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ORNITHINE DECARBOXYLASE ACTIVITY AND THE GROWTH OF NEUROBLASTOMA CELLS

THE EFFECTS OF BROMOACETYLCHOLINE, BROMOACETATE AND 1,3-DIAMINOPROPANE

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Abstract—The activity of ornithine decarboxylase (EC 4.1.1.17; L-ornithine carboxylase) of C1300 neuroblastoma cells was decreased significantly in the presence of cytolytic concentrations of bromoacetylcholine and bromoacetate. The inhibition of neuroblastoma cell replication by the ornithine decarboxylase inhibitor, 1,3-diaminopropane, lends credence to the suggestion that the mechanism of the potent cytolytic action of bromoacetylcholine and of bromoacetate may be related to the inhibition of this enzyme.

Bromoacetylcholine has been shown to inhibit the growth of C1300 neuroblastoma cells *in vitro* [1] and in an experimental animal model of human disease [2]; it is hoped that the drug can be used to treat this malignant tumor. The only known mechanism of action of bromoacetylcholine at the molecular level is its binding to specific receptors. This cholinergic agonist, when incubated with cell membranes for 15 min or longer, binds irreversibly to the cholinergic receptor at the nicotinic site but not at the muscarinic site [3, 4]. The relationship between the cytolytic effect of bromoacetylcholine and its receptor binding is not understood. Since bromoacetate, the hydrolysis product of bromoacetylcholine, has been shown to inhibit neuroblastoma in cell culture and in A/Jackson mice [2], its action was also the focus of the present study.

Although it is not known exactly how the cell transfers information from its surface receptors to its nucleus or cytoplasm, there is evidence that the activity of the enzyme, ornithine decarboxylase (L-ornithine carboxylase, EC 4.1.1.17), is affected by changes at the membrane level; this enzyme has been implicated as a metabolic mediator in rapidly proliferating tissues [5, 6].

Chen *et al.* [7] have shown that the growth of mouse leukemia L1210 cells was inhibited by agents which act at cellular surfaces (e.g. colchicine, vinblastine, and antibodies to L1210 membrane proteins); the inhibition of growth was associated with a decrease in ornithine decarboxylase activity.

Recently, analogs of the substrate (ornithine) or of the product (putrescine) have been synthesized in an effort to develop more specific inhibitors of ornithine decarboxylase [8]. α-Methyl ornithine was shown to be a potent competitive inhibitor of the enzyme and to block the proliferation of rat hepatoma cells in culture [9], and 1,3-diaminopropane produced a profound decrease in the synthesis of DNA in the regenerating rat liver [10, 11]. Although better and more specific inhibitors of ornithine decarboxylase are needed before their clinical applications can be realized, the results with these experimental agents further suggest that ornithine decarboxylase activity is associated with rapid growth.

It was the aim of the present study to see if the cytolytic effect of bromoacetylcholine or of the metabolite, bromoacetate, was associated with a decrease in the activity of ornithine decarboxylase.

Bachrach [12] has demonstrated that the activity of ornithine decarboxylase in mouse C1300 neuroblastoma clone N115 subcultures is very low during the stationary phase and very high in rapidly growing cells. He further showed that the activity of the enzyme in nongrowing cells rises precipitously when they are fed with a medium containing either 10% calf serum or solutions containing agents which elevate cyclic AMP levels. In this respect, the neuroblastoma culture resembles other cell lines in which ornithine decarboxylase activity is increased dramatically when cultured cells are transformed from the resting to the growing state by the addition of fresh medium, or by various agents that affect the cell growth [12–17].

In the present work, bromoacetylcholine or bromoacetate was added either during the log growth phase, or during the stationary phase after adding fresh medium containing serum. Possible cytolytic effects of 1,3-diaminopropane were studied during the log growth phase to compare the activity of bromoacetylcholine to a known ornithine decarboxylase inhibitor.

MATERIALS AND METHODS

Materials. DL-[1-^{14}C]ornithine (49.9 mCi/m-mole) was obtained from New England Nuclear, Boston,

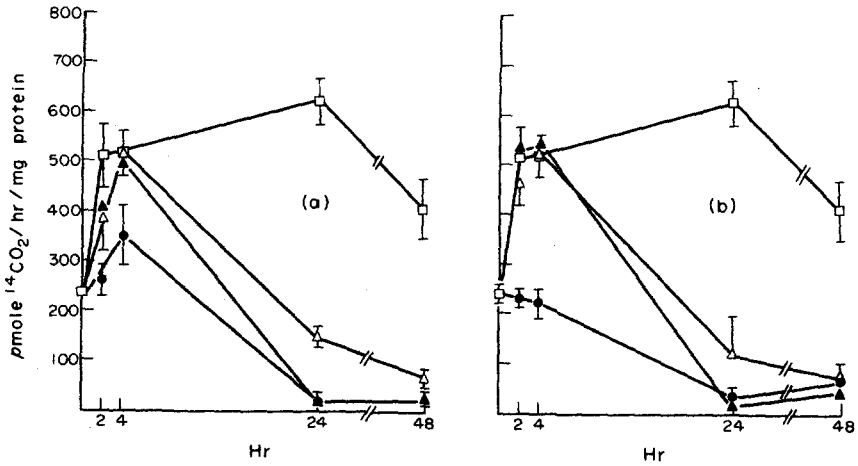

Fig. 1. Inhibition of neuroblastoma cell replication by bromoacetylcholine and bromoacetate. Drugs were added to the culture medium 24 hr after seeding (time 0) and left in the medium throughout the experiment. Key: (□), control; (△), 1×10^{-5} M; (▲), 3×10^{-5} M; and (●), 1×10^{-4} M. (A) The effects of bromoacetylcholine; (B) the effects of bromoacetate.

MA. [^{3}H]leucine (6 Ci/m-mole) and [^{3}H]uridine (8 Ci/m-mole) were purchased from Schwarz/Mann, Orangeburg, NY. Bromoacetate and 1,3-diaminopropane were products of Aldrich Chemical Co., Milwaukee, WI. Bromoacetylcholine was synthesized in this laboratory by the method published previously [18, 19].

Murine C1300 neuroblastoma cells were obtained from the American Type Culture Collection, Rockville, MD. Modified Eagle medium (Catalog No. F-11) and fetal calf serum (Catalog No. 614) were purchased from Grand Island Biological Co., Grand Island, NY.

Drug treatment. The effect of the various agents on ornithine decarboxylase activity was studied by adding less than 60-μl quantities of drug solutions to neuroblastoma cultures in 100-mm tissue culture dishes (Falcon) containing 12 ml of Modified Eagle medium supplemented with 50 units/ml of penicillin, 50 μg/ml of streptomycin, 20 mM L-glutamine and 10% fetal calf serum [2]. In studies of confluent cells, fresh media containing drugs were added on day 4 after seeding (day 5). In studies of cells in the log growth phase, the cells were fed on the day of seeding and the drugs were added 24 hr later (time zero). Cells were harvested at time intervals indicated in Results.

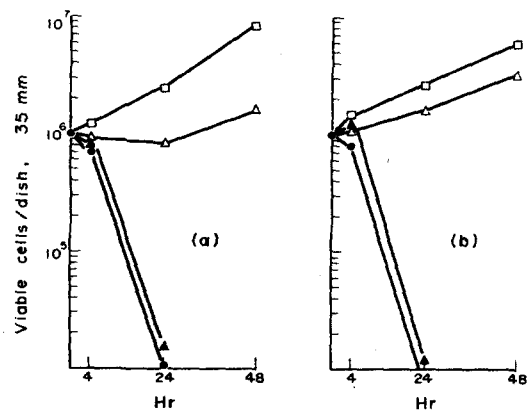

Fig. 2. Bromoacetylcholine and bromoacetate inhibition of ornithine decarboxylase in neuroblastoma cells during log growth. Drugs were added to the culture medium as described in Fig. 1. Key: (□), control; (△), 1×10^{-5} M; (▲), 3×10^{-5} M; and (●), 1×10^{-4} M. (A) The effects of bromoacetylcholine; (B) the effects of bromoacetate. Bars represent S. E. M. of six to ten values.

The cytolytic effect of 1,3-diaminopropane on neuroblastoma was studied by adding this ornithine decarboxylase inhibitor in medium containing serum 24 hr after the cells were plated. Viable cells were counted at 0, 4, 24, 48 and 72 hr after drug treatment.

RNA and protein synthesis. Neuroblastoma cells were cultured for 24 hr in the absence of drug and then for an additional 24 hr in the presence of either bromoacetylcholine or bromoacetate. At the end of the 24 hr of drug treatment, [^{3}H]uridine (10 μCi ml^{-1} of 8 Ci m-mole^{-1}) or [^{3}H]leucine (10 μCi ml^{-1} of 6 Ci m-mole^{-1}) was added and the incorporation was measured 6 hr later. The culture dishes were washed six times and the cells were harvested, solubilized with NCS Solubilizer, and placed in scintillation solution [dioxane, 1000 ml; naphthalene, 100 g; 2,5-diphenyloxazole (PPO), 7 g]. The radioactivity was counted in a Beckman scintillation counter. The number of viable cells was determined in order to express the RNA and protein synthesis in cpm/10^5 cells/6 hr.

Ornithine decarboxylase assay. After growth medium was removed from the cells, they were washed with ice-cold phosphate/saline (0.8% NaCl; 0.02% KCl; 0.115% Na_2HPO_4; and 0.02% KH_2PO_4, pH 7.2). To each plate, 1.0 to 1.6 ml of assay buffer (0.05 M Na–K phosphate, pH 7.2, containing 1 mM dithiothreitol, 0.25 mM EDTA, and 3 μM pyridoxal phosphate) was added. Cells were then scraped off the plate with a Teflon policeman and frozen and thawed three times. After centrifugation at 4500 *g* for 15 min, 0.2 ml of the supernatant solution was incubated at 37° for 15 min in 100 × 16 mm centrifuge tubes equipped with rubber stopper-supporting

Table 1. Effects of bromoacetylcholine and bromoacetate on the incorporation of [^{3}H]uridine and [^{3}H]leucine into neuroblastoma cells

Drug concn (M)	Bromoacetylcholine		Bromoacetate	
	[^{3}H]uridine (%)	[^{3}H]leucine (%)	[^{3}H]uridine (%)	[^{3}H]leucine (%)
0	100*	100†	100*	100†
1×10^{-5}	43 ± 8	29 ± 5	79 ± 17	5 ± 1
3×10^{-5}	15 ± 2	0	2 ± 1	0

* Rate is 30,104 cpm/10^5 cells/6 hr; N = 8.
† Rate is 1447 cpm/10^5 cells/6 hr; N = 8.

polyethylene center wells (Kontes Glass Co.). The assay was initiated with the addition of 45.5 μl of 0.6 mM [1-^{14}C] L-ornithine (10 μCi/mole of L-ornithine). After incubation at 37° for 30 min, 0.2 ml of ethanolamine-2-methoxyethanol (2:1, v/v) was injected into each center well and the tubes were incubated for an additional 30 min. The reaction was stopped with the addition of 0.3 ml of 1 M citric acid and the tubes were agitated for 30 min to release the CO_2. The center wells were then removed and placed in 12 ml of a scintillation mixture (10 ml of toluene omniflour plus 2 ml ethanol) and counted in a Beckman scintillation counter. Blank tubes contained supernatant solutions which had been heated at 65° for 10 min prior to the assay. Proteins were assayed according to the method of Lowry *et al.* [20], with corrections made for the effect of dithiothreitol on the protein assay.

RESULTS

Effect of bromoacetylcholine and bromoacetate on ornithine decarboxylase activity and on cell replication during the log growth phase. When either bromoacetylcholine or bromoacetate was added to cell cultures 24 hr after seeding, the results in Figs. 1 and 2 were obtained. A significant inhibition of cell replication was observed in 24 hr with concentrations of 1×10^{-5} M of either drug; with higher concentrations, the cell number was reduced below the original level (Fig. 1).

In parallel experiments, both bromoacetylcholine and bromoacetate were shown to inhibit ornithine decarboxylase activity (Fig. 2). A significant inhibition was observed in 24 hr with 1×10^{-5} M bromoacetylcholine or bromoacetate; inhibition with the highest drug concentration (1×10^{-4} M) was apparent within the first 4 hr.

Effect of bromoacetylcholine and bromoacetate on RNA and protein synthesis. To determine if changes in RNA or protein synthesis could be observed after 24-hr drug inhibition of ornithine decarboxylase, [^{3}H]uridine or [^{3}H]leucine was added at 24 hr and the incorporation was measured 6 hr later. The results in Table 1 show a significant inhibition of RNA synthesis (as measured by [^{3}H]-uridine incorporation) and of protein synthesis (as measured by [^{3}H]leucine incorporation) in the 6 hr after the inhibition of ornithine decarboxylase.

Effect of 1,3-diaminopropane on cell replication during the log growth phase. When the ornithine decarboxylase inhibitor, 1,3-diaminopropane, was added to

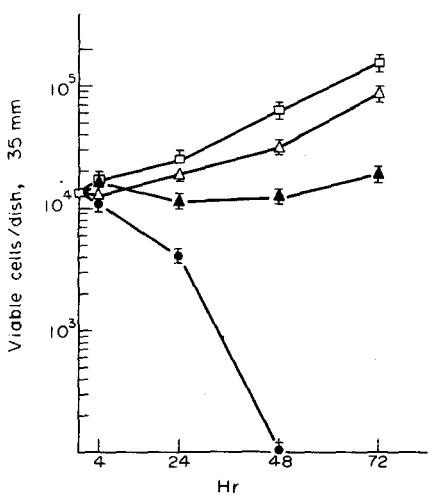

Fig. 3. Inhibition of neuroblastoma cell replication by 1,3-diamino-propane. Drug was added to the culture medium as described in Fig. 1. Key: (□), control; (△), 6.7×10^{-5} M; (▲), 6.7×10^{-4} M; and (●), 2×10^{-3} M.

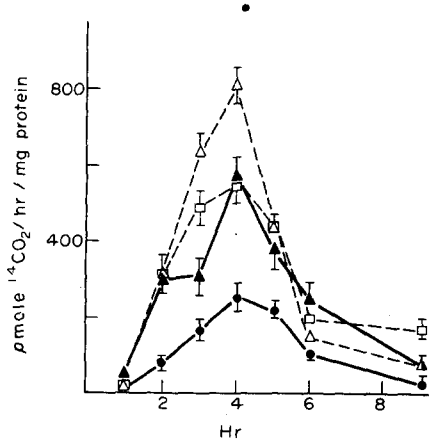

Fig. 4. Bromoacetylcholine inhibition of ornithine decarboxylase in confluent cells diluted with fresh media containing serum. Fresh media was added on day 4 after seeding, and various concentrations of bromoacetylcholine were added to the confluent cells (time 0). Key: (□), control; (△), 1×10^{-5} M; (▲), 3×10^{-5} M; and (●), 1×10^{-4} M. Each point is a mean of six values, and bars represent S. E. M.

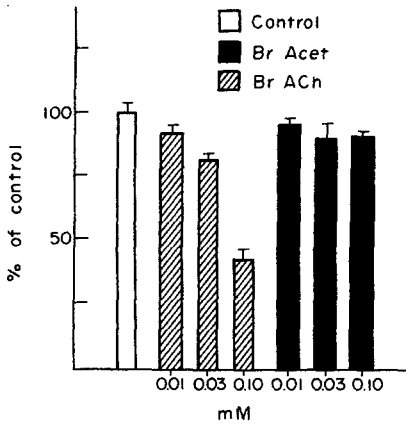

Fig. 5. Effect of bromoacetylcholine and bromoacetate on ornithine decarboxylase activity in a cell-free system. Drugs were added directly to ornithine decarboxylase assays containing supernatant solutions from untreated neuroblastoma cultures. BACh, bromoacetylcholine; BAcet, bromoacetate. Control ornithine decarboxylase activity was 595.2 pmoles $^{14}CO_2$/mg of protein/hr.

cell cultures 24 hr after seeding, the results in Fig. 3 were obtained. Inhibition of neuroblastoma cell replication was observed at concentrations of 6.7×10^{-4} M and higher. These results indicate that inhibition of the enzyme by a known ornithine decarboxylase inhibitor does contribute to the suppression of neuroblastoma growth.

Effect of bromoacetylcholine on the induction of ornithine decarboxylase activity in confluent cells after the addition of fresh medium. Ornithine decarboxylase activity was induced precipitously by adding fresh medium containing serum to confluent cells on day 4 after seeding (day 5) (Fig. 4). This induction was enhanced at 3 and 4 hr after dilution when a low concentration of bromoacetylcholine (1×10^{-5} M) was added to the fresh medium. In contrast, no enhancement could be observed at 3×10^{-5} M bromoacetylcholine, and a further increase in the concentration of the drug (1×10^{-4} M) resulted in an inhibition of decarboxylase induction.

Effect of bromoacetylcholine and bromoacetate on ornithine decarboxylase activity in a cell-free system. To determine the direct effect of cytolytic concentrations of bromoacetylcholine and bromoacetate on ornithine decarboxylase activity, the drugs were added to enzyme assays containing neuroblastoma supernatant solutions from untreated cells. Unlike the results with intact cells, no inhibition of enzyme activity was observed with cytolytic concentrations of bromoacetate; inhibition was observed only with the highest (1×10^{-4} M) concentration of bromoacetylcholine (Fig. 5).

DISCUSSION

This study clearly shows that ornithine decarboxylase activity in neuroblastoma cells is markedly altered in the presence of cytolytic concentration of either bromoacetylcholine or bromoacetate. The inhibition of neuroblastoma by the ornithine decarboxylase inhibitor, 1,3-diaminopropane, lends credence to the suggestion that the mechanism of the potent cytolytic action of bromoacetylcholine and of bromoacetate may be related to the inhibition of this enzyme.

Recent experimental evidence has shown that ornithine decarboxylase is activated during tumor development [5, 21–23] and that the induction of the enzyme is one of the earliest effects of tumor promoters [24–26]. Heby *et al.* [22] have shown a high correlation between the specific growth rate of rat brain tumor cells and the synthesis of the polyamines; their results were interpreted to indicate that polyamine biosynthesis is involved in the process of cell replication.

Our preliminary results do not indicate the mechanism by which bromoacetylcholine or bromoacetate inhibits ornithine decarboxylase activity. It could be suggested that the inhibition occurs through alterations of the enzyme levels since cytolytic concentrations (1×10^{-5} and 3×10^{-5} M) of either drug did not inhibit cell-free ornithine decarboxylase activity *in vitro* (Fig. 5). An exception would be the inhibition noted with very high concentrations (1×10^{-4} M) of bromoacetylcholine. In this situation, the presence of such a large cation pool might be expected to cause inhibition. Chen *et al.* [7] have demonstrated a similar cation inhibition with 10–20 mM Na^+, K^+ or Mg^{2+}. The significance of this cation effect is minimized *in vivo* since intracellular concentrations of bromoacetylcholine of this magnitude would not be achieved.

In our experiments, bromoacetylcholine altered the activity of ornithine decarboxylase both in rapidly growing cells and in cells which had been transformed from the resting to the growing state by the addition of fresh medium containing serum. A number of studies have indicated that the changes in ornithine decarboxylase activity during the two experimental growth situations mentioned above are mediated by changes in the activity of cyclic AMP protein kinase [12, 27–29]. Russell and Stambrook [30] demonstrated a correlation between ornithine decarboxylase activity and cellular cAMP levels during the growth of synchronized Chinese hamster cells; Bachrach [12] has shown that the induction of ornithine decarboxylase activity in neuroblastoma cells supplemented with serum could be mimicked by the addition of agents known to increase the level of cAMP. Work is in progress in our laboratories to determine the effects of bromoacetylcholine and bromoacetate on this cyclic AMP-dependent activation of ornithine decarboxylase.

Pösö and Jänne [10, 11] have shown that injections of 1,3-diaminopropane, a close structural analogue of putrescine, inhibited both the synthesis of DNA and the activity of ornithine decarboxylase in regenerating rat liver. Unlike the results with structural analogues of ornithine, in which enzyme activity was shown to be competitively inhibited *in vitro* [2, 31–33], their results indicated that 1,3-diaminopropane inhibited the synthesis of ornithine decarboxylase. In our experiments, this compound produced a marked inhibition of the growth of neuroblastoma cells at a concentration of 6.7×10^{-4} M. These results further indicate that ornithine decarboxylase activity is involved in the regulation of neuroblastoma cell growth.

In other studies of rapid growth processes, Costa *et al.* [34, 35] have shown that a tight temporal sequence of biochemical events occurs in drug-induced liver hypertrophy. The administration of inducing agents (e.g. Aroclor-1254, phenobarbital and 3-methylcholanthrene) causes: (1) an early activation of cyclic AMP-dependent protein kinase, (2) an increase in the activity of ornithine decarboxylase, (3) an elevation of RNA polymerase I activity, and (4) an increased liver weight to body weight ratio. Further studies are necessary to determine if neoplastic growth is associated with a similar sequence of enzyme inductions, but our results indicate that the drug-induced changes in ornithine decarboxylase activity were accompanied by changes in RNA and protein synthesis (Table 1). There is a need for future work to determine possible biochemical interactions of cytolytic agents such as bromoacetylcholine and bromoacetate with other steps in this growth sequence.

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