Polyamine starvation causes accumulation of cadaverine and its derivatives in a polyamine-dependent strain of Chinese-hamster ovary cells

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Starvation of the polyamine-dependent Chinese-hamster ovary cells for ornithine or ornithine-derived polyamines in serum-free culture resulted in the formation of cadaverine and its aminopropyl derivatives, *N*-(3-aminopropyl)cadaverine and *NN'*-bis(3-aminopropyl)cadaverine. The synthesis of these unusual amines was inhibited by treatment of the cells with DL-2-difluoromethylornithine, a specific inhibitor of ornithine decarboxylase (EC 4.1.1.17). In the absence of ornithine (the normal substrate), ornithine decarboxylase thus appeared to catalyse the decarboxylation of lysine to cadaverine. Cell proliferation was markedly inhibited by ornithine deprivation of the cells, and further depressed by exposure of the cultures to difluoromethylornithine.

The polyamines putrescine, spermidine and spermine are organic cations which are required for normal proliferation of both prokaryotic and eukaryotic cells (see Tabor & Tabor, 1976; Jänne *et al.*, 1978; Morris & Harada, 1980; Heby, 1981). Studies with the recently introduced polyamine-dependent strains of Chinese-hamster ovary (CHO) cells (Pohjanpelto *et al.*, 1981; Steglich & Scheffler, 1982) suggest that these compounds are vital for animal cell replication. The importance of polyamines is not hard to understand in the light of our observations that the polyamine-deprived CHO cells, A7, show major chromosome aberrations (Pohjanpelto & Knuutila, 1982) and disappearance of actin filaments and microtubules (Pohjanpelto *et al.*, 1981). However, certain unusual amines, particularly cadaverine and its aminopropyl derivatives, can partly replace polyamines in supporting growth, as demonstrated in cells depleted of polyamines by difluoromethylornithine (Mamont *et al.*, 1978; Hölttä *et al.*, 1979; Alhonen-Hongisto *et al.*, 1982a).

There are only few reports (see Rosengren *et al.*, 1981; Persson, 1981) on the accumulation of cadaverine in animal tissues. Biosynthesis of cadaverine from lysine in mammalian tissues was originally reported in mouse kidney stimulated to grow by an anabolic steroid (Henningson *et al.*, 1976). For cultured animal cells there is no firm evidence for the accumulation of cadaverine, except for the mycoplasma-infected cultures (Alhonen-Hongisto *et al.*, 1982b).

In the present paper we report the formation of cadaverine and its aminopropyl derivatives in the ornithine-starved A7 cells, which are unable to synthesize enough ornithine and polyamines, owing to insufficient arginase activity (Hölttä & Pohjanpelto, 1982). We show further that cadaverine is formed from lysine most probably through decarboxylation by ornithine decarboxylase.

Materials and methods

Chemicals

L-[U-¹⁴C]Lysine (sp. radioactivity 341 Ci/mol) was obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). L-Ornithine was purchased from Fluka A.G. (Buchs, Switzerland), and putrescine, cadaverine, spermidine and spermine were from Calbiochem (San Diego, CA, U.S.A.). *N*-(3-Aminopropyl)cadaverine was synthesized from cadaverine by the spermidine synthase reaction. DL-2-Difluoromethylornithine was generously given by the Centre de Recherche Merrell International (Strasbourg, France).

Cell culture

The A7 cells were cultured in serum-free medium on plastic Petri dishes coated with gelatin (Pohjanpelto *et al.*, 1981). The medium was a 1:1 (v/v) mixture of Eagle's minimal essential medium and Ham's nutrient mixture F12 (Gibco, Paisley, Scotland, U.K.), supplemented with 0.1% bovine serum albumin.

The cells were regularly checked for mycoplasma contamination both by autoradiography with [^3H]thymidine and by fluorescence staining with the Hoechst 33258 dye (Russell *et al.*, 1975). All the tests were negative.

Analytical methods

Polyamines were determined by the dansylation method (Seiler, 1970) as described previously (Hölttä *et al.*, 1979). The dansylated amine fractions were eluted with ethanol, and their radioactivity was measured in a liquid-scintillation spectrometer. DNA was determined by the method of Giles & Myers (1965). Cells were counted in an electric cell counter (Coulter Counter).

All the results were confirmed by repeating the

experiments at least twice. Despite some variation (up to 25%) of the basal values from experiment to experiment, the pattern of the changes under particular conditions was quite consistent, with less than 10% variation in them. In each experiment analysis of the different parameters from duplicate dishes gave values that agreed within 10%.

Results

Starvation of the A7 cells for ornithine and polyamines in serum-free medium caused appearance of three new amines in the cells. One of them migrated like authentic cadaverine and the other like authentic *N*-(3-aminopropyl)cadaverine on t.l.c. in our solvent systems (Hölttä *et al.*, 1979), containing

Table 1. Incorporation of radioactivity from [$U\text{-}^{14}\text{C}$]lysine into cadaverine with its derivatives and protein in the A7 cells grown in the absence and presence of ornithine and difluoromethylornithine

Cells were plated on Petri dishes (diam. 9 cm) in a 1:1 mixture (7 ml) of minimal essential medium and F12 medium (without putrescine). Thereafter ornithine (0.1 mM), difluoromethylornithine (5 mM) and [$U\text{-}^{14}\text{C}$]lysine (10 μCi) were added to the cultures. After 24 h of labelling, the cells were scraped off the plates, washed once with phosphate-buffered saline (Dulbecco's formula), and extracted with 250 μl of 0.2 M-HClO₄. A sample (25 μl) of the HClO₄ supernatant was counted for the total radioactivity in acid-soluble fraction, and the rest was analysed for the label in the amine fractions by the dansylation technique (see the Materials and methods section). The HClO₄ precipitate was subjected to acid hydrolysis for DNA analysis, and the remaining protein precipitate was dissolved in 0.5 M-NaOH and counted for the radioactivity. The DNA contents of the cultures were 13.2 (no additions), 22.3 (+ornithine) and 13.8 (+difluoromethylornithine) $\mu\text{g}/\text{plate}$. The values are the means for two cultures.

Additions	Radioactivity incorporated (c.p.m./ μg of DNA)				
	Acid-soluble fraction	Protein	Cadaverine	Aminopropyl-cadaverine	Bis(aminopropyl)-cadaverine
—	13 949	71 167	60	215	25
Ornithine	13 836	92 983	15	<3	<3
Difluoromethylornithine	17 584	69 844	10	70	7

Table 2. Polyamine contents in the A7 cells grown in the absence and presence of ornithine and difluoromethylornithine

Cells were seeded on Petri dishes (diam. 9 cm) in 15 ml of polyamine-free medium (see Table 1), and ornithine (0.2 mM) or difluoromethylornithine (5 mM) was added to the indicated cultures. The medium together with the additions was changed on the second day. Cells were scraped off the plates, washed once with minimal essential medium and extracted with 0.2 M-HClO₄ for analysis of the amine contents. The results are the means for duplicate determinations (for 1-day samples, cells from two dishes were combined).

Additions	Time of incubation (days)	Content (nmol/mg of DNA)					DNA ($\mu\text{g}/\text{plate}$)
		Putrescine	Cadaverine	Spermidine	Aminopropyl-cadaverine	Spermine	
—	1	1.1	2.8	3.8	17	82	15.6
Ornithine	1	21	1.9	85	12	120	16.4
Difluoromethylornithine	1	0.6	1.3	3.7	11	45	14.0
—	2	1.2	4.8	2.9	27	80	22.8
Ornithine	2	34	3.2	180	9.0	190	51.6
Difluoromethylornithine	2	0.8	1.3	2.9	12	65	19.4
—	3	1.2	5.6	2.8	40	89	27.9
Ornithine	3	10	3.0	140	9.0	230	59.7
Difluoromethylornithine	3	0.6	1.4	2.6	11	68	24.4

Fig. 1. Effect of ornithine starvation and difluoromethylornithine on growth of the A7 cells

On each plate (diam. 30mm) 10^4 cells were seeded in ornithine-free medium (see Table 1). Ornithine or difluoromethylornithine was then added to the indicated cultures. Cells, detached with trypsin, were counted in an electric cell counter. O, No additions; ●, +ornithine (0.1 mM); ▲, +difluoromethylornithine (5 mM). Values represent the means for duplicate dishes.

chloroform/dioxan/butan-1-ol (48:1:1, by vol.) or chloroform/butan-1-ol (49:1, v/v). The third, minor, fraction migrated slightly ahead of spermine, and presumably is *NN'*-bis(3-aminopropyl)cadaverine. The identity of the new amines was further confirmed by labelling the cells with radioactive lysine, which is decarboxylated to cadaverine, and this in turn is transformed into its aminopropyl derivatives through the subsequent actions of spermidine synthase and spermine synthase (Käpyaho, 1980; Pegg *et al.*, 1981). Table 1 shows that in the ornithine-starved cells the radioactivity from [U- 14 C]-

lysine was incorporated into the fractions representing cadaverine, *N*-(3-aminopropyl)cadaverine and *NN'*-bis(3-aminopropyl)cadaverine. Formation of cadaverine and its congeners was inhibited by the addition of 0.1 mM-ornithine, 5 mM-difluoromethylornithine (Table 1) or 1 μ M-putrescine (results not shown) to the cultures. Also, there was a slight decrease in the incorporation of the label from lysine into protein in the ornithine-starved cells, in both the absence and the presence of difluoromethylornithine, as compared with the ornithine-containing control cultures.

Table 2 illustrates the amounts of the normal and unusual polyamines in the cells cultured for different periods of time in the absence or presence of ornithine and difluoromethylornithine. In the ornithine-deprived cultures the concentrations of cadaverine and aminopropylcadaverine increased with time. The cells supplemented with ornithine had high contents of putrescine, spermidine and spermine, but the formation of cadaverine derivatives was inhibited. Some cadaverine was still accumulated in the presence of 0.2 mM exogenous ornithine, but the content of aminopropylcadaverine was decreased. Addition of 5 mM-difluoromethylornithine, an irreversible inhibitor of ornithine decarboxylase (Metcalf *et al.*, 1978), to the ornithine-starved cultures inhibited not only the synthesis of polyamines but also the formation of cadaverine and aminopropylcadaverine. There was a distinct decrease in the concentrations of putrescine and spermine in the cultures exposed to difluoromethylornithine as compared with the cells starved only for ornithine (Table 2).

Fig. 1 depicts the effects of ornithine starvation and difluoromethylornithine on the proliferation of the A7 cells. Ornithine deprivation of the cells caused a marked decrease in the proliferation rate, which was further depressed by difluoromethylornithine. Conceivably, inhibition of the synthesis of both the residual polyamines and the cadaverine-based amines (Table 2) contributes to the additional growth inhibition caused by difluoromethylornithine.

Discussion

In the present paper we show that starvation of the A7 cells for ornithine and putrescine resulted in the accumulation of the unusual polyamines cadaverine and aminopropylcadaverine. Polyamine starvation of a polyamine-auxotrophic strain of *Escherichia coli* is also known to cause the formation of cadaverine and aminopropylcadaverine (Dion & Cohen, 1972). Accumulation of these amines is further reported by mycoplasmas (Alhonen-Hongisto *et al.*, 1982b). However, the mechanisms involved in the formation of these

lysine-descended amines in the A7 cells are different from those in bacteria and mycoplasma-infected animals cells. In *E. coli* cadaverine is synthesized from lysine by lysine decarboxylase (Leifer & Maas, 1973), and mycoplasmas appear also to have lysine decarboxylase activity, which is responsible for the formation of cadaverine in the cells harbouring mycoplasmas (Alhonen-Hongisto *et al.*, 1982b). In the A7 cells, on the other hand, the production of cadaverine and its aminopropyl derivatives was inhibited by difluoromethylornithine, a highly specific inhibitor of ornithine decarboxylase (Metcalf *et al.*, 1978), indicating that ornithine decarboxylase catalysed the formation of cadaverine from lysine. Purified ornithine decarboxylase from rat liver (Pegg & McGill, 1979) and mouse kidney (Persson, 1981) is known to catalyse the decarboxylation of both ornithine and lysine, although the affinity of the enzyme for ornithine is about 100-fold higher than for lysine (Pegg & McGill, 1979). Separate ornithine decarboxylase and lysine decarboxylase activities have not been found in mammalian cells. Our results with the A7 cells also support the view that a single enzyme, ornithine decarboxylase, is responsible for the decarboxylation of both ornithine and lysine.

Since ornithine decarboxylase favours ornithine as a substrate, lysine concentration must be relatively high in comparison with that of ornithine for cadaverine formation to occur. As an essential amino acid, lysine is always present in the tissue-culture media, but ornithine is not included in the commonly used media. However, most cells are cultured in the presence of serum, which contains arginase, which catalyses the formation of ornithine from arginine (Hölttä & Pohjanpelto, 1982). This explains why cadaverine is not normally found in the serum-containing cultures.

The role of cadaverine and its higher polyamine analogues in animal cells is not known, but apparently they can partly substitute for the normal polyamines in the maintenance of cellular growth (Mamont *et al.*, 1978; Hölttä *et al.*, 1979; Alhonen-Hongisto *et al.*, 1982a). The ornithine decarboxylase-catalysed formation of cadaverine in the absence of ornithine and polyamines may be considered as a defence mechanism which helps to keep the cells alive during the polyamine-deficient condition.

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A receipt paper (Paulus *et al.*, 1982), which appeared after submission of the present paper, shows that ornithine deprivation causes formation of cadaverine and aminopropylcadaverine in an arginase-deficient *Neurospora crassa* mutant as well.

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