Selective regulation of *S*-adenosylmethionine decarboxylase activity by the spermine analogue 6-spermyne

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Polyamine-biosynthesis activity is known to be negatively regulated by intracellular polyamine pools. Accordingly, treatment of cultured L1210 cells with 10 μ M-spermine rapidly and significantly lowered ornithine decarboxylase (ODC) and *S*-adenosylmethionine decarboxylase (AdoMetDC) activities in a sequential manner. By contrast, treatment for 48 h with 10 μ M of the unsaturated spermine analogue 6-spermyne lowered AdoMetDC activity, but not ODC activity. An initial decrease in ODC activity at 2 h was attributed to a transient increase in free intracellular spermidine and spermine brought about through their displacement by the analogue. Thereafter, ODC activity recovered steadily to control values as 6-spermyne pools increased and spermidine and spermine pools decreased owing to analogue suppression of AdoMetDC activity. The apparent ability of 6-spermyne to regulate AdoMetDC, but not ODC, activity suggests an interesting structure–function correlation and demonstrates that the typical co-regulation of these enzyme activities can be dissociated. This, in turn, may reflect the existence of independent regulatory binding sites for the two enzymes.

INTRODUCTION

Of the four major enzymes comprising the polyamine-biosynthesis pathway, those most critically involved in its regulatory control are ornithine decarboxylase (ODC) and *S*-adenosylmethionine (AdoMet) decarboxylase (AdoMetDC). Probably not coincidentally, they are 'lead-in' enzymes for substrates into the pathway and are distinguished by having half-lives in the order of 20–90 min (Pegg, 1986). In addition to being highly inducible by growth-promoting stimuli, the decarboxylases are well known for their responsiveness to negative regulatory control by changes in intracellular polyamine pools. Decreases in polyamine pools such as those induced by specific enzyme inhibitors cause significant increases in either or both enzyme activities, according to the inhibitor used (Porter & Sufrin, 1986). By contrast, increases in polyamine pools, such as those resulting from uptake of polyamines or their analogues, typically result in decreases in enzyme activities (Porter & Sufrin, 1986). Whether the activity of ODC alone or of both enzymes is decreased depends on the polyamine involved; putrescine usually decreases only ODC activity, whereas spermidine and spermine decrease both (Porter & Bergeron, 1988). The apparent overall purpose of these regulatory controls is to enable cells to maintain a balanced and constant supply of putrescine, spermidine and spermine for cellular functions related to cell growth.

We have sought to exploit this regulatory control for anti-proliferative purposes by designing polyamine analogues which negatively regulate polyamine biosynthesis and, at the same time, are incapable of substituting for polyamines in growth-dependent functions (Porter & Bergeron, 1988). In attempting to identify the structural

Fig. 1. Structural representation of 6-spermyne in which the carbon hydrogen atoms were omitted

features of polyamine molecules which account for their various cellular effects, we have evaluated several polyamine analogues. It was observed that the combined regulation of ODC and AdoMetDC which is typically elicited by exogenous spermine does not occur with the unsaturated spermine analogue 6-spermyne (Fig. 1); rather, the analogue appears to regulate only AdoMetDC activity. The finding reveals that co-regulation of ODC and AdoMetDC can be uncoupled and provides insight into the structural determinants responsible for the control of these enzymes.

MATERIALS AND METHODS

Materials

The unsaturated spermine analogue 6-spermyne was synthesized as a hydrochloride salt by using schemes and methodologies described elsewhere (Bergeron, 1986).

Cell culture

Murine L1210 leukaemia cells were maintained in RPMI (Roswell Park Memorial Institute) 1640 medium containing 2% (w/v) Hepes/Mops buffer and 10% (v/v) NuSerum IV (Collaborative Research, Lexington, MA, U.S.A.) as a semi-defined serum substitute. Treatment was initiated with cells in exponential growth and in the presence of 1 mM-aminoguanidine as an inhibitor

Abbreviations used: AdoMet, *S*-adenosyl-L-methionine; AdoMetDC, AdoMet decarboxylase; BESm, *N*¹*N*¹²-bis(ethyl)spermine; ODC, ornithine decarboxylase.

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of serum diamine oxidase (Shore & Cohn, 1960). Cells were grown in 75 cm² tissue-culture flasks in a total volume of 50 ml, under a humidified atmosphere containing 5% CO₂ at 37 °C. Cell number was determined by electronic particle counting with a Cell-Dyne 300 haematology counter (Sequoia-Turner Co., Mountain View, CA, U.S.A.).

Enzyme activities

ODC and AdoMetDC were extracted from cells and their activities assayed precisely as described by Pegg & Seely (1983) and Pegg & Poso (1983) respectively. Activities were expressed as nmol of CO₂ released/h per mg of protein as determined by the Bio-Rad assay kit (Bio-Rad Laboratories, Richmond, CA, U.S.A.). Spermidine and spermine synthase assays were performed as described by Raina *et al.* (1983). Substrate for the reaction, decarboxy-Ado[³⁵S]Met, was kindly provided by Dr. Anthony Pegg (Hershey, PA, U.S.A.).

Intracellular polyamines and 6-spermyne

A sample of 10⁷ cells was taken for polyamine determinations after extraction with 0.6 M-HClO₄. The extract was analysed by h.p.l.c. with a system based on cation exchange and post-column derivative formation with *o*-phthalaldehyde as described by Porter *et al.* (1985). Polyamines and 6-spermyne were quantified against a mixture of standards run on the same day.

RESULTS

As observed previously (Porter & Bergeron, 1988; Porter *et al.*, 1987b), exposure of L1210 cells to exogenous spermine or the anti-proliferative spermine analogue *N*¹*N*¹²-bis(ethyl)spermine (BESm) effectively suppresses both ODC and AdoMetDC activities by regulatory mechanisms not involving direct enzyme inhibition (Porter *et al.*, 1987a,b). By 48 h, both enzyme activities were lowered by approx. 90% and polyamine pools were altered accordingly (Table 1). Unexpectedly, 6-spermyne had a differential effect on the enzymes. By 48 h, 10 µM-6-spermyne lowered AdoMetDC activity by 90%, but decreased that of ODC by only 5%. Intracellular polyamine pools reflected these enzyme effects: putrescine pools were increased slightly, whereas those of spermidine and spermine were decreased substantially, as would be expected for selective interference with AdoMetDC activity. It should be noted that, under the

Fig. 2. Dose-response plot illustrating the effects of increasing concentrations of spermine and 6-spermyne on AdoMetDC extracted from untreated L1210 cells

Drugs were included in the standard enzyme assay system during a 60 min incubation.

conditions of these experiments, cell growth was not affected by either 6-spermyne or spermine, whereas BESm decreased growth by 40%. In separate experiments, the dose of 6-spermyne giving 50% inhibition was found to be > 100 µM in the presence of 0.1 mM-aminoguanidine and 10 µM in its absence during 48 h incubations, indicating that the analogue is a substrate for serum oxidases. Aminoguanidine was used at 0.1 mM in all of the present studies.

To determine whether the decrease in AdoMetDC activity brought about by 6-spermyne was due to direct inhibition of the enzyme, the analogue or spermine was included in the enzyme assay during a standard 60 min incubation. At concentrations up to 1 mM, 6-spermyne was no more effective than spermine itself in inhibiting AdoMetDC (Fig. 2). At concentrations above that expected to be present in cells in unbound form (i.e. 1.0 mM), 6-spermyne inhibited AdoMetDC by 24%, as compared to 18% for spermine. It therefore seemed likely that 6-spermyne, like spermine, suppresses AdoMetDC activity in intact cells by regulatory mechanisms inherent to the polyamine-biosynthesis pathway [see Porter & Bergeron (1988) for review].

Table 1. Effects of spermine and selected analogues on L1210-cell polyamine biosynthesis

Control ODC activity was 10.1 nmol of CO₂/mg of protein, and for AdoMetDC, 3.4 nmol of CO₂/mg of protein. Abbreviations: PUT, putrescine; SPD, spermidine; SPM, spermine.

Treatment (48 h)	Growth inhibition (% of control)	Decarboxylase activity (% of control)		Polyamine pools (pmol/10 ⁶ cells)		
		ODC	AdoMetDC	PUT	SPD	SPM
None	100	100	100	270	2180	960
10 µM-SPM	90	9	12	nd	240	1980
10 µM-BESm	60	2	11	nd	40	430
10 µM-6-Spermyne	110	95	10	360	560	110

Fig. 3. Time-dependence of suppression of decarboxylase activities by 10 μ M-spermine (●) or 6-spermyne (■) in L1210 cells

Note that, after an initial period of suppression, ODC activity recovers in 6-spermyne-treated cells.

The kinetics of decarboxylase suppression during 0–48 h of treatment were compared for spermine and the analogue 6-spermyne (Fig. 3). In light of the 48 h data (Table 1), it was unexpected to observe that 6-spermyne produced a rapid decrease in ODC activity in a manner similar to spermine but that, after an initial maximal effect at 2 h, enzyme activity recovered steadily so that, by 48 h, it returned to control values, as observed previously (Table 1). ODC activity in cells treated with spermine remained depressed during the entire 48 h period. Suppression of AdoMetDC was nearly identical for 6-spermyne and spermine (Fig. 3). Activity was near-maximally decreased by about 12 h and fell only slightly more during the next 36 h.

The unexpected fall and rise in ODC activity with 6-spermyne treatment gave rise to suspicion that the compound might be degraded with time, although the sustained suppression of AdoMetDC seemed inconsistent with this possibility. 6-Spermyne was detectable by h.p.l.c. in a mixture of polyamine standards as a distinct peak just ahead of spermine, with a retention time of 23.50 min. In chromatograms of extracts from 6-spermyne-treated cells, the same peak was apparent, with a retention time of 23.39 min (Fig. 4). Presumably the peak represented the compound in intact form, since it was located precisely where the analogue would be predicted to appear from past experience with the system and other analogues. In cells treated with 6-spermyne, this peak was found to increase substantially during the

Fig. 4. H.p.l.c. of extracts from L1210 cells treated and not treated with 10 μ M-spermine or 6-spermyne for 2 h (upper panel) or 24 h (lower panel)

Note that 6-spermyne is detectable as a distinct peak just ahead of spermine. The elution time of 6-spermyne was 23.39 min in cell extracts and 23.50 min in a preparation of polyamine standards. Abbreviations: PUT, putrescine; SPD, spermidine; SPM, spermine.

period of 2–48 h (Table 2), indicating that the rise in ODC activity is not due to a decrease in intracellular 6-spermyne. It is significant to note that the recovery in ODC activity paralleled a decrease in spermidine and spermine pools and an increase in intracellular 6-spermyne (Table 2).

The possibility that 6-spermyne might decrease spermidine and spermine pools by inhibiting spermidine synthase and/or spermine synthase was examined. Co-incubation of an enzyme-containing extract from untreated cells with 100 μ M-6-spermyne during the enzyme assay decreased both synthase activities by only ~20%, whereas spermine decreased spermidine synthase by 23% and spermine synthase by 81%. Since 6-spermyne is less inhibitory towards either enzyme than is spermine itself, it seems doubtful that this relatively weak inhibitory effect on the enzymes is responsible for the alterations in spermidine and spermine pools.

Table 2. Time-dependence of 6-spermyne and spermine effects on polyamine biosynthesis in cultured L1210 cells relative to cellular accumulation

Treatments were in the presence of 0.1 mM-aminoguanidine. Cell growth was similar to control under all treatment conditions. Abbreviations as in Table 1; n.d., not detectable.

Treatment	Time (h)	Decarboxylase activity (% of control)		Polyamines (pmol/10 ⁶ cells)			
		ODC	AdoMetDC	PUT	SPD	SPM	Analogue*
None	2	100	100	515	2075	915	
10 μ M-6-Spermyne	2	24	53	200	1830	875	445
10 μ M-SPM	2	19	66	190	1805	1405	
None	24	100	100	660	2505	1245	
10 μ M-6-Spermyne	24	65	11	555	905	475	1720
10 μ M-SPM	24	10	5	130	455	3425	
None	48	100	100	265	1920	860	
10 μ M-6-Spermyne	48	95	13	345	505	155	2210
10 μ M-SPM	48	12	11	n.d.	195	2060	

* Presumed to be 6-spermyne from comparisons with a mixture of polyamine standards containing 6-spermyne.

DISCUSSION

It is well known that polyamine-biosynthesis activity is under negative regulatory control by intracellular polyamine pools and that manipulations which increase these pools tend to lower critical enzyme activities (Porter & Bergeron, 1988). Thus treatment of cells with 10 μ M-spermine rapidly suppressed both ODC and AdoMetDC activities in a sequential manner. By contrast, 6-spermyne decreased both enzymes at 2 h, after which ODC activity recovered to control values by 48 h, whereas AdoMetDC remained suppressed. Neither spermine nor 6-spermyne displayed a direct inhibitory effect on either enzyme at concentrations up to 1 mM, indicating that both compounds probably affected enzyme activity through regulatory mechanisms inherent to the polyamine-biosynthesis pathway as it functions in intact cells. Since 6-spermyne accumulated in cells with time, and since, unlike ODC, AdoMetDC activity did not recover, metabolism of 6-spermyne was discounted as a possible basis for recovery of ODC activity. Instead, polyamine-pool data suggest that ODC recovery occurs because, unlike spermine, 6-spermyne is unable to regulate the enzyme. The initial suppression of ODC activity is therefore probably not directly due to 6-spermyne, but rather to an increase in free intracellular spermidine and spermine resulting from their displacement by the analogue at various binding sites. Thus after 2 h ODC activity recovers steadily while, at the same time, 6-spermyne pools increase and those of spermidine and spermine decrease as a result of AdoMetDC suppression. As a consequence of these changes, ODC activity could recover because the amount of spermidine and spermine available for binding at the ODC regulatory site becomes limiting, and/or 6-spermyne blocks spermidine and spermine binding by ineffectively occupying a critical proportion of the sites. In either case, the analogue is unable to sustain the initial suppression of ODC activity, even though it is successful in suppressing AdoMetDC activity in a manner similar to spermine. Thus the activity of AdoMetDC remains suppressed throughout

the 48 h period, whereas that of ODC recovers to control values. It should be pointed out that, during the 48 h treatment with 6-spermyne, ODC activity rises only to control values despite a decline in spermidine and spermine pools, which ordinarily causes substantial increases in ODC to several times control values. Thus, although 6-spermyne apparently fails to suppress ODC, it does prevent the compensatory increase in enzyme activity (at least up to 48 h).

Negative regulation of ODC and AdoMetDC activities is a well-recognized phenomenon in cells treated with a variety of polyamines or their analogues (Porter & Bergeron, 1988; Porter *et al.*, 1987b; Poso *et al.*, 1977). Whether enzyme suppression or enzyme induction is considered, ODC is usually more responsive to regulatory control than is AdoMetDC. Thus, by restricting time or dose during polyamine exposure, or by using polyamines or analogues with apparently diminished binding affinities, it is readily possible to suppress ODC activity selectively without affecting AdoMetDC activity. As an example of the latter, bis(ethyl) derivatives of the polyamines can be used to obtain a spectrum of enzyme effects (Porter *et al.*, 1987b), including minimal suppression of ODC activity and an increase in AdoMetDC activity with the putrescine analogue ('BEP'), suppression of ODC activity and a minimal effect on AdoMetDC activity with the spermidine analogue ('BES'), and suppression of both enzyme activities with the spermine analogue ('BESm'). The interpretation of such findings is difficult, since they may indicate that the two enzymes are separately regulated, or that ODC is more sensitively controlled than AdoMetDC. The present finding that AdoMetDC, but not ODC, activity, is suppressed by 6-spermyne represents a significant departure from the usual trends with these enzymes. Accordingly, it provides strong indication that the two enzymes are regulated by two distinct and functionally separable sites, at least insofar as enzyme suppression is concerned. The same may not hold true for enzyme induction in response to trophic and regenerative influences (Porter & Bergeron, 1988).

The findings with 6-spermyne suggest an interesting structure-function relationship which may be exploited in designing regulatory polyamine analogues selective for AdoMet DC as potential anti-proliferative agents. We have shown previously (Porter *et al.*, 1987b) that, by negatively regulating both ODC and AdoMetDC, the spermine analogue BESm exerts a potent anti-proliferative activity. The ethyl groups minimize degradation of the analogue by serum oxidases (Porter *et al.*, 1987b) and, more importantly, prevent its substitution for the natural polyamines in growth-related functions. Presumably, the bis(ethyl) analogue of 6-spermyne might produce a different profile of growth inhibition by selectively suppressing only AdoMetDC activity. In this regard, it is noteworthy that, even at growth-permissive concentrations (10 μM), 6-spermyne is effective at decreasing spermidine and spermine pools (Table 2) in a manner consistent with predictions based on pathway function and inhibition [i.e. depletion of spermidine and spermine pools, but not putrescine pools (Pegg *et al.*, 1988)]. Despite this effect on spermidine and spermine pools, growth is unaffected, presumably because the analogue substitutes for the polyamines in partially supporting cell growth. As an indication of this ability, we have observed that 6-spermyne prevents growth inhibition by α -difluoromethylornithine as effectively as does spermine over a 48 h incubation (results not shown). Thus bis-*N*-alkylation of the analogue should render it less suitable as a polyamine substitute in growth-dependent functions and results in a correlation between spermidine and spermine depletion and growth inhibition.

The relatively high concentrations of 6-spermyne required for inhibiting cell growth (50% inhibition at > 100 μM), even though AdoMetDC is markedly suppressed, may reflect the ability of the compound to substitute for spermidine or spermine in cell functions required for growth. As with BESm, the presence of ethyl groups at the terminal nitrogen atoms would probably prevent this substitution without interfering with the enzyme-regulatory activity of the analogue, and thereby confer more potent anti-proliferative activity.

We have not determined whether, by virtue of an internal double bond, '6-spermene' might have regulatory effects similar to those of 6-spermyne. However, Sarhan *et al.* (1987) have reported the polyamine pools of tissues treated with the putrescine analogue 2-diamino-1,4-butene. The compound is readily converted into a spermidine and spermine analogue, and both spermidine and spermine pools are decreased. The latter could be the result of AdoMetDC suppression by the spermidine and/or spermine analogues which were synthesized by the cells.

6-Spermyne offers some insight into the structural specificity of the ODC and AdoMetDC regulatory sites, above and beyond the realization that the two seem to be functionally independent entities. In this regard, it may be relevant that the portion of the 6-spermyne molecule contributed by the ODC reaction (the putrescine moiety) is modified, whereas those portions contributed by the product of the AdoMetDC reaction, the aminopropyl moieties, are unaltered. Although speculative, it is tempting to consider that these particular portions of the spermine molecule might be differentially responsible for regulating the two decarboxylases.

Kameji & Pegg (1987) have provided strong evidence

that, at least in acellular systems, suppression of ODC and AdoMetDC activities by polyamines and their analogues seems to occur by selective interference with the translation of these two enzyme proteins. Likewise, the preponderance of cellular studies involving ODC also suggest that the enzymes are regulated at the level of translation (Dirks *et al.*, 1986; Sertich & Pegg, 1987; Persson *et al.*, 1986; Kahana & Nathans, 1985) and/or by post-translational mechanisms (Heller *et al.*, 1976; McCann *et al.*, 1979; Seely & Pegg, 1983; Murakami & Hayashi, 1986). As a further test of this possibility, it would be interesting to determine whether 6-spermyne negatively regulates translation of AdoMetDC mRNA, but not ODC mRNA, in the reticulocyte-lysate translation system *in vitro* (Kameji & Pegg, 1987). Conceivably, 6-spermyne will have other useful applications in studies characterizing the regulatory control of these unique enzymes.

Since the submission of this manuscript, Nagarajan & Ganem (1987) have reported on the synthesis of 6-spermyne and related analogues as potential inactivators of certain mammalian polyamine-oxidative enzymes. We have found no indication under the conditions of the present study that the analogue might act in this manner.

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