subtype ratios are activated at different agonist concentrations. The relatively limited selectivity of the agonists and antagonists currently available (100–500-fold) prevents further pharmacological separation of these responses at this point. It will be necessary to use other techniques to examine the convergence of the subtypes on this signaling system to clearly understand these interactions.

In summary, we studied the relationship between density and response for β_1ARs and β_2ARs in C_6 glioma cells using inducible expression vectors. Increases in the density of β_1ARs caused predictable increases in agonist potency but no change in maximal response, which is consistent with a preexisting β_1AR reserve. However, increases in β_2AR density decreased the maximum agonist response and caused only smaller increases in potency. In cells expressing predominantly β_2ARs, both subtypes seemed to contribute to the ISO response in a synergistic manner. These results suggest that there are complex interactions between coexisting β_1ARs and β_2AR subtypes and that the relationship of these two subtypes depends on their relative densities and ratio, as well as the concentration and selectivity of the agonist. Because β_1ARs and β_2ARs are known to coexist on a number of cell types, such studies have important functional implications.

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