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Down-Regulation of Gonadotropin and β-Adrenergic Receptors by Hormones and Cyclic AMP

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Loss of gonadotropin receptors in murine Leydig tumor cells and of β-adrenergic receptors in rat glioma C6 cells occurred following exposure of the cells to human chorionic gonadotropin and isoproterenol, respectively. Down-regulation of receptors was mimicked in part by other agents that elevated cyclic AMP levels in the cells such as cholera toxin and dibutyryl cyclic AMP. Whereas agonist-mediated receptor loss was rapid and almost total, down-regulation by cyclic AMP was slower and less extensive. Down-regulation of receptors did not appear to be accompanied by loss of the regulatory and catalytic components of adenylate cyclase. Hormone-mediated down-regulation was preceded by desensitization of hormone-stimulated adenylate cyclase. In contrast, there was no evidence that cyclic AMP caused desensitization. Finally, loss of receptors induced either by agonists or cyclic AMP required protein synthesis as cycloheximide inhibited down-regulation. We conclude that down-regulation of receptors in these cells is a complex process involving both cyclic AMP-independent and -dependent events.

Key words: adenylate cyclase, hormone receptors, cyclic AMP, down-regulation, desensitization, β-adrenergic receptors, gonadotropin receptors

The initial event in the stimulation of adenylate cyclase by hormones is their binding to high-affinity, specific receptors on the cell surface. Following activation of the enzyme, there often is a rapid attenuation of the response (desensitization) followed by a slower loss of the receptors (down-regulation). It is generally believed that down-regulation involves a process of receptor-mediated endocytosis [1,2]. The relationship between desensitization and down-regulation and the role of cyclic AMP

Abbreviations used: hCG, human chorionic gonadotropin; DHA, dihydroalprenolol; CYP, cyanopindolol; IBMX, isobutylmethylxanthine; PBS, phosphate-buffered saline; ISO, isoproterenol; K_{act}, concentration of agonist required for half-maximal stimulation; IC_{50}, concentration of inhibitor required for 50% inhibition; EGF, epidermal growth factor.

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in both phenomena are unclear. In the present paper, we address these issues using two different cell lines. One, a murine Leydig tumor cell line (MLTC-1), binds and responds to human chorionic gonadotropin (hCG) [3]. The other, rat glioma C6, has β-adrenergic receptors and a catecholamine-sensitive adenylate cyclase [4].

MATERIALS AND METHODS

Materials

Purified hCG was obtained and iodinated as described previously [3]. (−)-[Propyl-1,2,3-^{3}H]dihydroalprenolol ([^{3}H]DHA) and (±)-[^{125}I]-iodocyanopindolol ([^{125}I]CYP) were obtained from New England Nuclear (Boston, MA). (−)-[^{125}I]-iodopindolol was prepared from (−)-pindolol (a generous gift from Dr. D. Hauser, Sandoz, Basel, Switzerland) as described by Barovsky and Brooker [5]. Cholera toxin was from Schwarz/Mann. N^6,$O^{2'}$-dibutyryl cyclic AMP, isobutylmethylxanthine (IBMX), (−)-isoproterenol HCl (ISO) and (±)-propranolol were from Sigma (St. Louis, MO).

Cells and Cell Culture

Rat glioma C6 cells (low passage) and MLTC-1 cells were grown as described previously [3,4]. The C6 cells were exposed to the various effectors in serum-free medium and washed several times with ice-cold phosphate-buffered saline, (PBS) pH 7.4. The MLTC-1 cells were exposed to the various effectors in complete medium and washed as above. In some experiments where bound hCG had to be removed to measure total remaining receptors the cells were washed twice with ice-cold glycine-buffered saline (pH 3.0) [6,7].

Assay of Receptors

hCG-receptors on intact MLTC-1 cells were measured as described previously [3,6,7]. Briefly, the cells were incubated for 30 min at 37°C in medium containing 2 nM [^{125}I]-hCG with or without 200 nM unlabeled hCG, washed three times with ice-cold PBS, dissolved in 2 M NaOH, and assayed for bound radioactivity and protein. Binding of iodopindolol to intact rat glioma C6 cells was asssayed as described previously [5] except the cells were incubated with 150–200 pM iodopindolol at 4°C for 2 hr. Rat glioma C6 cells were lysed in 5 mM Hepes-1 mM $MgSO_4$ (pH 8.0); membranes were prepared from C6 cells as described previously [4]. Binding of labeled antagonists to the lysates and membranes was determined by established methods [4]. Nonspecific binding was determined in the presence of 2 μM (±)-propranolol.

Assay of cAMP and Adenylate Cyclase

The cAMP content of intact cells was determined by extracting the cells with 0.1 M HCl and analyzing the extract using a radioimmune assay [7]. Adenylate cyclase activity in MLTC-1 membranes was determined as described elsewhere [3]. Activity in rat glioma C6 lysates and membranes was assayed using the method of Salomon et al [8].

RESULTS

When MLTC-1 cells were exposed to a saturating concentration of hCG, there was a rapid and essentially complete loss of hCG receptors from the cell surface (Fig. 1A). When cells were exposed to a subsaturating dose of hCG (0.4 nM for 30 min), 75% of the cell surface receptors became occupied (Fig. 1B). Subsequent changes in total and unoccupied surface receptors were then determined. Initially, the number of unoccupied receptors was low (25% of total) but increased with time, reaching a maximum between 6 and 8 hr. Presumably this increase represents the appearance of new receptors on the cell surface, which will remain unoccupied in the absence of free hormone. The number of total receptors decreased with time until 6 hr. At this time, the number of unoccupied receptors equalled the number of total receptors, thus indicating that all of the occupied receptors had been removed from the cell surface (Fig. 1B, dashed line). With all of the occupied receptors gone, the down-regulation process appeared to stop for several hours and then resumed, reaching a new plateau by 16 hr. During this phase, the loss of total receptors paralleled the loss of unoccu-

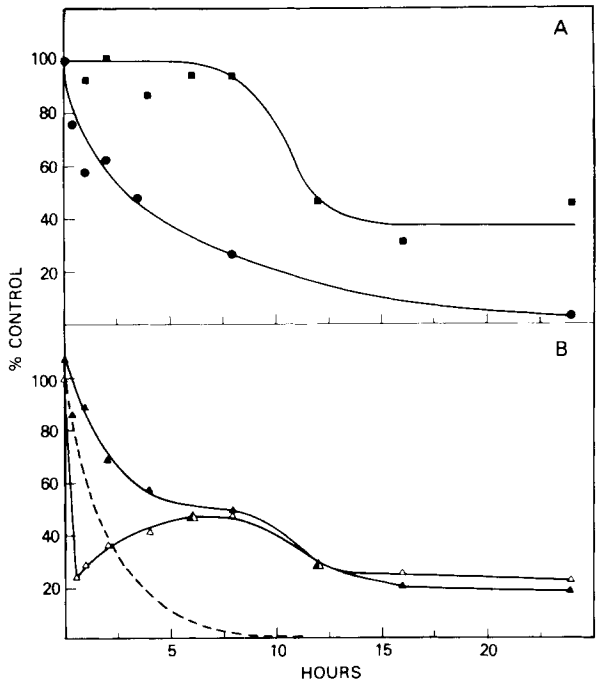

Fig. 1. Down-regulation of hCG receptors in MLTC-1 cells. A) cells were incubated with 2 nM hCG (●) or 1 mM each dibutyryl cAMP and IBMX (■) and assayed for total remaining hCG receptors at the indicated times. The cells were washed first with glycine-buffered saline (pH 3.0) to remove any remaining bound hCG as described in Materials and Methods. B) cells were incubated with 0.4 nM hCG for 30 min, washed, and incubated in fresh medium. At the indicated times, the cells were assayed for unoccupied (△) and total (▲) remaining hCG receptors. The number of occupied receptors at any time was calculated from the difference between total and unoccupied receptors (- - - -). Values were determined as fmol [^{125}I]-hCG specifically bound per mg cell protein and are expressed as percent of control cells (42–62 fmol/mg protein). Similar results were obtained in two additional experiments.

pied ones. Thus, under conditions of partial occupancy, down-regulation or hCG receptors appeared to be biphasic, the initial phase corresponding to loss of occupied receptors and the second phase to loss of unoccupied ones.

Since hCG elevates cAMP levels in these cells [3], we tested the possibility that the second phase of down-regulation is due to cAMP. Cells exposed to dibutyryl cAMP also exhibited a loss of hCG receptors but only after a lag of 8 hr (Fig. 1A). Similar results were observed with rat glioma C6 cells (Fig. 2). The agonist ISO induced a more rapid and extensive loss of β-receptors than did dibutyryl cAMP. With the latter agent, there was a lag of several hours before any loss of β-receptors was observed. Other agents that elevated cAMP such as cholera toxin also caused receptor loss in both cell types (Table I).

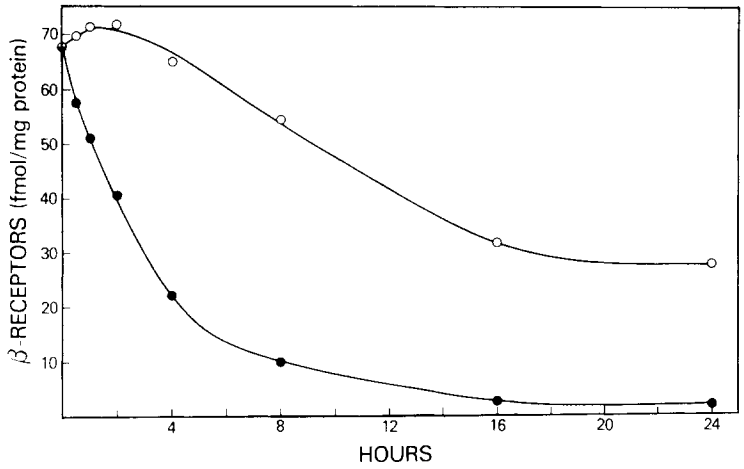

Fig. 2. Down-regulation of β-receptors in rat glioma C6 cells. Cells were incubated with 5 μM ISO (●) or 1 mM dibutyryl cAMP (○) for the indicated times, washed, and assayed for remaining β-receptors by binding of iodopindolol to the intact cells as described in Materials and Methods.

TABLE I. Effect of Agents That Elevate cAMP on Down-Regulation of Receptors*

Treatment	hCG receptors in MLTC-1 cells[a] (% remaining)	β-receptors in C6 cells[b] (% remaining)
None	100	100
Agonist	2	7
Cholera toxin	22	48
Dibutyryl cAMP plus IBMX	13	27

*Binding to receptors was measured on intact cells as described in Materials and Methods.

[a]Cells were incubated for 24 hr with no addition, 2 nM hCG, 12 nM cholera toxin, or 1 mM each of dibutyryl cAMP and IBMX.

[b]Rat glioma C6 cells were incubated for 20 hr with no addition, 1 μM ISO, 10 nM cholera toxin, or 1 mM each of dibutyryl cAMP and IBMX.

In a previous study [4], it was shown that C6 cells exposed to ISO for 24 hr exhibited no loss in guanine nucleotide-, NaF-, or cholera toxin-activated adenylate cyclase activity. We also observed no loss in NaF-stimulated activity in membranes from cells treated with dibutyryl cAMP ± IBMX (96% and 107% of control, respectively). Similar results were obtained for MLTC-1 cells down-regulated by hCG [7]. There was no loss of Mn^{2+} and NaF-stimulated activity in MLTC-1 membranes, and detergent extracts of control and down-regulated cells were equally effective in reconstituting cyclase activity in S49 cyc^- membranes. Thus, down-regulation did not appear to induce a loss of the regulatory and catalytic components of adenylate cyclase in these cells.

We recently showed that deglycosylated hCG is an antagonist for hCG in MLTC-1 cells and has the same high affinity for hCG receptors on these cells as the native hormone [6]. MLTC-1 cells exposed to saturating concentrations of deglycosylated hCG did not exhibit any loss of hCG receptors even after 6 hr (Table II). Thus, receptor occupancy does not appear to be sufficient for down-regulation. We also observed that down-regulation did not occur at low temperatures in MLTC-1 cells or C6 cells (Table III). This is consistent with down-regulation being a process of receptor-mediated endocytosis.

TABLE II. Effect of Deglycosylated hCG on Receptors of MLTC-1 Cells*

Treatment	hCG receptors (% remaining)
None	100
hCG	40
Deglycosylated hCG	95

*Cells were incubated with no addition or 100 ng/ml of either hCG or deglycosylated hCG for 6 hr at 37°C and assayed for remaining receptors as described in Materials and Methods.

TABLE III. Effect of Low Temperature on Down-Regulation of Receptors*

Temperature (°C)	hCG receptors in MLTC-1 cells[a] (% remaining)	β-receptors in C6 cells[b] (% remaining)
37	65	52
15	99	ND[c]
4	125	99

*Binding to receptors was determined on intact cells.
[a]MLTC-1 cells were incubated with no addition or 2 nM hCG at the indicated temperatures for 2 hr.
[b]Rat glioma C6 cells were incubated with no addition or 1 μM ISO at the indicated temperatures for 2 hr.
[c]ND, not determined.

We also found that down-regulation required protein synthesis. Exposing MLTC-1 cells to 10 μg/ml of cycloheximide caused an immediate and total block in incorporation of [^{3}H]leucine into trichloroacetic acid-precipitable material. Cycloheximide had a similar effect on C6 cells; incorporation was inhibited 93% after 1 hr and 95% after 24 hr. Prior exposure of MLTC-1 and rat glioma C6 cells to cycloheximide inhibited both agonist- and cAMP-mediated loss of hCG receptors and β-receptors, respectively (Table IV). The drug appeared to be more effective in blocking down-regulation by cAMP than by the agonists. As the latter agents cause more extensive down-regulation than cAMP, the significance of this observation may be difficult to assess. We also found that the longer the cells were preincubated with cycloheximide the more effective it was in blocking receptor loss. Thus, MLTC-1 cells exposed to the drug for 24 hr prior to adding hCG lost only 20% of their receptors after an additional 8 hr compared to 90% loss for control cells. This would be consistent with a slowly turning over component being required for receptor down-regulation.

It is well known that a short exposure of cells to agonists results in desensitization of the agonist-stimulated adenylate cyclase activity. It was shown in rat glioma C6 cells that ISO-induced desensitization also lowered the affinity for agonist of both the enzyme [9] and the β-receptor [4]. We explored the possibility that cAMP caused down-regulation but not desensitization in these cells (Table V). Cells exposed to ISO exhibited an increase both in the K_{act} and the IC_{50} for ISO, whereas cells exposed to IBMX exhibited no change or even a decrease. A similar decrease in the IC_{50} was observed in membranes from cells exposed to dibutyryl cAMP or cholera toxin (data not shown). When competition binding was measured in the presence of GTP, the affinity for ISO was reduced tenfold. This is consistent with previous studies [4] and has been interpreted to reflect the uncoupling of the receptor from the regulatory component to form a low-affinity state of the receptor [4,10–12]. We were unable to do similar experiments with MLTC-1 cells as hCG affinity for the receptor was unchanged after hCG-mediated desensitization and was not modulated by guanine

TABLE IV. Effect of Cycloheximide on Down-Regulation of Receptors

Treatment	hCG receptors in MLTC-1 cells[a] (% of control)	β-receptors in C6 cells[b] (% of control)
Agonist	4.5	4.1
plus cycloheximide	54	49.9
Cyclic AMP	35	46.6
plus cycloheximide	100	92.1

[a]MLTC-1 cells were incubated with and without 10 μg/ml of cycloheximide for 12 hr; then 2 nM hCG or 1 mM each dibutyryl cAMP and IBMX was added to some of the cultures and binding to remaining receptors was measured on intact cells after an additional 20 hr.

[b]Rat glioma C6 cells were incubated with and without 10 μg/ml of cycloheximide for 5 hr; then 5 μM ISO or 1 mM dibutyryl cAMP was added to some of the cultures and binding to remaining receptors was measured on intact cells after an additional 19 hr.

TABLE V. Effect of Agonist and cAMP Treatment on Agonist Affinity*

Treatment	β-receptors[a] (fmol/mg protein)	K_{act} for ISO[b] (nM)	IC_{50} for ISO[c] (nM)
None	213	5.5	17
ISO	149	16.2	71
IBMX	159	6.7	10

*Rat glioma C6 cells were incubated with no addition, 100 nM ISO for 2 hr of 1 mM IBMX for 20 hr.
[a]Receptor number was determined by measuring binding of [^{125}I]CYP to membranes prepared from cells treated as indicated.
[b]The K_{act} for ISO-stimulated adenylate cyclase activity was determined by incubating intact cells with increasing concentrations of ISO and measuring cAMP production.
[c]The affinity for ISO was determined by competition binding assays. Membranes were incubated with [^{3}H]DHA in the presence of increasing concentrations of ISO and the concentration required for 50% inhibition (IC_{50}) was determined.

nucleotides. Others also have found that hCG binding to gonadotropin receptors was not affected by guanine nucleotides [13].

Finally, we explored the specificity of these agents on receptor down-regulation. As cholera toxin binds specifically to a membrane glycolipid, the ganglioside GM1 [14], we measured toxin receptors in both cell lines after exposure to the various agents. MLTC-1 cells, exposed for 24 hr to hCG (1 nM) or cholera toxin (50 pM), actually exhibited a 60–65% increase in toxin receptors [15]. Rat glioma C6 cells treated for 17 hr with ISO (10 μM), cholera toxin (50 pM), dibutyryl cAMP (1 mM), IBMX (1mM), or both of the latter together, varied in the number of toxin receptors by ± 15% from untreated cells. Thus, elevation of cAMP in these cells either by agonists or other agents does not appear to cause a generalized increase in membrane turnover or loss of cell surface components.

DISCUSSION

We have shown in two different cell lines that hormone receptors can be down-regulated either by the appropriate agonist or by other agents that elevate cAMP levels in the cells. Whereas agonist-mediated receptor loss is rapid and essentially complete, that induced by cAMP appears to be slower and incomplete. Down-regulation was time-dependent and temperature-sensitive and required protein synthesis. It is generally believed that down-regulation involves a process of receptor-mediated endocytosis and that the receptors are ultimately degraded in the lysosomes. We have found that the rates of hCG degradation and hCG receptor down-regulation are similar in MLTC-1 cells [6,7]. In addition, cells exposed to methylamine internalized but did not degrade the bound hCG, and the detergent-extracted hCG sedimented as an hCG receptor complex on a sucrose density gradient [7]. More recently, we have found that chloroquine inhibits agonist-mediated loss of β-receptors in C6 cells as well as in HeLa cells [Zaremba and Kassis, unpublished observations].

We observed that receptor loss was not accompanied by loss of the regulatory and catalytic components of adenylate cyclase. Other groups have reported that in cells exposed to ISO the β-receptors appear in a light membrane [12,16] or vesicle fraction [17] that is deficient in these cyclase components. Thus, as the receptors are lost from the cell surface, they may appear in endosomes or receptosomes [2], which eventually fuse with lysosomes where the receptors are degraded.

We also found that down-regulation of β-receptors in C6 cells induced by cAMP did not cause desensitization of agonist-sensitive adenylate cyclase. The affinity for ISO was not reduced in cells exposed to dibutyryl cAMP, IMBX, or cholera toxin, whereas the affinity was reduced in cells exposed to agonist. Thus, cAMP does not appear to induce desensitization in rat glioma C6 cells, which is consistent with previous work from this laboratory [4]. Others also have found that cAMP causes loss of β-receptors in rat glioma C6-2B cells [18]. These latter cells appear to become refractory to ISO when exposed to ISO or other agents that elevate cAMP [19,20]. This phenomenon was measured in intact cells and may not be observed when membranes are assayed for ISO-stimulated adenylate cyclase activity [4,21]. Cyclic AMP also has been found to induce desensitization of ISO-stimulated adenylate cyclase activity in turkey erythrocytes [22]. These cells appear to behave very differently from C6 cells, as there is no loss of β-receptors and there is a loss of guanine nucleotide- and NaF-stimulated activity.

The ability for cAMP to induce down-regulation of hormone receptors raises the possibility that protein phosphorylation is involved in the process. The relationship between agonist-induced and cAMP-induced receptor loss, however, is not yet clear. It has been shown that a number of receptors have protein kinase activity associated with them and that ligand binding results in a phosphorylation of the receptor [23–28]. The best-studied receptors are those for insulin and epidermal growth factor (EGF). Murine Leydig tumor cells also have EGF receptors and EGF induces down-regulation of hCG receptors in these cells [29]. One possible hypothesis that is consistent with all of these observations is the following. Agonist binding to the receptor not only stimulates adenylate cyclase but activates a cAMP-independent protein kinase that phosphorylates the receptor. The receptor is now desensitized and marked for down-regulation. Elevated levels of cAMP in the cell activate cAMP-dependent protein kinases, which can also phosphorylate the receptor (at a separate site) and mark it for down-regulation. Experiments designed to isolate the down-regulated receptors and analyze them for phosphorylation will answer these questions.

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