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DIFFERENTIAL DOWN-REGULATION OF β_1- AND β_2-ADRENERGIC RECEPTOR mRNA IN C_6 GLIOMA CELLS

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SUMMARY: C_6 glioma cells possess both β_1- and β_2-adrenergic receptors. In response to exposure to isoproterenol, these cells down-regulated the mRNA for both β_1- and β_2-adrenergic receptors in a manner that indicates an independent regulatory mechanism for each subtype. In particular, the mRNA species for the β_1-adrenergic receptor initially increased two-fold during the first hour of exposure before decreasing to 40% of initial levels at 4 hours after exposure. In contrast, the β_2 mRNA species decreased rapidly and monotonically to 20% of initial levels by 2 hours. The unique response to isoproterenol of each subtype was blocked by the appropriate subtype-specific antagonists, betaxolol and ICI 118,551. In addition, β-adrenergic receptor mRNA down-regulation was observed in association with contact inhibition, suggesting that events other than receptor occupancy can regulate β-adrenergic receptor mRNA levels.

Desensitization of effector-coupled plasma membrane receptors is a general phenomenon that occurs in virtually every biological system. In many cells responsive to β-adrenergic agonists, exposure to these agents causes a rapid uncoupling of β-adrenergic receptors from adenylate cyclase and a slower down-regulation of receptor numbers (1). Down-regulation is seen initially as a disappearance of receptors from the extracellular membrane surface by internalization, but in time, total numbers of receptors decrease as well. Hadcock & Malbon and others have reported that the loss of β_2-adrenergic receptors in a smooth muscle cell line is associated with the down-regulation of β_2-adrenergic receptor mRNA (2,3). We report here the simultaneous, but differential, down regulation of β_1- and β_2-adrenergic receptor mRNA in the rat C_6 glioma cell line in response to isoproterenol. We find, in addition, that down-regulation of these two receptors mRNA species occurs in response to or in conjunction with contact inhibition in this cell line.

METHODS

C_6 cells from a single 75-cm^2 flask (passage numbers 46-52) were scraped and subcultured into 35-mm petri dishes. Cells were grown for 0-5 days in

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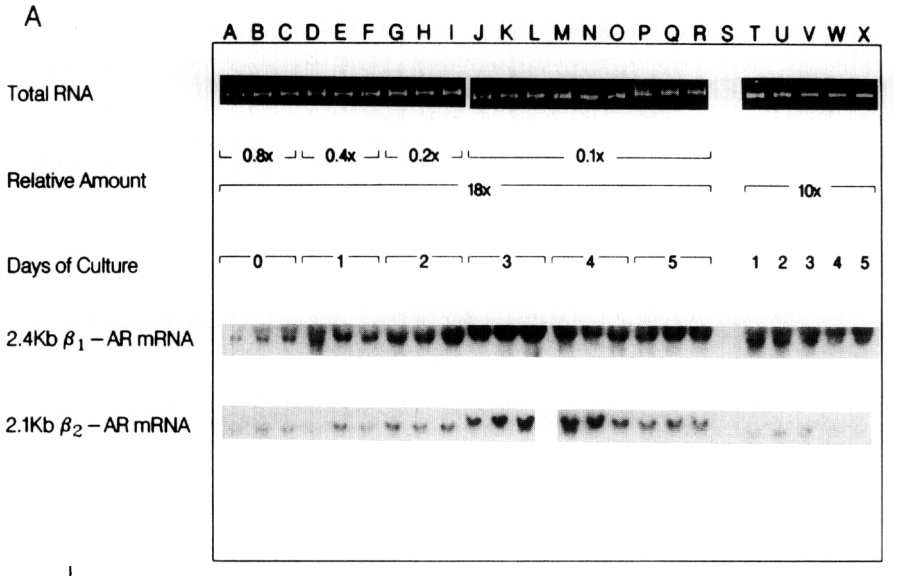
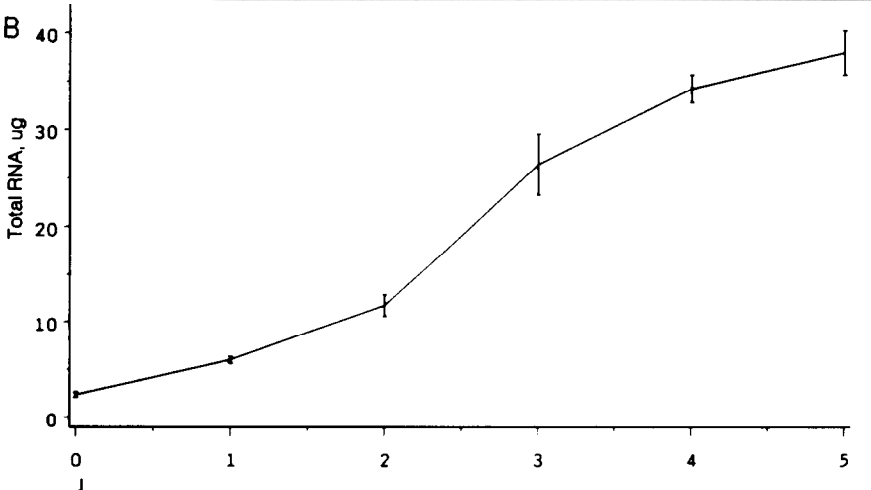
Dulbecco's Modified Eagles medium, 10% fetal calf serum, 50 units/ml of penicillin, 50 µg/ml of streptomycin at 37° C in 5% CO_2. When treated, the cells were used on the third day after subculture. The RNA contained within each culture dish was isolated separately essentially by the method of Chirgwin et al. (4). Once the RNA had been pelleted through 5.7 M CsCl, however, the number of manipulations of the RNA was minimized. The RNA pellet was rinsed gently with 75% ethanol-water, dried, and dissolved in 20 µl of formaldehyde loading buffer (4.4 M formaldehyde, 50% formamide, 20 mM MOPS pH 8.0, 0.02% tracking dye). The RNA samples were transferred to capped 1.5 ml tubes prior to electrophoresis on a 1% agarose-formaldehyde gel. An aliquot of 2 µl of each RNA sample was reserved for quantitation of total RNA. Total RNA was quantitated by laser densitometry of photographs of 1% agarose gels containing 2 µg/ml ethidium bromide on which a fraction of each RNA sample had been electrophoresed in parallel with E. coli ribosomal RNA quantitative standards. The primary RNA samples electrophoresed on agarose-formaldehyde gels were blotted onto nitrocellulose and probed with nick-translated fragments of cDNA clones of the human β_1-adrenergic receptor (5) and hamster β_2-adrenergic receptor (6) genes by standard methods used (7). In our experience, cells which have reached confluence in these dishes exhibit a high degree of reproducibility in number of total cells, protein and total RNA content. This is borne out by the precision of the triplicate determinations of each point in the figures.

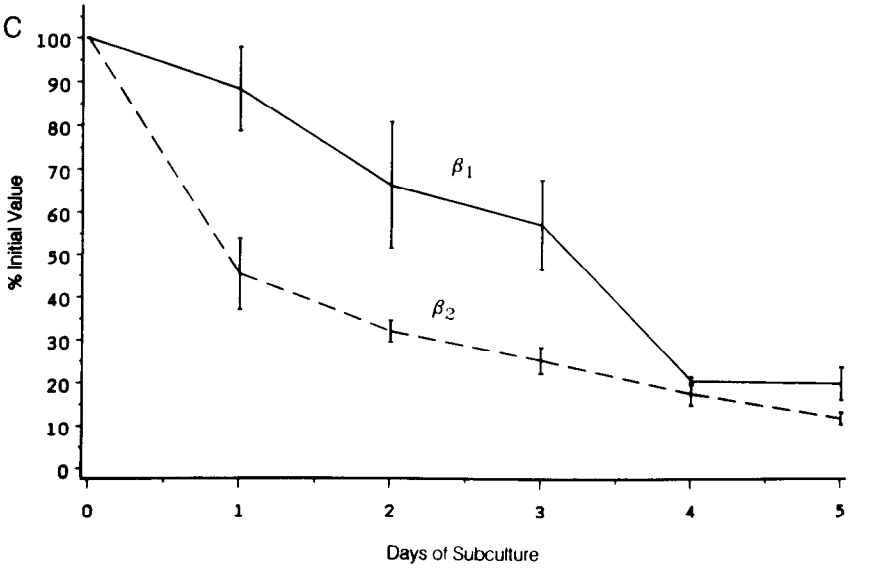
RESULTS

The net expression of β_1- and β_2-adrenergic receptor mRNA in C_6 cells increased as a function of time after subculture until day 3, after which it declined (Figure 1A). This occurred in parallel with increases of total RNA over the entire time course (Figure 1B). Total RNA levels increased exponentially until day 3. After this, the rate of net RNA synthesis decreased with each additional day. Confluence of the cells, as determined by inspection of the dishes under a light microscope, occurred between day 2 and 3. The expression of the two receptor mRNAs differed reproducibly over time. The rate of synthesis of β_2-adrenergic receptor mRNA during log phase growth of the cells lagged significantly behind that of β_1-adrenergic receptor and total RNA. Relative to total RNA (and hence to cell number), the levels of β_1- and β_2-adrenergic receptor mRNA both decreased with time after subculture (Figure 1C). For comparison, samples from another experiment in which equal amounts of RNA have been electrophoresed in each lane is given (Figure 1A).

In response to exposure to 1 µM isoproterenol, both β_1- and β_2-adrenergic receptor mRNAs were rapidly down-regulated in C_6 glioma cells (Figure 2A). The time course and pattern of down-regulation for the two receptor mRNA levels differed (Figure 2B). β_2-adrenergic receptor mRNA decreased rapidly and monotonically to 20% of its initial value by 2 hours. β_1-adrenergic receptor mRNA reproducibly increased (1.86-fold ± 0.44, n=8) over the first hour before decreasing to 40% of its initial value by 4 hours. The apparent half-times of decay for the β_1- and β_2-adrenergic receptor mRNA species were


A
A B C D E F G H I J K L M N O P Q R S T U V W X
Total RNA
0.8x
0.4x
0.2x
0.1x
Relative Amount
18x
10x
Days of Culture
0 1 2 3 4 5 1 2 3 4 5
2.4Kb β_1 – AR mRNA
2.1Kb β_2 – AR mRNA


B
Total RNA, ug
0
10
20
30
40
0 1 2 3 4 5


C
% Initial Value
0
10
20
30
40
50
60
70
80
90
100
β_1
β_2
0 1 2 3 4 5
Days of Subculture

3 and 0.5 hours, respectively. Neither the level of recovered ribosomal RNA, as seen in ethidium bromide stained gels (Fig. 1A), nor the expression of β-actin changed significantly in response to isoproterenol in these cells within this time (not shown). The β_1-specific antagonist, betaxolol, at 1 μM, largely reversed the isoproterenol-induced increase in β_1-adrenergic receptor mRNA at 1 hr (Fig. 3A) and attenuated the decrease at 4 hr (data not shown) but enhanced the isoproterenol-induced decrease in β_2-adrenergic receptor mRNA. Similarly, the β_2-specific antagonist, ICI 118,551, at 1 μM, reversed the isoproterenol-induced down-regulation at 1 hour of β_2-adrenergic receptor mRNA but actually enhanced the increase in β_1-adrenergic receptor mRNA (Fig. 3B).

DISCUSSION

Hadcock and Malbon have shown a down-regulation of β_2-adrenergic receptor mRNA in DDT_1 MF-2 hamster vas deferens cells in response to isoproterenol stimulation (2). We find this to be true in C_6 glioma cells for both β_1- and β_2-adrenergic receptor mRNA. The half-time of decay of β-adrenergic receptor mRNA in C_6, however, is much shorter, and is similar to that of the disappearance of total receptor number detected by ^{125}I-iodocyanopindolol binding (1). The increase in β_1-adrenergic receptor mRNA 1 hr after treatment with isoproterenol was reproducible and could be reversed by the β_1-specific antagonist, betaxolol. Collins et al. have reported early up-regulation of the β_2-adrenergic receptor mRNA in DDT1 MF-2 cells and have shown this to be due to increased transcription of the β_2-adrenergic receptor gene enhanced by one or more cAMP responsive elements 5′ to the site of transcription intiation (3). The functional significance of this transient increase in receptor mRNA synthesis remains to be elucidated.

Figure 1. Time Course of β-adrenergic receptor mRNA Expression after Subculture. **A.** β_1- and β_2-adrenergic receptor mRNA as well as total RNA from cultures of C_6 glioma cells on the day of subculture and each day thereafter for up to 5 days. One tenth aliquots of samples prepared for Northern blotting were diluted ten-fold and applied to a 1% agarose gel in amounts representing 0.8x,0.4x,0.2x, and 0.1x μg total RNA, as shown, where x is the concentration of the original sample in μg/μl RNA. The remaining original samples of RNA (18 μl) were electrophoresed on a formaldehyde agarose gel as described in Methods. RNA samples (from a separate experiment) whose concentration had been adjusted so as to allow the application of equal amounts of total RNA (10 μg), were included on the gel for comparison. Each lane represents a separate culture; each time point was done in triplicate; a duplicate experiment of identical result was obtained. **B.** Increase of total RNA with culture time. Estimation of total RNA shown in **A** was achieved as described in Methods. **C.** Changes in levels of β_1- (solid line) and β_2-adrenergic receptor (dotted line) mRNA with time of culture. The level of β_2-adrenergic receptor mRNA was estimated by densitometric scans of autoradiograms taken of the Northern blot described and shown in **A**. In the case of β_1-adrenergic receptor mRNA, only the area of the 2.4 Kb band was used. The area of each band was divided by the amount of RNA in that lane as determined in **B** and expressed as a fraction of the mean initial value (day 0 of subculture).

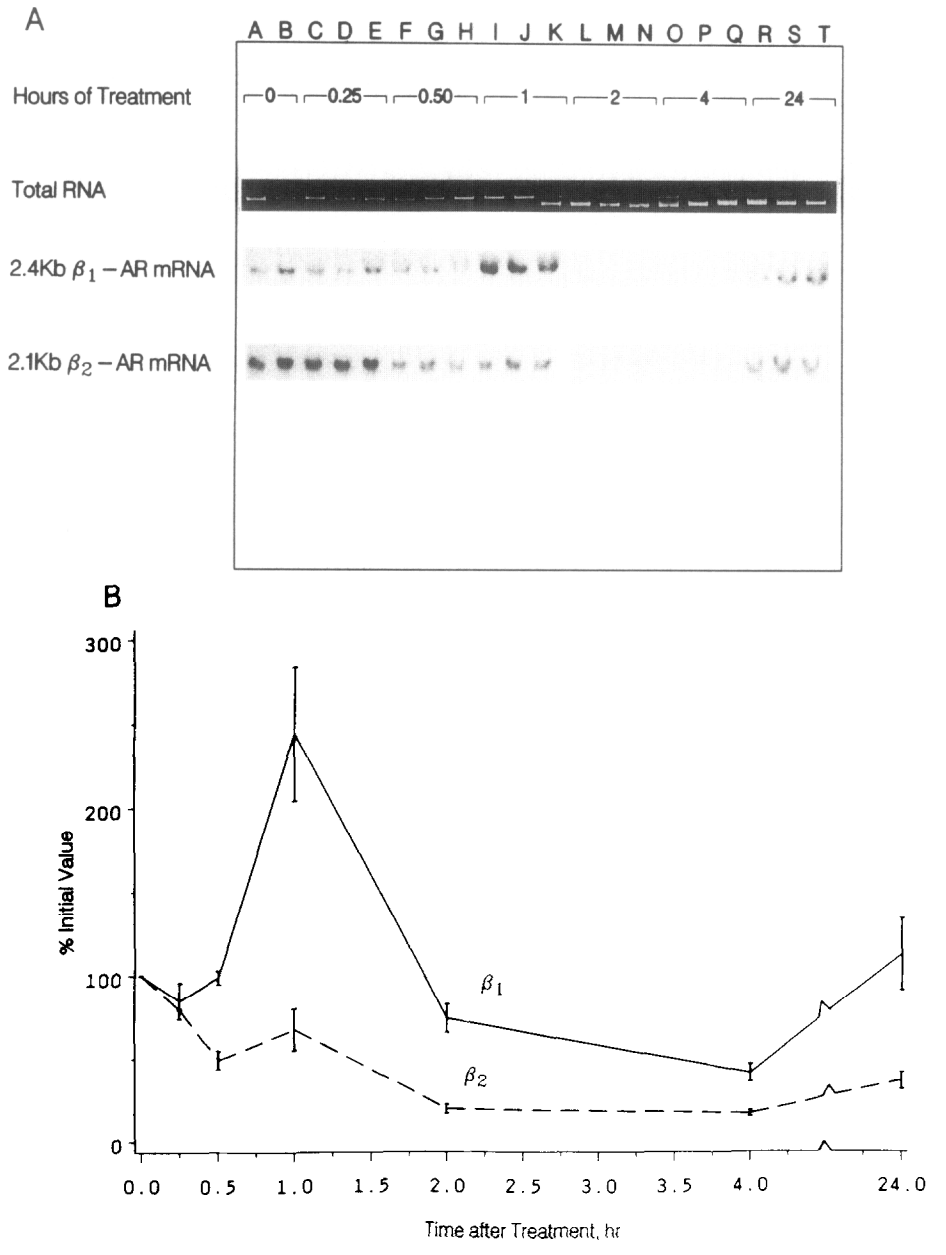


Figure 2. Time Course of β-adrenergic receptor mRNA Down-Regulation after Exposure to Isoproterenol. **A.** β_1- and β_2-adrenergic receptor mRNA as well as total RNA from C_6 glioma cultures treated with 1 μM (-)-isoproterenol for 0.25,0.5,1,2,4 and 24 hours. The total RNA shown was obtained as described (Fig. 1) except that equal amounts were applied to the gel in all cases. Each lane represents a different culture; time points are the average of triplicate samples; a duplicate experiment with identical results was obtained. **B.** Changes in levels of β_1- (solid line) and β_2-adrenergic receptor mRNA (dotted line) with time of isoproterenol treatment. The level of β-adrenergic receptor mRNA was estimated, normalized, and expressed as described (Fig. 1).

The differential regulation of β_1- and β_2-adrenergic receptor mRNA has been suggested by reports of selective regulation of β_1- and β_2-adrenergic receptor binding in C_6 glioma (1) and rat cerebral cortex (8) in response to

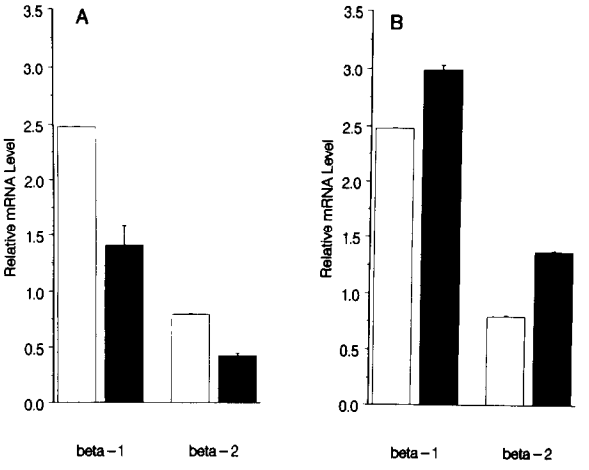


Figure 3. Blockade of Isoproterenol-Induced Changes in β-Adrenergic mRNA by Subtype-Specific Antagonists. **A.** Levels of β_1- and β_2-adrenergic receptor mRNA induced by 1 hr exposure to 1 μM isoproterenol in the presence (filled) and absence (unfilled) of 1 μM of the β_1-specific antagonist, betaxolol relative to control (no treatment). **B.** Levels of β_1- and β_2-adrenergic receptor mRNA induced by 1 hr exposure to 1 μM isoproterenol in the presence (filled) and absence (unfilled) of the β_2-specific antagonist, ICI 118,551, relative to the untreated control. Results shown are the average of duplicate determinations. A duplicate experiment of identical results was obtained.

atypical or partial agonists. Since both β_1- and β_2-adrenergic receptors are coupled to stimulation of adenylate cyclase, the differential down-regulation of mRNA specific for β_1- and β_2-adrenergic receptor in response to isoproterenol suggests that generation of cyclic AMP is not the sole factor involved in the control of β-adrenergic receptor mRNA levels. This is underscored by the complexity with which C_6 cells responded to the co-presence of isoproterenol and betaxolol or ICI 118,551. Furthermore, the fact that β_1-adrenergic receptor mRNA is up-regulated in C_6 glioma cells and β_2-adrenergic receptor mRNA is up-regulated in DDT_1 MF-2 hamster vas deferens cells suggests that differences in the regulation of β_1- and β_2-adrenergic receptor mRNA levels may not be due to differences in their protein sequences (and hence to differences in their function). In addition, the down-regulation of β-adrenergic receptor mRNA accompanying contact inhibition of C_6 cells argues that β-adrenergic receptor mRNA levels may be controlled by events other than β-adrenergic receptor stimulation. Such events may include the activation and/or membrane translocation of protein kinase C as suggested by the ability of phorbol esters to induce β-adrenergic receptor down-regulation (9). The overall decay of β-adrenergic receptor mRNA with time implies a relative loss of β-adrenergic receptor with passage number (10) unless receptor expression is induced by the loss of contact inhibition. The location of a protein kinase C translocation site to loci of cell adhesion (11) suggests that β-adrenergic receptor mRNA down-regulation by

contact inhibition may be mediated by protein kinase C. We are currently exploring this possibility.

ACKNOWLEDGMENTS

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