Characterization of putrescine- and spermidine-transport systems of a rat pancreatic acinar tumoral cell line (AR4-2J)

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Polyamines are polycationic molecules essential for cell growth and differentiation. Recent work has focused on cell polyamine-transport systems as a way to regulate intracellular polyamine levels. In this study, we demonstrate the presence of two different active transporters for putrescine and spermidine in a rat tumoral cell line (AR4-2J). The first has a K_m of 3.1 μM and a V_{max} of 3.7 pmol/15 min per μg of DNA for putrescine and the second a K_m of 0.42 μM and a V_{max} of 4.7 pmol/15 min per μg of DNA for spermidine. Competition studies performed between the polyamines confirm the difference between these two carriers; one has an equal affinity for the three main polyamines, and the other has a lower affinity for putrescine. Amino acids do not share this transport system, which is Na^+ -independent. Choline chloride inhibits selectively and in a dose-responsive manner the uptake of putrescine without affecting that of spermidine. These data demonstrate that AR4-2J cells possess two polyamine transporters; one is specific for aminopropyl groups (spermidine and spermine), and the other is choline-sensitive, but cannot discriminate between aminobutyl (putrescine) and aminopropyl groups.

INTRODUCTION

The polyamines putrescine, spermidine and spermine are polycationic molecules widely distributed in prokaryotic and eukaryotic cells. They are essential for cell growth and differentiation, and their biosynthesis is acutely regulated. This regulation greatly depends on ornithine decarboxylase (ODC), the key enzyme of the polyamine-biosynthetic pathway (for reviews see [1,2]).

Polyamines have been shown to exert a feedback control on ODC activity [3]. Furthermore, various studies emphasized the importance of a polyamine-transport system in several cell types [4–8]. Investigators have demonstrated the induction of this transport system after stimulation of proliferation [9,10]. Polyamine uptake would be an adaptative mechanism to regulate intracellular polyamine levels during cell proliferation. It could be also a possible response to some pharmacological events [polyamine-biosynthesis-blocking agents such as difluoromethylornithine (DFMO)] [11].

Polyamines have been proposed to play an important role in cancer. In human colon cancer, concentrations of polyamine are 3–4 times greater in tumoral tissues than in the adjacent normal mucosa [12]. This suggests an increase in biosynthesis and/or uptake in tumoral cells, and argues for physiological significance of these molecules during normal or pathological proliferative stimuli [12].

In the gastrointestinal tract, polyamine transport has been mainly studied by using intestinal preparations whose rapid turnover accounts for the polyamine-uptake abilities [6,7]. Pancreas has little regenerative capacity in comparison with intestinal mucosa. Nevertheless, some studies have shown the important role of ODC in pancreatic growth, for normal and tumoral tissues [13,14].

We have previously shown that growth of a rat acinar pancreatic cell line (AR4-2J) is dependent on intracellular polyamine contents [15], and could be stimulated by growth factors through an increase in ODC activity [16]. These characteristics confer on this cell line growth abilities dependent

on polyamines. However, the molecular basis of their transport system is still unknown.

The aim of the present work is to analyse polyamine uptake in AR4-2J cells and to characterize the transport system involved in this uptake.

MATERIALS AND METHODS

Materials

Media and reagents for cell culture were obtained from Gibco Brl (Cergy Pontoise, France), and polyamines (free bases) were from Sigma Chimie (La Verpillière, France). [^3H]Putrescine ([1,4(n)- ^3H]putrescine, 24 Ci/mmol) and [^{14}C]spermidine (117 mCi/mmol) were purchased from Amersham (Les Ulis, France); Aqualuma-Plus scintillation liquid was from Lumac Application Laboratory (Landgraaf, The Netherlands), and CH_3CN was from Info-Labo (Bordeaux, France).

Cell culture

AR4-2J cells, originally developed by Jessop & Hay [17], were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal-calf serum. Cells were plated at 2×10^5 cells/ml, and the medium was changed every 2 days. For uptake measurements, cells were plated into 35 mm-diam. dishes and allowed to attach overnight. At 24 h before the start of experiments, cells were deprived of serum. Serum deprivation has been shown to induce AR4-2J cells synchronization in the G_0 – G_1 phase [15]. In these conditions AR4-2J-cell metabolism and polyamine uptake are not modified (results not shown).

Uptake assay

Polyamine incorporations were started by adding unlabelled polyamines (putrescine, spermidine) in various concentrations with 2 μCi of [^3H]putrescine or 0.1 μCi of [^{14}C]spermidine to the amino-acid-free culture medium. For experiments with lower Na^+ concentration, two different media were used: a DMEM without NaHCO_3 in which final Na^+ concentrations were 25, 50,

Abbreviations used: ODC, ornithine decarboxylase; DFMO, difluoromethylornithine; DMEM, Dulbecco's modified Eagle's medium.

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75 mM, or a Hepes buffer (pH adjusted to 7.4 with Tris) with various Na^+ concentrations (5, 25, 50, 75, 140 mM). Osmolarity was maintained by corresponding amounts of choline chloride or sucrose.

Incubations were carried out either at 37 °C or 4 °C for various times. The medium was then discarded, and cells were rinsed with 2×1 ml of serum-free medium and disrupted by addition of 1 ml of 0.3 M- HClO_4 . The cell lysate was centrifuged at 1700 *g* for 10 min (4 °C); 500 μl of the supernatant was added to 10 ml of scintillation liquid, and radioactivity was determined by liquid-scintillation spectroscopy (Minaxi Tri-Carb 4000, Packard). DNA measurements were performed on the 1700 *g* pellets as described by Burton [18], and results are expressed as pmol of polyamines/ μg of DNA.

Non-specific uptake was measured in a parallel set of dishes at 4 °C and 37 °C by adding 2.5 mM of unlabelled polyamine to the radiolabelled polyamine. Under these conditions, the non-specific uptake was the same at 37 °C or 4 °C, and was always less than 20% of total uptake at 15 min or 90 min.

Cell polyamine content

Intracellular polyamines were extracted in 0.3 M- HClO_4 and dansylated by the procedure of Newton *et al.* [19]. Reverse-phase h.p.l.c. separation of the dansylated derivatives was carried out on a Zorbax C_8 column (Dupont de Nemours, Paris, France) with a solvent composed of triethylammonium phosphate/ CH_3CN (pH 3.5), in the ratio 1:1 (v/v) for solvent A and 1:4 for solvent B. Samples were eluted at a flow rate of 2 ml/min in the gradient mode by using concave gradient no. 9 (Waters model 660). The gradient changed from 100% solvent A to 100% solvent B in 15 min. Fluorimetric detection used a model 420 (Waters) equipped with 280 nm and 338 nm filters for excitation and emission respectively. Peak area were calculated on a Waters integrator model 740 by using a two-points calibration curve.

Measurement of intracellular volume

Cells were incubated in a Hepes buffer with 3-*O*-methyl-D-[^3H]glucose (5 $\mu\text{Ci}/\text{ml}$). At 30 min (equilibrium), cells were washed twice in 0.1 M- MgCl_2 and disrupted by 0.3 M- HClO_4 . Cell extracts were centrifuged at 1700 *g* (10 min). Radioactivity of the supernatant was determined by liquid-scintillation spectroscopy, and was considered as the intracellular distribution of this molecule.

Fig. 1. Time-course curves of polyamine uptakes in AR4-2J cells in the absence or presence of 1 mM-DFMO

AR4-2J cells were incubated for the indicated times in the presence of 5 μM -putrescine (■) or -spermidine (△). The effect of DFMO (□) was analysed by preincubating cells with DFMO (1 mM) 24 h before polyamine additions. Values represent the mean incorporation of radiolabelled polyamines in triplicate samples.

Calculation methods

K_m and V_{max} were determined by Lineweaver-Burk transformation of kinetics studies. K_i values were calculated by log/logit analysis of inhibition studies. Results are expressed as means $\pm$ S.D.

RESULTS

Uptake characteristics

As shown in Fig. 1, uptake of putrescine and spermidine is time-dependent, linear up to 60 min and stable until 120 min. In addition, results show a greater accumulation of spermidine than of putrescine. Lowering the temperature inhibits both uptakes by 95% (results not shown), demonstrating temperature-dependency of this mechanism.

Pretreatment of cells with DFMO (1 mM) blocks ODC activity and induces intracellular putrescine and spermidine depletion. In such conditions, putrescine uptake increases by 2.5-fold at 90 min.

Figs. 2 and 3 indicate that, in the presence of increasing concentrations of polyamine, uptake of both putrescine and spermidine is saturable. Kinetic parameters obtained by Lineweaver-Burk transformations of the data are K_m 3.1 ± 0.7 μM , V_{max} 3.7 ± 1.4 pmol/15 min per μg of DNA for putrescine uptake, and K_m 0.42 ± 0.3 μM , V_{max} 4.7 ± 1.4 pmol/15 min per μg of DNA for spermidine uptake.

H.p.l.c. analysis of intracellular radiolabelled polyamines shows that 90% of putrescine and 80% of spermidine incorporated remain in their native form after 15 min (incubation time for kinetic studies).

The intracellular volume of AR4-2J cells is 0.15 ± 0.05 $\mu\text{l}/\mu\text{g}$ of DNA. Intracellular concentrations of putrescine, spermidine and spermine are 49.3, 679.3 and 223.3 μM respectively. They were measured at 15 and 90 min and were found not to be affected by the uptake of polyamine. These different concentration values were for putrescine and spermidine respectively 9.9-fold and 135.9-fold greater than the extracellular polyamine concentration used, 5 μM .

Fig. 2. Kinetic parameters of putrescine uptake

AR4-2J cells were incubated for 15 min with increasing concentrations of unlabelled putrescine. The concentration of [^3H]putrescine in the medium was 40 nM. Data shown are means $\pm$ S.D. of three separate experiments performed in duplicate. Inset: Lineweaver-Burk transformation of the data. Intercepts of the line with the x and y axes are $-1/K_m$ and $1/V_{max}$, respectively. These values, as determined by linear-regression analysis, are $K_m = 3.1$ μM and $V_{max} = 3.7$ pmol/15 min per μg of DNA. Correlation coefficient of the line is 0.998.

Fig. 3. Kinetic parameters of spermidine uptake

AR4-2J cells were incubated for 15 min with increasing concentrations of unlabelled spermidine. Concentration of [14 C]spermidine in the medium was 0.4 μ M. Data shown are means $\pm$ S.D. of three separate experiments performed in duplicate. Inset: Lineweaver-Burk transformation of the data. Intercepts of line with the x and y axes are $-1/K_m$ and $1/V_{max}$, respectively. These values as determined by linear-regression analysis are $K_m = 0.42 \mu$ M and $V_{max} = 4.7$ pmol/15 min per μ g of DNA. Correlation coefficient of line is 0.989.

Table 1. Effect of polyamines on putrescine and spermidine uptake

Uptake assays were performed as described in the Materials and methods section. K_i values for competitive inhibition were determined over a 90 min incubation period by varying the inhibitor concentration in the presence of 5 μ M-putrescine or -spermidine. Values are means $\pm$ S.D. of four separate experiments performed in duplicate.

Inhibitor	Putrescine uptake (μ M)	Spermidine uptake (μ M)
Putrescine	K_m 3.1 $\pm$ 0.7	K_i 26.4 $\pm$ 8.1
Spermidine	K_i 1.1 $\pm$ 0.4	K_m 0.4 $\pm$ 0.3
Spermine	K_i 1.8 $\pm$ 0.5	K_i 3.1 $\pm$ 1.2

Table 2. Effect of amino acids on [3 H]putrescine and [14 C]spermidine uptake

Cells were incubated during 15 min as described in the Materials and methods section in the presence of amino acids (1 mM). Values are means $\pm$ S.D. of three separate experiments performed in duplicate, and are expressed as percentages of control uptake obtained in an amino-acid-free standard medium (DMEM with 140 mM- Na^+).

Amino acid	Uptake (% of control)	
	Putrescine	Spermidine
Leucine	117 $\pm$ 15	107 $\pm$ 10
Lysine	117 $\pm$ 12	93 $\pm$ 12
Asparagine	125 $\pm$ 19	103 $\pm$ 5

Competition studies

Competition studies were performed at plateau phase with various inhibitor concentrations and in the presence of 5 μ M of the polyamine studied. As shown in Table 1, putrescine and spermidine uptakes are inhibited by the other polyamines.

Table 3. Effect of various choline chloride concentrations and effect of sucrose on putrescine and spermidine uptake with incubation in DMEM (a) or in Hepes buffer (b)

Uptake of putrescine (Pt) and spermidine (Spd) was measured as described in the Materials and methods section with a concentration of 5 μ M of each polyamine and during a 15 min incubation period. Media of various NaCl concentrations were added at the beginning of the uptake studies. Osmolarity is maintained with a corresponding amount of either choline chloride or sucrose. Values are expressed as percentages of control uptake obtained in a standard medium (DMEM with 140 mM- Na^+).

(a) DMEM (Na^+ conc.)	Uptake (% of control)	
	Pt	Spd
75 mM	54 $\pm$ 7	94 $\pm$ 6
50 mM	48 $\pm$ 5	86 $\pm$ 10
25 mM	33 $\pm$ 4	84 $\pm$ 8

(b) Hepes buffer	Uptake (% of control)	
	Pt	
5 mM- Na^+ + 135 mM-choline $^+$	36 $\pm$ 6	
25 mM- Na^+ + 115 mM-choline $^+$	37 $\pm$ 4	
25 mM- Na^+ + 230 mM-sucrose	94 $\pm$ 8	
50 mM- Na^+ + 90 mM-choline $^+$	48 $\pm$ 7	
75 mM- Na^+ + 65 mM-choline $^+$	54 $\pm$ 4	
140 mM- Na^+ + 0 mM-choline $^+$	106 $\pm$ 10	
140 mM- Na^+ + 10 mM-choline $^+$	77 $\pm$ 6	
140 mM- Na^+ + 20 mM-sucrose	95 $\pm$ 9	

Putrescine uptake is inhibited with the same potency by spermidine and spermine (K_i 1.1 $\pm$ 0.4 μ M and 1.8 $\pm$ 0.5 μ M respectively), and spermine is more efficient than putrescine in inhibiting spermidine uptake (K_i 3.1 $\pm$ 1.2 μ M and 26 $\pm$ 8 μ M respectively).

Effects of amino acids

As shown in Table 2, the amino acids asparagine, leucine and lysine, representing each of the three groups of amino acids transporters, have no effect on putrescine or spermidine uptake.

Effects of Na^+

Incubation of cells with decreasing Na^+ concentrations inhibits putrescine uptake without any significant effect on that of spermidine. This effect was identical in DMEM or in Hepes buffer (Tables 3a and 3b). When choline is replaced by an uncharged compound (sucrose) in an amount to maintain osmolarity, putrescine uptake is no longer affected. The inhibition by choline chloride is dose-dependent and reaches a maximum at 25 mM- Na^+ . Furthermore, addition of choline chloride (10 mM) to a Hepes buffer containing 140 mM- Na^+ inhibits putrescine uptake by 23 %, whereas sucrose added in a corresponding amount to mimic the osmolar change has no significant effect on this uptake.

DISCUSSION

At least two different mechanisms regulate intracellular polyamine levels: firstly polyamine biosynthesis, which is mainly regulated by ODC, and secondly, the ability of cells to take in exogenous polyamines as an alternative source. Various studies have focused on characterization of the polyamine-transport

system in a variety of cell types [4–8]. Intracellular polyamine levels regulate both ODC activity [20,21] and their uptake [22]. These multiple ways provide cells with a precise regulation of their polyamine content.

We have previously demonstrated that DFMO depletes polyamine content and inhibits growth of AR4-2J cells. Polyamine addition to the culture medium reverses this inhibition, suggesting an uptake by the cells [15].

In our study we find that putrescine or spermidine uptake is a time- and temperature-dependent process, and occurs against a concentration gradient. Kinetic studies show that putrescine and spermidine transports are saturable. Thus all these parameters are characteristic of an active mechanism. Moreover, in cells depleted of putrescine and spermidine with DFMO, putrescine uptake is increased by 2.5-fold, pointing out the relation between ODC activity, intracellular polyamine content and polyamine-transport mechanism.

Parameters derived from Lineweaver–Burk transformations (Figs. 2 and 3) are consistent with either a single transporter having different affinity for putrescine and spermidine, or with two transporters. In order to investigate these hypotheses, the following protocols were carried out.

Firstly, lysine, leucine and asparagine, which are representative of the three main classes of amino acid transporters, have no inhibitory effect on the uptake at a concentration of 1 mM. These data are in agreement with those previously published on other cell types [5,6,22].

Secondly, competition studies show that spermine and spermidine are equally potent to inhibit putrescine uptake (K_i 1.1 and 1.8 μ M respectively), whereas spermine is 9 times more potent than putrescine to inhibit spermidine uptake (K_i 3.1 and 26 μ M respectively).

Thirdly, replacement of NaCl by a corresponding amount of choline chloride dose-dependently inhibits putrescine uptake without significantly affecting that of spermidine. These results confirm the presence of two distinct transporters. However, they do not preclude the possibility that they share the Na⁺-transport system. In fact, replacement of choline chloride by sucrose abolishes the inhibition observed of putrescine uptake. Furthermore, choline chloride (10 mM), added to a 140 mM-Na⁺ medium, lowers putrescine uptake. In the same condition, sucrose has no effect.

These data demonstrate the presence of two types of transporters in AR4-2J cells. Such results are partially in agreement with Kumagai & Johnson [5,6], who concluded a direct interaction of charged compounds (choline⁺, Li⁺ etc.) with polyamine carriers. However, in AR4-2J cells the inhibition concerns only putrescine uptake.

Byers *et al.* [22] identified two types of polyamine transporters, by means of the aminopropyl or aminobutyl affinity and their Na⁺-dependency. In their work, transport of putrescine, which

represents aminobutyl groups, is Na⁺-dependent. Our data are consistent with this distinction between aminopropyl and aminobutyl groups, but without Na⁺-dependency.

In conclusion, this work demonstrates the presence of a double polyamine-transport system in AR4-2J cells: one which is specific for aminopropyl groups (spermidine and spermine), and the other which cannot discriminate the two chemical forms but is choline chloride-sensitive. This point is in favour of a difference in biological and molecular structures between these two carriers.

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