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Control of Ornithine Decarboxylase Activity in Stimulated Human Lymphocytes by Putrescine and Spermidine

By JOHN E. KAY and VALERIE J. LINDSAY
Biochemistry Group, School of Biological Sciences, University of Sussex, Brighton BN1 9QG, U.K.

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Ornithine decarboxylase activity in extracts of phytohaemagglutinin-stimulated human lymphocytes is rapidly and extensively inhibited by additions of micromolar concentrations of putrescine or spermidine to the culture medium. This inhibition is not due to feedback inhibition of the enzyme by putrescine, spermidine or their metabolites. Inhibition is dependent on the continuation of protein synthesis, but does not require RNA synthesis. The effect of putrescine is abolished when its conversion into spermidine by the cells is prevented.

The conversion of many types of mammalian cells from the resting into the rapidly dividing state is accompanied by a marked increase in the activity of ornithine decarboxylase, the first enzyme in the pathway for the synthesis of polyamines (Cohen, 1972,). This enzyme has an unusually short half-life (Russell & Snyder, 1969), and it has been suggested that the rapid fluctuations in its activity may control important determinants of the growth rate of the cells, such as the rate of ribosomal RNA synthesis (Bachrach, 1970; Russell, 1971).

Little attention has been paid to the ways in which the enzyme activity is itself controlled. Kay *et al.* (1972) showed that in some circumstances marked changes in the enzyme activity can result from changes in its rate of degradation, whereas in others the changes seem to be mainly in its rate of synthesis or activation (Kay & Lindsay, 1973). We report here that the enzyme activity in extracts of phytohaemagglutinin-stimulated human lymphocytes is greatly decreased after addition of low concentrations of its product, putrescine, or its indirect product, spermidine to the culture medium. We suggest that this may be a physiological control.

Experimenta

Materials

Phytohaemagglutinin used in the experiments was batch Q1, kindly given by Dr. J. A. C. Parke of the Wellcome Research Laboratories, Beckenham, Kent, U.K. L-[1-^{14}C]Ornithine (58mCi/mmol), *S*-adenosyl-L-[1-^{14}C]methionine (60mCi/mmol) and [1,4-^{14}C]-putrescine (17.8mCi/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K., and unlabelled ornithine, putrescine and spermidine were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Putrescine was not detectably contaminated by spermidine, but the spermidine contained trace amounts (<0.5%) of putrescine and spermine. Methylglyoxal bis(guanylhydrazone) was obtained from Aldrich Chemical Co., Milwaukee, Wis., U.S.A. Eagle's minimal essential medium, penicillin and streptomycin were obtained from Flow Laboratories Ltd., Irvine, Ayrshire, U.K.

Methods

Preparation and incubation of lymphocyte cultures. Lymphocytes were prepared from human venous blood essentially as described by Cooper (1968). Erythrocytes were removed by sedimentation, and phagocytic cells by passing the leucocyte-rich plasma down a column of cotton wool warmed to 37°C. The lymphocytes were collected by sedimentation and resuspended at 2×10^6/ml in Eagle's minimal essential medium (Eagle, 1959) supplemented with 10% autologous plasma, 50μg of streptomycin/ml and 50 units of penicillin/ml. At least 98% of the nucleated cells in the cultures were small lymphocytes. Ornithine decarboxylase activity was induced by incubation of lymphocytes with 2μg of phytohaemagglutinin/ml for 40–48h.

Determination of ornithine decarboxylase activity. Ornithine decarboxylase activity was determined by a modification (Kay & Lindsay, 1973) of the method of Pegg & Williams-Ashman (1968*a*). This method depends on trapping in Hyamine $^{14}CO_2$ evolved from [1-^{14}C]ornithine by the action of the enzyme. All determinations were performed in triplicate, and replicate values were normally within ±2.5% of the mean. Activity was proportional to the enzyme concentration and followed linear kinetics throughout the period of the assay, and the substrate concentration used (0.125mM) was close to the saturating value

(Kay & Lindsay, 1973). Activity was expressed as pmol of ornithine decarboxylated/h per 10^6 lymphocytes used to prepare the extract. As noted by Kay & Lindsay (1973), the activity of ornithine decarboxylase was much more variable between experiments than most other parameters of lymphocyte stimulation. The reason for this variation is not clear, but possibly the short half-life of the enzyme makes its activity particularly susceptible to minor variations in culture conditions.

Determination of S-adenosylmethionine decarboxylase. Determinations of *S*-adenosylmethionine decarboxylase activity depended on trapping in Hyamine $^{14}CO_2$ released from *S*-adenosyl[1-^{14}C]methionine by the procedure of Kay & Lindsay (1973). We chose to assay this enzyme at subsaturating substrate concentrations, when non-enzymic putrescine-independent decarboxylation was insignificant. The activity was proportional to the enzyme concentration and followed linear kinetics throughout the assay (Kay & Lindsay, 1972).

Determination of [^{14}C]phenylalanine incorporation into protein. To determine the rate of [^{14}C]phenylalanine incorporation into protein, 1 μCi of L-[U-^{14}C]-phenylalanine (482mCi/mmol) was added to 0.5ml cultures containing 10^6 lymphocytes in culture tubes (12mm × 100mm) 2h before harvest. Incubation was terminated by the addition of 3ml of ice-cold 0.15M-NaCl. The cells were then washed twice with 0.15M-NaCl, resuspended in 3ml of 10% (w/v) trichloroacetic acid containing 1mM-phenylalanine (unlabelled) and the precipitate was collected by filtration on glass-fibre filters. The filters were washed with 10% (w/v) trichloroacetic acid, dried and transferred to vials containing 3ml of toluene-based scintillator (Kay & Korner, 1966) and radioactivity was determined in a Packard Tri-Carb model 3320 liquid-scintillation spectrometer.

Determination of incorporation of [^{3}H]uridine and [^{3}H]thymidine into nucleic acids. To determine the rate of incorporation of these precursors into nucleic acids, 10 μCi of [5-^{3}H]uridine (25Ci/mmol) or 1 μCi of [*methyl*-^{3}H]thymidine (5Ci/mmol) was added to 0.5ml cultures containing 10^6 lymphocytes 1h before harvest. Incubation was terminated by the addition of 3ml of ice-cold 0.15M-NaCl. The cell pellet was washed with successive 3ml batches of 0.15M-NaCl, 10% (w/v) trichloroacetic acid (twice) and methanol, and then dissolved in 0.2ml of 1M-Hyamine. The Hyamine was then mixed with 3ml of toluene-based scintillator and radioactivity was determined by liquid-scintillation spectrometry as above.

Results

Addition of 2mM-putrescine to cultures of unstimulated or phytohaemagglutinin-stimulated lymphocytes did not affect their rates of incorporation of [^{14}C]phenylalanine into protein, [^{3}H]uridine into RNA or [^{3}H]thymidine into DNA for at least 22h (Table 1). Addition of spermidine at concentrations up to 100 μM had little effect on the rate of incorporation of [^{3}H]thymidine into DNA or [^{14}C]phenylalanine into protein, but a concentration of 1mM caused significant inhibition (Fig. 1). Toxic effects of high concentrations of polyamines on other types of cultured cells have been reported and attributed to aldehydes produced by serum amine oxidases (Cohen, 1972). In some experiments 1mM-spermidine also caused marked attachment of the cells to the culture vessels. This effect was never seen with spermidine concentrations of 100 μM or less.

Incubation of cultures of phytohaemagglutinin-stimulated lymphocytes with either putrescine or spermidine greatly decreased the ornithine decarboxylase activity of their extracts (Fig. 2). More than 50% inhibition was found after incubation with putrescine concentrations as low as 5 μM for 3h, and 2mM-putrescine caused 95% inhibition. Spermidine was effective at even lower concentrations and caused 75% inhibition at a concentration of 1 μM. Putrescine and spermidine added directly to the enzyme assays

Table 1. *Effect of putrescine on incorporation of [^{14}C]phenylalanine into protein, [^{3}H]uridine into RNA and [^{3}H]-thymidine into DNA*

Cultures were incubated with or without phytohaemagglutinin for a total of 40h before addition of label. Putrescine was added to a final concentration of 2mM at 18h after the beginning of culture.

		Incorporation (c.p.m./10^6 lymphocytes)	
Phytohaemagglutinin	Label	Control	2mM-Putrescine
−	[^{14}C]Phenylalanine	881	846
+	[^{14}C]Phenylalanine	5523	5130
+	[^{3}H]Uridine	143482	155060
+	[^{3}H]Thymidine	15979	15838

also caused some inhibition of enzyme activity, but only at much higher concentrations. With the conditions used, 50% inhibition required a putrescine concentration of about 4mM, whereas the highest concentration of spermidine used (5mM), caused only 35% inhibition (Fig. 2). These concentrations are at least three orders of magnitude greater than those giving rise to the same degree of inhibition when added to intact cells in culture.

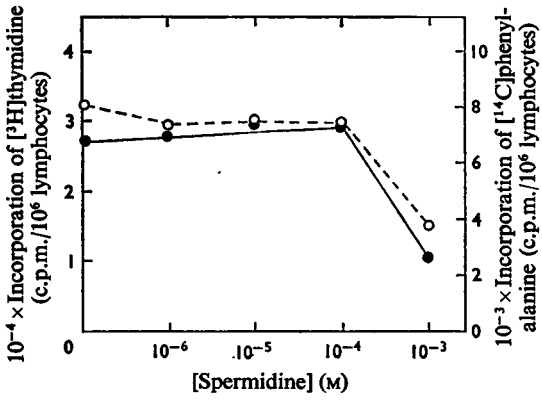

Fig. 1. *Effect of spermidine on the incorporation of [^{3}H]thymidine into DNA and [^{14}C]phenylalanine into protein*

Cultures were incubated with phytohaemagglutinin and the concentrations of spermidine indicated for 46h before the addition of label. ●, [^{3}H]Thymidine incorporation; ○, [^{14}C]phenylalanine incorporation.

The inhibition seen after addition of putrescine to cultures was not due to concentration of the added putrescine in the extracts used to determine enzyme activity. When 1.75mM putrescine containing tracer [^{14}C]putrescine was added to the cultures, the resultant putrescine concentration in the extracts was less than 20μM, a concentration that would not cause significant inhibition. This result was confirmed, and the inhibition was shown not to be due to some more strongly inhibitory metabolites of the added putrescine or spermidine, by mixing extracts of cells incubated with or without 2mM-putrescine or 1mM-spermidine (Table 2). Mixed extracts always had intermediate rates of ornithine decarboxylation activity. Ornithine decarboxylase activity was not recovered after dialysis of extracts, and addition of putrescine immediately before harvest was not inhibitory. Addition of 1mM-spermidine to lymphocytes 3h before harvest inhibited *S*-adenosylmethionine decarboxylase activity by about 60%, but the inhibition was much less pronounced than the inhibition of ornithine decarboxylase activity (Table 3). Addition of putrescine did not inhibit the activity of this enzyme, and in some experiments stimulated activity by up to 50%.

The kinetics of the decay of the activity of ornithine decarboxylase after the addition of 100μM-spermidine or 2mM-putrescine were compared with the rates of decay after inhibition of protein and RNA synthesis (Fig. 3). Activity was lost more slowly than after

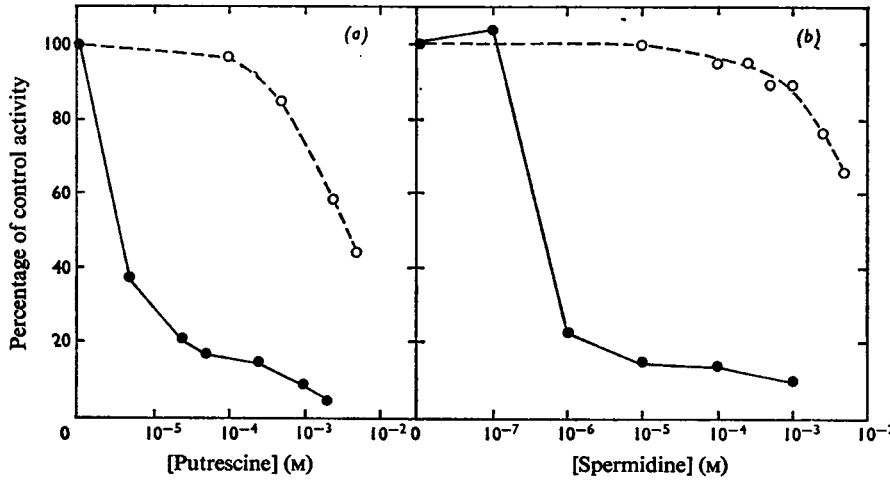

Fig. 2. *Effect of putrescine and spermidine on ornithine decarboxylase activity*

Ornithine decarboxylase activity of phytohaemagglutinin-stimulated lymphocytes was determined either 3h after the addition of putrescine (*a*) or spermidine (*b*) to the cells in culture (●) or after their addition directly to the assay (○).

Table 2. *Effect on ornithine decarboxylase activity of mixing extracts of phytohaemagglutinin-stimulated lymphocytes incubated with or without putrescine or spermidine*

Cultures of phytohaemagglutinin-stimulated lymphocytes were incubated with or without 2mM-putrescine or 1mM-spermidine for the final 3h of culture.

Expt. no.	Control extract (ml)	Extract+putrescine (ml)	Extract+spermidine (ml)	Ornithine decarboxylase activity (pmol/h per 10^6 lymphocytes)
1	0.5	—	—	58.1
	—	0.5	—	2.5
	0.25	0.25	—	27.4
2	0.5	—	—	147.2
	—	—	0.5	14.2
	0.25	—	0.25	80.9

Table 3. *Effect of putrescine and spermidine on S-adenosylmethionine decarboxylase activity*

2mM-Putrescine or 1mM-spermidine were added to cultures of phytohaemagglutinin-stimulated lymphocytes 3h before harvest.

Expt. no.	Additions	*S*-Adenosylmethionine decarboxylase activity (pmol/h per 10^6 lymphocytes)
1	—	5.51
	2mM-Putrescine	5.62
2	—	10.47
	1mM-Spermidine	4.64

inhibition of protein synthesis by cycloheximide (Figs. 3*a* and 3*b*), but more rapidly than after inhibition of RNA synthesis by actinomycin (Fig. 3*c*). Actinomycin did not inhibit enzymic decay in the presence of putrescine (Fig. 3*c*).

To determine whether putrescine and spermidine inhibited ornithine decarboxylase activity in the absence of protein synthesis we incubated cells with these agents and with or without 100μg of cycloheximide/ml for 45min. Each of these compounds alone caused partial inhibition after this time-interval (Figs. 3*a* and 3*b*), but neither putrescine nor spermidine caused significant additional inhibition in the presence of cycloheximide (Table 4).

Putrescine can be converted into spermidine by the addition of a propylamine group, which is derived from *S*-adenosylmethionine by the action of *S*-adenosylmethionine decarboxylase (Pegg & Williams-Ashman, 1968*b*). *S*-Adenosylmethionine decarboxylase is inhibited by methylglyoxal bis(guanylhydrazone), which thus blocks the conversion of putrescine into spermidine (Williams-Ashman & Schenone, 1972). Methylglyoxal bis(guanylhydrazone) at a concentration of 1μM inhibits lymphocyte *S*-adenosylmethionine decarboxylase almost completely, and at 1mM inhibits the conversion of [^{14}C]putrescine into spermidine by phytohaemagglutinin-stimulated lymphocytes by at least 97%, without affecting the rate of protein synthesis significantly within 3h of its addition (Kay & Pegg, 1973). Incubation with methylglyoxal bis(guanylhydrazone) for 3h inhibited ornithine decarboxylase activity by 30%, but increased by two orders of magnitude the concentration of putrescine required to inhibit the activity of this enzyme (Fig. 4).

Discussion

The inhibition of lymphocyte ornithine decarboxylase activity by high concentrations of putrescine and spermidine added directly to the assay is similar to that found in rat prostate (Pegg & Williams-Ashman, 1968*a*) and liver (Ono *et al.*, 1972). However, the much greater inhibition of enzyme activity by addition of very low concentrations of these compounds to the intact cells has not been reported previously. This inhibition is unlikely to be due to the presence of a feedback inhibitor of the enzyme derived from these polyamines, as such an inhibitor would have been expected to inhibit the activity of the control enzyme when extracts from cells incubated with and without the polyamines were mixed. The inhibition caused by putrescine requires the conversion of the putrescine into spermidine, but the active agent could be either spermidine itself or some further metabolite.

This inhibition of ornithine decarboxylase activity must occur at some post-transcriptional step, as the inhibition becomes apparent more rapidly after the addition of polyamines than after inhibition of RNA synthesis by actinomycin. It could operate either through inhibition of the synthesis of ornithine decarboxylase or by acceleration of its degradation. It seems rather improbable that the very extensive loss of activity after incubation with these polyamines

could be due entirely to a decrease in the stability of the enzyme, but this is obviously rather difficult to determine directly at times when the enzyme activity is severely inhibited. The experiments in which cells were incubated with putrescine or spermidine in the presence of cycloheximide for 45min showed that these agents were unable to cause additional inhibition in the absence of protein synthesis when alone they did cause partial inhibition. The results with putrescine are most impressive, as it inhibited ornithine decarboxylase activity by 47%, but caused only 5% inhibition in the presence of cycloheximide. These results would be expected if the polyamines inhibited ornithine decarboxylase activity by decreasing the rate of synthesis of the enzyme. They do not, however, altogether eliminate the possibility that the polyamines act by accelerating the rate of degradation of the enzyme, as the effect on degradation could itself require the synthesis of some other protein.

We have not yet been able to determine the concentrations of polyamines in cultured lymphocytes, either under normal conditions or after the addition

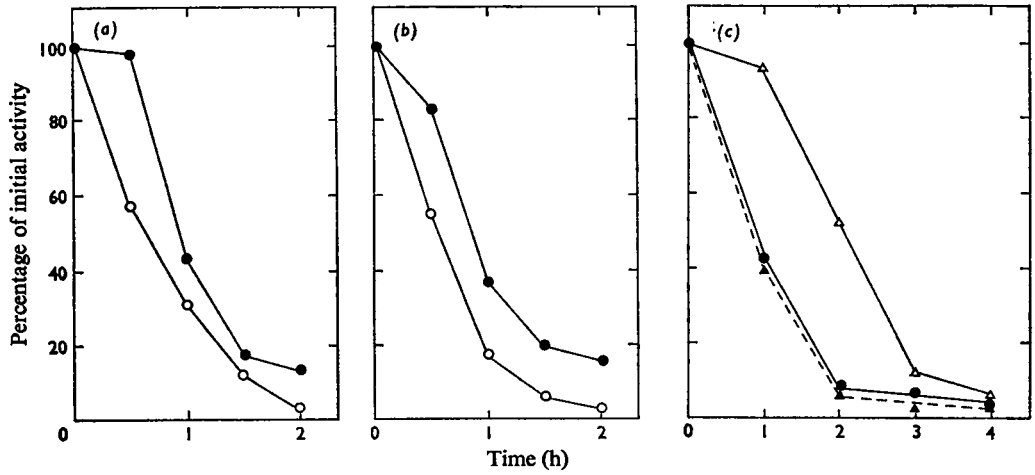

Fig. 3. *Effect of incubation with putrescine, spermidine, cycloheximide or actinomycin on ornithine decarboxylase activity*

Cultures of phytohaemagglutinin-stimulated lymphocytes were incubated for the time indicated before harvest with 100μM-spermidine (●, Fig. 3*a*); 2mM-putrescine (●, Figs. 3*b* and 3*c*); 100μg of cycloheximide/ml (○); 5μg of actinomycin/ml (△); or 2mM-putrescine and 5μg of actinomycin/ml (▲).

Table 4. *Effect of inhibition of protein synthesis on the inhibition of ornithine decarboxylase activity by putrescine and spermidine*

Cultures of phytohaemagglutinin-stimulated lymphocytes were incubated with 2mM-putrescine, 100μM-spermidine or 100μg of cycloheximide/ml for 45min.

Expt. no.	Cycloheximide	Putrescine	Ornithine decarboxylase activity (pmol/h per 10^6 lymphocytes)	Inhibition by putrescine (%)
1	−	−	25.6	
	−	+	13.5	47
	+	−	6.9	
	+	+	6.6	5
	Cycloheximide	Spermidine		Inhibition by spermidine (%)
2	−	−	23.0	
	−	+	17.6	23
	+	−	5.5	
	+	+	5.2	5

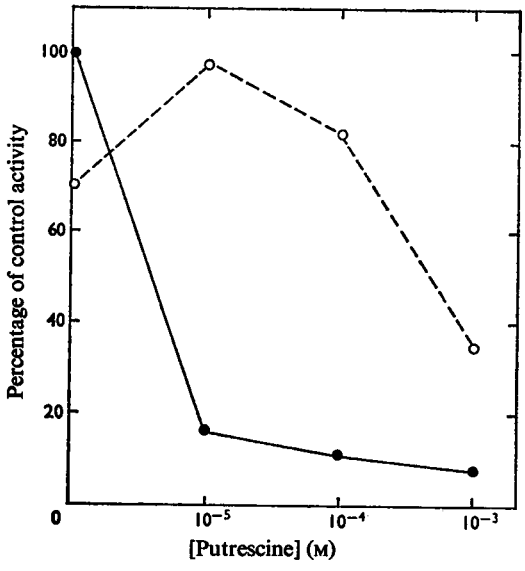

Fig. 4. *Effect of methylglyoxal bis(guanylhydrazone) on the inhibition of ornithine decarboxylase by putrescine*

Putrescine was added to cultures of phytohaemagglutinin-stimulated lymphocytes 3h before harvest. Where indicated 1mM-methylglyoxal bis(guanylhydrazone) was added 30min before putrescine. ●, Putrescine alone; ○, putrescine and methylglyoxal bis(guanylhydrazone).

of spermidine and putrescine to the culture medium. However, preliminary studies on the uptake of [^{14}C]-putrescine by stimulated lymphocytes have shown that only a very small proportion of the radioisotope added enters the cells within 3h, so that there cannot be any great concentration of this compound from the culture medium. The intracellular concentration of spermidine or its metabolites required to inhibit ornithine decarboxylase activity is thus likely to be low enough for this inhibition to be of physiological significance.

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