Transport systems for polyamines in the established renal cell line LLC-PK₁

Polarized expression of an Na⁺-dependent transporter

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We present evidence for the existence of an Na⁺-dependent transporter and an Na⁺-independent transporter for polyamines in LLC-PK₁ cells. Both transporters could be discriminated by their sensitivity to inhibitors, particularly *p*-chloromercuriphenyl sulphate and various polycationic molecules. By using cell monolayers grown on a permeable filter support, we have found that the Na⁺-dependent polyamine uptake occurred preferentially from the basolateral side. The Na⁺-independent uptake, on the other hand, occurred to the same extent from either the apical or the basolateral side.

INTRODUCTION

Both prokaryotic and eukaryotic cells are dependent on polyamines for growth and differentiation. Although virtually all cells are capable of synthesis of polyamines *de novo*, it was also found that polyamine import via specific polyamine transporters is widely distributed in a variety of cells [1]. The significance of polyamine transport is emphasized by the demonstration that the uptake is greatly stimulated in cells in which the synthesis of polyamines is prevented by DL-2-difluoromethylornithine (DFMO), an irreversible inhibitor of the synthesis pathway [2–4]. The polyamine transporters have not yet been fully characterized at the biochemical level. We have recently shown [2] that in the established renal cell line LLC-PK₁ there are at least two different transporters for putrescine: an Na⁺-dependent one and an Na⁺-independent one. Both transporters had a similar substrate-specificity, but the Na⁺-dependent transporter had an about 6-fold higher substrate affinity. The presence of an Na⁺-dependent transporter and an Na⁺-independent transporter for the same substrate is very common in epithelial cells, where a polarized localization of these transporters is a prerequisite for transcellular transport. The epithelial cell line LLC-PK₁ has been studied extensively with respect to the polarized expression of several Na⁺-coupled transporters. The transporters for D-glucose [5], phosphate [6], acidic amino acids [7] and lactate [8] are located on the apical surface, those for neutral amino acids [9,10] and the Na⁺/Ca²⁺ exchanger [11] are located on the basolateral surface, and two pharmacologically different Na⁺/H⁺ antiporters are located on respectively the apical surface and the basolateral surface [12].

LLC-PK₁ cells can be grown on a permeable support, e.g. nitrocellulose filters or collagen-coated polycarbonate filters [13], so that direct access to the apical or the basolateral surface is possible. In the present study

we have examined the polarized uptake of polyamine using LLC-PK₁ monolayers grown on nitrocellulose filters. It was found that the Na⁺-dependent pathway is localized on the basolateral surface whereas the Na⁺-independent pathway was distributed non-preferentially on both apical and basolateral surfaces. We furthermore observed that both transporters are very effectively inhibited by other polycationic compounds such as gentamycin, Ruthenium Red and poly-D-lysine.

EXPERIMENTAL

LLC-PK₁ cells (A.T.C.C. CRL 1392/CL 101, obtained from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K.) were subcultured weekly and used between passages 193 and 205. Cells were cultured at 37 °C in a humidified 5% CO₂ atmosphere. The medium was α -Minimal Essential Medium (Flow Laboratories) supplemented with 3.5 mM-L-glutamine, 87 μ g of streptomycin/ml, 87 i.u. of penicillin/ml and 10% (v/v) fetal-calf serum. For experiments, cells were seeded either in 12-well tissue-culture clusters of 3.8 cm² (Costar Europe Ltd., Badhoevedorp, The Netherlands) or in 30 mm-diameter tissue-culture inserts (Millicell HA; Millipore Corporation, Bedford, MA, U.S.A.) placed in six-well clusters. The bottom of the insert consists of a surfactant-free microporous membrane filter. Care was taken that no hydrostatic pressure gradient existed over the microporous bottom separating the 'apical' side or inside of the insert and the 'basolateral' side or outside of the insert. DFMO, an irreversible inhibitor of polyamine biosynthesis (Merrell Dow Research Center, Strasbourg, France), was added to the culture medium at a final concentration of 5 mM. The timing of DFMO addition is indicated in the legends to the Figures.

For cells grown on plastic, the uptake studies were done just before confluency. For cells grown on filters, on the other hand, uptake studies were done after

Abbreviations used: DFMO, DL-2-difluoromethylornithine; HBSS, Hanks balanced salt solution.

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confluency was reached. This was verified by measuring the increase in transepithelial resistance using the Millicell-ERS epithelial voltammeter (Millipore Corporation). Typically the electrical resistance after confluency reached a value of $> 40 \Omega \cdot \text{cm}^2$.

The wells were fixed on a thermostatically controlled plate (37 °C) placed on a mechanical shaker. The monolayers were rinsed twice with Na^+ -free Hanks balanced salt solution (HBSS) (Na^+ was replaced *N*-methyl-D-glucamine). The monolayers were then preincubated during 20 min at 37 °C with HBSS with or without Na^+ , and containing 20 mM-dithiothreitol. Dithiothreitol was added since we have found previously [2] that preincubation with this compound stimulated the Na^+ -dependent putrescine uptake by about 30 %. Uptake was initiated by addition of the labelled polyamines [^3H]-putrescine, [^{14}C]spermidine or [^{14}C]spermine from a stock solution in Na^+ -free HBSS. The final concentration of these compounds was 5 μM . The specific radioactivity was 0.3 mCi/ μmol for putrescine and 0.1 mCi/ μmol for spermidine and spermine. For experiments with cells grown on filters, the radiolabelled polyamine was added only to one side, either apical or basolateral, as indicated. The uptake was stopped by aspirating the medium and washing the monolayer (both sides in the case of cells grown on filters) with ice-cold HBSS containing 10 μM unlabelled substrate. The monolayers were then solubilized in 2 % (w/v) SDS. Radioactivity was measured with the use of Insta-Gel II (Packard Instruments S.A.) as scintillation liquid. We have corrected our uptake values for the contribution of the radioactivity that is retained in the filter and in the extracellular space. This correction was made using the radioactivity (d.p.m.) values for [^{14}C]mannitol, a compound that is not taken up by the cells.

Protein was measured by the method of Lowry *et al.* [14], with BSA as standard, after precipitation of the proteins in ice-cold 10 % (w/v) trichloroacetic acid.

Biochemicals were from Sigma Chemical Co. The radioactive labelled products were from Amersham International, Amersham, Bucks., U.K.

RESULTS AND DISCUSSION

Putrescine uptake in LLC-PK₁ cells is strongly up-regulated by treatment of a growing monolayer with 5 mM-DFMO, an irreversible inhibitor of the polyamine synthesis (Fig. 1). Putrescine uptake in DFMO-treated monolayers is linear up to 30 min (results not shown). In our standard conditions we used monolayers that had been pretreated with 5 mM-DFMO for 4–5 days. As shown in Fig. 1, putrescine uptake in these conditions was partially dependent on the presence of Na^+ in the uptake medium. The Na^+ -independent part of the uptake was obtained by substituting *N*-methyl-D-glucamine (150 mM) for Na^+ . The finding that part of the polyamine uptake is Na^+ -dependent is consistent with observations in other cell types such as mouse neuroblastoma cells [15], bovine adrenocortical cells [16] and mouse embryonic palatal mesenchymal cells [17]. In a few recent studies on rat enterocytes [18,19] and colon cancer cells [20] the inhibitory effect on polyamine uptake by substitution of *N*-methylglucamine for Na^+ has been explained as a direct inhibition of the polyamine uptake by *N*-methylglucamine, since no such effect was obtained upon substitution with uncharged compounds. In con-

Fig. 1. Initial uptake of putrescine in LLC-PK₁ cell monolayers

Cells were seeded in 12-well clusters at 18 500 cells/cm². DFMO (5 mM) was added with fresh medium at day 3 and day 5 after seeding. The uptake medium was HBSS (▲ and △) or HBSS with *N*-methylglucamine instead of Na^+ (■ and □). ▲ and ■, Controls; △ and □, DFMO-treated cells. The monolayers were pretreated with 20 mM-dithiothreitol (20 min) and the uptake was started by addition of 5 μM (final concentration) labelled substrate. The values are means of two observations. The spread was always less than 15 %.

trast with those studies, which were based on polyamine uptakes for 4 h, we have measured initial uptakes of polyamines and we have observed essentially the same inhibition whether sucrose or *N*-methylglucamine was substituted for Na^+ (results not shown).

Our previous data [2] clearly demonstrated that the Na^+ -dependent and the Na^+ -independent parts of the polyamine uptake in LLC-PK₁ cells represent two different transport systems. Both transporters are saturable with respect to the substrate concentration. They have a similar substrate-specificity, but the Na^+ -dependent transporter has a considerably higher affinity (K_m 4.7 μM) than the Na^+ -independent transporter (K_m 29.8 μM). We have as yet no evidence on whether the Na^+ -dependent part of polyamine transport in LLC-PK₁ cells is due to a direct coupling between Na^+ and polyamine, e.g. by an Na^+ /substrate co-transport mechanism.

We have grown LLC-PK₁ cell monolayers on permeable filter inserts. This system allowed us to measure polyamine uptake from the basolateral or the apical side. The experimental conditions were essentially the same as for the uptake experiments with cells grown on plastic, except that the experiments were done after confluency was reached. The experimental protocol was critically dependent on the timing for DFMO addition, since we had to find a balance between two effects provoked by DFMO: the up-regulation of the carrier on one hand and the drastic decrease of the growth rate of the monolayer on the other hand [2]. DFMO was therefore applied not earlier than 1 day before confluency. The experiment in Fig. 2 shows the uptake of putrescine, spermidine and spermine in LLC-PK₁ cell monolayers after application of these substrates to either the basolateral or the apical side. The Na^+ -dependent uptake,

Fig. 2. Polarized uptake of polyamines in LLC-PK₁ cell monolayers

Cells were seeded on nitrocellulose filters in tissue-culture inserts at 25000 cells/cm². DFMO (5 mM) was added together with fresh medium at day 4 and day 6 after seeding. The uptake medium was HBSS or Na⁺-free HBSS. The monolayers were pretreated with 20 mM-dithiothreitol and the uptakes were started by addition of 5 μ M (final concentration) labelled substrate. The initial uptake rates (5 min) are shown for three substrates: putrescine (▨), spermidine (▤) and spermine (■). The Na⁺-dependent and the Na⁺-independent parts of the uptake are shown with the radioactive substrate added at either the apical or the basolateral side. The error bars indicate the S.E.M. values for three observations. The significance of the differences between the basolateral and the apical uptake was determined by a Student's *t* test and is indicated as follows: **P* < 0.05; ***P* < 0.01.

obtained as the difference between the total uptake and the uptake with *N*-methylglucamine substituting for Na⁺, was much higher when the substrates were applied from the basolateral side. This effect was observed for all the three polyamines. The relatively low Na⁺-dependent uptake, when the radioactive labelled substrates were applied to the apical side, varied somewhat from experiment to experiment, but was usually less than 20% of the uptake from the basolateral side. It is very probable that this residual Na⁺-dependent uptake is due to a finite diffusion of the polyamines through the monolayer and subsequent uptake from the basolateral side. The Na⁺-independent uptake (Fig. 2), on the other hand, was not polarized. There was no significant difference in the Na⁺-independent uptake from either the apical or the basolateral side.

It should be pointed out that the rate of Na⁺-dependent putrescine uptake for cells grown on filters (520 pmol/5 min per mg of protein) was considerably higher than for cells grown on plastic (150 pmol/5 min per mg of protein). Although there were fluctuations from experiment to experiment, this difference was consistently found and probably indicates that, for monolayers grown on plastic, only some of the Na⁺-dependent transporters are available for the substrate. In addition, we have observed that uptake in cells grown on plastic decreased with increasing confluency of the monolayer

Table 1. Effect of *p*-chloromercuriphenyl sulphate on putrescine uptake in LLC-PK₁ cell monolayers

The cells were seeded on filters in culture inserts. The control conditions were as described in the legend to Fig. 2. In the conditions with *p*-chloromercuriphenyl sulphate (PCMPS), dithiothreitol was omitted and 100 μ M-*p*-chloromercuriphenyl sulphate (final concentration) was applied to both sides of the monolayer. *p*-Chloromercuriphenyl sulphate was added 20 min before the uptake. The values given for the putrescine uptake are means $\pm$ S.E.M. (*n* = 3). ** indicates a significant difference between the basolateral and the apical uptake (*P* < 0.01) as determined by a Student's *t* test.

	Putrescine uptake (pmol/5 min per mg of protein)	
	Control	PCMPS
Na ⁺ -dependent		
Basolateral side	540.4 $\pm$ 86.7**	211.4 $\pm$ 28.9
Apical side	77.2 $\pm$ 65.5	118.9 $\pm$ 12.1
Na ⁺ -independent		
Basolateral side	204.0 $\pm$ 40.2	201.8 $\pm$ 30.6
Apical side	186.7 $\pm$ 9.6	211.3 $\pm$ 11.5

(results not shown). No significant difference between monolayers grown on plastic or on filters was found with respect to the Na⁺-independent transporter.

It is conceivable that the polarized expression of the polyamine transporters only occurs in confluent monolayers after the formation of junctional complexes. The mechanism and time course of this process, however, are unknown. It is therefore not possible to decide from our experiments if the observed polarization for the Na⁺-dependent transporter and the lack of polarization for the Na⁺-independent transporter represent the final situation for fully differentiated cells. It is also possible that a further polarization with respect to the Na⁺-independent transporter may eventually occur.

The Na⁺-dependent and Na⁺-independent transporters differ with respect to their sensitivity to thiol-group-modifying reagents [2]. The Na⁺-dependent uptake from the basolateral side is strongly inhibited by *p*-chloromercuriphenyl sulphate (Table 1). The Na⁺-independent uptake on the other hand, was not affected at all, either from the apical or from the basolateral side.

The two transporters are relatively specific [2], and polyamines do not share amino acid transport mechanisms ([19] and references cited therein). In agreement with observations made by Hauser & Cook [21], we have found that the polyamine transporters in LLC-PK₁ cells are very effectively inhibited by other polycationic compounds. In Table 2, the concentrations for half-maximal inhibition by gentamycin, Ruthenium Red and poly-D-lysine are given. Apparently, compounds with multiple cationic sites were required, since 100 μ M-La³⁺ was not effective. Although both transporters were inhibited by the polycationic compounds, there were quantitative differences. Ruthenium Red was much more effective against the Na⁺-dependent transporter, whereas gentamycin was more effective against the Na⁺-independent transporter. These observations further confirm the different nature of the Na⁺-dependent and Na⁺-independent transporters.

Table 2. Inhibition by polycationic compounds of polyamine transport in LLC-PK₁ cell monolayers

The cells were seeded in 12-well clusters. The monolayers were treated with 5 mM-DFMO as described in the legend to Fig. 1. The inhibitors were applied from a stock solution in Na⁺-free HBSS. In the experiment with Ruthenium Red, the treatment with dithiothreitol was omitted since Ruthenium Red is reduced by this compound. The substrate (putrescine) concentration was 5 μ M. The concentrations of the inhibitors needed for half-maximal inhibition of the Na⁺-dependent and the Na⁺-independent transporters are listed. The S.E.M. values were obtained from the variance-co-variance matrix of a computerized non-linear-regression analysis.

Inhibitor	Concentration for half-maximal inhibition (μ M)	
	Na ⁺ -dependent transporter	Na ⁺ -independent transporter
Ruthenium Red	0.9 $\pm$ 0.1	10.9 $\pm$ 2.8
Gentamycin	36.0 $\pm$ 2.7	10.3 $\pm$ 1.1
Poly-D-lysine	1.2 $\pm$ 0.2	1.1 $\pm$ 0.2

A preferential uptake of polyamines from the basolateral side was also found for colon cancer cells [20], but in that study only one transporter was described and the transport was not dependent on Na⁺. A polarized localization of polyamine transport in epithelial cells may have a physiological role with respect to transepithelial transport. In renal epithelia a polarized localization of a high-affinity transporter at the basolateral side and the presence of a low-affinity transporter at the apical side would favour transepithelial secretion of polyamines.

It has been shown in rodents [22,23] and in humans [24] that normally a very significant proportion of the polyamines does not appear in the urine because of catabolism, the excreted amount being strongly dependent on the activity of the catabolic reactions. From experiments with specific inhibitors of the oxidative catabolic pathways it was concluded, however, that catabolism may represent a dispensable way of polyamine inactivation. It was found that urinary excretion was capable of taking over the functional role of oxidation in polyamine disposal [25]. It is therefore possible that the polarized distribution of the polyamine transporters described here is involved in a urinary polyamine excretion pathway.

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