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Adenosine Cyclic 3′,5′-Monophosphate in Fetal Rat Brain Cell Cultures

I. Effect of Catecholamines

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SUMMARY

The effects of various neurohormones and other chemical agents on intracellular levels of adenosine cyclic 3′,5′-monophosphate were evaluated in surface cultures of fetal rat brain cells. Treatment of cells with dopamine, prostaglandin $F_{2\alpha}$,5-hydroxytryptamine, or KCl was without effect on cyclic AMP levels. Increases were found following exposure to adenosine, prostaglandin E_1, norepinephrine, or isoproterenol. The catecholamines were considerably more effective than the other compounds, causing up to a 100-fold increase in cyclic AMP content. This maximal response was not affected by 1 mM theophylline, was blocked by the *beta* adrenergic antagonists sotalol and dichloroisoproterenol, and was maximal after 5–15 min of exposure of the cells to the catecholamine. The response to isoproterenol developed during culture; its magnitude increased to a maximum after about 14 days in culture.

INTRODUCTION

Adenosine cyclic 3′,5′-monophosphate plays a significant role in the regulation of differentiated functions of many tissues. Slice preparations from mammalian brain accumulate cyclic AMP intracellularly when treated with some putative neurotransmitters and other chemical agents (1, 2). Because of the extreme heterogeneity of brain as a tissue, it is difficult to correlate accumulations of the cyclic nucleotide with specialized activities of individual cell types. Recently cyclic AMP concentrations have been determined in clonal lines of mouse neuroblastoma (3) and rat and human glioma (4, 5) cells. In the glial cell lines, cyclic AMP levels found in response to treatment with norepinephrine and isoproterenol were much greater than those seen in brain slice preparations from the same species. This suggested that glia may be responsible for a large proportion of the accumulation of cyclic AMP stimulated by *beta* adrenergic effectors in brain, although tumor cells may not accurately reflect normal glial function.

It has been shown (6) that cells from normal brains of fetal mice in reaggregation cultures accumulate cyclic AMP in response to catecholamines, in a manner similar to

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brain slice preparations. Such cultures presumably contain relatively natural proportions of neuronal and glial cells, and multiplication of most or all cells is limited (7). In contrast, culture of brain cells on polystyrene surfaces results in a manyfold increase in cells (8, 9). In both systems the maintenance and/or development of certain biochemical and morphological characteristics has been demonstrated (6–11). Surface cultures of cells from normal mammalian brain could ultimately offer the potential for determining the cyclic AMP responses of individual cell types, as well as the molecular events prior and subsequent to these responses. As a first step toward these goals, this work presents data on the accumulation of cyclic AMP by cultures of unpurified cells from fetal rat brain in response to catecholamines and other agonists. These responses were qualitatively similar to those of rat brain slices (12), but the magnitude of the catecholamine response approached that in cultured glial tumor cells.

MATERIALS AND METHODS

Rat brain cell cultures. Whole brains of Fisher rat fetuses (3.1–4.8 g in body weight; 20–21 days of gestation) were obtained and dissociated to single cells by sieving, as previously described (9). The tissue was expressed through a single thickness of 210-μm-pore Nitex nylon mesh cloth (Tobler, Ernst and Traber, Elmsford, N. Y.) into isosmotic solution D (8). The resulting suspension of cells and cell clumps was then filtered by gravity flow through a single thickness of 130-μm-pore Nitex cloth. Cell number and viability were determined as described (8). Cells were inoculated into 60-mm-diameter (21 cm^2 surface area) Falcon polystyrene culture dishes, at the densities given for the individual experiments, with 3.5 ml/dish of 90% Dulbecco-Vogt modification of Eagle's medium (Grand Island Biological Corporation), 10% fetal bovine serum (Colorado Serum Company), 10 units/ml of sodium penicillin G, and 10 μg/ml of streptomycin sulfate. Dishes were incubated in an atmosphere of 10% CO_2–90% air at 37° and 98% humidity. Careful attention to inoculation procedures (13) resulted in ±10% reproducibility in protein content and choline acetyltransferase activity of replicate cultures. Medium changes were performed on the fourth day in culture, every second day thereafter, and 16–24 hr before each hormone incubation.

Hormone incubations. Dishes and their contents were washed twice with 5 ml of medium 1 (Dulbecco-Vogt minimal essential medium without serum, antibiotics, or glutamine) at room temperature. After removal of the second wash, 4 ml of medium 2 (medium 1 without $NaHCO_3$, but containing 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.2, with or without 1 mM theophylline) were added to each dish.

The dishes were incubated for 30 min in a covered 37° water bath. Hormones or other chemical agents were then added to the dishes. After a further 15-min incubation (except where indicated), the medium was removed and immediately replaced with 1.0 ml of 5% (w/v) trichloroacetic acid. This and a second 0.5–1.0-ml trichloroacetic acid wash were allowed to extract cyclic AMP and other soluble materials from the cells for a total of 15–30 min, and the trichloroacetic acid extracts were then assayed for cyclic AMP content as previously reported (14). Values obtained on replicate dishes were within 15% of the mean.

The material precipitated on the culture dishes was dissolved in 0.2 N NaOH and assayed for protein by the method of Lowry *et al.* (15).

When the effects of blocking agents were evaluated, the blocking agent was placed in the dish after the 30-min incubation with medium 2 and theophylline. After another 10 min of incubation, hormone addition and analysis proceeded as before.

Choline acetyltransferase activity in the cultures was determined by the method of Schrier and Shuster (16), as modified by Wilson *et al.* (8).

RESULTS

The effects of several neurohormones and other chemical agents on brain cells cultured for 28 days are shown in Table 1. The cyclic AMP levels were increased more than 50-fold after treatment with a high concentration of

TABLE 1

Effect of chemical agents on cyclic AMP levels of cultured fetal rat brain cells

Brain cells from 19–20-day-gestational (3.11-g) fetuses were plated at 1.5×10^6 viable cells/60-mm dish and grown for 28 days. Dishes were incubated without theophylline, and the cyclic AMP contents and protein were determined as detailed in the text. Each value represents the average of two dishes.

Hormone	Concentration	Cyclic AMP
	mM	*pmoles/mg protein*
None	0	7
l-Isoproterenol	0.1	407
Histamine	0.1	6
Adenosine	0.1	85
5-Hydroxytryptamine	0.1	5
KCl	40[a]	7
Prostaglandin E_1	0.003	211
Prostaglandin $F_{2\alpha}$	0.003	6

[a] Medium 2 (see MATERIALS AND METHODS) diluted to 260 mOsM with water and KCl added to 40 mM and 340 mOsM.

isoproterenol, 12-fold in response to adenosine, and nearly 30-fold after prostaglandin E_1 exposure. Histamine, KCl, serotonin, and prostaglandin $F_{2\alpha}$ were ineffective in this experiment, although histamine did appear to stimulate cyclic AMP accumulation slightly in another experiment performed at an earlier time in culture. The nature of the responses to adenosine and prostaglandin E_1, and the significance of the histamine response, will be evaluated in a subsequent report.

An evaluation of the effects of various concentrations of isoproterenol with and without theophylline (Fig. 1) showed that the *maximum* level of the response was unaffected by the presence of the cyclic nucleotide phosphodiesterase inhibitor. This situation is reminiscent of that in brain slices, where such effects of theophylline are not apparent, despite the high levels of phosphodiesterase in the organ (1). At low concentrations of isoproterenol (10–30 nM), theophylline potentiated the effect of the catecholamine 2-fold. The maximal concentration of cyclic AMP seen in this experiment was approximately 90 times that in control cells.

The response to isoproterenol was maximal after 5–15 min of exposure to the catecholamine in the presence of theophylline (Fig. 2), and the cyclic AMP concentration was still approximately 20 times the control levels after 60 min of incubation. It has not been determined whether theophylline prolongs the response to isoproterenol.

A comparison of the relative activities of dopamine, norepinephrine, and isoproterenol (Table 2) revealed that dopamine was essentially ineffective in raising cyclic AMP levels in this system. This result was in keeping with its effects reported to date in brain slices (1). While it was apparent that norepinephrine was very effective in stimulating cyclic AMP accumulation in this system, the level of cyclic AMP attained with 1 μM norepinephrine was only 18% of

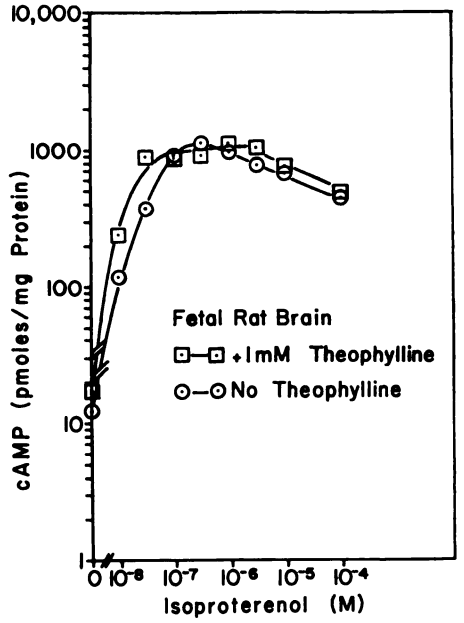

FIG. 1. *Effect of isoproterenol and theophylline on cyclic AMP levels*

Brain cells from 20-day-gestational (3.6-g) fetuses were plated at 4.7×10^6 viable cells/60-mm dish, grown for 14 days, and tested for adenosine cyclic 3′,5′-monophosphate (cAMP) response to various concentrations of *l*-isoproterenol (15-min incubation), with and without prior incubation with theophylline, as described in the text. Each point represents a single dish.

that seen with the same concentration of isoproterenol. These data are consistent with the hypothesis that the effect is mediated by *beta* adrenergic receptors.

Various adrenergic blocking agents were also employed to determine their effects on the isoproterenol stimulation of cyclic AMP production (Table 3). The *alpha* adrenergic blocker phentolamine appeared to have little effect on the response, while two *beta* adrenergic blocking agents, sotalol and dichloroisoproterenol, caused significant inhibition at 1 μM and nearly complete inhibition at 100 μM concentrations. Dichloroisproterenol appeared to be somewhat more potent than sotalol, and the relative potencies of the antagonists were very similar to those observed with glial tumors (4). This result confirmed the involvement of *beta* adrenergic receptors in the response.

The response to isoproterenol was evaluated at several different times during the development of fetal brain cells in culture (Fig. 3). In agreement with the data of Schmidt *et al.* (12), no effect of isoproterenol was seen with cells prepared from embryonic brain. However, a significant response was present by the third day of culture, and the magnitude of the response continued to increase until about 14 days. Interestingly, the increase in the "specific activity" of the response paralleled the increase in the total protein of the cultures.

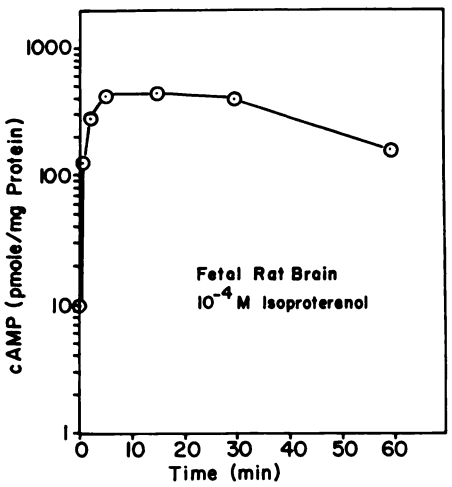

FIG. 2. *Time course of response to isoproterenol*
Brain cell cultures like those described in the legend to Fig. 1, at 14 days of culture, were incubated first with 1 mM theophylline and then for 0–60 min with 100 μM *l*-isoproterenol and theophylline, and assayed for adenosine cyclic 3′,5′-monophosphate (cAMP) levels and protein as described in the text. Each point represents the average of two dishes.

TABLE 2
Comparison of effects of isoproterenol, norepinephrine, and dopamine on cyclic AMP levels of cultured rat brain fetal cells
Brain cells from 20-day-gestational (3.69-g) fetuses were plated at 4×10^6 viable cells/60-mm dish and grown for 14 days. Cultures were incubated first with theophylline and then with the indicated compound, and finally were assayed for cyclic AMP content and protein as detailed in the text. Each value represents the content of one culture dish.

Hormone	Concentration	Cyclic AMP	Increase over control
	μM	*pmoles/mg protein*	*-fold*
None	0	10	
Dopamine	0.01	12	1
	0.1	13	1
	1	19	2
l-Norepinephrine	0.01	14	1
	0.1	15	2
	1	162	17
l-Isoproterenol	0.01	28	3
	0.1	658	69
	1	891	94

The activity of choline acetyltransferase was determined in four of these replicate cultures at 14 days. This was done to show that these cultures were similar to fetal rat brain cell cultures previously described (9). The specific activity of the enzyme was 193 pmoles/min/mg of protein, which is consistent with the value anticipated at this plating density.[1]

DISCUSSION

The data reported above show that mixed cell populations from fetal rat brain, grown in surface culture, respond to treatment with catecholamines, adenosine, and a prostaglandin with marked increases in intra-

[1] D. L. Shapiro and B. K. Schrier, unpublished observations.

TABLE 3

Effects of blocking agents on response to isoproterenol

The cultures described in the legend to Table 2 were used in this study at 14 days. Levels without blocking agent, with or without isoproterenol, each represent the average of two culture dishes. Other values represent single culture dishes for each determination.

Blocking agent	Concentration	*l*-Isoproterenol	Cyclic AMP	Increase over control[a]	Inhibition
	μM	(1 *μM*)	*pmoles/mg protein*	*-fold*	%
None		−	8		
None		+	632	77	
Regitine	1	−	10	1	
	100	−	10	1	
	1	+	506	53	20
	100	+	879	93	
Sotalol[b]	1	−	10	1	
	100	−	11	1	
	1	+	474	49	25
	100	+	27	3	96
Dichloroisoproterenol	1	−	14	2	
	100	−	13	2	
	1	+	220	16	65
	100	+	16	1	98

[a] Levels without isoproterenol are compared with control incubations without blocking agents or isoproterenol. Levels due to isoproterenol plus a blocking agent are compared with those for the corresponding concentration of blocking agent alone.

[b] *dl*-4(2-Isopropylamino-1-hydroxyethyl)methanesulfonanilide.

cellular concentrations of cyclic AMP. The stimulation by catecholamines appeared to be mediated by *beta* adrenergic receptors and was similar in many respects to that obtained with glial tumor cells. The pattern of development of the catecholamine response in these cultures mimicked that reported for rat brain slice preparations.

The cultures studied here presumably contain all the cell types of brain, including glial cells, neurons, and some fibroblasts of meningeal origin, as demonstrated by biochemical and morphological examinations[1] (8, 9). The degree of representation of each of these cell types in surface cultures varies at least with inoculation density, time in culture, and age of the source animal,[1] and cannot be reliably determined with methods presently available. The specific activity of choline acetyltransferase (presumably a neuronal marker) in the cultures of Fig. 3 was nearly twice that of brain homogenates from 19–20-day rat fetuses (9), indicating a significant representation by cells producing this activity. However, the cells for these cultures were obtained from fetuses close to parturition, an age at which much neuronal multiplication *in vivo* has been completed (17, 18) and a rapid increase in the glial to neuron ratio has begun (19). It is therefore reasonable to assume that accumulations of protein and cells in surface cultures obtained from such animals were due in large part to non-neuronal cell types, probably including glial cells. Hence, in culture, as opposed to the situation *in vivo*, a relative abundance of glial cells over neurons would be anticipated. In contrast, in reaggregated brain cell cultures, in which little cell division occurs, the proportions of cell types probably are similar to those of normal brain. Support for this hypothesis is found in comparing the specific activities of two enzymes responsible for neurotransmitter synthesis, choline acetyltransferase and glutamic acid decarboxylase, both of which reach higher levels in normal brain and reaggregation cultures (7) than in surface cultures[1] (8, 9). That surface cultures showed a much greater catecholamine-stimulated accumulation of cyclic AMP than

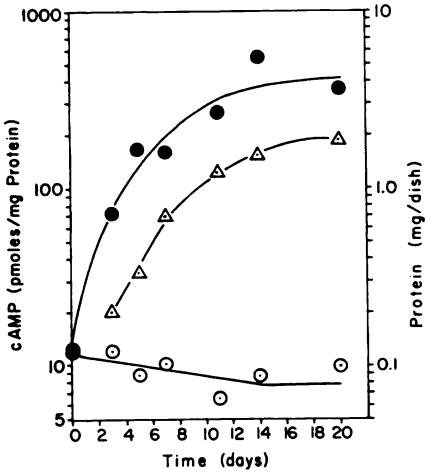

FIG. 3. *Development of response to isoproterenol during culture*

Brain cells from 21-day-gestational (4.8-g) fetuses were plated at 4×10^6 viable cells/60-mm dish, grown, and tested for adenosine cyclic 3′,5′-monophosphate (cAMP) responses as detailed in the text. All preliminary incubations included 1 mM theophylline. The *l*-isoproterenol concentration throughout was 10 μM. ●, isoproterenol-treated cultures; ⊙, control dishes; each point represents the average of two cultures. △, protein content per dish; each point represents the average of four or eight (14-day point) dishes.

either brain slices or reaggregation cultures supports the contention that this response in brain is mediated to a large extent by glial cells. Cultured fibroblasts have occasionally been found to show small stimulatory effects of catecholamines on cyclic AMP levels[2] (20), but stimulatory effects of prostaglandins are usually more prominent (20, 21).

The characteristics of the response to catecholamine were strikingly similar in surface cultures of brain cells and glial tumor cells (4). Since the brain cells were not transformed in these cultures, the similarities may be adduced as evidence in favor of the catecholamine response as a differentiated function in the glial tumor cells. Further support is lent to this argument by the similarity of the time-dependent development of this response in brain cell cultures and brain slice preparations.

[2] A. G. Gilman, unpublished observations.

Another important feature of this surface culture system would appear to be the large responses to adenosine and to prostaglandin E_1, neither of which was found in the glial clones. Experiments in progress, using the presently available systems as well as more purified preparations of normal neurons and glia, may help to assign specific responses to individual cell types of the mixed cell cultures and of the nervous system. These crucial steps may help to decipher the metabolic and/or functional role(s) of cyclic AMP in the brain.

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REFERENCES

1. T. W. Rall and A. Sattin, *in* "Role of Cyclic AMP in Cell Function" (P. Greengard and E. Costa, eds.), p. 113. Raven Press, New York, 1970.
2. T. W. Rall and A. G. Gilman, *Neurosci. Res. Program Bull.* **8**, 221 (1970).
3. A. G. Gilman and M. Nirenberg, *Nature* **234**, 356 (1971).
4. A. G. Gilman and M. W. Nirenberg, *Proc. Nat. Acad. Sci. U. S. A.* **68**, 2165 (1971).
5. R. B. Clark and J. P. Perkins, *Proc. Nat. Acad. Sci. U. S. A.* **68**, 2757 (1971).
6. N. W. Seeds and A. G. Gilman, *Science* **174**, 292 (1971).
7. N. W. Seeds, *Proc. Nat. Acad. Sci. U. S. A.* **68**, 1858 (1971).
8. S. H. Wilson, B. K. Schrier, J. L. Farber, J. E. Thompson, R. N. Rosenberg, A. J. Blume and M. W. Nirenberg, *J. Biol. Chem.* **247**, 3159 (1972).
9. B. K. Schrier, *J. Neurobiol.* In press.
10. G. R. DeLong, *Develop. Biol.* **22**, 563 (1970).
11. G. R. DeLong and R. L. Sidman, *Develop. Biol.* **22**, 584 (1970).
12. M. J. Schmidt, E. C. Palmer, W. D. Dettbarn and G. A. Robison,[1] *Develop. Psychobiol.* **3**, 53, (1970).
13. R. D. Cahn, H. G. Coon and M. B. Cahn, *in* "Methods in Developmental Biology" (F. H. Wilt and N. Wessels, eds.), p. 493, Crowell, New York, 1967.

14. A. G. Gilman, *Proc. Nat. Acad. Sci. U. S. A.* **67,** 305 (1970).
15. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193,** 265 (1951).
16. B. K. Schrier and L. Shuster, *J. Neurochem.* **14,** 977 (1967).
17. J. Altman and G. D. Das, *J. Comp. Neurol.* **126,** 337 (1966).
18. S. Zamenhof, E. van Marthens and F. L. Margolis, *Science* **160,** 322 (1968).
19. K. R. Brizzee, J. Vogt and X. Kharetchko, *Progr. Brain Res.* **4,** 136 (1963).
20. M. H. Makman, *Proc. Nat. Acad. Sci. U. S. A.* **68,** 2127 (1971).
21. V. Manganiello and M. Vaughan, *Proc. Nat. Acad. Sci. U. S. A.* **69,** 269 (1972).