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Characterization of spermine uptake by Ehrlich tumour cells in culture

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Summary. Spermine is taken up by Ehrlich ascites tumour cells through a specific, saturable, temperature and energy-dependent transport system with a remarkably low affinity constant for spermine (around 1 μM). In the absence of a potassium ion gradient through the plasma membrane, spermine uptake remains saturable but the value of the K_m for spermine is much higher (153 μM). Difluormethylornithine treatment (3 mM for 48 h) induces significant increases in V_{max} values (up to 9-fold) and changes in the K_m values with scarce statistical significance. Among the biogenic amines tested, only spermidine and, partly, agmatine seem to share the same transport system with spermine. No difference is observed in the rate of spermine transport when assays are carried out in the presence of 50-fold excess of ornithine or calcium, or 100-fold excess of glutamine.

Keywords: Amino acids – Polyamines – Spermine – Uptake – Ehrlich cells

Introduction

Polyamines behave as organic aliphatic polycations at physiological pH. From bacteria to mammalian cells, polyamines are essential for maintaining cell viability and active macromolecular synthesis through their interaction with nucleic acids, proteins and cellular membranes (Seiler, 1990; Heby and Persson, 1990). The intracellular concentration of a polyamine depends on both its rates of synthesis and degradation and on its rates of uptake and release (Grillo and Colombatto, 1994). Some cells appear to have a single transporter for putrescine, spermidine, and spermine; others seem to have more than one transport system for polyamine uptake (Seiler and Dezeure, 1990; Seiler et al., 1996). It is well established that cells and tissues with a high demand for polyamines, such as prostate, tumours, or normal, rapidly proliferating cells in fact take up polyamines at much higher rates than other not so rapidly proliferating cells (Seiler and Schröder, 1970; Clark and Fair, 1975; Volkow et al., 1983).

For the last ten years, our group has studied polyamine metabolism in the experimental tumour model Ehrlich ascites carcinoma (Matés et al., 1989, 1991; Márquez et al., 1989; Sánchez-Jiménez et al., 1992; Urdiales et al., 1992; Morata et al., 1994; Viguera et al., 1994; Medina et al., 1995). We have also previously studied the transport of the amino acid ornithine, a direct precursor of polyamines, in plasma membrane vesicles isolated from Ehrlich cells (Medina et al., 1991). Although Jänne's group studied some aspects of polyamine uptake in Ehrlich cells in the early 1980's (Alhonhen-Hongisto et al., 1980, 1982; Sepännen et al., 1981a,b), the kinetic characterization of the transport system(s) has not been reported so far. In the present work, we describe the kinetic characterization of spermine transport by intact Ehrlich ascites tumour cells grown as adherent cells in culture dishes.

Material and methods

Material

Sterile plasticware was from Nunc. DMEM medium, fetal calf serum, trypsin-versane and antibiotics were from Bio-Whittaker. [^{14}C]-Spermine (117 mCi/mmol) was supplied by Amersham. Ecoscint H liquid scintillation counting solution was from National Diagnostics. All other reagents were from Sigma.

Cell culture

Ehrlich ascites tumour cells adapted for their maintenance as cell cultures were obtained from the American Type Culture Collection. Ehrlich cells were grown in DMEM medium supplemented with 10% fetal calf serum and antibiotics (penicillin, streptomycin and amphotericin B) at 37°C in 90 mm diameter tissue culture dishes under 5% CO_2 atmosphere. When cells were confluent, they were detached with trypsin-versane and subcultured at a split ratio of 1:5.

Transport assays

The cells used for transport experiments were seeded into 24-well cluster dishes and they were used when subconfluent. The buffered media used in transport experiments were phosphate buffered salines containing 0.15 M sodium (PBS-Na), potassium (PBS-K), or choline (PBS-choline) chlorides, or 0.3 M mannitol (PB-mannitol). Serum-containing DMEM was retired from wells, cells were washed twice with PBS and transport was initiated by adding 0.5 mL of the buffered medium containing different concentrations of spermine. Experiments with PBS-Na and PBS-K at both 37°C and 0°C were always carried out in parallel. After 20 min of incubation, the uptake was terminated by removing the transport medium and washing with 2 × 0.5 mL aliquots of ice-cold PBS supplemented with 2 mM non-radiolabelled spermine. Plates were drained and 0.4 mL of 0.5% Triton X-100 were added. Cells were then removed with a cell scraper and suspended in this medium, 300 μL samples were dissolved in 10 mL of scintillation solution for counting and protein was determined using the method described by Bradford (1976). Cell counting in replica wells was carried out by using a Coulter counter. The non-saturable, linear component of uptake due to simple diffusion measured in the experiments carried out at 0°C was always substrated from the total amount of spermine transported at 37°C.

The endogenous polyamine pool of cells used for these experiments were: putrescine, 2.74 ± 0.10 pmol/μg protein; spermidine, 25.94 ± 0.82 pmol/g protein; spermine 11.08 ± 0.08, pmol/μg protein. The biogenic amines histamine and serotonine were below the detection level of the HPLC procedure used (Matés et al., 1992).

Results and discussion

Ionic dependence of spermine transport

We carried out kinetic experiments making the usual assumption that polyamine uptake has two components: one saturable, corresponding to the carrier-mediated uptake, and the other linear, corresponding to non-specific uptake, that is, passive diffusion following Fick's law. Since polyamine transport is energy dependent, diffusional uptake at 0°C is a measurement of non-specific uptake, including binding to cell surface (Seiler et al., 1996). For this reason, we always carried out parallel experiments at both 37°C and 0°C.

Figure 1 shows that the specific uptake of spermine (uptake at 37°C minus uptake at 0°C) in Ehrlich ascites tumour cells incubated in PBS-Na is a saturable process. In the conditions used, non-specific uptake accounted for less than 5% of total uptake at spermine concentrations lower than 20μM (results not shown).

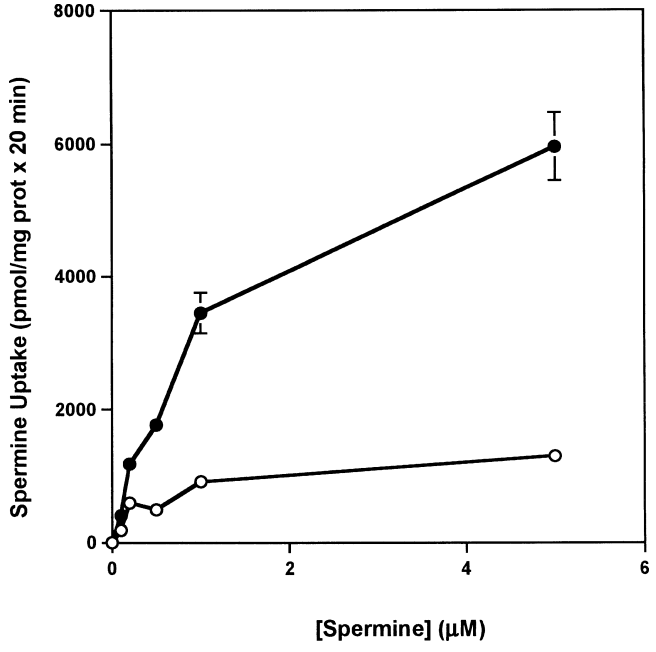

Fig. 1. Spermine uptake by Ehrlich cells is saturable. Specific spermine uptake by Ehrlich ascites tumour cells pretreated (closed circles) or not (open circles) with 3 mM DFMO for 48 h was assayed three independent times. Data from a representative experiment are depicted and they are means ± SD of three determinations in the same experiment. Experimental points not showing their SD had SD values smaller than the size of the circles used in the figure. Dilution factors of radiolabelled spermine were 1, 1/2, 1/5, 1/10 and 1/10 for 0.1, 0.2, 0.5, 1 and 5μM spermine final concentration, respectively

Figure 1 also shows that spermine uptake is highly increased in cells pretreated for 48 h with 3 mM DFMO. Under these conditions, intracellular pool of putrescine and spermidine in Ehrlich cells have been reported to decrease by more than 90%, with small changes in spermine concentrations (Alhonen-Hongisto et al., 1980).

The potential Na^+-dependence of spermine transport in Ehrlich cells was examined by isosmotically substituting NaCl with either KCl, choline chloride or mannitol in the assay medium. Table 1 shows that substitution of sodium ions with choline or potassium ions decreased spermine uptake. On the contrary, spermine uptake did not decrease by replacing NaCl with an osmotically equivalent concentration of mannitol.

In spite of the fact that some authors consider a "Na^+-dependent" uptake as the fraction of total uptake in the presence of sodium ions which is inhibited by isoosmotical concentrations of choline chloride (Rinehardt and Chen, 1984; Rannels et al., 1989), the correct definition for a Na^+-dependent transport system is that secondary active transport in which the substrate is taken up in symport with sodium ions. Since NaCl can be substituted by isosmotical concentrations of mannitol in our system, we cannot talk of a true Na^+-dependent uptake. In fact, in the literature, spermine uptake use to be described as a Na^+-independent transport (Seiler and Dezeure, 1990; Seiler et al., 1996). In breast cancer cells, membrane potential is primarily determined by a potassium-ion diffusion potential (Poulin et al., 1995). Therefore, increasing the concentration of extracellular potassium-ions should depolarize the plasma membrane, in accordance with the Nernst equation. As shown in Table 1, uptake of 10 μM spermine by Ehrlich cells incubated in PBS-Na is decreased by more than 60% in PBS-K. Thus, the "Na^+-dependent" uptake could be described here more precisely as a plasma membrane potential-dependent uptake (Nicolet et al., 1990; Morgan, 1992; Poulin et al., 1995, 1998).

Uptake in PBS-K is not significant at spermine concentrations lesser than 2 μM, but it becomes more and more important with increasing concentrations

Table 1. Ionic effects on the transport of spermine by Erhlich ascites tumour cells. Transport experiments were carried out in parallel in different phosphate buffered media containing isosmotic concentrations of either sodium, potassium or choline chloride, or mannitol. In all the cases, spermine concentration was 10 μM

Assay medium	Spermine uptake (%)
PBS-Na	100 ± 7
PBS-K	39 ± 6**
PBS-choline	62 ± 26*
PB-mannitol	143 ± 45

Data are percentages of specific transport, taking the values in PBS-Na as 100%, and they are given as means ± S.D. of the means values of three determinations in three independent experiments.
* Significant ($p < 0.05$) versus PBS-Na values according to a Student's t test. ** Significant ($p < 0.01$) versus PBS-Na values according to a Student's t test.

Table 2. Kinetic parameters of spermine uptake by Ehrlich ascites tumour cells pretreated or not with DFMO

Pretreatment	−DFMO		+DFMO	
	K_m (μM)	V_{max}[a]	K_m (μM)	V_{max}[a]
Plasma membrane potential-dependent uptake	1.08 ± 0.39	570 ± 94	1.54 ± 0.09	5,212 ± 1,228*
Uptake in the absence of potassium gradient	153 ± 4	1,585 ± 464	182 ± 61	3,736 ± 1,723*

Data are given as means ± S.D. of the means values of three determinations in, at least, three independent experiments.
[a] Values of velocities are given as pmol per mg of protein and per 20 min. * Significant ($p < 0.01$) versus control (−DFMO) values according to a Student's t test.

of spermine. We carried out kinetic experiments in PBS-K at higher spermine concentrations than those used in the kinetic experiments in PBS-Na. We found that this second transport mechanism is also saturable but at spermine concentrations with scarce physiological significance.

Determination of kinetic parameters

Table 2 summarizes the calculated kinetic parameters for both saturable spermine uptake mechanisms in both non-pretreated or DFMO-pretreated Ehrlich cells. These data allow us to conclude that, in fact, spermine can be taken up by two different specific mechanisms in Ehrlich cells: a plasma membrane potential-dependent mechanism, which shows high affinity, and a transport in the absence of potassium-ion gradient, which shows very low affinity for spermine. In both cases, the depletion of intracellular putrescine and spermidine contents by suicide inhibition of ornithine decarboxylase with DFMO induced very significant increases in V_{max} values without affecting the values of K_m. DFMO pre-treatment increased V_{max} values of plasma membrane potential-dependent spermine transport 9-fold, and V_{max} values of spermine transport in the absence of a potassium-ion gradient 2.4-fold. These data are in very good agreement with the 9-fold increase in spermine uptake after 48 h of 6 mM DFMO treatment in Ehrlich cells previously reported (Alhonen-Hongisto et al., 1980).

In any case, it seems clear that only the plasma membrane potential-dependent spermine uptake is physiologically relevant. For this reason, we decided to carry out our additional studies on this transport only.

Increased accumulation of evidence points to calcium-ion as a pivotal element in the regulation of polyamine transport (Seiler et al., 1996). In human breast cancer cells, depletion of the extracellular calcium ions significantly inhibits putrescine uptake, and the addition of 420 μM $CaCl_2$ in the extracellular medium induces a 2-fold increase of putrescine uptake

Table 3. Spermine transport by Ehrlich ascites tumour cells in the presence of calcium or pretreated with glutamine

Assay	Spermine uptake (%)
Control	100 ± 7
+ Calcium chloride 0.5 mM	73 ± 24
Pretreatment (1 mM glutamine for 3 h)	92 ± 19

Data are percentages of specific transport, taking the values in PBS-Na as 100%, and they are given as means ± S.D. of the means values of three determinations in three independent experiments.

(Poulin et al., 1995). In rabbit intestinal brush border membrane vesicles, addition of calcium-ions also increases putrescine uptake (Brachet and Tomé, 1992). On the contrary, when we compared 10 μM spermine transport in the absence or presence of 0.5 mM $CaCl_2$ in the assay medium, we found a decrease in total spermine uptake in the presence of calcium in the external medium, although this decrease had scarce statistical significance (Table 3).

It has also been shown that putrescine uptake by mouse neuroblastoma cells can be stimulated by a 3 h preincubation in the presence of system A amino acids (Rinehart and Chen, 1984). In Ehrlich cells, we have found that a 3 h preincubation in the presence of 1 mM glutamine induces no significant change in spermine uptake (Table 3).

Substrate specificity

Finally, we also decided to study the substrate specificity of spermine uptake by Ehrlich cells in PBS-Na. Figure 2 shows that, among polyamines and analogs, only spermidine could share the same transport system as spermine. Neither putrescine, nor histamine, serotonin and others could be taken up by the specific plasma membrane potential-dependent spermine transport system. Interestingly, apart from spermidine, only agmatine could partially inhibit spermine uptake. Agmatine, the product of arginine decarboxylation, has been recently detected in animal tissues, mainly brain and kidney, but not very much is known about it in animals (Li et al., 1994; Lortie et al., 1996). The cationic amino acid ornithine, from which polyamines are derived, could not inhibit spermine transport by Ehrlich cells, in contrast with the interference of diamines in ornithine transport by Ehrlich cell plasma membrane vesicles (Medina et al., 1991).

In conclusion, we have kinetically characterized a high affinity, temperature and energy dependent spermine transport system in Ehrlich cells which behaves as a highly specific carrier for spermine and, at least in part, spermidine. We have also shown that in the absence of a potassium ion gradient across the plasma membrane, spermine transport remains a saturable process but it behaves as a very low affinity transport system.

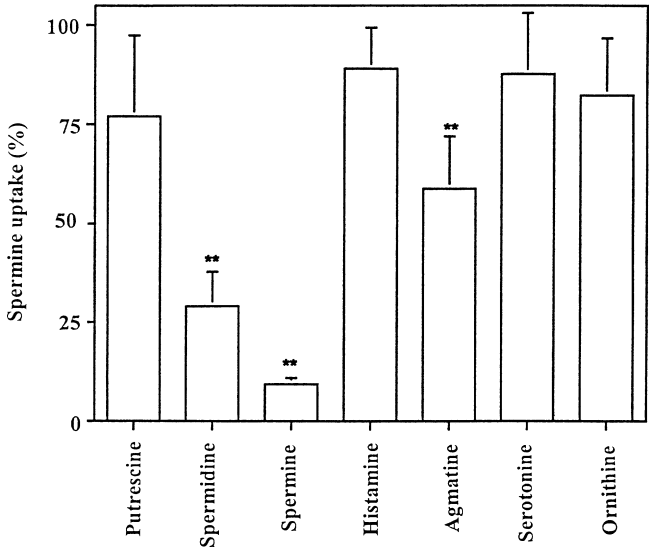

Fig. 2. Substrate specificity of spermine transport by Ehrlich ascites tumour cells. Uptake experiments were carried out in the presence of 1μM (0.1μM radiolabelled + 0.9μM unlabelled) spermine as the substrate and 50μM unlabelled polyamines and analogs as inhibitors. Data are percentages of specific transport, taking the control values as 100%, and they are given as means ± S.D. of the means values of three determinations in three independent experiments. **Significant ($p < 0.01$) versus control values according to a Student's t test

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