Regulation of $\beta_2$-Adrenergic Receptor mRNA and Gene Transcription in Rat C6 Glioma Cells: Effects of Agonist, Forskolin, and Protein Synthesis Inhibition

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SUMMARY

Incubation of rat C6 glioma cells with $\beta$-adrenergic receptor (BAR) agonist or with agents that increase cAMP levels results in down-regulation of the $\beta_2$AR, as measured by the loss of radioligand binding sites. In the present study, the role of $\beta_2$AR mRNA expression and stability in the down-regulation of $\beta_2$AR sites in C6 cells was examined. Isoproterenol or forskolin treatment decreased $\beta_2$AR mRNA levels in a time-dependent manner, with maximal loss of $\sim 50\%$ being observed after 2 hr. Pretreatment of the cells with a potent protein synthesis inhibitor, Pseudomonas exotoxin A, completely blocked isoproterenol- and forskolin-mediated down-regulation of $\beta_2$AR mRNA.

Exposure to agonist did not significantly influence the half-life of $\beta_2$AR mRNA, which was $\sim 60$ min. In contrast, isoproterenol treatment for 2 hr significantly decreased the rate of $\beta_2$AR gene transcription, as determined by nuclear run-on analysis. Based on these results, we propose that agonist regulation of $\beta_2$AR mRNA in C6 cells is mediated by activation of the cAMP system and occurs at the level of $\beta_2$AR gene transcription, not mRNA stability. In addition, the observed requirement for protein synthesis indicates that down-regulation of $\beta_2$AR mRNA may be mediated by expression of a repressor of $\beta_2$AR gene transcription.

It is well established that down-regulation of the $\beta_2$AR occurs in response to agonists or other agents that elevate cAMP levels (1, 2). Loss of receptors involves multiple mechanisms, including down-regulation of receptor mRNA (3, 4). In both hamster smooth muscle DDT,MF-2 and mouse lymphoma S49 cells, $\beta_2$AR mRNA levels decrease by 40% over a 24-hr period in response to isoproterenol or forskolin (5, 6). In DDT,MF-2 cells, this effect is accompanied by a decrease in $\beta_2$AR mRNA half-life but not gene transcription rate (7). In contrast, Collins et al. (8) reported that, in DDT,MF-2 cells exposed to agonist or agents that elevate cAMP, there is first an increase in levels of $\beta_2$AR mRNA and gene transcription and then a decrease of receptor mRNA levels; no change in $\beta_2$AR mRNA half-life was reported in that study.

A similar biphasic change in $\beta_1$AR mRNA is observed when rat C6 glioma cells are exposed to agonist or forskolin (9–11). We recently demonstrated that the initial rapid up-regulation of $\beta_1$AR mRNA is accompanied by an increased rate of gene transcription, whereas the subsequent slower down-regulation of receptor mRNA is correlated with a decreased rate of transcription, with no change in $\beta_1$AR mRNA stability (11). C6 cells also express endogenous $\beta_1$AR (11, 12), and $\beta_2$AR mRNA levels are reported to be down-regulated by agonist treatment (9), although the roles of the cAMP system, mRNA half-life, and gene transcription rate have not been examined.

Because $\beta_2$AR mRNA expression may be under the control of different regulatory mechanisms in different cell types and tissues, we examined the mechanisms that mediate the regulation of $\beta_2$AR mRNA in C6 glioma cells. We found that agonist or forskolin treatment decreased steady state levels of $\beta_2$AR mRNA and that this effect was accompanied by a decrease in $\beta_2$AR gene transcription rate but not mRNA stability. Moreover, inhibition of protein synthesis blocked the down-regulation of $\beta_2$AR mRNA, suggesting that the decrease in the transcription rate is mediated by induction of an inducible repressor. These results indicate that the mechanisms that underlie agonist regulation of $\beta_2$AR mRNA differ

ABBREVIATIONS: BAR, $\beta$-adrenergic receptor(s); CRE, cAMP response element; ICER, inducible cAMP early repressor(s); bp, base pair(s); EGTA, ethylene glycol bis($\beta$-aminoethyl ether)-N,N',N'-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
depending on the cell line under examination, and they suggest that different mechanisms may control the regulation of βAR mRNA expression in different tissues in vivo.

Experimental Procedures

Materials. [α-32P]UTP (3000 Ci/mmol) and [α-32P]CTP (800 Ci/mmol) were obtained from DuPont-New England Nuclear. Forskolin and actinomycin D were purchased from Calbiochem, Pseudomonas exotoxin A from List Biological Laboratories (Campbell, CA), and (-)-isoproterenol and (-)-alprenolol from Sigma. CGP 20712A and ICI 118,551 were generous gifts from Ciba-Geigy (Summit, NJ) and ICI (Macclesfield, UK), respectively. The rat βAR cDNA (13) was generously provided by Dr. J. C. Venter (Institute of Genomic Research, Gaithersburg, MD).

Cell culture. The culture of rat C6 glioma cells was carried out as described previously (11). Cells were plated at 5–7 × 10⁵ cells/175-cm² flask, in 50 ml of medium. After 4 days of culture the medium was changed to serum-free medium, and on the following day the cells were exposed to isoproterenol (1 μM) or forskolin (10 μM) for the times indicated. Where indicated, cells were also pretreated with exotoxin A (0.3 μg/ml) for at least 4 hr before addition of the stimulators. Cells (4–5 × 10⁶/flask) were collected by removal of the supernatants. Cells (4–5 × 10⁶/flask) were detached with 0.5% sodium dodecyl sulfate, 300 μg/ml salmon sperm DNA, and 0.5% 2-mercaptoethanol. The resulting suspension was frozen at −70°C for subsequent RNA isolation.

RNA extraction. After homogenization of the cells in the buffered guanidine thiocyanate solution, total RNA was isolated by centrifugation at 150,000 × g for 2 hr at 4°C, through a 5.7 M cesium chloride gradient (14). RNA was then resuspended in 0.5 M sodium acetate, pH 5.2, and precipitated with ethanol, and the concentration was determined by spectrophotometry at 260 nm.

Riboprobe and cRNA preparation. A 207-bp fragment of the rat βAR cDNA (positions +1099–1305) was amplified by polymerase chain reaction using the forward and reverse primers GGAT-GCGCTTCAGAGGCTTCTG and GGCTAGACCTGACCTGAC-GAGCAGGCTTCTG and was cloned into pBluescript II SK− (Strategene). The cDNA was linearized by EcoRI digestion to the insert, and uniformly radiolabeled riboprobes corresponding to the antisense strand were synthesized with T7 RNA polymerase, as described previously (11, 15). The specific activity of a typical riboprobe was ~1 × 10⁶ dpm/μg. Unlabeled sense strand cRNA was also prepared from the same plasmid and was used as a hybridization standard. The plasmid was linearized 3’ to the DNA insert, and uniformly radiolabeled riboprobes corresponding to the antisense strand were synthesized with T7 RNA polymerase (16). Unlabeled sense strand was then purified, quantified by spectrophotometric analysis at 260 nm, and frozen in aliquots at −70°C.

RNase protection assay. RNase protection analysis was carried out as described previously (11, 15). Briefly, 30-μg aliquots of total RNA were hybridized with 32P-labeled riboprobe (10⁶ cpm/sample) at 63°C for 16–18 hr. The samples were then digested with RNase at 37°C for 45 min. For the filtration assay, 10% trichloroacetic acid was added and then the samples were filtered through GF/C glass filters (Whatman). The filters were then extensively and sequentially washed with cold 5% trichloroacetic acid and then 95% ethanol and quantified by liquid scintillation counting. For polycrylamide gel analysis, samples were treated in a similar manner, with modifications (11, 15), and then analyzed on 6% polycrylamide/8% urea denaturing gels. The gels were dried, and labeled bands were detected by autoradiography.

mRNA stability analysis. To determine the half-life of βAR mRNA, the cells were incubated with actinomycin D to block transcription, as described previously (11, 17). Cells were incubated in the absence or presence of isoproterenol as described above; actinomycin D (2 μg/ml) was then added to the medium and the cells were harvested at different times (0–120 min). Total cellular RNA was extracted at each of the time points, and βAR mRNA levels were quantified by the RNase protection assay as described above. The concentration of actinomycin was shown to inhibit RNA synthesis by >98% (11).

Nuclear run-on analysis. Nuclei were isolated by Dounce homogenization using the alternate protocol described by Greenberg and Bender (18), and nuclear run-on analysis was conducted as described previously (11). Briefly, nuclei were incubated for 30 min at 30°C in a transcription mixture containing 1 μM unlabeled ATP, CTP, and GTP and 250 μCi of [α-32P]UTP. The newly transcribed RNA was extracted as described previously (18). The radiolabeled RNA was denatured and hybridized (at 42°C for 3 days) to βAR or cyclophilin cDNA immobilized on nitrocellulose membranes (5 μg/ml), in the hybridization buffer-N described previously (14), with the addition of 0.5% sodium dodecyl sulfate, 300 μg/ml salmon sperm DNA, and 20 μg/ml yeast tRNA. Filters were washed, dried, and subjected to autoradiography, with two intensifying screens, at −70°C for 5 days. Quantitative results were obtained by densitometric scanning.

Other methods. Levels of intracellular cAMP were determined by radioimmunoassay (19).

Results

Characterization of βAR mRNA by RNase protection analysis. A 207-bp fragment of the rat βAR cDNA, corresponding to coding region positions +1099–1305, was subcloned into pBluescript, and 32P-labeled antisense riboprobes were synthesized using T7 RNA polymerase. The radiolabeled riboprobe was hybridized with trNA, sense strand cRNA synthesized with T3 RNA polymerase, or total RNA isolated from C6 cells (Fig. 1). The riboprobe and sense cRNA contain additional vector sequences and are larger than the 207-bp fragment of the βAR coding region. The RNase-resistant hybrid resulting from hybridization with
tissue was determined by linear regression, using a 132AR sense strand ferent times. The half-life of 2A.R mRNA, determined from
tangent hybrids were precipitated and then collected by filtration over glass quired for down-regulation of 2AR mRNA was
sense strand cRNA dose dependent; the half-maximally effective concentration
was hybridized with different amounts of 132AR

2-390 ng) as described in Experimental Procedures. The RNase-resis-
was -5 nM, and the maximal concentration of agonist re-

was demonstrated by the lack of protected hybrids after reqll treatment on mRNA stability was examined. C6 cells

-90% by 6 hr, forskolin causes only ~50% loss, indicating that receptor down-regulation is mediated, in part, by the cAMP system. Exposure of C6 cells to isoproterenol (Figs. 1B and 4A) or forskolin (Fig. 4B) resulted in time-dependent down-regulation of 2AR mRNA by ~50–60%. In addition, isoproterenol-induced down-regulation of 2AR mRNA was dose dependent; the half-maximally effective concentration was ~5 nm, and the maximal concentration of agonist re-

ied that receptor down-regulation of 2AR mRNA. However, the
time courses for these two events were different. This is not


to activation of cAMP-dependent protein kinase in C6 cells

mRNA levels from the reports by Hosoda and Duman (15) and Hosoda et al. (11). As

shown in Fig. 3, levels of 2AR and 1AR mRNA ranged from

1 to 10 and from 0.5 to 5 amol/μg of total cellular RNA, respectively (Fig. 3). The relative levels of 2AR and 1AR
mRNA and their ratios compare favorably with the levels of 2AR and 1AR determined by ligand binding studies (20–
22). The C6 cells used in these studies have ~70% 2AR and 30% 1AR (11, 12, 23), which is the same as the distribution of
the respective receptor subtype mRNAs.

Isoproterenol- and forskolin-induced down-regulation of 2AR mRNA in C6 cells. Previous studies have
demonstrated that incubation of C6 cells with isoproterenol or forskolin, which stimulates cAMP formation, results in a coordinate, time-dependent, down-regulation of both 2AR subtypes (11, 12). Whereas isoproterenol mediates a receptor loss of ~90% by 6 hr, forskolin causes only ~50% loss, indicating that receptor down-regulation is mediated, in part, by the cAMP system. Exposure of C6 cells to isoproterenol (Figs. 1B and 4A) or forskolin (Fig. 4B) resulted in time-dependent down-regulation of 2AR mRNA by ~50–60%. In addition, isoproterenol-induced down-regulation of 2AR mRNA was dose dependent; the half-maximally effective concentration was ~5 nm, and the maximal concentration of agonist re-
quired for down-regulation of 2AR mRNA was ~100 nm (Fig. 5).

Effect of inhibition of protein synthesis on the reg-
ulation of 2AR mRNA expression. Agonist stimulation of cAMP production was also time and dose dependent (Fig. 6). The concentrations of isoproterenol required for half-maximal (~10 nm) and maximal (~100 nm) stimulation of cAMP production were similar to those re-
quired for down-regulation of 2AR mRNA. However, the
time courses for these two events were different. This is not

surprising, because receptor stimulation of cAMP production is a relatively rapid event, whereas down-regulation of recep- tor mRNA, which is dependent on activation of intracellular signal transduction pathways and is limited by the half-life (~60 min) of 2AR mRNA (Fig. 7), proceeds more slowly. We
have previously demonstrated that agonist treatment leads to activation of cAMP-dependent protein kinase in C6 cells
(11), which could represent the initiating event for down-
regulation of 2AR mRNA expression.

Effect of inhibition of protein synthesis on the reg-
ulation of 2AR mRNA in C6 cells. To examine the role of de novo protein synthesis in the down-regulation of 2AR
mRNA, C6 cells were pretreated with the potent and selective protein synthesis inhibitor Pseudomonas exotoxin A before exposure to isoproterenol or forskolin (Fig. 4). Pretreatment
with exotoxin alone increased levels of 2AR mRNA by approxi-
mately 30% (1.45 ± 0.26 and 1.91 ± 0.14 amol/μg of
RNA in control and treated cells, respectively, mean ± stand-
ard error). Moreover, exotoxin pretreatment completely
blocked the down-regulation of 2AR mRNA in response to
either isoproterenol or forskolin (Fig. 4).

Effect of agonist treatment on 2AR mRNA stability in C6 cells. To further explore the mechanism(s) by which
2AR mRNA is down-regulated, the influence of isoprotere-
nol treatment on mRNA stability was examined. C6 cells
were incubated in the absence or presence of isoproterenol for
2 hr, actinomycin D (an inhibitor of DNA transcription) was
then added to the medium, and cells were harvested at dif-
ferent times. The half-life of 2AR mRNA, determined from
the rate of 2AR mRNA degradation in the presence of acti-
nomycin D, reflects the stability of mRNA. As shown in Fig.
7, the half-life of 2AR mRNA was ~60 min in control C6

Fig. 4. Agonist- and forskolin-mediated regulation of $\beta_2$AR mRNA in rat C6 glioma cells. C6 cells were incubated with 1 $\mu$m isoproterenol (Iso) (A) or 10 $\mu$m forskolin (Fsk) (B) for the indicated periods of time. After the cells were collected, the levels of $\beta_2$AR mRNA were determined by RNase protection analysis as described in Experimental Procedures. In some experiments, cells were first treated for 16 hr with 0.3 $\mu$g/ml exotoxin A (Ex), to inhibit protein synthesis, and then exposed to stimulator. The results are presented as percentage of control and are the mean ± standard error of three or four separate determinations.

cells, and isoproterenol treatment did not significantly influence the half-life. Thus, regulation of mRNA stability does not appear to play a role in the down-regulation of $\beta_2$AR mRNA induced by agonist treatment. We have reported that $\beta_2$AR subtype mRNA has a similar half-life (61 min) in these same cells and that $\beta_2$AR mRNA half-life is not influenced by agonist treatment (11).

**Agonist-mediated regulation of $\beta_2$AR gene transcription.** To determine the influence of isoproterenol on the rate of $\beta_2$AR gene transcription, nuclear run-on analysis was conducted on nuclei isolated from control and isoproterenol-treated C6 cells (Fig. 8). Briefly, radiolabeled nascent RNA transcripts were generated from the isolated nuclei and used for hybridization with $\beta_2$AR cDNA, which had been blotted onto nitrocellulose filters. As a control, cyclophilin cDNA was also blotted onto the nitrocellulose filters. Incubation with isoproterenol for 90 min significantly decreased levels of nascent $\beta_2$AR RNA transcripts but did not influence levels of cyclophilin gene transcription (Fig. 8).

**Discussion**

Our results demonstrated that incubation of C6 cells with isoproterenol decreased levels of $\beta_2$AR mRNA. This effect was mimicked by incubation with forskolin, indicating that down-regulation of $\beta_2$AR mRNA expression is mediated by activation of the cAMP system. Agonist-induced down-regulation of $\beta_2$AR mRNA is similar to that reported previously in C6 cells (9), S49 cells (6), and DDT1MF-2 cells (5, 8). In DDT1MF-2 cells the down-regulation of $\beta_2$AR is preceded by a rapid transient elevation of $\beta_2$AR mRNA. This increase has been shown to result from activation of a CRE (8, 24); a CRE has been found in the promoters of the human, hamster, mouse, and rat $\beta_2$AR genes (24, 25). The lack of a transient elevation of $\beta_2$AR mRNA levels in C6 cells is surprising, because we have observed a transient up-regulation of $\beta_2$AR mRNA in this cell line (11). One possibility is that the rate of $\beta_2$AR gene transcription is under a greater degree of negative control in C6 cells than in other cell lines. This possibility is supported by the observation that incubation with a protein synthesis inhibitor increased basal $\beta_2$AR mRNA levels. These variations suggest that the mechanisms for regulation of $\beta_2$AR mRNA differ between cell lines and under different culture conditions.

Down-regulation of $\beta_2$AR mRNA in DDT1MF-2 cells is reported to be mediated by decreased stability of receptor mRNA (7). The decreased $\beta_2$AR mRNA stability in DDT1MF-2 cells is accompanied by induction of $\beta_2$AR mRNA-binding proteins that could mediate the destabilization of receptor mRNA (26). In the present study, the half-life of $\beta_2$AR mRNA was determined to be ~60 min in C6 cells, shorter than that of 12 hr reported by Hadcock et al. (7) but similar to that reported by Collins et al. (8) in DDT1MF-2 cells and by Kiely et al. (27) in C6 cells. Incubation of C6 cells with isoproterenol for 2 hr did not influence $\beta_2$AR mRNA half-life, suggesting that regulation of mRNA stability is not
the mechanism by which agonist treatment decreases the expression of β2AR mRNA in this cell line. Given that the half-life was determined in the same manner in all three studies, the variations in mRNA half-life observed in control and agonist-treated cells provide additional evidence that the mechanisms that regulate the levels of mRNA differ between cell lines and among cell lines cultured under different conditions.

The lack of effect of agonist treatment on β2AR mRNA half-life suggests that down-regulation of receptor mRNA occurs at the level of gene transcription. To test this hypothesis, we carried out run-on analysis in nuclei isolated from control and isoproterenol-treated C6 cells. We found that agonist treatment significantly decreased the rate of β2AR gene transcription in C6 cells, by ~25%, consistent with the hypothesis that gene transcription, and not mRNA stability, mediates the down-regulation of β2AR mRNA in C6 cells. The smaller magnitude of the change in transcription rate, relative to levels of β2AR mRNA, could be a reflection of the technical complexity of the nuclear run-on assay (e.g., maintenance of the mechanisms that control basal and agonist-regulated levels of transcription elongation in nuclei isolated from different groups of cells). We have reported similar levels of regulation for β1AR mRNA and gene transcription rates in C6 cells after agonist treatment (11).

The mechanisms that underlie the down-regulation of β2AR gene transcription by agonist treatment are not known. The observation that down-regulation of β2AR mRNA in response to agonist or forskolin incubation is blocked by inhibition of protein synthesis suggests that induction of an inhibitory transcription factor may be involved. Some forms of modulatory CRE-binding proteins, referred to as ICER, are rapidly induced by activation of the cAMP system and are negative regulators of CRE-mediated gene transcription (28, 29). In this way, it has been suggested that ICER acts as a negative feedback regulator and turns off, or inhibits, those genes that are rapidly induced by stimulatory CRE-binding proteins (29). We have found that isoproterenol or forskolin treatments increase the expression of ICER in C6 cells, consistent with the possibility that ICER induction mediates the down-regulation of β2AR gene expression. Blockade of ICER, or another repressor, could reveal CRE-mediated activation of β2AR gene expression, like that reported for β1AR (11). In fact, there was a tendency for levels of β2AR mRNA to be increased by isoproterenol and forskolin treatments in (11).

Regulation of receptor mRNA is one additional mechanism by which levels of β2AR binding sites are regulated by agonist treatment, as well as a mechanism for heterologous regulation via other receptors that regulate the cAMP system. Moreover, it is important to point out that multiple

mechanisms may be involved in the regulation of βAR mRNA levels in different cells and tissues. One mechanism elucidated in the present study, as well as our previous study (11), involves decreased rates of βAR gene transcription. A second mechanism appears to be decreased β2AR mRNA stability (26). A third mechanism involves increased βAR gene transcription via activation of CRE-binding proteins (8, 11, 24).

Identification of the different mechanisms underlying the regulation of β2AR expression could prove useful in future studies to identify disorders associated with altered levels of these receptors and to develop strategies for correction of such disorders.

References


