Glucocorticoids down-regulate β_1 -adrenergic-receptor expression by suppressing transcription of the receptor gene

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The expression of β_2 -adrenergic receptors is up-regulated by glucocorticoids. In contrast, β_1 -adrenergic receptors display glucocorticoid-induced down-regulation. In rat C6 glioma cells, which express both of these subtypes of β -adrenergic receptors, the synthetic glucocorticoid dexamethasone stimulates no change in the total β -adrenergic receptor content, but rather shifts the β_1 : β_2 ratio from 80:20 to 50:50. Radioligand binding and immunoblotting demonstrate a sharp decline in β_1 -adrenergic receptor expression. Metabolic labelling of cells with [³⁵S]-methionine in tandem with immunoprecipitation by β_1 -adrenergic-receptor-specific antibodies reveals a sharp decline in the synthesis of the receptor within 48 h for cells challenged with glucocorticoid. Steady-state levels of β_1 -adrenergic-receptor

mRNA declined from 0.47 to 0.26 amol/ μ g of total cellular RNA within 2 h of dexamethasone challenge, as measured by DNA-excess solution hybridization. The stability of receptor mRNA was not influenced by glucocorticoid; the half-lives of the β_1 - and β_2 -subtype mRNAs were 1.7 and 1.5 h respectively. Nuclear run-on assays revealed the basis for the down-regulation of receptor expression, i.e. a sharp decline in the relative rate of transcription for the β_1 -adrenergic-receptor gene in nuclei from dexamethasone-treated as compared with vehicle-treated cells. These data demonstrate transcriptional suppression as a molecular explanation for glucocorticoid-induced down-regulation of β_1 -adrenergic receptors.

INTRODUCTION

The regulation of the expression of β -adrenergic receptors is not thoroughly understood (Stiles et al., 1984; Wang et al., 1990). These receptors are subdivided into three subtypes, β_1 -, β_2 - and β_3 -, each subtype encoded by a separate gene (for references, see Emorine et al., 1989). β_1 - and β_2 -adrenergic receptors have been differentiated by pharmacological (Lands et al., 1967), structural (Cubero and Malbon, 1984; Graziano et al., 1985; Moxham et al., 1986; Wang et al., 1989) and genetic (Dixon et al., 1986; Frielle et al., 1987; Bahouth et al., 1988) criteria. Comparison of the sequences of the β_1 - and β_2 -receptor cDNAs revealed only a 55% similarity in the coding region (Dixon et al., 1986; Frielle et al., 1987). This divergence in nucleotide sequence permitted the construction of subtype-specific DNA probes for use in measuring mRNA levels (Guest et al., 1990). Glucocorticoid and thyroid hormones have been shown to regulate the expression of β -adrenergic receptors (Malbon, 1980; Sibley and Lefkowitz, 1985; Davies and Lefkowitz, 1984; Hadcock and Malbon, 1988a; Collins et al., 1988, 1991). Similarly, cyclic AMP and retinoic acid also regulate β -adrenergic receptor expression (Hadcock and Malbon, 1988a; Hadcock et al., 1989a; Galvin-Parton et al., 1990). β_2 -Adrenergic receptor expression increases in many cell types in response to glucocorticoids (for references, see Collins et al., 1988; Hadcock and Malbon, 1988b; Malbon and Hadcock, 1988). In contrast, far less is known about the regulation of β_1 -adrenergic receptors by steroids. Recent studies of mouse NIH 3T3-L1 and 3T3F442A cells demonstrated that the expression of β_1 - and β_2 -adrenergic receptors is altered as these cells progress through differentiation induced by glucocorticoids in combination with other factors (Guest et al., 1990; Feve et al., 1990). To examine regulation of β_1 -adrenergic receptors by gluco-

corticoids independent of those effects accompanying differentiation, we investigated regulation of β_1 -adrenergic receptors by glucocorticoids in terminally differentiated rat C6 glioma cells. The data are the first to provide a molecular explanation, i.e. transcriptional suppression, for the effect of glucocorticoids on the expression of β_1 -adrenergic receptors.

MATERIALS AND METHODS

The hamster β_2 -adrenergic-receptor cDNA (Dixon et al., 1986) was a gift from Dr. R. A. F. Dixon, Merck, Sharp and Dohme, West Point, PA, U.S.A. The sources of all other material is detailed elsewhere (Hadcock and Malbon, 1988a) and in the text. The human β_1 -adrenergic-receptor cDNA was generously given by Dr. R. J. Lefkowitz, HHMI, Duke University, Durham, NC, U.S.A.

Cell culture

Rat C6 glioma cells were grown to confluence as monolayers in Dulbecco's modified Eagle's medium (DMEM), supplemented with 5% fetal-bovine serum, streptomycin (60 mg/l) and penicillin (60 mg/l), as described by Fishman et al. (1981). Stock solutions of steroids were prepared in an ethanol vehicle and then diluted directly in DMEM. Untreated (control) cells were grown in the same DMEM to which only the vehicle was added.

Membrane preparation

Membrane fractions were prepared as described by Fishman et al. (1981), a lysis buffer composed of 2 mM Tris/HCl (pH 7.4), 1 mM EDTA, aprotinin (5 μ g/ml) and leupeptin (5 μ g/ml).

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; [¹²⁵I]CYP, (–)-[¹²⁵I]iodocyanopindolol; GRE, glucocorticoid response element.

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Radioligand binding

β -adrenergic receptors were measured in a crude membrane fraction of the post-nuclear supernatant, obtained by first depleting the broken-cell preparation of nuclei with a low-speed centrifugation (5000 *g*, 5 min), followed by a high-speed centrifugation (48 000 *g*_{av}) for 20 min at 4 °C. The resultant pellet collected by this final centrifugation step was resuspended in binding-assay buffer. Equilibrium radioligand-binding analysis was performed as described by Fishman et al. (1981) with the antagonist ligand (–)-[¹²⁵I]-iodocyanopindolol ([¹²⁵I]CYP; 50–100 pM). Non-specific [¹²⁵I]CYP binding insensitive to competition by 10 μ M propranolol represented about 10% of the radioligand binding. Binding data were corrected for the non-specific component. Specific binding of [¹²⁵I]CYP to β_1 -adrenergic receptors was measured by competition binding with 0.1 μ M of the β_1 -selective antagonist CGP-20712A (Bahouth and Malbon, 1987). This concentration of CGP-20712A has been shown to displace virtually all β_1 -receptor binding in membranes of C6 glioma cells (Zhong and Minneman, 1993).

Immunoblotting

Crude membranes were solubilized in 0.125 M Tris/HCl (pH 6.8) containing 4% SDS, 5% sucrose and 20 mM dithiothreitol, and incubated at 37 °C for 30 min. The samples were alkylated with 45 mM iodoacetamide and the solubilized proteins were separated by electrophoresis on 10%-acrylamide gels. The resolved proteins were transferred electrophoretically to nitrocellulose (Moxham et al., 1986). The nitrocellulose blots were stained with a β_1 -adrenergic-receptor-specific antibody (SB-03) prepared against a synthetic peptide corresponding to a sequence unique to this subtype (Bahouth, 1991), at a final dilution of 1:200. The blots were then probed with phosphatase-labelled or [¹²⁵I]-labelled goat anti-rabbit IgG, and the immune complexes were made visible by phosphatase-catalysed staining or autoradiography, respectively (Smith et al., 1987). Characterization of this antibody is provided elsewhere (Bahouth, 1991).

Metabolic labelling of cells

Cells, cultured as described above, were washed twice with PBS and then cultured in methionine-free DMEM containing 5% fetal-calf serum. To each batch of 10⁷ cells, [³⁵S]methionine (200 μ Ci) and either vehicle or dexamethasone (500 nM) was added. Cells were harvested at the times indicated, washed twice with PBS, and then used as a source for the preparation of crude membranes. Immunoprecipitation from detergent extracts of the crude membranes was performed as described by Hadcock et al. (1990).

Extraction of RNA

RNA was extracted by the guanidine isothiocyanate/ethanol precipitation method (Chirgwin et al., 1979). The integrity of the RNA was assessed by gel electrophoresis on 1.2% agarose in 3% formaldehyde (Sambrook et al., 1989).

Synthesis of solution probes H β 110 (β_1 -receptor) and H β 170 (β_2 -receptor)

The solution hybridization probe (β AR 170) was constructed as described previously (Hadcock and Malbon, 1988a; Hadcock

et al., 1989b). A DNA fragment corresponding to the sequence +265 to +376 of the human β_1 -adrenergic-receptor cDNA coding region was subcloned into the phagemid Bluescript SK⁺. The single-stranded probe, H β 110, was generated by the VSC M₁₃ helper-phage procedure of Vierra and Messing (1987). The probes, H β 110 and H β 170, were prepared as described by Williams et al. (1986), as modified by Bahouth et al. (1988). The probes were uniformly radiolabelled with [α -³²P]dCTP (sp. radioactivity 222 Ci/mmol) by primer extension, followed by digestion with *Sma*I. Single-stranded probes were separated from double-stranded DNA by gel chromatography on hydroxyapatite using a step gradient of sodium phosphate (20–220 mM), followed by desalting on NEN-sorb columns (New England Nuclear).

DNA-excess solution-hybridization assay of receptor mRNA levels

Uniformly radiolabelled probe [1.5 fmol (100 pg) per sample] was incubated with known amounts of template DNA (used as a standard), total cellular RNA, or alone for 60 h at 68 °C in 20 mM Hepes (pH 7.0)/0.3 M NaCl/1 mM EDTA containing 100 μ g/ml denatured salmon sperm DNA. S1 endonuclease (150 units/ml), denatured salmon sperm DNA (50 μ g/ml) and S1-endonuclease buffer (0.28 M NaCl, 4.5 mM ZnSO₄, 50 mM sodium acetate, pH 4.5) were then added to each sample, and the mixture was incubated for 90 min at 42 °C. The samples were treated with trichloroacetic acid (7.5%) and precipitated on ice for 10 min. The S1-endonuclease-resistant hybrids were collected by vacuum filtration on Whatman GF/C filters. The extent of internal resistance of the probe to S1-endonuclease treatment was typically less than 3% for each assay. Data from DNA-excess solution-hybridization assays were calculated as described previously (Hadcock and Malbon, 1988a; Williams et al., 1986) and expressed either as amol of β -adrenergic-receptor mRNA per μ g of total cellular RNA, or '% of control'.

Receptor-mRNA stability

The half-life of β -adrenergic-receptor mRNA was determined by the method of Rodgers et al. (1985), as previously reported (Hadcock et al., 1989a). Cells were exposed to dexamethasone (500 nM) or vehicle for 2 h. Actinomycin D (5 μ g/ml) was added thereafter, and RNA was isolated from samples taken over the next 6 h period. The content of β_1 - and β_2 -adrenergic-receptor mRNA in the total cellular RNA was quantified by DNA-excess solution hybridization.

Nuclear run-on transcription assays

Cells were cultured in DMEM supplemented with 5% fetal-bovine serum depleted of steroids by prior treatment with 0.1% activated charcoal. Dexamethasone (250 nM) or vehicle was added to the culture medium for 2 h, and the cells were then washed twice with ice-cold PBS. Initial studies of transcription by the method of Greenberg and Ziff (1984) using frozen nuclei provided no clear effect of the glucocorticoid treatment in run-on assays using nuclei from steroid-treated cells. An alternative modification was employed, as described by Bahouth (1991). To each 100 mm dish, 1 ml of buffer I (10 mM Tris/HCl, pH 8.0, 10 mM NaCl, 2.5 mM MgCl₂ and 5 mM dithiothreitol) was added for 10 min. A 1 ml portion of buffer I containing 0.6 M sucrose and 0.6% Triton X-100 was then added, and the cells were scraped into a chilled Dounce homogenizer. The cells were homogenized by six strokes with a type A pestle, and the homogenate was layered over 0.6 M sucrose in buffer I. Nuclei were collected by centrifugation at 2000 *g*_{av} for 10 min and

resuspended in glycerol buffer (50 mM Tris/HCl, pH 8.3, 40 % glycerol, 5 mM $MgCl_2$ and 0.1 mM EDTA) for immediate assay (Greenberg and Bender, 1989). Frozen nuclei were found to be unsuitable for the nuclear run-on assays. To detect nascent transcripts, nuclei $[(2-3) \times 10^7]$ in 200 μ l were added to 200 μ l of a reaction buffer composed of 10 mM Tris/HCl, pH 8.0, 5 mM $MgCl_2$, 0.3 M KCl, 5 mM dithiothreitol, 0.5 mM unlabelled GTP, ATP and CTP, and 10 μ l of [α - ^{32}P]UTP (New England Nuclear, 800 Ci/mmol; final concn. 0.5 mM UTP) for a 60 min reaction at 30 °C. Newly transcribed labelled RNA was extracted (Greenberg and Ziff, 1984) and then hybridized for 36 h at 65 °C with plasmid DNA immobilized on nitrocellulose. After hybridization, each sample was washed twice with $2 \pm$ SSC (SSC is 150 mM NaCl/15 mM sodium citrate, pH 7.0) for 60 min at 65 °C. The samples were then treated with RNAase A for 30 min at 37 °C, followed by a wash with $2 \times$ SSC at 37 °C for 60 min. The filters were dried and subjected to autoradiography for 5 days with an intensifying screen. Relative changes in transcription were assessed by scanning densitometry of the autoradiogram. The absorbance of the β_1 -adrenergic receptor band was normalized against that of the α -tubulin band, to correct for any differences in hybridization.

The plasmid harbouring the rat β_1 -adrenergic-receptor cDNA was generated by subcloning the 1765-bp *SlyI* fragment of the rat β_1 -adrenergic-receptor gene (nucleotides -274 to +1418 relative to the translational start site) into pBluescript SK⁺ (Shimomura and Terada, 1990). The rat β_2 -adrenergic-receptor plasmid RHB-DAR was purchased from the American Type Culture Collection (Rockville, MD, U.S.A.). This plasmid contains the sequence -101 to +1878 relative to the translational start site (Cocayne et al., 1987). The avian α -tubulin cDNA in plasmid pT₁ was generously given by Donald W. Cleveland (HHMI, Johns Hopkins University Medical Center). The β -adrenergic-receptor plasmids were linearized by *EcoRI* and 10 μ g of plasmid/lane was used. The pT₁ plasmid was linearized by *PstI*, and 5 μ g/lane was employed.

Presentation of data

Unless noted otherwise, data are presented as mean $\pm$ S.E.M. Statistical significance was determined by Student's *t* test.

RESULTS

Rat C6 glioma cells express both β_1 - and β_2 -adrenergic receptors (Table 1). The ratio of β_1 - to β_2 -subtype was determined by competition with the β_1 -adrenergic-receptor-selective antagonist CGP-20712A (Bahouth and Malbon, 1987). Under standard culture conditions, C6 glioma cells at confluence displayed 235 ± 50 fmol of β_1 -adrenergic receptors/mg of protein (mean $\pm$ S.E.M., $n = 10$), of which nearly 80 % (185 ± 40 fmol/mg) was β_1 and the remaining 20 % (52 ± 9 fmol/mg) was β_2 -subtype. Treatment of these cells with the synthetic glucocorticoid dexamethasone (500 nM) for 48 h shifted the ratio of β_1 : β_2 markedly, from 80:20 to 50:50. Total receptor content was unchanged ($P = 0.31$ for the difference between steroid-treated and control cells). Thus, glucocorticoids induce a 45 % decline (to 105 ± 18 fmol/mg; $P < 0.05$) in the β_1 component, whereas the β_2 -receptor population increases by about 2-fold (to 102 ± 18 fmol/mg; $P < 0.01$).

The ability of glucocorticoids to up-regulate β_2 - and down-regulate β_1 -adrenergic receptors prompted us to investigate the effects of steroid analogues and hormones such as retinoic acid

on expression of β -adrenergic receptors in rat C6 glioma cells (Table 2). The oestrogen β -oestradiol (500 nM), the progestin progesterone (500 nM) and the androgen testosterone (500 nM) altered neither the total number of β -adrenergic receptors nor the ratio of receptor subtypes (β_1 : β_2). Likewise, both the progesterone antagonist RU486 (1 μ M) and retinoic acid (100 nM) had no significant effect on β -adrenergic-receptor expression by these cells.

Immunoblots of crude membranes prepared from C6 cells and stained with a β_1 -adrenergic-receptor-specific antiserum (SB-03; Bahouth, 1991) revealed a prominent band of immunoreactivity corresponding to M_r 65000 (Figure 1, 'Blot'). Exposure of C6 glioma cells to dexamethasone (500 nM) for 48 h was accompanied by a sharp decline in the amount of immunostaining of C6 glioma cell membranes with anti-receptor antibodies (Figure 1, 'Blot'). Confirming the glucocorticoid-induced decline in β_1 -receptor radioligand binding, the results of the immunoblotting displayed a decline in β_1 -adrenergic-receptor immunoreactivity in blots prepared from glucocorticoid-treated cells. The immunoblotting data suggest a greater apparent decline in receptor immunoreactivity than that observed indirectly by radioligand binding with the β_1 -receptor-selective antagonist CGP-20712A.

Glucocorticoid-induced up-regulation of the expression of the β_2 -adrenergic receptors has been demonstrated at the level of mRNA and protein (Haddock and Malbon, 1988b; Collins et al., 1988; Malbon and Haddock, 1988; Haddock et al., 1989a).

Table 1 Glucocorticoids down-regulate β_1 - and up-regulate β_2 -adrenergic receptors in rat C6 glioma cells

Cells were exposed to vehicle (control) or dexamethasone (500 nM) for 48 h and then employed as a source for crude membranes. β -Adrenergic receptors were measured in these membranes by [125 I]CYP binding in competition with propranolol ($\beta_1 + \beta_2$) or CGP-20712A (β_1 -selective). The data presented are means $\pm$ S.E.M. from 10 independent experiments, each assayed in triplicate. Difference from control values (paired Student's *t* test): * $P = 0.026$, ** $P = 0.003$.

	[125 I]CYP binding (fmol/mg of protein)	
	Control	Dexamethasone
Total β -adrenergic receptors	235 $\pm$ 50	210 $\pm$ 25
β_1 -Adrenergic receptors	185 $\pm$ 40	105 $\pm$ 18*
β_2 -Adrenergic receptors	52 $\pm$ 09	102 $\pm$ 18**

Table 2 Glucocorticoids, but not oestradiol, progesterone, testosterone or retinoic acid, down-regulate β_1 - and up-regulate β_2 -adrenergic receptors

C6 glioma cells were treated with vehicle alone (C) or with dexamethasone (500 nM), oestradiol (500 nM), progesterone (500 nM), retinoic acid (100 nM), testosterone (500 nM) or RU-486 (1.0 μ M) for 48 h before harvest. β -Adrenergic receptors were measured in crude membranes prepared from these cells, by [125 I]CYP binding in competition with propranolol ($\beta_1 + \beta_2$) or CGP-20712A (β_1 -selective). The data presented are means $\pm$ S.E.M. from 6 independent experiments, each assayed in triplicate. Abbreviation: AR, adrenergic receptors.

Treatment	Total β -AR (fmol/mg of protein)	β_1 AR (%)	β_2 AR (%)
Control	268 $\pm$ 30	73 $\pm$ 5	27 $\pm$ 4
Dexamethasone	225 $\pm$ 50	48 $\pm$ 2	52 $\pm$ 3
Oestradiol	260 $\pm$ 35	68 $\pm$ 8	32 $\pm$ 7
Progesterone	237 $\pm$ 42	67 $\pm$ 11	33 $\pm$ 6
Retinoic acid	265 $\pm$ 105	65 $\pm$ 10	35 $\pm$ 9
Testosterone	255 $\pm$ 20	65 $\pm$ 5	35 $\pm$ 8
RU-486	268 $\pm$ 26	65 $\pm$ 4	35 $\pm$ 4

Figure 1 Glucocorticoids down-regulate β_1 -adrenergic receptors: analysis of steady-state receptor levels and receptor synthesis

Blot: cells were exposed to vehicle (—, control) or dexamethasone (DEX, 500 nM) for 48 h and then used as a source for crude membranes. The membranes (0.2 mg/lane) were subjected to SDS/PAGE and the resolved proteins transferred to nitrocellulose blots. The blots were probed with anti- β_1 -adrenergic receptor antibody SB-03 (1:200 dilution) and then with calf alkaline phosphatase-linked goat anti-(rabbit IgG) antibody (1:1000 dilution). The blot is representative of three separate analyses performed on different preparations of cells. The M_r of protein markers is shown in the margin. Metabolic labelling: C6 glioma cells were metabolically labelled with [35 S]methionine in the absence or presence of dexamethasone (DEX, 500 nM) for 4, 24 or 48 h. Immunoprecipitations were performed with solubilized crude membrane fractions from cells, with either non-immune (NI) antisera or anti- β_1 -adrenergic receptor antiserum SB-03, as described in the Materials and methods section. Immune complexes were subjected to SDS/PAGE. The gels were fixed, dried, and subjected to autoradiography. An autoradiogram from a single experiment replicated twice more is displayed. The position of the β_1 -adrenergic receptor is indicated by ' β_1 '.

Metabolic labelling of cells with [35 S]methionine, followed by immunoprecipitation of the β_1 -adrenergic receptors, revealed a decline in the relative amount of receptor synthesis in cells challenged with dexamethasone for 24 and 48 h (Figure 1, 'Metabolic labelling'). Taken together, the results of radioligand binding, immunoblot analysis of steady-state receptor expression, and metabolic-labelling studies clearly demonstrate a glucocorticoid-induced down-regulation of β_1 -adrenergic receptors.

Analysis of glucocorticoid-induced down-regulation of β_1 -adrenergic receptors at the level of mRNA required the use of DNA-excess solution hybridization (Haddock et al., 1989b). This technique offers greater sensitivity than Northern-blot analysis and permits molar quantification of low-abundance or rare mRNA species (Williams et al., 1986). The β_1 -specific probe (H β 110) was derived from a region in human β_1 -adrenergic-receptor cDNA that was > 95% identical with the corresponding sequence in the rat gene. S1-endonuclease-protection studies demonstrated hybridization of the probe H β 110 to rat β_1 -adrenergic-receptor mRNA (Figure 2). The RNA:DNA hybrids formed are protected from degradation by the single-strand-specific S1 endonuclease. Poly(A) $^{+}$ -selected RNA from rat C6 glioma cells (lane 3), RNA from human placenta that is rich in β_1 -adrenergic receptors (Bahouth and Malbon, 1987) (lane 4), mRNA of rat β_1 -adrenergic receptors transcribed *in vitro* in reticulocyte lysates (lane 5), and the cDNAs for the human and the rat β_1 -adrenergic receptors (lanes 6 and 7 respectively) all hybridized to the probe and thus protected the probe from digestion by S1 endonuclease. In contrast, the β_2 - and α_1 -adrenergic-receptor cDNAs failed to protect H β 110 from degradation by S1 endonuclease (results not shown). These data validated the use of the probe for hybridization studies with the β_1 -receptor mRNA.

DNA-excess solution hybridization with H β 110 was standardized against DNA template for the probe (Figure 3), and the curve generated was used to calculate the content of receptor mRNA in RNA samples prepared from human placenta, rat C6 glioma cells and hamster DDT $_1$ MF-2 smooth-muscle cells. RNA

Figure 2 Analysis of β_1 -adrenergic-receptor mRNA by using probe H β 110 and S1-endonuclease protection

Uniformly radiolabelled H β 110 was hybridized either to 10 μ g of yeast tRNA (lanes 1 and 2), or to 5 μ g of poly(A) $^{+}$ -selected RNA from C6 glioma cells (lane 3), 50 μ g of human placental RNA (lane 4), 1 μ g of rat β_1 -adrenergic-receptor RNA transcribed *in vitro* (lane 5), 1 μ g of human β_1 -adrenergic-receptor RNA transcribed *in vitro* (lane 6) and 10 μ g of human β_1 -adrenergic-receptor cDNA (lane 7). After 18 h at 48 $^{\circ}$ C, the samples were treated with (lanes 1, 3–7) or without (lane 2) S1 endonuclease (100 units/ml) at 42 $^{\circ}$ C for 1 h and subjected to electrophoresis on 6% polyacrylamide gels in 8 M urea. After drying, the gel was subjected to autoradiography. The number of bases, indicated in the margin, was established by reference to digests of pBR322 DNA with *mspi* or of ϕ X174 DNA with *Hae*III (New England Biolabs). The digests were 32 P-end-labelled and detected by autoradiography.

extracted from fresh human placenta displayed 1.2 ± 0.05 ($n = 5$) amol of β_1 -adrenergic-receptor mRNA/ μ g of RNA, whereas that from C6 glioma cells displayed 0.47 ± 0.01 ($n = 4$) amol of β_1 -adrenergic-receptor mRNA/ μ g of total cellular RNA. A linear relationship between input RNA and the amount of S1-endonuclease-resistant hybrids was obtained in solution-hybridization assays of total cellular RNA from C6 glioma cells and placenta, but not in assays of RNA from hamster DDT $_1$ MF-2 cells, which do not express significant amounts of β_1 -adrenergic receptor (Figure 3).

Glucocorticoids promoted a rapid decline in steady-state levels of β_2 -adrenergic-receptor mRNA. Within 2 h of challenge with the steroid, β_1 -receptor mRNA levels declined by 45% (0.47 ± 0.01 and 0.26 ± 0.04 amol/ μ g of total cellular RNA for control and glucocorticoid-treated cells respectively). This steroid-induced decline persisted for up to the first 24 h after treatment with dexamethasone, β_1 -receptor mRNA levels remaining at 0.27 ± 0.05 amol/ μ g of total cellular RNA ($n = 4$). mRNA levels for β_2 -adrenergic receptors in C6 glioma cells, in contrast, remained relatively unaffected at 2, 4 and 24 h after challenge with dexamethasone (results not shown).

The dose-response relationship between dexamethasone and steady-state levels of β_1 -adrenergic-receptor mRNA in C6

Figure 3 β_1 -Adrenergic-receptor mRNA levels: analysis by DNA-excess solution hybridization

(a) Standard curve of H β 110 probe hybridized with template DNA; (b) hybridization of H β 110 probe with total cellular RNA from rat C6 glioma (C6 RNA), hamster smooth-muscle cells DDT₁MF-2 (DDT-1 RNA) or human placenta (Placental RNA). A representative assay is displayed.

Figure 4 Glucocorticoids down-regulate β_1 - but not β_2 -adrenergic-receptor mRNA

Rat C6 glioma cells were incubated in the absence or presence of dexamethasone at the indicated concentration for 2 h. Total cellular RNA was collected, and mRNA levels for β_1 - and β_2 -adrenergic receptors (β_1 AR and β_2 AR) were assayed by DNA-excess solution-hybridization assay. The results displayed are means $\pm$ S.E.M. from 4 independent experiments, each assayed in triplicate.

glioma cells was analysed at 2 h after challenge with steroid (Figure 4). Dexamethasone at 10 nM, the lowest concentration tested, promoted a $38 \pm 9\%$ ($n = 4$) decline in β_1 -adrenergic-receptor mRNA levels. In this set of experiments, 500 nM steroid induced a $64 \pm 4\%$ decline in β_1 -receptor mRNA, whereas at higher concentrations (1 μ M) of dexamethasone, the decline in receptor mRNA levels was 45%. A similar effect of dexamethasone on β_1 -receptor-mediated activation of cyclic AMP accumulation by C6 glioma cells was observed by Zhong and Minneman (1993) at μ M concentrations of the steroid (i.e., the effects of dexamethasone were less at 5 μ M than at 0.5 μ M). Compared with DDT₁MF-2 smooth-muscle cells (Hadcock and Malbon, 1988b), C6 glioma cells displayed a similar elevation in β_2 -adrenergic-receptor binding in response to glucocorticoid. mRNA levels for

Figure 5 β -Adrenergic-receptor mRNA stability is not altered in response to glucocorticoid

Rat C6 glioma cells were incubated with vehicle (○) or 500 nM dexamethasone (●) for 2 h, then concurrently with actinomycin D (5 μ g/ml). Cells were harvested at 0, 1, 2, 3 or 4 h after the addition of actinomycin D. β_1 -Adrenergic-receptor (β_1 AR) mRNA levels were assayed in triplicate by DNA-excess solution hybridization with probe H β 110. The data are expressed as mean values of triplicates from a single experiment, representative of 4 replicate experiments.

Figure 6 Transcriptional suppression of the β_1 -adrenergic-receptor gene by dexamethasone

(a) C6 glioma cells grown in a steroid-deficient medium were exposed to 250 nM dexamethasone (+) or vehicle (−) for 2 h. The cells were harvested and nuclei prepared as outlined in the Materials and methods section. Elongation of the transcripts [(2–3) $\times 10^7$ nuclei per assay] was allowed to continue in the presence of [α - 32 P]UTP and unlabelled nucleotides. After elongation, equal amounts of radiolabelled RNA ($\sim 6 \times 10^6$ c.p.m.) were hybridized to a plasmid harbouring the full-length rat β_1 - or β_2 -adrenergic-receptor gene (10 μ g/slot), or to a plasmid harbouring the avian α -tubulin cDNA (5 μ g/slot). Autoradiograms (5-day exposure) were analysed by laser scanning densitometry, and the absorbances of the bands for β_1 -adrenergic receptor (β_1 AR), β_2 -adrenergic receptor (β_2 AR) and α -tubulin transcripts were quantified. (b) For each condition, the absorbance of the β_1 -adrenergic-receptor band was normalized among the samples to that of α -tubulin to correct for differences in hybridization conditions. The ratio in the dexamethasone-treated lane (+) was divided by the corresponding value in the vehicle-treated (−) lane to calculate the percentage change in the transcriptional rate of the β_1 -adrenergic-receptor gene in response to dexamethasone. The data are expressed as means $\pm$ S.E.M. of three separate experiments.

β_2 -adrenergic receptors at 2 h after challenge with dexamethasone were not significantly altered (Figure 4), although by 48 h of exposure of 500 nM dexamethasone β_2 -receptor mRNA levels have been shown to be elevated (Zhong and Minneman, 1993).

The stability of β_1 - and β_2 -adrenergic-receptor mRNAs was assessed in C6 glioma cells challenged with dexamethasone or vehicle by using the technique of Rodgers et al. (1985), which takes advantage of the RNA polymerase II inhibitor, actinomycin D. These experiments probed whether or not post-transcriptional regulation played some role in the effect of dexamethasone on β_1 -adrenergic-receptor mRNA levels (Figure 5). β_1 - and β_2 -adrenergic-receptor mRNA levels in C6 glioma cells declined by

~ 80 % within 4 h of exposure to actinomycin D (5 μ g/ml). The half-life ($t_{1/2}$) of the β_1 -adrenergic-receptor mRNA was found to be 1.67 ± 0.22 and 1.83 ± 0.30 h for vehicle-treated and dexamethasone-treated cells respectively (mean $\pm$ S.E.M., $n = 3$). A typical result of the analysis of β_1 -adrenergic-receptor mRNA stability is shown in Figure 5. The $t_{1/2}$ of β_2 -adrenergic-receptor mRNA was found to be similar to that of the β_1 -adrenergic-receptor mRNA (1.5 h) and also was unaffected by dexamethasone as previously observed in DDT₁MF-2 smooth-muscle cells (Hadcock et al., 1989a).

The stability of β_1 -adrenergic-receptor mRNA was unaffected by glucocorticoids. Using nuclear run-on assays, we investigated whether the steroid mediated its effect on receptor mRNA by a transcriptional mechanism (Figure 6). The rates of transcription for β_1 - and β_2 -adrenergic-receptor genes relative to that of α -tubulin were determined by using nuclei prepared from vehicle-treated and dexamethasone (250 nM)-treated C6 glioma cells (Figure 6a). The transcription of the β_1 -adrenergic-receptor gene declined by $47 \pm 6\%$ ($n = 3$) in nuclear run-on assays prepared with nuclei from dexamethasone-treated cells compared with vehicle-treated cells (Figure 6b). The relative rate of transcription of the β_2 -adrenergic-receptor gene, in contrast, was elevated slightly for nuclei from steroid-treated as compared with control cells (1.58 ± 0.23 -fold; Figure 6b).

DISCUSSION

G-protein-linked receptors displayed a rich diversity in physiological regulation, including that by hormones such as thyroid hormones, retinoids and steroids (for references, see Malbon et al., 1988; Wang et al., 1990; Morris et al., 1991; Bahouth and Malbon, 1994). β_2 -Adrenergic receptors have been the focus of intense study, and remain an important general model for G-protein-linked receptors. Our knowledge about the regulation of β_1 -adrenergic receptors is far more limited. Thyroid hormones have been shown to regulate the expression of cardiac β_1 -adrenergic-receptors at the level of mRNA (Lazar-Wesley et al., 1991; Bahouth, 1991), a reflection of transcriptional activation (Bahouth, 1991). Several features about β_1 -adrenergic receptor regulation have been illuminated through studies of differentiation of mouse 3T3-L1 (Guest et al., 1990) and 3T3-F442A (Feve et al., 1990) cells. These cells express both β_1 - and β_2 -subtypes of adrenergic receptors and display differential regulation of their expression during adipogenesis. Glucocorticoids stimulate the differentiation of 3T3-L1 cells and decrease the ratio of β_1 - to β_2 -adrenergic receptors in adipocytes (Guest et al., 1990). Similarly, glucocorticoids suppressed β_2 -adrenergic receptor expression in 3T3-F442A cells (Feve et al., 1990). Because differentiation can mask proximal effects of steroids, rat C6 glioma cells were adopted as a model system with which to explore the regulation of β_1 -adrenergic receptors by glucocorticoids and other steroids.

Glucocorticoids induce a down-regulation of β_1 -adrenergic receptors in rat C6 glioma cells, extending the previous report in mouse 3T3-L1 cells of a decline in β_1 -adrenergic receptors in response to glucocorticoid alone (Guest et al., 1990). The C6 glioma cells displayed an interesting phenotypic response to glucocorticoids, shifting the ratio of β -adrenergic-receptor subtypes β_1 : β_2 from 80:20 to 50:50, while maintaining the β -adrenergic-receptor population constant. The increase in β_2 -component confirms previous studies demonstrating that glucocorticoids induce up-regulation of receptor expression (Hadcock and Malbon, 1988b; Hadcock et al., 1989a). In the present paper, we analysed glucocorticoid-induced down-regulation of β_1 -adrenergic receptor by several independent techniques: radio-

ligand binding, immunoblotting and metabolic labelling. Our results confirm the detailed radioligand-binding analysis of C6 glioma cells reported by Zhong and Minneman (1993). Glucocorticoids were shown to decrease β_1 -receptor expression, although the molecular basis for this steroid-induced effect was not provided (Zhong and Minneman, 1993). In the present study, we report receptor synthesis and the steady-state level of receptor expression to be suppressed in cells challenged with glucocorticoids. The results from the immunoblotting suggest that the competition studies with the β_1 -selective ligand CGP-20712A may not offer the same precision, although their results are qualitatively in agreement internally and with the recent report of Zhong and Minneman (1993) with respect to radioligand binding.

Glucocorticoids (Hadcock and Malbon, 1988b; Collins et al., 1988), thyroid hormones (Lazar-Wesley et al., 1991; Bahouth, 1991), testosterone (Collins et al., 1991) and retinoids (Galvin-Parton et al., 1990) act as modulatory hormones for transmembrane signalling events propagated via G-protein-linked receptors. With the exception of glucocorticoids, these hormones, as well as oestrogen, progesterone and its antagonist RU-486, failed to influence the expression of either β_1 - or β_2 -adrenergic receptors in C6 glioma cells.

The glucocorticoid-induced up-regulation of the β_2 -adrenergic receptor is preceded by a sharp transient increase in receptor mRNA levels (Collins et al., 1988; Hadcock and Malbon, 1988b; Hadcock et al., 1989a). In hamster smooth-muscle DDT₁MF-2 cells, a 4-fold increase in β_2 -adrenergic-receptor mRNA level occurs within 4 h of challenge with glucocorticoid. For β_1 -adrenergic receptors, mRNA levels of C6 glioma cells are shown to decline by 45–60 % within 2 h of dexamethasone challenge. Zhong and Minneman (1993), using the less-sensitive Northern-blot assay, were forced to analyse poly(A)⁺-selected RNA and were unable to define a change in β_1 -receptor mRNA in dexamethasone-treated C6 glioma cells. In the present study, nuclear run-on and DNA-excess solution-hybridization assays clearly demonstrate glucocorticoid-induced suppression of β_1 -receptor mRNA, providing a molecular explanation in concert with the steroid-induced decline in receptor expression. At the lowest concentration of dexamethasone tested (10 nM), a 40 % decline in β_1 -adrenergic-receptor mRNA could be observed with this approach.

Phenomenologically, the glucocorticoid-induced down-regulation of β_1 -adrenergic-receptor mRNA appears similar to β -adrenergic-agonist-induced down-regulation of β_2 -receptor mRNA levels discovered previously (Hadcock and Malbon, 1988a; Hough and Chuang, 1990). Destabilization of pre-existing mRNA provided the basis for agonist-induced down-regulation of β_2 -adrenergic-receptor mRNA (Hadcock et al., 1989a, 1990). Although steroids often exert their regulation at the level of gene transcription (Ringold, 1985), stability of mRNA plays an important role, as in the case of oestrogen regulation of ovalbumin mRNA (Palmiter and Carey, 1974). Analysis of the half-life of β_1 -adrenergic-receptor mRNA revealed two important observations. First, glucocorticoids failed to alter receptor mRNA stability, eliminating one likely explanation from further consideration. Second, the half-life of β -adrenergic receptors can be quite variable: for the β_2 -receptor it is 12 h in DDT₁MF-2 smooth-muscle cells and ~ 1.5 h in C6 glioma cells (Hadcock et al., 1989a; the present work). Whereas agonist-induced down-regulation of β_2 -adrenergic receptors in DDT₁MF-2 cells promotes a decline in mRNA half-life from ~ 12 to ~ 7 h, dexamethasone-induced down-regulation of β_1 -adrenergic receptors in C6 glioma cells occurs in the absence of any significant change in receptor mRNA stability.

Nuclear run-on assays revealed a sharp decline in the apparent rate of transcription for the β_1 -adrenergic receptor gene in C6 glioma cells that are challenged with dexamethasone. For β_2 -adrenergic receptors, glucocorticoids act as transcriptional activators operating via glucocorticoid response elements (GREs) located in the receptor genes (Malbon and Hadcock, 1988). The glucocorticoid receptor is a member of the zinc-finger family of transcription factors and binds as a homodimer to cognate GRE sequence (O'Malley, 1990; Perlmann et al., 1990). The sequence AGAACANNNTGTTCT binds the glucocorticoid-hormone-receptor complex, as well as other ligand-hormone-receptor complexes (Yamamoto, 1985). There exists considerable variability in the sequence of naturally occurring GREs. For example, the TGTCT portion of the sequence is highly conserved in GREs, but the left-most hexanucleotide sequence (AGAACA) can be quite variable (Lucas and Granner, 1992). The sequence TGTCT has been identified at position -1936 relative to the initiation of translation and at position -1084 on the reverse strand of the human β_1 -adrenergic-receptor gene (Collins et al., 1993). In the rat β_1 -adrenergic-receptor gene, a TGTCT sequence has been identified at positions (relative to the site of initiation of translation) -943 on the reverse strand (Shimomura and Terada, 1990) and -2767 (C. Machida, unpublished work). The consensus sequence AGAACANNNTGTTCT is a GRE by which glucocorticoids typically increase the rate of transcription. The consensus sequence(s) for GREs by which glucocorticoids suppress transcription, as is shown here for glucocorticoid-promoted suppression of the transcription of the β_1 -adrenergic-receptor gene, remains to be established. In addition, the changes in β_1 -receptor mRNA may explain, only in part, glucocorticoid effects on the expression of receptor, as changes in protein turnover and/or translational effects may occur also.

In summary, we demonstrate that glucocorticoids suppress the transcription of the gene encoding the β_1 -adrenergic receptor in rat C6 glioma cells. Both steady-state levels of receptor mRNA and receptor synthesis decline in response to glucocorticoids, yielding a down-regulation of β_1 -adrenergic-receptor expression. Thus glucocorticoids can promote markedly different effects on transcription of β_1 - and β_2 -adrenergic-receptor genes, suppressing the β_1 - while activating the β_2 -receptor genes.

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