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DIFFERENTIAL REGULATION OF PUTRESCINE UPTAKE IN *TRYPANOSOMA CRUZI* AND OTHER TRYPANOSOMATIDS

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Summary. Putrescine uptake in Trypanosoma cruzi epimastigotes is 10 to 50-fold higher than in Leishmania mexicana or Crithidia fasciculata. Polyamine transport in all these trypanosomatids is an energy-dependent process strongly inhibited by the presence of 2,4-dinitrophenol or KCN. Putrescine uptake in T. cruzi and L. mexicana was markedly decreased by the proton ionophore carbonylcyanide m-chlorophenylhydrazone but it was not affected by ouabain, a Na^+-K^+ pump inhibitor. The depletion of intracellular polyamines by treatment of parasite cultures with α-difluoromethylornithine elicited a marked induction of putrescine uptake in L. mexicana and C. fasciculata by increasing considerably the Vmax of this process. Conversely, the uptake of putrescine in T. cruzi was essentially unchanged by the same treatment. The differential regulation of putrescine transport in T. cruzi might be related to some distinctive features of polyamine metabolism in this parasite.

High levels of intracellular polyamines have been observed in a wide variety of actively proliferating cells (1-4). The regulation of polyamine endogenous concentrations is accomplished through the biosynthesis, degradation, uptake from the external medium and excretion of these compounds. Therefore, all these processes must be evaluated in order to understand the control of physiological conditions necessary for cell growth.

Different types of eucaryotic cells show a remarkable induction of ornithine decarboxylase (ODC) activity in

Abbreviations: ODC, ornithine decarboxylase; DFMO, α-difluoromethylornithine; 2,4 DNP, 2,4 dinitrophenol; CCCP, carbonylcyanide m-chlorophenylhydrazone.

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response to growth factors and other stimuli (5-8). An increase of polyamine uptake has also been observed in cells stimulated to proliferate (9-11).

Studies intended to set up conditions to deplete intracellular polyamines in order to block cell proliferation have been recently carried out with mammalian cells and protozoa (12-14). The results obtained so far have clearly indicated that when polyamine biosynthesis was abolished with specific inhibitors of ornithine decarboxylase, polyamine uptake from the external media became essential for cell growth (14).

Although the polyamine transport systems have been characterized in various cells (15,16), the regulation of the corresponding processes has been scarcely investigated.

In the present work we describe a comparative study of putrescine transport systems in Trypanosoma cruzi epimastigotes, Leishmania mexicana promastigotes and Crithidia fasciculata choanomastigotes. Our results indicate that the regulation of putrescine uptake in T. cruzi shows some distinctive properties not observed in the other trypanosomatids.

MATERIALS AND METHODS

Chemicals: Brain heart infusion, yeast extract and tryptose were supplied by Difco Laboratories, Detroit, Michigan. Minimal essential medium (SMEM) and amino acids were obtained from Gibco; vitamins, haemin, unlabeled polyamines, 2,4 dinitrophenol, carbonylcyanide m-chlorophenylhydrazone (CCCP), ouabain and antibiotics were purchased from Sigma. 1,4 [^{14}C]-putrescine dihydrochloride (110 mCi/mmol), [Terminal methylenes ^{3}H(N)] spermidine trihydrochloride (17.6 Ci/mmol) and [2,3 ^{3}H(N)] putrescine dihydrochloride (40.3 Ci/mmol were from New England Nuclear, Boston, M.A. Difluoromethylornithine (DFMO) was a generous gift of Marion Merrell Dow Inc.

Parasite cultures: Epimastigotes of Trypanosoma cruzi Dm28c, promastigotes of Leishmania mexicana mexicana MHOM/BZ/82/BEL21 (kindly provided by Prof. D.A. Evans, London, School of Hygiene and Tropical Medicine) and Crithidia fasciculata (ATCC 11745) were grown with shaking at 26-28°C in a rich (Warren's modified medium) or a defined medium (HOSMEM II) as described previously (14,17). Some cultures were carried out in the presence of DFMO as indicated. Parasite growth was followed by cell counting.

Polyamine uptake: Exponentially growing parasites were harvested by centrifugation at room temperature for 5 min at

1,000 g. Cells were washed with PBS and resuspended in the same buffer at a concentration of 2 x 10^7 parasites/ml. The cell suspension was incubated with radioactive polyamines under the conditions indicated in each case and 1 ml aliquots were taken at different times. After dilution with three volumes of PBS containing 1 mM concentration of the corresponding unlabeled polyamine, samples were quickly filtered under vacuum through Millipore membranes. Filters were washed three times with 3 ml 1 mM putrescine or spermidine in PBS, dried and counted in a liquid scintillation spectrometer.

RESULTS AND DISCUSSION

We have recently observed that the addition of DFMO to cultures of Leishmania mexicana grown in rich media elicited only a partial depletion of intracellular polyamines without any alteration of growth in spite of the fact that ODC was almost completely inhibited (14). On the other hand, when the parasite was cultivated in a defined medium free of

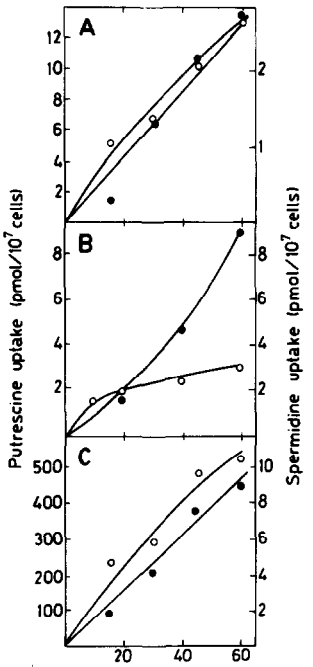

Fig. 1. Putrescine and spermidine uptake in trypanosomatids. Exponentially growing L. mexicana (A), C. fasciculata (B) and T. cruzi (C) were harvested and resuspended in PBS. The cell suspension was incubated with [^{3}H]-putrescine (1 µCi/ml, 2.5 µM) or [^{3}H]-spermidine (5 µCi/ml, 2.5 µM) and the accumulation of polyamines inside the cells was measured in 1 ml aliquots taken at different times. Putrescine (•) and spermidine (o) values are the average of duplicate experiments. All other details are as described in Materials and Methods.

polyamines, the same treatment with the specific inhibitor of putrescine biosynthesis led to the arrest of proliferation. These results seem to indicate that polyamine uptake from the extracellular medium is required to maintain growth under certain conditions. Due to the relevance of polyamine transport systems on the possible control of parasite proliferation we have measured different parameters related to putrescine and spermidine uptake in Leishmania mexicana, Crithidia fasciculata and Trypanosoma cruzi.

Fig. 1 shows that putrescine uptake in L. mexicana and T. cruzi was 5 to 50 times higher than that of spermidine, while in Crithidia the transport of both compounds was of the same order of magnitude. Furthermore, the uptake of putrescine in T. cruzi was considerably higher than in the other trypanosomatids.

Putrescine uptake in T. cruzi and L. mexicana is a saturable, temperature dependent process showing a maximal value at 37°C (Fig. 2).

Uncouplers of the respiratory chain like KCN or 2,4 dinitrophenol (2,4 DNP) completely abolished the uptake of putrescine by T. cruzi, while a residual transport of 20 to 40% was still detected in L. mexicana treated with the

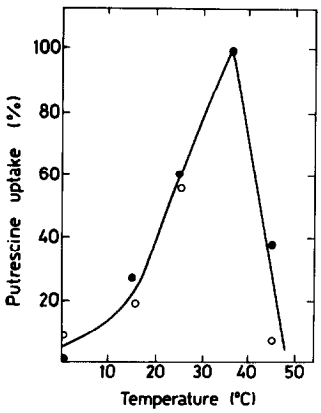

Fig. 2. Temperature dependency of putrescine uptake in T. cruzi and L. mexicana. All assays were performed in duplicate using exponentially growing T. cruzi epimastigotes (•) and L. mexicana promastigotes (o). The results shown are the percentages of the uptake values obtained at 37°C (6.3 and 0.21 pmol.min^{-1}/10^7 cells for T. cruzi and L. mexicana, respectively).

Table 1. Effects of metabolic inhibitors and ionophores on putrescine uptake in T. cruzi and L. mexicana

Additions	Concentration	Putrescine uptake (% of control)	
		T. cruzi	L. mexicana
None	--	100	100
KCN	1 mM	3	26
2,4 DNP	1 mM	5	37
CCCP	10 µM	38	83
CCCP	40 µM	4	7
Ouabain	200 µM	92	119

Parasites were cultivated in rich medium for 72 h. The indicated compounds were added to the cell suspensions before the uptake measurements. Results are given as percentages of the corresponding control values obtained in the absence of inhibitors (1.9 and 0.1 pmol/min/10^7 cells for T. cruzi and L. mexicana, respectively). All other details as in Materials and Methods.

same inhibitors (Table 1). The addition of the ionophore for protons CCCP at 10 µM concentration markedly decreased putrescine uptake in T. cruzi, whereas the transport of this polyamine by L. mexicana was almost unaffected. However, higher concentrations of the ionophore suppressed putrescine uptake in both trypanosomatids. On the other hand, the transport of the same polyamine was not altered in T. cruzi or L. mexicana in the presence of ouabain, a well known inhibitor of the Na^+-K^+ pump. All these results indicate that putrescine uptake in T. cruzi and L. mexicana requires the cell metabolic energy. Although the Na^+-K^+ pump is probably not implicated in the transport of polyamines by the trypanosomatids, this process seems to be related to the potential generated across the parasite membrane.

Putrescine uptake was almost completely inhibited in L. mexicana as well as in T. cruzi by the sulfhydryl reagents sodium p-chloromercuribenzoate and N-ethylmaleimide (results not shown). Preincubation with 0.5 mM asparagine, leucine or lysine did not affect putrescine uptake in T. cruzi or Leishmania, indicating that polyamine uptake in

Table 2. Effects of DFMO on the induction of putrescine uptake in several trypanosomatids

DFMO incubation period	Putrescine uptake pmol/min/10^7 cells	%
	L. mexicana	
--	0.04	100
24 h	0.05	125
72 h	0.25	625
	C. fasciculata	
--	0.17	100
24 h	1.01	594
72 h	2.15	1,265
	T. cruzi	
--	2.6	100
24 h	2.4	92.3
72 h	2.2	84.6

Cultures of *L. mexicana*, *C. fasciculata* and *T. cruzi* were grown in the absence or presence of 5 mM DFMO for the indicated periods of time and putrescine uptake was determined as indicated in Materials and Methods.

these parasites is not regulated by A, L or Ly^+ amino acids transport systems (18).

Many studies carried out in a variety of mammalian cells as well as in the protozoa *Trypanosoma brucei* and *Leishmania infantum* have shown that polyamine transport was markedly enhanced when putrescine biosynthesis was blocked by DFMO treatment (18-22).

In order to investigate the regulation of putrescine uptake in several trypanosomatids, we have measured the accumulation rate of the radioactive polyamine in *T. cruzi*, *L. mexicana* and *C. fasciculata* when cultures of these parasites were incubated for different times in the presence of DFMO. The values obtained (Table 2) indicate that upon DFMO treatment putrescine uptake increased 5-to 10-fold in *L. mexicana* and *C. fasciculata* while it remained essentially unchanged in *T. cruzi*. At the same time spermidine transport was slightly reduced in all cases (results not shown).

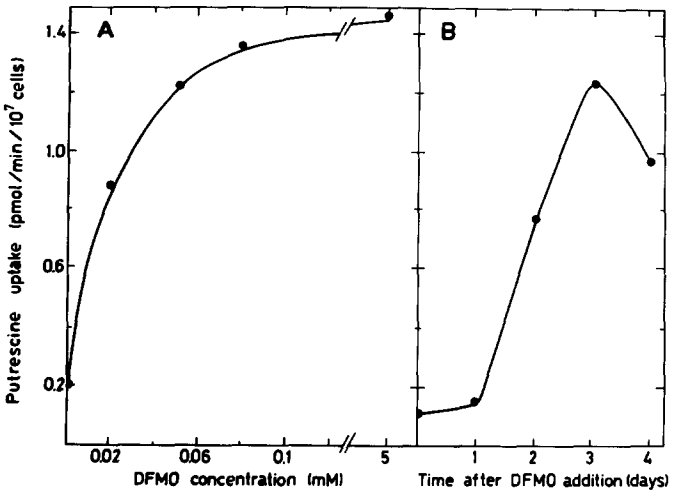

Fig. 3. Effect of DFMO treatment on putrescine uptake by L. mexicana promastigotes. Parasite cultures were incubated for 72 h at different concentrations of DFMO (A) or for different periods of time in the presence of 5 mM DFMO (B) and the corresponding putrescine uptake rates were measured in duplicate experiments.

The strong stimulation of putrescine transport observed in L. mexicana and C. fasciculata increased with the concentration of DFMO (Fig. 3A) and reached a maximum after incubation with the inhibitor for a period equivalent to 2-3 duplication times (Fig. 3B).

Table 3. Kinetic parameters of putrescine uptake in L. mexicana, C. fasciculata and T. cruzi

Parasite	DFMO treatment	Km µM	Vmax pmol/min/10^6 cells
L. mexicana	-	10.7	0.04
	+	27	1.66
C. fasciculata	-	66	1.0
	+	50	12.5
T. cruzi	-	5.7	1.1

Parasites were cultivated as indicated in Table 1 in the absence or presence of 5 mM DFMO. Uptake assays were performed and kinetic constants were calculated from the initial rates of putrescine uptake.

The enhancement in the rate of putrescine uptake caused by DFMO in L. mexicana and C. fasciculata was due to a marked increase of the corresponding Vmax values rather than to a significant decrease of the apparent affinity constants as shown in Table 3 by the kinetic parameters corresponding to the transport process. These results seem to indicate that the depletion of polyamines evoked by the inhibition of their biosynthesis in Leishmania and Crithidia gives rise to a compensatory mechanism producing a remarkable induction of the putrescine transporter(s) synthesis. On the other hand, ODC has not been detected in T. cruzi (23); this fact might explain why DFMO is not able to induce a similar regulation involving the enhancement of putrescine uptake in this parasite. Therefore, most of polyamine requirements of T. cruzi seem to be provided by the extracellular medium through a constitutive and active transport system.

Since uptake and efflux of polyamines can control the intracellular levels of these substances, further investigation on the regulation of these processes in trypanosomatids will certainly contribute to the search for ways to control parasite proliferation.

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