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Adenosine 3′,5′-Monophosphate in Glial Tumor Cells Treated with Glucocorticoids

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SUMMARY

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The effect of various steroids on adenosine 3′,5′-monophosphate in C-6 glial tumor cell cultures was examined. Glucocorticoids increased basal and norepinephrine-stimulated concentrations of cyclic AMP in intact cells 2-fold. Exposure of cells to 2 μM corticosterone for at least 48 hr was required to obtain a maximal effect. The concentration of norepinephrine needed for half-maximal activation and the time course of the cyclic AMP response were unaltered. A 2-fold increase in adenylate cyclase activity of broken cell preparations similarly dependent upon corticosterone concentration and time was observed. The apparent K_m for ATP was not changed but the maximum velocity was doubled, suggesting that the steroid induces the synthesis of additional adenylate cyclase. The activity of a second enzyme localized in the surface membrane, ($Na^+ + K^+$)-ATPase, was unaltered in glucocorticoid-treated cells. Also, no differences in the activities of cyclic nucleotide phosphodiesterases were detected.

INTRODUCTION

Steroid hormones modulate many cell functions which are mediated primarily by adenosine 3′,5′-monophosphate. For example, glucocorticoids modulate the glycogenolytic response of liver to epinephrine by controlling the sensitivity of the system to cyclic AMP (1), and they increase the concentration of cyclic AMP generated in response to epinephrine in hepatoma cells (2) and lymphocytes (3). Adenylate cyclase activity is increased by progesterone action on the oviduct (4) and by testosterone action on seminal vesicles (5), but estradiol abolishes norepinephrine activation of adenylate cyclase of the pineal (6). Abundant evidence exists for steroid hormone (7) and cyclic nucleotide (8) participation in specialized functions of the central nervous system, as well as in basic controls over cell growth and differentiation (9), but understanding of interactions between the two classes of compounds at the cellular level in brain is extremely limited.

The C-6 cell line, which was cloned from a chemically induced astrocytoma in the rat, is known to respond to catecholamines and glucocorticoids (10). Activation of adenylate cyclase coupled to a *beta* adrenergic receptor causes the cells to accumulate high concentrations of cyclic AMP (11). Glucocorticoid

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treatment of the cells results in doubling of the glycerol phosphate dehydrogenase activity, apparently through a mechanism independent of cyclic AMP (10). However, the occurrence of steroid hormone interactions with cyclic nucleotides was not excluded. The possibility that such interactions could be identified in C-6 cells and that they might pertain to brain function and to control of cell growth and differentiation led to the present experiments.

METHODS AND MATERIALS

Cell culture. The C-6 clonal line of rat astrocytoma cells was obtained from the American Type Culture Collection. Monolayer cultures were grown in Falcon plastic flasks (25 cm^2) in Ham's F-10 medium containing 10% fetal calf serum under a humidified atmosphere of 95% air–5% CO_2. The medium was replaced on Monday, Wednesday, and Friday. Removal of cells for subculturing was performed at 14-day intervals. Monolayers were washed with 3 ml of Hanks' balanced salt solution (12) and incubated at 37° for 5 min with 1 ml of 0.5 mM EDTA containing 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, and 1.1 mM glucose. Stock or experimental cultures were then plated at a density of 2×10^4 cells/cm^2 and were fed 48–72 hr after subculture. Under these conditions a doubling time of 21 hr and a stationary phase density of 3×10^5 cells/cm^2 were obtained. Stationary phase cultures were generally obtained 10–12 days after seeding. Penicillin and streptomycin were added to experimental cultures to concentrations of 25 units/ml and 50 μg/ml, respectively. Steroids were dissolved in water by stirring at room temperature for 18 hr, then filter-sterilized, and added directly to the cells at the times indicated. Concentrations of steroid solutions were determined by ultraviolet absorption.

Measurement of cyclic AMP in intact cells. Monolayers were washed twice with 3 ml of Hanks' balanced salt solution. Then 3 ml of Ham's F-10 medium minus serum were added, and the cultures were incubated at 37° with or without norepinephrine. At the end of the incubation the medium was removed by means of a Pasteur pipette attached to a suction line, and the monolayer was covered with 1 ml of 5% trichloracetic acid. The bottles were allowed to stand at room temperature for 20 min. The trichloracetic acid supernatant fractions were then removed, acidified with 100 μl of 1 N HCl, and extracted five times with 2 ml of water-saturated ether. The trichloracetic acid-free solutions were lyophilized and assayed for cyclic AMP according to a modification (13) of Gilman's method. Incubation of samples prepared from steroid-treated or untreated cells in the presence of bovine heart phosphodiesterase resulted in values indistinguishable from the blanks. The trichloracetic acid-extracted monolayers were dissolved in 1 ml of 1 N NaOH and analyzed for protein content according to Lowry *et al.* (14).

Assay of adenylate cyclase. Adenylate cyclase was determined by a modification of the method of Krishna *et al.* (15). Cells were washed twice with 3 ml of Hanks' balanced salt solution and once with 2 ml of the 0.5 mM EDTA solution described above. The monolayers were then incubated at 37° with 1 ml of EDTA solution for 5 min, and 1 ml of Hanks' solution was added. Suspensions were centrifuged for 3 min at 600 × *g*. The pellets were gently suspended in a hypotonic medium containing 50 mM Tris-chloride (pH 8.0), 3 mM $MgCl_2$, 10 mM theophylline, 12 mM phosphoenolpyruvate, 3.8 mM cyclic AMP, 6.2 mM dithiothreitol, and 25 μg/ml of pyruvate kinase. Significantly lower levels of activity were obtained when cells were broken by homogenization or sonication. [^{3}H]ATP in 2 mM ATP (specific activity, 30 cpm/pmole) was added to initiate the reaction, and the incubation was carried out for 10 min in a total volume of 100 μl. The reaction was terminated by the addition of 500 μl of 5% trichloracetic acid, and the samples were centrifuged at 5000 × *g* for 15 min. Aliquots (0.5 ml) of the supernatant fractions were acidified with 50 μl of N HCl, extracted five times with 1 ml of water-saturated ether, and neutralized with 60 μl of 1 M Tris-hydroxide. Then 50 μl of a 0.3 M solution of barium hydroxide were added, and the samples were allowed to stand for 10 min. Fifty microliters of 0.3 M $ZnSO_4$ were added, and the samples were centrifuged at 8000 × *g* for 15 min. The precipitation procedure with barium and zinc was re-

peated twice. Then 500-μl aliquots of the final supernatant fractions were passed through 0.6 × 10 cm AG50W-X8 columns equilibrated with 1 mM phosphate buffer, pH 7.3. The columns were washed with 6 ml of phosphate buffer and 1 ml of water. The cyclic AMP was eluted with 5 ml of water. This column treatment provided better separation of cyclic AMP from ATP than did elution with water alone, and resulted in values for enzyme activity which were somewhat lower than reported previously for this cell line (16). The solutions were then lyophilized, suspended in 1 ml of water, and counted in 10 ml of Aquasol. Treatment of final solutions with bovine heart phosphodiesterase resulted in values equivalent to those of zero-time control blanks.

In order to verify that glucocorticoid treatment did not introduce an analytical artifact, both control and corticosterone-treated cells were disrupted in the usual way and adenylate cyclase activity as a function of time and of protein concentration was measured. The assay was linear to 15 min and to 600 μg of protein for both control and corticosterone-treated cells. Variations of the incubation mixture which failed to achieve higher rates included additions of ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid, F^-, Ca^{++}, phosphatidylserine, and phosphatidylinositol (data not shown).

Assay of cyclic AMP phosphodiesterase. Phosphodiesterase activity was determined according to a modification of the method of Brooker *et al.* (17). Cells were suspended and centrifuged as described above, resuspended in 10 mM imidazole buffer, pH 7.6, containing 1 mM $MgCl_2$, and sonicated for 10 sec at minimum intensity with a Branson Sonifier cell disrupter, model 185D. Assays were conducted at 37° for 30 min. The reaction was initiated by the addition of enzyme and terminated by incubation for 1 min in a boiling water bath. The 5′-AMP was then converted to adenosine by the addition of 0.5 μg of alkaline phosphatase and incubation for 15 min at 30°. Then 1 ml of a slurry of AG1-X8 resin (1:2, v/v, in water) was added to sequester unconverted cyclic AMP, and the samples were centrifuged at 1000 × *g* for 10 min. Aliquots (0.5 ml) of the supernatant fractions were removed for scintillation counting. Values were corrected for controls incubated without enzyme. Kinetic experiments performed in this laboratory indicated that a phosphodiesterase with a K_m for cyclic AMP of 1 μM and a second activity with a K_m of 60 μM existed in this cell line.

Assay of $(Na^+ + K^+)$*-ATPase.* The sodium- and potassium-stimulated, ouabain-sensitive, ATPase activity was measured by a modification of the method of Post and Sen (18). Cells were washed, removed from culture bottles, and centrifuged as described above. The pellet of cells was suspended in a buffer containing 30 mM imidazole, 30 mM glycylglycine, 0.75 mM tetrasodium EDTA, and 7.5 mM of $MgCl_2$, pH 7.6. Cells were disrupted in a glass homogenizer at 0°. Buffered homogenates containing 250 μg of protein were incubated for 10 min with or without 0.1 mM ouabain. Sodium chloride and potassium chloride were then added to all samples to concentrations of 150 mM and 30 mM, respectively. The reaction was initiated by the addition of 10 μl of 30 mM ATP containing [^{32}P]ATP (specific activity, 420 cpm/nmole) in the buffer described above. The final volume of the reaction mixture was 130 μl. After 30 min of incubation at 37°, the reaction was terminated by the addition of 500 μl of 0.6 N HCl containing 2% Norit A. The suspensions were centrifuged at 8000 × *g* for 5 min, and an aliquot of the supernatant fraction was analyzed for radioactivity in Aquasol. A zero-time control was used as a blank. At most 15% of the ATP was hydrolyzed under these conditions.

DNA measurement. DNA was determined according to the fluorometric method of Kissane and Robins (19).

Materials. Ham's F-10 medium was obtained from Flow Laboratories, Inc., Rockville, Md.; fetal calf serum, from Grand Island Biological Company; corticosterone and hydrocortisone acetate, from Nutritional Biochemicals; estradiol-17β, testosterone acetate, progesterone, pregnenolone, and calf intestinal mucosa alkaline phosphatase (350 units/mg of protein), from Sigma Chemical Company; AG50W-X8 and AG1-X8, 200–400 mesh, from Bio-Rad Laboratories; disodium adenosine triphosphate and

pyruvate kinase, from Boehringer/Mannheim; ouabain, from Calbiochem; and Aquasol, [^{3}H]adenosine 3′,5′-monophosphate (randomly labeled), and [^{3}H]adenosine 5′-triphosphate (randomly labeled), from New England Nuclear Corporation. Triamcinolone was a gift from American Cyanamid, and *l*-norepinephrine bitartrate monohydrate from Sterling-Winthrop Research Institute. [γ^{32}P]Adenosine 5′-triphosphate was prepared by the method of Glynn and Chappell (20), using carrier-free [^{32}P]phosphate obtained from New England Nuclear Corporation.

RESULTS

Cells were grown for 11 days in the presence or absence of various steroids, and cyclic AMP was measured with or without an exposure to norepinephrine. Testosterone, estradiol, progesterone, and pregnenolone were without effect, but cells grown with hydrocortisone, corticosterone, or with the synthetic glucocorticoid triamcinolone had higher basal concentrations of cyclic AMP and were capable of elevating cyclic AMP in response to norepinephrine 2–3 times that of untreated cells (Table 1).

Measurements of DNA and protein content of cultures treated with 3 μM glucocorticoid were compared with controls. Typically protein and DNA values for treated cultures were 85% of controls, whether the cells were taken in the early logarithmic (5 days post-seeding) or stationary (14 days post-seeding) phase. A 10–20% inhibition of cell growth and replication was seen if the steroid was added from the time of seeding or during the logarithmic phase. The increase in cyclic AMP concentration of treated cells in response to norepinephrine was seen to occur under each condition described above. These observations suggest that the effects of the glucocorticoid on this cell line are independent of the age of the culture. Most experiments reported here were performed on cells in late log phase (6–10 days post-seeding), since confluent monolayers have been shown to be required for a cyclic AMP response to norepinephrine (16).

Response of glucocorticoid-treated cells to norepinephrine. Cells incubated in the presence of increasing concentrations of norepinephrine attained a half-maximal effect at 0.7 μM and a maximal cyclic AMP con-

TABLE 1

Effects of steroids on cyclic AMP in intact cells and on adenylate cyclase

Two sets of experiments are represented. Values given are the means ± ranges of two determinations. In A, cells were grown for 10 days with 3 μM steroid. Cyclic AMP concentrations were measured without neurohormone treatment or after 10 min of incubation with 100 μM norepinephrine. In each instance cells from three steroid-treated and three control cultures were analyzed. No differences among the control cultures were noted, and so the control values were pooled. In B, cells were grown for 11 days with 2 μM steroid, then lysed in hypotonic medium and assayed for adenylate cyclase activity in the absence or presence of 100 μM norepinephrine. Values represent the average and range of duplicate determinations on homogenates prepared from two bottles of cells grown under each condition.

Steroid	A. Cyclic AMP		B. Adenylate cyclase	
	Basal	+Norepinephrine	Basal	+Norepinephrine
	pmoles/mg protein		*pmoles cyclic AMP/mg protein/min*	
None	7 ± 1	1354 ± 485	4.7 ± 1.1	20 ± 0.02
Corticosterone	11 ± 2	4570 ± 542	9.9 ± 0.3	33 ± 1.8
Cholic acid			3.7 ± 0.2	21 ± 1.0
Estradiol-17β	5 ± 2	1450 ± 135	5.3 ± 0.4	18 ± 2.0
Hydrocortisone	10 ± 3	4565 ± 585		
Pregnenolone	6 ± 2	1500 ± 280	4.1 ± 0.5	22 ± 2.0
Progesterone	8 ± 1	1210 ± 230	4.0 ± 0.3	16 ± 0.5
Testosterone	5 ± 2	1068 ± 125	4.4 ± 1.0	17 ± 1.8
Triamcinolone	14 ± 4	3750 ± 227	9.1 ± 1.3	32 ± 3.0

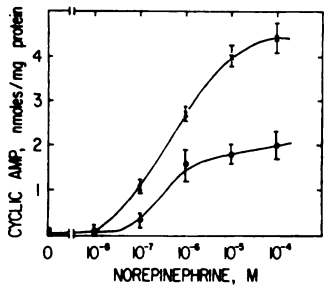

FIG. 1. *Response of control and hydrocortisone-treated cells to norepinephrine*

Cultures were grown for 8 days with (×——×) or without (●——●) 3 μM hydrocortisone and incubated for 10 min with or without norepinephrine, and the cyclic AMP content was determined. Indicated at each point are the mean and range of determinations made on two bottles of cells. Each bottle contained 700–800 μg of protein.

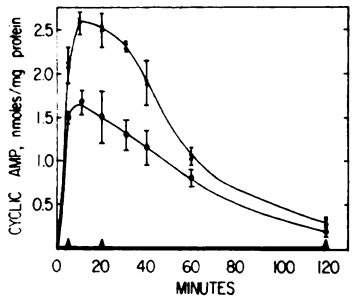

FIG. 2. *Time course of norepinephrine responses of control and triamcinolone-treated cells*

Cultures were grown for 8 days in the presence (×) or absence (●) of 3 μM triamcinolone, and cyclic AMP was measured after incubation for various times in the presence (——) or absence (▲——▲) of 100 μM norepinephrine. Indicated at each point are the mean and range of determinacions made on two bottles of cells. The protein tontent per bottle was 700–900 μg.

centration above 10 μM catecholamine (Fig. 1). Hydrocortisone-treated cells elevated their cyclic AMP concentrations in response to norepinephrine to approximately twice that of control cells. However, the norepinephrine concentration required for half-maximal effect was the same for steroid-treated cells as for controls.

Both control cells and cells grown in the presence of glucocorticoid showed maximal responses after 10 min of incubation with the incubation with the catecholamine, followed by a decline to 10% of the maximum after 2 hr (Fig. 2). However, greater amounts of cyclic AMP were obtained at all time points with cells grown in the presence of triamcinolone.

In order to determine the length of time steroid treatment required for the optimal effect, corticosterone was added to cultures at various times prior to assay with norepinephrine. The capacity of C-6 cells to elevate their cyclic AMP concentrations in response to norepinephrine increased linearly during incubation for 4–48 hr with corticosterone (Fig. 3). The optimum effect was achieved by 2 or more days of incubation with steroid.

Cultures were incubated for 72 hr with varying concentrations of corticosterone prior to assay with norepinephrine. The optimal effect was reached at 2 μM steroid (Fig. 4). Half-maximal effect was achieved at approximately 0.6 μM corticosterone.

Adenylate cyclase. The concentration of cyclic AMP in cells is determined by the relative rates of synthesis and removal by hydrolysis or transport. In considering these possibilities, glucocorticoid effects on adenylate cyclase were examined first. Adenylate cyclase activity of steroid-treated cultures exhibited an increase dependent upon corticosterone concentration (Fig. 5) and time (Fig. 6), which closely resembled the cyclic AMP response of intact cells. Corticosterone

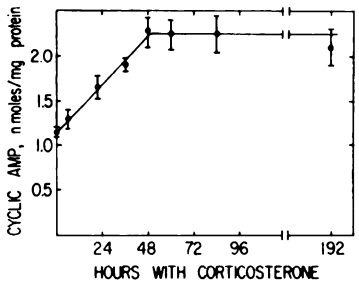

FIG. 3. *Effect of period of exposure to corticosterone on cyclic AMP concentrations of cells in response to norepinephrine*

Six-day-old cultures, incubated for the additional periods of time indicated with 3 μM corticosterone, were assayed for cyclic AMP in response to 100 μM norepinephrine for 10 min. Indicated at each point are the mean and range of determinations made on two bottles of cells. The protein content per bottle was 800–900 μg.

treatment increased both basal and norepinephrine-stimulated adenylate cyclase activities 2-fold (Fig. 5). The norepinephrine effect was noted to be a 4-fold increase throughout the range of corticosterone concentrations employed.

The increase in adenylate cyclase activity brought about by steroid treatment could be due either to the synthesis of more enzyme or to the production of an altered enzyme. In an attempt to distinguish between these possibilities, adenylate cyclase activities of control and steroid-treated cultures were measured with varying substrate concentrations. The apparent K_m for ATP of adenylate cyclase, calculated from the data of Fig. 7, was the same (3×10^{-4} M) for both corticosterone-treated and control cells. However, the V_{max} obtained from corticosterone-treated cells was estimated to be twice that of control cells. These results suggest that more enzyme, rather than one with changed substrate affinity, was synthesized in the presence of glucocorticiod.

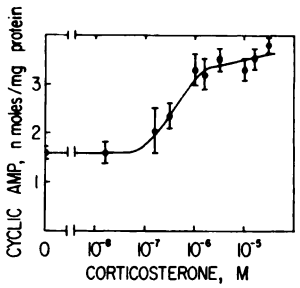

FIG. 4. *Effect of corticosterone concentration on cyclic AMP content of cells in response to norepinephrine*

Five-day-old cultures, incubated for an additional 72 hr with varying concentrations of corticosterone, were assayed for cyclic AMP in response to 10 min of incubation with 100 μM norepinephrine. Indicated at each point are the mean and range of determinations made on three bottles of cells. Each bottle contained 800–900 μg of protein.

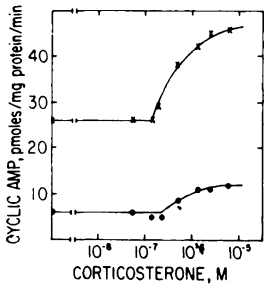

FIG. 5. *Adenylate cyclase activity of cultured cells as a function of corticosterone concentration*

Six-day-old cultures were treated for an additional 72 hr with the indicated concentration of corticosterone. Reaction mixtures contained 240 μg of protein and were incubated for 10 min with (×——×) or without (●——●) 100 μM norepinephrine.

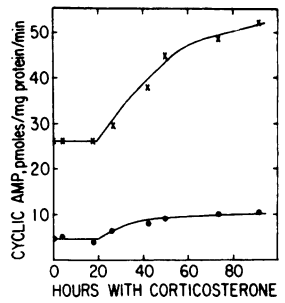

FIG. 6. *Adenylate cyclase activity of cells as a function of time with corticosterone*

Twelve-day-old cultures, treated for the additional periods of time indicated with 3 μM corticosterone, were assayed for enzyme activity with (×——×) or without (●——●) 100 μM norepinephrine. Reaction mixtures contained 300–400 μg of protein and were incubated for 15 min.

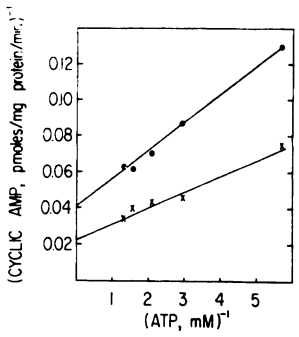

FIG. 7. *Effect of ATP concentration on initial velocity of adenylate cyclase*

Adenylate cyclase activity was measured in 6-day-old cultures treated for an additional 72 hr with (×——×) or without (●——●) 2 μM corticosterone. Reaction mixtures contained 395 μg of protein and 100 μM norepinephrine and were incubated for 15 min.

The reversibility of the steroid effect was assessed (Fig. 8). The elevation of activity in cultures from which corticosterone had been removed remained unchanged for 24 hr, but declined during the next 48 hr. Seventy-two hours after steroid removal the enzyme activity was equivalent to that of untreated cultures.

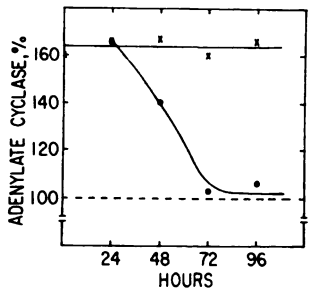

FIG. 8. *Effect of removal of corticosterone on adenylate cyclase activity*

Ten-day-old cultures were treated for an additional 72 hr with or without 3 μM corticosterone. The medium was then replaced by fresh medium. Half of the steroid-treated cultures were retreated with the same concentration of corticosterone. Adenylate cyclase activity was measured at points over a 96-hr period. Results are expressed as a percentage of the activity of control, untreated cells. ×——×, cultures treated continuously with steroid; ●——●, cells from which steroid was removed. Reaction mixtures contained 200–500 μg of protein and 100 μM norepinephrine and were incubated for 10 min.

Glucocorticoid specificity for stimulating adenylate cyclase was revealed when cultures were grown in the presence of various steroids and then assayed for basal and norepinephrine-dependent adenylate cyclase activity (Table 1). Of the steroids tested, only corticosterone and triamcinolone were effective in increasing the activity of the enzyme. The bile salt cholic acid was also ineffective, suggesting that the steroid action was not that of a detergent.

Cyclic nucleotide phosphodiesterase. No significant differences in the maximum velocities obtained for either high- or low-K_m phosphodiesterase activities were observed in cells grown in the presence of corticosterone (Table 2).

(Na^+ + K^+)-ATPase. The possibility that glucocorticoids caused an elaboration of the plasma surface membrane, rather than a specific enhancement of adenylate cyclase activity, was examined by estimating the activity of another membrane-associated enzyme. (Na^+ + K^+)-activated, ouabain-sensitive ATPase, which is believed to be part of the active transport system of the surface membrane, was measured in corticosterone-treated and in control cultures (Table 2). The level of activity of steroid-

TABLE 2

Lack of effect of corticosterone on cyclic nucleotide phosphodiesterase and (Na^+ + K^+)-ATPase

Two sets of experiments are represented. In A, 6-day-old cultures were grown for an additional 72 hr with or without 3 μM corticosterone, then assayed for cyclic nucleotide phosphodiesterase activity. The maximum velocity for the high-K_m enzyme was determined using 180 μg of protein and cyclic AMP concentrations ranging from 8 to 600 μM. The maximum velocity for the low-K_m enzyme was determined using 18 μg of protein and cyclic AMP concentrations ranging from 0.3 to 1.5 μM. In B, 7-day old cultures were grown for an additional 72 hr without or with 3 μM corticosterone, then assayed for (Na^+ + K^+)-ATPase. Values represent the average and range of duplicate determinations on homogenates prepared from five bottles of cells grown under each condition.

Culture	A. Phosphodiesterase activity, maximum velocity		B. (Na^+ + K^+)- ATPase		
	High-K_m activity	Low-K_m activity	−Ouabain (a)	+Ouabain (b)	(b) a–b
	pmoles 5'-AMP/mg protein/min		*nmoles P_i/mg protein/min*		
Control	360	34	10.1 ± 0.2	7.1 ± 0.1	3.0
Corticosterone-treated	380	38	9.6 ± 0.3	6.5 ± 0.05	3.1

treated cultures was equivalent to that of control cultures.

DISCUSSION

In these experiments we have found that C-6 glial tumor cells treated with glucocorticoids possess elevated basal concentrations of cyclic AMP and an augmented cyclic AMP response to norepinephrine. Increased adenylate cyclase is probably responsible for the increments in cyclic AMP observed in intact cells. The steroid incubation time required to increase adenylate cyclase activity coincided with that for increased cyclic AMP response of intact cells to norepinephrine. Only glucocorticoids were effective in both systems of assay, and the half-maximal effective concentration of corticosterone was the same for augmenting the cyclic AMP response of intact cells and for increasing adenylate cyclase. The time course of the cyclic AMP response to norepinephrine for glucocorticoid-treated cultures did not differ from that of control cultures. In addition, cyclic nucleotide phosphodiesterase activity of cell lysates measured at several concentrations of cyclic AMP was unchanged in glucocorticoid-treated cells. Kinetic studies also support the hypothesis that more adenylate cyclase is synthesized in the presence of glucocorticoids. The apparent K_m for ATP was unaltered after treatment with corticosterone, but the V_{max} was doubled. Enzyme induction in many other systems typically results in elevations of enzyme activity much greater than this. The possibility exists that most adenylate cyclase synthesis in C-6 cells is under the control of the glucocorticoids and that the presence of these substances in serum is responsible for activity in the absence of added steroid. Clearly, experiments with inhibitors of DNA and RNA synthesis will be necessary to establish that glucocorticoids induce synthesis of adenylate cyclase at the level of transcription or translation.

Stationary phase cultures were able to degrade adenylate cyclase activity for which added steroid was responsible. Seventy-two hours after removal of corticosterone from previously treated cultures the level of activity was equivalent to that in controls. However, it was possible to surmise only that the rate capacity for removal of the enzyme was at least as great as that observed, because clearance of glucocorticoid from the culture was not measured.

Control and glucocorticoid-treated cultures were compared under phase contrast microscopy. Cells treated with the steroid appeared to be flattened, with their margins less clearly defined than controls. Morphological changes were apparent in both early log and stationary phase cultures of treated cells, provided that the steroid had been present for at least 48 hr. However, mitotic figures were present in both control and treated cultures. Cells grown with corticosterone clearly exhibited increased clumping in calcium-free medium during harvesting. It is well established that morphological changes occur in cell lines following glucocorticoid treatment. De Vellis *et al.* (21) have reported that hydrocortisone depletes C-6 cells of electron-dense material and causes the nucleus to appear polymorphic. The surface membrane of cultured hepatoma cells is known to be altered with respect to adhesiveness, electrophoretic mobility, and antigenic properties after treatment with glucocorticoids (22). HeLa cells incubated with prednisolone become enlarged and accumulate sialopeptides at the cell periphery (23). These observations brought into consideration several alternative explanations for the glucocorticoid effect on cyclic AMP.

Adenylate cyclase of several tissues has been localized primarily in the plasma surface membrane of cells. If the effect of the glucocorticoid on C-6 cultures were simply an elaboration of membranous structures, a consequence of steroid treatment would be a relative enrichment in adenylate cyclase. However, a second enzyme, which is also localized primarily in plasma surface membranes, was not increased in corticosterone-treated cultures. The ouabain-sensitive $(Na^+ + K^+)$-ATPase activity of treated cells did not differ from that of controls, suggesting that elaboration of cellular membranes would not itself account for the observed increase in adenylate cyclase activity. Yet on the basis of the morphological appearance of treated cultures and of the

behavior of these cells in calcium-free medium, it is possible that steroid treatment altered the cell membranes in some manner with variable effects on the enzymes localized therein.

It is unlikely that the beta adrenergic receptor was primarily affected by glucocorticoid, because the concentration of norepinephrine required for half-maximal stimulation of adenylate cyclase and of intact cells did not change after steroid treatment. Gilman and Minna (24) have shown by means of hybrids between C-6 cells and other cell lines that the unusually high concentrations of cyclic AMP generated in C-6 cells are attributable to the loss of a heritable factor which normally limits the amount of *beta* adrenergic receptor or constrains the efficiency of coupling the receptor to adenylate cyclase. Those findings led us to consider whether glucocorticoids would enhance still further the largely unrestrained coupling mechanism in C-6 cells. That possibility appears not to explain the steroid effect in the present experiments, because the ratio of basal to catecholamine-dependent activity measured in both intact and broken cell systems was unaffected by glucocorticoid treatment.

Cyclic AMP concentrations in several cell lines are lowered by the addition of serum (25). The mechanism of this phenomenon is not known, but in view of the cell surface changes associated with glucocorticoid action we wondered whether those agents might elevate cyclic AMP by inducing resistance to entry of the serum factor into the cell. We observed that the ratio between rates of cellular cyclic AMP accumulation and of adenylate cyclase activity were closely similar in control as compared to glucocorticoid-treated cells. Although such evidence is indirect and incomplete, it suggests that the steroid effect does not restrain entry of a serum factor which lowers cyclic AMP in the cell.

We wished to know whether the enhanced cyclic AMP response to glucocorticoid-treated cells could be explained as an indirect consequence of slowing the growth rate. Several lines of rapidly dividing cells exhibit lower concentrations of cyclic AMP than do slowly dividing cell lines (9), which may reflect in part the fact that amounts of cyclic AMP at mitosis are about one-half of those during interphase (26, 27). However, glucocorticoids were at least as effective with stationary phase 12-day-old cultures of C-6 cells as with cultures growing in the log phase, suggesting that the increase in cyclic AMP and adenylate cyclase was not due to a difference in the rate of cell growth but resulted from enzyme induction.

Although the rate at which cyclic AMP is transported out of the cells was not measured in the present experiments, a steroid-induced alteration in that parameter is not likely to explain increased cyclic AMP concentrations, because C-6 cells grown in the absence of glucocorticoids fail to release detectable amounts of cyclic AMP into the medium during a 10-min interval when stimulated by 100 μM norepinephrine (16).

The present findings may have a few implications for future studies of glial functions in the nervous system. C-6 tumor cells retain several differentiated properties. For example, the cells resemble astrocytes morphologically and possess the capacity to synthesize S-100 protein, a characteristic of normal astrocytes (28). C-6 cells are capable of active uptake of γ-aminobutyric acid (29).

In the brain astrocytes are in intimate contact with norepinephrine-containing axon terminals and in some locations exhibit specialized contacts with them (30). Astrocytes are abundantly represented in those brain regions which selectively accumulate corticosterone (31), so that it seems certain that these cells are exposed *in situ* to both catecholamines and glucocorticoids.

Of the diverse functions which have been attributed to glia (32), a role in morphogenesis and in control of the neuronal microenvironment may have plausible counterparts among the characteristics of C-6 cells which are linked to cyclic AMP. For example, the morphology of C-6 cells is altered by dibutyryl cyclic AMP (16). Increased concentrations of cyclic AMP are associated with acceleration of glycogenolysis (33). Cultured C-6 cells which have been grown with [^{14}C]glucose and then exposed to norepinephrine release labeled metabolites into

the medium concurrently with elevations of cyclic AMP concentrations (34). To the degree that C-6 cells resemble normal astrocytes, the present results imply that cellular activities controlled by cyclic AMP in astrocytes *in situ* would occur at lower concentrations of norepinephrine in a defined group of cells, or involve a larger cell population for a given amount of norepinephrine released, when the cells are concurrently exposed to glucocorticoids. Finally, some events in the central nervous system now ascribed to glucocorticoid action may be understood more precisely by considering the concurrent actions of other neurohumoral factors which alter cyclic AMP concentrations of glia.

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