PROTEIN INHIBITOR OF ORNITHINE DECARBOXYLASE DOES NOT ACCOUNT FOR EFFECT OF PUTRESCINE ON 3T3 CELLS

Jeffrey L. Clark and Joyce L. Fuller

Department of Biological Chemistry
University of California
Irvine, California 92717

Received October 7,1976

SUMMARY

Putrescine and other amines are known to rapidly reduce or prevent increases in ornithine decarboxylase activity in a number of systems. We have confirmed reports of a nondialyzable inhibitor of the enzyme in serum-starved H-35 hepatoma cells exposed to serum and putrescine. In contrast, we detected little if any nondialyzable inhibitor in serum-limited Swiss 3T3 cells treated similarly. Also, evidence of a dissociable enzyme-inhibitor complex was found in H-35 cells but not in 3T3 cells. These results suggest that assimilated putrescine can reduce ornithine decarboxylase activity by mechanisms not involving a macromolecular inhibitor.

Ornithine decarboxylase (EC 4.1.1.17), the first enzyme in polyamine biosynthesis, has an activity half-life far shorter than that reported for any other mammalian enzyme (1). This property renders the enzyme uniquely suited for control by mechanisms which change the amount, rather than activity, of the enzyme (2). Immunological evidence that enzyme protein varies with total activity has recently been obtained with regenerating rat liver (3,4) and HTC cells (4); thus regulation at the level of enzyme concentration is established.

However, there are indications that the activity of preformed enzyme is also modulated. Although no small molecule effectors (besides polyamines) have been identified, multiple forms of the enzyme have been detected in Physarum polycephalum (5), 3T3 cells (6), and rat liver (7). In Physarum, the forms are to some degree interconvertible (5), and in 3T3 cells their relative abundance changes with the growth state (6). Also, Canellakis and coworkers have reported a protein inhibitor (M.W. 26,500) of ornithine decarboxylase. This

inhibitor (called "antizyme") is produced in stimulated cultures of serum-starved H-35 hepatoma (8) and other (9) cell lines which are simultaneously exposed to 5-10 mM putrescine, spermidine, or spermine. Such an inhibitor was not detected by us (10) or other investigators in earlier studies employing a variety of systems treated with exogenous polyamines. We have now examined several cell lines more closely in an attempt to access the importance of this mode of ornithine decarboxylase regulation.

MATERIALS AND METHODS

Unless otherwise indicated, Swiss 3T3 cells were grown to confluency as previously described (10,11) and then cultured in medium containing 0.5% calf serum for 24 hr before addition of 10% calf serum and 10 mM putrescine (final). Cultures of polyoma-transformed 3T3 cells were handled similarly, except they were placed in serum-free medium when at very high density. H-35 hepatoma cells, a gift of Dr. Francis Kenney, Oak Ridge, were cultured and serum-starved as described by Fong et al. (8) before 10% fetal calf serum and 10 mM putrescine addition. Human foreskin fibroblasts of 20-30 passages were cultured similarly except that calf serum was used. Synthetic media were from Flow Laboratories and sera were from Irvine Scientific.

Three hours after serum and putrescine were added, all cell lines were harvested and processed as previously described for 3T3 cells (11), except that the buffer was 50 mM Tris-HCl, pH 7.2, 0.1 mM EDTA, 0.05 mM pyridoxal-5'-phosphate, 5 mM dithiothreitol, and 50 mM KCl. The 30,000 xg supernatant solution was usually dialyzed against a 500-fold excess of the same buffer before use as the source of ornithine decarboxylase and inhibitor.

1.5 x 28 cm columns of Sephadex G-75 equilibrated with harvest buffer were calibrated with blue dextran, bovine serum albumin (BSA) (67,500), chymotrypsinogen A (25,000), and cytochrome c (12,400). Supernatant solution containing 6 to 10 mg of protein was concentrated to 0.5 ml (Amicon Minicon) before layering. Flow rates were 3 to 4 ml/hr and 0.52 ml fractions were collected. When supernatant solution was made 20% with $(NH_4)_2SO_4$ before chromatography in order to dissociate the inhibitor/enzyme complex (9), 6 ml of buffer containing 10% $(NH_4)_2SO_4$ was passed into the column immediately before the treated extract was applied (9). Column fractions were assayed for enzyme and inhibitor immediately after collection of 30 ml.

Ornithine decarboxylase was assayed by CO_2 capture (10) or by a new ion-exchange paper assay (12). Inhibitor was assayed in similar fashion except that 1.8 units[1] of 3T3 ornithine decarboxylase purified 30 fold from cell extracts by affinity chromatography (unpublished) were added.

RESULTS

When extracts from serum-stimulated 3T3, polyoma-transformed 3T3, or human fibroblasts were assayed for ornithine decarboxylase in combination with extracts from homologous putrescine-treated cultures (which contain negligible

[1] One unit of enzyme is that amount which catalyzes the release of 1 nmol CO_2 or putrescine/hr. One unit of inhibitor inhibits one unit of enzyme.

TABLE 1

NONDIALYZABLE INHIBITION OF ORNITHINE DECARBOXYLASE ACTIVITY IN VARIOUS CELLS*

Extract	Treatment	Activity**	Inhibition***
Swiss 3T3	none (a)	0.65	
	calf serum (b)	15.6	
	serum + putrescine (c)	0.44	
	1/2 (b) + 1/2 (a)	8.32	0%
	1/2 (b) + 1/2 (c)	7.86	2%
Human Fibroblasts	none (a)	0.51	
	calf serum (b)	1.26	
	serum + putrescine (c)	0.08	
	1/2 (b) + 1/2 (a)	1.08	0%
	1/2 (b) + 1/2 (c)	0.71	0%
H-35 Hepatoma	none (a)	0.09	
	fetal calf serum (b)	4.85	
	serum + putrescine (c)	< 0.01	
	1/2 (b) + 1/2 (a)	2.32	6%
	1/2 (b) + 1/2 (c)	1.07	55%
Polyoma-Transformed Swiss 3T3	none (a)	2.1	
	calf serum (b)	22.5	
	serum + putrescine (c)	1.0	
	1/2 (b) + 1/2 (a)	11.6	6%
	1/2 (b) + 1/2 (c)	13.1	0%

* Extracts were dialyzed against a 500-fold excess of buffer and assayed alone or together. Each value is the average of nine assays from three experiments.

** Units per mg of soluble protein.

*** The percent of the activity in extract (b) inhibited by the companion extract, assuming no reciprocal inhibition or activation.

enzyme activity), occasionally less than additive activity was seen (data not shown). However after dialysis little or no inhibition remained (Table 1). The dialyzable inhibitor is presumably putrescine assimilated by the cells (10). In contrast, substantial inhibitory activity (1.4 units/mg soluble protein) remained after dialysis of H-35 cell extract (Table 1), in confirmation of the findings of Canellakis and coworkers (8,9). Results were the same for all cell lines whether or not putrescine-treated cultures were also stimulated with serum.

In attempts to see if under other conditions inhibitor could be detected

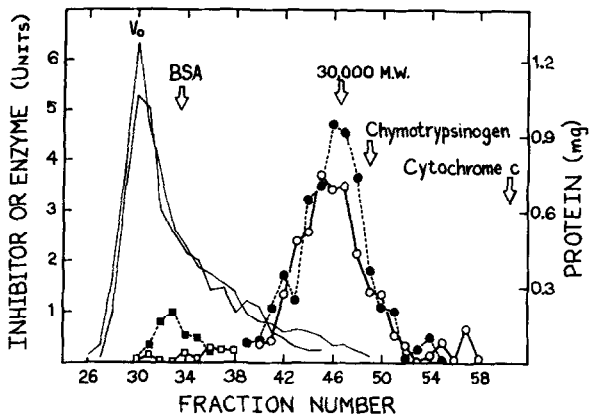

Figure 1. Sephadex G-75 chromatography of extract from putrescine-treated and stimulated H-35 cells. Concentrated extracts from 2 to 4 x 10^7 cells were divided into two equal aliquots. One portion (open symbols) was layered directly on a G-75 column and chromatographed (see Methods). The other (closed symbols) was treated with $(NH_4)_2SO_4$ before layering, as described in Methods. The data is a composite of three experiments. Inhibitor, circles; ornithine decarboxylase, squares. The smaller of the protein peaks (light lines) is for the salt-treated extracts.

---

in 3T3 cells, various sera and pituitary growth factors (11) were used singly and in combination as stimulants, with or without prior serum limitation. Also, extracts from cells exposed to putrescine and/or stimulant(s) for up to ten hours were used since the maximal enzyme activity in stimulated 3T3 cells occurs several hours later than in H-35 cells (8,11). All of these variations were carried out with 3T3 cells at subconfluent as well as confluent densities. No evidence of nondialyzable inhibitor was found.

When extract from putrescine-treated H-35 cells was chromatographed on Sephadex G-75, 2.3 to 3.7 units/mg of inhibitor were recovered in three experiments (Figure 1, open circles). This is more than the average 1.4 units/mg found in the mixing experiments (Table 1). As enzyme activity was still not detectable (Figure 1, open squares) this higher yield does not seem to be due to dissociation of an enzyme-inhibitor complex.

Heller et al. (9) have shown that the inhibitor-enzyme complex can be

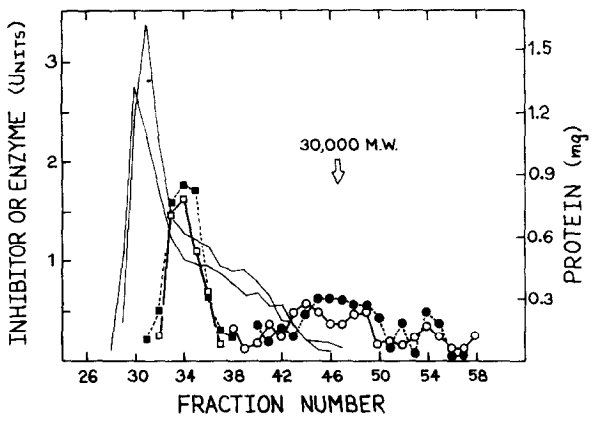

Figure 2. Sephadex G-75 chromatography of extract from putrescine-treated and stimulated 3T3 cells. See legend to Fig. 1 and Methods.

---

dissociated by treatment with 20% $(NH_4)_2SO_4$ and the components resolved on Sephadex G-75. We used this method to demonstrate that in extract from putrescine-treated H-35 cells, additional inhibitor (0.7 units/mg) was apparent after $(NH_4)_2SO_4$ treatment and that there was now a measurable amount (0.4 units/mg) of enzyme activity (Figure 1, solid symbols). As this is about 12% of the enzyme activity which is found in chromatographs of extract from stimulated cultures not containing added putrescine (data not shown), it appears that at least part of the decrease in enzyme activity in H-35 cells upon putrescine treatment is due to macromolecular inhibition of preformed enzyme.

Little or no inhibitor was detected by chromatography of the analogous 3T3 cell extract, with or without prior 20% $(NH_4)_2SO_4$ treatment (Figure 2). In other experiments, use of 10% or 30% $(NH_4)_2SO_4$ gave similar results. There was additional enzyme activity in chromatographed salt-treated 3T3 cell extracts, but this increment ($\sim$ 0.15 units/mg soluble protein) was small when compared to the enzyme activity (12.1 units/mg) present in chromatographs of extract from stimulated but nonputrescine-treated cells (not shown).

DISCUSSION

In 1968 Pett and Ginsberg (13) observed that ornithine decarboxylase activity in human KB cells rapidly decreased when the cells were cultured in 1 mM

putrescine. Schrock et al. (14) observed a similar phenomenon with rat liver *in vivo* in 1970. Since that time, additional work with liver and cultured cells has suggested that putrescine, other diamines, and polyamines reduce enzyme activity by a post-transcriptional mechanism which does not involve direct inhibition by the amines (15,16,11,10,4,17). We have postulated that polyamines inhibit translation of the enzyme (10).

Recently, Fong et al. (8) and Heller et al. (9) reported a protein inhibitor ("antizyme") of ornithine decarboxylase produced in H-35 hepatoma, L1210, and N18 neuroblastoma cells, as well as normal rat liver, in response to exogenous polyamines. This discovery adds an intriguing dimension to the regulation of ornithine decarboxylase. We have reproduced some of these results with H-35 cells and in addition have recovered a small amount of enzyme activity, apparently from an inactive complex, in extracts of putrescine-treated cells. However, we have found at most only a small amount of nondialyzable inhibitor in 3T3, polyoma-transformed 3T3, or human fibroblast cells. Therefore we are continuing to seek evidence that polyamines directly or indirectly block translation of the enzyme message in 3T3 cells (10).

ACKNOWLEDGEMENT

This work was supported in part by grant GM-21854 of the NIH.

REFERENCES

1. Raina, A., and Jänne, J. (1975) Med. Biol. 53, 121-147.
2. Berlin, C.M., and Schimke, R.T. (1965) Mol. Pharmacol. 1, 149-156.
3. Hölttä, E. (1975) Biochim. Biophys. Acta 399, 420-427.
4. Canellakis, Z.N., and Theoharides, T.C. (1976) J. Biol. Chem. 251, 4436-4441.
5. Mitchell, J.L.A., Campbell, H.A., and Carter, D.D. (1976) FEBS Lett. 62, 33-37.
6. Clark, J.L., and Fuller, J.L. (1976) Eur. J. Biochem. 67, 303-314.
7. Prouty, W.F., and Obenrader, M.F. (1976) Feb. Proc. 35, 1687.
8. Fong, W.F., Heller, J.S., and Canellakis, E.S. (1976) Biochim. Biophys. Acta 428, 456-465.
9. Heller, J.S., Fong, W.F., and Canellakis, E.S. (1976) Proc. Nat. Acad. Sci. USA 73, 1858-1862.
10. Clark, J.L., and Fuller, J.L. (1975) Biochemistry 14, 4403-4409.
11. Clark, J.L. (1974) Biochemistry 13, 4668-4674.
12. Clark, J.L. (1976) Anal. Biochem. 74, 329-336.
13. Pett, D.M., and Ginsberg, H.S. (1968) Fed. Proc. 27, 615.
14. Schrock, T.R., Oakman, N.J., and Bucher, N.L.R. (1970) Biochim. Biophys. Acta 204, 564-577.
15. Kay, J.E., and Lindsay, V.J. (1973) Biochem. J. 132, 791-796.
16. Jänne, J., and Hölttä, E. (1974) Biochem. Biophys. Res. Commun. 61, 449-456.
17. Pösö, H., and Jänne, J. (1976) Biochem. Biophys. Res. Commun. 69, 885-892.