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Regulation of Adenosine 3′:5′-Cyclic Monophosphate Concentration in Cultured Human Astrocytoma Cells by Catecholamines and Histamine

(growth cycle/propranolol/theophylline)

RICHARD B. CLARK AND JOHN P. PERKINS

Department of Pharmacology, University of Colorado Medical Center, Denver, Colo. 80220

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ABSTRACT **Norepinephrine, epinephrine, and histamine cause a rapid increase in the concentration of adenosine 3′:5′-cyclic monophosphate (cAMP) in a tumor astrocyte cell line derived from a primary culture of a human glioblastoma multiforme. The catecholamine-induced increase in cAMP is dependent on the cell density, being far greater in cells in the log phase of growth than in cells near terminal density. The response to norepinephrine is inhibited 50% by 0.01 μM propranolol, a blocking agent of β-adrenergic receptors. In contrast, the effect of histamine on cAMP concentration varies only slightly from log-phase growth to terminal density, and is not inhibited by 10 μM propranolol. The results suggest that astrocytoma cells have independent receptors for catecholamines and histamine. Further, if the astrocytoma cell is an adequate model of the normal glial cell, these results suggest that astrocytes in human cerebral cortex may be sensitive to norepinephrine and histamine.**

All of the known components of the cAMP regulatory system, adenylate cyclase (1), a cAMP-dependent protein kinase (2), and a cAMP-specific phosphodiesterase (3), are demonstrable in brain. Furthermore, the adenylate cyclase of brain generates cAMP in response to various effectors: e.g., putative neurotransmitters such as norepinephrine, histamine, and serotonin (4), depolarizing agents such as K^+ and veratridine (5), and the nucleoside, adenosine (6). However, elucidation of the exact role of cAMP has been hampered because of the anatomic and cellular complexity of brain. For example, there is great variation in the response of the adenylate cyclase system to these effectors within different brain regions (7), within the same region in different species (5), and at different stages of brain development (8). Further, since brain is composed of a number of cell types, primarily neurons and glia, measurements of enzyme activities in homogenates or of cAMP concentrations in slices will include contributions from both of these cell populations. To our knowledge, no report has appeared describing attempts to differentiate the response of the adenylate cyclase system in the two major cell types. Since neurons and glia probably have distinctly different functional capacities, an increase in the intracellular concentration of cAMP would presumably be mediated by cell-specific stimuli. In view of these considerations, studies with homogeneous cell populations of either neurons or glia should reveal whether one cell type or both are responsive to the various effectors, and ultimately should provide a rational approach to the determination of the role of cAMP in brain function. For this reason, we are characterizing the cAMP regulatory system of a human astrocytoma cell line, 1181N1, maintained in continuous culture. Adenylate cyclase, cAMP phosphodiesterase, and cAMP-dependent protein kinase are present (9), and the growth pattern and morphology of the cells is altered by N^6, $O^{2'}$-dibutyryl cAMP (10). Here, we report the effects of catecholamines and histamine on the intracellular concentrations of cAMP. A preliminary report of some of the results of this investigation has appeared (11).

MATERIALS AND METHODS

The cloned tumor astrocyte line, 1181N1, was originally derived from a human cerebral glioblastoma multiforme (12). The cells are grown in Eagle's minimal essential medium–Earle's basic salt solution with glutamine (Schwarz–Mann) supplemented with 10% fetal-calf serum (Microbiological Associates Rockville, Md.), 100 μg/ml of streptomycin sulfate, and 150 units/ml of penicillin G, under an atmosphere of CO_2–air 5:95 at 37°C. Unless otherwise indicated, Falcon plastic Petri-dishes of 60-mm diameter were innoculated with about 4×10^5 cells and grown 12–14 days to multilayer density (about 6×10^6 cells per dish). Fresh medium was added twice a week; experiments were performed 4 days after the last addition of medium except in later experiments (as indicated). Immediately before an experiment, the growth medium was removed by aspiration and the attached cells were gently washed twice, then incubated at 37°C in growth medium minus serum and antibiotics.

Norepinephrine(L-arterenol-D-bitartrate, Schwarz–Mann), histamine·HCl (Sigma), and propranolol (Ayerst Laboratories) were dissolved in 0.01 N HCl and diluted 100-fold into the incubation media. After the appropriate incubation period, the medium was removed by aspiration and 1 ml of hot (75°C) 5% Cl_3CCOOH was added. The suspension was transferred to glass vessels and immediately homogenized. An aliquot was removed for protein determination by the biuret method, and the chilled suspension was then centrifuged at 17,000 × *g* for 10 min. The supernatant fraction was acidified by the addition of 0.1 ml of 1.0 N HCl/ml of the supernatant fraction, extracted five times with water-saturated ether, lyophilized, resuspended in 50 mM sodium acetate (pH 4.0), and frozen. The cAMP in samples prepared and stored in this way was stable for at least 1 month. The recovery of cAMP through the extraction procedure was evaluated by addition of [^{3}H]cAMP to the Petri dish immediately after the incubation medium was removed, and just before the addition of Cl_3CCOOH. Recovery was 90–100%. Values reported are not corrected for any loss of cAMP.

Cyclic AMP was measured by the direct binding, isotope dilution method of Gilman (13). On selected samples, the specificity of the assay for cAMP was tested by incubation of the sample, containing added [^{3}H]cAMP, with purified

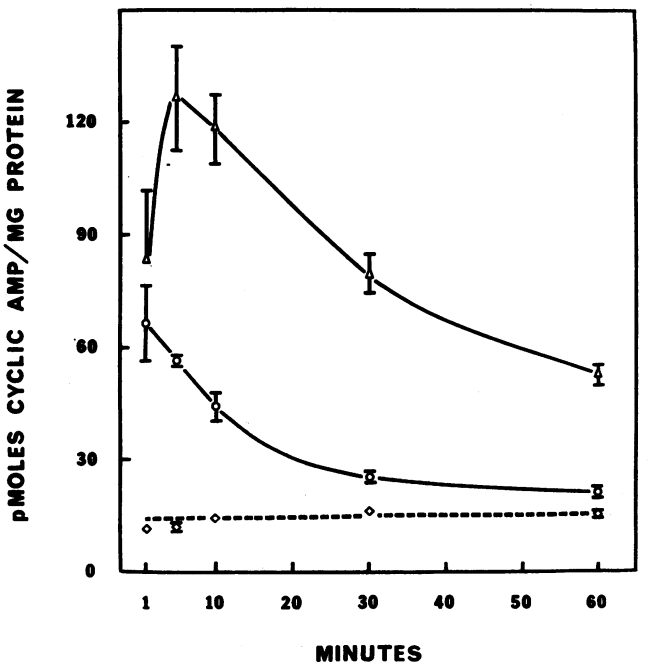

FIG. 1. Rate of increase in cAMP concentration in cultured astrocytoma cells. Cells were incubated with 30 μM norepinephrine, O–O; 30 μM histamine, Δ–Δ; or with no additions, ◇–◇. Values for cAMP are given as the mean ±SE, $n \geq 3$. Control values that have no SE are the average of two determinations.

beef-heart phosphodiesterase (3) for different time intervals. This treatment was found to reduce assayable cAMP in the sample at the same rate as [^{3}H]cAMP was destroyed.

RESULTS

The rate of change in cellular concentrations of cAMP in response to the addition of 30 μM norepinephrine or histamine is shown in Fig. 1. In each case, the generation of cAMP was rapid, and appeared to be maximally elevated by 1 min with norepinephrine and by 5 min with histamine. With stimulation by norepinephrine the cAMP concentrations decreased to near basal concentrations by 60 min, while with histamine the levels at 60 min were still four times the basal concentrations. The concentration of cyclic AMP in control cells (no amine added) did not change significantly during the 60-min incubation period. In 30 different measurements of control concentrations of cAMP from 12 separate experiments with cells at multilayer density, the mean value was 11.5 pmole per mg of protein, with a standard error of ±0.9. The range of cAMP concentrations in these experiments was 4–22 pmol cAMP per mg of protein.

A complete explanation for the reduction of the elevated concentrations of cAMP elicited by norepinephrine is not apparent. However, the following experiment suggests that this reduction is not simply a result of destruction of norepinephrine. Astrocytoma cells were incubated with 30 μM norepinephrine for 60 min, after which time the medium was removed and either fresh norepinephrine or histamine was added for a further 5-min incubation. The readdition of norepinephrine resulted in no change of the cAMP concentrations, whereas histamine produced a 3-fold rise.

The effect of various concentrations of norepinephrine and histamine on cAMP concentrations is shown in Fig. 2. Determinations were made after a 5-min incubation and represent the optimal time for histamine and a near-optimal time for norepinephrine. Under these experimental conditions, 0.3 mM norepinephrine produced an 8-fold rise in cAMP concentration, and histamine produced a more than 20-fold increase. The concentration of amine causing a half-maximal response was 6 μM for norepinephrine and 9 μM for histamine. Significant increases in cAMP were observed with either amine at 1 μM.

Experiments such as those shown in Figs. 1 and 2 and Table 2, were performed with cells at a high density that had not undergone a medium change for 96 hr. When experiments were performed with cells not yet at saturation density, the magnitude of the norepinephrine and histamine effects were considerably altered. The experiments shown in Table 1 illustrate the variation in the rise of cAMP concentrations in response to norepinephrine, epinephrine and histamine at different cell densities and in different nutritional states. 30 μM norepinephrine caused a 150-fold rise in the cAMP concentration in cells growing logarithmically, a 40-fold rise in cells in a confluent monolayer, and a 10-fold rise in cells that had grown to the multilayer stage. In the two nutritional states represented in the 11-day experiments, the percentage increase in cAMP concentration in response to norepinephrine was similar; however, both the control concentrations of cAMP and the total cAMP concentration

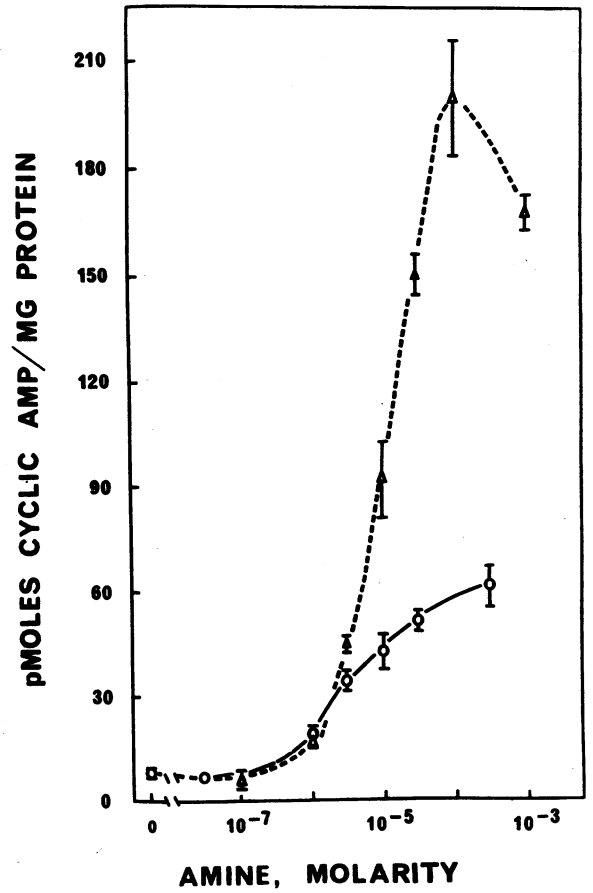

FIG. 2. Increase in cAMP concentrations of cultured astrocytoma cells. Cells were incubated 5 min with various concentrations of norepinephrine, O–O; or histamine, Δ–Δ. Values for cAMP are given as the mean ±SE ($n \geq 3$).

TABLE 1. *Cyclic AMP response to catecholamines and histamine as a function of cell density*

Days of cell growth	Number of cells	Total mg of protein	pmol of cAMP per mg of protein*			
			Controls	Norepinephrine	Epinephrine	Histamine
5†	1.6×10^6	0.31 ± 0.01‡	18.2 ± 0.79	2682 ± 269	2089	64.9 ± 5.5
		(10)	(3)	(3)	(1)	(3)
8*	2.9×10^6	0.82 ± 0.024	14.4 ± 1.3	609 ± 49	860	65.0 ± 6.9
		(10)	(3)	(3)	(1)	(3)
11*	4.8×10^6	1.47 ± 0.032	24.4 ± 3.2	240 ± 20	341	83.2 ± 7.6
		(10)	(3)	(3)	(1)	(3)
11§	3.7×10^6	1.24 ± 0.030	12.0 ± 0.95	129 ± 21	148	83.5 ± 10.0
		(10)	(3)	(3)	(1)	(3)

* Norepinephrine and histamine were 30 μM. Epinephrine was 40 μM. All incubations were 5 min.

† Cells were inoculated at day 1 with 9×10^5 cells/plate; medium was changed at days 4, 7, and 10, and 4 hr before the experiment.

‡ Values throughout the table are $\pm$SE, with n given in parentheses.

§ Cells of this group were identical to the other 11-day group, except that the medium was not changed after day 7 or before the experiment on day 11.

generated by norepinephrine in the cells exposed to fresh medium were twice the concentrations in the deprived cells. Epinephrine produced responses similar to norepinephrine.

In contrast to the effects of norepinephrine, the response to histamine showed almost no variation in the cells at 5, 8, and 11 days; in the cells deprived of fresh medium, histamine caused a greater percentage increase in the cAMP concentration.

The variation of the response of the adenylate cyclase system to catecholamines and histamine as a function of cell density suggests that the receptors for norepinephrine and histamine are independent. In a further attempt to characterize these receptors, the effects of the β-adrenergic blocking agent, propranolol, were examined (Table 2). 0.01 μM propranolol caused a 50% inhibition of the increase caused by 30 μM norepinephrine, while the increase elicited by 30 μM histamine was not significantly inhibited by 10 μM propranolol. The blocking agent had no effect on basal concentrations when added alone, even at concentrations as high as 1 mM.

Methlyxanthines, inhibitors of cAMP-phosphodiesterase, can mimic the effects of hormones in certain tissues (14). However, 0.5 mM theophylline had no significant effect on cAMP concentrations in the 1181N1 astrocytes, nor did it change the response to 30 μM norepinephrine during a 5-min incubation.

DISCUSSION

The results reported here demonstrate that the intracellular concentration of cAMP in human astrocytoma cells undergoes a marked, rapid increase in the presence of norepinephrine, epinephrine, and histamine. It is now apparent that these tumor-derived astrocytes contain all of the known components of the cAMP regulatory system, i.e., an effector-responsive adenylate cyclase, a cAMP-phosphodiesterase (9), and a cAMP-dependent protein kinase (9). Certain characteristics of the adenylate cyclase system of the astrocytoma cells are similar to those of brain slices and of other cell types. These similarities are important to a consideration of the validity of the astrocytoma cells as a useful model for a study of the role of cAMP in glial function.

Upon addition of norepinephrine to the 1181N1 cells, the cAMP concentration rapidly rises, but the concentrations quickly decay to control values. The decay has been ascribed to a loss of responsiveness of the adrenergic receptor and a turnover of cAMP due to phosphodiesterase action. A similar phenomenon was observed by Kakiuchi and Rall (4), in that cAMP concentrations in brain slices rose upon the addition of either norepinephrine or histamine but subsequently fell. After prolonged incubation, addition of the other agonist, but not the same agonist, would evoke a second rise in the cAMP concentration. Thus, the fall in cAMP concentrations was not due simply to the destruction of the agonist, but more likely resulted from a loss of receptor sensitivity. Makman (15) demonstrated that incubation of mouse fibroblasts in the presence of epinephrine led to the

TABLE 2. *Inhibition by propranolol of norepinephrine- and histamine-induced increases in cAMP*

Agent	Propranolol (μM)	pmols of cAMP per mg of protein	Inhibition (%)
Expt. *1*			
Controls	None	15.7 ± 1.7 (3)*	—
Norepinephrine (30 μM)	None	72.6 ± 7.3 (3)	—
Norepinephrine (30 μM)	10	11.9, 12.1	100
Norepinephrine (30 μM)	1.0	11.1, 15.8	100
Norepinephrine (30 μM)	0.1	23.8, 35.3	76
Norpinephrine (30 μM)	0.01	41.8, 43.3	53
Expt. *2*			
Controls	None	14.6 ± 0.9 (8)	—
Histamine (30 μM)	None	102.6 ± 6.5 (5)	—
Histamine (30 μM)	10	97.5, 96.3	6

Incubation time was 5 min.

* Values are $\pm$SE where (n) was ≥ 3. Where only two values were obtained, they are both shown.

selective loss of adenylate cyclase sensitivity to epinephrine when enzyme activity was subsequently measured in cell homogenates. The effect was selective in the sense that basal or NaF-stimulated activity was not altered. Such observations suggest that the adenylate cyclase systems in brain, fibroblasts, and astrocytoma cells, when exposed to catecholamines for a sufficient period of time, become less responsive to the agonist. The decay of the system appears to be limited to the regulatory component and, at least in brain and the 1181N1 astrocytes, is receptor-specific, i.e., stimulation by an agonist that acts on another receptor will still elicit a rise in cAMP concentration.

Two lines of evidence suggest that the receptors for norepinephrine and histamine in the astrocytoma cells are different. First, the magnitude of the rise in cAMP elicited by norepinephrine is markedly different in the various stages of the growth cycle of the culture, while the effects of histamine are essentially the same throughout the cycle. Second, propranolol causes a selective inhibition of the effects of norepinephrine, even through the concentrations of norepinephrine and histamine causing a half-maximal effect are similar, i.e., 6 and 9 μM, respectively. The inhibitory effect of propranolol suggests that the catecholamine receptor is of the β-adrenergic class (16).

The lack of effect of theophylline in combination with submaximal concentrations of norepinephrine is somewhat unique. In most tissues that respond to catecholamines with a rise in cAMP concentration, the coaddition of theophylline potentiates the response. The notable exception to this generality is the response to norepinephrine of brain slices prepared at room temperature (17), wherein methylxanthines elicit little or no effect. Theophylline is a significantly less effective inhibitor *in vitro* of the cAMP-phosphodiesterase of the astrocytoma cells than of this enzyme activity of the rat cerebrum (9).

In summary, there are three observations that suggest a similarity between the adenylate cyclase system(s) of brain and of the astrocytoma cells. First, both astrocytoma cells and brain slices respond to histamine. Although many cell types respond to catecholamines with an increase in cAMP concentrations, only brain and heart (18) have been shown to respond to histamine. Second, the loss of responsiveness of the adrenergic receptor is somewhat unique and is comparable to observations in brain slices. Third, the lack of potentiation of the norepinephrine effect by theophylline is rare, but again comparable to observations made with brain slices. Such comparisons, although suggestive, are by no means proof of the validity of the use of the astrocytoma cell as a model for normal glia. In an attempt to further demonstrate the validity of this model, we are currently attempting to measure the effects of norepinephrine and histamine on various preparations of glia isolated from the brains of small laboratory animals.

It is not apparent what effects an increase in the concentration of cAMP in glial cells might have, since it is not at all clear what role the glia play in brain function. A possible effect of catecholamines on glial function is suggested by the report of DeVellis (19) that catecholamines induce lactate dehydrogenase (EC 1.1.1.27) activity in the C-6 rat glial cell tumor in culture, although the significance of an increase in this enzyme activity is not readily apparent. However, we can speculate that in glia, as in the target cells of hormones, cAMP serves as an intracellular mediator of an extracellular effector. The presence in glia of a norepinephrine-responsive adenylate cyclase suggests a mechanism whereby neurons might communicate with glia. Namely, if norepinephrine released from nerve endings led to elevated concentrations of cAMP in adjacent glia, then neuronal activity could cause an alteration in glial metabolism, perhaps directed towards some support function. Such an idea is speculative, but is similar in essence to a recent hypothesis of Kuffler and Nicholls (20), who found that impluse conduction down an axon released K^+ into the intercellular clefts to such an extent that the surrounding glia were depolarized. They felt that the degree of depolarization of the glia "could be a register of the amount of general, nonspecific activity in the neighborhood of a glial cell in the brain." They speculated further that K^+ could trigger a trophic response in the glia, and that a feedback to the neurons was an ultimate possibility. The possibility exists that both K^+ depolarization and neurotransmitter-specific effects could combine to evoke greater increases in the concentration of cAMP in glia. It has been shown by Huang *et al.* (21) that K^+ acts synergistically with norepinephrine and histamine to generate large increases in cAMP concentration in brain slices from the guinea pig.

Our results suggest that the function of astrocytes in brain may be influenced by neurotransmitter-substances, mediated by an increase in the intracellular concentration of cAMP.

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