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FEEDBACK REPRESSION OF POLYAMINE UPTAKE INTO MAMMALIAN CELLS REQUIRES ACTIVE PROTEIN SYNTHESIS

John L.A. Mitchell, Roger R. Diveley, Jr., and Aviva Bareyal-Leyser

Department of Biological Sciences,
Northern Illinois University, DeKalb, IL 60115

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SUMMARY: Two mammalian cell lines, rat hepatoma (HTC) and Chinese hamster ovary (CHO), were fed 10 to 50 μM spermidine while changes were monitored in intracellular polyamine levels and spermidine uptake activity. Normal feedback control preventing excessive polyamine uptake was found to be completely blocked by the addition of inhibitors of protein synthesis at the time of polyamine exposure. Under these conditions the cells accumulated abnormally high, toxic concentrations of spermidine. Further, continuous protein synthesis was needed to maintain repression of polyamine transporter proteins that had been inhibited previously by normal or elevated intracellular polyamines. These results suggest that a major factor in the regulation of polyamine uptake is the rapid, reversible inactivation of existing polyamine carrier molecules by an unstable protein whose synthesis is stimulated by intracellular polyamines.

Activity of the polyamine uptake system in mammalian cells is very sensitive to cellular polyamine levels. Alhonen-Hongisto et al. (1) and others (2,3) established that deprivation of intracellular polyamines by the use of the inhibitor α-difluoromethylornithine (DFMO) results in an increase in the rate of polyamine incorporation. Induced increases in polyamine transport require new protein synthesis and are not associated with alterations in the affinity of the transporter for the various polyamines (4-8), suggesting that these responses are due to an increase in transporter quantity, rather than a modification of existing transport protein molecules.

Polyamine-deprived cells rapidly incorporate exogenous polyamines until intracellular spermidine levels are replenished, generally within 1-3 h of polyamine addition, and then uptake is abruptly terminated (1,4,5,8-10). The signal for this feedback response appears to be excess intracellular spermidine or spermine, and not putrescine or other natural polyamine derivatives (3,5,8), however the mechanism of this polyamine-induced feedback has not been elucidated.

In the studies presented here, we have established for the first time that this polyamine-induced repression of transport is mediated by a short-lived regulatory protein, and not by the polyamines directly. This spermidine-induced inhibitor of polyamine transport appears to play a

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prominant role in rapid, large amplitude variations in polyamine uptake. Further characterization of this inhibitor is fundamental to our understanding of the mechanism of regulation of cellular polyamine levels and the action of cell signaling agents that have been shown to alter polyamine incorporation activity, such as phorbol esters, Ca^{+2}, plasma and EGF (11).

MATERIALS AND METHODS

Chemicals. [^{14}C]Spermidine was purchased from NEN/Dupont. o-Phthalaldehyde was from Pierce Chemicals. The polyamines, aminoguanidine, dithiothreitol, cycloheximide, Brij-35, puromycin and bovine serum albumin were from Sigma Chemical Co., and 2-mercaptoethanol and inorganic chemicals from Fisher Scientific Co. DFMO (α-difluoromethylornithine) was generously provided by Marion Merrell Dow Research Institute, Marion Merrell Dow Inc.

Cell culture. Rat hepatoma (HTC) cells were grown in monolayer and suspension cultures in Swim's-77 medium containing 10% calf serum. Chinese Hamster (CHO) cells were maintained in Swim's-77 medium containing 2% fetal calf serum and 6% calf serum. Aminoguanidine (3 mM) was added to culture medium at the time of addition of any polyamines.

Polyamine analysis. Extracts were made by placing 1-2x10^6 cells in 1 ml of 0.2 N perchloric acid, heating at 60°C for 30 min and then cooling to 4°C. The suspensions were centrifuged for 20 min at 10,000 rpm in an Eppendorf microfuge and the supernatants analyzed for polyamines while the pellets were assayed for protein content using the Bio-Rad Protein Assay with bovine serum albumin standards. Portions (100 μL) of the supernatant were analyzed for polyamines as described previously (12).

Spermidine incorporation. The rate of spermidine uptake was determined by removing 6 ml samples of culture (3-6x10^6 cells) to pre-warmed 30 ml, round-bottom plastic centrifuge tubes containing small stir bars. Aminoguanidine (3 mM) and unlabeled spermidine (10 μM) were added if these were not in the parent culture already. Immediately [^{14}C]spermidine was added to make a final specific activity of 7,000 dpm/nmol. Multiple samples were withdrawn after 5 and 25 min and placed in ice-cold phosphate-buffered saline containing 0.5 mM unlabeled spermidine. These cells were centrifuged at 1500 rpm, decanted and washed an additional 3 times. Eventually the pellets were suspended in distilled water and quantitatively transferred to scintillation vials for counting. The difference in ^{14}C incorporated in the 20 min period from 5 to 25 min was expressed in relation to protein determinations made on similar samples from these cultures.

RESULTS

In the period 19-24 h after refeeding, suspension cultures of HTC cells are capable of incorporating spermidine at the rate of 2-8 nmol/h/mg protein, utilizing the common polyamine transport system. As shown in Fig 1A, the addition of 50 μM spermidine to such an HTC culture resulted in a sharp increase in the intracellular level of this polyamine. Within 2 h of spermidine addition, however, the increase in cellular polyamines ceased (Fig 1A), and by 3 h the rate of spermidine uptake was reduced by over 80 % (Table I).

This polyamine-induced repression of spermidine uptake was completely prevented by addition of the protein synthesis inhibitors cycloheximide (Fig 1B) or puromycin (Table I) at the same time that spermidine was added to the medium. In the absence of this feedback repression of transport, spermidine levels continued to increase (Fig 1B) until toxic concentrations were exceeded (12), and cell lysis ensued (not shown). Inhibition of protein synthesis in the absence of exogenous spermidine had relatively little effect

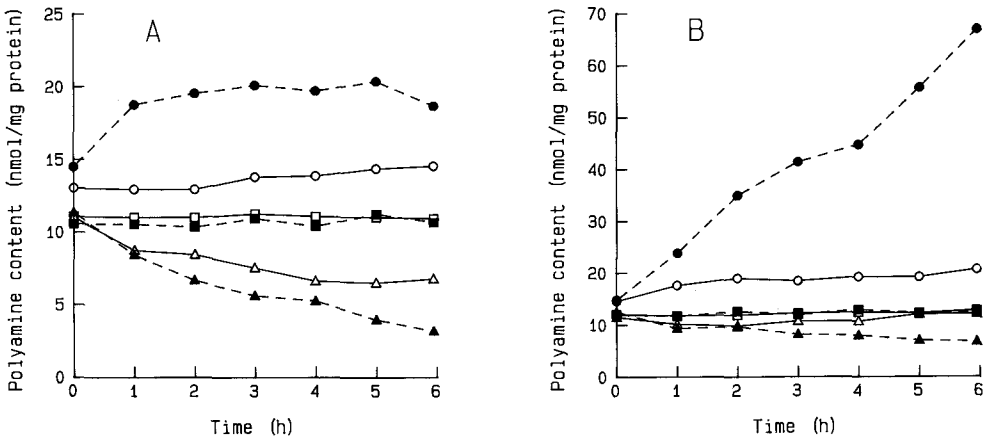

Fig.1. Changes in cellular polyamine content in response to spermidine addition to the culture medium. HTC cultures 19 h after fresh media addition were treated with 50 μM spermidine (solid figures) or not treated with spermidine (open figures) either in the absence (panel A) or presence (panel B) of cycloheximide (0.2 mM). Intracellular levels of putrescine (△,▲), spermidine (○,●) and spermine (□,■) were monitored over the next 6 h as described in Methods. Note the scale compression in panel B.

on cellular spermidine levels (Fig 1B), however the potential activity of the polyamine uptake system was enhanced by this inhibition (Table I). The fact that new protein synthesis appears to be essential for the spermidine-induced inactivation of polyamine uptake suggests the involvement of a, as yet uncharacterized, polyamine-induced transport repressor protein.

The experiment described in Fig 2 suggests that interaction between this hypothetical repressor protein and the polyamine transport mechanism is reversible. In this study cycloheximide was added to cultures of HTC cells

TABLE I. Effect of inhibitors of protein synthesis on feedback regulation of polyamine uptake. Suspension cultures of HTC cells 12 h after resuspension were treated with 15 μM spermidine (SPD), 0.2 mM cycloheximide and 200 μg/ml puromycin as indicated. After 3 h these cultures were analyzed for spermidine uptake as described in Methods. Rates are the average of at least 3 determinations (± S.D.).

Treatment	Spermidine Uptake Velocity	
	nmol/h/mg prot	% of control
control (no additions)	7.19 ± 0.37	100
+ cycloheximide	10.55 ± 1.21	147
+ puromycin	11.09 ± 0.40	154
+ SPD	1.37 ± 0.08	19
+ SPD + cycloheximide	10.51 ± 0.87	146
+ SPD + puromycin	11.95 ± 0.41	166

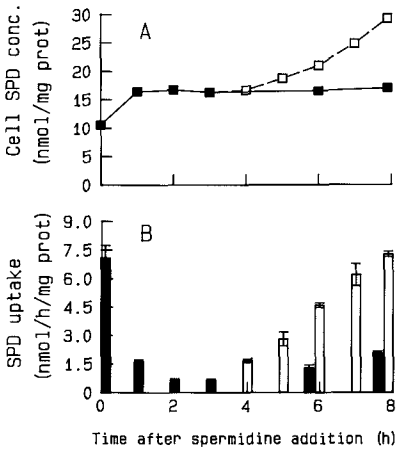

Fig. 2. Reversal by cycloheximide of previously inhibited spermidine uptake. HTC cultures as in Fig. 1 were exposed to 10 μM spermidine and after 3 h the culture was split and cycloheximide added to half. Panel A shows the resultant changes in intracellular spermidine following cycloheximide addition (□). Panel B shows the values for spermidine uptake ± S.D. in these same cultures. The open bars represent the culture to which cycloheximide was added at 3 h.

that had been preincubated with 50 μM spermidine for 3 h. Although the spermidine level in these cells was stable at the time cycloheximide was added, it started to increase within 2 h of this addition (Fig 2A). Intracellular spermidine continued to climb in this culture, paralleling the uncontrolled increase in the culture to which spermidine and cycloheximide were added simultaneously.

The changes in intracellular polyamines observed in Fig 2A were directly attributable to variations in the activity of the polyamine transporter. As shown in Fig 2B, the velocity of spermidine incorporation in these cultures decreased sharply in the first hour after spermidine was added, and remained low for at least 8 h. In the cultures to which cycloheximide was added after 3 h incubation with spermidine, the polyamine transporter regained activity progressively over the next 4 h, and eventually achieved the velocity exhibited before the addition of spermidine. Since it is not likely that new polyamine carrier protein was synthesized in the presence of cycloheximide, the increase in uptake velocity must have been due to activation of existing transport proteins. The transport system itself has been shown to be long lived (8), and therefore may only be transiently inactivated by the putative repressor. Accordingly, this regulatory protein must have a relatively short effective life in order for the repression to be reversed so quickly after cycloheximide addition.

The involvement of an unstable polyamine-induced feedback protein in the regulation of polyamine uptake is not restricted to HTC cells. Under experimental conditions similar to those used with HTC cells above, the spermidine uptake pathway of Chinese hamster ovary (CHO) cells responded in an equivalent manner (Table II). At the start of this experiment approx. 88% of

TABLE II. Inhibition of protein synthesis blocks feedback regulation of spermidine uptake in CHO cells. Suspension cultures of CHO cells 19 h after fresh media addition were treated with 10 μM spermidine (SPD) and 0.2 mM cycloheximide as indicated. After 3 h spermidine uptake activity was determined and, where indicated, cycloheximide was added to cultures with and without previous exposure to spermidine. Spermidine uptake in the appropriated cultures was evaluated after an additional 4 h. Uptake values are the average of 3 determinations (± S.D.) and are also expressed with respect to the maximum value observed, following a 3 h exposure to cycloheximide.

Time (h)	Treatment	Spermidine Uptake Velocity nmol/h/mg prot	% of maximum
3	control (no additions)	1.12 ± 0.32	12
3	+ cycloheximide	9.36 ± 0.30	100
3	+ SPD	0.63 ± 0.15	7
3	+ SPD + cycloheximide	8.19 ± 0.17	94
7	control (no additions)	1.57 ± 0.38	17
7	+ cycloheximide at 3 h	9.29 ± 2.10	99
7	+ SPD at 0 h	1.55 ± 0.23	17
7	+ SPD at 0 h + cycloheximide at 3 h	9.26 ± 0.45	98

the existing polyamine transporter protein was inhibited. This repression was reversed by the addition of cycloheximide regardless of the presence or absence of spermidine in the medium. Cycloheximide promoted activation of spermidine uptake even when added 3 h after incubation with spermidine.

From the increases in spermidine transport velocity induced by 3 h exposure to cycloheximide noted in Tables I and II it appears that a variable and potentially large fraction of the existing polyamine transport protein is normally maintained in an inactive conformation, perhaps a reserve, in exponentially growing cells. Past studies have suggested that extended periods of cellular polyamine deprivation are required for maximal polyamine transport activity. The question then emerges as to how much of the variation in transport activity observed is due to changes in the amount of transport protein, as opposed to changes in the degree of repression of existing transporters. In Table III we show a comparison of spermidine uptake activity in normal and polyamine-depleted cells with and without exposure to cycloheximide. Under the normal growth conditions a majority (52 % of HTC and 75 % of CHO) of the available polyamine transport protein was observed to be in an inactive state, apparently repressed by the labile spermidine-induced inhibitor. By contrast, the cultures that were polyamine deprived by treatment with DFMO showed very little repressed polyamine transport protein. This 48 h treatment with DFMO resulted in approximately a 2-fold increase in available transporter, whereas greater than 7-fold variations in the activity of this transporter have been observed to be due to interaction with the spermidine-induced repressor protein.

DISCUSSION

The functional characteristics of an apparent polyamine-induced transport repressor have been deduced from studies correlating polyamine transport

TABLE III. Proportion of polyamine transport activity that is repressed in normal and polyamine-deprived cells. Suspension cultures of HTC and CHO cells were grown in the presence or absence of 4 mM difluoromethylornithine (DFMO) for 48 h. 19 h after the last medium change the cultures were divided into 2 flasks, to which either 0.2 mM cycloheximide or no additions were made. After an additional 6 h of culture, spermidine uptake activity was assayed as previously described. Any increase in activity due to cycloheximide addition is considered to represent transport protein that had been inhibited by the labile, polyamine-induced repressor protein.

Culture	Treatment	Spermidine Uptake (nmol/h/mg prot)	Repression (%)
HTC	no additions	3.78 ± 0.13	52
	+ cycloheximide	7.83 ± 0.28	
HTC + DFMO	no additions	18.43 ± 0.48	0
	+ cycloheximide	18.41 ± 1.05	
CHO	no additions	4.36 ± 0.07	75
	+ cycloheximide	17.62 ± 0.55	
CHO + DFMO	no additions	31.85 ± 1.22	7
	+ cycloheximide	34.27 ± 0.14	

activity with intracellular polyamine levels. Within 1-2 h of spermidine addition to culture medium, polyamine transport into HTC cells was effectively inhibited. This feedback response required new protein synthesis, indicating that this response was mediated by a regulatory protein. Since polyamine transport activity that had been inhibited by this regulatory protein was extensively reactivated following the addition of cycloheximide, it appears that the function of the regulator is to block, but not destroy, existing transport molecules. Furthermore, the rapidity with which this repression was lost following protein synthesis inhibition would suggest that the activity of this regulatory protein was short-lived.

These results are consistent with the conclusion, reached by Byers and Pegg (8), that polyamine transport proteins are stable; however, our results suggest that transport activity can be rapidly and reversibly modulated by a polyamine-induced regulatory protein, whereas these researchers did not observe this phenomenon in their studies on CHO cells. Although dissimilar regulatory mechanisms may be utilized in different organisms, our studies with CHO cells (Tables II & III) suggest that these mammalian cells are similarly regulated.

There is precedence for the involvement of a labile protein in the regulation of polyamine transport. Davis et al. (13) studied the effects of extracellular Ca^{+2} and cycloheximide on putrescine transport into Neurospora crassa mycelia and a mutant cell type with abnormal polyamine transport characteristics. Similar to our observations in mammalian cells, they found that exposure of Neurospora to cycloheximide resulted in the accumulation of abnormal levels of polyamines from the medium. They suggest that polyamine uptake in Neurospora crassa normally may be restricted by an unstable Ca^{+2}-binding protein. The mammalian polyamine transport system is also known to be sensitive to Ca^{+2} (11,14), but the mechanics of this association have yet to be elucidated.

The polyamine-stimulated, unstable regulatory protein suggested by this study is similar, in many respects, to antizyme, a regulatory protein that purportedly facilitates inactivation and degradation of ornithine decarboxylase. Synthesis of antizyme is enhanced greatly by spermidine, it acts by binding reversibly to a target enzyme, and it has a very short functional half-life (15-20). In addition to these similarities it is interesting to note that the DH23b cell, an HTC variant that overproduces ODC, is concurrently defective in both ODC degradation (21) and the rapid feedback inhibition of polyamine transport (12).

The observation that active protein synthesis must occur in order for the polyamine uptake system to be turned off has potentially beneficial implications. In particular, there is much current interest in the use of the polyamine transport system to incorporate polyamine analogs and related compounds that appear to show promise as cytotoxic clinical agents (2,11,22,23). Many of these compounds, however, stimulate the feedback response, and thereby limit their own uptake. By temporary exposure to an inhibitor of protein synthesis the incorporation of such test compounds could be greatly enhanced.

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