Agonist-induced Destabilization of β-Adrenergic Receptor mRNA

ATTENUATION OF GLUCOCORTICOID-INDUCED UP-REGULATION OF β-ADRENERGIC RECEPTORS*

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β-Adrenergic receptor expression and receptor mRNA levels are down-regulated by β-adrenergic agonists and up-regulated by glucocorticoids. The interaction between these two opposing regulatory pathways was investigated at the levels of receptor and receptor mRNA in DDT, MF-2 hamster vas deferens cells. Dexamethasone blunted a marked decrease in receptor expression induced by isoproterenol alone, as made visible by indirect immunofluorescence using anti-receptor antibodies. Receptor mRNA levels were quantified by DNA-excess solution hybridization. Dexamethasone stimulated a sharp increase in receptor mRNA at 4 h following the addition of steroid in either the absence or the presence of isoproterenol. By 12 h, dexamethasone treatment resulted in a new steady-state level of receptor mRNA double that observed in untreated cells. Isoproterenol blunted the dexamethasone effect observed at 12 h. Cells treated with isoproterenol and dexamethasone in combination displayed a new steady-state level only 30% greater than untreated cells. Measured by nuclear run-on assays, transcription rates of the receptor gene were unaffected in cells challenged with isoproterenol alone. Dexamethasone, in contrast, stimulated a 4-fold increase in β-adrenergic receptor gene transcription. Isoproterenol and dexamethasone in combination promoted a transcription rate comparable to dexamethasone alone. The half-life of receptor mRNA in untreated and dexamethasone-treated cells was 12 h. In contrast, β-adrenergic receptor mRNA half-life declined to 5 h in cells that were treated with isoproterenol in the presence or absence of dexamethasone. Agonist-promoted destabilization and steroid-induced transcription provide mechanisms for the interplay of two opposing pathways controlling receptor mRNA levels.

Glucocorticoids and β-adrenergic agonists play important roles in the regulation of the hormone-sensitive adenylate cyclase (Malbon et al., 1988). The β-adrenergic receptor is a prominent locus for permissive hormone effects (Davies and Lefkowitz, 1984). Glucocorticoids stimulate a 2-3-fold increase in receptor expression and sensitivity to stimulation by β-agonist (Mano et al., 1979; Lee and Reed, 1977; Scarpace et al., 1985; Fraser and Venter, 1980; Lai et al., 1981; Foster and Harden, 1980; Sharma et al., 1989). Recently, we and others have reported that glucocorticoids increase the steady-state levels of β-adrenergic receptor mRNA and expression in DDT, MF-2 hamster vas deferens cells (Haddock and Malbon, 1988b; Collins et al., 1988) and CHO-K1 cells (Malbon and Haddock, 1988a). Increased transcription of the β-adrenergic receptor gene has been implicated as a basis for the induction of receptor mRNA by glucocorticoids in DDT, MF-2 cells (Collins et al., 1988b; Haddock and Malbon, 1988b).

In addition to regulation by permissive hormones, the β-adrenergic receptor is regulated by agonist stimulation. Treatment of cells with β-adrenergic agonists leads to an "uncoupling" of the receptor from the stimulatory G-protein,1 G, the G-protein that mediates hormonal stimulation of adenylate cyclase, and a loss of radioligand binding (Sibley and Lefkowitz, 1985). Chronic stimulation of DDT, MF-2 cells with agonist promotes down-regulation of β-adrenergic receptor and receptor mRNA levels. β-Adrenergic agonists promote a down-regulation of receptor mRNA, establishing a new steady-state level of message at 18 h of challenge which is 40–50% lower than that observed in untreated cells (Haddock and Malbon, 1988a).

β-Adrenergic agonists promote bronchodilation and represent important therapeutic agents for the treatment of asthma. Chronic use of β-adrenergic agonists, however, leads to adaptation, manifest by a reduced ability of β-adrenergic agonists to maintain bronchodilation (Nelson, 1986). β-Adrenergic receptors in lung are down-regulated by chronic stimulation with β-agonists. This agonist-promoted down-regulation can be reversed by treatment with glucocorticoids (Davies and Lefkowitz, 1984). Exposure to glucocorticoids, in vivo or in vitro, restores the responsiveness of isoproterenol-stimulated cAMP accumulation in leukocytes desensitized by chronic incubation with β-adrenergic agonists (Lee and Reed, 1977; Logson et al., 1972). The reversal of agonist-induced down-regulation of the β-adrenergic receptor by glucocorticoids has been well characterized in DDT; MF-2 cells at the level of radioligand binding. Scarpace et al. (1986) have demonstrated that glucocorticoid treatment of cells previously exposed to agonist "resensitizes" the cells to stimulation by β-agonists and promotes recovery of β-adrenergic receptors to levels greater than that observed in control cells. The recovery of receptors is associated with a complete restoration of agonist-stimulated adenylate cyclase.

DNA-excess solution hybridization assays have been uti-

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The abbreviations used are: G-protein, guanine nucleotide-binding regulatory protein; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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**Experimental Procedures**

**Materials**—The hamster β2AR cDNA (Dixon et al., 1986) was a gift from Dr. R. A. F. Dixon, Merck, Sharp and Dohme, West Point, PA. The sources of all other materials are detailed elsewhere (Haddock and Mabon, 1988a, 1988b; Collins et al., 1988). A more complete understanding of the interaction between agents with opposing actions on β-adrenergic receptor expression was sought in the present work. At the level of mRNA, DNA-excess solution hybridization and nuclear run-on transcription assays were employed. At the level of receptor protein, radioligand binding and indirect immunofluorescence were used to study the expression of β-adrenergic receptors.

**Cell Culture**—DDT1 MF-2 cells were grown to confluence as monolayers in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum, 3.75 g/liter sodium bicarbonate, streptomycin (50 μg/ml), and penicillin (50 μg/ml), as described by Scarpace et al. (1985). Nuclei were isolated from cultured cells in 0.5 M sucrose, 10 mM Tris-HCl, pH 8.3, 5% glycerol, 5 mM MgCl₂, 0.1 mM EDTA, 4°C for 3 h. The resulting pellets were washed and resuspended in lysis buffer composed of 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, and 0.5% Nonidet P-40. Nuclei were collected by centrifugation at 500 × g for 10 min. The nuclei pellets were washed and resuspended in glycol buffer (50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM MgCl₂, 0.1 mM EDTA) for storage and maintained at −70°C. Nascent transcripts were detected as described by Greenberg and Ziff (1984). Nuclei (200 μl) were added to 200 μl of a reaction buffer composed of 10 mM Tris-Cl, pH 8.0, 5 mM MgCl₂, 0.5 mM KCl, 5 mM dithiobetrol, unlabeled CTP, ATP, and 100 μl of [β-32P]UTP (Du Pont-New England Nuclear, 800 Ci/mmol). Newly transcribed labeled RNA was extracted (Greenberg and Ziff, 1984) and then incubated for 36 h at 65°C with plasmid DNAs immobilized on Nitrex (Schleicher & Schuell). After hybridization, each sample was washed two times with 2 × SSC (1 × SSC is 15 mM sodium citrate, pH 7.0, 0.15 M NaCl) for 60 min at 65°C. The samples were then treated with RNase A for 30 min at 37°C followed by a wash with 2 × SSC at 37°C for 60 min. The filters were dried and subjected to autoradiography for 72 h with an intensifying screen. Relative changes in transcription were assessed by scanning densitometry of the autoradiograms.

**Indirect Immunofluorescence of β-Adrenergic Receptors**—Indirect immunofluorescence of β-adrenergic receptors was performed on intact DDT1 MF-2 cells grown on glass chamber slides. The cells were fixed with paraformaldehyde and probed with either the anti-receptor antisera (George et al., 1988; Haddock and Mabon, 1988b) or preimmune serum, at 1:200 dilutions. The fixed cells were then stained by rhodamine-conjugated goat anti-rabbit IgG (Boehringer Mannheim) diluted 1:1000. Phase-contrast and epifluorescence microscopy were performed with a Zeiss Axioshot system and photographed with hypersensitized Kodak 2415 film (George et al., 1988).

**Results**

The steady-state levels of β-adrenergic receptor mRNA in control untreated cells were found to be 0.63 ± 0.05 amol β-adrenergic receptor mRNA/μg of total cellular RNA (amol/μg of RNA). When cells were treated with dexamethasone and isoproterenol, β-adrenergic receptor mRNA levels increased to 1.7 ± 0.2 amol/μg of RNA by 4 h (Fig. 1A). By 12 h, a new steady-state level of 1.3 ± 0.15 (n = 5) amol/μg of RNA was achieved. This new steady-state remained essentially constant for the next 24 h. Challenging cells with dexamethasone and a β-agonist isoproterenol similarly produced a sharp increase in the steady-state level of receptor mRNA at 4 h, rising from 0.63 to 1.53 ± 0.17 (n = 3) amol/μg of RNA. In contrast to the sustained elevation of receptor mRNA which occurred at later times (>4 h) in response to steroid alone, receptor mRNA levels were not sustained in cells treated concurrently with dexamethasone and isoproterenol. By 12 h, β-adrenergic receptor mRNA levels declined from 1.3 to 0.85 ± 0.12 (n = 3) amol/μg of RNA. Challenge with isoproterenol alone induced a progressive decline in β-adrenergic receptor mRNA levels. After a lag of 4 h, receptor mRNA of isoproterenol-treated cells declined to 50-60% of their original levels (Fig. 1B). Dexamethasone induced a sharp increase in receptor mRNA levels in control cells (Fig. 1A) as well as in isoproterenol-treated cells (Fig. 1B). In which agonist had prompted a substantial down-regulation of receptor mRNA. By 4 h, steroid increased mRNA levels in isoproterenol-treated cells from 0.38 ± 0.09 (n = 3) to 0.9 ± 0.12 (n = 3) amol/μg of RNA (Fig. 1B). This new steady state in receptor mRNA induced by dexamethasone remained elevated over the next 12 h.

Was the attenuated dexamethasone response in isoproterenol-treated cells a result reduced sensitivity of the cells to...
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2.00 -4
1.75 * T
0-0 untreated
0-0 dexamethasone

FIG. 1. Interplay of agonist- and glucocorticoid-induced modulation of β-adrenergic receptor mRNA levels. A, DDT, MF-2 cells were incubated for the indicated times with vehicle (untreated, ○—○), with 500 nM dexamethasone (□—□), with 500 nM dexamethasone followed by concurrent addition of 10 μM isoproterenol (■—■), and with 10 μM isoproterenol simultaneously with 500 nM dexamethasone (iso/dex, ▲—▲). B, DDT, MF-2 cells were incubated for the indicated times with vehicle (untreated, ○—○), 10 μM isoproterenol (●—●), and 10 μM isoproterenol followed by concurrent addition of 500 nM dexamethasone at 24 h (iso then dex at 24 hr). Cells were harvested, RNA was extracted, and DNA-excess solution hybridization was performed as described under “Experimental Procedures.” The results displayed are the means (±S.E.) of three to five separate determinations. Each determination was performed in duplicate.

the steroid? The dose dependence of the steroid-induced increase of receptor mRNA was examined in control cells and cells challenged with agonist (10 μM isoproterenol for 24 h). In both control and isoproterenol-treated cells alike, the maximal response in receptor mRNA occurred at ~250 nM dexamethasone. Half-maximal stimulation by steroid was achieved at equivalent concentrations of steroid in control and isoproterenol-treated cells (Fig. 2). Isoproterenol and dexamethasone regulation of β-adrenergic receptor mRNA levels was explored at the level of transcription by nuclear run-on assays. Dexamethasone (4 h, 500 nM) enhanced the relative rates of transcription of the β2-adrenergic receptor gene by 4-fold (Fig. 3). Isoproterenol, in contrast, failed to alter the relative rate of transcription of the β2-adrenergic receptor gene. When cells were challenged with dexamethasone and isoproterenol simultaneously, transcription rates of the β2-adrenergic receptor gene were enhanced to levels essentially equivalent to those of cells treated with steroid alone.

One possible mechanism by which the steady-state receptor mRNA levels might be down-regulated by agonist was evalu-

Dexamethasone but not isoproterenol modulates the rate of transcription of the β-adrenergic receptor gene: analysis by nuclear run-on assays. DDT, MF-2 cells were treated with vehicle (basal), 1 μM dexamethasone (dex) alone, 10 μM isoproterenol (iso) alone, or dexamethasone and isoproterenol in combination (dex/iso) for 4 h. The cells were harvested, and nuclei were isolated as described under "Experimental Procedures." Transcription elongation was allowed to continue in the presence of [α-32P]UTP and unlabeled nucleotides. After elongation, radiolabeled RNA was hybridized either to plasmid harboring the β-adrenergic receptor cDNA (BAR) or to the plasmid lacking the receptor cDNA insert (pUC). Autoradiograms (72-h exposure) were analyzed by scanning densitometry to quantify the relative changes in the rate of transcription. The left panel is a representative autoradiogram. The right panel is the mean (± the range) of values derived from two independent experiments.
ated by investigating the stability of receptor mRNA. Cells were treated with vehicle, isoproterenol (10 μM, 24 h) alone, dexamethasone alone (500 nM, 12 h), or isoproterenol and dexamethasone in combination for 24 h and then concurrently with actinomycin D for 12 h (Fig. 4). Cells were then harvested at 0, 4, 8, and 12 h following the addition of actinomycin D. Using the approach of Rodgers et al. (1985), we estimated the half-life of receptor mRNA. Receptor mRNA displayed a half-life of 12 h in control cells. Interestingly, receptor mRNA half-life was observed to decline from 12 to 5 h in cells challenged with β-agonist. Receptor mRNA half-life was 5 h in cells treated with isoproterenol and dexamethasone in combination. Dexamethasone, in contrast to β-agonist, failed to alter the half-life of receptor mRNA (Fig. 4).

Receptor expression at the protein level in response to steroid and β-agonist was probed by two independent methods, radioligand binding and indirect immunofluorescence with antireceptor antibodies. By 72 h of treatment, dexamethasone doubled the steady-state expression of receptors as measured by \(^{125}\)I-hydroxycyanopindolol binding (Table I). Isoproterenol reduced β-adrenergic by half and, in addition, attenuated the glucocorticoid-induced increase in receptor expression. Indirect immunofluorescence revealed a similar set of observations (Fig. 5). Challenging cells with β-agonist reduced the epifluorescence, suggesting a loss in receptor expression (compare Figs. 5, A and C). Glucocorticoids, in contrast, were shown to increase epifluorescence (Fig. 5F). These data agree well with those observed by radioligand binding. Isoproterenol blunted the glucocorticoid-induced increase in receptor expression when cells were challenged simultaneously with both steroid and β-agonist (Fig. 5G).

**Figure 4. β-Adrenergic receptor mRNA stability: effects of agonists and glucocorticoid-response elements.** DDT, MF-2 cells were incubated with vehicle (○——○), 10 μM isoproterenol alone (iso, △——△), 500 nM dexamethasone (dex, ■——■), or simultaneously with isoproterenol and 500 nM dexamethasone (dex + iso, ▲——▲), then concurrently with actinomycin D (5 μg/ml). Cells were harvested at 0, 4, 8, and 12 h after the addition of actinomycin D. β-Adrenergic receptor mRNA levels (amol/μg of total cellular RNA) of cells were as follows: control, 0.66; isoproterenol-treated, 0.30; dexamethasone-treated, 1.23; and dexamethasone + isoproterenol-treated, 0.76. The data are mean values of three separate experiments except the dexamethasone and isoproterenol in combination, which is the average of two separate experiments. Each separate determination of mRNA was performed in duplicate.

**Table I**

Radioligand binding to DDT, MF-2 cell membranes

| Treatment             | Receptor levels of ICPY binding | Significance with respect to control agonist |
|-----------------------|---------------------------------|---------------------------------------------|
|                       | fmol/mg protein | p value | Control | Agonist |
| Control               | 37 ± 5             | <0.02   |         |         |
| Isoproterenol (10 μM) | 18 ± 2             | <0.02   | <0.01   |         |
| Dexamethasone (500 nM)| 81 ± 12            | <0.02   |         |         |
| Dexamethasone/isoproterenol | 46 ± 3       | NS      | <0.05   |         |

Glucocorticoids “up-regulate” the expression of β-adrenergic receptors (Haddock and Malbon, 1988b; Collins et al., 1988), whereas agonists “down-regulate” the expression of these G-protein-linked receptors (Haddock and Malbon, 1988a). The goal of the present work was to explore the counterregulation of agonist and glucocorticoids at the levels of both receptor protein and mRNA. Chronic treatment of asthmatic conditions with β-agonists promotes an adaptive response that compromises the beneficial aspects of this therapy over an extended period (Nelson, 1986). Glucocorticoids have been shown to be effective in treating this agonist-induced adaptation (Davies and Lefkowitz, 1984). These observations prompted us to explore the underlying molecular basis for the counterregulatory effects of glucocorticoids and agonists on receptor expression.

Radioligand binding and in situ indirect immunofluorescence provided a compelling picture of the interplay between glucocorticoids and β-agonist stimulation at the level of receptor expression. As observed previously (Haddock and Malbon, 1988b), glucocorticoids increased β-adrenergic receptor levels in DDT, MF-2 cells. In vivo, glucocorticoids promote the expression of β-adrenergic receptors in lung and other tissues (Davies and Lefkowitz, 1984; Sharma et al., 1989). Chronic stimulation by agonist promoted a loss of radioligand binding as well as a reduction in the epifluorescence signal of cells stained with antireceptor antibodies. Interestingly, the punctiform patterns of staining were not grossly altered by either up- or down-regulation. When cells were exposed to both agents simultaneously, a clear reduction in epifluorescence signal and radioligand binding was evident when compared with the unopposed action of glucocorticoid alone. The epifluorescence and binding data do suggest, however, that glucocorticoids rescue, to a limited extent, the cells from the agonist-induced down-regulation of receptors. With respect to expression of receptor, neither glucocorticoid-induced up-regulation or agonist-promoted down-regulation predominates. Agonists remain capable of down-regulating receptor expression even in the presence of glucocorticoids. Glucocorticoids are capable of up-regulating receptor levels even in cells chronically stimulated by agonist.

Analysis at the level of receptor mRNA provided both a basis for the counterregulatory effects of agonist and glucocorticoids as well as a molecular explanation for the reduction in mRNA promoted by chronic stimulation by agonist. A sharp transient peak of receptor mRNA was induced by glucocorticoid alone and by glucocorticoid in combination with β-agonist. Thus, the early phase of the glucocorticoid response appears to be largely unaffected by chronic stimulation by agonist. The second phase, establishing a new steady-state level of receptor mRNA in response to glucocor-
ticoid, in contrast, was sensitive to chronic stimulation with agonist. Isoproterenol stimulation reduced the steady-state level of receptor mRNA in response to steroid back to nearly control levels. Regulation of the expression of receptor mRNA and protein remains sensitive to both of these two opposing forces.

Analysis of the relative rate of transcription by nuclear run-on assays revealed two important features about the counter-regulation by agonist and glucocorticoids. First, agonist treatment does not appear to alter the rate of transcription of the \(\beta\)-adrenergic receptor gene. The presence of cAMP-response elements in the gene and the ability of \(\beta\)-agonists to increase intracellular cAMP prompted the suggestion that these elements may be involved in the negative control of transcription (Haddock and Malbon, 1988a). This would have been a novel mode of regulation as cAMP-response elements have been shown to enhance rather than suppress transcription rates of target genes (Roesler et al., 1988). Our data clearly demonstrate that agonist stimulation does not suppress transcription of the receptor gene. Second, isoproterenol treatment did not alter the ability of glucocorticoids to enhance transcription of the receptor gene. These observations prompted us to evaluate the stability of receptor mRNA under the influence of agonist and steroid.

The half-life of the \(\beta\)-adrenergic receptor mRNA was examined in control cells and cells chronically stimulated with steroid. As reported previously (Haddock and Malbon, 1988b), the half-life for receptor in control cells was \(-12\) h. Glucocorticoids did not alter the half-life of receptor message. Several consensus sequences for glucocorticoid-response elements exist in both the hamster and human \(\beta\)-adrenergic receptor genes (Kobilka et al., 1987; Emorine et al., 1987). These potential glucocorticoid-response elements have been identified in the 5′-noncoding, coding, and 3′-noncoding regions of the genes. Evidence has been presented that the glucocorticoid-response element(s) in the 5′-noncoding domain of the \(\beta\)-adrenergic receptor gene are obligate for glucocorticoid responsiveness (Malbon and Haddock, 1988). In sum, these studies suggest that it is primarily the enhanced rate of transcription at a glucocorticoid-response element in the 5′-noncoding portion of the gene which is responsible for the steroid-induced up-regulation of receptor mRNA. Message stability appears to play no major role in the glucocorticoid effect.

Finally, the present work reveals a molecular explanation for agonist-promoted down-regulation of receptor mRNA, message destabilization. The half-life of receptor mRNA declined from \(-12\) h in control cells to \(-5\) h under the influence of stimulation with \(\beta\)-agonist. The half-life of receptor mRNA in glucocorticoid-stimulated cells was similarly reduced to \(-5\) h.

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**Fig. 5.** Agonist and glucocorticoid regulation of \(\beta\)-adrenergic receptor expression in DDT, MF-2 cells: analysis in situ by indirect immunofluorescence. DDT, MF-2 cells were incubated for 72 h in medium alone (panels A and B), medium plus 10 \(\mu\)M isoproterenol (panels C and D), medium plus 500 \(\mu\)M dexamethasone (panels E and F), or medium plus 500 \(\mu\)M isoproterenol (panels G and H). Phase-contrast (panels B, D, F, and H) and epifluorescence (panels A, C, E, and G) images are shown. Fixed intact cells were stained with antireceptor antiserum CMS-3 (panels A–H). The results displayed are from a single experiment, representative of four separate experiments. (Bar = 0.25 \(\mu\)M).
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h following stimulation by β-agonist. Study of the time course of receptor mRNA levels in glucocorticoid-treated cells demonstrated that in the second phase of steroid induction, isoproterenol was able to reduce mRNA levels rapidly without altering the rate of transcription. Thus, by promoting destabilization of message, agonist can maintain counterregulation of the glucocorticoid response. These studies provide a more detailed picture of the dynamic regulation of the expression of a G-protein-linked receptor in response to counterregulation by agonist and steroid. Via the interplay of enhanced transcription (steroid effect) and altered mRNA stability (agonist effect) the steady-state level of receptor message and protein can be modulated dynamically by two opposing forces.

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