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Glucocorticoids Increase GTP-Dependent Adenylate Cyclase Activity in Cultured Fibroblasts

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SUMMARY

Glucocorticoid treatment of cultured fibroblasts increases intracellular cyclic AMP accumulation induced by isoproterenol or cholera toxin. This increase in agonist activity is not a direct action of glucocorticoids on cyclic AMP metabolism since about 2 days are necessary for maximal effect. Basal cyclic AMP levels are not changed. In membrane preparations, GTP-dependent adenylate cyclase activity is increased, basal adenylate cyclase activity is unchanged, and NaF-stimulated activity is decreased. The number of *beta*-adrenergic receptors is unchanged, but the affinity of receptor for the antagonist dihydroalprenolol is increased about 3-fold. This change in affinity is probably not responsible for the increased response to isoproterenol since the augmented response is noted at 0.1 mM isoproterenol, a concentration much larger than the apparent K_D (about 5 nM). The results suggest that an alteration in some component in the GTP-dependent regulatory complex is responsible for the increase in agonist response.

INTRODUCTION

Glucocorticoid steroids affect cyclic AMP metabolism in many different types of cells (1–8). The nature of the effect is cell type-dependent, and several components in the cyclic AMP system have been reported to change with steroid treatment. Cyclic nucleotide phosphodiesterase activity decreases in some (2, 5) but not all (3, 4) cells, and the *beta*-receptor content is sensitive to glucocorticoids in some cells (8–10). Adenylate cyclase is also a target for glucocorticoid action (1, 3, 4, 8). In rat glioma (4) and human astrocytoma cells (8), basal, fluoride-, and hormone-stimulated activities increase, suggesting that in these cells the catalytic unit of cyclase is affected by the glucocorticoid treatment.

Here we report that glucocorticoid treatment of cultured fibroblasts increases the amount of cyclic AMP produced in intact cells during cyclase stimulation by isoproterenol or cholera toxin. In membrane preparations GTP-stimulated activity is increased, basal activity is unchanged, and NaF-stimulated activity is decreased. The results are not explained by changes in catalytic unit or agonist receptor, but rather another factor—probably a component in the regulatory, GTP-dependent complex—is a target for glucocorticoid action in these cells.

MATERIALS AND METHODS

Cell culture. NRK[3] (11), SV-NRK, and HSV-NRK cells were grown as previously described (12, 13). These cell lines were chosen because they have been used previously in studies on cyclic AMP metabolism (12–14). Syrian golden hamster cells (2° cultures) were obtained from Dr. C. Evans (National Cancer Institute) and had been prepared as previously described (15). All cells were grown in Dulbecco-Vogt modified Eagle's medium with 10% calf serum (Colorado Serum Company, Denver, Colo.). For steroid treatment, cells were planted in medium without steroids, and the next day fresh media containing steroids were added. Steroids were dissolved in ethanol, and aliquots were added to the media. MIX was added directly to culture media.

Cyclic AMP analysis and adenylate cyclase assay. Crude membranes were prepared and adenylate cyclase was assayed as previously described (12, 14). Cyclic AMP was extracted and quantitated by the acetylation modification of the radioimmunoassay (13). Each extract was assayed in triplicate and the average of the three assays was used for tabulation. Cultures were rinsed once with serum-free medium prior to extraction of basal cyclic AMP, since residual medium on cells after aspiration contains enough cyclic AMP to affect significantly basal level determinations (13). Protein content was determined in companion cultures (16).

[^3H]*DHA binding assay. Beta*-adrenergic receptors were measured under equilibrium conditions essentially as described by Mallorga *et al.* (17). Cells were rinsed twice with cold phosphate-buffered saline [0.01 M sodium phosphate (pH 7.4)/0.15 M NaCl] followed by one rinse with cold binding buffer (50 mM Tris-HCl, pH 7.0). Cells were scraped into

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[3] The abbreviations used are: NRK, normal rat kidney cells; HSV-NRK, Harvey sarcoma virus-transformed cells; HEF, hamster embryo fibroblasts; MIX, 1-methyl,3-isobutylxanthine; DHA, dihydroalprenolol; PGE_1, prostaglandin E_1.

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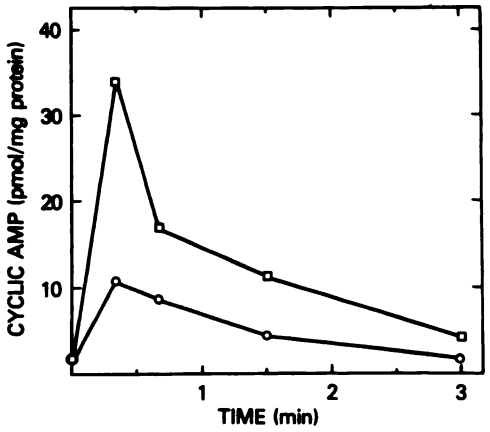

FIG. 1. *Time course for cyclic AMP accumulation and decay during isoproterenol stimulation of adenylate cyclase*

NRK cells were grown for 45 hr in normal medium (0) or medium containing dexamethasone (0.5 μg/ml) (□). Isoproterenol (0.1 mM) was added and cyclic AMP was extracted at the indicated times.

binding buffer and homogenized in a Dounce homogenizer (20 strokes with a tight pestle). The homogenate was centrifuged at 12,000 × *g* for 10 min and the pellet was resuspended in binding buffer to a concentration of 2–4 mg of protein per milliliter. Crude membranes were incubated with [^{3}H]DHA (1–10 nM) in binding buffer in a total volume of 0.5 ml. After 10 min at 30°, 7 ml of cold binding buffer were added to each tube, and the samples were filtered through glass-fiber filters (Whatman GF/C). Two 7-ml rinses followed. Filters were placed in vials containing 10 ml of aqueous counting solution and radioactivity was determined in a liquid scintillation counter. Background binding is the amount of [^{3}H]DHA bound in the presence of 5 μM (±)-propranolol. Specific binding, accounting for 40–60% of total, is the difference between the total binding and the background. Scatchard analysis of binding (18) provided measures of *beta*-receptor number and affinity for the labeled ligand.

Materials. Cholera toxin, L-isoproterenol-D-bitartrate, MIX, dexamethasone, hydrocortisone, triamcinolone acetonide, corticosterone, progesterone, and β-estradiol were obtained from Sigma Chemical Company (St. Louis, Mo.). 11-α-Hydroxycortisol was obtained from Dr. E. B. Thompson (National Cancer Institute). L-[*propyl*-1,2,3-^{3}H]DHA-HCl (43 Ci/mmole) was obtained from New England Nuclear Corporation (Boston, Mass.).

RESULTS

Treatment of NRK cells with dexamethasone for 45 hr resulted in a 2 to 3-fold increase in the ability of isoproterenol to increase cyclic AMP levels (Fig. 1). This increase was not a result of a slower desensitization process, since cyclic AMP levels quickly fell after reaching a maximum. Basal cyclic AMP levels were unchanged (2.15 and 2.30 pmoles/mg of protein in control and dexamethasone-treated cultures, respectively). An increase in the agonist response was observable with dexamethasone at concentrations as low as 5 ng/ml and was essentially maximal at about 50 ng/ml (0.13 μM) (Fig. 2). Other steroids with glucocorticoid activity (triamcinolone, corticosterone, and hydrocortisone) were effective (Table 1), whereas non-glucocorticoid steroids (progesterone and β-estradiol) were not (Fig. 1). Also, 11 α-hydroxycortisol, the isomer of hydrocortisone which does not have glucocorticoid activity (19), was ineffective (data not shown). Glucocorticoids apparently did not affect cyclic AMP metabolism directly, since about 2 days were required for maximal respones (Fig. 3).

NRK cells treated with glucocorticoids were somewhat flatter in morphology but appeared healthy, with no obvious cell death. Growth was slowed, with a range of 10–30% fewer cells and less protein per culture after 45 hr of treatment. The extent of altered agonist response was not correlated with extent of growth inhibition by dexamethasone treatment in different experiments. Also, similar decreases in growth were observed with progesterone and β-estradiol treatment where the isoproterenol response was unchanged.

Responses of other activators of adenylate cyclase were tested in NRK, SV-NRK, HSV-NRK, and HEF (3° cultures) treated with dexamethasone. No consistent pattern of response was observed (Table 2). The dexameth-

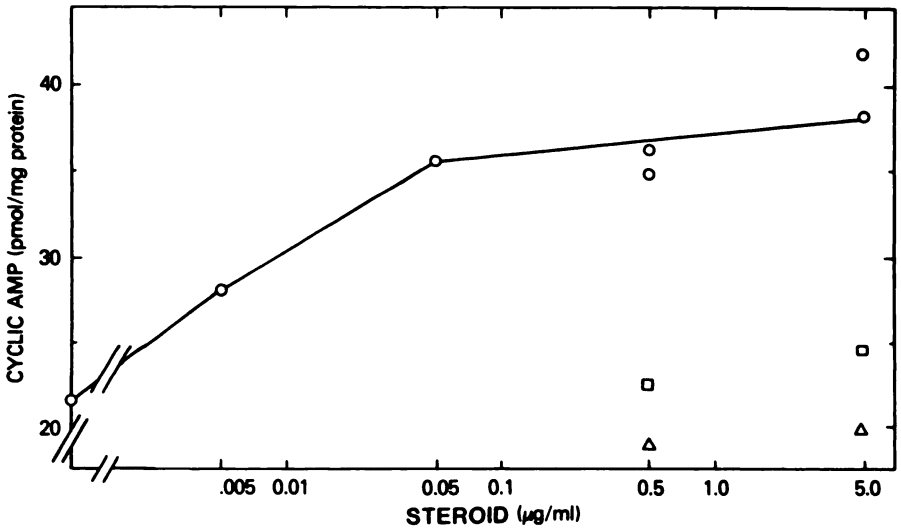

FIG. 2. *Steroid concentration dependence*

NRK cells were grown for 45 hr in media containing dexamethasone (○) at the indicated concentration. Cyclic AMP was extracted after isoproterenol (0.1 mM) treatment for 20 sec. For comparison, the isoproterenol responses in cells treated with progesterone (□) or β-estradiol (△) are shown.

TABLE 1

Isoproterenol response in NRK cells treated with glucocorticoid steroids

Cells were grown for 45 hr in media containing the indicated steroid (0.5 μg/ml). Isoproterenol (0.1 mM) was added for 20 sec and cyclic AMP was extracted. Ratio of treated to control is given. Results are presented as mean ± standard error of the mean of the number of experiments in parentheses.

Treatment	Cyclic AMP	Ratio
	pmoles/mg protein	
None	32.8 ± 0.8 (3)	1.0
Dexamethasone	67.9 ± 1.8 (3)	2.1
Triamcinolone	64.4 (2)	2.0
Corticosterone	47.9 (2)	1.5
Hydrocortisone	51.3 (2)	1.6

asone-induced increase in cholera toxin response in NRK and HSV-NRK (about 20%) was smaller than the increase in isoproterenol response (about 60%). In SV-NRK, the cholera toxin response was about 2-fold increased, whereas the isoproterenol response was unchanged. Contrary to our previous observations (12, 13), NRK and SV-NRK did not respond to PGE_1 in this series of experiments (data not shown). The reasons for this lack of response are not known. In HEF, the increases in response to isoproterenol and cholera toxin were 60% and 200%, respectively, whereas the PGE_1 response was unchanged by dexamethasone treatment (Table 3).

To determine the enzymatic basis for the alterations in cyclic AMP metabolism, adenylate cyclase activity was analyzed. Hormone-responsive cyclase was affected, as shown by 50% and 90% increases in GTP-stimulated and GTP plus isoproterenol-stimulated activities in the average of four experiments (Table 4). In contrast, basal adenylate cyclase was unchanged and fluoride-stimulated activity was decreased about 25% in crude membranes from dexamethasone-treated NRK cells (Table 4). These results indicate that glucocorticoid treatment increases hormone-stimulated cyclase through alterations in a GTP-dependent component but not in the catalytic subunit of adenylate cyclase.

Cyclic nucleotide phosphodiesterase activity was ana-

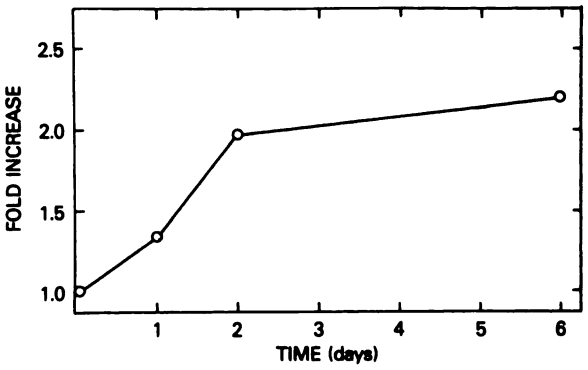

FIG. 3. *Time dependence for increase in isoproterenol response*

NRK cells were grown for the indicated times in media dexamethasone (0.5 μg/ml). Isoproterenol was added for 20 sec, and cyclic AMP was extracted. For the 6-day treatment, cultures were trypsinized and replanted on day 3.

TABLE 2

Isoproterenol and cholera toxin response in NRK and its transformed derivatives treated with dexamethasone

Cells were grown for 45 hr in control medium or medium containing dexamethasone (0.5 μg/ml). Isoproterenol (0.1 mM) was added for 20 sec. For cholera toxin treatment, media were removed, and cultures rinsed with serum-free medium and serum-free medium with or without dexamethasone plus cholera toxin (50 ng/ml) were added for 5 hr. Results are presented as the mean and standard error of the mean of the number of experiments in parentheses.

Cell line	Dexamethasone	Cyclic AMP	
		+Isoproterenol	+Cholera toxin
		pmoles/mg protein	
NRK	–	63.0 ± 7.2 (4)	64.2 ± 2.8 (4)
	+	102 ± 7.5 (4)	73.8 ± 6.0 (4)
SV-NRK	–	30.0 ± 3.4 (6)	676 ± 76 (6)
	+	26.8 ± 3.6 (6)	1280 ± 180 (6)
HSV-NRK	–	40.6 (2)	700 (2)
	+	77.5 (2)	824 (2)

lyzed indirectly by use of the inhibitor, MIX (Fig. 4). The results indicate that the increased response to isoproterenol (Fig. 1) is not a result of changes in phosphodiesterase activity. First of all, basal cyclic AMP levels were 3-fold increased by MIX, but not by dexamethasone. Second, cyclic AMP levels increased for at least 3 min when phosphodiesterase was inhibited by MIX, whereas they fell quickly after reaching a maximum in dexamethasone-treated cells, when MIX was not added.

The increased response to isoproterenol by dexamethasone treatment (Fig. 1) could result, in part, from changes in the *beta*-adrenergic receptor. In previous studies it has been observed that *beta*-receptor content is influenced by glucocorticoids (8–10). To evaluate this possibility, *beta*-adrenergic receptors were measured by assaying the binding of [^{3}H]DHA in membranes from control and dexamethasone-treated NRK cells. Scatchard analysis of the binding data (Fig. 5) demonstrated that the number of binding sites was not altered in membranes from treated cells. However, the apparent dissociation constant was 3-fold decreased by dexamethasone treatment (7.5 nM in control versus 2.6 nM in treated cell membranes), indicating that glucocorticoids alter the affinity of the *beta*-adrenergic receptor for the labeled antagonist. A change in the affinity is in contrast to previous observations in other cells, where glucocorticoids changed receptor number, but not affinity (8–10). These differences further demonstrate variations in response to glucocorticoids in different cell types.

TABLE 3

Isoproterenol, PGE_1, and cholera toxin response in HEF treated with dexamethasone

Cells were grown and treated as described in Table 2. The PGE_1 treatment was 10 μg/ml for 15 min.

Dexamethasone	Cyclic AMP				
	+Isoproterenol			+Cholera toxin	+PGE_1
	0.5 min	1 min	2 min		
	pmoles/mg protein				
–	43.9	47.1	37.4	28.0 ± 4.0 (3)	190 (2)
+	67.8	81.4	61.4	52.0 ± 6.0 (3)	182 (2)

TABLE 4

Adenylate cyclase activities

Crude membranes were prepared from control cells and cells treated with dexamethasone (0.5 μg/ml) for 45 hr. Assays were carried out in triplicate. The mean ± standard error of the mean for a representative experiment and the ratios of activities in dexamethasone-treated cell membranes to those in control membranes from four experiments are presented. Concentrations were as follows: GTP, 0.1 mM; isoproterenol, 0.2 mM; NaF, 10 mM.

Treatment	Adenylate cyclase activity			
	Basal	+GTP	+GTP and isoproterenol	+NaF
	pmoles/mg protein/10 min			
Control	97.0 ± 18.0	120 ± 5	637 ± 15	3590 ± 30
Dexamethasone	62.6 ± 1.2	158 ± 1 ($p < 0.02$)	1050 ± 30 ($p < 0.01$)	2440 ± 30 ($p < 0.01$)
	Ratio (dexamethasone/control)			
Average	0.94 ± 0.22	1.54 ± 0.33	1.91 ± 0.17	0.76 ± 0.05

It is unlikely that the increased agonist response in dexamethasone-treated cells is a result of observed changes in receptor affinity, since the cyclic AMP accumulation shown in Fig. 1 was in response to 0.1 mM isoproterenol, a concentration much greater that the apparent dissociation constant (about 5 nM). Also, the cyclic AMP accumulation during a 20-sec treatment with isoproterenol at concentrations ranging from 10^{-7} to 10^{-5} M was augmented similarily by dexamethasone treatment (data not shown).

DISCUSSION

Glucocorticoid treatment increases adenylate cyclase activity in fibroblasts. Several lines of evidence suggest that the altered enzymatic activity is due to a more effective GTP-dependent coupling complex (20). First of all, basal adenylate cyclase and basal cyclic AMP levels were unchanged and NaF-stimulated activity was decreased, indicating that the catalytic unit is not a target for glucocorticoid action. Second, *beta*-adrenergic receptor affinity was increased by dexamethasone treatment, but this change was apparently not responsible for the increased agonist response since the increase was observed with 0.1 mM isoproterenol, a concentration much greater than the apparent K_D (about 5 nM). Third, in membrane homogenates, GTP plus isoproterenol-stimulated activity was increased. Fourth, the response to cholera toxin, an agent which activates cyclase through a mechanism involving an ADP-ribosylation of a 42,000-dalton protein in the regulatory complex (20, 21), was increased by dexamethasone treatment. The nature of this change in the regulatory component is not known. It is probably not due simply to the presence of more of the

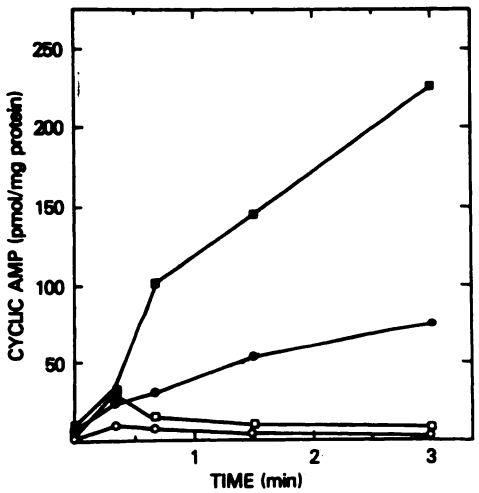

FIG. 4. *Effect of dexamethasone and MIX on isoproterenol response*

NRK cells were grown for 45 hr in control medium (○, ●) or medium containing dexamethasone (0.5 μg/ml) (□, ■). MIX (1 mM) was added to one-half of the cultures (●, ■) for the last 6 hr. Isoproterenol (0.1 mM) was added and cyclic AMP was extracted at the indicated times.

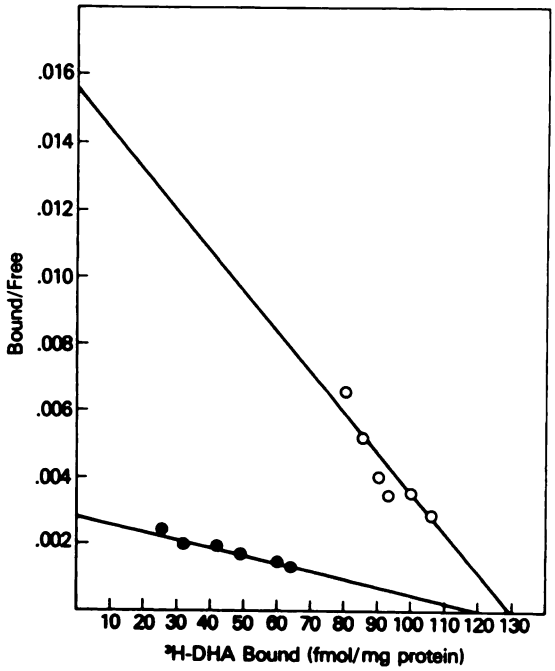

FIG. 5. *Scatchard analysis of [³H]DHA binding to crude membranes from control and dexamethasone-treated NRK cells*

Binding assays were performed as described under Materials and Methods. Cells were treated with dexamethasone (0.5 μg/ml) for 45 hr prior to the assay. Data points represent the mean of triplicate determinations. K_D values for control (●) and dexamethasone-treated (○) cells were 7.5 and 2.6 nM, respectively, and the total numbers of DHA binding sites were 118 fmoles of DHA per milligram of protein for control and 128 fmoles of DHA per milligram of protein for dexamethasone-treated cells.

regulatory component, since NaF-stimulated activity was not increased, and also there was a lack of parallel increase in response to all agonists (Tables 2 and 3). It is puzzling why the responses to isoproterenol, PGE_1, and cholera toxin were not increased in parallel. Components in the regulatory complex in addition to the *N* or *G/F* protein (20) may be involved. Further studies with glucocorticoids should be useful in learning more about the complex events in the regulation of adenylate cyclase activity. Another steroid, progesterone, inhibits adenylate cyclase in *Xenopus* oocytes. Interestingly, this inhibition appears to involve changes in the regulatory component of cylase (22, 23).

Glucocorticoid treatment could result in loss of endogenously bound guanine nucleotide, and many of the observed affects could be explained by this mechanism: (a) If less of the inactive or inhibitory guanine nucleotide, GDP, is present, GTP added in the assay could be more effective in activating cyclase. (b) It is well known from many studies that guanine nucleotide bound to the regulatory component decreases the affinity of receptor for agonist (20). A decrease in bound guanine nucleotide may increase affinity. (c) In turkey erythrocyte membranes, GMP plus isoproterenol pretreatment decreases NaF stimulation, an effect overcome by GTP. This observation has been interpreted to suggest that GTP bound to the regulatory component is required for NaF activation of cyclase (24). NaF stimulation is decreased in dexamethasone-treated cell membranes.

We have previously reported that agonist response and GTP-stimulated adenylate cyclase activity are increased in cells treated with certain drugs (12, 13). The relationship of these earlier observations to those with glucocorticoids in the present study are not clear, but the observations differ in one aspect in that the NaF response in membranes from dexamethasone-treated cells is decreased, whereas in membranes from cells treated with other drugs the NaF response is unaffected or slightly increased. Whether or not glucocorticoids and the drugs alter cyclase by a common mechanism, the results of the present study are consistent with our earlier proposal that the functioning of the GTP-dependent component of cyclase is subject to regulation in intact cells (12, 13) and in addition suggest that glucocorticoids may play a role in this regulation.

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