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Altered Adenosine Cyclic 3',5'-Monophosphate Synthesis and Degradation by C-6 Astrocytoma Cells Following Prolonged Exposure to Norepinephrine

EDWARD T. BROWNING, CHARLES O. BROSTROM, AND VINCENT E. GROPPI, JR.

Department of Pharmacology, Rutgers Medical School, College of Medicine and Dentistry of New Jersey, Piscataway, New Jersey 08854

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SUMMARY

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C-6 astrocytoma cells which were incubated with norepinephrine for 3 hr lost the ability to respond to the hormone by accumulating high concentrations of adenosine cyclic 3',5'-monophosphate (cAMP). The altered response of the treated cells was not attributable to a greater release of cAMP from the cells into the incubation fluid. Comparison of the rates of cAMP accumulation by and disappearance from intact cells provided evidence that cells treated with norepinephrine hydrolyzed cAMP more rapidly than did naive cells. Increased cyclic nucleotide phosphodiesterase activity was found in homogenates of treated cells. The increase in phosphodiesterase activity was prevented by cycloheximide and actinomycin D, indicating that an induction of new enzyme synthesis was responsible for the increase in enzyme activity. The increased phosphodiesterase activity was apparent with cAMP but not with guanosine cyclic 3',5'-monophosphate (cGMP) as substrate in the enzyme assay, suggesting that the induced form of the enzyme did not hydrolyze cGMP. The increased enzyme activity did not require Ca^{2+} for activity. Cycloheximide only partially prevented the effect of norepinephrine treatment on cAMP accumulation by intact cells. Further experimentation revealed that the norepinephrine-stimulated adenylate cyclase was decreased in cells treated with norepinephrine in the presence of cycloheximide.

INTRODUCTION

The clonal line C-6 of astrocytoma cells responds to norepinephrine by increasing their content of adenosine cyclic 3',5'-monophosphate more than 200-fold upon initial exposure to this neurohormone (1). Upon repeated exposure to norepinephrine the cells exhibit tachyphylaxis in this response (2, 3). After a 3-hr initial exposure to norepinephrine and a 1-hr recovery period without norepinephrine, the cells respond to norepinephrine with only a 4–6-fold increase of cAMP[1] (3). Interestingly, slices of rabbit cerebellar cortex increase cAMP in response to norepinephrine, but within minutes the cAMP concentration returns toward the basal value even though the slices are in the continued presence of the agonist (4).

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[1] The abbreviations used are: cAMP, adenosine cyclic 3',5'-monophosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; MIX, 1-methyl-3-isobutylxanthine; cGMP, guanosine cyclic 3',5'-monophosphate; CDR, calcium-dependent regulator; dibutyryl cAMP, N^6,$O^{2'}$-dibutyryladenosine cyclic 3',5'-monophosphate.

The present communication analyzes the adaptive changes in cAMP metabolism occurring in C-6 astrocytoma cells following 3 hr of exposure to norepinephrine. Rates of cAMP accumulation and disappearance in cells not previously exposed to norepinephrine (naive cells) and in cells treated for 3 hr with norepinephrine (treated cells) were compared, as were the activities of adenylate cyclase and cyclic nucleotide phosphodiesterase.

Portions of the present work have been presented in preliminary form (5, 6).

MATERIALS AND METHODS

Materials. Unless specified below, materials were obtained as described previously (3). Sotalol was obtained from Regis Chemical Company. Cycloheximide and actinomycin D were purchased from Sigma Chemical Company. Scintiverse scintillation mixture was obtained from Fisher Scientific Company. [2,8-^{3}H]ATP was obtained from New England Nuclear Corporation. The Ca^{2+}-binding protein of Wolff and Siegel (7) was a gift of Dr. D. J. Wolff.

Methods. With the exception of cells grown for adenylate cyclase experiments, cells were grown on glass coverslips and manipulated as previously described (3). A brief description of the procedures employed in experiments which utilized cells grown on coverslips appears in the legend to Table 1.

For adenylate cyclase experiments cells were grown on the surface of glass roller bottles. Cells were seeded at a density of 600 cells/cm^2 of bottle surface and grown at 37° in Ham's F-10 medium plus 10% fetal calf serum, 0.16 ml/cm^2. Growth medium was replaced 3, 5, and 7 days after subcultures, and cells were used for experimentation on the 10th day after subculture. Cells grown on the surface of roller bottles exhibited properties similar to those of cells grown on coverslips, as evidenced by the amount of cAMP released into the incubation fluid during a 3-hr incubation with norepinephrine.

Assay of cyclic nucleotide phosphodiesterase. Phosphodiesterase measurements were performed by a modification (8) of the procedure described by Thompson and Appleman (9). Boiled phosphodiesterase controls were identical with controls without enzyme, which exhibited 10–30% of the radioactivity obtained in the presence of active enzyme. Ca^{2+} and EGTA at the concentrations used in these experiments did not affect the binding of nonhydrolyzed cAMP to the AG1 resin. The AG1-X8 resin used in the phosphodiesterase assay was found to bind a significant but constant proportion of the adenosine (39.2%) and guanosine (40.5%) occurring in assay samples. Data have been corrected for nucleoside binding. Measurements were conducted under conditions in which product formation was linear with time.

Adenylate cyclase activity. Enzyme was incubated for 10 min at 37° in a final volume of 100 μl which contained 0.5 mM [2,8-^{3}H]ATP (62 cpm/pmole), 1 mM 1-methyl-3-isobutylxanthine, 7 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mg of bovine serum albumin, 1 μg of pyruvate kinase, 5 μg of myokinase, 2 mM phosphoenolpyruvate, 50 mM glycylglycine (pH 7.5), and, as indicated, 0.1 mM norepinephrine. Incubation was initiated with 20 μl of enzyme. At the end of the incubation 100 μl of 1.4 mM cAMP were added and samples were placed in a boiling water bath for 2 min. Then 0.8 ml of H_2O was added and protein was removed by centrifugation. Supernatant material was purified on columns of AG50W-X4 resin as described by Solomon *et al.* (10). The column eluate was applied to a 0.5 × 2 cm QAE-Sephadex column.[2] The columns were washed with 2 ml of 50 mM NH_4HCO_3, and cAMP was eluated with 5 ml of 50 mM NH_4HCO_3. cAMP recovery was determined spectrophotometrically (259 nm). Samples were taken to dryness overnight at 80°. Then 0.5 ml of H_2O and 5 ml of Scintiverse were added to the residues, and radioactivity was determined by scintillation counting.

The presence or absence of unlabeled cAMP during the enzyme incubation did not alter the recovery of radioactive cAMP. Unincubated enzyme blanks contained approximately 200 cpm compared to 600–1000 cpm for samples incubated in the absence of norepinephrine and 4000–5000 cpm for samples incubated in the presence of norepinephrine. cAMP recovery after

[2] Y.-C. Huang, personal communication.

the column separations ranged between 75% and 85%. The identity of the radioactive product as cAMP was verified by thin-layer chromatography (11). The adenylate cyclase assay was linear with time when observed over minutes of incubation, but the enzyme activity may not represent truly initial rates comparable to those obtained by incubation of coverslip samples of cells for periods of only a few seconds.

RESULTS

The decreased capacity of norepinephrine-treated cells to accumulate cAMP might be due to a decreased rate of cAMP synthesis, an increased rate of cAMP hydrolysis, or an increased rate of cAMP release by the cells.

cAMP release into incubation fluids. Naive cells incubated in the presence of norepinephrine for 10 min released 814 pmoles of cAMP per milligram of protein into the incubation fluid; treated cells released only 48 pmoles of cAMP per milligram of protein (Table 1). In a control experiment hydrolysis of radioactive cAMP added to the incubation fluid of coverslip samples was examined and found to be insignificant. Therefore, increased cAMP release by treated cells did not account for the decreased cAMP accumulation within the cells. Subsequent experiments examined only the cAMP present in coverslip samples of cells.

Addition of the cyclic nucleotide phosphodiesterase inhibitor MIX to the incubation fluid led to the accumulation of increased quantities of intracellular cAMP in both naive and treated cells (Table 1). However, decreased quantities of cAMP were recovered from the incubation fluid of naive cells incubated in the presence of MIX. The quantity of cAMP recovered from the incubation fluid of treated cells was increased only 3-fold compared to the 20-fold increase observed in the cell extracts. Interference by MIX with the cAMP binding assay was excluded in a control experiment. The difference in the amount of cAMP which was recovered from the incubation fluid of naive cells incubated with or without MIX was significant statistically ($p < 0.01$; t-test). Perhaps

TABLE 1

Effect of norepinephrine treatment on cAMP accumulation in C-6 astrocytoma cells and incubation fluid

Coverslip samples of cells were grown to postconfluent density for 8 days (3), then washed four times with 5 ml of Ham's F-10 medium to remove serum proteins. The samples were incubated in 3 ml of Ham's F-10 medium in the absence or presence of 0.1 mM norepinephrine for 3 hr, then washed as before to remove norepinephrine and incubated for 1 hr in 3 ml of Ham's F-10 medium to restore the basal cAMP content of the cells. The coverslip samples were then transferred to 3 ml of Ham's F-10 medium containing the additions indicated below and incubated for 10 min. Incubations were terminated by transferring the coverslip samples to 1 ml of 5% trichloracetic acid at 0°. After 10 min in trichloracetic acid, coverslip samples and adherent protein were transferred to 1 ml of 1 N sodium hydroxide at room temperature. The trichloracetic acid extracts were ether-extracted and lyophilized (12) prior to analysis of their cAMP content by the procedure of Brostrom and Kon (13). The protein content of the sodium hydroxide extracts was determined by the method of Lowry *et al.* (14), using bovine serum albumin as standard. Further details of the above procedure are presented elsewhere (3). For cAMP analysis of the incubation medium, 1-ml aliquots of the medium were adjusted to 5% in trichloracetic acid by the addition of 50 μl of 100% (w/v) trichloracetic acid, either-extracted, and lyophilized. Data are means ± standard errors of four incubation samples.

3-hr norepinephrine treatment	10-min test incubation		cAMP	
	MIX, 1 mM	Norepinephrine, 0.1 mM	Incubation fluid	Cells
			pmoles/mg protein	
−	−	+	814 ± 42[a]	3380 ± 220
+	−	+	48 ± 15	53 ± 5
−	+	+	498 ± 11	5076 ± 490
+	+	+	166 ± 31	1036 ± 190

[a] cAMP present in the incubation fluid from control samples which were incubated for 10 min in the absence of norepinephrine was undetectable because of the large ratio of incubation fluid volume to intracellular volume. cAMP present within these cells was 7 ± 2 or 6 ± 2 pmoles/mg of protein, respectively, for naive or treated cells (means ± standard errors of four incubation samples).

MIX interferes with the mechanism by which cAMP leaves the cells.

Rate of cAMP accumulation. Substantial amounts of cAMP accumulated in treated cells upon re-exposure to norepinephrine for 10 min if MIX was present (3) (Table 1). Data in Fig. 1 compare the rates of cAMP accumulation by C-6 cells in the presence and absence of MIX during the first 30 sec of exposure to norepinephrine. When MIX was present, naive and treated cells accumulated cAMP at approximately equal rates for the first 5 or 10 sec of incubation with norepinephrine (Fig. 1). After 20 sec the mean content of cAMP in the treated cells was less than that present in the naive cells; by 30 sec the difference in accumulated cAMP was significant statistically ($p < 0.02$). In the absence of MIX, treated cells accumulated cAMP more slowly than similarly tested naive cells over the entire 30 sec of the experiment (Fig. 1).

Rate of cAMP disappearance. Cells which had accumulated cAMP in the presence of norepinephrine and MIX were used to compare the rates of cAMP decay in naive and treated cells (Fig. 2). Treated cells hydrolyzed cAMP at the highest rate during the first 15 sec after transfer to fresh medium (Fig. 2). Naive cells hydrolyzed cAMP slowly at first, achieving a maximal rate only after the first minute of incubation.

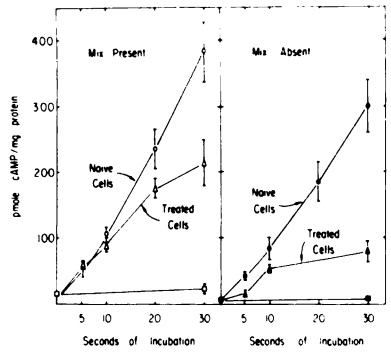

FIG. 1. *Initial rates of cAMP accumulation by C-6 astrocytoma cells in response to norepinephrine*

Cells were incubated as described in Table 1, except that after the 3-hr norepinephrine treatment cells were incubated in Ham's F-10 medium for 30 min to restore basal cAMP, and then transferred to 3 ml of Ham's F-10 medium with or without 1 mM 1-methyl-3-isobutylxanthine (MIX) and incubated in this fluid for 30 min prior to the second norepinephrine addition.

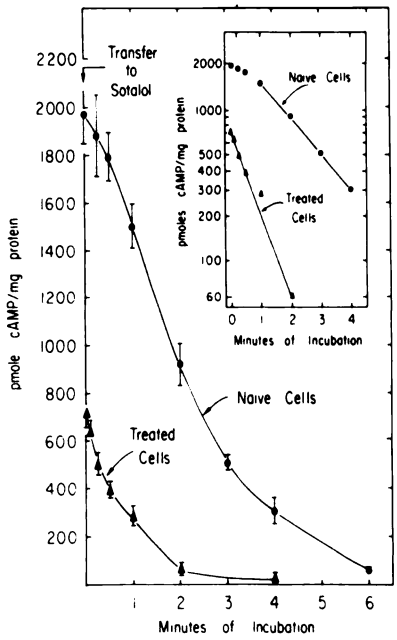

FIG. 2. *Rate of cAMP breakdown in C-6 astrocytoma cells*

Cells were treated as described in Table 1 with respect to the 3-hr norepinephrine treatment and 1-hr recovery. Next coverslip samples were exposed to 10 μM norepinephrine and 1 mM MIX for 4 min. This incubation was terminated by transferring the coverslip samples to 10 ml of Ham's F-10 medium containing 0.1 mM sotalol, which blocks *beta* adrenergic receptors of these cells (1), and the samples were incubated in this solution for the times indicated. The Ham's F-10 medium–sotalol incubation was terminated by transferring the samples to 1 ml of 5% trichloracetic acid, and samples were then handled as described in Table 1. The data represent means of four to six incubation samples derived from three experiments.

The rates of cAMP breakdown in the two groups of cells were compared at similar intracellular cAMP concentrations, those at which adaptive changes in cAMP accumulation had occurred in the treated cells. The treated cells hydrolyzed cAMP at a rate of 840 ± 113 pmoles/min/mg of protein during the first 15 sec of incubation, when the cAMP content decreased from 710 to 500 pmoles/mg of protein. The naive cells hydrolyzed cAMP at a rate of 390 ± 120 pmoles/min/mg of protein during

the third minute of incubation, when the cAMP content decreased from 910 to 520 pmoles/mg of protein. The difference in rate of cAMP disappearance was significant statistically ($p < 0.05$; t-test).

The possibility was considered that the slower initial rate of cAMP disappearance from naive cells was due to a lower rate of migration of MIX out of these cells compared to the treated ones. A control experiment was performed in which cycloheximide was used in place of MIX as a means of inducing cAMP accumulation in treated cells (see below). Naive cells exhibited a 10-sec lag prior to the onset of decay in the concentration of cAMP whereas treated cells exhibited no lag. In another control experiment, conducted without cycloheximide or MIX, the lag was shown to be independent of the duration of exposure of naive cells to norepinephrine. Apparently the slow initial rate of cAMP decay was attributable to something other than the presence of MIX.

Cyclic nucleotide phosphodiesterase activity. Cyclic nucleotide phosphodiesterase was assayed with cAMP or cGMP as substrate, under conditions in which Ca^{2+}-dependent and Ca^{2+}-independent forms of the enzyme would be detected. Ca^{2+}-independent enzyme activity was measured in the presence of EGTA. Ca^{2+}-dependent enzyme activity was measured as the increase in enzyme activity observed in the presence of Ca^{2+} in excess of EGTA. Measurements were conducted with and without saturating concentrations of a Ca^{2+}-dependent regulator from pig brain. CDR from pig brain activates the Ca^{2+}-dependent phosphodiesterase of these cells as does endogenous CDR (8).

With cAMP as substrate a 2-fold increase in Ca^{2+}-independent enzyme activity was observed following 3 hr of exposure of the cells to norepinephrine (Table 2). The increase in enzyme activity was observed at three enzyme concentrations and at 10 cAMP concentrations between 0.25 and 25 μM (data not shown). The increase in phosphodiesterase activity was not observed if cycloheximide was present during the 3-hr exposure to norepinephrine (Table 2). In other experiments cycloheximide was shown not to change the phosphodiesterase activity observed after a 3-hr incubation in the absence of norepinephrine. It was concluded that cycloheximide inhibited the increase in the Ca^{2+}-independent phosphodiesterase activity. Actinomycin D, 3 μg/ml, also inhibited the increase in Ca^{2+}-independent phosphodiesterase which was induced by 3 hr of norepinephrine treatment (data not shown). With cGMP as the substrate in the phosphodiesterase assay no increase in the Ca^{2+}-independent phosphodiesterase activity was observed (Table 2).

Ca^{2+}-dependent phosphodiesterase activity was little affected by the 3-hr norepinephrine treatment. This enzyme activity was increased by approximately 20% in the present experiment if cAMP was the substrate (Table 2), but this increase was not routinely seen in other experiments. With cGMP as the substrate in the assay, no change in Ca^{2+}-dependent phosphodiesterase activity was observed.

The enzyme activity observed in the presence of added CDR was greater than that observed in its absence with either substrate. However, added CDR uniformly increased the Ca^{2+}-dependent activity observed with or without norepinephrine treatment. The amount of endogenous CDR associated with the phosphodiesterase therefore appeared unchanged by the norepinephrine treatment.

Cycloheximide and tachyphylaxis in intact cells. Because cycloheximide inhibited the increase in cyclic nucleotide phosphodiesterase activity induced by 3 hr of norepinephrine treatment, this agent was tested for its ability to inhibit the development of tachyphylaxis in the cAMP response to norepinephrine in intact cells. Cycloheximide partially inhibited the development of tachyphylaxis, but the amount of inhibition varied between 8% and 40%, depending upon the batch of serum in which the cells were grown; six batches were tested. Data in Table 3 demonstrate the inhibition observed when a representative batch of serum was used. A 22% inhibition was observed in this experiment. The effectiveness of cycloheximide as an inhibitor of protein synthesis was tested by measuring the incorporation of L-[4,5-^{3}H]lysine into trichloracetic acid-insoluble residues of

TABLE 2

Effect of norepinephrine treatment on cAMP and cGMP hydrolysis by cyclic nucleotide phosphodiesterase of C-6 astrocytoma cells

Cells were grown, washed free of serum proteins, and incubated for 3 hr as described in Table 1. Norepinephrine was present during the 3-hr incubation as indicated. At the end of the incubation coverslip samples of cells were transferred to 5 ml of 150 mM sodium chloride–25 mM Tris-chloride, pH 7.4, at room temperature, and then into 1 ml of 10 mM imidazole–1 mM magnesium chloride, pH 7.4. Cells were scraped from the coverslips, and the cellular suspension was placed in a 1.5-ml Eppendorf centrifuge tube and sonicated for 10 sec at minimum power in a Heat Systems model W185D sonicator equipped with a microprobe. Sonic extracts were analyzed for cyclic nucleotide phosphodiesterase as described in MATERIALS AND METHODS. The cAMP or cGMP concentration was 25 μM; the enzyme volume was 25 μl. Data represent means of duplicate assay samples from a representative experiment.

3-hr norepinephrine treatment	Cycloheximide, 10 μg/ml	Cyclic nucleotide	Additions to assay			Total activity
			EGTA	Ca^{2+}	Ca^{2+} + CDR	
			pmoles/min/mg protein			
−	−	cAMP	170	192	266	436
+	−	cAMP	341	238	325	666
+	+	cAMP	179	213	297	476
−	−	cGMP	82	266	331	413
+	−	cGMP	82	239	330	412
+	+	cGMP	77	259	331	408

the cells. Lysine incorporation was inhibited 87%, 93% or 95% by 1, 10, or 50 μg of cycloheximide per milliliter, respectively. Because inhibition of protein synthesis and phosphodiesterase induction (Table 2) were nearly complete and inhibition of the development of tachyphylaxis was not more than 40%, the conclusion was drawn that the tachyphylaxis involved a component in addition to phosphodiesterase induction.

TABLE 3

Effect of cycloheximide on cAMP content of naive and norepinephrine-treated C-6 astrocytoma cells

Experiments were conducted as described in Table 1. Cells were incubated with cycloheximide as indicated, for 30 min prior to initiation of the 3-hr incubation by norepinephrine as indicated. Cycloheximide was included in all subsequent wash and incubation fluids for the appropriate samples. Control samples which were not exposed to norepinephrine during the 10-min final incubation contained 5 pmoles of cAMP per milligram of protein. Data are means ± standard errors of four incubation samples.

Cycloheximide	cAMP following 10-min exposure to norepinephrine	
	Naive cells	Treated cells
μg/ml	*pmoles/mg protein*	
0	2180 ± 150	49 ± 4
1	2130 ± 170	216 ± 47
10	2170 ± 47	378 ± 37
50	2340 ± 41	468 ± 54

Adenylate cyclase activity. Cells were incubated in the presence of cycloheximide with or without norepinephrine for 3 hr. Adenylate cyclase activity was measured on a particulate fraction prepared from homogenized cells. Particulate fractions from naive and treated cells exhibited approximately the same adenylate cyclase activity in the absence of norepinephrine (Table 4). In the presence of norepinephrine, particulate fractions from naive cells exhibited a 10-fold increase in adenylate cyclase activity whereas fractions from treated cells exhibited only 5-fold increases in activity. The amount of norepinephrine-sensitive adenylate cyclase activity observed in the naive cells, 280 pmoles/min/mg of protein, was less than the rate of cAMP accumulation observed in intact cells (Fig. 1). The rate of the hormone-stimulated adenylate cyclase observed in the present experiments was similar to or greater than other reported values for C-6 cells (16, 17). Adenylate cyclase activities reported in Table 4 are representative of values observed in the present series of experiments, but variability in adenylate cyclase activity occurred from experiment to experiment with respect to both basal and hormone-

TABLE 4

Effect of norepinephrine treatment on adenylate cyclase activity of C-6 astrocytoma cells

Cells were cultured under the same conditions used for the other experiments, except that culture was conducted in roller bottles. The confluent cultures were washed three times with 100 ml of Ham's F-10 medium and incubated for 30 min in 125 ml of Ham's F-10 medium containing 50 μg/ml of cycloheximide. Norepinephrine (0.1 mM) was added as indicated, and the cells were incubated for 3 hr. The cells were then washed with 100 ml of Krebs bicarbonate buffer ($CaCl_2$ omitted) and were resuspended with 100 ml of EDTA solution (0.5 mM EDTA, 137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, and 1.1 mM glucose). The cell suspension was decanted into 100 ml of Hanks' balanced salt solution (15), and the cells were collected by centrifugation. The pellets were resuspended in 4 ml of homogenizing buffer containing 250 mM sucrose, 10 mM glycylglycine, 2 mM $MgCl_2$, 1 mM EDTA, and 3 mM dithiothreitol, pH 7.5, and were homogenized by nine up-and-down strokes in a Teflon-glass homogenizer. The homogenates were centrifuged at 20,000 × *g* for 10 min. Pellets were resuspended in 4 ml of homogenizing buffer. Adenylate cyclase assays were conducted on this material or on similarly prepared samples which had been quick-frozen by rapidly pipetting the particulate suspension onto the inner surfaces of Pyrex test tubes which had been chilled in ethanol–Dry Ice. Fresh and quick-frozen enzyme activities were similar. The data presented were derived from quick-frozen material.

0.1 mM norepinephrine present during assay	Adenylate cyclase	
	Naive cells	Treated cells
	pmoles cAMP/min/mg protein	
−	26	24
+	280	150

stimulated activities. However, within each experiment treated cells consistently exhibited lower norepinephrine-stimulated adenylate cyclase than did naive cells.

Maguire *et al.* (18) reported that norepinephrine undergoes extensive, nonspecific, irreversible binding to a membrane fraction prepared from C-6 cells. This binding was inhibited by reducing agents such as ascorbate or sodium metabisulfite. A control experiment in the present study indicated that these reducing agents, at concentrations of 100 μM, did not alter the development of tachyphylaxis in C-6 cells. In another control experiment tachyphylaxis was induced by compounds with *beta* adrenergic activity (epinephrine, norepinephrine, and isoproterenol) and not by other compounds (dopamine, phenylephrine, and catechol). Therefore nonspecific binding by oxidation products of norepinephrine appears not to explain the development of tachyphylaxis.

DISCUSSION

Tachyphylaxis to norepinephrine by C-6 astrocytoma cells results in part from increased cAMP hydrolysis and in part from decreased cAMP synthesis. Increased rates of cAMP hydrolysis were observed in intact cells (Fig. 2) and were detected as an increase in the cyclic nucleotide phosphodiesterase activity of sonically disrupted cells (Table 2). The decreased rate of cAMP synthesis was observed as a decrease in the norepinephrine-stimulated adenylate cyclase activity (Table 4). Observations of the rate of cAMP accumulation by intact cells during the first 30 sec of incubation with norepinephrine (Fig. 1) were consistent with the interpretation that cAMP synthesis was decreased in treated cells, but other interpretations were possible.

The rate of cAMP hydrolysis by intact cells was estimated by following the decay in cAMP content of the cells upon termination of norephinephrine-stimulated cAMP synthesis. Treated cells hydrolyzed cAMP at more than twice the rate of naive cells at cAMP concentrations where adaptive changes in cAMP metabolism had occurred in treated cells. cAMP breakdown followed first-order kinetics in both groups of cells in this range of cAMP concentrations. Therefore the increased enzyme activity of treated cells appeared to have an affinity for cAMP which was similar to that operating in naive cells in the concentration range examined.

The increase in cyclic nucleotide phosphodiesterase activity observed in treated cells was inhibited by cycloheximide and actinomycin D, indicating a requirement for RNA and protein synthesis for the increase in phosphodiesterase activity to occur and suggesting that the increase in enzyme activity was due to an induction of new phosphodiesterase synthesis. Related

observations have been made by others in cultured cells. Dibutyryl cAMP induces phosphodiesterase in fibroblasts (19), and phosphodiesterase activity of fibroblasts is increased after treatment with prostaglandins E_1 and E_2 (20). Norepinephrine induces the synthesis of one peak of electrophoretically separated phosphodiesterase activity in the C-2A line of rat glioma cells, and the induction is blocked by propranolol (21). In C-6 cells phosphodiesterase activity is increased 24 hr after treatment with dibutyryl cAMP (22). Concurrently with our present studies, Schwartz and Passonneau have reported that dibutyryl cAMP and norepinephrine induce phosphodiesterase in C-6 cells (23). They observed increases in enzyme activity which were apparent at 1.4 μM and 1 mM cAMP and were inhibited by cycloheximide, actinomycin D, and propranolol. Present results extend the findings of Schwartz and Passonneau in demonstrating that the induced phosphodiesterase hydrolyzes cAMP but not cGMP and that the induced activity does not require calcium. Russell and Pastan have recently reported induction of a form of phosphodiesterase in chicken embryonic fibroblasts which hydrolyzes cAMP but not cGMP, and they concluded that the enzyme was under separate genetic control from the cGMP phosphodiesterase of those cells (24).

In the present studies cycloheximide only partially reversed the effect of 3 hr of norepinephrine treatment (Table 3). This was interpreted to mean that some factor other than phosphodiesterase induction, which did not require protein synthesis, contributed to the tachyphylaxis in the effect of norepinephrine on cAMP concentration. The present results are in contrast to a similar experiment by Schwartz and Passonneau in which nearly complete reversal of the tachyphylaxis was observed in the presence of cycloheximide along with norepinephrine (23). Presumably some difference in experimental conditions accounts for the difference in results. Factors which might account for the difference are presently under investigation.

De Vellis and Brooker have reported that the development of tachyphylaxis to norepinephrine is partially inhibited by cycloheximide in the 2B subclone of the C-6 line of cells (25). This subclone did not induce cyclic nucleotide phosphodiesterase in response to norepinephrine treatment. The effect of cycloheximide treatment was attributed to a change in cAMP synthesis. The present observations are not inconsistent with the finding of de Vellis and Brooker. Experiments conducted with the C-6 line cannot easily demonstrate changes in the rate of cAMP synthesis which require new protein synthesis, because phosphodiesterase induction presents a second variable which complicates the interpretation of results.

Modulation of cAMP concentration in cells may be achieved by at least two classes of mechanisms. The selective loss of sensitivity to catecholamines or to prostaglandin E_1 by fibroblasts (26) or glial tumor cells (27) in response to initial exposure to the respective stimulus represents an agonist-specific form of control. The control of cAMP metabolism which is expressed as induction of new phosphodiesterase synthesis represents a more generally expressed control, in that cAMP generated as a result of any activator of the adenylate cyclase system would be hydrolyzed.

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