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Regulation of ornithine decarboxylase activity by spermidine and the spermidine analogue N^1N^8-bis(ethyl)spermidine

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Polyamine biosynthesis in intact cells can be exquisitely controlled with exogenous polyamines through the regulation of rate-limiting biosynthetic enzymes, particularly ornithine decarboxylase (ODC). In an attempt to exploit this phenomenon as an antiproliferative strategy, certain polyamine analogues have been identified [Porter, Cavanaugh, Stolowich, Ganis, Kelly & Bergeron (1985) Cancer Res. **45**, 2050–2057] which (*a*) lower ODC activity in intact cells, (*b*) have no direct inhibitory effects on ODC, (*c*) are incapable of substituting for spermidine (SPD) in supporting cell growth, and (*d*) are growth-inhibitory at micromolar concentrations. In the present study, the most effective of these analogues, N^1N^8-bis(ethyl)SPD (BES), is compared with SPD in its ability to regulate ODC activity in intact L1210 cells and in the mechanism(s) by which this is accomplished. With respect to time and dose-dependence of ODC suppression, both polyamines closely paralleled one another in their response curves, although BES was slightly less effective than SPD. Conditions of minimal treatment leading to near-maximal ODC suppression (70–80%) were determined and found to be 3 μM for 2 h with either SPD or BES. After such treatment, ODC activity was fully recovered within 2–4 h when cells were re-seeded in drug-free media. By assessing BES or [^{3}H]SPD concentrations in treated and recovered cells, it was possible to deduce that an intracellular accumulation of BES or SPD equivalent to less than 6.5% of the combined cellular polyamine pool was sufficient to invoke ODC regulatory mechanisms. Decreases in ODC activity after BES or SPD treatment were closely paralleled by concomitant decreases in ODC protein. Since cellular ODC mRNA was not similarly decreased by either BES or SPD, it was concluded that translational and/or post-translational mechanisms, such as increased degradation of ODC protein or decreased translation of ODC mRNA, were probably responsible for regulation of enzyme activity. Experimental evidence indicated that neither of these mechanisms seemed to be mediated by cyclic AMP or ODC-antizyme induction. On the basis of the consistent similarities between BES and SPD in all parameters studied, it is concluded that the analogue most probably acts by the same mechanisms as SPD in regulating polyamine biosynthesis.

INTRODUCTION

The ability of naturally occurring polyamines to regulate intracellular polyamine biosynthesis in cultured cells has been recognized for some time (Pett & Ginsberg, 1968; Kay & Lindsay, 1973; Clark & Fuller, 1975). Addition of micromolar concentrations of polyamines to cell cultures, for example, typically results in a rapid and near-total depletion of ornithine decarboxylase (ODC). A number of regulatory mechanisms have been proposed (McCann, 1980) and they are not necessarily mutually exclusive. They include: (*a*) post-transcriptional control of enzyme synthesis (Kay & Lindsay, 1973; Clark & Fuller, 1975; Canellakis & Theoharides, 1976; Kallio *et al.*, 1977; McCann *et al.*, 1979); (*b*) changes in the rate of enzyme turnover (Morris & Fillingame, 1974); (*c*) modification of the enzyme by covalent attachment of phosphate (Atmar & Kuehn, 1981) or putrescine (PUT) groups (Russell, 1981); (*d*) interconversion of the enzyme protein from an active to an inactive form (Mitchell & Sedory, 1974; Mitchell *et al.*, 1985); and (*e*) induction of an inhibitory protein, termed ODC antizyme, which complexes with the enzyme and inactivates it (Heller *et al.*, 1976; Fong *et al.*, 1976; McCann *et al.*, 1977, 1979; Heller & Canellakis, 1981). Evidence has been obtained for each of these mechanisms over the past several years in a wide variety of biological systems. At present, the preponderance of these findings favours a combination of mechanisms in which post-translational modification via antizyme binding to ODC contributes to an enhanced degradation of the enzyme protein (Seely & Pegg, 1983; Murakami & Hayashi, 1985; Murakami *et al.*, 1985; Canellakis *et al.*, 1985; Kanamoto *et al.*, 1986). Evidence has been presented to indicate that synthesis of the ODC polypeptide might be decreased at the level of ODC mRNA translation (Kahana &

Abbreviations used: AdoMet, *S*-adenosyl-L-methionine; AdoMetDC, AdoMet decarboxylase; BES, N^1N^8-bis(ethyl)spermidine; DFMO, α-difluoromethylornithine; ODC, ornithine decarboxylase (EC 4.1.1.17); PBS, phosphate-buffered saline (0.8% NaCl/0.115% Na_2HPO_4/0.02% KH_2PO_4,$2H_2O$/0.02% KCl, pH 7.4); PUT, putrescine; RPMI-1640, Roswell Park Memorial Institute Medium 1640; SPD, spermidine; SPM, spermine.

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Nathans, 1985*b*; Höltta & Pohjanpelto, 1986). Certain unique features of the ODC gene, such as an unusually long 5′ leader sequence (Kahana & Nathans, 1985*a*; Gupta & Coffino, 1985) lend plausibility to this proposal. It should be noted, however, that evidence for this mechanism was, in both studies (Kahana & Nathans, 1985*a*; Höltta & Pohjanpelto, 1986), derived from ODC-overproducing cell lines, which may not be normal in their ODC-regulatory mechanisms.

In a biological evaluation of their cellular effects of a series of N^1N^8- and N^4-SPD analogs (Porter *et al.*, 1982, 1985), we have identified certain N^1N^8-SPD derivatives, particularly N^1N^8-bis(ethyl)SPD (BES; Fig. 1), which appear to regulate ODC activity (and hence polyamine biosynthesis) in much the same manner as exogenous SPD. Unlike SPD, however, these analogues are incapable of substituting for SPD in function(s) related to growth, and consequently demonstrate meaningful antiproliferative activity *in vitro*. Comparison studies between BES and the specific ODC inhibitor, α-difluoromethylornithine (DFMO; Mamont *et al.*, 1978), in which the kinetics of growth inhibition, polyamine depletion and drug effects on other growth-related parameters were found to be nearly identical (Porter *et al.*, 1986), tend to support a causal relationship between polyamine depletion brought about by the regulatory effects of BES on ODC activity and inhibition of cell growth. This, however, remains to be substantiated by more direct evidence.

Although the effects of BES on ODC activity, polyamine biosynthesis and cell growth are very similar to those of DFMO (Porter *et al.*, 1986), the two agents differ with respect to various compensatory reactions elicited in the cell. Specifically, whereas DFMO increases AdoMetDC activity, and hence decarboxylated AdoMet pools (Pegg *et al.*, 1982; Mamont *et al.*, 1982), and also brings about an increase in the cellular uptake of polyamines (Alhonen-Hongisto *et al.*, 1982), BES does not (Porter *et al.*, 1986). Since these effects could compromise the effectiveness *in vivo* of inhibitors of ODC and since, on a concentration basis, BES is a more potent inhibitor of cell growth (Pera *et al.*, 1986; Porter *et al.*, 1986), we have elected to pursue the development of BES as a potential antiproliferative agent. Inherent in the pursuit of this goal is the need for elucidation of the mechanism(s) by which BES suppresses ODC activity. Accordingly, the present studies compare BES and SPD in their effects on ODC activity, protein and mRNA under conditions of presumed primary drug effect. In all parameters assessed, including the probable mechanism(s) of action, BES behaved identically with SPD. The data reveal a unique mode of action for polyamine analogues which may be exploited in their development as antiproliferative agents (Porter & Sufrin, 1986).

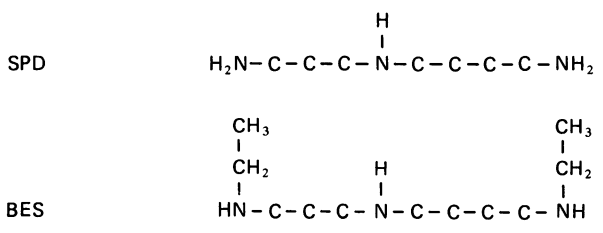

Fig. 1. Structural representations of SPD and N^1N^8-bis(ethyl)-SPD (BES), in which the hydrogen atoms of the aliphatic carbons in the SPD moiety have been purposely omitted

Note that, unlike SPD, BES has no primary amino groups.

MATERIALS AND METHODS

Materials

BES was synthesized as a hydrochloride salt via schemes and methodologies described elsewhere (Bergeron, 1986). Forskolin was obtained commercially from Boehringer Mannheim Chemical Co. (Indianapolis, IN, U.S.A.).

Culture conditions

Murine L1210 leukaemia cells were grown as a suspension culture in RPMI-1640 medium containing 2% Hepes/Mops as a buffer system, 1 mM-aminoguanidine as an inhibitor of serum diamine oxidase and 10% Nu Serum IV (Collaborative Research Inc., Lexington, MA, U.S.A.) as a semi-defined serum substitute. Cells were grown in 75 cm^2 tissue-culture flasks in a total volume of 15 or 50 ml respectively, under a humidified atmosphere containing 5% CO_2 at 37°C. Cell number was determined by electronic particle counting (model ZF Coulter Counter; Coulter Electronics, Hialeah, FL, U.S.A.).

Cell treatment

L1210 cells were seeded in 75 cm^2 plastic tissue-culture flasks and allowed to grow for 16 h before addition of either BES or SPD. The seeding inoculum was adjusted so that the cell density at the time of treatment was approx. 3×10^5 cells/ml. While cells were in exponential growth (16 h after seeding), BES or SPD was carefully introduced into the media with minimal disturbance of culture conditions. At 0.5, 1, 2, 4, 6 or 24 h after addition of either 3 μM or increasing concentrations of BES or SPD, cells were removed, centrifuged (1000 ***g*** for 5 min) and washed twice with ice-cold PBS. In certain experiments, cells were treated with SPD or BES in the presence of 50 μM-cycloheximide. The final cell suspension was counted for radioactivity and divided into portions for determination of ODC activity, protein or mRNA or for quantification of intracellular BES or [^{3}H]SPD when it was substituted for SPD. For recovery studies, cells were treated as above for 2 h with 3 μM-BES or -SPD, washed once with warm PBS and reseeded in drug-free medium at 1.5×10^5 cells/ml. Cells were then harvested 1, 2, 4 or 24 h later for determination of ODC activity, protein or mRNA or for quantification of intracellular BES or [^{3}H]SPD.

ODC activity and protein

Extracts for measurement of ODC activity and ODC protein were prepared by sonicating control or treated cells in 10 mM-Tris/HCl (pH 7.2) containing 0.5 mM-Na_2EDTA, 5 mM-dithiothreitol and 50 μM-pyridoxal 5′-phosphate, followed by centrifugation at 20000 ***g*** for 20 min. ODC activity was determined by measuring the release of $^{14}CO_2$ from L-[1-^{14}C]ornithine in the presence of saturating concentrations of pyridoxal phosphate (0.1 mM) in accordance with the methods of Pegg & Seely (1983). ODC protein was quantified as described by Seely & Pegg (1983), with a monospecific rabbit antiserum to

mouse ODC and ODC labelled by reaction with [^{3}H]DFMO as the radioactive ligand.

Analysis of ODC mRNA

Total RNA was isolated by the guanidinium chloride method (Cox, 1968). To measure ODC mRNA, 10 mg of RNA was fractionated on 1.5% formaldehyde/agarose gels, transferred to nitrocellulose and hybridized to ^{32}P-labelled pODC934 DNA, a plasmid which contains sequences complementary to mouse kidney ODC mRNA (Berger *et al.*, 1984). Hybridization was observed by autoradiography.

Intracellular polyamines

A sample of 10^7 cells was taken for polyamine determinations after extraction with 0.6 M-$HClO_4$. The extract was analysed by h.p.l.c. with a system based on cation exchange and post-column derivative formation with *o*-phthaldehyde as described elsewhere (Porter *et al.*, 1985).

Intracellular BES

Because BES lacks primary amino groups and is, therefore, minimally reactive with *o*-phthaldehyde (Benson & Hare, 1975), intracellular concentrations of the analogues were measured by h.p.l.c. after pre-column derivative formation with danysl chloride by L. J. Marton and colleagues (University of California, San Francisco), by using a polyamine-analysis system established as described elsewhere (Kabra *et al.*, 1986).

Cyclic AMP

Cyclic AMP present in acid-soluble extracts of cells was quantified by the competition binding assay kit of Amersham (Arlington Heights, IL, U.S.A.). Nucleosides were first separated from perchlorate salts by Freon/alamine extraction (Khyme, 1975).

RESULTS

In order to increase the probability of characterizing a primary (and possibly singular) mechanism responsible for polyamine regulation of ODC, conditions of minimal treatment in terms of time and concentration were determined. Time-dependence was first established by treating cells for 0.5–24 h with 10 μM-BES or -SPD (Fig. 2). This concentration was chosen as one known from previous studies (Porter *et al.*, 1985) to be non-toxic during a 24 h incubation. During the first 2 h of treatment, ODC activities fell rapidly to about 30% of control values with BES and 20% with SPD. After 2 h, ODC activity rose slightly to a small peak at 4 h and then declined slowly to approx. 25% of control at 24 h for both polyamines. Over the entire 24 h period the response curve for BES closely paralleled that for SPD, although BES was slightly less effective than SPD in suppressing ODC activity. On the basis of these curves, 2 h was selected as the minimum treatment time yielding a maximum suppression of ODC activity.

Next, the minimum polyamine concentration was determined by treating cells with increasing concentrations of BES or SPD for the 2 h period derived above (Fig. 2). Suppression of ODC first became apparent at 0.1 μM-SPD and 0.3 μM-BES and reached a near maximum at 1.0 μM and 3.0 μM respectively. Concentrations as high as 100 μM failed to suppress ODC substantially beyond that achieved with these lower concentrations. As with time, the dose–response curve for BES closely paralleled that for SPD, and it was consistently slightly less effective. On the basis of the response curves in Fig. 2, treatment with 3 μM-BES or -SPD for 2 h was adopted for all subsequent studies. Under such conditions, AdoMetDC activity was only slightly lowered by either polyamine (see Table 3).

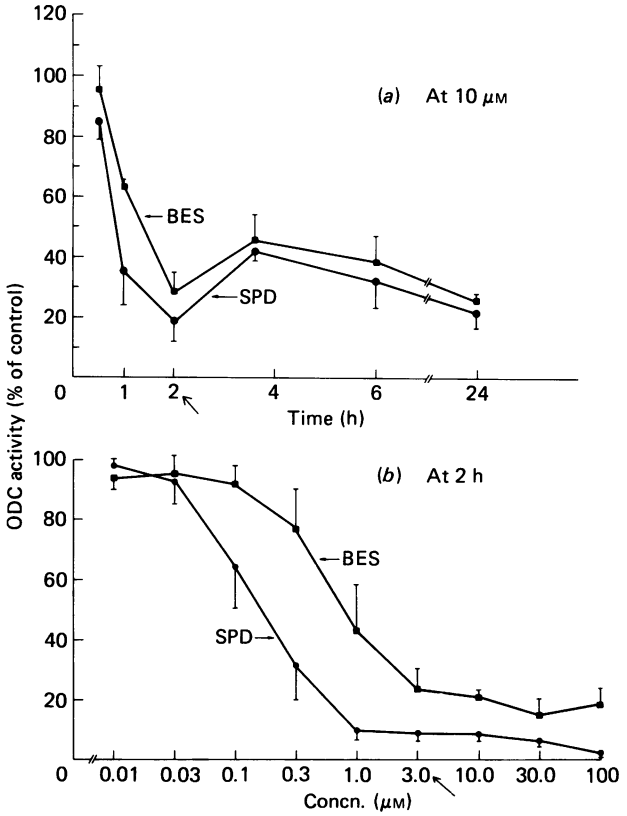

Fig. 2. Characterization of the dependence of ODC regulation of L1210 cells on time (*a*) and drug concentration (*b*)

On the basis of these findings, conditions for treatment were defined as 3 μM-BES or -SPD for 2 h (see arrows). Note that, with both time and concentration, BES closely parallels SPD in its ability to suppress ODC activity and that it is slightly less effective. Mean control ODC activity for these experiments was 11.2±3.1 nmol of CO_2/h per mg of protein.

As an additional comparison between BES and SPD, the ability of cells treated with 3 μM-BES or -SPD for 2 h to recover control ODC activity when placed in polyamine-free medium was characterized with respect to time (Fig. 3). Again the recovery curves for both polyamines closely paralleled one another. By 2 h after re-seeding in drug-free medium, cells treated with either polyamine had surpassed the ODC activities in control cells processed identically. ODC activity remained at slightly greater than control values for the 22 h recovery period. It should be noted that, owing to perturbations of culture conditions during re-seeding, control cell ODC activity was not as great as that of control cells in exponential growth at the time of BES or SPD treatment (see legend to Fig. 3). Thus restoration of enzyme activity was found to be a rapid phenomenon, and a recovery period of 4 h (where experimental variation was

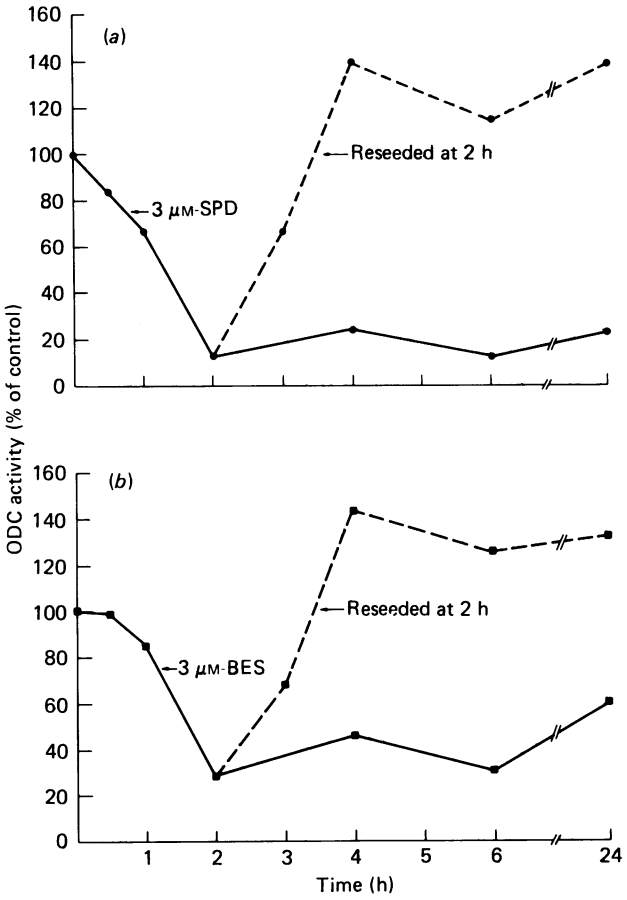

Fig. 3. Kinetics of ODC suppression of SPD (*a*) or BES (*b*)

Over the period 0.5–24 h (continuous line), at 3 μM-BES or -SPD, suppression of ODC activity reaches a maximum at 2 h and does not significantly decrease further with time. If, after 2 h of treatment, the cells are re-seeded into drug-free media (broken line), ODC activity recovers to greater than control values within 2 h for both drugs. Control ODC activity for treated cells (2 h) averaged 10.8 ± 3.8 nmol/h per mg, whereas, owing to perturbations of culture conditions during re-seeding, control activity for re-seeded cells (6 h) averaged 8.1 ± 2.7 nmol/h per mg.

less of a factor than at 2 h) was adopted for subsequent studies.

It became of obvious interest to determine the concentration of exogenous BES or SPD in cells at the time of ODC suppression of recovery from suppression at the conditions defined above. Intracellular BES was quantified by h.p.l.c. After 2 h, there was no evidence of further conversion of BES into a SPM analogue, nor of its retro-conversion into a PUT analogue (results not shown). This may derive from a lack of primary amino groups (Fig. 1), which are likely to be critical determinants for such enzyme reactions. Thus the BES detected by h.p.l.c. was taken to represent the total amount of analogue that had entered the cell during treatment. In the case of SPD, [^{3}H]SPD was substituted in order to follow cellular accumulation. The intracellular radioactivity was characterized by collecting h.p.l.c. fractions, counting their radioactivity by scintillation techniques and comparing the data with those obtained by using known standards of [^{3}H]PUT, [^{3}H]SPD and [^{3}H]SPM. During the 2 h period, less than 4% of the total cellular radioactivity was found to be associated with SPM, and none was associated with PUT, as might occur by sequential acetylation and oxidation via the recycling pathway (Pegg & McCann, 1982). Thus 96% of the total radioactivity associated with cells treated with [^{3}H]SPD was taken to represent exogenous SPD accumulated during the 2 h incubation. Intracellular [^{3}H]SPD content during longer (i.e. 6 h) incubations was not similarly quantified, because of a more significant conversion into SPM.

In the course of a 2 h incubation, BES accumulated to a total of 550 pmol/10^6 cells and SPD to 670 pmol/10^6 cells (Table 1). When compared with control amounts of SPD (2070 pmol/10^6 cells), the newly accumulated BES or [^{3}H]SPD amounted to an increase of approx. 27% and 32% respectively. After recovery for 4 h in polyamine-free medium, when ODC activity surpassed control values, the cellular BES content fell from 550 to 380 pmol/10^6 cells. The difference of 170 pmol/10^6 cells constitutes only 8% of the total SPD pool. Likewise with [^{3}H]SPD, cellular radioactivity declined from 670 to 445 pmol/10^6 cells, a difference of 225 pmol/10^6 cells or about 11% of the total cellular SPD. Thus relatively

Table 1. Intracellular polyamine pools under defined conditions of suppression and recovery of ODC activity by BES or [^{3}H]SPD

Values for polyamine pools are from four experiments performed in duplicate. In all cases, variation in polyamine pools relative to control cells was less than 10% between experiments.

Conditions	Polyamine pools (pmol/10^6 cells) PUT	SPD	SPM	BES or [^{3}H]SPD (pmol/10^6 cells)	(% of control SPD pool)
2 h treatment					
Control	330	2070	670	–	–
3 μM-BES	85	2500	820	550	27
3 μM-[^{3}H]SPD	80	2705	855	670*	32
2 h treatment, 4 h recovery					
Control	320	2155	625	–	–
3 μM-BES	305	2195	755	380	18
3 μM-[^{3}H]SPD	300	1870	860	445	21

* Of the total cellular radioactivity, 96% was found to be present as SPD and only 4% as SPM.

small fluctuations (i.e. approx. 10%) in the total SPD pool resulting from the uptake of exogenous SPD or its analogue, BES, seem sufficient to account for substantial decreases or increases in cellular ODC activity.

The polyamine pools in treated and recovered cells were also analysed. For the most part, fluctuations in PUT pools in SPD-treated cells reflected a decrease or recovery in ODC activity, whereas those of SPD reflected an accumulation of exogenous polyamine (Table 1). In cells treated with BES, both SPD and SPM pools increased by nearly as much as in cells treated with [^{3}H]SPD. The basis for this is not clear. It could not be accounted for by the conversion of BES into SPD or SPM, since neither of the probable intermediate analogues (i.e. N^1- or N^8-ethyl-SPD) was detected by h.p.l.c. In addition, the time course (2 h) seems too short for such conversions.

In order to gain further indication that BES might be acting mechanistically similarly to SPD in regulating ODC activity, effects on ODC protein and mRNA contents were compared under the treatment and recovery conditions defined above. The decline in ODC activity seen at 2 h with 3 μM-BES or -SPD correlated in both cases with a concomitant decline in ODC protein as followed by immuno-detection (Table 2). When compared with control cells, the ODC activity/protein ratio was very similar, in the range of 0.6–0.8 unit/ng of protein. Likewise, during the recovery of ODC activity in polyamine-free media, ODC protein increased in parallel so that, again, the ODC activity/protein ratio remained about the same for control and pre-treated cells (0.7–0.8 unit/ng). It should be noted that, during removal of drug for recovery studies, the culture conditions were markedly perturbed. Thus both ODC activity and protein of control cells before re-seeding were considerably higher than in control cells after re-seeding (Table 2).

To ascertain whether decreases in gene transcription might account for polyamine-induced decreases in ODC activity and protein, ODC mRNA was assayed under conditions of treatment and recovery by Northern-blot analysis (Fig. 4). Despite a substantial decrease in both ODC activity and protein after a 2 h treatment with 3 μM-BES or -SPD, ODC mRNA was not similarly affected. A faint 2.6-kilobase and a major 2.1-kilobase

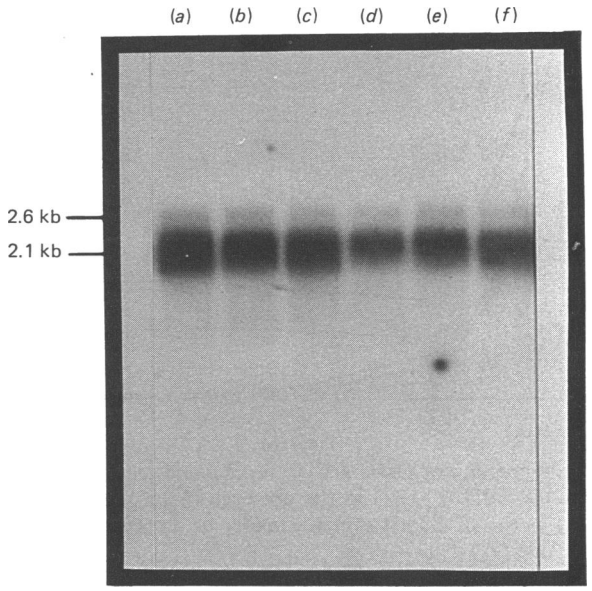

Fig. 4. Northern-blot analysis using pODC934 DNA (Berger *et al.*, 1984) as a probe for the ODC mRNA content of control L1210 cells (lane *a*) or cells treated for 2 h with 3 μM-BES (lane *b*) or SPD (lane *c*)

Lanes (*d*)–(*f*) represent ODC mRNA from cells treated as above and re-seeded in drug-free medium for 4 h. Specifically, these include control cells (lane *d*) and cells pretreated with BES (lane *e*) or SPD (lane *f*). Note that both the minor 2.6-kilobase (kb) and the major 2.1-kilobase transcript species of ODC mRNA are similarly affected in all cases.

Table 2. Effects of SPD and BES on intracellular activity and protein

A unit of ODC equals 1 nmol/30 min.

Conditions	ODC activity (units/mg)	ODC protein (ng/mg)	Activity/protein ratio (units/ng of protein)
2 h treatment			
Control	10.6	12.8	0.8
3 μM-SPD	0.4	0.7	0.6
3 μM-BES	2.1	3.8	0.6
2 h treatment, 4 h recovery			
Control	6.0	7.3	0.8
3 μM-SPD	6.1	8.3	0.7
3 μM-BES	6.0	8.5	0.7

ODC mRNA were detected, and both transcripts remained unchanged by exposure to the polyamines. After re-seeding, the treated cells were observed to contain more ODC mRNA than did control cells. The difference seems to be due to a decrease in transcription in control recovered cells, which in turn may be related to the effects of perturbation of culture conditions during re-seeding. For example, after re-seeding, control ODC protein decreased from 12.8 to 7.3 ng/mg of cellular protein (Table 2).

Overall, the ODC protein and mRNA data are consistent with two mechanisms of SPD and BES actions: increased degradation of the ODC protein and/or decreased translation of the ODC mRNA. Studies of suppression of ODC activity by PUT and SPD indicate that both mechanisms may be operative (Seely & Pegg, 1983; Murakami *et al.*, 1985; Kahana & Nathans, 1985*b*; Hölttä & Pohjanpelto, 1986). Concomitant treatment of cells with BES or SPD and cycloheximide did not antagonize the ability of these polyamines to suppress ODC activity (Fig. 5) and, in fact, increased it slightly. Thus rapid synthesis of antizyme or any similar protein does not seem to play a role in either of the above-mentioned possibilities.

Finally, we became intrigued by the possibility that the regulation of cellular ODC activity by small quantities of exogenous BES or SPD relative to the amount already in the cell may be mediated by membrane mechanisms involving cyclic AMP (Canellakis *et al.*, 1979). In order to test this possibility, intracellular cyclic AMP was determined in cells treated with BES or SPD. In addition, ODC and AdoMetDC activity was assayed in

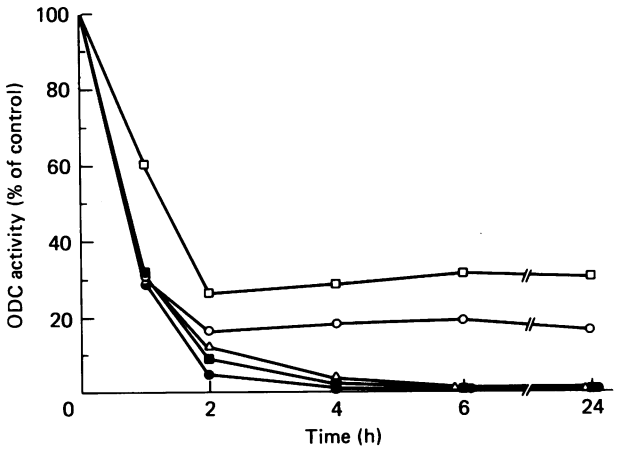

Fig. 5. Suppression of ODC activity by 3 μM-BES (□, ■) and 3 μM-SPD (○, ●) in the presence (■, ●) or absence (□, ○) of 50 μM-cycloheximide, or by cycloheximide alone (△)

With both BES and SPD, suppression of ODC activity is enhanced by cycloheximide during the 1–24 h period studied.

Table 3. Comparison of the effects of BES and SPD with those of dibutyryl cyclic AMP and forskolin on intracellular cyclic AMP and ODC or AdoMetDC activity in L1210 cells

Control cyclic AMP was 4.4 pmol/10^7 cells. Control ODC activity was 18.5 nmol/h per mg, and control AdoMetDC activity, 7.25 nmol/h per mg.

Treatment (2 h)	Cyclic AMP (% of control)	Decarboxylase activity (% of control	
		ODC	AdoMetDC
3 μM-SPD	107	15	82
3 μM-BES	96	33	86
3 μM-Dibutyryl cyclic AMP	135	63	94
3 μM-Forskolin	160	71	102
30 μM-SPD	98	5	63
30 μM-BES	89	15	77
30 μM-Dibutyryl cyclic AMP	150	40	101
30 μM-Forskolin	164	42	77

cells after exposure to dibutyryl cyclic AMP and to forskolin, a stimulator of adenylate cyclase (Metzger & Lindner, 1981; Seamon & Daly, 1983). Since neither SPD nor BES was found to increase intracellular cyclic AMP (Table 3), its involvement as a mediator of enzyme regulation was excluded. However, it was noteworthy that the increases in cyclic AMP brought about by dibutyryl cyclic AMP or forskolin resulted in a 60% decrease in ODC activity.

DISCUSSION

It has been previously demonstrated that the ODC activity in extracts from untreated cells is not directly inhibited by concentrations of BES ranging up to 10 mM (Porter *et al.*, 1985, 1986). Accordingly, the rationale for the present inquiry was based on the probability that, in the context of an intact cell, BES behaves similarly to SPD in the well-recognized ability of the latter polyamine to regulate ODC activity negatively. Consistent with this premise, close similarity in the kinetics of ODC suppression by BES and SPD was observed. With respect to both time and concentration, the two polyamines closely paralleled one another. This parallel was also observed in the recovery kinetics of ODC activity after drug removal. Thus, whatever mechanism(s) are invoked by BES and SPD in suppressing ODC activity, they are quite comparable in their rapidity of onset and reversibility.

This similarity was also extended to all parameters examined in characterizing the molecular mechanism(s) of ODC regulation. Under defined conditions of minimal drug treatment designed to identify probable primary drug actions, BES and SPD were observed to elicit a decrease in ODC protein proportional to ODC activity without changing the amount of ODC mRNA. The findings are consistent with the current literature indicating that ODC suppression by polyamines may involve decreased translation of the ODC mRNA (Kahana & Nathans, 1985*b*; Höltta & Pohjanpelto, 1986), possibly together with post-translational mechanisms related to ODC degradation (Seely & Pegg, 1983; Murakami & Hayashi, 1985; Murakami *et al.*, 1985; Canellakis *et al.*, 1985; Kanamoto *et al.*, 1986). Whether or not, under the conditions defined in the present study, the latter involves binding of ODC antizyme to the enzyme was not discerned. The fact that concomitant treatment of cells with cycloheximide as well as SPD or BES did not decrease the extent of enzyme suppression suggests that, if antizyme was involved in the process, it was preformed and released by polyamines, as opposed to being rapidly induced by them.

It was particularly interesting to quantify the intracellular concentrations of BES or SPD associated with suppression of ODC activity. Possibly because BES lacks primary amino groups, it was not metabolized forward to a SPM analogue or backward to a PUT analogue. Thus its intracellular concentration could be related directly to effects on enzyme activity. Because the conditions of treatment (i.e. 3 μM for 2 h) were minimized, over 96% of the radioactivity in cells treated with [^{3}H]SPD was recovered by h.p.l.c. as SPD. Thus, under the conditions of minimal treatment which yielded a near-maximal suppression of ODC activity, the intracellular concentration of both BES and SPD could be quantified and was found to be 27 and 32%, respectively, of the total SPD pool present in control cells. Since the primary events in ODC suppression were initiated much earlier than the 2 h period of observed maximal suppression in ODC activity, the concentrations required to trigger the effect were undoubtedly substantially less than those given above. An estimate of this amount was deduced by subtracting the amount of BES or SPD present in cells permitted to recover ODC activity in drug-free medium for 4 h. When this is done, it was found that an increase of about 10% in the intracellular SPD pool by either BES or SPD seems sufficient to regulate ODC. This percentage becomes even lower if one relates it to total polyamine pools including PUT, SPD and SPM, since all three are

capable of regulating ODC activity. Thus an increase of less than 6.5% in total polyamines would be required to elicit an effect on ODC. Assuming an even distribution within the cell (which is probably unlikely), this represents about 200–300 μM, as compared with cellular concentrations of 0.5 mM-PUT, 3.1 mM-SPD and 1 mM-SPM [concentrations were calculated by using cell volume determinations reported previously for L1210 cells (Pera *et al.*, 1986)].

It is somewhat paradoxical that such a small amount of exogenous polyamine relative to the amount of total polyamines already in the cell is sufficient to bring about such a response. At least two explanations can be offered: (*a*) intracellular polyamines are largely bound or compartmentalized and unavailable for such an effect, or (*b*) exogenous polyamines may bind at sites associated with sites at the outer surface of the plasma membrane and trigger enzyme regulation through additional mechanisms (Canellakis *et al.*, 1979). As a test of this latter possibility, we examined the possible involvement of the plasma-membrane second messenger, cyclic AMP, in the phenomenon of ODC regulation. Experiments revealed that neither BES nor SPD increased intracellular cyclic AMP, and therefore tend to exclude a role for it in polyamine-mediated ODC regulation. Paradoxically, however, two agents which increased intracellular cyclic AMP, forskolin and dibutyryl cyclic AMP, also suppressed ODC activity, so that different mechanisms for enzyme regulation may exist.

The relatively small amount of polyamine or polyamine analogue required for the intracellular regulation of polyamine biosynthesis, together with the presence of an active transport mechanism (Porter *et al.*, 1982), argues favourably for the use of polyamine analogues as an antiproliferative strategy (Porter & Sufrin, 1986). This regulatory approach is further strengthened by the finding that such a physiological intervention of polyamine biosynthesis is not likely to invoke the same compensatory reactions elicited with enzyme inhibitors. Specifically, BES does not result in the same increases in AdoMetDC, decarboxylated AdoMet pools and polyamine uptake as observed with ODC inhibitors such as DFMO (Porter *et al.*, 1986). However, although it is tempting to propose that growth inhibition by BES derives exclusively from negative regulation of polyamine biosynthesis, the possibility must be considered that analogue binding at sites otherwise occupied by SPD or other polyamines might also play a role.

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