The Modulation of the Induction of Ornithine Decarboxylase by Spermine, Spermidine and Diamines

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ABSTRACT Extremely low concentrations of putrescine, spermidine and spermine added to the extracellular medium of cultures of mammalian cells inhibit the induction of ornithine decarboxylase activity despite 100- to 1,000-fold greater intracellular polyamine concentrations. The diamines, 1,2-diaminoethane, 1,3-diaminopropane, 1,5-diaminopentane, 1,7-diaminooctane, 1,10-diaminodecane, 1,12-diamindodecane also inhibit ornithine decarboxylase at all concentrations tested (greater than 10^-7 M). In contrast, 10^-4 M to 10^-6 M 1,8-diaminooctane, the alkR analog of spermidine, enhances ornithine decarboxylase activity.

The concentration of putrescine required to inhibit the activity of ornithine decarboxylase by 50% is a characteristic of each cell line; however, it varies by as much as 1,000-fold among the five cell lines we have tested (L1210 leukemia, H35 hepatoma, N18 neuroblastoma, W256 carcinosarcoma and 3T3 fibroblasts). The antizyme to ornithine decarboxylase can be induced in all these cells by high (di)(poly)amine concentrations.

Based on these and other experiments we suggest a working hypothesis that the polyamines regulate ornithine decarboxylase activity through two different sites that may be interrelated; a sensitive membrane-mediated site that responds to minute fluctuations of extracellular polyamine levels and a more-coarse site which may be intracellular or membrane associated that responds to larger fluctuations of intracellular polyamine levels. The consequences of such a control mechanism operating within the whole organism are discussed.

The physiologically occurring (di)(poly)amines, putrescine, spermidine, and spermine, inhibit the activity of ornithine decarboxylase (ODC) in mammalian cells in cell culture (Pett and Ginsberg, '86; Kay and Lindsey, '73; Clark and Fuller, '75; Fong et al., '76; Helfer et al., '76; McCann et al., '77) and in the rat liver in vivo (Janné and Hóvta, '74; Pass and Janné, '76a). We have shown that they induce the synthesis of a non-competitive protein inhibitor of ODC (Fong et al., '76; Helfer et al., '76). Because of the specificity of this protein and its apparent ubiquity, we have suggested the name ODC antizyme; we furthermore proposed that the interplay between the physiological (di)(poly)amines, the ODC antizyme and ODC could participate in a sensitive modulation of intracellular ODC activity (Helfer et al., '75; Canelakis et al., '76).

The induction of ODC antizymes by other cells in culture as well as in rat liver has been confirmed and extended by other laboratories (McCann et al., '77; Jefferson and Pepp, '77).

The synthetic analogues, 1,3-diaminopropene and 1,6-diaminohexane are also effective inhibitors of intracellular ODC activity (Pass et al., '77; Pass and Janné, '76b; Janné and Hóvta, '74; Cuna and Janné, '77). In order to investigate the mechanism of this inhibition and to detail further the mechanism of the regulation of ODC activity we have examined the effect of spermidine and spermine and of a series of diamines, of the general structure \( \text{NH}_2\big[\text{CH}_2\big]_n\text{NH}_2 \) (where \( n = 2,3,4,7,10,12 \)), on the induction of ODC activity and on the induction of the ODC antizyme. A preliminary

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report of these results has been presented (Heller et al., '77).

**MATERIALS AND METHODS**

L1210 cells were grown in Fischer's medium plus 10% horse serum as described previously (Tsai et al., '79). The cells were grown to the plateau phase (9.5 x 10^6 cells/ml) and then diluted with fresh medium plus 10% horse serum, to about 3 x 10^6 cells/ml. The neutralized 5,6-Diamino-2,3-dihydrophosphonate, or polyamine, was added to 15 ml portions of the diluted cells to give the final concentrations indicated in the figure legends. After a 4-hour incubation at 37°C, the cells were harvested by centrifugation and washed twice with phosphate-buffered saline. The cell pellets were resuspended in 0.5 ml of assay buffer (10 mM Tris-HCl, 0.1 mM EDTA, 5 mM dithiothreitol and 0.05 mM pyridoxal phosphate) pH 7.2 rapidly frozen-thawed twice and dialyzed overnight against 10 mM Tris HCl, 0.1 mM EDTA, 0.05 mM pyridoxal phosphate, 5 mM dithiothreitol, final pH 7.2 at 4°C. The contribution little or no ODC activity during dialysis.

H-35 rat liver hepatoma cells were grown in Eagle's minimal essential medium supplemented with 10% fetal calf serum; ODC activity was induced by starvation for serum overnight and refedding with medium supplemented with 10% fetal calf serum as previously described (Fong et al., '76; Heller et al., '76). N-18 mouse neuroblastoma cells were grown in Dulbecco's medium supplemented with 10% fetal calf serum: ODC activity was induced with prostaglandin E and 3,4,5-dihydroxy-6-n-butylamine.

**Enzyme reactions were carried out in 17-mm x 108-mm polystyrene culture tubes (Falcon 2057), sealed with polyethylene caps. A 1/2 filter paper disc (Schleicher and Schuell, Inc., New Hampshire) impregnated with 0.1 ml of NCS (Amersham-Buchler) diluted 1:1 with toluene, transferred with an 18-gauge syringe needle through the cap was present during the incubation. The reactions contained 0.15 M of the dialyzed cell homogenate, 0.015 ml of substrate 0.888 µmol, 0.75 µCi/mole DL-2,4-Diaminobenzoic acid and 0.01 ml of 0.5 M Tris-HCl buffer; final pH was 7.2 at 37°C. The reaction was incubated for one hour at 37°C, stopped by injecting 0.1 ml of 10% trichloroacetic acid into each reaction tube. The reaction was stopped and incubated at 37°C for an additional 1 hour. The liberated radioactive CO_2 was determined by placing the paper disc in 8 ml of ACS (Amersham-Buchler) scintillation fluid and counting in a Packard Tri-carbon liquid scintillation spectrometer. The values presented in the figures represent the averages of duplicate samples of two to four experiments with standard deviations of less than 10%.

ODC activity in the cell homogenate was assayed by adding known amounts of partially purified rat liver ODC activity to portions of the homogenate and measuring the recovery of this added activity. The inhibition, as previously described (Fong et al., '76; Heller et al., '76), is defined as that amount which inhibits one unit of the latter is equivalent to 1 nm per 60 minutes. The (di)polyamines (B. Chemical Corp.) were purified scale using 20 cm x 100 plates 6 F-254 (Eastman-following thermographic aminomethane, 1,3-diaminopropane, amorphous n-butyl acryclic acid: water (30:20:50:50 no:ethanol) in n-butyric acid: water (40:10:50:50; spectrorime in chloroform: methanol (55:40:50:50). The relevant species and the amines used in the experiments are described in the text.
recovery of this added activity and therefore the inhibition, as previously described (Fong et al., '76; Helfer et al., '76). One unit of inhibitor is defined as that amount of inhibitor which inhibits one unit of ODC activity; this latter is equivalent to 1 n mole of CO\textsubscript{2} released per 90 minutes.

The (di/poly)amines (300 mg) (Aldrich Chemical Corp.) were purified on a preparative scale using 20 cm \times 20 cm silica gel plates 60 F-254 (Eastman-Kodak Co.) in the following chromatographic solvents: (1,2-di-aminoethane, 1,3-diaminopropane and 1,5-di-amino-pentane) in n-butyl alcohol: pyridine: acetic acid: water (30:20:6.24:0.18), (1,7-diaminoheptane) in n-butyl alcohol: acetic acid: water (40:10:50 v/v); spermidine and spermine in chloroform: methanol: acetic acid: water (55:40:5:5 v/v). The relevant amine bands were eluted and the amines crystallized. We were unable to resolve the impurities in spermidine and spermine with some batches of these plates for no apparent experimental reason. Putrescine was purified by vacuum distillation.

RESULTS

In each of the following experiments, the induced value of ornithine decarboxylase (ODC) activity four hours after dilution of stationary L1210 cells (MATERIALS AND METHODS) is taken as 100% while the 0% value represents no detectable ODC activity; the numerical correspondence to each 100% value is provided in the legend of each figure.

The addition of the physiologically occurring (di/poly)amines to the medium at the time of dilution of L1210 cells affects the induction of ODC activity as shown in figure 1. Their common characteristic is to cause a precipitous fall of ODC activity at concentrations between 10^{-5} and 10^{-4} M. Spermidine, added at an external concentration as low as 5 \times 10^{-6} M, causes a nominal 5% inhibition of the induction of ODC activity; this inhibition becomes accentuated as the external concentration of spermidine is raised. The presence of 10^{-4} M spermine inhibits the induction of ODC activity by 40% while 10^{-3} M putrescine is required to cause a similar inhibition.

Radioactive putrescine (10^{-5} M) added to the medium following dilution of stationary L1210 cells is concentrated ten-fold, after a 4-hour exposure, resulting in an intracellular concentration of 6 \times 10^{-5} M radioactive putrescine. These increases in the intracellular concentrations of putrescine and of spermidine are well below the total extractable levels of spermidine and
putrescine contained in the L1210 cells under these conditions. These intracellular concentrations approximate $1 \times 10^{-3} \text{ M}$ for spermidine and $1.5 \times 10^{-3} \text{ M}$ for putrescine.

Consequently, the existing intracellular spermidine and putrescine levels of L1210 cells are several orders of magnitude greater than the amount that has been concentrated by the cells from the extracellular medium. Therefore, the decrease in ODC activity following the addition of minute concentrations of these amines to the medium is not the result of a substantial decrease of the intracellular polyamines from polyamines in the medium.

On the other hand, a corroboration relationship between alterations in the intracellular putrescine concentration and ODC activity can be detected, as shown in figure 2. When ODC activity is induced in N-16 neuroblastoma cells, the time sequence of increase in ODC activity shows the attainment of a maximum ODC activity which then decreases as the intracellular putrescine concentration increases (fig. 2). Such tautomer changes have been obtained both in intact animals (Hayaishi et al., '71) as well as in cell culture (Maurieski et al., '79). It appears that the decrease in ODC activity may be related to the increase in intracellular putrescine concentration.

Examination of figure 1 shows that a striking characteristic of the inhibition of ornithine decarboxylase activity by spermine, in contrast to that incurred by putrescine, is that over a broad range of extracellular spermine concentrations, only relatively minor fluctuations of ornithine decarboxylase activity can be detected. This effect is most prominent for spermine and for this reason we have termed it the "spermine effect" (Canellakis et al., '76). A qualitatively similar response of ornithine decarboxylase activity occurs in response to a 500-fold range of extracellular spermidine concentrations. In this case, the plateau level of the inhibited ornithine decarboxylase activity is maintained at much lower, but substantially above zero, levels.

The "spermine effect" is also reproduced by the addition of L-4-diaminopropionate and L-4-diaminobutyrate to the diluted medium of L1210 cells. Figure 3 shows that after an initial inhibition of ornithine decarboxylase activity at low concentrations of these two diamines, less than $10^{-3} \text{ M}$, there is a maintenance of $
Fig. 3 The effects of varying concentrations of 1,2-diaminocyclohexane, G. ( ), 1,3-diaminopropane, C. ( ), 1,4-diaminobutane, G. ( ), 1,6-diaminohexane, G. ( ), and 1,7-diaminoheptane, G. ( ) added to the medium on the induction of GDC activity in L1210 cells. The 100% value for GDC activity is 20.9 mmol/hr. The experimental details are as described in MATERIALS AND METHODS.

Fig. 4 The effects of 1,6-diaminohexane, G. ( ), 1,7-diaminoheptane, G. ( ), and 1,12-diaminododecane C. ( ) added to the medium on the induction of GDC activity in L1210 cells. The 100% value for GDC activity is 21.1 mmol/hr. The experimental details are as described in MATERIALS AND METHODS.
constant ODC activity over a 100- to 1,000-fold range of added diamines; further increases of these two diamines result in complete inhibition of ODC activity. 1,2-Diaminoethane, 1,5-diamino-2-pentane, 1,10-di-aminodecane and 1,12-diaminododecane inhibit the induction of ODC activity without eliciting the "spermine effect" (figs. 3, 4). The enhancement of ODC activity that is elicited by concentrations of 1,8-diaminooctane over a 1,000-fold range, from 1 × 10^(-3) M to 1 × 10^(-5) M is notable (fig. 3). This observation is particularly interesting in that 1,8-diaminooctane is the alkyl analog of spermine, and because spermidine in these concentration ranges is extremely inhibitory. The next closest analogs that we have studied, 1,7-diaminohexane and 1,10-diaminodecane, produce only inhibitory effects. The determination of the site and the mechanism of action of 1,8-diaminooctene should provide interesting information on the regulation of ornithine decarboxylase.

The detailed mechanism through which low concentrations of di(poly)amines inhibit the induction of ODC activity remains to be established. One, as yet unproven possibility, is that this occurs through the induction of ODC anti-enzyme. On the other hand, the experimental evidence is clearly definitive that higher concentrations of these amines will induce the appearance of free ODC anti-enzyme. The concentrations of di(poly)amines at which ODC anti-enzyme is detected in L1210 cells range from 5 × 10^(-5) M to spermine to 1 × 10^(-2) M for 1,8-diaminooctane. The appearance of free ODC anti-enzyme can be detected in the cell extracts where the added amines have produced a total inhibition of ODC activity.

For each of these diamines and for each of the cell lines described below, the free ODC anti-enzyme was characterized: (a) as a non-competitive inhibitor of ODC; (b) by its molecular weight (Sephadex gel filtration) 26,500 ± 2,000; (c) by its general properties which were comparable to those previously reported (Fong et al., '76; Heller et al., '76); (d) by heat labile, sensitive to chymotrypsin and to trypsin but not to RNase and/or to DNase. It was assayed by adding partially purified rat liver ODC to extracts of the cells and measuring the subsequent extent of inhibition of ODC as previously described (Fong et al., '76; Heller et al., '76; MATERIALS AND METHODS). Under standardized conditions of exposure of L1210 cells to 10 mM "poly"-diamines, the amount of free ODC anti-enzyme formed is presented in table 1. These values are presented only as evidence that the ODC anti-enzyme is formed subsequent to the exposure of L1210 cells to the diamines and not as a measure of the relative potential of these "poly"-diamines to produce ODC anti-enzyme. Such a quantitation will require more detailed experiments, including variable times of exposure, "poly"-amine concentration curves, etc. Because of their insolubility, the higher, "poly"-diamines were not tested for their ability to produce ODC anti-enzyme.

We have detected the production of ODC anti-enzyme in all cell lines tested so far, i.e. L1210, P388, W256, neuroblastoma N/1, rat hepatoma H35 and mouse 3T3 fibroblasts. Figure 5 shows the extent of production of ODC anti-enzyme by 3T3 cells with time of exposure to 10 mM putrescine. It is apparent that free ODC anti-enzyme can be detected subsequent to the total inhibition of ODC activity; ODC anti-enzyme then progressively increases in total units. It has been claimed that these cells do not produce free ODC anti-enzyme (Clark and Fuller, '76). However, figure 5 emphasizes that unless the time of exposure to the inducer, i.e., time, concentration, etc., are such as to eliminate the ODC activity completely, it is patently impossible to detect the existence of the free form of ODC anti-enzyme, a non-competitive inhibitor of ODC. The inhibitory effect of increasing concentrations of putrescine on the induction of ODC activity in rat sarcoma 180 W-256, leukemia L1210, 3T3 fibroblasts, neuroblastoma N-18 and hepatoma H-35 cells is shown in figure

<table>
<thead>
<tr>
<th>Compound</th>
<th>ODC anti-enzyme formed (%)</th>
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<tbody>
<tr>
<td>Putrescine</td>
<td>50</td>
</tr>
<tr>
<td>Diaminooctane</td>
<td>30</td>
</tr>
<tr>
<td>Diaminopentane</td>
<td>10</td>
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<tr>
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<td>5</td>
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<tr>
<td>Diamino-2-hexane</td>
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Fig. 3 Time course of ODC activity in L1210 cells after induction with 10 mM putrescine (10 mg/ml). The cells were plated at 100,000 cells per ml in RPMI 1640 medium supplemented with 10% FBS and induced with 10 mM putrescine. The free ODC activity was determined at various time points.

Fig. 6 The effect of putrescine on ODC activity in L1210 cells. The values are the mean ± SD of four determinations. The experiments were performed in triplicate. The mean values were determined for each time point.

The experiment was performed as described in MATERIALS AND METHODS.
Fig. 5 Time course of the appearance of ODC enzyme activity following the exposure of 3T3 cells to 10 mM pyruvate. 3T3 cells were starved for serum overnight; at time zero the medium was replaced with medium supplemented with 10% fetal calf serum plus 10 mM pyruvate. At the time indicated, cells were harvested with trypsin as described by Leuchten (74) and the cell pellet resuspended with phosphate-buffered saline. The pellet was frozen thawed with 0.1 M Tris-glycine buffer and the homogenate reextracted at 0.005% Triton X-100. ODC activity in the supernatant was assayed as described by Feng et al. (73) and Heller et al. (136). One unit of ODC activity equals 1 nmol of CO2 released per hour and one unit of ODC activity corresponds to the inhibition of one unit of ODC activity.

Fig. 6 The effect of varying concentrations of pyruvate on the induction of ODC in L1210 R, W-356 C3H, B-106 in 3T3 cells. The 100% values for ODC activity are 4.55 nmol/mg hr, W-356: 14.1 nmol/mg hr, L-1210: 20.5 nmol/mg hr, B-106: 4.5 nmol/mg hr. The experimental details are as described in MATERIALS AND METHODS.
6. These results emphasize that there is a 1,000-fold increase in the putrescine concentrations necessary to produce 50% inhibition of the induction of ODC activity in these two different cell lines; most sensitive to inhibition are the L1210 cells while the 3T3 fibroblasts proved to be the most resistant.

DISCUSSION

These results suggest that a cellular homeostatic mechanism for the control of ODC activity exists in cells. It is emphasized by the sensitivity of the induction of ODC activity to minute variations of extracellular concentrations of spermidine in the face of high intracellular concentrations of spermidine. This apparent contradiction could be accommodated for by assuming that different cellular sites exist for the control of ODC activity.

Some experimental evidence exists for the presence of membrane-associated sites that affect ODC activity. Chen et al. (75) have shown that agents known to affect the membrane via the cytoskeleton (colchicine, cytochalasin, vinblastine) inhibit the induction of ODC, rabbit activator prepared against L1210 plasma membranes inhibit the induction of ODC (K. Y. Chen and E. S. Canellakis, unpublished results); furthermore, Quash et al. (76) have reported that putrescine may be associated with sites on the surface of embryonic cells.

Based on these results, we suggest as a working hypothesis that there is a homeostatic mechanism for the control of ODC activity. At low levels of extracellular polyamines, the induction of ODC activity can be inhibited through sensitive membrane-mediated sites. This decrease in ODC activity results in a decreased rate of putrescine synthesis; this decreased rate of putrescine synthesis may elicit an increase in the activity of ODC. The existence of such a counterregulatory feedback would explain the presence of a constant level of ODC activity over a range of inhibitory extracellular spermidine and spermine concentrations; we have referred to this buffering region as "the spermine effect." When the extracellular putrescine concentrations are further increased, raising at the same time the intracellular polyamine concentrations, the second site which may be either intracellular or membrane associated becomes predominant. The cell now becomes geared toward minimizing the further synthesis of polyamines consequent on the trend would now be to limit any further increase in ODC activity; in fact, to inhibit the activity of ODC. It is possible that, at this time, the ODC antizyme comes into play. However, it is also possible that these mechanisms, at all times, act through the induction of ODC antizyme, and that the appearance of a free ODC antizyme is only the extreme consequence of maximal titration of ODC by non-competitive inhibitor. Such a two-site hypothesis fits both the inhibition of ODC activity by minute amounts of extracellular ODC as well as the decay of the induced ODC activity that occurs when the intracellular polyamine concentration increases.

A considerable and definitive body of literature emphasizes that ornithine decarboxylase activity fluctuates and is inducible in a number of tissues in vivo (Tabor and Tabor, 74; Moise and Fillingame, 74; Tabor and Tabor, 76; Sefton and Janas, 75; Hopkins et al., 73; Hanafi et al., 72; Tanagi et al., 72). The range of putrescine concentrations necessary for 50% inhibition of the induction of ODC activity in the various cell lines and the differential response of the L1210 cells to a variety of a,omega-disamines indicates a differential sensitivity which is probably not unique to cells in culture; it is likely that it is also represented among the normal cells of different tissues (kidney, liver, spleen, etc.). Furthermore, the experimental evidence indicates that the levels of spermidine in human lymphocytes are in the range of the 1 x 10^-14 to 10^-13 M (Benett et al., 76; Lundgren et al., 76) and in the range of 1 x 10^-13 M in human serum (Bartoe et al., 72). These two values are approximately 5 and 50 times higher than the concentrations required for 50% inhibition of ODC activity in L1210 cells, our most sensitive line.

It is therefore apparent that the normal human serum levels of spermidine are such that they could tend to minimize the induction of ODC activity in cells. However, if normal or tumor cells synthesize excessive amounts of polyamines, there will occur an additional stimulation of the level of polyamines. This increase in the serum level of polyamines may act to further inhibit the induction of ODC activity in both the high polyamine producing cells as well as in the large mass of non-tissue. As a consequence of this inhibition of ODC activity, a decreased output of these tissues will occur. This net level of urinary polyamine is therefore not simply an indication of production of polyamines by a cell, it also mirrors the absolute mass of non-tissue mass to respond in serum polyamine levels, by output of polyamines. The direction will depend on the mass of non-tissue mass to respond in ODC to respond to the increase in urinary polyamine levels.

If we accept the that these paper are not peculiar to cells in culture, but have certain cells of an organism, it becomes the urinary output cannot be of a high polyamine product, Bouch, 76; Durie et al., 77; Bartoe, 71; Russell et al., 77; Towseman et al., 76. The urinary output will vary depending on the ability of an individual tissue to carboxylate to respond to urinary polyamines. This would provide compensation for the production and would be a variable output.

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fore not simply an indication of the high level of production of polyamines by a discrete tissue; it also mirrors the ability of the large mass of host tissues to respond to the increase in serum polyamine levels, by decreasing their output of polyamines. The degree of compensation will depend on the mass of available tissue, on the differential sensitivity of ODC in various tissues and on the ability of the tissue ODC to respond to the increased serum polyamine levels.

If we accept that the results presented in this paper are not peculiar characteristics of cells in culture, but have counterparts in the cells of an organism, it becomes apparent that the urinary output cannot be a reliable index of a high polyamine producing tumor (Bachrach, '70; Durie et al., '77; Lipton et al., '76; Russell, '71; Russell et al., '71; Russell, '77; Townsend et al., '76). The urinary polyamine output will vary depending on the differential ability of an individual tissue's ornithine dehydrogenase to respond to the serum polyamine levels. This would result in a differential compensation for the increased polyamine production and would therefore result in an unpredictable and variable urinary polyamine output.

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