0026-895X/93/061085-09$03.00/0

MOLECULAR PHARMACOLOGY, 44:1085-1093



# Close Reciprocal Regulation of $\beta_1$- and $\beta_2$-Adrenergic Receptors by Dexamethasone in $C_6$ Glioma Cells: Effects on Catecholamine Responsiveness

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Received June 23, 1993; Accepted September 10, 1993

**SUMMARY**

We studied the regulation of $\beta$-adrenergic receptor (AR) subtypes co-existing in rat $C_6$ glioma cells to clarify the importance of subtype ratio in responses to catecholamines. Radioligand binding studies with [$^{125}$I]-cyanopindolol showed that $\beta_1$- and $\beta_2$-ARs co-existed in this cell line in approximately an 80:20 ratio. Norepinephrine (NE) and epinephrine (EPI) were equally potent in increasing cAMP accumulation, consistent with a primarily $\beta_1$-response, although both $\beta_1$- and $\beta_2$-components of the response could be isolated using selective agonists (NE and zinterol), and antagonists (CGP 20712A and ICI 118,551). Little or no evidence of $\beta_3$-ARs could be found in this cell line. Treatment of cells with 500 nM dexamethasone (DEX) for 48 hr increased the proportion of $\beta_2$-ARs (20 to 60%). However, a reciprocal decrease in $\beta_1$-ARs resulted in no change in total $\beta$-ARs. Studies on the time- (12 to 72 hr) and concentration- (5 nM to 5000 nM) dependence of DEX treatment showed that increases in $\beta_2$-ARs were closely linked to decreases in $\beta_1$-ARs with little or no change in total receptor density observed at any time or in any concentration studied. Treatment with DEX also increased $\beta_2$- and decreased $\beta_1$-mediated cAMP responses, but did not alter the response to the nonselective agonist, isoproterenol. Northern blot analysis showed a 2- to 3-fold increase in $\beta_2$-AR mRNA, but no change in $\beta_1$-AR mRNA, after exposure to 50 or 500 nM DEX for 48 hr. Surprisingly, after DEX treatment, NE and EPI were still equally potent in activating cAMP accumulation, although responses to the $\beta_2$-selective agonist, zinterol, were increased. These studies show a close reciprocal regulation by DEX of the relative proportions of $\beta_1$- and $\beta_2$-AR subtypes in $C_6$ cells. The functional significance of the changing subtype ratios does not appear to be related to catecholamine responsiveness.

Many neurotransmitters and hormones exert their effects through large families of often closely related receptor subtypes. At least nine AR subtypes have been identified (1–6), mediating the effects of NE and EPI. These ARs are grouped into three families, called $\alpha_1$, $\alpha_2$, and $\beta$. There are at least three distinct subtypes within each family that have similar ligand binding properties and homologous structures and appear to activate similar signalling mechanisms.

The existence of distinct $\beta_1$- and $\beta_2$-AR subtypes was recognized first from the different potencies of EPI and NE in activating responses in different tissues (7–8). EPI and NE are equally potent at $\beta_1$-ARs, but EPI is about 20 times more potent than NE at $\beta_2$-ARs (5). Recently, a third $\beta$-AR subtype has been identified ($\beta_3$) where NE is about 20 times more potent than EPI (6). All three $\beta$-AR subtypes activate adenylate cyclase through Gs (5–6), increasing cAMP accumulation, but it is not yet clear whether they do so with similar coupling efficiencies. It is also not yet known whether other signalling mechanisms might also be activated by these different receptor subtypes.

AR subtypes often co-exist in the same tissue (9–10) and can be co-expressed on the same cells (11–13). The functional and therapeutic implications of such co-existence are only now beginning to be studied. The co-existence of different subtypes on the same cell may cause opposite, additive, redundant, or synergistic responses to a single neurotransmitter. For example, co-existing $\beta_1$- and $\alpha_2$-ARs cause opposing effects on cAMP formation in primary cultures of rat glial cells (14), but co-existing $\beta_1$- and $\beta_3$-ARs cause redundant increases in cAMP formation in human SK-N-MC neuroblastoma cells (15).

Glucocorticoids such as DEX selectively regulate the density and responsiveness of certain AR subtypes (16–24). Glucocorticoids increase $\beta_2$-AR receptor density by binding to a glucocorticoid response element in the 5′ noncoding region of the gene and increasing transcription (22, 24). However, glucorticoid treatment usually diminishes both $\beta_1$- and $\beta_3$-AR receptor

This work was supported by NIH grant NS21325.

**ABBREVIATIONS:** AR, adrenergic receptor; NE, norepinephrine; EPI, epinephrine; DEX, dexamethasone; EDTA, ethylenediaminetetraacetic acid; CGP, CGP 20712A; ICI, ICI 118,551; $^{125}$ICYP, [$^{125}$I]-cyanopindolol; ISO, isoproterenol, KRB, Krebs-Ringer bicarbonate buffer; IBMX, 3-isobutylmethylxanthine; PBS, phosphate-buffered saline; ZINT, zinterol; $EC_{50}$, median effective concentration.

expression (16, 19). We wondered if glucocorticoids would inversely regulate $\beta_1$- and $\beta_2$-ARs co-existing on the same cell, thereby changing the subtype ratio and responsiveness to the endogenous catecholamines EPI and NE. Because $\beta_1$- and $\beta_2$-ARs are known to be co-expressed in rat $C_6$ glioma cells (11, 25–27), we studied the effect of DEX on $\beta_1$- and $\beta_2$-AR density and catecholamine responsiveness in these cells.

## Experimental procedures

**Materials.** Rat $C_6$ glioma cells were obtained from the American Type Culture Collection (Rockville, MD). Materials were obtained from the following sources: horse serum, fetal bovine serum, and trypsin-EDTA (GIBCO BRL, Grand Island, NY), [$^3$H]adenine (~25 Ci/mmol, Du Pont NEN, Boston, MA), [$^{32}$P]dCTP (~6000 Ci/mmol, Amersham, Chicago, IL), ICI (Cambridge Research Biochemicals, Macclesfield, UK), Ham's F-10 medium, (−)NE, (−)ISO, (−)EPI, (−)propranolol, DEX, IBMX, cAMP, and all other agents (Sigma Chemical Co., St. Louis, MO). ZINT was kindly donated by Mead Johnson Pharmaceuticals (Evansville, IN). CGP 20712A and CGP 12177 were kindly donated by Ciba Geigy (Summit, NJ).

**Cell cultures.** $C_6$ cells were grown in Ham's F-10 medium supplemented with 15% horse serum, 2.5% fetal bovine serum, 20 mg/l streptomycin and 20 mg/l penicillin in a humidified atmosphere containing 7% $CO_2$ at 37°. Cells were grown to confluency and the medium was changed every 2 to 3 days. Confluent cells were subcultured at a ratio of 1:4 into Primaria flasks. For cAMP assays, 2 ml of cells ($10^4$ cells/ml) were subcultured into 35-mm Primaria plates and grown to confluency. For membrane radioligand binding assays, 10 ml of cells of the same concentration were added to 100-mm plates and grown to confluency.

**cAMP accumulation.** cAMP accumulation was measured by the [$^3$H]adenine prelabeling technique (28), as described previously (14). Briefly, confluent cells in 35-mm plates were prelabeled with [$^3$H]adenine (1 $\mu$Ci/2 ml) for 2 hr. Culture medium was then removed and cells were washed twice with 1 ml of KRB at 37° and replaced with 1 ml KRB. Drugs were added and cells were returned to the 37° incubator and incubated for 10 min. At the end of the incubation, 100 $\mu$l of 77% trichloroacetic acid was added to terminate the reaction, and 50 $\mu$l of unlabeled cAMP (10 mM) was added as a carrier. Cells were scraped and centrifuged at 30,000 × $g$ for 10 min. A 50 $\mu$l aliquot of the supernatant was taken for counting the incorporation of [$^3$H]adenine and [$^3$H]cAMP isolated from the rest of the supernatant by sequential DOWEX and alumina chromatography (29, 30). Results were expressed as a percentage of the conversion of [$^3$H]ATP to [$^3$H]cAMP.

**Radioligand binding assay.** Binding of $^{125}$ICYP to $C_6$ cell membrane preparations was assessed as described previously (31). Briefly, confluent cells were washed twice in PBS (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). Membranes (0.1 ml) were incubated with $^{125}$ICYP in a final volume of 0.25 ml PBS at 37° for 1 hr with or without competing drugs. For Scatchard analysis, increasing concentrations (maximal 300 pM) of $^{125}$ICYP were used. For competition binding, 50,000 cpm $^{125}$ICYP (50 pM) was used. At the end of the incubation, the binding was terminated by adding 10 ml 10 mM TrisHCl (pH 7.4) and filtered through glass fiber filters (Schleicher and Schuell, #30, Keene, NH) under vacuum. Filters were washed with an additional 10 ml buffer, dried, and counted in a gamma counter. Nonspecific binding was defined as binding in the presence of 10 $\mu$M ISO.

**mRNA analysis.** The cDNA encoding the human $\beta_1$-AR (3.0 $K_b$ subcloned in pBC12) (32) was kindly provided by Dr. R. J. Lefkowitz (Duke University, Durham, NC). The cDNA encoding the human $\beta_2$-AR (2 $K_b$ in pUC18) (33) was obtained from American Type Culture Collection (Rockville, MD). Full length inserts were labeled with [$^{32}$P]dCTP by the random primer method (Prime-It, Stratagene, La Jolla, CA). Northern blot analysis of mRNA was performed as described previously (31). Confluent $C_6$ cells were incubated with 0, 50, or 500 nM DEX for 48 hr. Cells were washed with cold PBS buffer and poly (A)$^+$ RNA was isolated (FastTrack, Invitrogen, San Diego, CA). After electrophoresis on 1.2% agarose/0.7% formaldehyde gel, RNA was blotted on Hybond N nylon membranes (Amersham, Chicago, IL) overnight. The blots were washed and baked for 1 hr at 80°. Membranes were prehybridized for 2 hr, and then hybridized with [$^{32}$P]-labeled cDNA probes for 16 hr at 42°. After hybridization, membranes were washed twice at room temperature in 2 × standard saline citrate with 0.1% SDS for 15 min each, followed by two 15-min washes in 0.2 × standard saline citrate with 0.1% SDS at 55°. Autoradiographs were made by exposure to Kodak X-Omat film (Kodak, Rochester, N.Y.) for 2 to 6 days. Blots were stripped and reexposed to film for 3 days before hybridizing with a different probe. Bands were quantitated using a video densitometer.

## Results

**$\beta_1$- and $\beta_2$-ARs co-exist in $C_6$ glioma cells.** The high affinity antagonist radioligand, $^{125}$ICYP, was used to characterize $\beta_1$- and $\beta_2$-ARs in $C_6$ cell membranes. Scatchard analysis of $^{125}$ICYP binding resulted in a linear plot (Fig. 1) with a $K_d$ of 25 pM and a $B_{max}$ of 172 fmol/mg protein. Competition for $^{125}$ICYP (50 pM) binding with the $\beta_1$-selective antagonist, CGP, and the $\beta_2$-selective antagonist, ICI, resulted in biphasic curves

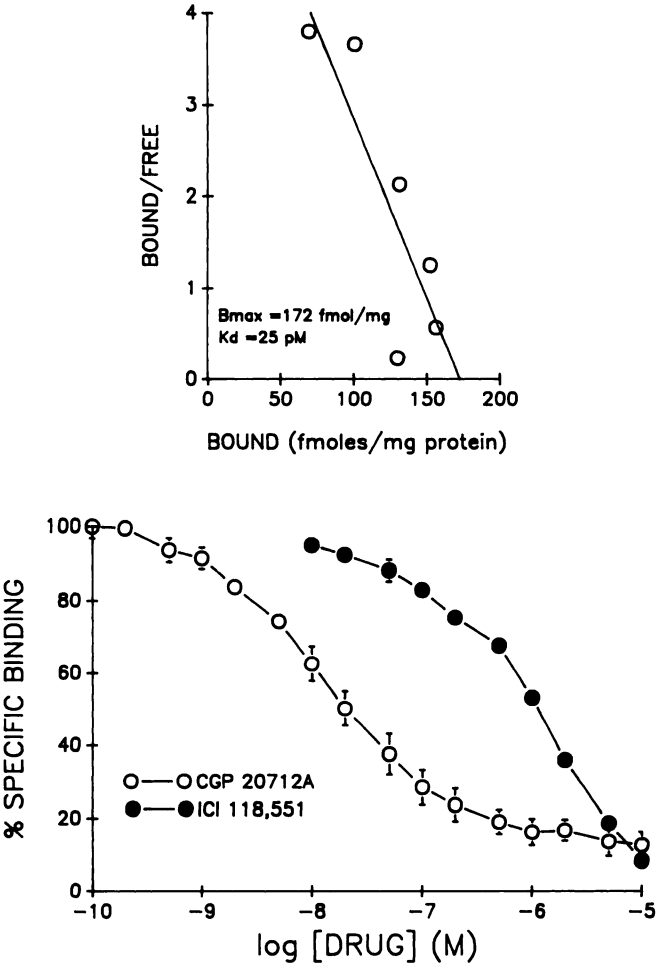


**Fig. 1.** $\beta_1$- and $\beta_2$-AR binding sites in membranes from rat $C_6$ glioma cells. *Upper*, Scatchard analysis of specific $^{125}$ICYP binding. *Lower*, inhibition of $^{125}$ICYP (50 pM) binding by the $\beta_1$-selective antagonist CGP and the $\beta_2$-selective antagonist ICI. Each value is the mean ± standard error of three or four experiments performed in duplicate.

indicative of two affinity sites. Nonlinear curve-fitting showed a ratio of 80:20 high/low affinity sites for the $\beta_1$-selective drug (CGP), while the ratio was reversed for the $\beta_2$-selective drug (ICI), indicating that $\beta_1$- and $\beta_2$-ARs co-exist in this cell line in an approximately 4:1 ratio. $K_d$ values calculated for CGP and ICI for binding to each subtype are listed in Table 1.

**Rank order of agonist potency indicates that the cAMP response is mediated primarily by $\beta_1$-ARs.** Concentration-response relationships in increasing cAMP formation in intact $C_6$ cells for the endogenous catecholamines NE and EPI, the nonselective agonist ISO, and the $\beta_2$-selective agonist ZINT are shown in Fig. 2. The rank order of potency of the catecholamines was ISO > NE = EPI, indicative of a primarily $\beta_1$-response. However, the $\beta_2$-selective agonist ZINT also caused a substantial (although submaximal) response, although the concentration-response relationship was shallow.

**Activation of either $\beta_1$- or $\beta_2$-ARs can increase cAMP formation.** We used the $\beta_1$-selective antagonist CGP and the $\beta_2$-selective antagonist ICI to define the contribution of $\beta_1$- and $\beta_2$-ARs to agonist-stimulated cAMP responses. Fig. 3 shows that CGP and ICI inhibited the cAMP response to ISO, NE and ZINT, but the relative potencies of these selective antagonists varied depending on the agonist and its concentration. CGP was more potent than ICI in blocking the cAMP response to 1 μM ISO (Fig. 3, *Upper Left*), indicating a primarily $\beta_1$-response. CGP was about 100 times more potent than ICI at inhibiting the cAMP response to 0.1 μM NE (Fig. 3, *Upper Right*), again indicating a $\beta_1$-response stimulated by this concentration of NE. At a relatively low concentration of ZINT (0.3 μM), ICI was substantially more potent than CGP in blocking the response (Fig. 3, *Lower Left*), consistent with a primarily $\beta_2$-response. However, at a higher ZINT concentration (100 μM), CGP and ICI were approximately equally potent in blocking the response (Fig. 3, *Lower Right*), indicating both $\beta_1$- and $\beta_2$-ARs contribute at higher ZINT concentrations.

**Responses to individual subtypes can be isolated with selective agonists and antagonists.** The highly $\beta_1$-selective antagonist CGP was used to define conditions where responses to each subtype could be quantitated. Fig. 4 (*Upper*) shows that increasing concentrations of CGP cause complex shifts in the concentration-response curve for the nonselective agonist ISO. At low CGP concentrations, the concentration-response curve for ISO was shifted to the right in a parallel manner, indicating a primarily $\beta_1$-type response. At higher CGP concentrations, the concentration-response curves for ISO became biphasic, with the upper portions continuing their parallel shift to the right, but the lower portions unaffected by further increases in CGP concentration. The concentration-response curve to the

TABLE 1

**Two-site analysis of displacement of specific $^{125}$ICYP binding by the $\beta_1$-selective antagonist CGP 20712A and the $\beta_2$-selective antagonist ICI 118,551**

Displacement curves (Fig. 1, *Lower*) were subjected to nonlinear regression analysis to obtain the best two-site fit for proportion of total sites and affinity. Each value is the mean ± SE of data from three experiments performed in duplicate.

| | Total sites | $K_d$ |
|---|---|---|
| | % | μM |
| CGP 20712A | | |
| $\beta_1$ | 82.5 ± 5.8 | 0.004 ± 0.001 |
| $\beta_2$ | 17.5 ± 5.8 | 2.48 ± 1.06 |
| ICI 118,551 | | |
| $\beta_1$ | 14.6 ± 1.8 | 0.009 ± 0.004 |
| $\beta_2$ | 85.4 ± 1.8 | 0.45 ± 0.05 |

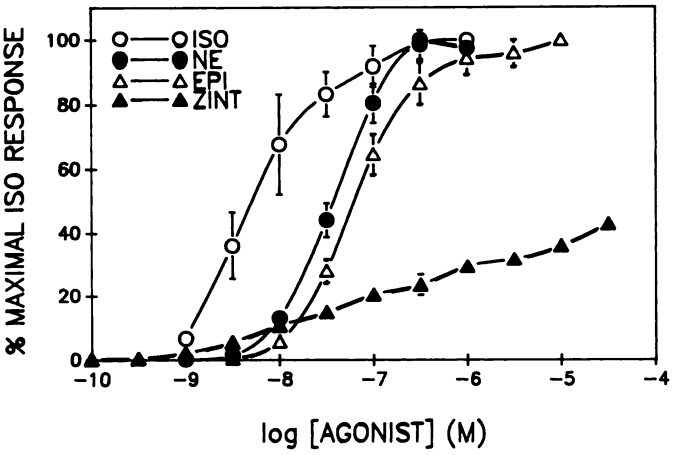


**Fig. 2.** Concentration-response curves for activation of cAMP accumulation by ISO, NE, EPI, and ZINT in intact $C_6$ cells. Data are expressed as a percent of the maximal response to ISO (Basal = 0.05 ± 0.01; maximal = 9.7 ± 2.1% conversion of [$^3$H]-ATP to [$^3$H]-cAMP). Each value is the mean ± standard error of three experiments performed in duplicate.

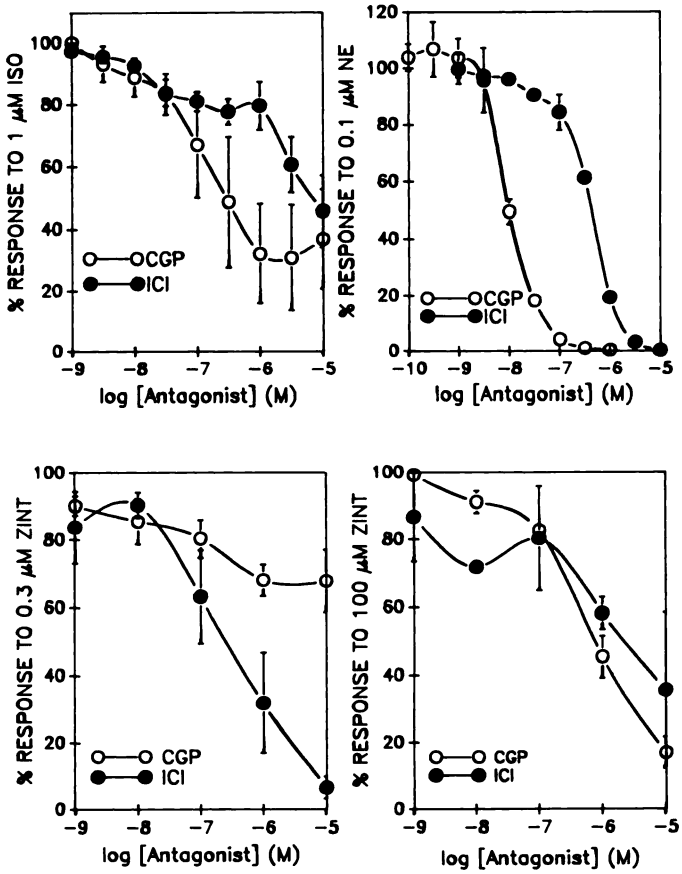


**Fig. 3.** Inhibition of cAMP responses to ISO (1 μM, *Upper, left*), NE (0.1 μM, *Upper, right*), a low concentration of ZINT (0.3 μM, *Lower, right*), or a high concentration of ZINT (100 μM, *Lower, left*) by the $\beta_1$-selective antagonist CGP and the $\beta_2$-selective antagonist ICI. Data are expressed as a percent of the response to each agonist in the absence of antagonist. 100% ISO-, 0.3 μM ZINT-, and 10 μM ZINT- stimulated responses of 5.15 ± 1.61, 2.09 ± 0.88, and 3.87 ± 1.04% conversion of [$^3$H]-ATP to [$^3$H]-cAMP, respectively. Basal values were 0.05 ± 0.01 and 0.11 ± 0.04% conversion, respectively. Each value is the mean ± standard error of three experiments performed in duplicate.

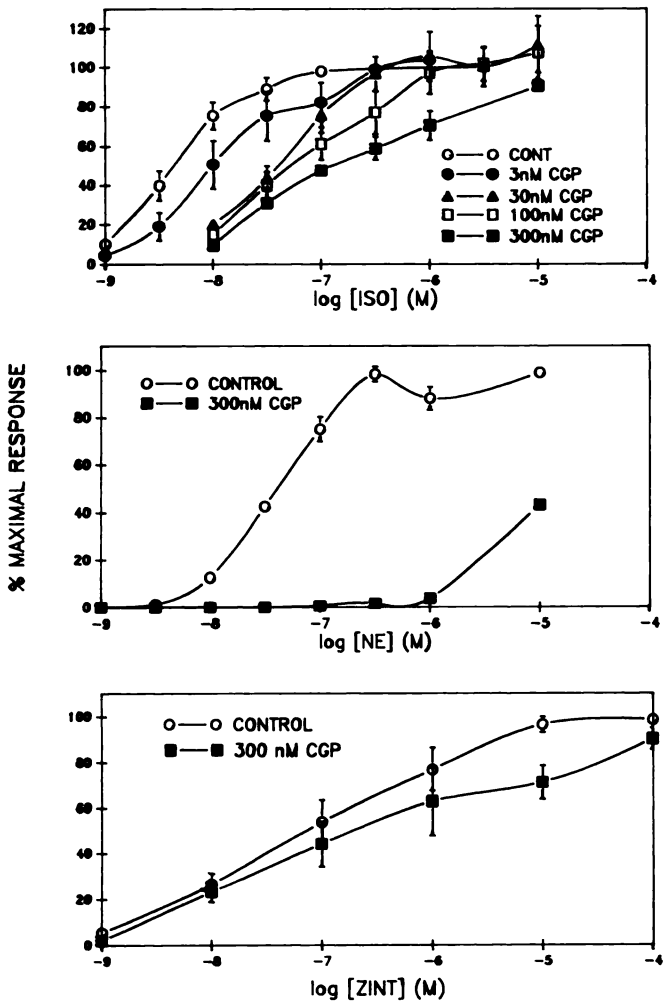


**Fig. 4.** Effect of the $\beta_1$-selective antagonist CGP on concentration-response curves for agonist-stimulated cAMP accumulation. Concentration-response curves for ISO (*Upper*), NE (*Middle*), or ZINT (*Lower*) in the absence or presence of various concentrations of CGP. Data are expressed as a percent of the maximum response to each individual agonist. 100% ISO response corresponds to 6.16 ± 0.62% conversion of $^3$H-ATP to $^3$H-cAMP. Basal response corresponds to 0.09 ± 0.03% conversion. Each value is the mean ± standard error of three experiments performed in duplicate.

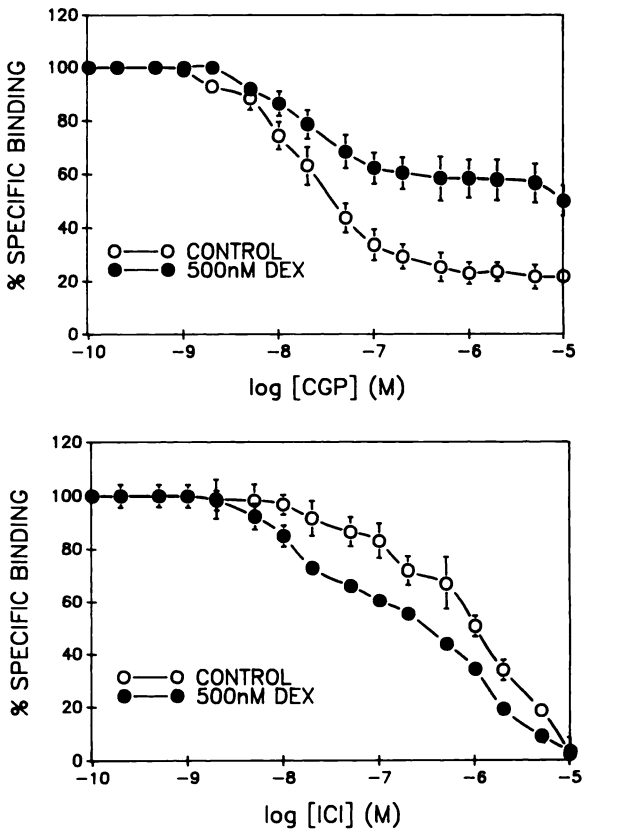


**Fig. 5.** Inhibition of $^{125}$ICYP binding (50 pM) by CGP (*Upper*) and ICI (*Lower*) in membranes from control and DEX- (500 nM, for 48 hr) treated $C_6$ cells. Each value is the mean ± standard error of three experiments performed in duplicate.

$\beta_1$-selective agonist NE, however, was almost completely blocked by inclusion of 300 nM CGP, indicating that it is mediated exclusively by $\beta_1$-ARs. In contrast, 300 nM CGP had no significant effect on the concentration-response curve to the $\beta_2$-selective agonist ZINT at concentrations of 1 $\mu$M or less, although some inhibition was observed at higher ZINT concentrations. This indicates that the response to ISO involves both $\beta_1$- and $\beta_2$-ARs in a complex interaction, the response to NE is mediated almost exclusively by $\beta_1$-ARs, while the response to relatively low concentrations of ZINT (<1 $\mu$M) are mediated almost exclusively by $\beta_2$-ARs. Based on these data, we chose responses to 0.1 $\mu$M NE and 0.1 $\mu$M ZINT as indices of $\beta_1$- and $\beta_2$-responses in further experiments.

**Treatment with DEX causes reciprocal changes in $\beta_1$- and $\beta_2$-AR density and responses.** In an attempt to alter the proportion of $\beta_1$- and $\beta_2$-ARs, we treated intact $C_6$ cells with the synthetic glucocorticoid DEX (500 nM) for 48 hr. Fig. 5 shows inhibition of $^{125}$ICYP binding by CGP ($\beta_1$-selective, *Upper*) and ICI ($\beta_2$-selective, *Lower*) in control and DEX-treated cells. DEX treatment significantly increased (20 to 60%) the proportion of the $\beta_2$-subtype (low affinity for CGP, high affinity for ICI) and decreased (80 to 40%) the proportion of the $\beta_1$ subtype (high affinity for CGP, low affinity for ICI) without significantly altering the total number of $^{125}$ICYP binding sites (not shown, see below). Similar results were observed when cAMP responses were compared in control and DEX-treated (500 nM, for 48hr) cells (Fig. 6). The total ($\beta_1$ + $\beta_2$) cAMP response was defined as the response to 1 $\mu$M ISO, the $\beta_2$-response as the response to 0.1 $\mu$M ZINT, and the $\beta_1$-response as the response to 0.1 $\mu$M NE (Fig. 3). DEX treatment decreased the $\beta_1$-response and increased the $\beta_2$-response without altering the total cAMP response (Fig. 6).

**Reciprocal changes show similar concentration- and time-dependence.** To further characterize the effects of DEX pretreatment, cells were treated with different concentrations of DEX for 48 hr, or with the same concentration of DEX for different times. Total $\beta$-ARs were measured by Scatchard analysis of specific $^{125}$ICYP binding, and $\beta_1$- and $\beta_2$-AR subtypes were distinguished with 300 nM CGP. Fig. 7 shows that DEX pretreatment did not change total $\beta$-AR density at any concentration tested (5 to 5000 nM) although the proportion of $\beta_1$- and $\beta_2$-AR subtypes changed dramatically. Scatchard analysis of $^{125}$ICYP binding in the presence of 10 $\mu$M CGP to block the $\beta_1$-subtype showed that DEX treatment caused $\beta_2$-AR to increase from about 20 fmol/mg protein to 50 fmol/mg protein, with an $EC_{50}$ for DEX around 5 nM (Fig. 7). However, a close reciprocal decrease in $\beta_1$-AR density (80 to 40 fmol/mg protein) resulted in little change in total $\beta$-AR density at any concen-

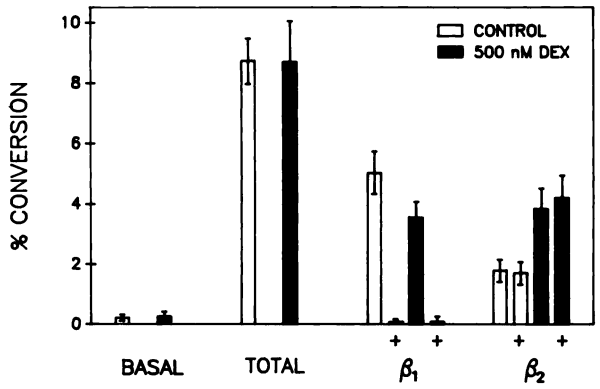


**Fig. 6.** Total, $\beta_1$-, and $\beta_2$-AR mediated cAMP responses in control (*blank bar*) and DEX- (*filled bar*) treated (500 nM, for 48 hr) cells. Total response is defined as the response to 1 μM ISO, $\beta_1$- as response to 0.1 μM NE, and $\beta_2$- as response to 0.1 μM ZINT. Note that the $\beta_1$-response is completely inhibited by 0.3 μM CGP (*bars with "+" sign under them*), but the $\beta_2$-response is unaffected by 0.3 μM CGP in either control or DEX-treated cells. Data are expressed as percent of conversion of [$^3$H]-ATP to [$^3$H]-cAMP. Each value is the mean ± standard error of data from three experiments performed in duplicate.

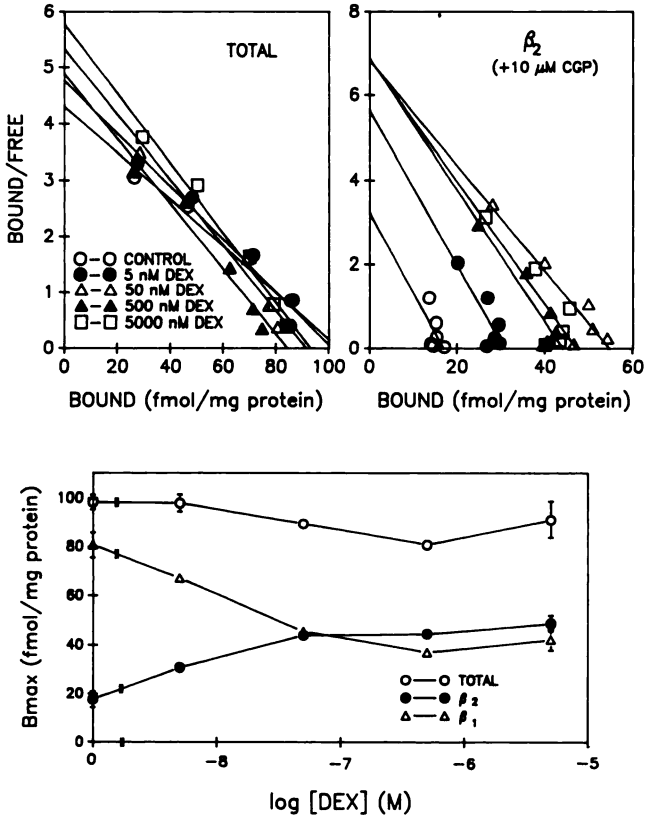


**Fig. 7.** Effect of increasing concentrations of DEX on total, $\beta_1$-, and $\beta_2$-ARs in $C_6$ cell membranes. Total β-AR density was determined by Scatchard analysis of specific $^{125}$ICYP binding (*Upper, left*) and $\beta_2$-ARs by Scatchard analysis of specific $^{125}$ICYP binding when $\beta_1$-ARs are blocked with 10 μM CGP (*Upper, right*). The lower panel shows the concentration-dependent effect of DEX treatment (48 hr) on total (*open circle*), $\beta_2$-AR (*filled circle*) and calculated $\beta_1$-AR (difference between total and $\beta_2$-AR density, *open triangle*) binding sites. Each value is the mean ± standard error of data from three experiments performed in duplicate.

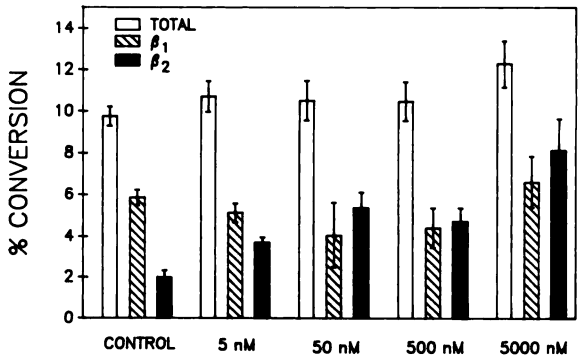


**Fig. 8.** Effect of different concentrations of DEX (48 hr) on total, $\beta_1$-AR-, and $\beta_2$-AR-mediated cAMP responses. Total, $\beta_1$-, and $\beta_2$-AR-mediated response are defined as in Fig. 6. Data are expressed as a percent of total incorporated [$^3$H]-ATP converted to [$^3$H]-cAMP. Each value is the mean ± standard error of data from three experiments performed in duplicate.

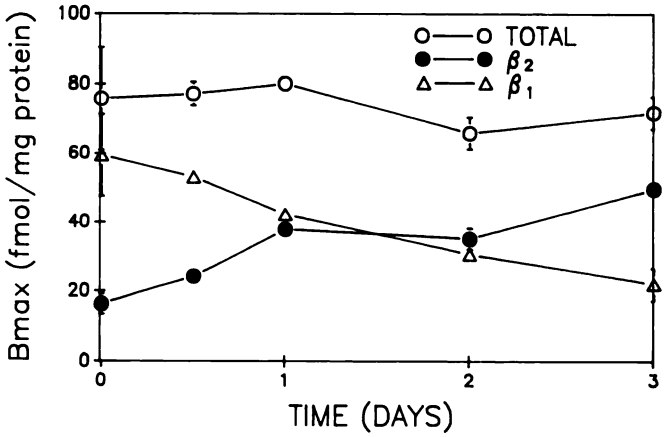


**Fig. 9.** Time course for effects of DEX (500 nM) treatment on total, $\beta_1$-, and $\beta_2$-AR binding sites in $C_6$ cells. Total, $\beta_1$-, and $\beta_2$-AR binding sites were measured or calculated as described in Fig. 7. Each value is the mean ± standard error of data from three experiments performed in duplicate.

tration of DEX examined. Fig. 8 shows the concentration-dependence of DEX pretreatment on cAMP responses to ISO and selective agonists. There was a concentration-dependent increase in the $\beta_2$-response and a reciprocal, although less robust decrease in the $\beta_1$-response. No significant change in the response to ISO was observed with any concentration of DEX examined.

Fig. 9 shows the time course for changes in $\beta_1$- and $\beta_2$-AR density induced by DEX (500 nM) pretreatment. There was a time-dependent increase in the density of $\beta_2$-ARs, and a reciprocal decrease in the density of $\beta_1$-ARs. Again, little change was observed in total β-AR density after any time of DEX exposure. Indeed, treatment with DEX for 3 days further increased the proportion of $\beta_2$-ARs to about 80%, essentially reversing the subtype ratio found in control cells. (Fig. 9).

**Effect of other steroids on $\beta_1$- and $\beta_2$-responses in $C_6$ cells.** To examine the specificity of the DEX-induced changes, we compared total, $\beta_1$-, and $\beta_2$-cAMP responses in $C_6$ cells pretreated for 48 hr with DEX (0.1 μM), the naturally occurring glucocorticoid hydrocortisone (1 μM), and the mineralocorticoid deoxycorticosterone acetate (1 μM). Table 2 shows that none of these treatments significantly affected the total response to 1 μM ISO or the $\beta_1$- response to 100 nM NE, however both

TABLE 2

**Effect of gluco- and mineralo-corticoid pretreatment on cAMP responses in $C_6$ glioma cells**

Cells were treated with no drug (Control), 1 μM desoxycorticosterone acetate (DOCA), 1 μM hydrocortisone, or 100 nM dexamethasone for 48 hr. Total, $\beta_1$- and $\beta_2$-AR mediated cAMP responses were determined as described in the legend to Fig. 6. Each value is the mean ± SE of data from three experiments performed in duplicate. Data are expressed as % conversion of $^3$H-ATP to $^3$H-cAMP.

| | Control | DOCA | Hydrocortisone | Dexamethasone |
|---|---|---|---|---|
| Basal | 0.27 ± 0.06 | 0.23 ± 0.03 | 0.23 ± 0.03 | 0.28 ± 0.04 |
| Total | 11.9 ± 2.8 | 11.9 ± 2.3 | 11.8 ± 2.21 | 14.3 ± 2.7 |
| $\beta_1$ | 8.6 ± 1.6 | 8.83 ± 2.04 | 7.8 ± 1.5 | 8.1 ± 1.6 |
| $\beta_2$ | 4.2 ± 0.8 | 4.95 ± 1.08 | 8.0 ± 1.8 | 8.4 ± 1.9 |

TABLE 3

**Non-additivity of cAMP responses stimulated by subtype-selective agonists (NE and ZINT) or by non-selective agonist ISO in DEX (500 nM, 48 hr)-treated cells**

The concentration of NE and ZINT used activated only $\beta_1$- and $\beta_2$-AR, respectively (Fig. 6). No significant differences were found between responses caused by ZINT and ZINT + NE, or by ISO, ISO + NE, and ISO + ZINT using a *t* test. Each value is the mean ± SE of data from at least two experiments performed in duplicate. Basal levels of response (0.08 ± 0.01) are subtracted from all the data. Data are expressed as % conversion of $^3$H-ATP to $^3$H-cAMP.

| Agonists | cAMP Response |
|---|---|
| 0.1 μM ZINT | 8.95 ± 0.77 |
| 0.1 μM NE | 4.33 ± 0.62 |
| 0.1 μM ZINT + 0.1 μM NE | 9.35 ± 0.50 |
| 3.0 μM ISO | 12.21 ± 1.58 |
| 3.0 μM ISO + 0.1 μM NE | 13.39 ± 1.0 |
| 3.0 μM ISO + 0.1 μM ZINT | 14.19 ± 2.4 |

DEX and hydrocortisone (but not deoxycorticosterone acetate) increased the $\beta_2$-response to 200 nM ZINT.

**$\beta_1$- and $\beta_2$-AR mediated cAMP responses are not additive.** The additivity of cAMP responses to selective agonists was examined in cells pretreated with 500 nM DEX for 48 hr, where substantial responses to both subtypes are observed. Table 3 indicates that when $\beta_1$- and $\beta_2$-selective agonists (NE and ZINT) are combined in DEX-pretreated cells, the overall cAMP responses are not additive, despite the fact that this response is less than the maximal response to ISO. Also, neither NE nor ZINT further increased the cAMP response to a maximal ISO concentration.

**DEX increases $\beta_2$-AR mRNA without altering $\beta_1$-AR mRNA.** Fig. 10 shows that treatment of $C_6$ cells with 50 or 500 nM DEX for 48 hr increased levels of $\beta_2$-AR mRNA by 2 and 3 times, respectively, consistent with the increase in $\beta_2$-AR density. However, DEX treatment had little effect on levels of $\beta_1$-AR mRNA, despite the reduction in $\beta_1$-AR density.

**Catecholamines still show a $\beta_1$-AR order of potency after DEX treatment.** To examine the effects of changing the β-AR subtype ratio on cellular responses to agonists, concentration-response curves for stimulation of cAMP formation by ISO, EPI, NE and ZINT were examined in DEX-pretreated (500 nM, for 48 hr) cells. Fig. 11 shows that responses to the $\beta_2$-selective agonist ZINT occur at lower concentrations and are larger relative to the ISO maximum in DEX-treated cells compared with control cells (Fig. 2). This is consistent with the DEX-induced increase in $\beta_2$-AR density documented above. Surprisingly, $EC_{50}$ values for ISO, EPI and NE were not changed by DEX pretreatment (Table 4). ISO was still most potent, and EPI and NE were still approximately equally potent and efficacious in causing cAMP accumulation after DEX

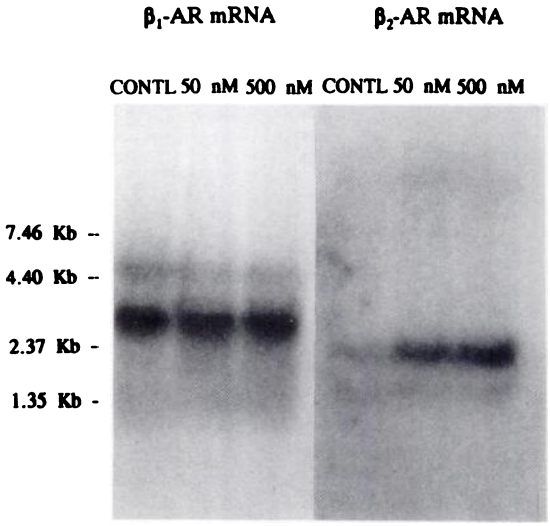


**Fig. 10.** Northern blot analyses of poly(A)$^+$ RNA from $C_6$ cells treated with 0 (Contl.), 50, or 500 nM DEX for 48 hr. The RNA blot was first hybridized with a random primed full length human $\beta_2$-cDNA probe and exposed to film for 6 days. After stripping clean, the blot was then hybridized with a random primed human $\beta_1$-cDNA probe and exposed to film for 3 days. Size was determined with a GIBCO RNA ladder.

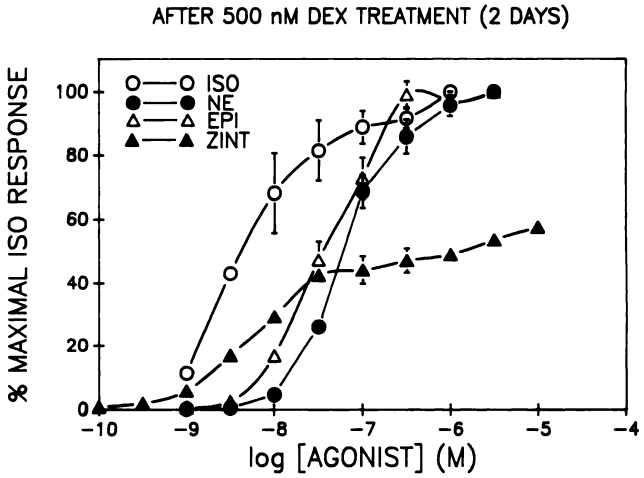


**Fig. 11.** Concentration-response curves for ISO-, NE-, EPI-, and ZINT-stimulated cAMP formation in DEX-treated (500 nM, for 48 hr) $C_6$ cells. Data are expressed as a percent of the maximal response to ISO. 100% ISO response corresponds to 10.70 ± 1.15% conversion of [$^3$H]-ATP to [$^3$H]-cAMP and basal response corresponds to 0.04 ± 0.003%. Each value is the mean ± standard error of data from three experiments performed in duplicate.

treatment (Fig. 11), despite the reversal in the proportion of $\beta_1$- and $\beta_2$-AR subtypes.

**Are $\beta_3$-ARs present in $C_6$ cells?.** To examine if $\beta_3$-ARs might also co-exist in $C_6$ cells, we studied the cAMP response to the $\beta_3$-selective agonist CGP 12177. As shown in Fig. 12, CGP 12177 gave a concentration-dependent increase in cAMP formation, but the maximal response was only 6% of the response to 1 μM ISO (Fig. 12, *Upper*). In addition, the cAMP response to 0.1 μM CGP 12177 was completely inhibited by propranolol (Fig. 12, *Lower*), indicating possible involvement of $\beta_1$- and $\beta_2$-AR in mediating the response. In DEX-pretreated cells (500 nM, 48 hr), the response to CGP 12177 was diminished to about 3% of the ISO response, and was still inhibited by propranolol (data not shown).

TABLE 4

**$EC_{50}$ values for NE-, EPI-, and ISO-stimulated cAMP responses in control and DEX (500 nM, 48 hr)-treated cells**

There were no significant difference between the $EC_{50}$ values of any agonist in control and DEX-treated cells by Student's *t* test. Each value is the mean ± SE of data from the number of experiments indicated in parentheses, performed in duplicate.

| Agonist | logEC₅₀ | |
|---|---|---|
| | Control | Dexamethasone-treated |
| | *M* | |
| (−)-NE | −7.39 ± 0.07 (*n* = 10) | −7.19 ± 0.06 (*n* = 5) |
| (−)-EPI | −7.13 ± 0.12 (*n* = 5) | −7.39 ± 0.09 (*n* = 4) |
| (−)-ISO | −8.22 ± 0.21 (*n* = 5) | −8.10 ± 0.05 (*n* = 3) |

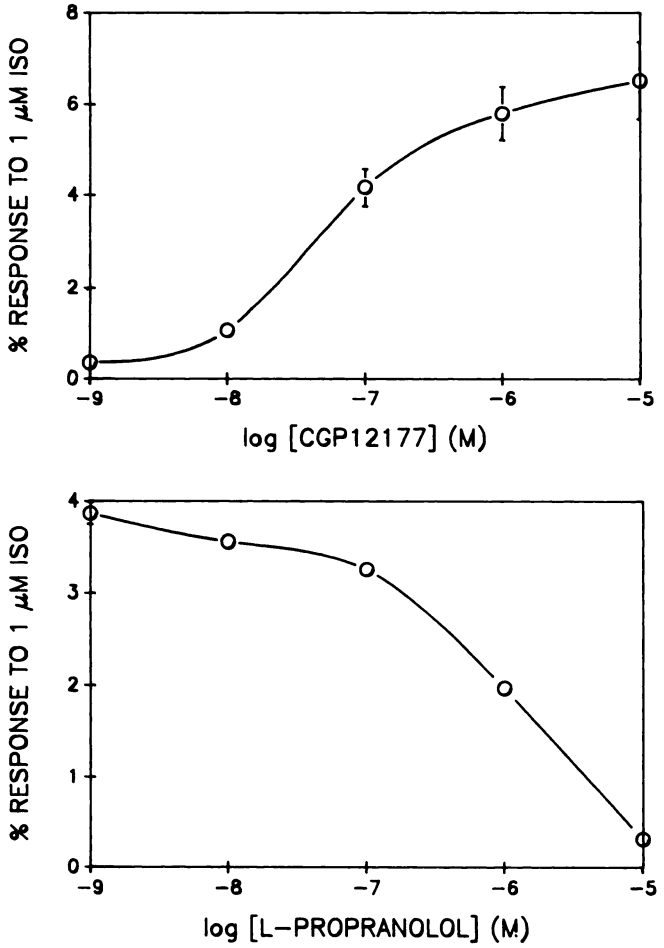


**Fig. 12.** Concentration-response curve for CGP 12177-stimulated cAMP formation (*Upper*) and inhibition of the response to 0.1 μM CGP 12177 by propranolol (*Lower*). Data are expressed as a percent of the maximal response to ISO (3 μM). Each value is the mean ± standard error of data from two experiments performed in duplicate.

## Discussion

Increasing numbers of distinct receptor subtypes are being identified for a variety of hormones and neurotransmitters. However, the functional significance of these multiple subtypes is not yet clear. Mixtures of receptor subtypes for a single neurotransmitter probably co-exist on many cells, and interactions between these subtypes are likely to be important in cell signalling. Because $\beta_1$- and $\beta_2$-ARs co-exist in $C_6$ glioma cells (11), we used this homogeneous cell population to study the interactions between closely related subtypes. Previous studies have examined the differential regulation of these two subtypes by atypical (25) and typical (26) agonist exposure, and their relative rates of reappearance after inactivation or down-regulation (27). However, the role of each subtype in cell signalling, and the importance of the ratio of subtypes in responses to transmitter have not been previously examined.

We found that $\beta_1$- and $\beta_2$-ARs co-exist in $C_6$ cells in approximately an 80:20 ratio, in agreement with Homburger *et al.* (11). A much higher proportion of the $\beta_2$- subtype (66%) had been reported by Neve *et al.* (25). However, Hough and Chang (26) also found substantially higher concentrations of $\beta_1$- than $\beta_2$-mRNA in these cells. Catecholamines activated cAMP accumulation with an order of potency expected for $\beta_1$-ARs (ISO > NE = EPI), consistent with this being the dominant subtype in these cells. However, activation of $\beta_2$-ARs also increased cAMP, because responses to low concentrations of the $\beta_2$-selective agonist ZINT were selectively blocked by the $\beta_2$-selective antagonist ICI.

The use of selective agonists and antagonists showed that the contribution of each subtype to the cAMP response depended on agonist selectivity and concentration. A low concentration of the $\beta_1$-selective antagonist CGP (3 nM) caused a parallel shift to the right in the concentration-response curve for the non-subtype-selective agonist ISO, indicating a primarily $\beta_1$-type response. However, at higher CGP concentrations, concentration-response curves for ISO became biphasic, and the lower portion of the curve became insensitive even to relatively high concentrations of CGP. These results indicate that $\beta_2$-ARs also contribute to ISO responses, particularly at higher ISO concentrations. The different concentrations of ISO required for activation of each subtype indicate that these two subtypes may have differential receptor reserves in these cells. The $\beta_1$-selective agonist NE, on the other hand, stimulated cAMP accumulation exclusively through the $\beta_1$-subtype at the concentrations tested, because this response was completely blocked by 0.3 μM CGP. These results show that different agonists can activate responses through different proportions of co-existing subtypes, depending on the selectivity and concentration of the agonist, and the receptor reserves for each subtype.

To compare responses with individual subtypes, selective agonists and antagonists were used to isolate responses to a single subtype. The $\beta_1$-response was defined as the response to 100 nM NE, which was totally inhibited by CGP; the $\beta_2$-response was defined as the response to 100 nM ZINT, which was not inhibited by CGP. Although not necessarily maximal, these responses can be used to monitor changing responses to individual subtypes, allowing comparison to the more complex responses to catecholamines.

Glucocorticoids are known to have prominent effects on ARs, and have been shown to up-regulate $\beta_2$-ARs in many tissues and cell lines. It is now known that glucocorticoids increase the rate of $\beta_2$-AR gene transcription (22, 23) through a glucocorticoid response element in the 5′ flanking region of the gene (24). Glucocorticoids also have prominent effects on other AR subtypes, reducing the density of $\beta_1$- (16, 17) and $\beta_3$- (19) ARs, and increasing the expression of $\alpha_{1B}$-ARs (18). A glucocorticoid response element has been identified in the 5′ flanking region

of the human $\beta_1$-AR gene (34). In 3T3-L1 preadipocytes and adipocytes, low concentrations of DEX increased expression of $\beta_2$-ARs but reduced expression of $\beta_1$-ARs, with a net 2- to 3-fold increase in $\beta$-AR density (16, 35). An inverse regulation of mRNA levels for the two subtypes by DEX was also observed (35). DEX-induced alterations in subtype ratio increased the potency of ISO 6- to 10-fold in activating adenylate cyclase, consistent with the increased total $\beta$-AR density, although responses to individual subtypes were not monitored. Later studies showed that $\beta_3$-ARs were also expressed in this cell line after differentiation (19), and that this subtype was also down-regulated by DEX treatment. Because of the prominent reciprocal effects of glucocorticoids on $\beta_1$- and $\beta_2$-AR densities, we used this approach to examine the importance of subtype ratio on responses to catecholamines.

We found that DEX treatment of $C_6$ cells dose- and time-dependently increased the density of $\beta_2$-ARs, but that this was associated with a close reciprocal decrease in the density of $\beta_1$-ARs. Thus, little or no change in total receptor density occurred after DEX treatment, despite the almost complete reversal in subtype ratio. Treatment with DEX also increased $\beta_2$- and slightly decreased $\beta_1$-mediated cAMP responses, but did not alter the response to the non-subtype-selective agonist ISO. There appeared to be a close link between decreases in $\beta_1$-ARs and increases in $\beta_2$-ARs in $C_6$ cells. This link was not observed in 3T3-L1 adipocytes where glucocorticoid-induced increases in the proportion of $\beta_2$-ARs were associated with a large increase in total $\beta$-AR density (16, 35). However, these cells are differentiating from preadipocyte to adipocyte-like character, which further complicates interpretation of the glucocorticoid-induced AR regulation. The situation is simpler in $C_6$ cells, where no such differentiation is induced by DEX. The close inverse regulation of $\beta_1$- and $\beta_2$-ARs in $C_6$ cells raises the possibility that there may be reciprocal mechanisms controlling the ratio of these subtypes, which may serve to control the overall response to catecholamines.

Similar to other systems studied previously, DEX treatment up-regulated $\beta_2$-AR mRNA levels, consistent with the increase in receptor density. However, the mechanism involved in the DEX-induced reduction in $\beta_1$-AR density remains to be defined. This mechanism does not seem to involve changes in transcription, because DEX caused substantial decreases in the density of $\beta_1$-ARs without any observable changes in mRNA levels. Although different processes appear to be involved in regulation of these two closely related subtypes, the close reciprocal relationship observed during treatment with DEX argues for some relationship between the two regulatory mechanisms.

Surprisingly, we found that DEX treatment did not alter catecholamine responses in $C_6$ cells. Despite the dominance of the $\beta_2$-subtype and the increased $\beta_2$-responses observed after DEX treatment, we found that the concentration-response curves to catecholamines were not substantially altered compared with control cells. There was no change in maximum response to ISO, EPI, or NE despite the increased response to the $\beta_2$-selective agonist ZINT. There was also no change in the relative or absolute potencies of the catecholamines in activating cyclic AMP accumulation. In particular, there was no decrease in the potency of NE, despite the fact that $\beta_2$-ARs, which numerically predominate after DEX treatment, have an affinity for NE 20 times lower than do $\beta_1$-ARs. This may be a result of the presence of differential receptor reserves for $\beta_1$- and $\beta_2$-ARs in $C_6$ cells. In control cells, some evidence indicates that $\beta_1$-ARs (about 80% of the total) have a larger receptor reserve than do $\beta_2$-ARs. If so, then decreases in $\beta_1$-AR density after DEX treatment may decrease, but not eliminate, this receptor reserve. In this case, $\beta_1$-selective agonists like NE could still maximally activate cAMP responses with a potency not substantially lower than that in control cells. Similarly, the increases in the $\beta_2$-AR number after DEX-treatment may not be large enough to allow increasing cAMP responses in the presence of an efficiently-coupled $\beta_1$-AR population. Such an explanation requires that $\beta_1$-ARs couple more efficiently to adenylate cyclase activation than do $\beta_2$-ARs in $C_6$ cells. It is interesting, however, that when $\beta_1$- and $\beta_2$-AR subtypes are transfected into CHO cells at similar densities, $\beta_2$-ARs couple more efficiently to G proteins and adenylate cyclase activation than do $\beta_1$-ARs (36). If this were true in $C_6$ cells, changes in the receptor should affect $\beta_2$-responses more than $\beta_1$-responses, which was not observed. Although further analysis is needed, these data indicate that changes in ratios of closely related subtypes will not necessarily predict catecholamine responsiveness.

The presence of $\beta_3$-ARs in $C_6$ cells may complicate interpretation of these experiments. Therefore, we performed functional studies with $\beta_3$-selective agonists. We found that a number of $\beta_3$-selective agonists (hydroxybenzylpindolol, pindolol, BRL 37344, and CGP 12177) could increase cAMP in these cells, although their responses were very small. For example, CGP 12177 gave a maximal response only 5% of the maximal ISO response, which was not increased after DEX pretreatment. Also, these responses were totally blocked by the $\beta_1$- and $\beta_2$- selective antagonist propranolol. Northern blot analysis with rat $\beta_3$-AR cDNA (37) showed faint hybridization bands after long exposure times which co-localized with those of either $\beta_1$- or $\beta_2$-ARs (data not shown). Although we cannot conclude that there are no $\beta_3$-ARs in $C_6$ cells, if they do exist they are a very minor component of the total $\beta$-AR population.

In summary, we found that $\beta_1$- and $\beta_2$-ARs co-existing in $C_6$ glioma cells both activate cAMP accumulation, although with different efficiencies and receptor reserves. DEX treatment caused a close reciprocal regulation of $\beta_1$- and $\beta_2$-ARs through different mechanisms, dramatically changing the subtype ratio without changing total receptor density. Alterations in subtype ratio paralleled changing responses to individual receptor subtypes, but did not alter responses to catecholamines. These results indicate that closely related subtypes co-existing on the same cell can contribute to the same biological response, and that subtype ratios can be altered by hormone treatment. However, the densities and ratios of closely related subtypes are not related in a simple manner to cellular responsiveness. The biological importance and therapeutic significance of such changing subtype ratios remain to be determined.

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