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Interaction of N^1,N^{12}-Diacetylspermine with Polyamine Transport Systems of Polarized Porcine Renal Cell Line LLC-PK$_1$

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LLC-PK$_1$ cells grown on porous membrane filters were employed as a model system to explore the renal transport of polyamines. The polarity of LLC-PK$_1$ monolayers was confirmed by the exclusive appearance of a Na^+-dependent α-methylglucoside transport system on the apical surface. The uptake of free polyamines from the basolateral side of monolayers was consistent with the existence of a single class of transport system, while the existence of two kinetically distinct polyamine transport systems with higher and lower affinities on apical membranes was suggested. The results of competition studies indicated that each of these transporters was able to interact with putrescine, spermidine and spermine. LLC-PK$_1$ cells incorporated monoacetylspermine from the apical surface of monolayers at about half the rate of spermine uptake. Monoacetylspermine inhibited spermidine uptake, indicating that free polyamine transport systems also recognized the monoacetylated derivative. In contrast, N^1,N^{12}-diacetylspermine did not inhibit spermidine uptake, nor was it incorporated into the cells, indicating the absence of transport systems that recognize N^1,N^{12}-diacetylspermine on the apical membranes of LLC-PK$_1$ cells. These results may be relevant as to our previous observation that the content of diacetylpolyamines in urine is relatively constant, and may explain the excellence of N^1,N^{12}-diacetylspermine as a tumor marker.

Key words: diacetylspermine, polyamine, renal cell, transport, tumor marker.

Abbreviations: DiAcSpm, N^1,N^{12}-diacetylspermine; PBS, phosphate-buffered saline; PCMPS, *p*-chloromercuriphenyl sulfate.

Recently, we developed a highly sensitive analytical system for urinary polyamines based on fractionation by HPLC, and reported that N^1,N^8-diacetylspermidine and N^1,N^{12}-diacetylspermine (DiAcSpm) are found in urine samples from healthy persons as relatively minor but regular constituents (*1*, *2*). Subsequent analysis indicated that these diacetylpolyamines were frequently and markedly increased in the urine of urogenital cancer patients, implying that the urinary level of diacetylpolyamines, DiAcSpm in particular, is useful for the diagnosis of urogenital malignancies (*3*, *4*). More recently, we developed an ELISA system for DiAcSpm determination involving a highly DiAcSpm-specific antibody preparation (*5*, *6*), and further substantiated that DiAcSpm serves as a sensitive and specific novel marker for many cancers including early and late stage colorectal and breast cancers (*7*). Urinary polyamines were first proposed by Russell (*8*) to be potential tumor markers. This evoked a surge of intensive studies, but later studies revealed that the clinical use of the total polyamines or the major urinary polyamine components including acetylputrescine and N^1- and N^8-acetylspermine in the urine as tumor markers is not feasible, since there were too many false negative as well as false positive cases, as judged based on this parameter (*9*).

The sharp increase in the level of DiAcSpm in the urine of cancer patients is not surprising in view of the requirement of polyamines for cell proliferation and activation of polyamine metabolism in rapidly growing tissues (*10*, *11*). However, the reason why only diacetylpolyamines, *i.e.* not monoacetylpolyamines, are highly sensitive and tumor-specific for various types of malignant diseases remains obscure.

To establish the usefulness of DiAcSpm as a tumor marker, it is important to define factors that determine the DiAcSpm content in the urine. One such factor would be the reabsorption of low molecular weight substances at the renal brush border. As a model for the transport systems in the renal brush border we investigated polyamine uptake by polarized monolayers of pig kidney epithelia–derived LLC-PK$_1$ cells. Measurement of polyamine transport using this system revealed that high and low affinity transport systems for free polyamines are expressed on the apical surface of the polarized cells, and that these systems were able to transport monoacetylspermine as well. In contrast, there was no sign of uptake of DiAcSpm by LLC-PK$_1$ cell monolayers. This may partly explain the excellence of DiAcSpm as a tumor marker.

MATERIALS AND METHODS

Materials—[2,3-^{3}H]Putrescine dihydrochloride (1,493 GBq/mmol) and [1,8-^{3}H]spermidine trihydrochloride

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(781 GBq/mol) were obtained from DuPont NEN (Boston, MA, USA). Methyl(α-D-[*U*-^{14}C]gluco)pyranoside (10.8 GBq/mol) was purchased from Amersham International. [1,10-^{3}H]Spermine tetrahydrochloride (1,480 GBq/mol) was a product of American Radiolabeled Chemicals Inc. (St. Louis, MO, USA). [1,12-^{3}H]N^{1},N^{12}-diacetylspermine (2.0 GBq/mol) was a kind gift from Dr. A. Shirahata at Jyosai University. [^{3}H]Monoacetylspermine was prepared by partial deacetylation of [^{3}H]diacetylspermine with acylpolyamine amidohydrolase, and subsequently purified by HPLC on an ODP column using 0.1 M phosphate buffer (pH 7.0) (*1*) . Other chemicals used were of the highest grade commercially available.

Cell Culture—LLC-PK$_1$ cells were obtained from the American Type Culture Collection (ATCC CRL-1392) and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum supplied by JRH Bioscience (Lenexa, KS, USA), 0.1 mg of streptomycin/ml, and 100 units of penicillin/ml. For uptake studies, the cells were seeded on collagen-coated membrane filters (3 μm pores, 4.71-cm^2 growth area) placed in a Transwell cell culture chamber (Coster, Cambridge, MA, USA) at a cell density of 1–2 × 10^6 cells/filter. The cell monolayers were fed with fresh medium every 2 days, and usually used on the 6th or 7th day for the uptake studies. At this stage, the cell monolayers showed transepithelial electrical resistance ranging from 230 to 320 Ω/cm^2, as measured by Millicell-EPR (Millipore, Bedford, MA, USA).

Uptake Assay—For measurement of polyamine uptake in LLC-PK$_1$ cells grown on filters, both sides of a monolayer were washed twice with phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$, and 0.5 mM $MgCl_2$), and then the cells were preincubated in PBS for 10 min at 37°C prior to uptake assays. Uptake was started by adding a ^{3}H-labelled polyamine substrate in PBS (2 ml) to either the apical or basolateral side of the monolayer. The monolayer was incubated at 37°C for the indicated time under a normal atmosphere. The medium was then removed, and the cells were washed twice with ice-cold PBS containing 0.1 mM of the unlabelled substrate polyamine. After the filter with the monolayer had been removed from the chamber, the cells were solubilized in 0.4 ml of 1 N NaOH at 70°C for 30 min. Following the addition of 0.1 ml of 4 N HCl, radioactivity was measured with a liquid scintillation counter. Protein was quantitated by the method of Bradford (*12*). In the case of Na^+-dependent α-methylglucoside uptake, 50 μM methyl(α-D-[*U*-^{14}C]gluco)pyranoside in PBS was added to either the apical or basolateral side. In Na^+-free medium, NaCl and Na_2HPO_4 in PBS were replaced with the same concentrations of choline chloride and K_2HPO_4.

For determination of the kinetic parameters of polyamine uptake, the polyamine concentration was varied by adding increasing concentrations of a non-radioactive substrate polyamine to a fixed amount of the tritium-labelled substrate polyamine. The Michaelis constant (K_m) and maximum velocity were determined by Eadie-Hofstee analysis. Competition for polyamine uptake was measured by adding increasing concentrations of competing polyamines in the presence of a fixed amount of [^{3}H]putrescine or [^{3}H]spermidine.

RESULTS

Polarity of LLC-PK$_1$ Cells as Assessed by the Na^+-Dependent Glucose Transporting Activity—A confluent LLC-PK$_1$ monolayer grown on a microporous membrane filter should exhibit polarity with the apical surface facing the upper compartment and the basolateral surface facing the lower compartment (*13*). To establish the validity of our experimental system as a model for renal epithelia, we measured the activity of Na^+-dependent glucose transporter on both sides of a monolayer (Fig. 1). This transporter serves as an appropriate marker for the apical surface judging from previous results obtained with this cell line (*14*). When 50 μM α-methyl-D-glucoside was placed on the apical side, LLC-PK$_1$ cells took up the glucose analogue at a rate of 20.7 nmol/mg protein/hr in the presence of Na^+, but not at all in its absence (0.43 nmol/mg protein/h). The uptake of the glucose analogue from the basolateral side in the presence and absence of Na^+ was 0.29 and 0.19 nmol/mg protein/h, respectively. These results indicate that a LLC-PK$_1$ monolayer exhibits the expected polarity with the preferential localization of Na^+-dependent glucose transporter on the apical surface. The maximal rate of the Na^+-dependent uptake of α-methyl-D-glucoside from the apical side was attained on the 4th day after seeding and was still maintained on the 10th day (data not shown).

Time Course of Polyamine Uptake by a LLC-PK$_1$ Monolayer—Figure 2 shows the characteristics of putrescine and spermidine uptake by polarized LLC-PK$_1$ cells. The cells took up both substrates from both the apical and basolateral sides at 37°C in a time-dependent manner. Lowering of the temperature to 6°C greatly redued the uptake of polyamines.

Kinetics of Polyamine Uptake by Polarized LLC-PK$_1$ Cells—To define kinetic parameters for putrescine and spermidine uptake by LLC-PK$_1$ monolayers, cells were incubated with increasing concentrations of these polyamines. As shown in Fig. 3A, Eadie-Hofstee analysis of putrescine uptake from the basolateral side gave a straight

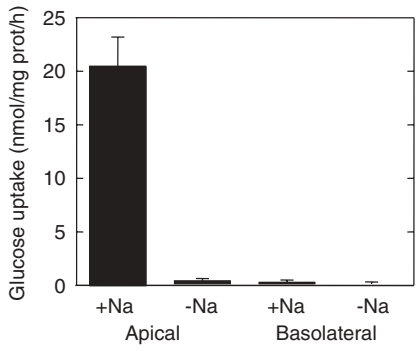

Fig. 1. **Uptake of α-methyl-D-glucose by LLC-PK$_1$ monolayers.** LLC-PK$_1$ cells were grown to confluence on collagen-coated filters. On the 6th day after seeding the cells were preincubated in PBS or Na^+-free medium at 37°C for 10 min, and then 50 μM α-methyl-D-[^{14}C]glucoside was added to either the apical or basolateral side in the presence and absence of Na^+. The uptake of α-methyl-D-[^{14}C]glucoside was measured as described under "MATERIALS AND METHODS" after incubation at 37°C for 1 h. Each column represents the mean ± SE for 4 independent measurements.

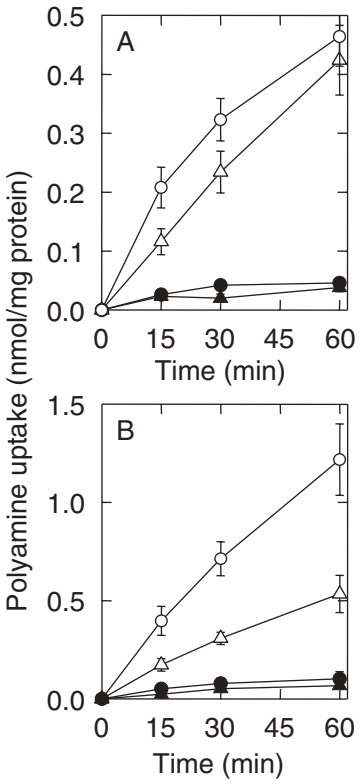

Fig. 2. **Time- and temperature-dependent uptake of spermidine and putrescine by polarized LLC-PK$_1$ cells.** LLC-PK$_1$ monolayers were incubated with 5 μM [^{3}H]putrescine (A) or 5 μM [^{3}H]spermidine (B) in PBS added to either the apical (open circles, solid circles) or basolateral side (open triangles, solid triangles) at 37°C (open circles, open triangles) or 6°C (solid circles, solid triangles). Cellular polyamine uptake was determined at the times indicated as described under "MATERIALS AND METHODS." Each point represents the mean ± SE for 3 experiments.

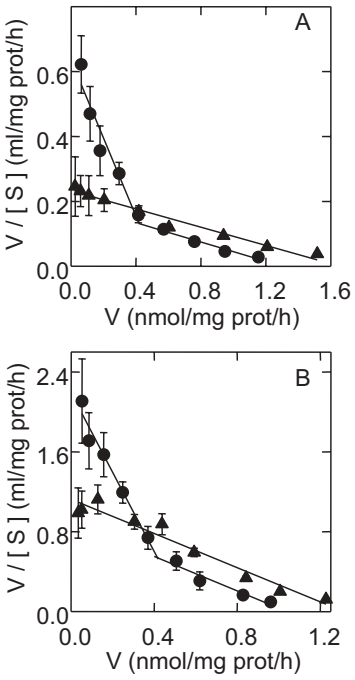

Fig. 3. **Eadie-Hofstee analysis of putrescine and spermidine uptake by polarized LLC-PK$_1$ cells.** LLC-PK$_1$ monolayers were incubated at 37°C for 30 min with various concentrations of [^{3}H]putrescine (A) or [^{3}H]spermidine (B) added to either the apical (solid circles) or basolateral side (solid triangles) of the monolayers Other conditions were as in Fig. 2. Each point represents the mean ± SE for 3 experiments.

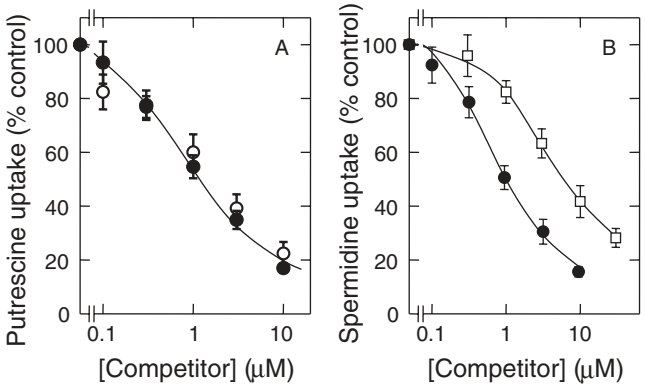

Fig. 4. **Specificity of putrescine and spermidine uptake by polarized LLC-PK$_1$ cells.** The uptake of 0.1 μM [^{3}H]putrescine (A) or 0.1 μM [^{3}H]spermidine (B) from the apical side of monolayers was measured in the presence of increasing concentrations of putrescine (open squares), spermidine (open circles), or spermine (solid circels). Other conditions were as in Fig. 3. Each point represents the mean ± SE for 3 independent experiments.

line characterized by single K_m and V_{max} values of 14 μM and 3.3 nmol/mg protein/h, respectively, which is consistent with the existence of a single class of transporter. In contrast, uptake of putrescine from the apical side was biphasic, suggesting the interaction of putrescine with two sites. The K_m and V_{max} values for the high and low affinity sites were estimated to be 2.3 μM and 1.1 nmol/mg protein/h, and 13 μM and 2.6 nmol/mg protein/h, respectively. Spermidine was also accumulated in the cells, the kinetic features being similar to as for putrescine (Fig. 3B), but the K_m values for spermidine were much lower than those for putrescine. K_m and V_{max} for basolateral uptake of spermidine were 2.3 μM and 2.6 nmol/mg protein/h, respectively. As to apical uptake, K_m and V_{max} were determined to be 0.52 μM and 1.1 nmol/mg protein/h for the high affinity site, and 2.3 μM and 2.1 nmol/mg protein/h for the low affinity site, respectively. The kinetic characteristics of spermine uptake by LLC-PK$_1$ monolayers were very similar to those for spermidine (data not shown), for which K_m = 2.5 μM and V_{max} = 2.9 nmol/mg protein/h for basolateral uptake, and K_m = 0.60 μM and V_{max} = 1.2 nmol/g protein/h for the high affinity site and K_m = 2.0 μM and V_{max} = 2.4 nmol/mg protein/h for the low affinity site for the apical uptake system.

Specificity of Polyamine Uptake by Polarized LLC-PK$_1$ Cells—The substrate specificity of the putrescine and spermidine uptake systems was assessed by analysis of the competition by other polyamine species (Fig. 4). When putrescine uptake was measured in the presence of increasing concentrations of spermidine or spermine, the uptake of putrescine from the apical side of the monolayers was inhibited by these polyamines (Fig. 4A). Spermidine uptake

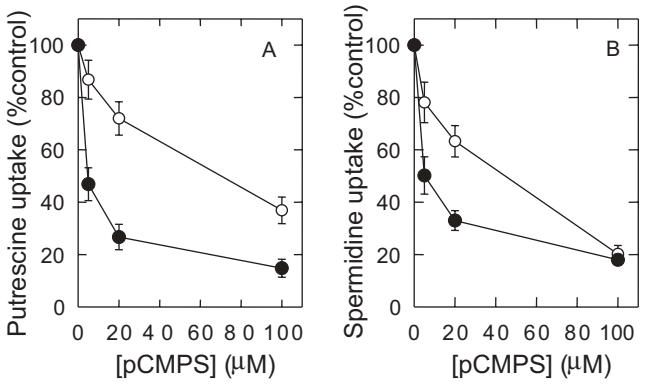

Fig. 5. **Inhibition of putrescine and spermidine uptake by PCMPS in polarized LLC-PK$_1$ cells.** LLC-PK$_1$ cells were preincubated with the indicated concentrations of PCMPS dissolved in PBS at 37°C for 10 min prior to the uptake assay. After washing the cells with PBS, 0.1 μM [^{3}H]putrescine (A) or 0.1 μM [^{3}H]spermidine (B) was added to either the apical (open circles) or basolateral (solid circles) side of the monolayers. Polyamine uptake was determined after incubation at 37°C for 30 min as described under "MATERIALS AND METHODS." Each point represents the mean ± SE for 3 experiments.

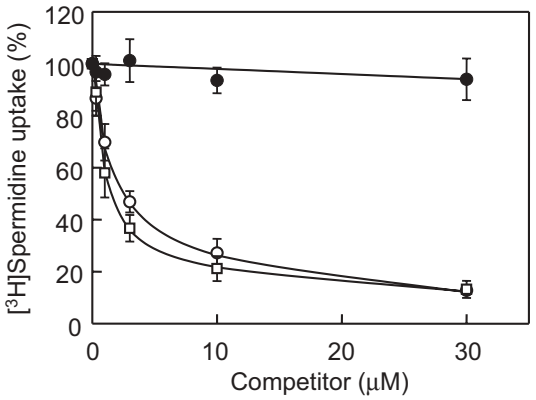

Fig. 6. **Effects of spermine and its acetylated derivatives on spermidine uptake by polarized LLC-PK$_1$ cells.** The uptake of 0.1 μM [^{3}H]spermidine from apical membranes was assayed in the presence of increasing concentrations of spermine (open squares), monoacetylspermine (open circles), or diacetylspermine (solid circles). Other conditions were as in Fig. 4. Each point represents the mean ± SE for 3 experiments.

from the apical surface also decreased in the presence of putrescine or spermine (Fig. 4B). The uptake of putrescine and spermidine from the basolateral surface was similarly affected mutually and also by spermine to that from the apical surface (data not shown). These results indicate that a common transport system(s) is present for the uptake of putrescine, spermidine, and spermine in the apical and basolateral membranes of LLC-PK$_1$ cells. Putrescine and spermidine uptake were inhibited by other polyamines in lower concentration ranges on the apical side than on the basolateral side, indicating the presence of a transport system with higher affinity for polyamines on the apical side. This is consistent with the data obtained in the kinetic study on polyamine transport shown above and in Fig. 3.

Effect of PCMPS on the Polyamine Uptake by Polarized LLC-PK$_1$ Cells—Figure 5 shows the effect of *p*-chloromercuriphenyl sulfate (PCMPS) on polyamine uptake by polarized LLC-PK$_1$ cells. The basolateral uptake of polyamines was inhibited by 50% with about 5 μM PCMPS, while the polyamine transport system on the apical side was much less sensitive to PCMPS. These results indicate that a polyamine transport system with lower sensitivity to PCMPS is present in the apical membranes of LLC-PK$_1$ cells. The transport system with lower PCMPS sensitivity likely represents the high affinity transport system, because polyamine uptake *via* the low affinity system constitutes only 20–30% of total polyamine uptake from the apical surface under the experimental conditions used in Fig. 5. The partial but significant inhibition of apical spermidine transport noted at a PCMPS concentration as low as 5 μM may represent the contribution by the low affinity transport system.

Uptake of Acetylated Derivatives of Spermine by LLC-PK$_1$ Cells—Figure 6 shows the effects of acetylated derivatives of spermine on the uptake of spermidine across the apical membranes of polarized LLC-PK$_1$ cells. Acetylspermine and spermine inhibited the uptake of spermidine from the apical side, suggesting their interaction with the spermidine transport systems in the apical membranes.

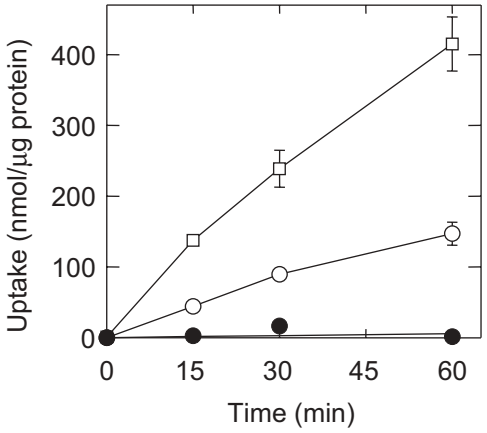

Fig. 7. **Time course of uptake of spermine and its acetylated derivatives by polarized LLC-PK$_1$ monolayers.** The uptake of 1.0 μM [^{3}H]spermine (open squares), [^{3}H]monoacetylspermine (open circles), and [^{3}H]diacetylspermine (solid circles) from apical membranes was assayed as described in Fig. 2. Each point represents the mean ± SE for 2 experiments.

We estimated K_i values for acetylspermine, assuming two competition sites in the apical transport system. They were 0.49 μM and 3.6 μM for the apical uptake systems, which were comparable to the K_m values of the apical transport systems for spermine. In contrast, DiAcSpm did not affect the spermidine uptake at all, indicating a lack of its interaction with these spermidine transport systems.

Figure 7 shows the time course of the uptake of mono- and diacetylspermine into polarized LLC-PK$_1$ cells from the apical membranes. Monoacetylspermine was taken up from the apical side of LLC-PK$_1$ monolayers, although the rate of uptake was about half that of spermine. Its uptake was temperature-sensitive, being more than 10 times lower at 6°C than at 37°C (data not shown). These results together with those in Fig. 6 indicate that monoacetylspermine is incorporated into LLC-PK$_1$ cells through transport systems that are common to free polyamines. In contrast, the uptake of DiAcSpm was not observed at

all, indicating the absence of a transport system that can carry DiAcSpm into the cytoplasm of LLC-PK$_1$ cells.

DISCUSSION

Polarized monolayers of LLC-PK$_1$ cells cultured on membrane filters were utilized as a model for the polyamine transport systems in renal epithelia. Using radiolabeled putrescine and spermidine as substrates, we were able to show the existence of one and two kinetically distinguishable transport systems on the basolateral and apical surfaces of the monolayers, respectively. Each of these transport systems was shown to interact with putrescine, spermidine and spermine as mutually competing transport substrates, judging from the results of competition experiments. The low affinity transport systems in the apical and basolateral membranes seem to be identical or closely similar judging from their K_m and V_{max} values for these three polyamine species.

Polyamine transport in mammalian cells has long been a matter of considerable interest (*15*), but attempts to identify the polyamine transporter genes in mammals have not yet been successful. Previous studies involving polarized cultures of LLC-PK$_1$ cells indicated the existence of Na^+-dependent and Na^+-independent polyamine transport systems in these cells (*16–18*). The former was inhibited by a low concentration of PCMPS, while the latter was relatively insensitive to PCMPS. In view of these reports it is tempting to assume that the uptake of polyamines from the basolateral surface is mainly carried out by a PCMPS-sensitive Na^+-dependent transporter, while the apical polyamine transport is due to both types of transporters. However, previous studies indicated that the Na^+-dependent polyamine transport system exhibits higher affinity for polyamines than the Na^+-independent system. We carried out the present polyamine transport study in the presence of 140 mM NaCl so that all polyamine transport systems present in the membranes were operating while we were examining the capacity of cellular polyamine transport systems to recognize polyamine conjugates including DiAcSpm. The identity of the PCMPS-sensitive basolateral polyamine transporter with lower affinity with the Na^+-sensitive polyamine transporter reported in the literature thus remains to be seen.

The most important finding in this study is that DiAcSpm did not inhibit any polyamine transport system expressed in the apical membranes of LLC-PK$_1$ cell monolayers, nor was it incorporated into the cells. In contrast, monoacetylspermine was able to interact with cellular polyamine transporters with an affinity almost as high as that of spermine, and was transported into the cells. This implies that renal brush border membranes may incorporate relatively low concentrations of unconjugated and monoacetylated polyamine species through their high affinity transport system, but are unable to transport DiAcSpm.

Polyamines excreted from various organs and tissues into the circulation are filtered through the glomerular basement membrane in the kidney, but a significant portion of monoacetylpolyamines as well as unconjugated polyamines is soon reabsorbed from the glomerular filtrate into tubular cells through polyamine transport systems as described above, and converted to free polyamines by cellular polyamine oxidase to be reutilized in the body (*19*). The present results indicate that this reabsorption route is not available for DiAcSpm. It is therefore likely that DiAcSpm excreted from cells in the body is recovered in the urine without significant loss, while the amount of urinary monoacetylpolyamines decreases by an unknown amount compared to that originally excreted from the cells due to renal reabsorption and reutilization. This may partially explain why the urinary level of DiAcSpm reflects the presence of cancer in the body with high sensitivity and at early clinical stages (*7*), while the urinary levels of monoacetylpolyamines do not (*3*, *4*).

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