Regulation of a high-affinity diamine transport system in *Trypanosoma cruzi* epimastigotes

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Trypanosoma cruzi epimastigotes take up exogenous [^{3}H]-putrescine and [^{3}H]cadaverine by a rapid, high-affinity, transport system that exhibits saturable kinetics (putrescine K_m 2.0 μM, V_{max} 3.3 nmol/min per 10^8 cells; cadaverine K_m 13.4 μM, V_{max} 3.9 nmol/min per 10^8 cells). Putrescine transport is temperature dependent and requires the presence of a membrane potential and thiol groups for activity. Its activity is altered in response to extracellular putrescine levels and as the cells proceed through the growth cycle. This transporter shows high specificity for the diamines putrescine and cadaverine, but low specificity for the polyamines spermidine and spermine. The existence of rapid diamine/polyamine transport systems whose activity can be adjusted in response to the growth conditions is of particular importance, as they seem unable to synthesize their own putrescine [Hunter, Le Quesne and Fairlamb (1994) Eur. J. Biochem. **226**, 1019–1027].

INTRODUCTION

Polyamines are ubiquitous, aliphatic polycation metabolites that are thought to be essential for normal cell proliferation, differentiation and macromolecular synthesis in most organisms [1,2]. Consequently polyamine levels tend to be highly regulated [3,4].

Most organisms synthesize their own putrescine from ornithine by the action of ornithine decarboxylase (ODC; EC 4.1.1.17) or, in plants and bacteria, from arginine (via agmatine) by the action of arginine decarboxylase (reviewed in [1]). However, many cells also contain energy-dependent di- and poly-amine uptake systems (reviewed in [5,5a]. These can be used to supplement cellular synthesis *de novo* with uptake of di- and poly-amines from the environment; in particular, on stimulation by a wide variety of growth factors, hormones and other stimuli that provoke cell growth or when *de novo* synthesis is blocked by the action of inhibitors of polyamine biosynthesis such as the ODC inhibitor, D,L-α-difluoromethylornithine (reviewed in [5]).

In contrast with other cells, the epimastigote (insect) stage of the parasitic protozoan *Trypanosoma cruzi* is unable to synthesize putrescine *de novo* from ornithine or arginine [6]. Furthermore, D,L-α-difluoromethylornithine treatment is ineffective against *T. cruzi* [6,7] and does not elevate putrescine uptake [8,9]. This is in contrast with other trypanosomatids containing an active ODC, such as *Leishmania donovani* and *Crithidia fasciculata*, where D,L-α-difluoromethylornithine treatment increases putrescine uptake [8,9]. Although trace amounts of arginine decarboxylase activity have been reported in the mammalian stages of *T. cruzi* [10], the levels are likely to be quantitatively insignificant, being approx. 10^{-5} that in *Escherichia coli* [11] and 1/500 of the ODC activity in *Trypanosoma brucei brucei* bloodstream trypomastigotes [12]. Furthermore, because the arginine decarboxylase inhibitor D,L-α-difluoromethylarginine has only a slight effect on their growth [13], it remains unclear to what extent this pathway contributes to polyamine biosynthesis in *T. cruzi*.

Another unusual aspect of polyamine metabolism in trypanosomatids is their ability to conjugate spermidine with glutathione to form trypanothione [N^1,N^8-bis(glutathionyl)spermidine] [14]. The trypanothione system replaces the glutathione system found in mammalian cells [15]. In contrast with other trypanosomatids, *T. cruzi* epimastigotes are also capable of converting cadaverine to the trypanothione analogue, homotrypanothione [N^1,N^9-bis(glutathionyl)aminopropylcadaverine] [6].

Hence the apparent lack of *de novo* putrescine synthesis in *T. cruzi* epimastigotes, coupled with previous work that indicates that these parasites are able to take up diamines [6,8,9], has led us to characterize the diamine (putrescine and cadaverine) transport system(s) of *T. cruzi* epimastigotes with respect to its/their kinetics, energy dependence, regulation and interaction with polyamine and amino acid systems.

EXPERIMENTAL

Materials

[1,4-^{3}H]Putrescine,2HCl (407 GBq/mmol) and [*terminal methylenes*-^{3}H(N)-]spermidine,3HCl (577 GBq/mmol) were purchased from New England Nuclear. [1,5-^{3}H]Cadaverine,2HCl (666 GBq/mmol) was prepared from the diacetyl derivative and purified as described previously [6]. [^{14}C]Inulin carboxylic acid (440 MBq/mmol; Amersham International) was dialysed against 0.9 % NaCl before use. All other reagents were of the highest purity available and purchased from Sigma unless otherwise stated. Tissue culture materials were purchased from Greiner Labortechnik and foetal calf serum was from Life Technologies.

Cell culture

T. cruzi epimastigotes, line MHOM/BR/78/Silvio (clone X10/6), were cultured at 28 °C in RTH medium, which contains trace amounts of polyamines (0.68 μM putrescine, 1.00 μM cadaverine, 1.09 μM spermidine and 0.48 μM spermine) [6]. Cells were harvested in the exponential phase of growth unless stated otherwise. Inhibitors were added from filter-sterilized stock solutions.

Abbreviation used: ODC, ornithine decarboxylase.

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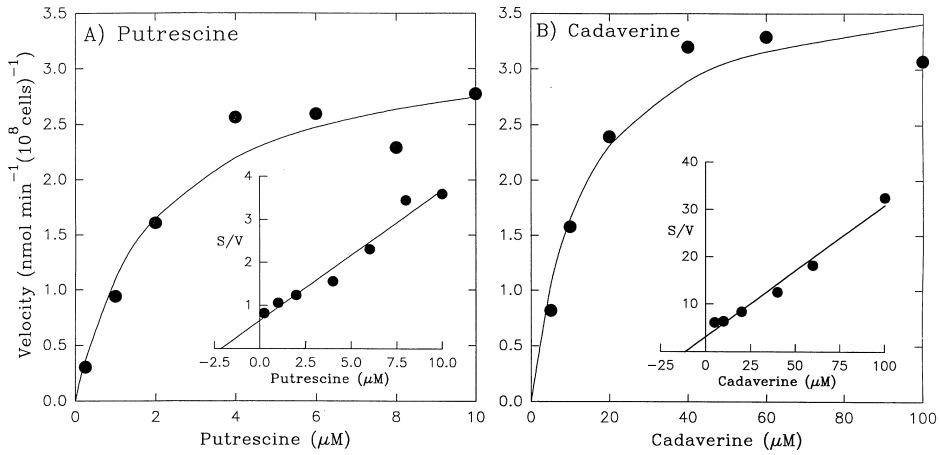

Figure 1 The kinetics of putrescine and cadaverine transport in *T. cruzi* epimastigotes

Rates of uptake were measured for (**A**) putrescine and (**B**) cadaverine as described in the Experimental section. Curves were fitted by non-linear regression to the Michaelis–Menten equation with the Enzfitter software package. The insets show a linear [S]/V versus [S] transformation of the data (Hanes–Woolf plot) with the line fitted from K_m and V_{max} values obtained from the non-linear plot.

Transport measurements through silicone oil

Transport was measured by using a rapid sampling technique [16] involving rapid mixing of the cells with radiolabelled polyamines, followed by separation by centrifugation through silicone oil. Uptake assays were performed in Carter's Balanced Salt Solution [17] supplemented with 100 i.u./ml penicillin and 100 μg/ml streptomycin at 28 °C. All operations with *T. cruzi* were performed in a Class II safety cabinet. After centrifugation, cells were stored on ice for up to 30 min without loss of label into the silicone oil. Subsequently the medium was aspirated and the region above the oil layer rinsed twice with PBS to remove any residual label before aspirating the silicone oil. The pellet was then extracted overnight with 0.1 ml of 1 M NaOH before liquid-scintillation counting in 1 ml of Pico-Fluor 40. Initial rates of uptake were determined by linear regression analysis on up to five time points measured at 5 s intervals over the first 20–25 s. This approach obviates the need to correct for non-specific binding to cells or carry-over of extracellular fluid through the silicone oil because the intercepts are not used in subsequent calculations. All rates of uptake have a regression coefficient of $r > 0.95$. K_m and V_{max} values were calculated by unweighted non-linear regression with the Enzfitter software package (Elsevier Biosoft, Cambridge, U.K.).

Short-term metabolism of ^{3}[H]putrescine and ^{3}[H]cadaverine

This was performed essentially by the method of Damper and Patton [18], where cells were mixed with radiolabel for 90 s before centrifuging through silicone oil into 10% (w/v) trichloroacetic acid to prevent further metabolic conversion. Extracts were taken from the trichloroacetic acid layer and their polyamine content was analysed as described below.

Intracellular polyamine content

T. cruzi epimastigotes (2×10^8 cells) were taken and prepared for separation by HPLC by using the dansyl chloride pre-column derivatization procedure as described previously [6], except that the samples were acid hydrolysed [19] before dansylation. For radiolabelling studies, fractions (1 min) were collected and their radioactivity was determined by scintillation counting.

Cell volume determination

With the [^{14}C]inulin exclusion method of Damper and Patton [20] the cell volume of *T. cruzi* epimastigotes was determined to be 5.5 ± 0.4 μl per 10^8 cells ($n = 4$).

Protein assay

This was performed by Peterson's modification of the micro-Lowry method (Sigma). BSA (fraction V) was used as the protein standard. From this method a protein content of 233 ± 19 μg per 10^8 cells ($n = 4$) was determined for *T. cruzi*.

RESULTS

Preliminary experiments established that, at the lowest concentration used, uptake of diamines by *T. cruzi* is linear for 20 s. In a separate experiment, it was found that over 90 s less than 1.5% of [^{3}H]putrescine is converted into spermidine and no metabolism of [^{3}H]cadaverine to aminopropylcadaverine occurs (results not shown). Saturable Michaelis–Menten-type kinetics is observed for the transport of both putrescine and cadaverine over the first 20–25 s in exponentially growing (day 3) *T. cruzi* cells. Putrescine is transported by a rapid high-affinity saturable carrier with a K_m of 2.0 ± 0.7 μM and a V_{max} of 3.3 ± 0.3 nmol/min per 10^8 cells, which is equivalent to 14.1 nmol/min per mg of protein (Figure 1A). Transport of cadaverine exhibits a similar V_{max} of 3.9 ± 0.3 nmol/min per 10^8 cells or 16.6 nmol/min per mg of protein (Figure 1B), but the K_m of 13.4 ± 3.8 μM is approx. 7-fold higher than the K_m for putrescine. Subsequent studies were carried out solely with [^{3}H]putrescine, as it is a better substrate (lower K_m) and more readily available than cadaverine. Maximum transport activity was determined at $5 \times K_m$ (10 μM).

An Arrhenius plot of log V against the reciprocal of tem-

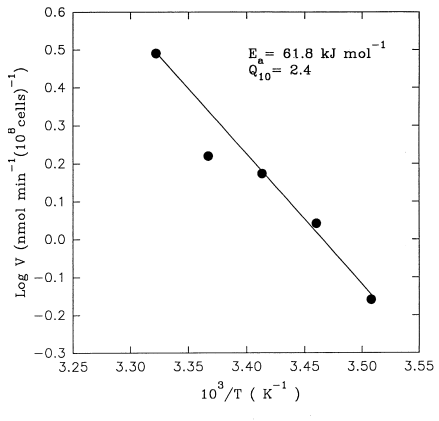

Figure 2 Arrhenius plot for putrescine transport

The rates of transport are mean values from two observations determined with 10 μM putrescine (5 × K_m). The 24 °C point was omitted in the determination of the E_a and Q_{10} values.

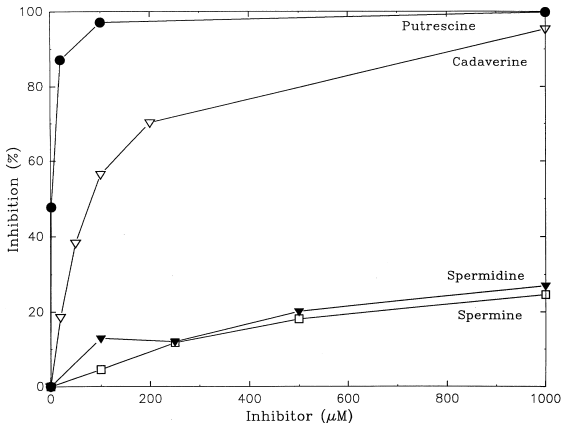

Figure 3 Inhibition of [^{3}H]putrescine transport by unlabelled putrescine, cadaverine, spermidine or spermine

Rates of uptake of 2 μM [^{3}H]putrescine transport were measured as described in the Experimental section with the addition of: unlabelled putrescine (●), cadaverine (▽), spermidine (▼) and spermine (□). The rate of putrescine transport in the absence of additions was 3.1 nmol/min per 10^8 cells.

Table 1 Effect of ionophores and thiol reagents on putrescine transport

In the absence of inhibitors, transport of 2 μM [^{3}H]putrescine was 3.0 nmol/min per 10^8 cells and represents 0% inhibition. The cells were preincubated with the inhibitor for 10 min (no asterisk) or 20 min (asterisk) before assaying [^{3}H]putrescine transport. Results are the means of two observations.

Inhibitor	Transport (% inhibition)
Ionophores:	
20 nM gramicidin[4] (Na^+/K^+)*	13
2 μM valinomycin (K^+)*	28
10 μM carbonyl cyanide *m*-chlorophenylhydrazone (H^+)*	90
2 μM calcimycin–A23187 (Ca^{2+})*	29
1 μg/ml oligomycin (mitochondrial ATPases)	−3
1 mM ouabain (Na^+/K^+ pump)	7
1 mM 2,4-dinitrophenol (H^+)	67
Thiol reagents:	
1 mM iodoacetate	37
1 mM *N*-ethylmaleimide	> 99
200 μM *p*-hydroxymercuribenzoate	> 99
200 μM *p*-chloromercuriphenylsulphonate	99

perature (Figure 2) indicates that the maximal rate of putrescine transport is highly temperature-dependent. From the slope of the graph ($-E_a/2.3R$) an activation energy, E_a, of 61.8 kJ/mol is obtained for putrescine. The Q_{10} value, which represents the increase in velocity observed when the temperature is raised by 10 °C, is 2.4 for the diamine transporter in the range 12–28 °C. These findings suggest that diamine transport in *T. cruzi* epimastigotes is an active energy-dependent process.

Transport of putrescine (2 μM) is not inhibited by a 100-fold excess of the following amino acids that are recognized by specific transport systems in mammalian cells: asparagine (N), serine (ASC), leucine (L), aspartate (x^-) or ornithine, lysine or arginine (y^+) (results not shown). Only agmatine is a weak inhibitor of putrescine transport (24% inhibition at a 100-fold excess). Thus putrescine is not taken up on any of the known amino acid transporters.

The effect of inhibitors on putrescine transport is summarized in Table 1. Of the ionophores, the protonophores carbonyl cyanide *m*-chlorophenylhydrazone [21] and 2,4-dinitrophenol [22] are the most potent inhibitors, suggesting that putrescine transport could be linked to a membrane potential. Of the thiol reagents, *N*-ethylmaleimide, *p*-hydroxymercuribenzoate and *p*-chloromercuribenzene sulphonate all inhibit transport by over 99%, whereas iodoacetate is much less effective (37%).

Unlabelled diamines inhibit [^{3}H]putrescine transport in a concentration-dependent manner, with putrescine and cadaverine being the most potent (Figure 3). Other diamines of the general formula $NH_2(CH_2)_nNH_2$, (where $n = 2$, 3 or 6–12) are much less effective (results not shown). Even at a 500-fold excess, spermidine and spermine both inhibit putrescine transport by less than 30% (Figure 3). Likewise methylglyoxal bis(guanylhydrazone) and paraquat, which share structural similarities with polyamines, only inhibit transport by approx. 20% (results not shown). Together these results suggest that this transporter has an optimal specificity for diamines containing four or five methylene groups and only weakly recognizes polyamines and their analogues.

While measuring the kinetics of diamine transport in *T. cruzi* it was noticed that cells exhibited similar K_m values but widely different V_{max} values depending on the stage of growth. When the kinetic parameters were measured on the same batch of cells harvested in the exponential, late exponential or stationary phase of growth it was found that the V_{max} decreased to one-twentieth as the cells went from exponential to stationary phase (Table 2). However, the K_m for putrescine remained essentially unchanged throughout growth (Table 2).

When stationary-phase cells were inoculated into fresh medium, after a lag of 8 h transport activity increased 10-fold to reach a maximum of 5.2 nmol/min per 10^8 cells (Figure 4A). Cells incubated with actinomycin D or cycloheximide were unable to up-regulate transport, indicating that *de novo* synthesis of RNA and protein was required. The effect of cycloheximide was reversible, because cells regained full transport capacity after culture in fresh medium for a further 48 h. When early log-phase cells expressing maximal transport were treated with cycloheximide, putrescine transport activity decreased exponentially with a half-life of approx. 18 h (Figure 4B).

Exogenous putrescine also inhibits up-regulation of putrescine

Table 2 Alterations in K_m and V_{max} values for putrescine and cadaverine transport with cell growth phase

Cell density is the mean value of two determinations; K_m (μM) and V_{max} (nmol/min per 10^8 cells) values were determined with the Enzfitter software package (means $\pm$ S.E.M., $n = 7$). Statistical significance: *, $P < 0.001$ compared with day 3 cells (Student's paired t-test). Abbreviation: ND, not determinable.

Time in culture (days)	Cell density (10^7cells/ml)	Putrescine transport		Cadaverine transport	
		K_m	V_{max}	K_m	V_{max}
3 (mid-exponential)	2.32	1.99 $\pm$ 0.67	3.29 $\pm$ 0.34	13.4 $\pm$ 3.9	3.86 $\pm$ 0.32
5 (late exponential)	6.53	2.06 $\pm$ 0.65	0.61 $\pm$ 0.06*	45.0 $\pm$ 19.3	1.01 $\pm$ 0.21*
7 (stationary)	5.89	2.69 $\pm$ 0.63	0.17 $\pm$ 0.01*	ND	ND

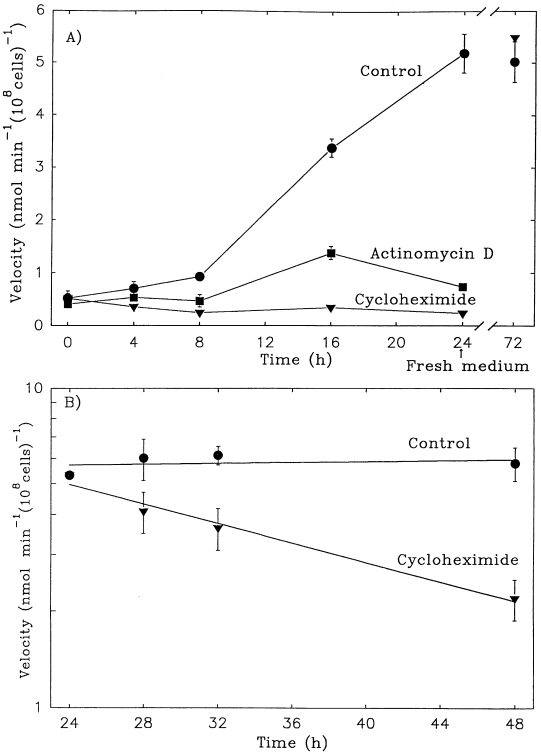

Figure 4 Regulation of putrescine transport during growth

(**A**) Up-regulation. *T. cruzi* cells were grown for 7 days and then diluted to 10^7 cells/ml into fresh medium either on its own (control cells) or supplemented with either 10 (or 100) μM cycloheximide (superimposable values) or 2 μM actinomycin D, and the velocity of 10 μM putrescine transport was measured. After 24 h, control and 10 μM cycloheximide-treated cells were washed once and resuspended at 10^7 cells/ml in fresh medium and incubated for a further 48 h. (**B**) The effect of cycloheximide on maximal putrescine transport activity. After 24 h the remaining control cells from (**A**) were divided into two and incubated for a further 24 h in the presence or absence of 100 μM cycloheximide. The velocity of transport of 10 μM putrescine was measured. Each point represents the mean $\pm$ S.D. for three determinations. From a transformation of the data to log V against time, a half life of 18 h is obtained for the loss of transporter activity.

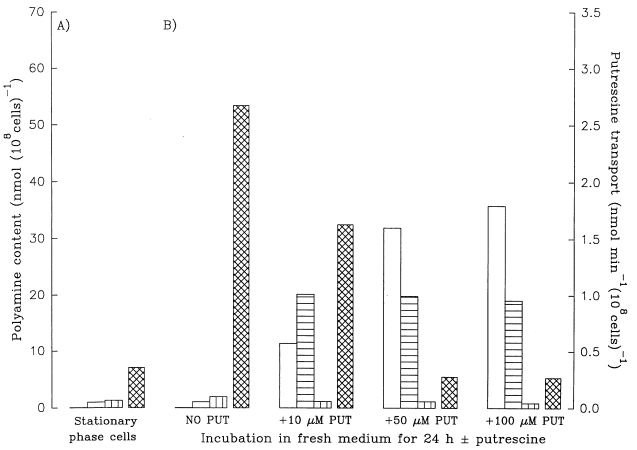

Figure 5 Effect of exogenously added putrescine (PUT) on up-regulation of *T. cruzi* putrescine transport and intracellular polyamine levels

(**A**) Stationary-phase cells. (**B**) Cells incubated in fresh medium in the presence or absence of putrescine for 24 h. The velocity of putrescine transport was determined as described in the Experimental section with 10 μM [^{3}H]putrescine (cross-hatched). Intracellular polyamine content: putrescine (open bars), spermidine (horizontal lines) and spermine (vertical lines). The results are the means for two determinations.

transport in a dose-dependent manner (Figure 5). Stationary-phase cells have low levels of both intracellular polyamines and putrescine transport (Figure 5A). After incubation in fresh growth medium for 24 h, transport increases approx. 8-fold with very little change in cellular polyamine content (Figure 5B). This induction of putrescine transport is partly or completely abolished by the addition of putrescine to the medium and is associated with striking changes in intracellular polyamine content. Putrescine levels increase, in a dose-dependent manner, from undetectable levels (less than 0.05 nmol per 10^8 cells) in controls, to a maximum of 36 nmol per 10^8cells in the presence of 100 μM putrescine. Spermidine increases from 1 to 19 nmol per 10^8 cells regardless of the amount of putrescine added. In contrast, spermine levels remain essentially unchanged.

Exogenous putrescine also down-regulates putrescine transport (Figure 6). Early exponential-phase cells show high levels of putrescine transport and low levels of intracellular polyamines (Figure 6A). This pattern does not change after culturing for a further 24 h in the absence of putrescine (Figure 6B). However, exposure to putrescine for 24 h results in a concentration-dependent decrease in putrescine transport down to basal levels (approx. 0.3 nmol/min per 10^8 cells), similar to those observed in stationary-phase cells. As in the previous experiment, intracellular putrescine increases in a dose-dependent manner, whereas spermidine increases from 0.8 nmol per 10^8 cells to a new constant level (13–17 nmol per 10^8 cells) independent of the exogenous putrescine concentration. Again spermine remains unaffected by exogenous putrescine.

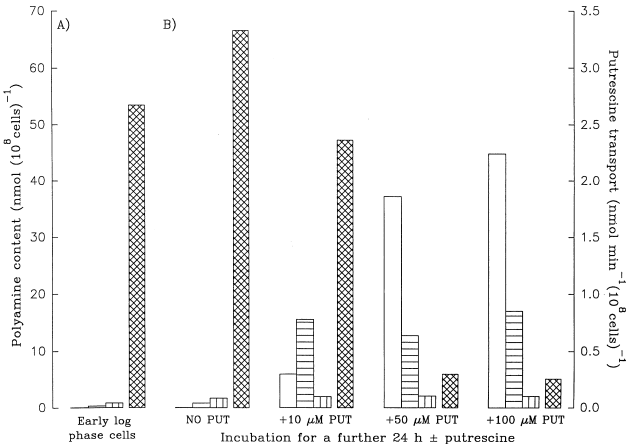

Figure 6 Effect of exogenously added putrescine (PUT) on down-regulation of *T. cruzi* putrescine transport and intracellular polyamine levels

(**A**) Stationary-phase cells that have been incubated in fresh medium for 24 h. (**B**) Cells from (**A**) that have been incubated for a further 24 h in fresh medium in the presence or absence of putrescine. The velocity of putrescine transport was determined as in Figure 5 at 10 μM putrescine (cross-hatched). Intracellular polyamine content: putrescine (open bars), spermidine (horizontal lines) and spermine (vertical lines). The results are the means for two determinations.

DISCUSSION

Our previous studies indicate that *T. cruzi* epimastigotes lack the ability to synthesize significant amounts of putrescine or cadaverine from amino acid precursors [6]. The results presented here indicate that these cells are able to accumulate putrescine and cadaverine rapidly from their surroundings on a high-affinity transport system that can be regulated in response to growth conditions and the polyamine content of the medium. The fact that the K_m values for putrescine and cadaverine in *T. cruzi* epimastigotes are in the micromolar range may be of physiological significance, because micromolar quantities of these diamines are present in the excreta of the *T. cruzi* insect vector, *Rhodnius prolixus*, after a blood meal [6].

Both active transport and facilitated diffusion systems have been described for the uptake of nutrients in the trypanosomatids (reviewed in [23]). The transport of diamines into *T. cruzi* seems to be an active temperature-dependent process that requires the presence of thiol groups. Inhibition by protonophores suggests that a membrane potential may be involved. However, in *E. coli* an uptake system preferential for spermidine has been described with a requirement for both a membrane potential and ATP [24].

Our results indicate the presence of at least two diamine/polyamine transport systems in *T. cruzi* epimastigotes. The system described here has high specificity for the diamines and low specificity for polyamines. As spermidine can also be taken up with high affinity [K_m 0.81 ± 0.22 μM, V_{max} 1.34 nmol/min per 10^8 cells (S. A. Le Quesne and A. H. Fairlamb, unpublished work)] there is likely to be at least one additional diamine/polyamine transporter. Because *T. cruzi* epimastigotes seem to be auxotrophic for diamines and/or polyamines [6], it is not altogether surprising that they contain more than one uptake system for such essential metabolites.

The activity of the *T. cruzi* diamine transporter seems to be regulated. It is induced in response to favourable growth conditions, where putrescine concentrations in the medium are low. A similar type of induction of both polyamine uptake (reviewed in [5]) and ODC activity (reviewed in [25]) can be observed in mammalian cells in response to various growth stimuli. In mammalian cells one type of regulation of both ODC and polyamine transport activity involves a small labile protein called antizyme (reviewed in [26]). Although it is not known whether *T. cruzi* contains antizyme, *T. b. brucei* does not seem to contain this protein [27]. Moreover, in breast cancer cells other regulatory mechanisms are operative, in addition to antizyme, in the feedback regulation of polyamine uptake [28].

Putrescine transport in *T. cruzi* epimastigotes is also responsive to exogenous putrescine. Our data show that exogenous putrescine can either block induction of putrescine transport from basal levels or promote down-regulation from maximal to basal rates in exponentially growing cells. In both cases the down-regulation of putrescine transport in the presence of exogenous putrescine coincides with a massive increase in total intracellular polyamine concentration. From our calculated value for the cell volume of *T. cruzi* epimastigotes of 5.5 μl per 10^8 cells, a change in the total intracellular polyamine concentration was observed (Figures 5 and 6) from less than 550 μM to more than 10 mM for cells grown for 24 h in the absence or presence respectively of 100 μM exogenous putrescine. Out of these total values, putrescine levels go from less than 10 μM to more than 6.5 mM in cells grown in the absence or presence respectively of 100 μM exogenous putrescine. Interestingly, spermine levels seem to be tightly regulated, varying only between 0.8 and 2.1 nmol per 10^8 cells (145–380 μM). These findings are similar to the changes that have been observed in putrescine transport activity and intracellular polyamine levels on addition of exogenous putrescine in Chinese hamster ovary cells [29].

As polyamines are required for growth [30], and as both replicating stages of *T. cruzi* (the epimastigote in the insect gut and the amastigote in mammalian cells) are normally bathed in micromolar levels of diamines and polyamines, it could be argued that these stages do not require a capacity for *de novo* synthesis when they can simply take them up from their surroundings. Perhaps then the activity of these transporters plays a central role in the overall control of intracellular polyamine levels in *T. cruzi* in a similar fashion to that observed with mammalian ODC, the dominant controlling factor of their entire polyamine pathway [31]. Alternatively a lack of tight regulation, similar to that observed with *T. b. brucei* ODC [32,33], could potentially be the downfall of *T. cruzi*.

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