REGULATION OF ORNITHINE DECARBOXYLASE BY ODC-ANTIZYME IN HTC CELLS

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SUMMARY

Low concentrations of putrescine (10^{-5}M) blocked ornithine decarboxylase (ODC) in rat hepatoma (HTC) cells in culture, but the lower homologue of putrescine, 1,3 diaminopropane, had no effect on ornithine decarboxylase at 10^{-5}M. Higher concentrations of both putrescine and 1,3 diaminopropane induced approximately the same amount of soluble ODC antizyme type inhibitor. When concentrated dialyzed supernatants of cells grown in 10^{-5}M putrescine were treated with 250 mM NaCl and chromatographed on a superfine Sephadex G-75 column, both ODC and inhibitor were recovered. Spermidine, spermine and cadaverine also induced the inhibitor suggesting a low specificity of induction by amines.

INTRODUCTION

It has been demonstrated in growing KB cells (1), in lymphocytes (2), in regenerating rat liver (3,4), and in 3T3 cells (6), that putrescine and spermidine have a rapid blocking effect on induction of ornithine decarboxylase (L-ornithine carboxy-lyase EC 4.1.1.7) (ODC)[1]. Spermine (10^{-5}M) and spermidine (6×10^{-5}M) can block increased synthesis of ODC in rat hepatoma (HTC) cells as measured by enzyme specific immuno-precipitation techniques (7) suggesting a post-transcriptional control of ODC by these amines. Contrarily, Canellakis and his group have now reported finding a non-competitive ODC inhibitor, termed antizyme, in rat hepatoma (H-35) cells (8) as well as in mouse leukemia

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[1] Abbreviations used are: ODC, ornithine decarboxylase; HTC, hepatoma tissue culture.

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(L 1210) cells, and neuroblastoma cells (9) which appears in the presence of higher concentrations of putrescine, spermidine, and spermine. However, this ODC inhibitor was not measurable in 3T3 cells after similar treatment with putrescine (10).

To reconcile some of these seemingly disparate findings we investigated the role of polyamines in the control of ODC activity. Our results show that free ODC antizyme or ODC-inhibitor complex can be detected in HTC cell extracts even when these are grown in the presence of low concentrations of putrescine.

MATERIALS AND METHODS

Rat hepatoma (HTC)[1] cells were grown in spinner culture in Swim's S-77 medium (Gibco) to a high density ($9x10^5$/ml) and diluted into fresh medium containing 10% calf serum at the beginning of each experiment to a final concentration of 1.5×10^5/ml as previously described (11,17). Cycloheximide (50 µg/ml), Actinomycin D (1 µg/ml), ornithine (1 mM), and polyamines (10^{-5}M) dissolved in phosphate-buffered saline were added 12-13 hours after dilution, as indicated. Aliquots of cells in medium ($1.5x10^6$ to $3.0x10^6$ cells) were washed by centrifugation twice with phosphate buffered saline at 4°C, sonicated and then assayed for ODC by the method previously described (11). Since absolute enzyme activities varied from experiment to experiment, controls were always carried out using cells from the same spinner culture that was subjected to experimental manipulations. Cell protein was measured by the fluorescamine method (18). Data for variation in ODC activity were plotted semi-logarithmically as a function of time after addition of inactivator to cell cultures. The half life of decrease in enzyme activity was calculated from the slope obtained by linear least-squares regression using that portion of the curve where activity was decreasing. Disappearance of activities were measured in at least three different experiments. There was less than 10% variation among the individual determinations.

ODC-inhibitory activity was assayed as described by Canellakis and his co-workers (8,9) where one inhibitor unit is defined as an amount which will inhibit one unit of ODC activity i.e. 1 nmole CO_2/hr/mg protein. Each inhibitor value represents the mean of at least two separate determinations. There was less than 5% variation among the individual determinations.

Separation of ODC and inhibitor was carried out on a superfine Sephadex G-75 column (1.6x24 cm) equilibrated with assay buffer (30 mM sodium phosphate pH 7.0, 0.1 mM EDTA, 5 mM dithiothreitol, 0.1 mM pyridoxal phosphate) and bovine serum albumin (2 mg/ml). Supernatants from 100,000 x g centrifugation were prepared from concentrated sonicated cell extracts ($1.5x10^8$ cells in 10 ml) and extensively dialyzed in assay buffer at 4°C. Two ml of the supernatants were put on the column in the absence or presence of 250 mM NaCl (added to the supernatant sample and also to the column buffer). Fractions of 0.30 ml were collected and 0.10 ml was used for each assay of enzyme and inhibitor. The fraction number 0 (Fig. 3) is the end of the void volume, which was 12 ml.

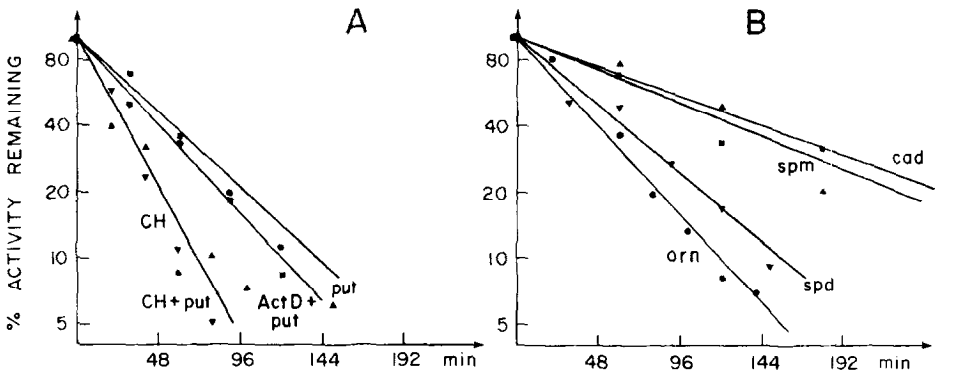

Figure 1. Rates of decrease in ODC activity in HTC cells caused by cycloheximide and polyamines.
A ▼, cycloheximide (50 µg/ml); ▲, cycloheximide (50 µg/ml) + putrescine (10^{-5}M); ●, Actinomycin D (1 µg/ml) + putrescine (10^{-5}M); ■, putrescine (10^{-5}M).
B ▼, spermidine (10^{-5}M); ■, spermine (10^{-5}M); ▲, cadaverine (10^{-5}M); ●, ornithine (1 mM).

RESULTS AND DISCUSSION

Putrescine added to HTC cells at a low concentration (10^{-5}M) caused a decrease of ODC activity. The half life of disappearance of ODC activity was 42 minutes and constant throughout the biphasic expression of ODC activity in the cell cycle (11). The effect of putrescine was independent of RNA synthesis. It was not blocked by the presence of 1 µg/ml of Actinomycin (Fig. 1) as was also previously reported in other cell systems (2,8). This confirms previous suggestions of a post-transcriptional control of ODC by putrescine (2,3,6,7,8). Exogenously added putrescine, therefore, might have a direct or indirect effect on enzyme activity or alter the degradative or synthetic rates of the enzyme.

Confirming previously published reports in other cell systems (6,7), we found putrescine to be a very weak competitive inhibitor of ODC from HTC cells with a $K_I = 2 \times 10^{-3}$M. Thus, it appears unlikely that 10^{-5}M exogenously added putrescine directly inhibits intracellular ODC. Furthermore, it is also unlikely that putrescine has an effect on ODC degradation. At a concentration of cycloheximide which blocks protein synthesis in HTC cells (14) (50 µg/ml), ODC half life was 22 minutes (Fig. 1), independent of

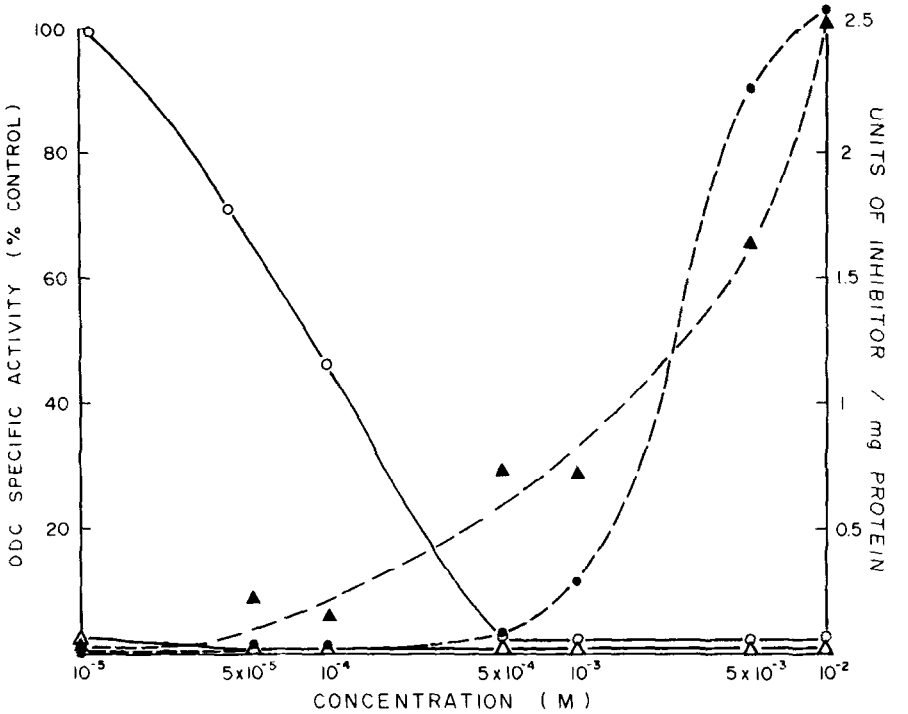

Figure 2. Dose response of varying concentrations of putrescine and 1,3 diaminopropane on cellular ODC, and on the induction of a soluble ODC inhibitor.
HTC cells were incubated for eight hours after induction of ODC by fresh medium. Then, they were incubated for an additional five hours in the presence or absence of increasing concentrations of polyamines. Aliquots of cells were taken and extracts were prepared and assayed for ODC as in Materials and Methods. Activities are expressed relative to the specific activity of control cells (5.7 nmoles CO_2/hr/mg protein) grown at the same time. Effect of putrescine Δ and 1.3 diaminopropane ○ on ODC activity, and the effect of putrescine ▲ and 1,3 diaminopropane ● on the appearance of ODC inhibitor.※

time after enzyme induction, contrary to a previous report (15).* This half life was not decreased by the presence of cycloheximide plus putrescine (Fig. 1) which was also the case in 3T3 cells (6).

In vitro incubation of rat hepatoma (H-35) cells (8,10) and other cell lines (9) with 10^{-2}M putrescine induced the formation

※ Assays for the inhibitor were also performed with both dialyzed HTC cell ODC and pH precipitated partially purified rat liver ODC (19) in combinations with dialyzed and non-dialyzed HTC cell extracts and the same results were obtained.

* Loss of ODC activity during inhibition of protein synthesis has been specifically correlated with loss of enzyme molecules in regenerating rat liver (13), where rapid decay of ODC activity observed after addition of cycloheximide was accompanied by a decrease in ODC immunoreactive protein.

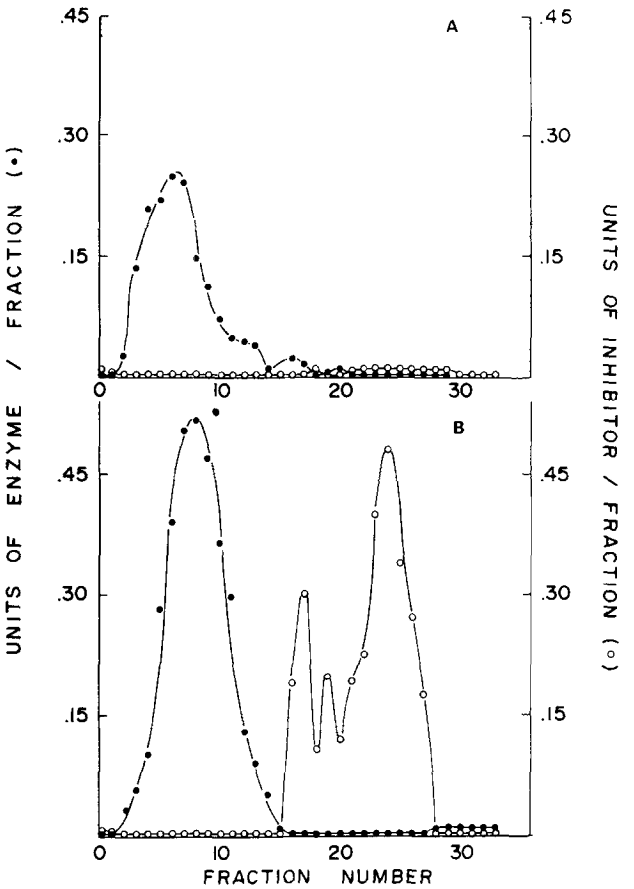

Figure 3. Chromatography of supernatant from HTC cells treated for 5 hours with 10^{-5}M putrescine after initial stimulation of ODC as in Fig. 2. Two ml of concentrated cell supernatants (3 x 10^7 cells) with approximately 5 units of residual ODC activity were layered onto a G-75 superfine Sephadex column and chromatographed in the absence (A) or presence (B) of 250 mM NaCl as described in Materials and Methods. (●), ODC activity; (○), inhibitor activity.

of a non-competitive ODC inhibitor protein. Putrescine induced the same inhibitory activity in rat liver (9). 1,3 Diaminopropane, the lower homologue of putrescine also blocked ODC in regenerating rat liver by an unknown mechanism (4,5). However, it seemed to us this could be also due to the induction of the ODC antizyme. Indeed, Fig. 2 shows the dose response of increasing concentrations of putrescine and 1,3 diaminopropane on intracellular ODC activity and on the appearance of free inhibitory activity in rat hepatoma (HTC) cells. After 5 hours, 10^{-5}M putrescine eliminated almost all intracellular ODC activity while 1,3 diaminopropane had no effect.

We prepared concentrated supernatants of cell extracts treated with 10^{-5}M putrescine and used Sephadex column chromatography after treatment of supernatants with salt (250 mM NaCl; H.G. Williams-Ashman, personal communication) to separate any inhibitor and ODC (9) which might be present as an inactive complex. Figure 3A shows that when 2 ml of supernatant ($3.0x10^7$ cells) were chromatographed in the absence of 250 mM NaCl only residual ODC from the supernatant was recovered. However, when 2 ml of the same supernatant was treated and chromatographed in the presence of 250 mM NaCl, three units of inhibitor and somewhat more ODC was recovered (Fig. 3B). This indicates that even at 10^{-5}M putrescine the ODC inhibitor is formed and is present in HTC cells.

Next we tested specificity of the regulation of ODC by low concentrations of other polyamines. Spermidine (10^{-5}M) caused loss of ODC activity similar to that caused by putrescine (Fig. 1). Spermine, and cadaverine, a polyamine not generally found in mammalian cells, both reduced ODC activity although at a much slower rate (Fig. 1). Ornithine (1mM) was effective (Fig. 1) contrary to a previous report (15), but its effect possibly was via conversion to putrescine. In other experiments we found $2x10^{-3}$M spermidine, spermine and cadaverine all induced 1.0-1.5 units of inhibitor/mg protein, suggesting a relatively low specificity of induction of this inhibitor by amines. It also appears unlikely that putrescine and spermidine regulate ODC only because of their cationic properties. It was reported (12) that 10^{-2}M of various cations (Mg^{++}, K^+, Na^+) blocked induction of ODC in rat hepatoma (H-35) cells, L 1210 and neuroblastoma cells. We found 10^{-5}M and $2x10^{-3}$M MgCl had no effect on pre-induced ODC (5.4 nmoles CO_2/hr/mg protein) in HTC cells, while 10^{-2}M $MgCl_2$ decreased ODC activity by only 40% five hours after addition, suggesting a different regulatory mechanism altogether for these cations.

We therefore confirm results in other cell systems (8,9,10) and in rat liver (9) by finding a soluble ODC inhibitor in HTC cells, and show that it can also be induced by 1,3 diaminopropane as well as by other polyamines. The fact that no free ODC inhibitor was found in 3T3 cells (10) can be explained by the fact that it could not exist in free form while ODC activity

is still measurable. However, the question still remains as to whether or not induction of the inhibitor is the only mechanism by which polyamines can regulate ODC. A preliminary report has shown that polyamines can block ODC synthesis as demonstrated by measurement of immunoprecipitable enzyme protein (7).

Finally, do both putrescine and spermidine actually intracellularly regulate ODC? The fact that exogenously added ornithine itself blocks ODC, probably by conversion to polyamines, might suggest that this is the case. Also the levels of putrescine correlate with ODC activity at various points in the cell cycle (11,17). Induction of the enzyme can occur when there are considerable amounts of polyamines present (16). This suggests perhaps that some compartmentalized pool of polyamines can regulate ODC either at the level of its synthesis or can control its catalytic activity via the induction of an inhibitory protein.

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