Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 5021–5025, July 1988
Biochemistry

Down-regulation of β-adrenergic receptors: Agonist-induced reduction in receptor mRNA levels

(cAMP/DNA-excess solution hybridization)

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Communicated by Alfred G. Gilman, April 1, 1988 (received for review January 22, 1988)

ABSTRACT **Incubation of DDT_1 MF-2 hamster vas deferens cells with β-adrenergic agonists results in a time- and concentration-dependent decrease in both β-adrenergic receptor (βAR) responsiveness and receptor number. Receptor mRNA levels were quantified by DNA-excess solution hybridization by using a 170-nucleotide single-stranded probe derived from the hamster β_2AR cDNA. RNA blot analysis of poly(A)$^+$-selected RNA with the solution probe revealed a 2.2-kilobase species. Digestion of the RNA/solution probe mixture with S1 endonuclease revealed a single species of RNA (170 bases) that was protected by the solution probe. DDT_1 MF-2 cells were found to contain 0.38 pg of βAR mRNA per μg of total cellular RNA. Incubation (16 hr) with isoproterenol decreased βAR mRNA levels in cells by 40%. This agonist-induced decrease in receptor mRNA levels was found to be dependent on the time of incubation and the dose of agonist. The decrease in βAR mRNA was half-maximal at 0.1–0.5 μM isoproterenol. The β-adrenergic antagonists CGP 20712A (β_1-selective) and ICI 118,551 (β_2-selective) blocked in a dose-dependent fashion the ability of isoproterenol to effect receptor mRNA levels. The β_2-adrenergic antagonist displayed a potency 25-fold greater than that of the β_1-adrenergic antagonist, in agreement with the subtype of receptor (β_2) expressed by these cells. For down-regulated cells in which receptor mRNA levels declined in response to agonist, the addition of the antagonist ligand (−)-propranolol (1 μM) was able to restore receptor mRNA levels to 90% of the control value within 12 hr. Full recovery of steady-state βAR mRNA was achieved within 60 hr. These studies provide a molecular explanation for the down-regulation of GTP-binding regulatory protein (G protein)-linked cell-surface receptors that accompanies desensitization.**

Desensitization, an attenuated response to chronic stimulation, is commonly observed in biological systems. Photoadaptation (1), as well as tolerance to therapeutic agents, such as insulin (2) and catecholamines (3), are important examples of desensitization. Many of the physiological effects of catecholamines are expressed via specific interaction with β-adrenergic receptors (βARs) (4). βARs, in fact, typify a large class of GTP-binding regulatory protein (G protein)-linked cell-surface receptors that generally display the phenomenon of desensitization (3).

Chronic incubation of cells with β-adrenergic agonists such as isoproterenol leads not only to desensitization, but also to a marked reduction (down-regulation) in receptor number (5, 6). In 1321N1 human astrocytoma and S49 mouse lymphoma cells, for example, agonist-induced desensitization has been shown to be accompanied by a down-regulation of βARs (7). This down-regulation of receptors is preceded by a readily reversible "uncoupling" of the receptor from its G protein, G_s (the G protein that mediates stimulation of adenylate cyclase) (7). Whereas the agonist-induced uncoupling of receptor from G protein occurs within 30 min, the down-regulation of receptors is demonstrable only after 4–12 hr (8). This general pattern of desensitization and down-regulation has been observed in many other cell types, including DDT_1 MF-2 cells (9–11). The molecular basis for agonist-induced down-regulation of G-protein-linked cell-surface receptors is poorly understood. Agonist-induced reduction in receptor number may be due to an inability of the activated receptor to bind ligand, to a sequestration of receptors from the cell surface to some other compartment of the cell, or to the degradation or actual loss of a full complement of cellular receptors. A role for agonist-induced phosphorylation of receptors in the densensitization of βARs has been proposed (9), although recent reports suggest this proposal remains controversial (12, 13).

The recovery of receptor number to normal levels after agonist-induced down-regulation in postconfluent cell cultures has been shown to require the *de novo* synthesis of receptors (8). In spite of a vast literature on the regulation of βARs, and the recent advances in our understanding of the structure of these receptors and their genes (14, 15), knowledge about the nature and regulation of the mRNAs for these proteins is limited. The G-protein-linked receptors studied to date are low-abundance proteins, and examination of their mRNAs by standard approaches such as RNA blot analysis suggests that mRNA levels are comparably low (14). Quantification of very low abundance mRNAs poses a serious problem to the study of mRNA regulation. In some circumstances, DNA-excess solution hybridization analysis has been shown to be of adequate sensitivity to permit quantification of mRNA levels for the low density lipoprotein receptor in control and estrogen-treated rabbits (16). In the present report, DNA-excess solution hybridization analysis is applied to the study of mRNA levels for a low abundance G-protein-linked receptor, the βAR. Agonist-induced receptor down-regulation is probed at the level of mRNA through the application of this technique.

EXPERIMENTAL PROCEDURES

Cell Culture. The hamster vas deferens DDT_1 MF-2 cells were grown to confluence as monolayers as described (11).

Measurement of βARs. βAR levels were measured in intact cells by equilibrium radioligand binding (11) with the antagonist ligand (−)-[^{125}I]iodocyanopindolol.

Preparation of RNA. Total cellular RNA was extracted as described (17). For RNA blot analysis, poly(A)$^+$-selected RNA was isolated by using oligo(dT)-cellulose (18). Form-

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Abbreviations: βAR, β-adrenergic receptor; G protein, GTP-binding regulatory protein; G_s, G protein that mediates stimulation of adenylate cyclase.

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aldehyde/agarose gel electrophoresis was used to assess the integrity of the RNA (18).

Construction of Solution Hybridization Probe βAR 170. The solution probe corresponding to nucleotides 222–392 (14) was constructed from the full-length hamster β_2AR cDNA in pUC8 (a generous gift of R. Dixon, Merck Sharp & Dohme). The βAR cDNA was sequentially digested with *Sma* I and *Taq* I. The resulting 170-base pair fragment (βAR 170) was gel isolated and ligated into M13mp18 replicative form that had been previously digested with *Sma* I and *Acc* I. This construct was used to transform *Escherichia coli* K-12 strain JM101. Sequence analysis (19) verified the sequence and orientation of the probe βAR 170.

Solution Hybridization Probe Synthesis. Synthesis of the probe βAR 170 was essentially as described by Williams *et al.* (20). After primer extension with dATP, dGTP, dTTP, and 180 μCi of [^{32}P]dCTP (800 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear), the probe was digested with 40 units of *Sma* I for 2 hr, and then subjected to 8 M urea/6% polyacrylamide gel electrophoresis. The probe was identified by autoradiography, excised, and electroeluted from the gel slice. The probe was then subjected to hydroxylapatite chromatography and eluted from the matrix by using a step-gradient of $NaPO_4$ (pH 6.5) (20–220 mM). The eluted probe was pooled and desalted with a Centricon-30 cartridge.

Solution Hybridization Assay. Uniformly radiolabeled probe (100 pg per sample) was incubated with known amounts of template DNA (used as a standard), with total cellular RNA, or alone for 60 hr at 68°C in 20 mM Hepes, pH 7.0/0.3 M NaCl/1 mM EDTA/100 μg of denatured salmon sperm DNA per ml. S1 nuclease (150 units), denatured salmon sperm DNA (50 μg/ml), and S1 nuclease buffer (0.28 M NaCl/4.5 mM $ZnSO_4$/50 mM sodium acetate, pH 4.5) were then added to each sample. The samples were then incubated for 90 min at 42°C. The samples were precipitated with trichloroacetic acid (7.5%) on ice for 10 min and the S1 nuclease-resistant hybrids were collected by vacuum filtration on Whatman GF/C filters (20). The S1 nuclease resistance of the probe was <1%.

S1 Endonuclease Protection of βAR mRNA. S1 nuclease mapping was performed by the method of Berk and Sharp (21), as modified by Shelness and Williams (22). βAR 170 (50,000 cpm) was incubated with total cellular RNA for 16 hr at 54°C in 80% formamide/250 mM NaCl/1 mM EDTA/40 mM Pipes, pH 6.4. After it was diluted 1:10 in S1 nuclease buffer, the mixture was supplemented with S1 nuclease (200 units/ml) and incubated again for 90 min at 42°C. The S1 nuclease-resistant hybrids were subjected to electrophoresis on 8 M urea/6% acrylamide gels. The gels were dried and subjected to autoradiography for 72 hr.

RNA Blot Hybridization. Poly(A)$^+$-selected RNA (25 μg) was subjected to electrophoresis on 1.2% agarose/3% formaldehyde gels (18). The RNA was transferred to nylon membranes by electroblotting (0.3 mA) for 14–18 hr in 25 mM $NaPO_4$ (pH 6.5) (23). After immobilization of the RNA by UV-catalyzed crosslinking, the blots were prehybridized for 6 hr in 0.6 M NaCl/75 mM sodium citrate, pH 7.0 (5× SSC) buffer containing 5% $NaDodSO_4$. The blots were next incubated for 12 hr at 42°C with βAR 170 (2 × 10^6 cpm/ml) in 5× SSC containing 5% $NaDodSO_4$. The blot was washed twice with 2× SSC/0.5% $NaDodSO_4$ for 15 min at 25°C and then four times with 0.2× SSC/0.5% $NaDodSO_4$ for 30 min at 55°C (18). The blot was dried and subjected to autoradiography for 4 days.

RESULTS

DDT_1 MF-2 hamster vas deferens cells display agonist-induced desensitization and receptor down-regulation (9–11). Challenging these cells with the potent β-adrenergic agonist (−)isoproterenol (10 μM) resulted in a reduction in the number of βARs, as determined by radioligand binding of [^{125}I]iodocyanopindolol to intact cells (Fig. 1). In control cells, the number of βARs was found to be 34,000 sites per cell, in agreement with earlier studies (9). Incubation with isoproterenol resulted in a decline in radioligand binding to 19,000 sites per cell within 1 hr. This 45% reduction in radioligand binding was maintained for 16 hr in the presence of isoproterenol. Based on these data and the earlier work of others (7, 9–11), the influence of chronic agonist stimulation upon receptor mRNA levels was investigated to explore the basis for the apparent loss of βARs.

Measurement of βAR mRNA levels was accomplished with DNA-excess solution hybridization analysis. Initially, RNA blot analysis with a nick-translated radiolabeled cDNA probe was used to evaluate mRNA levels. The yield of poly(A)$^+$-selected RNA from DDT_1 MF-2 cells was such that a liter of cells had to be processed to isolate enough mRNA for just a few RNA blots. Solution hybridization, in sharp contrast, can be used to quantify changes in the steady-state levels of mRNAs with a sensitivity capable of detecting one molecule per cell (20). The 170-nucleotide probe (βAR 170) used in the studies described below was constructed from the hamster cDNA for the β_2AR (14) corresponding to nucleotides 222–392 of the cDNA. Before its use in DNA-excess solution hybridization, the probe was evaluated with respect to several criteria using RNA from DDT_1 MF-2 cells. RNA blot hybridization of 25 μg of poly(A)$^+$ RNA (Fig. 2*A*) with βAR 170 revealed a single band of 2.2 kilobases, in agreement with previous reports (24). A more sensitive assay designed to determine the specificity of βAR 170 for the βAR mRNA, protection of mRNA from digestion by S1 nuclease, was performed with 150 μg of total cellular RNA from cells treated with 10 μM isoproterenol (Fig. 2*B*, lane 4) and from untreated cells (lane 5). A single prominent species of RNA that was 170 bases long was protected specifically from digestion by S1 nuclease in both the isoproterenol-treated and untreated cells (lanes 4 and 5). However, the amount of S1 nuclease-resistant hybrids was clearly less in assays of RNA from the isoproterenol-treated cells. These data demonstrate the high specificity of βAR 170 as measured against total cellular RNA.

DNA-excess solution hybridization using βAR 170 was evaluated next as a means to quantify the levels of receptor mRNA in DDT_1 MF-2 cells. A standard curve in which the amount (cpm) of S1 nuclease-resistant hybrids generated in the presence of an increasing concentration of template DNA was plotted against the amounts of template DNA used in the hybridizations (Fig. 3*A*). The amount of S1 nuclease-resistant

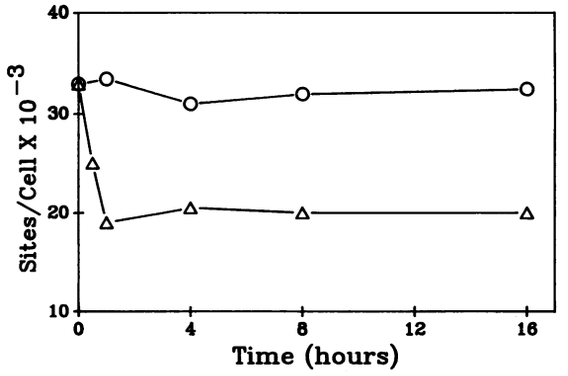

FIG. 1. Down-regulation of βARs in DDT_1 MF-2 cells. Cells were incubated in either the presence (○) or absence (△) of 10 μM isoproterenol for 0–16 hr. Whole-cell binding was performed with [^{125}I]iodocyanopindolol in the presence or absence of 1 μM alprenolol.

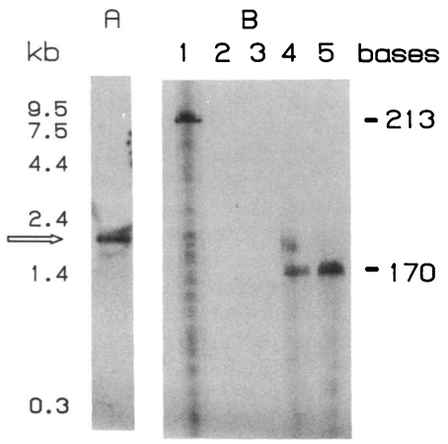

FIG. 2. RNA blot and S1 nuclease protection analyses of βAR mRNA. (*A*) RNA (25 μg) was subjected to RNA blot analysis and probed with βAR 170. The positions of marker RNAs in the autoradiogram (4-day exposure) are shown in kilobases (kb). Arrow marks position of the βAR mRNA (*B*) S1 nuclease analysis. The uniformly labeled probe βAR 170 was hybridized alone (lanes 1 and 2), with 10 μg of yeast tRNA (lane 3), and 150 μg of total cellular RNA from DDT_1 MF-2 cells that were treated for 16 hr with 10 μM isoproterenol (lane 4), and 150 μg of total cellular RNA from untreated cells (lane 5). All samples except in lane 1 were digested with S1 nuclease (150 units/ml) and were subjected to electrophoresis on 8 M urea/6% polyacrylamide gels. A representative autoradiogram is shown.

hybrids (cpm) in RNA samples was compared to this standard curve and was found to increase in a linear fashion when either increasing amounts of standard template DNA or DDT_1 MF-2 RNA were used in the assay (Fig. 3*B*). The theoretical values (assuming 100% hybridization of probe with template) were calculated for the amount of S1 nuclease-resistant hybrids. The actual values obtained with template DNA over the range from 0 to 40 pg agree well with the theoretical values (Fig. 3*A*). The amount of βAR mRNA in units of pg of mRNA per μg of total cellular RNA was calculated by taking into account parameters such as the differences in the length of the probe and that of the full-length mRNA and the presence of M13 sequences in the template DNA that do not contribute to the actual signal (20).

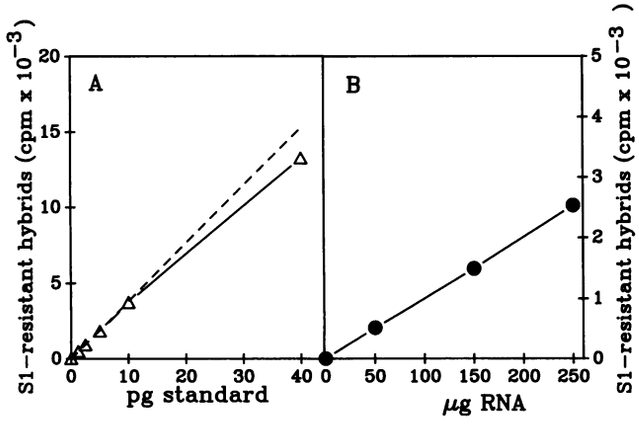

FIG. 3. Measurement of βAR mRNA by DNA-excess solution hybridization. (*A*) Standard curve of βAR 170 solution hybridization probe (100 pg) and template DNA (0–40 pg) complementary to the probe. The values for S1 nuclease-resistant hybrids determined experimentally were compared to the theoretical values (dashed line). (*B*) Total cellular RNA (0–250 μg) extracted from untreated DDT_1 MF-2 cells was hybridized with βAR 170 and then digested with S1 nuclease (150 units/ml). The resulting S1 nuclease-resistant hybrids were then collected and quantified.

For DDT_1 MF-2 cells, a value of 0.38 ± 0.014 pg of βAR mRNA per μg of total cellular RNA (n = 4) was determined.

The time course for the effect of isoproterenol on the level of βAR mRNA was investigated (Fig. 4). After an apparent lag of 4 hr, the βAR mRNA levels were observed to decline rapidly. In DDT_1 MF-2 cells exposed to chronic stimulation with isoproterenol, βAR mRNA levels declined to 60% of their control values, to 0.23 ± 0.011 pg per μg of RNA (n = 5) by 20 hr. No further reduction in receptor mRNA levels was observed in cells that were challenged by isoproterenol for periods of 20–72 hr, the longest time period examined.

To determine whether this agonist-induced reduction in receptor mRNA levels was a reversible process, antagonist ligand was added to isoproterenol-treated cells and the levels of βAR mRNA were analyzed (Fig. 4). The βAR antagonist propranolol (1 μM) was used in combination with washing of the down-regulated cells to ensure blockade of the receptors from any residual agonist (11). When cells exposed to 10 μM isoproterenol for 12 hr were washed and then incubated with fresh medium containing 1 μM propranolol, receptor mRNA levels were observed to recover to 90% of control levels within 12 hr. Full recovery of receptor mRNA levels for cells that had undergone agonist-induced (10 μM isoproterenol) down-regulation was achieved within 60 hr of the washout of agonist and simultaneous challenge with propranolol.

The concentration dependence of the agonist-induced decline in receptor mRNA levels was investigated (Fig. 5*A*). Concentrations of isoproterenol as low as 1 nM were capable of producing a significant decline in the steady-state levels of receptor mRNA at 20 hr of incubation. Half-maximal reduction of βAR mRNA by agonist was achieved at 0.1–0.5 μM isoproterenol, a value that agrees well with that reported for half-maximal stimulation of cAMP accumulation in DDT_1 MF-2 cells in response to isoproterenol (11).

The pharmacology of the agonist-induced decline in receptor mRNA was explored with βAR antagonist ligands that, unlike propranolol, display selectivity for either β_1- or β_2AR (Fig. 5*B*). The β_2-selective antagonist ligand ICI-118,551 inhibited the reduction in receptor mRNA levels stimulated by isoproterenol (10 μM) half-maximally at 20 nM. The β_1-selective antagonist ligand CGP-20712A (25), in contrast, displayed half-maximal inhibition of agonist-induced reduction in receptor mRNA at 0.5 μM. These data demonstrate that the character of the receptors mediating this effect of agonist on receptor mRNA levels in DDT_1 MF-2 cells is β_2 in nature, in agreement with the βAR subtype identified in DDT_1 MF-2 cells (9, 11).

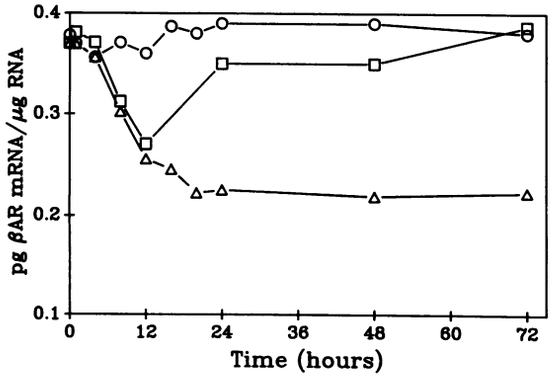

FIG. 4. Time course of down-regulation of βAR mRNA induced by agonist: analysis by DNA-excess solution hybridization. Cells were untreated (○); incubated with 10 μM isoproterenol (△); or incubated with 10 μM isoproterenol for 12 hr, washed, and treated with 1 μM propranolol (□). Each value represents the average of two experiments, each performed in duplicate.

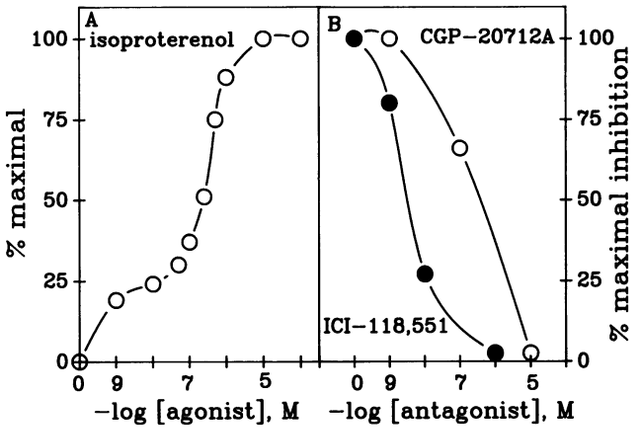

FIG. 5. Agonist-stimulated down-regulation of βAR mRNA; dose–response to isoproterenol and analysis of receptor subtype. (*A*) RNA was prepared from cells that had been incubated with 0–100 μM isoproterenol for 20 hr. Untreated cells and cells stimulated with 100 μM isoproterenol contained 0.38 and 0.227 pg of βAR mRNA per μg of total cellular RNA, respectively. The extent to which the indicated concentrations could stimulate the down-regulation of βAR mRNA is presented as the percentage of the maximal decrement. (*B*) Cells were incubated for 20 hr with 10 μM isoproterenol and the indicated concentrations of either ICI-118,551 (0–1 μM) (●) or CGP-20712A (0–10 μM) (○), and total cellular RNA was prepared. The values were calculated as described in *A*. Results are averages of two experiments, each performed in duplicate.

It was important to determine whether the decline in βAR mRNA observed in response to a βAR agonist ligand could be mimicked by other agents, including those that do not alter cAMP levels. DDT_1 MF-2 cells were incubated for 20 hr with various agents and the βAR mRNA levels were then measured (Fig. 6). The α_1AR agonist ligand phenylephrine (10 μM) as well as somatostatin (100 μM), agents that do not alter cAMP levels in these cells, failed to effect the steady-state levels of βAR mRNA. No change in [^{125}I]iodocyanopindolol binding was detected in cells treated with either phenylephrine or somatostatin (data not shown). Forskolin, a diterpene that activates adenylate cyclase directly, increases intracellular cAMP levels. Incubation with forskolin (10 μM) resulted

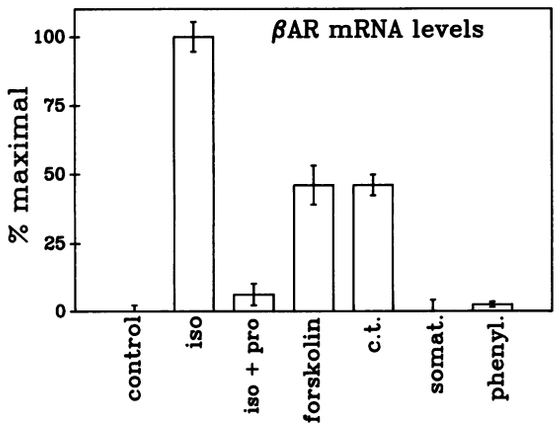

FIG. 6. Effect of various agents on the steady-state levels of βAR mRNA. Cells were incubated for 20 hr with the following agents and total cellular RNA was prepared: none (control); 10 μM isoproterenol (iso); 10 μM isoproterenol + 1 μM propranolol (iso + pro); 10 μM forskolin (forskolin); 10 ng of cholera toxin per ml (c.t.); 100 μM somatostatin (somat.); and 10 μM phenylephrine (phenyl.). Data are presented as percentage of the down-regulation of receptor mRNA observed in response to 10 μM isoproterenol (0.229 pg of βAR mRNA per μg of RNA). Untreated cells contained 0.374 pg of βAR mRNA per μg of RNA.

in a decline in βAR mRNA levels. Cholera toxin, an agent that catalyzes the ADP-ribosylation and activation of G_s, also increases intracellular cAMP levels. Like forskolin, cholera toxin produced a significant decline in βAR mRNA levels. In cells treated with either forskolin or cholera toxin for 20 hr, specific [^{125}I]iodocyanopindolol binding declined by 21% and 17%, respectively. The decline in steady-state βAR mRNA levels in response to either forskolin or cholera toxin, however, was only half that observed in response to the βAR agonist, isoproterenol.

cAMP accumulation was found to be equivalent in DDT_1 MF-2 cells that had been incubated for 40 hr either with forskolin (10 μM) alone (16.5 pmol of cAMP per 10^5 cells) or with forskolin for the entire 40-hr period and isoproterenol (10 μM) for the final 20 hr (17.0 pmol of cAMP per 10^5 cells). The decline of receptor mRNA was found to be 2-fold greater in the cells exposed to forskolin and isoproterenol than in cells exposed to forskolin alone. Although stimulating equivalent levels of cAMP, the treatment with isoproterenol produced a significantly greater decline in βAR mRNA levels.

DISCUSSION

Chronic exposure of cells to βAR agonist leads to agonist-specific desensitization and down-regulation of βARs. Initially, short-term challenge with agonist has been shown to result in an uncoupling of the receptor from G_s. The values for the $t_{1/2}$ of this uncoupling of receptor and G_s have been reported within the range of 7–15 min in 1321N1 human astrocytoma, S49 mouse lymphoma, DDT_1 MF-2 smooth muscle, and BC3H1 smooth muscle cell lines (7, 9, 26). After the initial desensitization process, there is a decrease in the complement of receptors at the cell surface, as detected by radioligand binding (3, 5, 6). The rapid initial phase of agonist-specific down-regulation of βARs may be due to a conformational change in the receptor such that these receptors can no longer bind ligand and therefore would not be detected by conventional radioligand binding techniques. A second possibility is that the receptor is rapidly sequestered away from the plasma membrane. Evidence in favor of receptor sequestration in response to agonist has been reported (27). After prolonged incubation with agonists (>6 hr), there is a decrease in the number of βARs in all fractions of the cell as defined by radioligand binding (7). The βARs in DDT_1 MF-2 cells display a similar pattern of down-regulation in response to agonist stimulation (refs. 9–11; Fig. 1). Agonist-induced down-regulation of receptors and an analysis of steady-state receptor mRNA levels are the focus of this communication.

DNA-excess solution hybridization is a highly sensitive technique for analyzing and quantifying steady-state levels of specific mRNAs. For the present work, this technique offered several advantages over alternative methods of RNA analysis. Because the RNA is not immobilized like that in an RNA or a dot-blot analysis, all the RNA is free to hybridize with the probe. Second, in solution assays the hybridization can be driven to completion. Third, the level of detection by solution hybridization far surpasses that of other standard types of RNA analysis (20).

Changes in the steady-state levels of receptor mRNAs have been shown to be regulated in cells challenged with ligand. In the presence of cholesterol, the steady-state mRNA levels for low density lipoprotein receptors, for example, have been shown to decline (28). Challenge of GH_3 cells by thyroid releasing hormone, likewise, has been shown to result in a decrease in the mRNA levels of the TRH receptor (29). The steady-state mRNA levels for epidermal growth factor receptors and for interleukin 2 receptors, in contrast, have been shown to increase in response to receptor stimulation (30,

31). Using DNA-excess solution hybridization assays, we have been able to quantify βAR mRNA levels and demonstrate a receptor-mediated modulation of steady-state levels of βAR mRNA. The decline in the steady-state βAR mRNA levels after challenge by agonist has been shown to be dependent both on the time of incubation and on the dose of agonist. After an apparent lag of 4 hr, the steady-state levels of βAR mRNA decline to 60% of control levels over the next 16 hr. Control cells contain 0.38 ± 0.014 pg of βAR mRNA per μg of RNA, whereas cells incubated with agonist for 20 hr contain only 0.23 ± 0.011 pg of βAR mRNA per μg of RNA.

The recovery of βAR levels after chronic exposure to agonist ligand has been detailed in a variety of cell lines (6). A 60–90% reduction in the steady-state level of βAR has been reported in cells exposed to agonist for 12–24 hr (3, 5, 6). The recovery of βARs in cells after chronic exposure to agonists does not reach the initial steady-state levels in many cases. A new steady-state level that is 30–50% lower than the original level has been reported after the "recovery" phase (8, 32). These data suggest that chronic stimulation with agonist results in an alteration in receptor metabolism, such as decreased synthesis or enhanced degradation of receptor, or perhaps both. The sensitivity of βAR recovery to cycloheximide reported in agonist-treated 1321N1, BC3H1, and human VA lung cells clearly implicates a central role for the *de novo* synthesis of receptor in this process (8, 26, 32). The $t_{1/2}$ for the recovery of receptors in agonist-treated cells has been reported to be 20–40 hr, whereas the $t_{1/2}$ for the apparent loss of receptors in these same cells was <2 hr (7, 8, 11). The receptor-mediated decline in βAR mRNA levels described in the present study may explain, in part, the agonist-induced down-regulation of βAR in DDT_1 MF-2 and these other cell lines (7, 8). Consistent with this notion, the time required for the recovery of the βAR mRNA levels in agonist-treated cells (12 hr) was found to be shorter than that required for the recovery of receptors in these same cells.

The extent to which changes in intracellular cAMP participate in the receptor-mediated decline of βAR mRNA levels and βAR down-regulation was explored in cells treated with forskolin or cholera toxin. Both of these agents stimulate intracellular cAMP accumulation through non-receptor-mediated mechanisms. Cholera toxin and forskolin, like isoproterenol, stimulated a decline in βAR mRNA levels. Heterologous down-regulation of βARs (i.e., down-regulation not mediated via βAR) has been reported to reach only 50% of the level observed in homologous or agonist-mediated down-regulation (3, 5, 33). Our data on heterologous down-regulation of βAR mRNA levels agree well with these previous studies. Heterologous down-regulation of receptor mRNA levels are shown in the present work to achieve only 50% of the decline observed in homologous or βAR agonist-mediated down-regulation of mRNA levels. It is of interest to note that in S49 cyc^- mutant cells lacking a functional G_s (34), chronic stimulation with isoproterenol leads to a down-regulation of βARs (27). We speculate that this down-regulation of βAR in S49 cyc^- reflects a receptor-mediated cAMP-independent regulation of βAR levels.

The data presented in this communication identify two components of down-regulation of βARs and their steady-state mRNA levels. The first appears to be a cAMP-mediated reduction in βAR mRNA levels. Agents that act to increase intracellular cAMP accumulation, such as forskolin, cholera toxin, or β-adrenergic agonists, decreased βAR mRNA levels. The modulation of receptor mRNA levels by βAR agonist display a second component of regulation that appeared in addition to the cAMP-dependent component. Several possible sites for the modulation of receptor mRNA levels can be envisioned. The rate of transcription, the stability of the receptor mRNA, or both, may be modulated by receptor activation. Analysis of the β_2AR gene revealed two sequences in close agreement to the consensus cAMP-responsive element (CRE) found in other genes (35). These CREs, found in close proximity to each other, exist in the coding region of the gene at a position close to the initiator methionine. Whether CREs present in the βAR gene could function in a manner that reduces rather than enhances transcription is a provocative and open question.

This work was supported by Public Health Service Grants DK30111, DK25410, and K04 AM00786 (to C.C.M.) from the National Institutes of Health.

1. Liebman, P. A. & Pugh, E. N. (1980) *Nature (London)* **287**, 734–736.
2. Rubin, C. S. & Rosen, O. M. (1975) *Annu. Rev. Biochem.* **44**, 831–887.
3. Sibley, D. R. & Lefkowitz, R. J. (1985) *Nature (London)* **317**, 124–129.
4. Graziano, M. P. & Gilman, A. G. (1987) *Trends Pharmacol. Sci.* **8**, 478–481.
5. Harden, T. K. (1983) *Pharmacol. Rev.* **35**, 5–32.
6. Mahan, L. C., McKernan, R. M. & Insel, P. A. (1987) *Annu. Rev. Pharmacol. Toxicol.* **27**, 215–235.
7. Su, Y.-F., Harden, T. K. & Perkins, J. P. (1980) *J. Biol. Chem.* **255**, 7410–7419.
8. Doss, R. C., Perkins, J. P. & Harden, T. K. (1981) *J. Biol. Chem.* **256**, 12281–12286.
9. Strasser, R. H., Benovic, J. L., Caron, M. G. & Lefkowitz, R. J. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 6362–6366.
10. Toews, M. L. (1987) *Mol. Pharmacol.* **31**, 58–68.
11. Scarpace, P. J., Baresi, L. A., Sanford, D. A. & Abrass, I. B. (1985) *Mol. Pharmacol.* **28**, 495–501.
12. Strader, C. D., Sigal, I. S., Blake, A. D., Cheung, A. H., Register, R. B., Rands, E., Zemcik, B. A., Candelore, M. R. & Dixon, R. A. F. (1987) *Cell* **49**, 855–863.
13. Kobilka, B. K., MacGregor, C., Daniel, K., Kobilka, T. S., Caron, M. G. & Lefkowitz, R. J. (1987) *J. Biol. Chem.* **262**, 15796–15802.
14. Dixon, R. A. F., Kobilka, B. K., Strader, D. J., Benovic, J. L., Dohlman, H. G., Frielle, T., Bolanowski, M. A., Bennett, C. D., Rands, E., Diehl, R. E., Mumford, R. A., Slater, E. E., Sigal, I. S., Caron, M. G., Lefkowitz, R. J. & Strader, C. D. (1986) *Nature (London)* **321**, 75–79.
15. Chung, F. Z., Lentes, K. U., Gocayne, J., Fitzgerald, M., Robinson, D., Kervalage, A. R., Fraser, C. M. & Venter, J. C. (1987) *FEBS Lett.* **211**, 200–206.
16. Ma, P. T. S., Yamamoto, T., Goldstein, J. L. & Brown, M. S. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 792–796.
17. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) *Biochemistry* **24**, 5294–5299.
18. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
19. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
20. Williams, D. L., Newman, T. C., Shelness, G. S. & Gordon, D. A. (1985) *Methods Enzymol.* **128**, 671–689.
21. Berk, A. J. & Sharp, P. A. (1984) *Cell* **12**, 721–732.
22. Shelness, G. S. & Williams, D. L. (1984) *J. Biol. Chem.* **259**, 9929–9935.
23. Shackelford, G. M. & Varmus, H. E. (1987) *Cell* **50**, 89–95.
24. Kobilka, B. K., Frielle, T., Dohlman, H. G., Bolanowski, M. A., Dixon, R. A. F., Keller, P., Caron, M. G. & Lefkowitz, R. J. (1987) *J. Biol. Chem.* **262**, 7321–7327.
25. Bahouth, S. W. & Malbon, C. C. (1987) *Biochem. J.* **248**, 557–566.
26. Hughes, R. J. & Insel, P. A. (1986) *Mol. Pharmacol.* **29**, 521–530.
27. Mahan, L. C., Koachman, A. M. & Insel, P. A. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 129–133.
28. Yamamoto, T., Davis, C. G., Brown, M. S., Schneider, W. J., Casey, M. L., Goldstein, J. L. & Russell, D. W. (1984) *Cell* **39**, 27–38.
29. Oron, Y., Straub, R. E., Traktman, P. & Gershengorn, M. C. (1987) *Science* **238**, 1406–1408.
30. Clark, A. J. L., Ishii, S., Richert, N., Merlino, G. T. & Pastan, I. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 8374–8378.
31. Depper, J. M., Leonard, W. J., Drogula, C., Kronke, M., Waldman, T. A. & Greene, W. C. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 4230–4234.
32. Fraser, C. M. & Venter, J. C. (1980) *Biochem. Biophys. Res. Commun.* **94**, 390–397.
33. Moylan, R. D., Barovsky, K. & Brooker, G. (1982) *J. Biol. Chem.* **257**, 4947–4950.
34. Northup, J. K., Smigel, M. D., Sternweis, P. C. & Gilman, A. G. (1983) *J. Biol. Chem.* **258**, 11369–11376.
35. Silver, B. J., Bokar, J. A., Virgin, J. B., Vallen, E. A., Milsted, A. & Nilson, J. H. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 2198–2202.