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Norepinephrine-Sensitive Properties of C-6 Astrocytoma Cells

EDWARD T. BROWNING, JOAN P. SCHWARTZ,[1] AND BRUCE MCL. BRECKENRIDGE

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

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SUMMARY

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C-6 astrocytoma cells respond to 0.1 mM norepinephrine by a greater than 200-fold elevation of adenosine cyclic 3′,5′-monophosphate (cAMP) and an 80% degradation of glycogen during 20 min of incubation in Ham's F-10 medium. Evaluation of the time course of these effects indicated that glycogenolysis occurred nonlinearly at a rate of 1–2 nmoles/min/mg of protein; cAMP was maximally elevated after 5 min of incubation and remained so for the 30-min experiment. Norepinephrine induced transformation of glycogen phosphorylase to the 5′-AMP-independent or *a* form during the first 30 sec of incubation. At the time when phosphorylase *a* formation had begun, 5 sec after norepinephrine addition, cAMP was elevated only 10-fold. During a 3-hr treatment of cells with norepinephrine, cAMP and glycogen concentrations returned to nearly control concentrations; yet incubation fluid retained sufficient norepinephrine to elevate cAMP as before in fresh samples of cells. Treatment of C-6 cells with norepinephrine for 3 hr followed by 1 hr of incubation without hormone largely suppressed the effect of a second norepinephrine treatment on cAMP concentration, but glycogenolysis occurred as before. 1-Methyl-3-isobutylxanthine, an inhibitor of cAMP phosphodiesterase, partially overcame the suppression of the norepinephrine effects when added with norepinephrine. It is concluded that C-6 astrocytoma cells contain a norepinephrine-activated system of enzymes for degrading glycogen and that norepinephrine treatment modifies the response of the cells to subsequent exposure to this neurohormone.

INTRODUCTION

Glial tumor cells which have been cloned and maintained in tissue culture afford advantages in studying certain problems of central nervous system function. For example, the heterogeneity of cell type, which characterizes brain samples of even minute size, is avoided and problems associated with diffusion barriers of organized tissue are minimized.

The C-6 line of rat astrocytoma cells retains the capacity to synthesize S-100 protein characteristic of neuroglia (1, 2). Stimulation of the *beta* adrenergic receptor of C-6 cells maintained in culture is known to increase greatly the cellular content of cAMP (3).

In brain the likelihood is high that astro-

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[1] Present address, National Institute of Neurological Diseases and Stroke, Section on Cellular Neurochemistry, Bethesda, Maryland 20014.

cytes in immediate proximity to norepinephrine-containing presynaptic structures are exposed to pulses of the neurotransmitter throughout adult life. For example, glial cells, including astrocytes, invest the dendritic and somatic surfaces of the Purkinje cells in the rat cerebellum (4), and norepinephrine-containing fibers terminate on Purkinje cells in this region (5). cAMP[2] mediated effects in numerous cell types occur both at a level of acute interaction, such as phosphorylase interconversion, and over more prolonged time periods at the level of new protein synthesis (6). Should the glia in the region of noradrenergic synapses exhibit a *beta* adrenergic receptor, one might expect them to undergo adaptive change as a result of exposure to norepinephrine.

The aim of the present study was to examine the cAMP-related effects of initial and repeated exposures of C-6 astrocytoma cells to norepinephrine.

MATERIALS AND METHODS

Materials. Ham's F-10 medium for cell culture was obtained from Flow Laboratories, Inc.; Ham's F-10 medium for cell incubations and fetal calf serum were obtained from Grand Island Biological Company. Ham's F-10 medium for cellular incubations was obtained as powdered material and made up on the day of the experiment. Norepinephrine, ATP, ADP, 5'-AMP, type III pig liver glycogen, and rabbit muscle phosphorylase *a* (α-1,4-glucan:orthophosphate glucosyltransferase, EC 2.4.1.1) were purchased from Sigma Chemical Company. Hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1), glucose 6-phosphate dehydrogenase (D-glucose-6-phosphate:NADP oxidoreductase, EC 1.1.1.49), creatine phosphokinase (ATP:creatine phosphotransferase, EC 2.7.3.2), pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40), myokinase (ATP:AMP phosphotransferase, EC 2.7.4.3), phosphoglucomutase (α-D-glucose-1,6-diphosphate:α-D-glucose-1-phosphate phosphotransferase, EC 2.7.5.1), NADP, and the tricyclohexylamine salt of phosphoenolpyruvate were purchased from Boehringer/Mannheim. Creatine phosphate was purchased from Calbiochem. NADH was purchased from P-L Biochemicals, Inc. Beef heart lactate dehydrogenase (L-lactate:NAD oxidoreductase, EC 1.1.1.27) was purchased from Worthington. 1-Methyl-3-isobutylxanthine was obtained from G. D. Searle Company.

Culture conditions. The C-6 line of rat astrocytoma cells was obtained in frozen form from the American Type Culture Collection and grown at 37° in monolayer culture in Ham's F-10 medium plus 10% fetal calf serum in Falcon plastic bottles under an atmosphere of 95% air–5% CO_2. Antibiotics were not used in these cultures. At 14-day intervals the confluent cultures of cells were resuspended by treatment with a solution of 0.52 mM EDTA and 137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, and 1.1 mM glucose (7), counted, and subcultured at a density of $8 \times 10^5 \pm 2 \times 10^5$ cells/bottle (25 cm^2). A proportion of the cells resulting at each subculture was used as "seed stock" for carrying the line, and the remainder were grown for experimentation.

Subcultures for experimentation. Cells for experimentation were grown as above, but in 60-mm Petri dishes into which two 25-mm round coverslips were placed as described by Gilman and Nirenberg (3). Each Petri dish contained Ham's F-10 medium plus 10% fetal calf serum in a 6-ml final volume. Cells, 4×10^5/Petri dish, were pipetted directly over the slips to ensure confluent cultures on the slips. Penicillin, to a final concentration of 25 units/ml, and streptomycin, 50 μg/ml, were added to these cultures in a 0.2-ml volume. The cells were fed 2, 4, and 7 days after subculture. Experiments were conducted on the eighth day.

After 8 days in culture a typical coverslip contained 1.5×10^6 cells, 36 μg of DNA, and 600 μg of protein.

Cell incubations. The confluent monolayer cultures, adherent to coverslips, were removed from the Petri dishes with forceps, and washed free of serum proteins by transferring the slips through four 5-ml aliquots

[2] The abbreviations used are: cAMP, adenosine cyclic 3',5'-monophosphate; MIX, 1-methyl-3-isobutylxanthine.

of Ham's F-10 medium in 30-ml plastic beakers. The cells were next incubated in a shaking incubator in 3 ml of Ham's F-10 medium at 37° under an atmosphere of 5% CO_2–95% air for the times indicated. If cells had been treated with norepinephrine, incubations were conducted as above; then the cells were again washed with four 5-ml volumes of Ham's F-10 medium to remove norepinephrine. A 1-hr incubation in the absence of hormone was performed, and a 10-min challenge with norepinephrine was conducted as indicated.

Termination of incubations. Cells were fixed by transferring coverslip samples into acid or liquid nitrogen. In the case of acid extractions, slips were placed in 1 ml of 5% (w/v) trichloracetic acid for 10 min at 0°, and the slips were transferred to 1 ml of 1 N NaOH at room temperature. The trichloracetic acid extracts were made 0.1 N in HCl and extracted five times with 2 volumes of water-saturated ether (8). Aliquots, 0.25 ml, of the resulting acidic extract were taken for cAMP assay, and the remainder was neutralized to pH 5.5–6.5 with 0.5 M potassium carbonate for analysis of other nucleotides and creatine phosphate. In the above procedure, coverslips were removed from the 1 N NaOH after 30 min, and sodium hydroxide-soluble material was stored at −80° until analysis for glycogen and protein. Measurements of the protein and glycogen content of trichloroacetic acid extracts indicated that these materials were not released from the slips by the trichloroacetic acid treatment. In a few early experiments perchloric acid was found to contribute significantly to the blank of the cAMP assays reported in Table 1; therefore trichloracetic acid was used routinely in later experiments. Perchloric and trichloracetic acids were approximately equally effective in releasing adenine nucleotides and creatine phosphate from the cells. Neither a second 10-min storage period of coverslip samples in trichloracetic acid nor sonication of the coverslip samples while in trichloracetic acid released significantly more of these materials from the coverslips. The acid extract from a single coverslip sample provided sufficient material for assay of cAMP, ATP, ADP, 5′-AMP, and creatine phosphate.

Incubations were terminated by freezing coverslip samples of cells in liquid nitrogen when phosphorylase activity was to be determined. Coverslip samples were removed from the incubation fluid with a tripod forceps (Triceps, Universal Technical Products, Inc., Forest Hills, N. Y.) 2 sec before freezing, excess fluid was blotted from the edge of the slip with a paper tissue during 1 sec, and the slip was frozen at the appropriate second. Samples were swirled in liquid nitrogen for approximately 5 sec before transfer to a freezer at −80° for storage. Control experiments indicated that a coverslip swirled for 1 sec in liquid nitrogen was sufficiently chilled to condense and freeze moisture from the air. Therefore most enzymatic processes would probably terminate in less than 1 sec by this technique.

Analytical techniques. Metabolites other than cAMP and protein were analyzed by fluorometric enzyme assay linked to nicotinamide adenine dinucleotide oxidation or reduction, using the analytical format described by Williamson and Corkey (9). ATP was determined using hexokinase and glucose-6-phosphate dehydrogenase (9). Creatine phosphate was measured in the same cuvette as ATP, as described by Lowry *et al.* (10), except that ADP was added prior to creatine kinase in order to allow reaction of contaminating ATP prior to the reaction of creatine phosphate. ADP and 5′-AMP were assayed as described by Williamson and Corkey (9). Glycogen was assayed according to Passonneau *et al.* (11).

Glycogen phosphorylase was assayed after frozen samples had been resuspended from coverslips by a modification of the glycogen assay of Passonneau *et al.* (11) (see below). Samples were resuspended by placing slips (−80°) in a beaker at 0° which contained 0.5 ml of 10 mM sodium phosphate, 3 mM sodium EDTA, 1 mM dithiothreitol, and 100 mM sodium fluoride, pH 7.0. Cells were disrupted by homogenization or by sonication as indicated in table and figure legends.

Phosphorylase was assayed using the experimental conditions for the glycogen assay (11), but the order of addition of phosphorylase (homogenate), substrates, and 5′-AMP was changed. Cuvettes were prepared to contain buffer [50 mM imidazole, 1 mM

ethylene glycol bis (β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA), 0.5 mM magnesium chloride, and 0.02% bovine serum albumin, pH to 7.0 with HCl] plus homogenate to give 2 ml. Ten microliters of NADP (12.6 mg/ml), 10 μl of 1 M potassium phosphate (pH 7), 10 μl of phosphoglucomutase (1.25 mg/ml), and 10 μl of glucose 6-phosphate dehydrogenase (0.2 mg/ml) were added, and a linear baseline was established. 5′-AMP-independent phosphorylase activity was determined after adding 20 μl of a 1% solution of freshly prepared rabbit liver glycogen in 1 mM sodium hydroxide. The glycogen solution had been heated for 10 min at 60° to disaggregate the glycogen. 5′-AMP-dependent phosphorylase activity was determined after addition of 20 μl of 100 mM 5′-AMP, pH 7.0, to the cuvette. The 5′-AMP content of assay reagents and cells was determined separately and totaled less than 50 nM at the dilutions occurring in the assay. The dependence of the observed reactions on added phosphate and glycogen was also determined separately. No assay reaction occurred in the absence of phosphate and glycogen when cells were suspended in a phosphate-free resuspending fluid.

Protein was determined by the method of Lowry *et al.* (12) with bovine serum albumin as standard. cAMP was determined according to Gilman (8) or according to a modification of this procedure by Brostrom and Kon (13).

RESULTS

cAMP and glycogen. As shown in Table 1, norepinephrine caused a greater than 200-fold increase in the cAMP content of the C-6 astrocytoma cells, in confirmation of early reports (3). The norepinephrine treatment promoted glycogenolysis to an extent which led to depletion of four-fifths of the glycogen stores of the cells after 20 min of incubation. Approximately one-fifth of the initial glycogen content of the cells was depleted in the absence of norepinephrine.

Adenine nucleotides and creatine phosphate. The cells maintain high levels of creatine phosphate and ATP and maintain creatine phosphate to ATP ratios near unity (Table 2). ATP:ADP ratios in the cells ranged between 11 and 22. Differences between these values are primarily a result of changes in the ADP content. Norepinephrine had little effect on the levels of these energy carriers.

TABLE 1

Effect of norepinephrine on cAMP and glycogen content of C-6 astrocytoma cells

Cells were grown to confluence in monolayer culture on 25-mm coverslips in 60-mm Petri dishes. On the eighth day after subculture, 1 day after the last feeding, slips were washed free of serum proteins by four 5-ml washes with Ham's F-10 medium and incubated at 37° for the times indicated in 3 ml of Ham's F-10 medium under a 95% air–5% CO_2 atmosphere. Norepinephrine was added as 20 μl of 15 mM norepinephrine HCl in 5 mM HCl. In control measurements this volume of acid was shown not to change the pH of the incubation mixture. Incubations were terminated by transferring the slips to 1 ml of 6% perchloric acid at 0°. Perchloric acid extracts were neutralized with potassium carbonate. cAMP was measured by the binding assay of Gilman (8). Glycogen was determined by the fluorometric procedure of Passonneau *et al.* (11).

Norepinephrine 0.1 mM	Incubation time	cAMP [a]	Glycogen [b]
	min	*pmoles/mg protien*	*nmoles glucosyl units /mg protien*
−	0	4	65 ± 11
−	20	15	50 ± 2
+	20	3500	13 ± 1

[a] cAMP data were derived from one of the two experiments in which the glycogen data were acquired.

[b] Values represent means ± standard errors for four coverslip samples.

Glycogenolysis. The breakdown of glycogen with time following norepinephrine treatment is shown in Fig. 1. Preliminary experiments indicated that rapid, transient changes in the glycogen content of the cells occurred during the first few minutes after cells were removed from growth medium and placed in fresh Ham's F-10 medium. The glycogen content rose initially, then fell. For this reason these cells were incubated for 10 min before hormone addition. During the 30 min of incubation the content of glycogen in control samples decreased somewhat (Fig. 1). Norepinephrine caused a more rapid decrease in glycogen content in the cells. The

TABLE 2

Effect of norepinephrine on adenine nucleotide and creatine phosphate content of C-6 astrocytoma cells

Data were derived from the same experiments as described in Table 1. Values represent means ± standard errors for four coverslip samples.

Norepinephrine, 0.1 mM	Incubation time	Creatine phosphate	ATP	ADP	5'-AMP	Total adenine nucleotides[a]	ATP:ADP
	min	*nmoles/mg/protein*					
−	0	27 ± 1.7	22 ± 1.8	1.0 ± 0.2	1.0 ± 0.1	24	22
−	20	23 ± 1.5	22 ± 0.7	2.3 ± 0.7	0.8 ± 0.2	25	10
+	20	25 ± 1.1	20 ± 1.0	1.1 ± 0.1	0.6 ± 0.1	25	17

[a] Sum includes nucleotide present as cAMP, which is presented in Table 1.

decrease in glycogen content which wa initiated by norepinephrine followed a smooth but nonlinear curve, which appeared to be initiated near the time of norepinephrine addition. The rate of glycogenolysis was approximately 1 nmole/min/mg of proten during the first 5 min of the experiment, and after 15 min the rate of fall in glycogen level was reduced to 0.4 nmole/min/mg of protein.

Measurement of the time course of the cAMP elevation in these experiments indicated that the cAMP had reached nearly a maximum level within 5 min and that it remained near this level for the remaining 25 min of the experiment (Fig. 2). The cAMP level seen after norepinephrine treatment in these experiments was approximately 1500 pmoles/mg of protein and was more typical of the results seen in this laboratory than was the 3500 pmoles/mg reported in Table 1. Control cAMP levels remained between 5 and 10 pmoles/mg of protein.

Glycogen phosphorylase. The mechanism by which glycogenolysis was activated in the above experiments was examined. First, the forms of phosphorylase present under control and hormone-treated conditions were characterized. Data in Table 3 indicate that prior to treatment with norepinephrine the enzyme activity was stimulated 6-fold by 5'-AMP. Cells which were incubated with norepinephrine for 5 min contained increased amounts of 5'-AMP-independent phosphorylase activity; yet the enzyme was stimulated 30% by this nucleotide. These data suggest that under control conditions

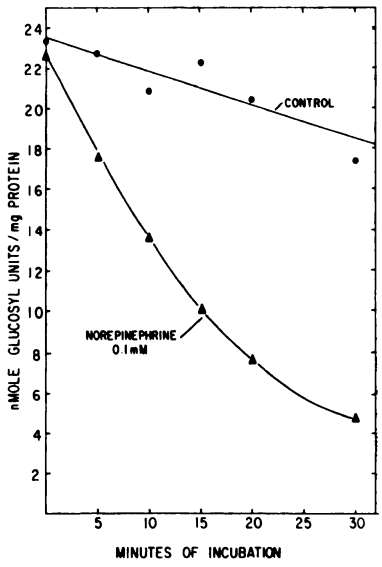

FIG. 1. *Effect of norepinephrine on glycogen content of C-6 astrocytoma cells*

Incubation conditions were the same as in Table 1, except that cells were incubated for 10 min prior to norepinephrine addition and incubations were terminated at the times indicated by transferring the coverslips to 5% trichloracetic acid at 0°.

phosphorylase *b* predominated in C-6 cells and that upon treatment with norepinephrine the enzyme was converted to phosphorylase *a*. The amount of enzyme activity present under assay conditions was several fold in excess of that needed to account for the rate of glycogenolysis in the previous experiments (Table 1 and Fig. 1). The small amount of apparent 5'-AMP-independent phosphorylase activity in the

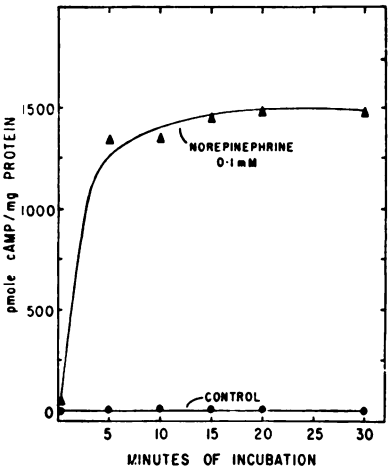

FIG. 2. *Effect of norepinephrine on cyclic AMP level in C-6 astrocytoma cells*

Data were derived from the trichloracetic acid extracts of the same experiments presented in Fig. 1.

TABLE 3

Glycogen phosphorylase activity of C-6 astrocytoma cells

The experiment was conducted as described in Table 1, except that cells were incubated for 10 min prior to norepinephrine addition and the incubation was terminated by freezing the coverslips in liquid nitrogen. Thawed, resuspended cells (see MATERIALS AND METHODS) were transferred to a Teflon-glass homogenizer, and cells remaining in the resuspension beaker were washed into the homogenizer with 0.5 ml of resuspension buffer. Cells were homogenized by hand, and 50 or 100 μl of homogenate were immediately analyzed for glycogen phosphorylase. Glycogen phosphorylase was assayed in the direction of glycogen breakdown as described in the text. Data represent the results of two experiments, and are presented as the means ± standard errors for four coverslip samples.

Conditions	Glycogen phosphorylase
	nmoles/min/mg protein
Untreated cells	1.4 ± 0.3
Untreated cells, 5′-AMP added to assay mixture	7.8 ± 0.3
Cells treated 5 min with norepinephrine	6.7 ± 0.4
Cells treated 5 min with norepinephrine; 5′-AMP added to assay mixture	8.8 ± 1.3

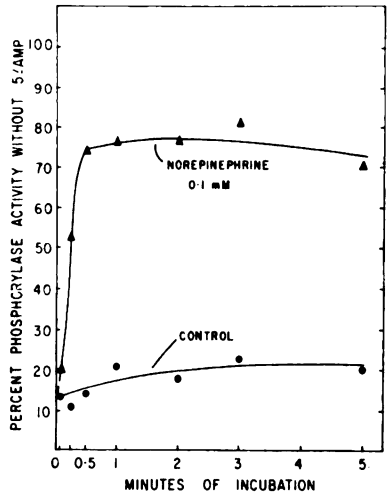

FIG. 3. *Effect of norepinephrine on phosphorylase a activity of C-6 astrocytoma cells*

Incubation conditions were the same as presented in Table 1, except that these cells were incubated for 10–15 min prior to norepinephrine addition and incubations were terminated by freezing the coverslips in liquid nitrogen. The data result from two experiments, each of which included duplicate incubation samples.

cells under control conditions may be accounted for in part by a small amount of phosphorylase *a* in the cells under control conditions and in part by 5′-AMP present in the tissue extracts and assay reagents (see MATERIALS AND METHODS).

The time course of phosphorylase interconversion after norepinephrine addition was determined (Fig. 3). The percentage of total phosphorylase activity present in the absence of 5′-AMP was used as an index of phosphorylase *a*. At 5 sec after norepinephrine addition, mean activity was slightly elevated. After 15 sec of incubation phosphorylase was more than half-converted to the *a* form, and by 30 sec the conversion was essentially complete. The enzyme remained in the *a* form for the remaining 4.5 min of the experiment.

The conversion of phosphorylase *b* to *a* within this short time period demonstrates that the large elevations of cAMP seen after several minutes of incubation with norepinephrine are not necessary for the full activation of glycogenolysis. It was not possible to measure cAMP concentrations

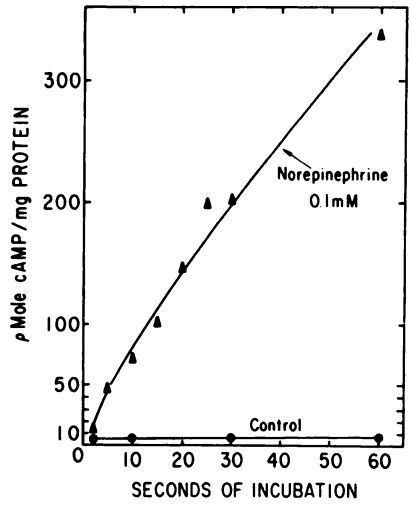

FIG. 4. *Effect of norepinephrine on cAMP content of C-6 astrocytoma cells*

Incubation conditions were as described in Table 1, except that these cells were incubated for 10–15 min prior to norepinephrine addition and incubations were terminated by freezing coverslips in liquid nitrogen. Frozen slips were later placed in 1 ml of 5% trichloracetic acid and extracted as described under MATERIALS AND METHODS.

in the above experiments, in which phosphorylase was assayed, since the extraction procedure for phosphorylase assay did not provide a suitable sample for cAMP estimation by the binding assay. Data from a separate experiment show the increase of cAMP during the first minute after norepinephrine addition (Fig. 4). After 5 sec of incubation with norepinephrine, the point at which phosphorylase activation had been initiated, the cAMP content of the cells was elevated from 4 to 46 pmoles/mg of protein. Therefore a concentration of cAMP far below the maximum concentration exhibited by these cells is sufficient to initiate phosphorylase activation. By 30 sec in the presence of norepinephrine, when phosphorylase was fully activated, the cAMP content was 200 pmoles/mg of protein, an amount which is only 13% of the 1500 pmoles/mg that is typically produced by norepinephrine treatment. Therefore the large elevations of cAMP obtainable in these cells upon longer incubation are not required to activate the glycogen phosphorylase system fully.

Successive exposure of cells to norepinephrine. The above experiments described first exposures of C-6 cells to norepinephrine for time periods up to 30 min. An additional group of experiments was performed to describe the effect of prior hormone treatment on subsequent treatments. C-6 cells were incubated in the presence or absence of norepinephrine for 3 hr, washed free of hormone, incubated for 1 hr without norepinephrine, and then incubated again with or without norepinephrine for 10 min. Ten minutes was chosen for the second treatment period to allow maximum elevation of cAMP as established in earlier experiments and to permit a moderate amount of glycogenolysis to occur. Increases or decreases in the amount of glycogen breakdown would therefore be detectable. Additional groups of samples were fixed after the 3-hr and 1-hr incubations to assess the cAMP and glycogen contents of the cells at these steps in the experiment.

Samples that were fixed at the end of the 3-hr incubation in the absence of norepinephrine contained amounts of glycogen consistent with earlier experiments, whereas the glycogen content of norepinephrine-treated samples was depressed by 30% at this time (Table 4, A). Earlier experiments had shown that after 20–30 min of treatment with norepinephrine the cells were largely depleted of glycogen (Table 1 and Fig. 1). It therefore appears that some compensatory mechanism had come into play which promoted regeneration of a substantial amount of glycogen by the end of 3 hr. Examination of the cAMP levels of the cells at the end of the 3-hr incubation period indicated that cAMP levels of untreated incubates remained low, and the cAMP levels of norepinephrine-treated samples were also relatively low (Table 4, A) compared with the greater elevations seen after a few minutes of hormone treatment (Fig. 2). The presence of norepinephrine in the incubation medium was established in a control experiment (data not shown), in which fresh coverslip samples were placed in a 3-hr incubation fluid for 10 min and the fluid caused the usual elevation of cAMP.

Cell samples incubated for 3 hr with norepinephrine, washed, and incubated again

TABLE 4

Effect of previous norepinephrine treatment on response of C-6 astrocytoma cells to norepinephrine

Coverslip samples of cells were incubated in Ham's F-10 medium according to the schedule outlined below. Incubation was terminated at the appropriate point by placing the coverslip in 5% trichloracetic acid at 0°. Data represent means ± standard errors for the number of coverslip samples shown in parentheses.

	Incubation procedure			cAMP	Glycogen
	3-hr incubation with (+) or without (−) norepinephrine	1-hr incubation without norepinephrine	10-min incubation with (+) or without (−) norepinephrine		
				pmoles/mg protein	*nmoles/mg protein*
A	−	0	0	3.1 ± 0.4 (6)	49.1 ± 1 (6)
	+	0	0	24 ± 2 (6)	32.0 ± 1.5 (6)
B	−	+	0	8.4 ± 1.3 (6)	43.2 ± 2.9 (6)
	+	+	0	5.4 ± 2.2 (3)	47.6 ± 3.2 (6)
C	−	+	−	7.4 ± 1.2 (5)	51.5 ± 4.7 (6)
	−	+	+	1300 ± 110 (6)	35.9 ± 3.7 (6)
D	+	+	−	6.3 ± 1.4 (3)	50.0 ± 2.2 (6)
	+	+	+	25 ± 3.7 (6)	36.2 ± 0.7 (6)

for 1 hr without hormone contained glycogen and cAMP levels which were not distinguishable statistically from similarly treated control samples (Table 4, B).

Cells which did not receive norepinephrine during the 3-hr incubation responded to the 10-min hormone treatment by accumulating cAMP to 1300 pmoles/mg of protein and by degrading glycogen to the extent of 15 nmoles/mg of protein (Table 4, C).

Cells that had previously been treated with norepinephrine responded to the second exposure by degrading nearly the same amount of glycogen, 14 nmoles/mg of protein, but their cAMP content was elevated only slightly, from 6 to 25 pmoles/mg of protein (Table 4, D). Interestingly, the cAMP elevation to 25 pmoles/mg of protein is nearly identical with the 24 pmoles of cAMP per milligram of protein present at the end of the 3-hr norepinephrine treatment. The conclusion is drawn that the first norepinephrine treatments of these cells altered the cells in a way which prevented a large increase in cAMP upon exposure to the hormone 1 hr later. A relationship of this observation to the depressed cAMP levels at the end of the 3-hr norepinephrine treatment period is suggested.

The state of the phosphorylase system at the end of the 3-hr norepinephrine treatment, at the end of the 1-hr recovery period, and after a second norepinephrine treatment was determined in separate experiments. Assay of the phosphorylase activity of cells incubated for 3 hr in the presence or absence of norepinephrine indicated that phosphorylase had returned almost entirely to the *b* form after 3 hr of hormone exposure (Table 5), despite the small elevation of cAMP seen in Table 4, A. The phosphorylase *a* content of cells incubated without norepinephrine for 3 hr was lower than that seen in earlier, shorter incubations. If cells were washed after the 3-hr incubation and incubated for an additional 1 hr (Table 5), the phosphorylase activity was unchanged compared to the 3-hr samples.

Cells which were re-exposed to norepinephrine partially converted phosphorylase to the *a* form (Fig. 5). Initiation of conversion was apparent by 10 sec after norepinephrine addition; maximum conversion was achieved after 2 min in the presence of

TABLE 5

Effect of previous norepinephrine treatment on state of activation of glycogen phosphorylase in C-6 astrocytoma cells

Cells were incubated as indicated in Table 4, except that incubations were terminated by freezing the coverslip samples in liquid nitrogen. After resuspension (see MATERIALS AND METHODS) cells were transferred to a 1.5-ml Eppendorf plastic centrifuge tube and dusrupted by sonication for 10 sec at minimum power, using a Heat Systems-Ultrasonics, Inc., model W185D Sonifier. Phosphorylase was determined immediately thereafter. Data represent the means of duplicate incubation samples from one typical experiment.

Incubation procedure		Phosphorylase activity		
3-hr incubation with (+) or without (−) norepinephrine	1-hr incubation without norepinephrine	−5′-AMP	+5′-AMP	Activity without 5′-AMP
		nmoles/min/mg protein		*% total*
−	0	0.08	6.6	1.2
+	0	0.09	6.2	1.6
−	+	0.08	6.4	1.3
+	+	0.12	6.9	1.7

hormone. The maximum was only 55% of total phosphorylase activity, compared to 75% achieved under other experimental conditions (Fig. 3).

Recently reported experiments demonstrate that several repeated treatments of C-6 cells with norepinephrine produced progressively smaller increases in the cAMP level and showed that inhibitors of cAMP phosphodiesterase caused the cAMP increase to remain larger upon repeated treatment with hormone (14). Therefore the effect of an inhibitor of cAMP phosphodiesterase, MIX, was tested for its effects on cAMP levels and glycogen under the conditions described in the previous experiment. If cells that had been incubated without norepinephrine were incubated in MIX for 10 min, the cAMP level rose 2-fold and significant glycogenolysis occurred (Table 6, B). Cells incubated for 10 min with norepinephrine exhibited the usual elevation of cAMP, and a reduction in glycogen content occurred which was equal to that seen with MIX. In cells which had been incubated for 3 hr with norepinephrine, MIX caused a 4-fold rise in cAMP; norepinephrine caused a 6-fold rise; and the two materials together caused a 100-fold rise in cAMP during the subsequent 10-min incubation. Therefore the *beta* adrenergic receptor of the cells remains at least partially active. MIX caused a reduction in glycogen content greater than that seen with norepinephrine, and the two agents together caused a reduction in glycogen which was no greater than that seen with MIX alone. Analysis of the 5′-AMP content of these cells (data not shown) demonstrated no increase in this nucleotide after treatment with MIX.

DISCUSSION

Experiments in the present paper extend previous observations on norepinephrine-initiated phenomena in C-6 astrocytoma cells (3, 14). In the absence of norepinephrine cAMP levels were low and remain so on prolonged incubation. Upon addition of the neurohormone to the cells, cAMP rose to 200 times the basal concentration within 5 min, and remained at this concentration for 30 min. Glycogen phosphorylase became

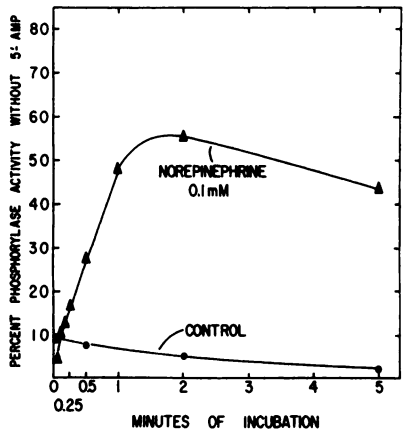

FIG. 5. *Effect of norepinephrine on phosphorylase a activity of C-6 astrocytoma cells previously treated with norepinephrine*

All cells were incubated for 3 hr with norepinephrine, followed by 1 hr without hormone as described in Table 4 and the text. Coverslip samples were then incubated with or without norepinephrine as indicated and frozen in liquid nitrogen at the appropriate times.

TABLE 6

Effect of previous norepinephrine treatment on response of C-6 astrocytoma cells to incubation with norepinephrine and 1-methyl-3-isobutylxanthine

Coverslip samples of cells were incubated according to the schedule outlined below. Because of the low solubility of MIX, this inhibitor was dissolved in Ham's F-10 medium and the 10 min incubation was initiated by transferring coverslip samples into fresh medium containing the additions indicated. Incubation was terminated at the appropriate point by placing the cover slip in 5% trichloracetic acid at 0°. Values represent means ± standard errors for the number of incubation samples shown in parentheses.

	Incubation conditions				cAMP	Glycogen
	3-hr incubation with (+) or without (−) norepinephrine	1-hr incubation without norepinephrine	10-min incubation with (+) or without (−) drug			
			Norepinephrine	MIX		
					pmoles/mg protein	*nmoles/mg protein*
A	+	+	0	0	3.4 ± 0.4 (6)	45 ± 5 (9)
B	−	+	−	−	4.1 ± 1.0 (6)	43 ± 5 (9)
	−	+	−	+	9.1 ± 1.2 (6)	26 ± 3 (9)
	−	+	+	−	1300 ± 97 (6)	26 ± 3 (9)
C	+	+	−	−	4.0 ± 0.8 (7)	43 ± 2 (10)
	+	+	−	+	15 ± 2.1 (5)	22 ± 3 (8)
	+	+	+	−	24 ± 3.7 (5)	35 ± 4 (8)
	+	+	+	+	410 ± 28 (7)	19 ± 2 (9)

fully converted to the *a* form within 30 sec after norepinephrine addition, long before cAMP reached its maximum concentration, in agreement with cAMP-activated phenomena in other systems (15).

Treatment of C-6 cells with norepinephrine for 3 hr caused cAMP to return nearly to control levels. That this result was not due to disappearance of norepinephrine during the incubation was revealed by placing a fresh coverslip sample of cells in the 3-hr incubation fluid and demonstrating a typical cAMP increase in the second sample. It would appear that some compensatory mechanism of these cells led to the return of their cAMP content to a low value. The nature of this mechanism remains to be determined. When cells treated for 3 hr with norepinephrine were washed, incubated for 1 hr without hormone, and again exposed to norepinephrine for 10 min, they increased cAMP only to the concentrations prevailing at the end of 3 hr in the presence of norepinephrine. Other cell samples which had been incubated without norepinephrine during the 3-hr period responded to the 10-min hormone exposure by increasing cAMP 180-fold. The suppression of the cAMP elevation due to norepinephrine, described above, is in agreement with results reported by Schultz, Hamprecht, and Daly (14) who showed that upon repeated 12-min treatments of C-6 cells with norepinephrine, followed by 15-min recovery periods, the cells responded by elevating their cAMP levels by progressively smaller amounts. In similar experiments these workers added papaverine, an inhibitor of cAMP phosphodiesterase, along with norepinephrine, and observed that the effect of norepinephrine on cAMP was better retained during repeated treatment (14). The same effect was observed here, using MIX as an inhibitor of the phosphodiesterase (Table 6). Papaverine produced a similar effect in our experiments but was found to inhibit respiration in the cells, as described in a following paper (16).

Other cellular systems respond to hormones with an initial increase of cAMP content, followed by a return toward the

normal value. Brain slices yield such results (17), as do adipose cells (18). The decrease in cAMP levels might be due to a decreased rate of cAMP synthesis or to an increased rate of cAMP destruction.

Previous results showed that C-6 cells grown for 40 hr in N^6-2′-*O*-dibutyryladenosine 3′,5′-monophosphate contain increased cAMP phosphodiesterase activity (19). Whether a similar increase in phosphodiesterase activity accounts for the present observation remains to be determined. A phenomenon of tachyphylaxis, whereby the *beta* adrenergic receptor has been "desensitized," has recently been described (20).

The additional observation that treating cells with norepinephrine a second time caused cAMP to rise to almost exactly the same value seen at the end of 3 hr in the hormone (Table 4) suggests that the adaptive phenomenon associated with return of the cAMP content toward normal had been completed after 3 hr in norepinephrine or that the adaptive process required the continual presence of the hormone and/or elevated cAMP.

Norepinephrine promoted glycogenolysis in the C-6 cells on first hormone treatments (Table 1) and upon repeated hormone treatments as well (Tables 4 and 6). The phosphorylase was rapidly converted to the *a* form, and glycogenolysis ensued. Phosphorylase kinase of skeletal muscle is activated at physiological pH by phosphorylation (21–25) and requires Ca^{++} (26). The enzyme from brain appears to have similar properties generally (27) and requires Ca^{++}.[3] Phosphorylation of phosphorylase kinase might be accomplished by a cAMP-stimulated protein kinase which has been reported to occur in these cells (28). The cytosolic Ca^{++} concentration of diverse cell types is normally low under basal conditions (29, 30). Should Ca^{++} be required for activity of phosphorylase kinase in C-6 cells and should the basal cytosolic Ca^{++} level be low as elsewhere, Ca^{++} might arise from extracellular fluid, endoplasmic reticulum, mitochondria, or membrane binding sites to stimulate phosphorylase kinase. In skeletal muscle the sarcoplasmic reticulum is the source of cytosolic Ca^{++} during muscle contraction (30), whereas in neurons Ca^{++} needed for neurotransmitter release arises outside the nerve endings (31).

Cells that had been treated with norepinephrine degraded glycogen to the same degree as cells that had been similarly incubated but without the hormone (Table 4). The cAMP in the norepinephrine-treated cells rose only to a level equal to that present at the end of the 3-hr hormone treatment. Phosphorylase was almost entirely in the *b* form (Table 5) at the end of the 3-hr treatment, but phosphorylase *a* formation occurred upon the second addition of norepinephrine. It would appear that factors other than the cAMP content of the cells per se control glycogenolysis in norepinephrine-treated cells. Ca^{++} has been proposed as a modulator of the phosphorylase kinase reaction (26, 32) and may be the other determinant of phosphorylase *b* to *a* conversion. The dissociation of phosphorylase activation from changes in cAMP level is in agreement with previous work in skeletal muscle (33, 34), in heart (35), and in brain slices (36).

The cellular contents of phosphorylase *a* and glycogen have been observed to fluctuate among experiments and within experiments in the absence of norepinephrine. The phosphorylase *a* activity in the absence of norepinephrine varied between 1 and 20% of total activity (Figs. 3 and 5; Table 5). Glycogen varied between 20 and 65 nmoles/mg of protein (Fig. 1; Tables 1, 4, and 6). In preliminary experiments rapid, transient changes in glycogen occurred following removal of coverslip samples of cells from growth medium, washing, and placing the cells in fresh Ham's F-10 medium. Consideration of the multiplicity of factors which govern the enzymes of glycogen metabolism in more widely studied cell types suggests that these fluctuations are not unlikely, but makes them difficult to interpret. That one or more factors other than the activity of the *beta* adrenergic receptor are involved with control of glycogen metabolism in the present cell types is emphasized by these results.

[3] C. Brostrom, personal communication.

The amount of phosphorylase *a* in C-6 cells after norepinephrine treatment was several fold in excess of that needed to account for the observed rate of glycogenolysis were the enzyme operating at maximum velocity. Differences in experimental conditions between the phosphorylase assay and the cellular incubations may account for part of the difference, but that this is not the entire explanation is indicated by the fact that papaverine causes glycogenolysis to occur at a 2–4-fold greater rate than was seen with norepinephrine (16). Thus some feature of the cellular milieu other than the enzyme per se limits the glycogenolytic flux. Substrate glycogen and phosphate levels may be limiting, or the rate of glycogen resynthesis may be significant. The state of the glycogen-synthetic reaction in these cells is unknown. However, glycogen synthetase is controlled by a phosphorylation-dephosphorylation mechanism which is complementary to the control of glycogen phosphorylase in most cells (37), and the rapid, complete activation of phosphorylase might suggest a complementary deactivation of the synthetase. The glycogen content of the present cells was similar to that found in experiments in which papaverine caused a faster rate of glycogenolysis, so that a difference in glycogen content does not account for the difference in flux. Inorganic phosphate concentration may differ in the two experimental situations. Under basal conditions high-energy phosphate stores are high in the cells and remain so after norepinephrine addition, whereas during incubation with papaverine the creatine phosphate and ATP levels are substantially decreased. It would seem likely that the concentration of inorganic phosphate is elevated in the papaverine incubation, accounting for the increased glycogenolytic flux in those cells, and accordingly that inorganic phosphate limits the rate of glycogenolysis in the norepinephrine-treated cells.

REFERENCES

1. Cicero, T. J., Cowan, W. M., Moore, B. W. & Suntzeff, V. (1970) *Brain Res.*, **18**, 25–34.
2. Perez, V. J., Olney, J. W., Cicero, T. J., Moore, B. W. & Bahn, B. A. (1970) *J. Neurochem.*, **17**, 511–519.
3. Gilman, A. G. & Nirenberg, M. (1971) *Proc. Natl. Acad. Sci. U. S. A.*, **68**, 2165–2168.
4. Eccles, J. C., Ito, M. & Szentagothai, J. (1967) *The Cerebellum as a Neuronal Machine*, p. 202, Springer, New York.
5. Bloom, F. E., Hoffer, B. J. & Siggins, G. R. (1971) *Brain Res.*, **25**, 501–521.
6. Wicks, W. D. (1971) *Ann. N. Y. Acad. Sci.*, **185**, 152–165.
7. Paul, J. (1970) *Cell and Tissue Culture*, Ed. 4, p. 218, Williams & Wilkins, Baltimore.
8. Gilman, A. G. (1970) *Proc. Natl. Acad. Sci. U. S. A.* **67**, 305–312.
9. Williamson, J. R. & Corkey, B. E. (1969) *Methods Enzymol.* **13**, 434–513.
10. Lowry, O. H., Passonneau, J. V., Hasselberger, F. X. & Schulz, D. W. (1964) *J. Biol. Chem.* **239**, 18–30.
11. Passonneau, J. V., Gatfield, P. D., Schulz, D. W. & Lowry, O. H. (1967) *Anal. Biochem.*, **19**, 315–326.
12. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265–275.
13. Brostrom, C. O. & Kon, C. (1974) *Anal. Biochem.*, in press.
14. Schultz, J., Hamprecht, B. & Daly, J. W. (1972) *Proc. Natl. Acad. Sci. U. S. A.*, **69**, 1266–1270.
15. Robison, G. A., Butcher, R. W. & Sutherland, E. W. (1971) *Cyclic AMP*, p. 31, Academic Press, New York.
16. Browning, E. T., Groppi, V. E., Jr. & Kon, C. (1974) *Mol. Pharmacol.*, **10**, 175–181.
17. Kakiuchi, S. & Rall, T. W (1968) *Mol. Pharmacol.*, **4**, 367–378.
18. Kuo, J. F. & De Renzo, E. C. (1969) *J. Biol. Chem.*, **244**, 2252–2260.
19. Schwartz, J. P., Morris, N. R. & Breckenridge, B. McL. (1973) *J. Biol. Chem.*, **248**, 2699–2704.
20. Fleisch, J. H. & Titus, E. (1972) *J. Pharmacol. Exp. Ther.*, **181**, 425–433.
21. De Lange, R. J., Kemp, R. G., Riley, W. D., Cooper, R. A. & Krebs, E. G. (1968) *J. Biol. Chem.*, **243**, 2200–2208.
22. Riley, W. D., De Lange, R. J., Bratvold, G. E. & Krebs, E. G. (1968) *J. Biol. Chem.*, **243**, 2209–2215.
23. Walsh, D. A., Perkins, J. P. & Krebs, E. G. (1968) *J. Biol. Chem.*, **243**, 3763–3765.
24. Mayer, S. E. & Krebs, E. G. (1970) *J. Biol. Chem.*, **245**, 3153–3160.
25. Walsh, D. A., Perkins, J. P., Brostrom, C. O., Ho, E. S. & Krebs, E. G. (1971) *J. Biol. Chem.*, **246**, 1968–1976.
26. Brostrom, C. O., Hunkeler, F. L. & Krebs, E. G. (1971) *J. Biol. Chem.*, **246**, 1961–1967.

27. Drummond, G. I. & Bellward, G. (1970) *J. Neurochem.*, **17**, 475–482.
28. Opler, L. A. & Makman, M. H. (1972) *Biochem. Biophys. Res. Commun.*, **46**, 1140–1145.
29. Bianchi, C. P. (1968) *Cell Calcium*, Appleton-Century-Crofts, New York.
30. Ebashi, S. & Endo, M. (1968) *Prog. Biophys. Mol. Biol.*, **18**, 123–183.
31. Katz, B. & Miledi, R. (1965) *Proc. R. Soc. Lond., Ser. B., Biol. Sci.*, **161**, 496–503.
32. Meyer, W. L., Fischer, E. H. & Krebs, E. G. (1964) *Biochemistry*, **3**, 1033–1039.
33. Posner, J. B., Stern, R. & Krebs, E. G. (1965) *J. Biol. Chem.*, **240**, 982–985.
34. Stull, J. T. & Mayer, S. E. (1971) *J. Biol. Chem.*, **246**, 5716–5723.
35. Namm, D. H., Mayer, S. E. & Maltbie, M. (1968) *Mol. Pharmacol.*, **4**, 522–530.
36. Kakiuchi, S. & Rall, T. W. (1968) *Mol. Pharmacol.*, **4**, 379–388.
37. Larner, J., Villar-Palasi, C., Goldberg, N. D., Bishop, J. S., Huijing, F., Wenger, J. I., Sasko, H. & Brown, N. B. (1968) *Adv. Enzyme Regul.*, **6**, 409–423.