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Effects of *S*-adenosyl-1,8-diamino-3-thio-octane and *S*-methyl-5′-methylthioadenosine on polyamine synthesis in Ehrlich ascites-tumour cells

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The rate-limiting enzymes in polyamine biosynthesis, ornithine decarboxylase (ODC) and *S*-adenosylmethionine decarboxylase (AdoMetDC), are negatively regulated by the polyamines spermidine and spermine. In the present work the spermidine synthase inhibitor *S*-adenosyl-1,8-diamino-3-thio-octane (AdoDATO) and the spermine synthase inhibitor *S*-methyl-5′-methylthioadenosine (MMTA) were used to evaluate the regulatory role of the individual polyamines. Treatment of Ehrlich ascites-tumour cells with AdoDATO caused a marked decrease in spermidine content together with an accumulation of putrescine and spermine. Treatment with MMTA, on the other hand, gave rise to a marked decrease in spermine, with a simultaneous accumulation of spermidine. A dramatic increase in the activity of AdoMetDC, but not of ODC, was observed in MMTA-treated cells. This increase appears to be unrelated to the decrease in spermine content, because a similar rise in AdoMetDC activity was obtained when AdoDATO was given in addition to MMTA, in which case the spermine content remained largely unchanged. Instead, we show that the increase in AdoMetDC activity is mainly due to stabilization of the enzyme, probably by binding of MMTA. Treatment with AdoDATO had no effects on the activities of ODC and AdoMetDC, even though it caused a precipitous decrease in spermidine content. The expected decrease in spermidine-mediated suppression of ODC and AdoMetDC was most probably counteracted by the simultaneous increase in spermine. The combination of AdoDATO and MMTA caused a transient rise in ODC activity. Concomitant with this rise, the putrescine and spermidine contents increased, whereas that of spermine remained virtually unchanged. The increase in ODC activity was due to increased synthesis of the enzyme. There were no major effects on the amount of AdoMetDC mRNA by treatment with the inhibitors, alone or in combination. However, the synthesis of AdoMetDC was slightly stimulated in cells treated with MMTA or AdoDATO plus MMTA. The present study demonstrates that regulation of neither ODC nor AdoMetDC is a direct function of the polyamine structure. Instead, it appears that the biosynthesis of the polyamines is feedback-regulated by the various polyamines at many different levels.

INTRODUCTION

It is well established that the polyamines putrescine, spermidine and spermine are essential for cell growth and differentiation (Pegg, 1986; Heby *et al.*, 1987). It has been demonstrated that the first enzyme in polyamine biosynthesis, ODC, is highly regulated. This regulation is carried out at the transcriptional as well as the translational level. Induction of cell growth is usually accompanied by an increase in the amount of ODC mRNA (Kontula *et al.*, 1984; Persson *et al.*, 1985; Olson & Spizz, 1986; Katz & Kahana, 1987; Sertich & Pegg, 1987) and in ODC synthesis. The increase in ODC mRNA content is prevented by actinomycin D (Butler & McDonald, 1987) and is most likely a result of increased transcription, although changes in mRNA half-life have been reported (Rose-John *et al.*, 1987).

When cells are depleted of their putrescine and spermidine contents by treatment with the ODC inhibitor 2-difluoromethylornithine, the translation of ODC mRNA increases without any change in the amount of ODC mRNA (Persson *et al.*, 1985, 1986). Conversely, when cells are supplemented with polyamines, ODC mRNA translation decreases. Again, there is no change in the content of ODC mRNA (Persson *et al.*, 1986, 1988; Sertich & Pegg, 1987). Hence it may be concluded that the polyamines exert feedback control of ODC synthesis by affecting the efficiency of ODC mRNA translation. However, it has not been possible to discriminate between the effects that each individual polyamine exerts on ODC, since they are rapidly interconverted (Pegg, 1986).

The effects of polyamines on AdoMetDC differ from those on ODC. Thus putrescine and spermidine depletion by treatment with 2-difluoromethylornithine causes an increase in the AdoMetDC mRNA content (Shirahata & Pegg, 1986; Persson *et al.*, 1989). Putrescine administration accelerates the conversion of the inactive proenzyme of AdoMetDC into the active form (Kameji & Pegg, 1987*a*; Pegg *et al.*, 1988), whereas spermidine and spermine appear to regulate AdoMetDC negatively at both the transcriptional and the translational level (Kameji & Pegg, 1987*b*; Pegg *et al.*, 1987; Persson *et al.*, 1989).

Abbreviations used: ODC, ornithine decarboxylase (EC 4.1.1.17); AdoMet, *S*-adenosylmethionine; AdoMetDC, AdoMet decarboxylase (EC 4.1.1.50); AdoDATO, *S*-adenosyl-1,8-diamino-3-thio-octane; MMTA, *S*-methyl-5′-methylthioadenosine.

In the present study, we have used *S*-adenosyl-1,8-diamino-3-thio-octane (AdoDATO) and *S*-methyl-5′-methylthioadenosine (MMTA), specific inhibitors of spermidine synthase and spermine synthase, to evaluate the importance of spermidine and spermine, respectively, in the regulation of polyamine biosynthesis.

EXPERIMENTAL

Materials

[^{35}S]Methionine and [^{14}C]methylated protein M_r markers were purchased from Amersham. L-[1-^{14}C]-Ornithine, DL-2-difluoromethyl[3,4-^{3}H]ornithine and *S*-adenosyl[*carboxy*-^{14}C]methionine (Ado[^{14}C]Met) were from New England Nuclear. All cell-culture reagents were from NordVacc, Stockholm, Sweden. AdoDATO and MMTA were prepared as described by Tang *et al.* (1980).

Cell culture

Ehrlich ascites-tumour cells were routinely cultivated in a 1:1 mixture of Eagle's minimum essential medium and Ham's F12 medium, supplemented with 0.2% bovine serum albumin (fraction V) and antibiotics. For the experiments, the cells were seeded at a density of 1.0×10^5/ml in the above medium lacking putrescine. AdoDATO and/or MMTA were added at the time of seeding to concentrations of 50 μM and 200 μM respectively. Cells were counted in a haemocytometer, and samples were collected every 24 h for polyamine determination and enzyme assays.

Determination of polyamine content

The cells [$(2–5) \times 10^6$] were sonicated in 0.5–1.0 ml of ice-cold 0.1 M-Tris/HCl, pH 7.5, containing 0.1 mM-EDTA and 0.5 mM-dithiothreitol. Samples of the homogenates were used for analysis of enzyme activities and polyamine content. For polyamine analysis 4% (w/v) sulphosalicylic acid containing 0.04% EDTA was added to each sample. After centrifugation at 12000 *g* for 2 min, the pH of the supernatant was adjusted to 2.0–2.5 with KOH. The polyamine content was determined with an automatic amino acid analyser (Biotronic LC 5001) equipped with a 3.2 mm × 75 mm column of cation-exchange resin. The citrate buffers used contained 0.4 M (pH 5.48; 5 min), 1.3 M (pH 5.54; 21 min), 2.0 M (pH 5.48; 10 min) and 2.5 M (pH 5.08; 11 min) K^+. The buffer flow rate was 0.43 ml/min. Eluted polyamines were quantified by reaction with *o*-phthaldialdehyde.

Enzyme-activity assays

After centrifugation at 20000 *g* for 20 min at 4 °C, the supernatant was used to determine ODC and AdoMetDC activities. ODC activity was determined by measuring the release of $^{14}CO_2$ from [*carboxy*-^{14}C]ornithine in the presence of 0.1 mM-pyridoxal 5′-phosphate and 0.5 mM-L-ornithine as described by Jänne & Williams-Ashman (1971). AdoMetDC activity was determined by measuring the release of $^{14}CO_2$ from Ado[^{14}C]Met in the presence of 2.5 mM-putrescine and 0.2 mM-AdoMet essentially as described by Shirahata *et al.* (1985). A unit of enzyme activity is defined as the amount of enzyme catalysing the formation of 1 nmol of CO_2/h.

Determination of enzyme half-lives

At 24 h after seeding, cycloheximide was added to a final concentration of 50 μg/ml. Cell samples were collected at 0, 30, 60, 90, 120 and 180 min and were further processed for determination of ODC and AdoMetDC activities as described above.

Analysis of ODC synthesis

Cells grown for 24 h in the absence or presence of AdoDATO (50 μM) plus MMTA (200 μM) were collected by centrifugation at 500 *g* for 5 min, and were reseeded at 1.0×10^6 cells/ml in the above medium lacking methionine. After a 5 min preincubation at 37 °C, [^{35}S]methionine was added at 10 μCi/ml. The cells were then incubated for 25 min, during which time the intracellular amount of methionine was sufficient to maintain a normal rate of protein synthesis, as determined by [^{3}H]leucine labelling. The incorporation of labelled methionine was terminated by adding an excess of unlabelled methionine and then cooling the cells on ice. After centrifugation at 2000 *g* for 10 min at 4 °C, the cell pellets were stored at −70 °C.

At the time of analysis, cell pellets were sonicated in 0.1 M-Tris/HCl, pH 7.5, containing 0.1 mM-EDTA and 0.5 mM-dithiothreitol. After centrifugation, samples of the supernatants were incubated with an excess of anti-ODC antiserum (Persson, 1982) for 30 min at room temperature. ODC–antibody complexes were precipitated with Protein A and washed in 10 mM-Tris/HCl, pH 7.5, containing 0.1 mM-EDTA, 0.05 mM-dithiothreitol, 0.1% bovine serum albumin, 0.1% Triton X-100 and 0.1% SDS. The immunoprecipitated material was fractionated by SDS/polyacrylamide-gel electrophoresis as described by Persson *et al.* (1984). [^{3}H]Difluoromethylornithine-labelled purified mouse ODC was used as M_r standard. The gels were subjected to fluorography after incubation in Amplify (Amersham).

Analysis of AdoMetDC synthesis

The rate of AdoMetDC synthesis was determined by pulse-labelling with [^{35}S]methionine and immunoprecipitation with a monospecific anti-AdoMetDC antibody (Persson *et al.*, 1989). The method was essentially the same as that described for measuring ODC synthesis, except that the cells were grown for 48 h before labelling. Pure rat prostate AdoMetDC (M_r = 33000), labelled with [*Me*-^{3}H]AdoMet essentially as described by Shirahata *et al.* (1985), and a mixture of [^{14}C]methylated proteins were used as M_r standards. The M_r of mouse AdoMetDC was somewhat lower than that of the rat enzyme (Fig. 5).

Determination of AdoMetDC mRNA content

Total RNA from cells grown in the absence or presence of AdoDATO and/or MMTA for 48 h was isolated by the method of Chomczynski & Sacchi (1987). The RNA was fractionated on formaldehyde-containing 1% agarose gels (Elliott & Berger, 1983), blotted on to Gene-Screen (New England Nuclear) and hybridized to the insert of pSDC-0.18 (Mach *et al.*, 1986) labelled by the oligonucleotide-priming method (Feinberg & Vogelstein, 1983).

RESULTS

Seeding of the cells in fresh medium gave rise to an increase in polyamine biosynthesis, as demonstrated in Fig. 1. Marked increases in ODC (> 300-fold) and AdoMetDC (5-fold) activities were seen during exponential growth (days 1–2). These increases were accompanied by elevated levels of putrescine and spermidine. The level of spermine, on the other hand, remained relatively constant during cell growth. Addition of AdoDATO to the cells not only prevented the normally occurring increase in spermidine content, but caused a major decrease. At the same time the cellular putrescine and spermine levels increased above controls.

When cells were instead treated with MMTA, their spermine content decreased significantly, to about one-third of that in untreated cells. Putrescine and spermidine, on the other hand, increased in MMTA-treated cells as compared with controls.

Surprisingly, the simultaneous addition of AdoDATO and MMTA did not decrease the cellular levels of spermidine and spermine below controls. Instead, the spermidine level increased and the spermine level remained largely unaltered. Not only spermidine, but also putrescine, increased markedly above control values during the combined treatment.

The changes in cellular polyamine levels caused by AdoDATO or MMTA did not affect the ODC activity of the cells. Notably, however, the combination of AdoDATO and MMTA caused a more than 2-fold increase in ODC activity, as compared with the control, 1 day after seeding. At 2 days the activity had returned to the control level. The increase in ODC activity caused by the combination of the two inhibitors was not attributable to stabilization of the enzyme, as shown in Fig. 2(*a*). Thus the half-life of ODC was only somewhat longer in cells treated with AdoDATO plus MMTA than in untreated cells, i.e. 70 as compared with 60 min. Instead, the elevated ODC activity in cells treated with AdoDATO plus MMTA was mainly due to an increase in ODC synthesis (Fig. 3).

The increase in AdoMetDC activity after seeding in

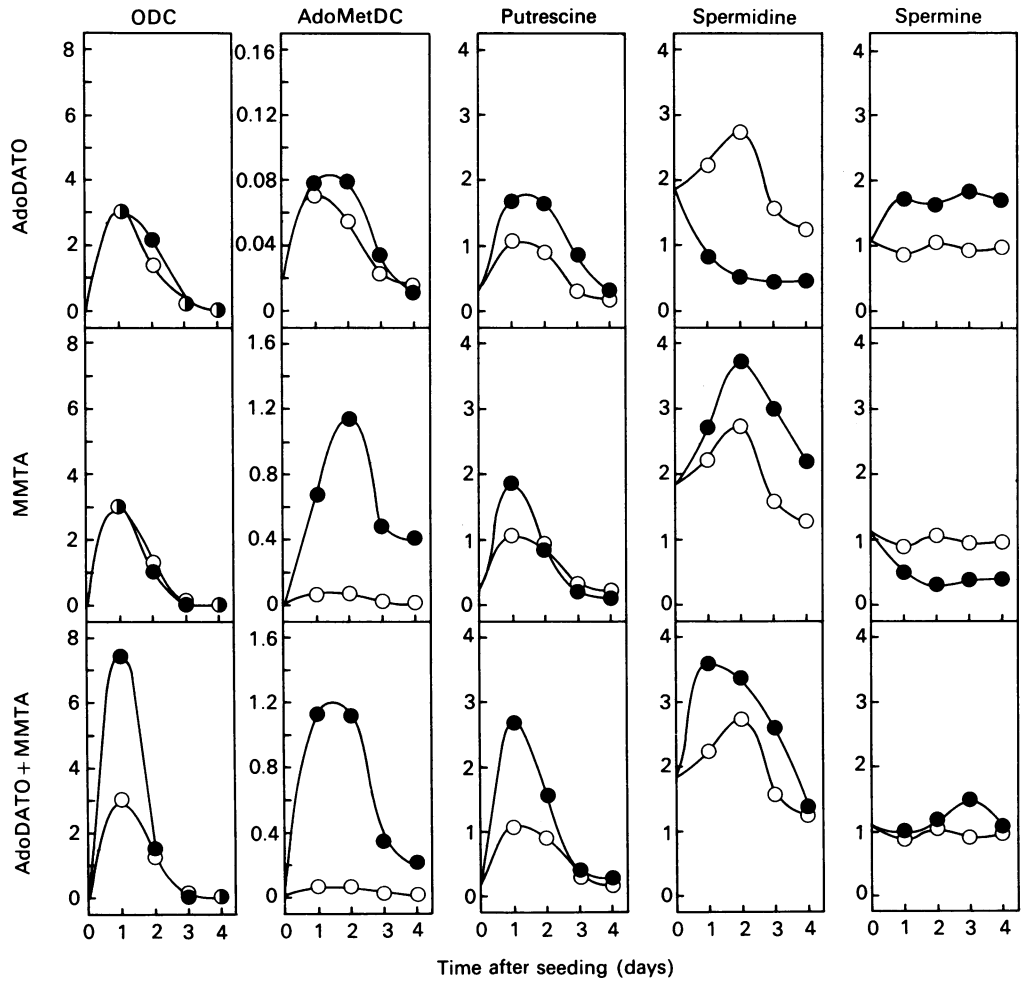

Fig. 1. Effects of AdoDATO, MMTA and their combination on ODC and AdoMetDC activities and polyamine levels in Ehrlich ascites cells

Cells (1.0×10^5/ml) were seeded in the absence (○) or presence (●) of 50 μM-AdoDATO and/or 200 μM-MMTA. Samples were removed every 24 h and were analysed for enzyme activities (units/10^6 cells) and polyamines (nmol/10^6 cells) as described in the Experimental section. Results are means of 3–4 experiments. Note the different scales on the axes representing AdoMetDC activity.

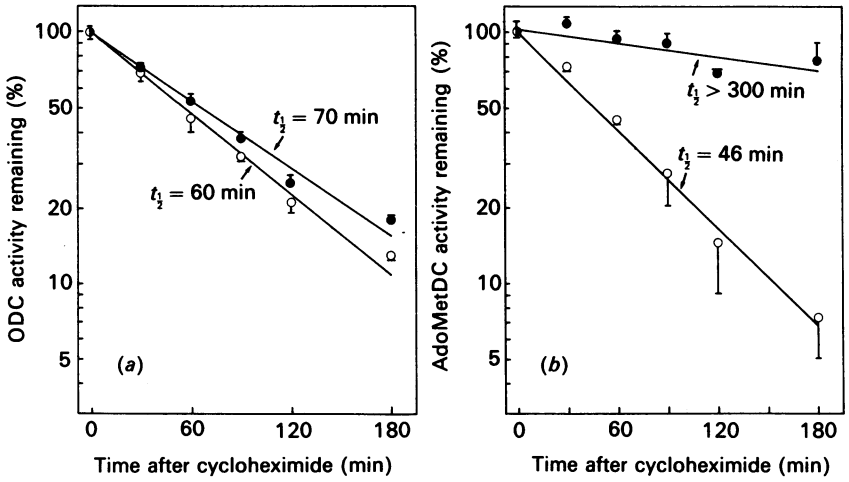

Fig. 2. Effects of the combination of AdoDATO plus MMTA on the half-lives ($t_{\frac{1}{2}}$) of ODC and AdoMetDC

Ehrlich ascites-tumour cells were seeded in the absence (○) or presence (●) of a combination of AdoDATO (50 μM) and MMTA (200 μM). After 24 h, cycloheximide was added and cell samples were removed after 0, 30, 60, 90, 120 and 180 min. The cell samples were analysed for their activities of ODC (*a*) and AdoMetDC (*b*) as described in the Experimental section. Results are means ± S.E.M. (n = 3).

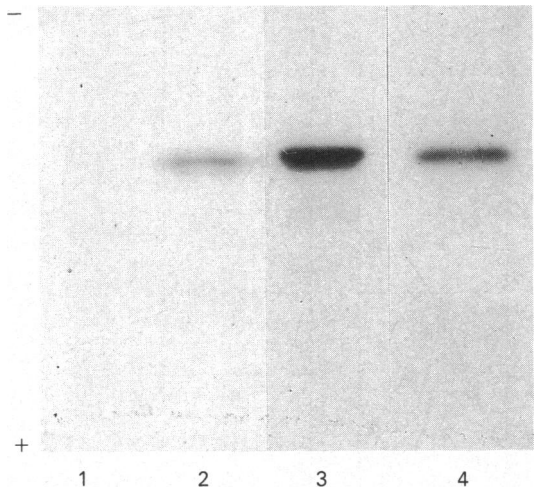

Fig. 3. Effects of AdoDATO, MMTA and their combination on ODC synthesis

Ehrlich ascites-tumour cells were seeded in the absence or presence of AdoDATO plus MMTA. After 24 h they were reseeded in methionine-free growth medium and pulse-labelled for 25 min with [^{35}S]methionine. Cytosolic ODC was precipitated with a monospecific anti-ODC antiserum and subjected to SDS/polyacrylamide-gel electrophoresis and fluorography. Lane 1, non-immune serum; lane 2, untreated control cells; lane 3, AdoDATO plus MMTA; lane 4, [^{3}H]difluoromethylornithine-labelled purified mouse ODC (M_r = 53000).

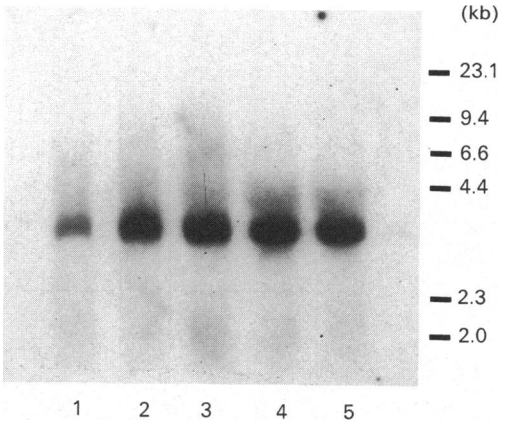

Fig. 4. Effects of AdoDATO and MMTA on AdoMetDC mRNA levels

Cells were seeded in the absence or presence of AdoDATO and/or MMTA. After 48 h the cells were harvested, and total RNA was isolated and separated on a 1% agarose gel. The RNA was blotted on to Gene-Screen and hybridized to the insert of pSDC-0.18. Lane 1, control cells, 0 h; lane 2, control cells, 48 h; lane 3, AdoDATO (50 μM), 48 h; lane 4, MMTA (200 μM), 48 h; lane 5, AdoDATO plus MMTA, 48 h. Migration of DNA molecular-size standards is indicated.

fresh medium was largely unaffected by the addition of AdoDATO. In contrast, treatment with MMTA (alone, or in combination with AdoDATO) gave rise to a dramatic increase in AdoMetDC activity (10–20-fold above control). This MMTA-mediated increase in AdoMetDC activity was not associated with any significant change in AdoMetDC mRNA as measured by Northern-blot analysis (Fig. 4). MMTA treatment only produced a slight increase in AdoMetDC synthesis as measured by [^{35}S]methionine pulse-labelling experiments (Fig. 5). Instead, the increase in AdoMetDC activity was mainly due to a stabilization of the enzyme. The half-life of AdoMetDC increased at least 7-fold (from 45 to more than 300 min) after treatment with the combination of AdoDATO and MMTA (Fig. 2*b*). The induction of AdoMetDC after seeding the cells in fresh medium was partly attributable to an increase (2–3-fold) in AdoMetDC mRNA content (Fig. 4). The Northern blots revealed one major band corresponding to a mRNA of 3.1 kb.

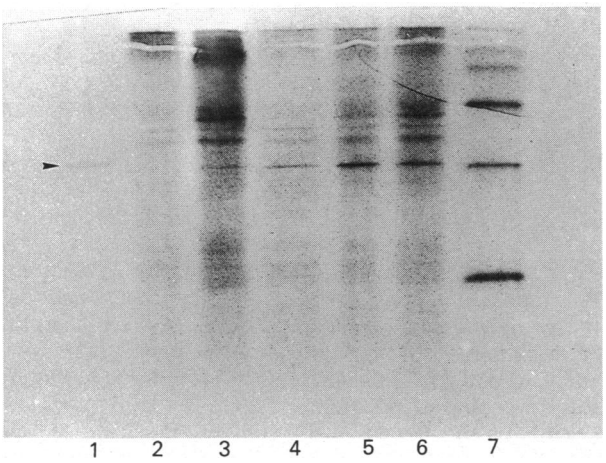

Fig. 5. Effects of AdoDATO, MMTA and their combination on AdoMetDC synthesis

Cells were seeded in the absence or presence of AdoDATO and/or MMTA. After 48 h the cells were reseeded in methionine-free growth medium and pulse-labelled for 25 min with [^{35}S]methionine. Cytosolic AdoMetDC was precipitated with a monospecific anti-AdoMetDC antiserum and subjected to SDS/polyacrylamide-gel electrophoresis and fluorography. Lane 1, pure rat prostate AdoMetDC labelled with [*Me*-^{3}H]AdoMet (M_r = 33000) (arrow); lane 2, untreated control cells, non-immune serum; lane 3, untreated control cells; lane 4, AdoDATO (50 μM); lane 5, MMTA (200 μM); lane 6, AdoDATO plus MMTA; lane 7, [^{14}C]methylated protein M_r markers (from the top to bottom, M_r 200000, 92500, 69000, 46000, 30000 and 14300).

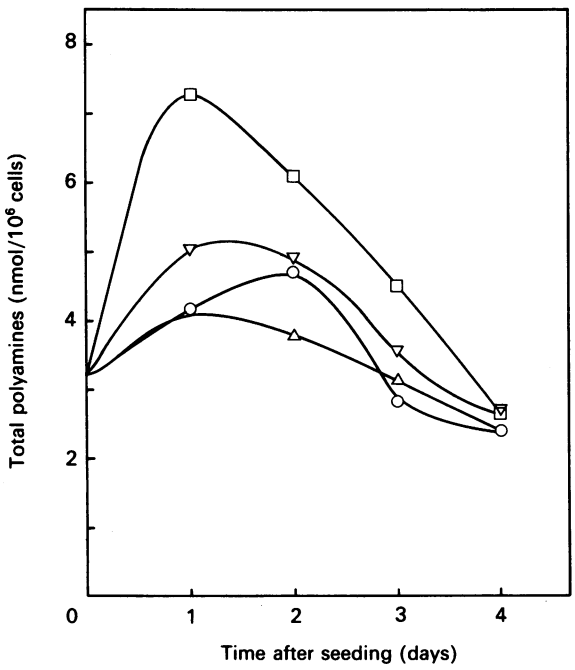

Fig. 6. Effects of AdoDATO, MMTA and their combination on the total endogenous polyamine content of Ehrlich ascites-tumour cells

The individual values for putrescine, spermidine and spermine (Fig. 1) were added. ○, Untreated control cells; △, AdoDATO (50 μM); ▽, MMTA (200 μM); □, AdoDATO plus MMTA.

The possibility that transitory changes in ODC and/or AdoMet DC activities occurred during the first day after the addition of the inhibitors was analysed by determining the enzyme activities at 4, 8 and 12 h. However, the results obtained (not shown) supported the curves drawn in Fig. 1.

The changes in cellular polyamine content caused by either AdoDATO or MMTA (or the combination) did not significantly affect the growth rate of the cells. This is probably because the total amount of polyamines in the cells was not decreased below the control level by the inhibitors (Fig. 6).

DISCUSSION

From the present study it is apparent that neither ODC nor AdoMetDC is specifically regulated by one particular polyamine. The decrease in spermidine level caused by the spermidine synthase inhibitor AdoDATO was accompanied by a rise in putrescine and spermine levels. These probably counteracted any potential regulatory effects that the decrease in spermidine might have exerted on the ODC or AdoMetDC activity. The rise in putrescine content was most probably due to the block in conversion of putrescine into spermidine, and the decrease in spermidine content a consequence of simultaneous inhibition of spermidine synthase and increased conversion of spermidine into spermine. The elevation in spermine content is likely to result from decreased competition by spermidine synthase (blocked by AdoDATO) for decarboxylated AdoMet, which is a substrate for both spermidine synthase and spermine synthase.

Inhibition of spermine synthase with MMTA caused a marked decrease in the cellular spermine level. This decrease was accompanied by increased putrescine (day 1) and spermidine levels. MMTA-treated cells exhibited a marked rise in AdoMetDC activity. At first sight the rise in AdoMetDC activity appeared to be connected with the decrease in cellular spermine content. However, this idea was contradicted by the fact that a similar rise in AdoMetDC activity was observed when MMTA was given in combination with AdoDATO, in which case the spermine content was largely unaffected. By analysing the half-life of AdoMetDC, we obtained evidence indicating that the increase in AdoMetDC activity caused by MMTA was mainly due to a stabilization of the enzyme, probably caused by binding of the inhibitor to the enzyme, thus rendering it less susceptible to degradation (Pegg *et al.*, 1987). The rate of AdoMetDC synthesis was only slightly stimulated by MMTA treatment. A somewhat different finding was reported by Pegg *et al.* (1987), using another cell line and a higher (0.4 mM) concentration of MMTA. The MMTA-mediated increase in AdoMetDC activity in SV-3T3 cells was found to be a result of both stabilization and increased synthesis of the enzyme (Pegg *et al.*, 1986, 1987). In the experiments on SV-3T3 cells, the increase in synthesis of AdoMetDC and part of the decreased degradation of AdoMetDC appeared to be due to the depletion of spermine, since the provision of this amine neutralized the effects of MMTA. At variance with the present study on Ehrlich ascites-tumour cells, MMTA caused no increase in spermidine in SV-3T3 cells (Pegg *et al.*, 1987). It is conceivable that the MMTA-mediated increase in spermidine content may counteract the

potential effects that spermine depletion may exert on AdoMetDC synthesis in Ehrlich ascites-tumour cells.

When the two inhibitors were combined, both putrescine and spermidine pools were increased, whereas the spermine pool was largely unaffected. The increase in spermidine may be due to a combination of effects, i.e. the inhibition of spermine synthase (the normal acceptor of spermidine) and the stabilization of AdoMetDC. As a result of the latter, there may be an increased pool of decarboxylated AdoMet, which may compete with AdoDATO for the active site in spermidine synthase. In addition, the combination of AdoDATO and MMTA caused an increase in ODC activity, thus producing more putrescine for spermidine synthesis. Putrescine has been shown to exert a direct stimulatory effect on AdoMetDC and to promote the conversion of the AdoMetDC proenzyme into the active form (Pegg *et al.*, 1988). Therefore, the concentration of decarboxylated AdoMet is likely to increase even further, thus neutralizing the adverse effects of AdoDATO and MMTA on spermidine and spermine synthesis.

The increase in ODC activity observed in cells treated with AdoDATO plus MMTA was not due to a decrease in polyamine-mediated suppression of ODC synthesis, since both putrescine and spermidine were increased after this treatment. The cause of this increase in ODC synthesis is unknown. Results from a study by Kramer *et al.* (1988) indicate that the cellular AdoMet pool may also affect the activity of ODC and AdoMetDC. Being analogues of AdoMet, it is conceivable that the combination of AdoDATO and MMTA may interfere with this regulatory mechanism and thus give rise to an induction of ODC activity.

The effects of AdoDATO and MMTA, and their combination, on cell growth were only minor. This is to be expected, because the total polyamine content of the cells was not decreased below that of untreated control cells. Notably, treatment with the combination of the two inhibitors caused a marked increase in total polyamine content. The present results demonstrate the difficulties in effectively depleting cells of their polyamines. Moreover, compartmentalization of the polyamines in the cell may further complicate the interpretation of the data. The biosynthesis of the polyamines is regulated at many different levels by the various polyamines, and more work is needed to clarify these regulatory mechanisms.

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