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Cyclic AMP-mediated induction of ornithine decarboxylase of glioma and neuroblastoma cells

(polyamines/phosphodiesterase inhibitors/neurohormones)

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ABSTRACT **The activity of ornithine decarboxylase (EC 4.1.1.17; L-ornithine carboxy-lyase) of C6-BU-1 glioma and N115 neuroblastoma cells increases significantly when confluent cultures are treated with compounds that increase cellular cAMP levels. These include norepinephrine or isoproterenol, and prostaglandin E_1 or adenosine, which stimulate ornithine decarboxylase activity in C6-BU-1 glioma and N115 neuroblastoma cells, respectively. Ornithine decarboxylase activity is also elevated in confluent C6-BU-1 glioma cells treated with dibutyrylcAMP and theophylline, or after the glioma cells are fed with a serum-depleted medium in the presence of catecholamines and inhibitors of cyclic nucleotide phosphodiesterase. The activity of the enzyme increases 500- to 1000-fold, 2–6 hr after stationary-phase N115 neuroblastoma cells are fed with a serum-free medium, supplemented with phosphodiesterase inhibitors, adenosine, or prostaglandin E_1. This stimulation is antagonized by carbamoyl choline and is blocked by actinomycin D or cycloheximide. These results suggest that the synthesis of ornithine decarboxylase of C6-BU-1 glioma and N115 neuroblastoma cells is controlled by cAMP.**

Studies of both bacterial cells and mammalian systems suggested that polyamine biosynthesis is one of the earliest events that occur during cell proliferation (1, 2). Ornithine decarboxylase (EC 4.1.1.17; L-ornithine carboxy-lyase), which catalyzes the formation of putrescine from ornithine, is the rate-limiting enzyme in polyamine biosynthesis. It has the most rapid turnover rate of any mammalian enzyme (3) and is elevated in tumor cells (4–6).

Numerous studies indicated that ornithine decarboxylase activity can be increased by hormones that affect growth (2). Since cyclic nucleotides are considered the second messenger between the receptor site on the cell membrane and the subsequent increase in biosynthetic activity, it was reasonable to assume that adenosine-3′:5′-cyclic monophosphate (cAMP) might induce decarboxylase activity.

Clonal lines derived from tumors of the nervous system may be very useful in elucidating the possible role of cAMP in ornithine decarboxylase induction. It is well known that neurons respond differently to environmental stimuli, which include hormones. Thus, neuroblastoma cells increase their intracellular levels of cAMP in the presence of prostaglandin E_1 (PGE_1) (7, 8) and adenosine (9), while rat glioma cells do so when exposed to catecholamines (10).

We have recently demonstrated† that the activity of the decarboxylase in C6-BU-1 glioma or N115 neuroblastoma cells fluctuates during the growth cycle, being low in stationary-phase and high in rapidly growing cells. We were able to show that when nongrowing cells are fed with a medium containing 10% calf serum, decarboxylase activity rises precipitously after a lag of approximately 2 hr. Five to six hours after feeding, the activity is maximal and increases 500- to 1000-fold.

Now, we wish to present evidence that treatment of C6-BU-1 glioma cells with dibutyryladenosine-3′:5′-cyclic monophosphate (Bt_2cAMP), norepinephrine, or isoproterenol results in an increased ornithine decarboxylase activity. Similarly, in N115 neuroblastoma cells, the enzyme activity increases after exposure to PGE_1 or adenosine.

Abbreviations: Ro20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; IBMX, 3-isobutyl-1-methylxanthine; Bt_2cAMP, N^6,O^2-dibutyryladenosine-3′:5′-cyclic monophosphate; PGE_1, prostaglandin E_1; cAMP, adenosine 3′:5′-cyclic monophosphate.

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† U. Bachrach, in preparation.

MATERIALS AND METHODS

Materials. DL-[*1*-^{14}C]Ornithine (32.2 mCi/mmol) was obtained from New England Nuclear. Cycloheximide, actinomycin D; *l*-norepinephrine, pyridoxal-5′-phosphate, *dl*-isoproterenol, guanosine-3′:5′-cyclic monophosphoric acid-sodium salt, and carbamoylcholine (carbachol) were from Sigma. PGE_1 was kindly provided by Dr. John Pike, Upjohn Co. Ro20-1724 [4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone] was from Hoffman LaRoche. IBMX (3-isobutyl-1-methylxanthine) was from Aldrich. Bt_2cAMP was from JEM Research Laboratory and dithiothreitol from P-L Biochemicals. Adenosine and theophylline were from Nutritional Biochemical.

Cell Lines. Rat glioma clone C6-BU-1 (11), derived from C6 (12) and mouse C1300 neuroblastoma clone N115 subcultures, were grown in Falcon 100-mm plastic dishes in Dulbecco's modified Eagle's minimum medium (GIBCO, Cat. no. H-21), supplemented with 10% fetal calf serum, in an atmosphere of 90% air/10% CO_2.

Ornithine Decarboxylase Assay. After growth medium was removed from the cells (5–7 mg of protein per 100-mm dish), they were washed with ice-cold phosphate/saline (0.8% NaCl; 0.02% KCl; 0.115% Na_2HPO_4; 0.02% KH_2PO_4, pH 7.2), then drained and stored at −20°. To each plate, 1.6 ml of assay buffer (50 μM ethylenediaminetetraacetic acid; 25 μM pyridoxal phosphate; 2.5 mM dithiothreitol in 25 mM Tris·HCl, pH 7.1), was added. Cells were than scraped off with a rubber policeman and frozen and thawed three times. After centrifuging at 4500 × *g* for 10 min, ornithine decarboxylase activity was determined by incubating 0.5-ml quantities of the supernatant solution with 100 μl of [*1*-^{14}C]ornithine (0.4 μCi; 12.5 nmol) in 16 × 125-mm plastic tubes equipped with a rubber stopper supporting a polyethylene center well (4). After incubation at 37° for 45 min, 0.2 ml of hydroxide of hyamine (Packard Instrument) was injected into each center well. Tubes were incubated at 37°

Table 1. Effect of cyclic nucleotide phosphodiesterase inhibitors and dibutyrylcAMP on ornithine decarboxylase induction in C6-BU-1 glioma cells

Treatment	Ornithine decarboxylase activity (pmol/mg protein·hr)
No addition	1.3
Ro 20-1724, 200 μM	62.0
40 μM	2.5
4 μM	1.3
IBMX, 250 μM	9.6
50 μM	1.2
5 μM	1.3
Theophylline, 1 mM	1.5
Theophylline, 1 mM + Bt_2cAMP, 1 mM	7.8

Inhibitors (100 μl) were added to 5-day-old cells (last fed 48 hr earlier) and ornithine decarboxylase activity was determined after incubation at 37° for 5 hr. Cells were preincubated with theophylline for 30 min to test the effect of Bt_2cAMP.

for another 15 min, followed by the injection of 0.2 ml of 6% perchloric acid to stop the reaction. To release bound CO_2, tubes were agitated for 15 min; thereafter, center wells were removed and their radioactivities determined. Proteins were assayed according to Lowry *et al.* (13).

Effect of Hormones. The effect of the various agents on ornithine decarboxylase activity was studied by adding 100-μl quantities of aqueous solutions (or PGE_1 in ethanol) to confluent cultures in 100-mm plastic dishes containing 10 ml of Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Medium was usually changed 48 hr prior to the beginning of the experiment. The effect of Bt_2cAMP on confluent C6-BU-1 glioma cells was studied under the above described conditions, except that cells were preincubated with 1 mM theophylline for 30 min at 37° and then exposed to the cyclic nucleotide. In some experiments, cultures were preincubated with 0.5 mM IBMX or Ro20-1724 (in ethanol) for 30 min at 37°. Ethanol (0.5% with either Ro20-1724, or PGE_1) had no adverse effect on decarboxylase activity. In parallel experiments, confluent cultures (in 100-mm plastic dishes) were washed 3 times with 5 ml of medium A [Dulbecco's modified Eagle's medium with 25 mM Hepes (*N*-2-hydroxyethyl piperazine-*N'*-ethanesulfonic acid) pH 7.4, instead of $NaHCO_3$, adjusted to 340 mosmol/liter with 1.1 g of NaCl/liter]. Cells were then treated with 100-μl quantities of the various agents in 10 ml of medium A, which is a serum-free medium.

RESULTS

The activity of ornithine decarboxylase in confluent C6-BU-1 cells was barely above the basal level. This activity increased 10- to 30-fold, 2–6 hr after the addition of either 1 μM norepinephrine or 0.1 μM isoproterenol to confluent 5-day-old C6-BU-1 glioma cells, without changing the medium (Fig. 1).

Cyclic nucleotide phosphodiesterase inhibitors, which are known to inhibit the degradation of intracellular cAMP, also activated ornithine decarboxylase. Table 1 shows that Ro20-1724 was most active, and at a concentration of 200 μM, increased the enzyme activity approximately 50-fold. IBMX was less active, and at a concentration of 250 μM, the enzyme activity was elevated by a factor of 7 only. Theophylline (1 mM) hardly affected the activity. On the other hand,

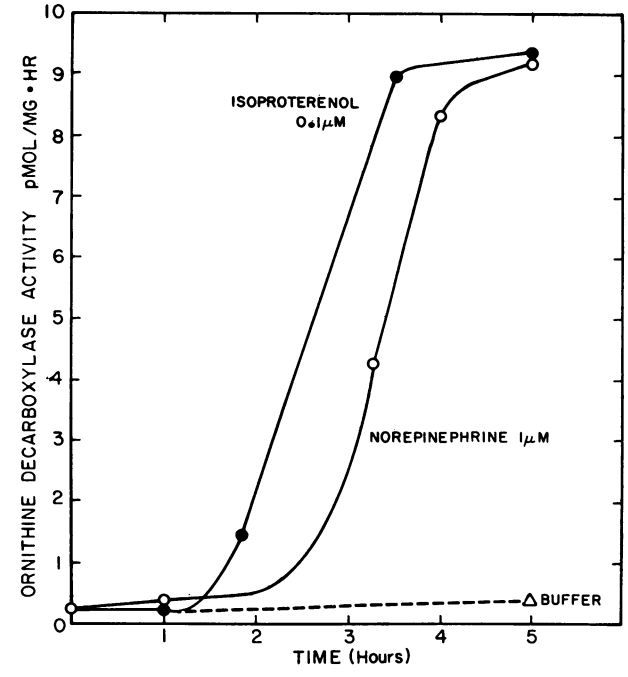

FIG. 1. Effect of catecholamines on ornithine decarboxylase activity. C6-BU-1 glioma cells were fed on the first and third day after seeding. The experiment was started on day 5, by adding 100-μl quantities of the reagents to the cells (6 mg of protein per plate) without changing the media. Ornithine decarboxylase activity at zero time was 0.3 pmol/mg of protein per hr.

a significant increase in enzyme activity was noticed when Bt_2cAMP (1 mM) was added to confluent C6-BU-1 glioma cells (without changing the medium), along with 1 mM theophylline (Table 1). Bt_2cAMP without added theophylline had no appreciable effect on ornithine decarboxylase, neither did the sodium salt of cGMP increase decarboxylase activity when added to C6-BU-1 glioma cells along with theophylline (not shown). Ornithine decarboxylase activity was markedly elevated when catecholamines or IBMX were added to confluent C6-BU-1 glioma cells, previously washed with medium A (lacking serum) and then incubated with the reagents made up in medium A. Fig. 2A shows that medium A alone brought about a moderate activation of ornithine decarboxylase after a lag of 2–3 hr. This activation was significantly enhanced by the addition of either 1 μM norepinephrine or 0.1 μM isoproterenol to the medium (Fig. 2A). The phosphodiesterase inhibitor, IBMX, also induced enzyme activity in confluent C6-BU-1 glioma cells, washed and fed with medium A. After incubation for 4 hr, IBMX increased the activity of the enzyme 370-fold, whereas incubation of C6-BU-1 glioma cells with IBMX and norepinephrine or isoproterenol increased the activity 580- and 460-fold, respectively (Fig. 2B).

While C6-BU-1 glioma cells are known to respond to catecholamines, N115 neuroblastoma cells increase their intracellular cAMP levels in the presence of PGE_1 or adenosine. It may be seen (Table 2) that confluent N115 neuroblastoma cells, in the old growth medium, barely responded to the addition of adenosine or PGE_1. On the other hand, PGE_1 significantly increased ornithine decarboxylase activity of N115 neuroblastoma cells after incubation with 0.5 mM IBMX. The phosphodiesterase alone also stimulated the activity of confluent N115 neuroblastoma cells that remained in the old growth medium (Table 2). Similar to the results depicted in Fig. 2B for C6-BU-1 glioma cells, N115 neuro-

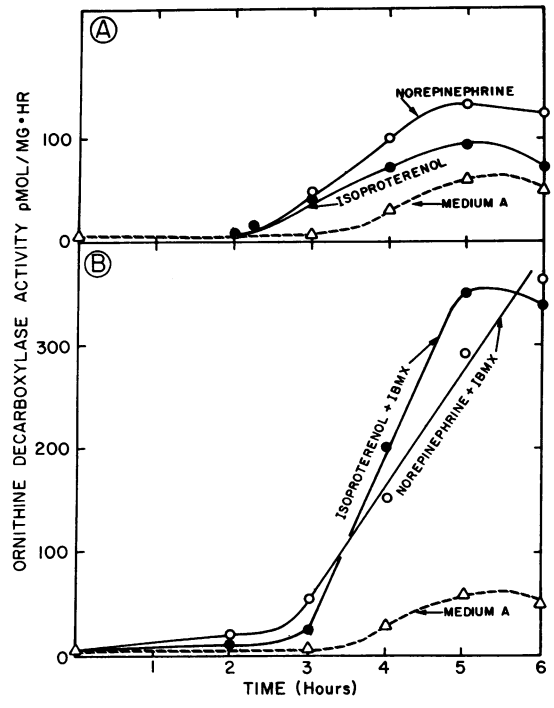

FIG. 2. Effect of catecholamines and IBMX on ornithine decarboxylase activity of C6-BU-1 glioma cells fed with serum-depleted medium. Five-day-old cells were used. On the day of the experiment, the old medium was removed and cells were washed with medium A (which lacks serum). Reagents (100 μl) were added to 10-ml quantities of medium A, and ornithine decarboxylase activity was determined. Enzyme activity at zero time was 0.6 pmol/mg of protein per hr. (A) No phosphodiesterase inhibitor added. (B) Cells were preincubated with 0.5 mM IBMX for 30 min prior to addition of the catecholamines.

blastoma cells were more responsive to hormones after confluent cells were washed with medium A. It may be seen (Fig. 3) that medium A slightly activated the decarboxylase of N115 neuroblastoma cells, whereas a 150-fold activation was observed when N115 neuroblastoma cells were incubated with PGE_1 (10 μM) in medium A. As expected, IBMX (0.5 mM) had a marked (activation by a factor of 500) effect

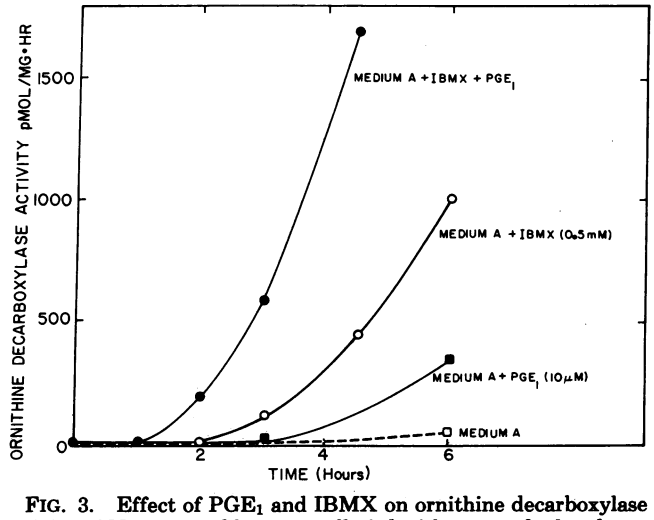

FIG. 3. Effect of PGE_1 and IBMX on ornithine decarboxylase activity of N115 neuroblastoma cells fed with serum-depleted medium. Five-day-old cells were used. On the day of the experiment, the old medium was removed, cells were washed with medium A, and ornithine decarboxylase activity was determined. Enzyme activity at zero time was 2 pmol/mg of protein per hr.

Table 2. Effect of various agents on ornithine decarboxylase activity of N115 neuroblastoma cells

	Treatment	Ornithine decarboxylase activity (pmol/mg of protein·hr)
(A)	No addition	8.2
	Adenosine, 0.5 mM	7.6
	PGE_1, 10 μM	6.7
	IBMX, 0.5 mM	14.0
(B)	IBMX + adenosine	16.0
	IBMX + PGE_1	46.0

(A) Reagents (100 μl) were added to 5-day-old cells (last fed 48 hr earlier), and ornithine decarboxylase activity was determined after incubation at 37° for 5 hr.
(B) Cells were preincubated with 0.5 mM IBMX at 37° for 30 min, followed by addition of the reagents.

on the enzyme, and a combination of IBMX and PGE_1 was most active (Fig. 3). Table 3 shows that the activation of decarboxylase in N115 neuroblastoma cells by PGE_1, was proportional to the concentration of the hormone (1–20 μM) when cells were preincubated with 0.5 mM IBMX in medium A.

Carbachol (carbamoyl choline), a relatively stable acetylcholine analog, antagonized the stimulatory effect of IBMX in the induction of ornithine decarboxylase (Table 4). Thus, after incubation for 3 hr, carbachol reduced IBMX-induced activity by approximately 75%. Carbachol has been shown to elevate cGMP and reduce cAMP levels in N115 neuroblastoma cells‡. Table 4 also shows that adenosine, which is known to elevate cAMP levels in N115 neuroblastoma cells‡, increased the decarboxylase activity of IBMX-treated cells. This induction was also antagonized by carbachol (Table 4).

Actinomycin D and cycloheximide inhibited the rise of decarboxylase activity when added to confluent N115 neuroblastoma cells along with a serum-containing growth medium (Fig. 4). Similar results were also obtained when IBMX and medium A were used instead of a serum-containing medium (not shown).

DISCUSSION

We have recently demonstrated† fluctuations in ornithine decarboxylase during the growth cycle of C6-BU-1 glioma and N115 neuroblastoma cells. These clones, derived from tumors of the nervous system, thus resemble other cell lines in which ornithine decarboxylase activity is stimulated after the addition of fresh medium (5, 14–19). Experiments described in this paper indicated that Bt_2cAMP induces the enzyme in C6-BU-1 glioma cells even in the absence of fresh growth medium. These findings are in line with the results of other studies, which showed that Bt_2cAMP can induce ornithine decarboxylase in baby hamster cells (17) and in the adrenal medulla and other rat tissues (20–24).

In the present study we used catecholamines, and PGE_1 and adenosine, which specifically elevate cAMP levels in glioma and neuroblastoma cells, respectively. All these reagents increased the activity of ornithine decarboxylase. The activation of the decarboxylase by adenosine and the reversal by carbachol strongly suggest that the enzyme is controlled by cAMP and not by cGMP. It has been established that in N115 neuroblastoma cells, adenosine increases the in-

‡ H. Matsuzawa and M. Nirenberg, in preparation.

Table 3. Effect of PGE_1 on ornithine decarboxylase activity of N115 neuroblastoma cells

Treatment	Ornithine decarboxylase activity (pmol/mg protein·hr)
No addition	2
Medium A (alone)	16
Medium A + IBMX, 0.5 mM	300
Medium A + IBMX + PGE_1, 1.0 μM	700
Medium A + IBMX + PGE_1, 5.0 μM	814
Medium A + IBMX + PGE_1, 10.0 μM	940
Medium A + IBMX + PGE_1, 20.0 μM	1202

Cells (7-day-old) were washed with medium A and incubated with 0.5 mM IBMX in 10-ml quantities of medium A. After incubation at 37° for 4 hr, ornithine decarboxylase activity was determined.

tracellular level of cAMP, while carbachol decreases cAMP levels and causes the accumulation of cGMP‡. Our findings that carbachol reverses the stimulatory action of IBMX or of adenosine (Table 4) conforms with the known inverse relationship of cAMP and cGMP.

There are conflicting data published regarding cAMP levels in growing cells. Most recently, Russell and Stambrook (25) demonstrated a good correlation between ornithine decarboxylase activity and cellular cAMP levels during the growth of synchronized chinese hamster cells. These results and the use of phosphodiesterase inhibitors prompted Byus and Russell (26, 27) to conclude that the synthesis of the decarboxylase is controlled by cAMP.

In the present study we showed that medium A alone, in the absence of serum, slightly activated the enzyme in C6-BU-1 glioma and N115 neuroblastoma cells. A similar activation of the enzyme by various amino acids (in the absence of serum) has been reported (15, 16).

Induction of ornithine decarboxylase in neuroblastoma and glioma cells by media supplemented with serum could be mimicked by the addition of medium A, supplemented with specific hormones or phosphodiesterase inhibitors. The same reagents had only a slight effect on the decarboxylase of cells maintained in the old growth medium. It is reasonable to assume that the old medium contains inhibitory products that interfere with enzyme induction. One candidate for such an inhibitor is putrescine, which is probably excreted into the growth medium during cellular proliferation. Preliminary experiments indeed indicated that putrescine inhibits decarboxylase induction in N115 neuroblastoma cells.

Table 4. Effect of carbachol, IBMX, and adenosine on ornithine decarboxylase activity of N115 neuroblastoma cells

Treatment	Ornithine decarboxylase activity (pmol/mg protein·hr)
No addition	1.5
IBMX, 0.5 mM	415
IBMX + adenosine, 0.5 mM	486
IBMX + carbachol, 1 mM	172
IBMX + adenosine + carbachol	293

Cells (5-day-old) were grown in DMEM medium supplemented with 10% calf serum. On the day of the experiment, cells were washed with medium A, and incubated with the reagents (in medium A) for 3 hr. Cells were then assayed for ornithine decarboxylase.

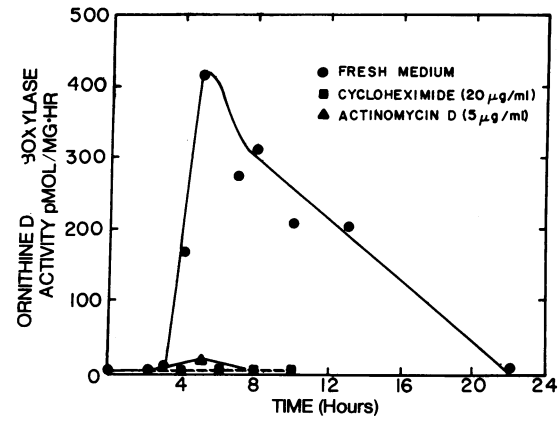

FIG. 4. Effect of fresh growth medium and inhibitors on ornithine decarboxylase activity of N115 neuroblastoma cells. Fresh Dulbecco's modified Eagle's medium supplemented with 10% calf serum was added to 5-day-old cells in the presence or absence of inhibitors. Ornithine decarboxylase activity was determined after incubation at 37° for times indicated.

Numerous studies indicated that ornithine decarboxylase activity increases approximately 2 hr after hormone administration and that this stimulation can be blocked by drugs that inhibit protein or RNA synthesis. Our results (Fig. 4) agree with these findings and imply a *de novo* synthesis of the enzyme. The effect of the inhibitors and the prolonged lag between stimulation and enzyme induction argue against an activation of the decarboxylase by a direct phosphorylation of the enzyme or its precursor through a cAMP-dependent protein kinase. It is conceivable that cAMP regulates the expression of ornithine decarboxylase at the transcriptional level, in analogy to its effect on the *lac* operon in *Escherichia coli*.

Our experiments indicate the involvement of cAMP in the induction of ornithine decarboxylase cultures of tumors of the nervous system. It appears that this is a general mechanism for activation of the decarboxylase, common to many mammalian cells. It is not clear as yet how the enzyme is related to cell growth. Manen and Russell (28) have recently demonstrated a correlation between ornithine decarboxylase and rat liver RNA polymerase I activities. Preliminary experiments in our laboratory corroborate these findings and show a parallel increase in enzyme and RNA synthesis in cultures of N115 neuroblastoma cells.

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