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The diversity of Na^+-independent uptake systems for polyamines in rat intestinal brush-border membrane vesicles

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Na^+-independent uptake rate and binding to the membrane surface of polyamines (spermine, spermidine and putrescine) have been characterized using rat small intestinal brush-border membrane vesicles. The uptake of spermine and spermidine was saturable (K_m = 30.4 μM and 148.1 μM, respectively), however, putrescine uptake was not saturable up to 8 mM. In contrast, the values of binding to the membrane surface of all polyamines were not saturable in the present studies. In Dixon plot analysis, spermine competitively inhibited the uptake rate of spermidine with a K_i value of 33.8 μM, while the putrescine inhibitory effect on the spermidine uptake rate was non-competitive (K_i = 3.28 mM). These uptake systems were not affected by the valinomycin-induced K^+-diffusion potential (inside negative). These results suggested that there were two different Na^+-independent uptake systems for spermine and spermidine, as well as for putrescine, on this membrane. However, they were not the same as the electric potential-dependent uptake system for monocationic compounds. Furthermore, this uptake system for spermine and spermidine might not be a carrier protein, because the intravesicular spermine exhibited no trans-stimulation effect on the uptake of spermidine.

Introduction

The polyamines (spermine, spermidine and putrescine), which play an important role in the growth, differentiation and multiplication of the cells [1,2], are regulated by the biosynthesis from ornithine and the transport of exogenous polyamine [3,4]. Although the gastrointestinal tract is considered to be the most important exogenous polyamine source for the tissues [5], the characteristics of polyamine transport system(s) in intestinal epithelial cells still remains unclear. Several investigations have dealt with the Na^+ dependence of these polyamine transport systems using LLC-PK_1 cells [6] and human erythrocytes [7].

On the other hand, putrescine has been reported to be taken up by a Na^+-independent mechanism in isolated enterocytes [8] and the human colon adenocarcinoma Lovo cell line [9]. It seems that such a discrepancy with regard to the effect of Na^+ on polyamine transport is due to the utilization of whole cells in almost all of these studies, for it was difficult to differentiate the transmembrane transport of polyamines from the binding to membrane surfaces. Additionally, the cell suspensions were not available to distinguish between the uptake from apical membrane and that from basolateral membrane.

We reported previously [10,11] that there is marked uptake of polyamines into rat intestinal brush-border membrane vesicles (BBMVs) and that the uptake was unchanged by an inward Na^+ gradient. Furthermore, these polyamine uptakes depended upon the pH of the medium, which was based on the interaction between polyamines and acidic phospholipids of plasma membrane. Recently, Quemener et al. reported that putrescine uptake by human glioblastoma cell line U-251, and putrescine binding to the surface of these cells are independent processes [12]. In the present study, to obtain more information as to the mechanism responsible for polyamines uptake by the small intestine, we distinguished between the uptake and the binding to the membrane surface, and characterized the uptake process of polyamines into rat intestinal BBMVs.

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Abbreviations: Tris, tris(hydroxymethyl)-aminomethane; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

Materials and Methods

Chemicals. [^{14}C]Spermine tetrahydrochloride (4.1 GBq/mmol), [^{14}C]spermidine trihydrochloride (4.07 GBq/mmol) and [^{14}C]putrescine dihydrochloride (4.07 GBq/mmol) were purchased from Amersham (Amersham, UK). Spermine tetrahydrochloride, spermidine trihydrochloride, putrescine dihydrochloride and valinomycin were obtained from Sigma (St. Louis, MO, USA). All other chemicals were of the highest grade available and used without further purification.

Isolation of the brush-border membrane from rat small intestine. Adult male Wistar rats, 180–220 g, were used in this study. Entire small intestine was excised under anesthesia (sodium pentobarbitone; 30 mg/kg body weight, i.p.). Brush-border membrane vesicles (BBMVs) were isolated using the calcium precipitation method of Kessler et al. [13] as described previously [10].

Uptake experiments. Uptake of ^{14}C-labelled polyamines was performed using the Millipore filtration technique as described previously [10]. In a routine assay, 20 μl of membrane suspension were added to 100 μl of incubation medium kept at 37°C. The composition of the incubation media are described in the legends of the figures. At selected time intervals, the uptake was stopped by diluting the incubation media with 2 ml of ice-cold 10 mM Hepes-Tris buffer (pH 7.5) containing 150 mM KCl. The mixture was immediately filtered through a Millipore filter (HAWP, 0.45 μm, 2.5 cm diameter). The filter was rinsed with 3 ml of the same buffer, and processed by counting the radioactivity. All experiments presented in this paper were repeated at least twice and were always performed in triplicate. As blanks, membrane-free incubation media were handled in an identical manner.

Analytical methods. The radioactivity of [^{14}C]spermine, [^{14}C]spermidine and [^{14}C]putrescine trapped on the filter were measured by standard liquid scintillation technique. Protein was determined by the method of Lowry et al. [14] using bovine serum albumin as a standard. Statistical analysis was performed using Student's t-test and a $P < 0.05$ was considered significant.

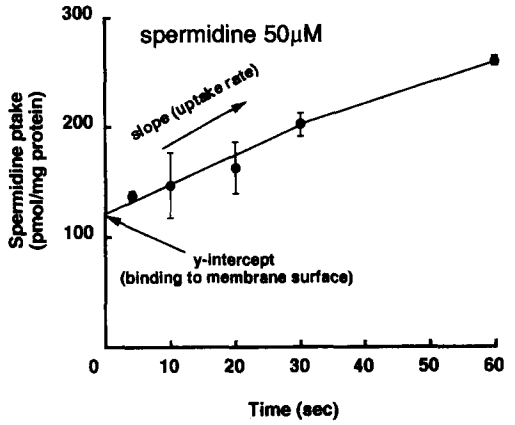

Fig. 1. Time-course of initial [^{14}C]spermidine uptake by rat intestinal brush-border membrane vesicles. Membrane vesicles (20 μl) were suspended in 100 mM KCl, 100 mM D-mannitol and 20 mM Hepes-Tris buffer (pH 7.5). Uptake studies were performed by adding incubation medium (100 μl) containing 20 mM Hepes-Tris buffer (pH 7.5), 60 μM [^{14}C]spermidine, 100 mM D-mannitol and 100 mM KCl. Each point represents as the mean ±S.D. of four determinations.

Results

Determination of the initial uptake rate by rat intestinal BBMVs

The initial (0 − 1 min) time-course of spermidine uptake in rat intestinal BBMVs (50 μM) is shown in Fig. 1. The uptake behavior was nearly linear until 30 s. The linearity of initial uptake behavior until 30 s is also proven in the other polyamine-uptake study (data not

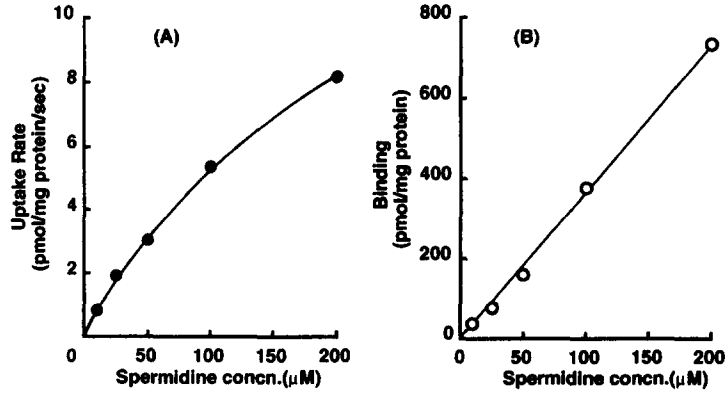

Fig. 2. Concentration dependence of the uptake rate (panel A) and binding to the membrane surface (panel B) for [^{14}C]spermidine by the intestinal brush-border membrane vesicles. Membrane vesicles (20 μl) were suspended in 100 mM KCl, 100 mM D-mannitol and 20 mM Hepes-Tris buffer (pH 7.5). Uptake studies were performed by adding an incubation medium (100 μl) containing 20 mM Hepes-Tris buffer (pH 7.5), 100 mM D-mannitol, 100 mM KCl and various concentrations of [^{14}C]spermidine. Each value of uptake rate or binding to the membrane surface was calculated from three time points (until 30 s) of three different experiments using a linear regression program to fit data.

shown). The initial uptake rate (pmol/mg protein per s) and the value of binding to the membrane surface (pmol/mg protein) can be obtained from the slope and y-intercept of this linear time-course of initial uptake, respectively. Thus, at least three points of the initial uptake (10–30 s) were used in this study to determine the uptake rate and the binding by the membrane vesicles.

Concentration dependence of the uptake rate and the binding of polyamines by BBMVs

Fig. 2 illustrates the effect of the spermidine concentration (up to 200 μM) on the uptake rate and the binding by BBMVs. The initial uptake rate was obviously saturable, however, the value of binding to the membrane surface was approximately linear under the conditions in these concentration ranges. As shown in Fig. 3, Lineweaver-Burk transformation of the corrected data from the uptake rate resulted in a value of 148.1 μM and 13.11 pmol/mg protein per s for K_m and V_{max}, respectively.

The uptake rate of spermine also exhibited saturation kinetics, K_m and V_{max} values were 30.4 μM and 22.53 pmol/mg protein per s, respectively (Fig. 4). On the other hand, the value of binding to membrane surface also did not saturate up to 80 μM (data not shown).

On the other hand, neither the binding nor the uptake rate of putrescine was saturable until 8 mM (Fig. 5).

Kinetic analysis for the inhibitory effect of spermine and putrescine on the spermidine uptake rate

In order to investigate whether these polyamines share an identical uptake system in the intestinal BBMVs or not, the effects of spermine and putrescine on the uptake rate of spermidine were examined. In Fig. 6, Dixon plot analysis demonstrated that spermine competitively inhibited the uptake rate of spermidine with a K_i value of 33.8 μM, nearly the same as the K_m value for spermine (30.4 μM). In contrast, the inhibition of putrescine on the spermidine uptake rate was non-competitive (Fig. 7).

These results suggested that there were two different Na^+-independent uptake systems for spermine and spermidine, as well as for putrescine on the intestinal BBMVs. Kumagai et al. mentioned that the uptake mechanism of putrescine in rat isolated enterocytes was different from that of spermine and spermidine

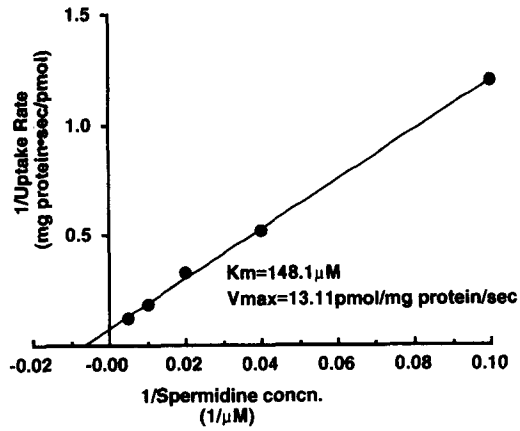

Fig. 3. Lineweaver-Burk plot of [^{14}C]spermidine uptake rate by the intestinal brush-border membrane vesicles. Data are corrected for Fig. 2A. A linear regression program has been used to fit data, the intercepts of line with the x- and y-axis are $-1/K_m$ (K_m = 148.1 μM) and $1/V_{max}$ (V_{max} = 13.11 pmol/mg protein/s).

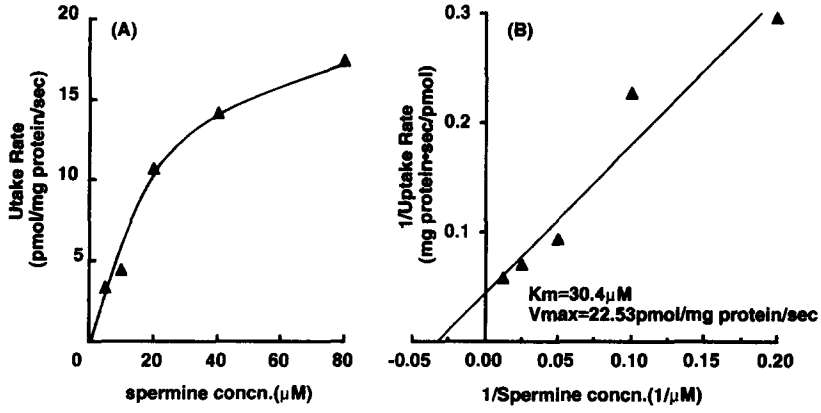

Fig. 4. Concentration dependence of the [^{14}C]spermine uptake rate (panel A) and Lineweaver-Burk plot of the uptake rate (panel B) by the intestinal brush-border membrane vesicles. Membrane vesicles (20 μl) were suspended in 100 mM KCl, 100 mM D-mannitol and 20 mM Hepes-Tris buffer (pH 7.5). Uptake studies were performed by adding an incubation medium (100 μl) containing 20 mM Hepes-Tris buffer (pH 7.5), 100 mM D-mannitol, 100 mM KCl and various concentrations of [^{14}C]spermine. Each value of uptake rate or binding to the membrane surface was calculated from three time points (until 30 s) of three different experiments using a linear regression program to fit the data. In panel B, a linear regression program has been used to fit data corrected for panel A. The intercepts of the line with the x- and y-axis are $-1/K_m$ (K_m = 30.4 μM) and $1/V_{max}$ (V_{max} = 22.53 pmol/mg protein/s).

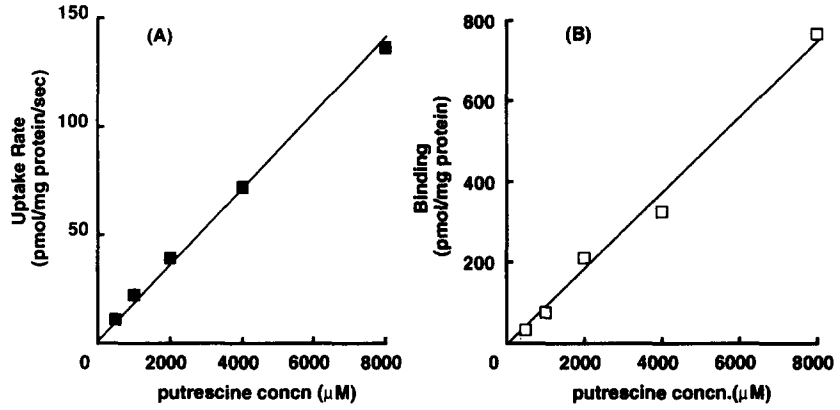

Fig. 5. Concentration dependence of [^{14}C]putrescine uptake rate (panel A) and binding to the membrane surface (panel B) by the intestinal brush-border membrane vesicles. Membrane vesicles (20 μl) were suspended in 100 mM KCl, 100 mM D-mannitol and 20 mM Hepes-Tris buffer (pH 7.5). Uptake studies were performed by adding an incubation medium (100 μl) containing 20 mM Hepes-Tris buffer (pH 7.5), 100 mM D-mannitol, 100 mM KCl and various concentrations of [^{14}C]putrescine. Each value of uptake rate or binding to the membrane surface was calculated from three time points (until 30 s) of three different experiments using a linear regression program to fit data.

[15]. However, the question remains as to the existence of these uptake systems and where they are located, in the apical or basal side. The present results agreed with the reports of Kumagai et al., and moreover, demonstrated that such uptake systems were located in the apical membrane of enterocyte.

Effect of a valinomycin-induced K^+-diffusion potential (inside negative) on the uptake of polyamines by BBMVs

Fig. 8 shows that there was no effect by a valinomycin-induced K^+-diffusion potential (inside negative) on the uptake of spermidine and putrescine. On the other hand, in our previous report [16], the uptake of propantheline, a cationic drug, was stimulated by the inside-negative K^+-diffusion potential after preloading of this drug for 10 min on rat intestinal BBMVs. Nevertheless, as shown in Fig. 9, there was also no effect indicated on polyamine uptake by the same assay.

Trans-stimulation effect of spermine on the uptake of spermidine by BBMVs

To confirm whether polyamine uptake into the intravesicular space participates in a carrier-mediated

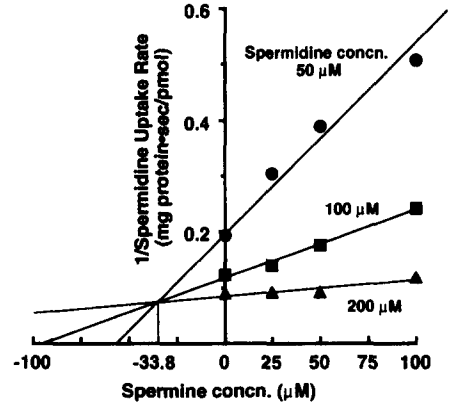

Fig. 6. Inhibition of [^{14}C]spermidine uptake rate by various concentrations of spermine into the intestinal brush-border membrane vesicles. Membrane vesicles (20 μl) were suspended in 100 mM KCl, 100 mM D-mannitol and 20 mM Hepes-Tris buffer (pH 7.5). Uptake studies were performed by adding an incubation medium (100 μl) containing 20 mM Hepes-Tris buffer (pH 7.5), 100 mM D-mannitol, 100 mM KCl, various concentrations of spermine and 60 μM (●), 120 μM (■) and 240 μM (▲) of [^{14}C]spermidine (final concentrations of spermidine are indicated in the figure). Each value of uptake rate or binding to the membrane surface was calculated from three time points (until 30 s) of three different experiments using a linear regression program to fit data. The data are presented as a Dixon plot.

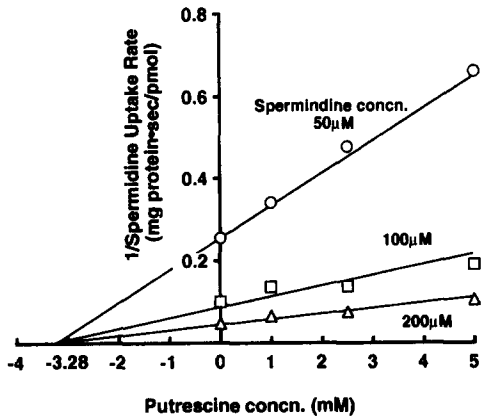

Fig. 7. Inhibition of the [^{14}C]spermidine uptake rate by various concentrations of putrescine into the brush-border membrane vesicles. Membrane vesicles (20 μl) were suspended in 100 mM KCl, 100 mM D-mannitol and 20 mM Hepes-Tris buffer (pH 7.5). Uptake studies were performed by adding an incubation medium (100 μl) containing 20 mM Hepes-Tris buffer (pH 7.5), 100 mM D-mannitol, 100 mM KCl, various concentrations of putrescine and 60 μM (○), 120 μM (□) and 240 μM (△) of [^{14}C]spermidine (final concentrations of spermidine are indicated in the figure). Each value of uptake rate or binding to the membrane surface was calculated from 3 time points (until 30 s) of 3 different experiments using a linear regression program to fit data. The data are presented as a Dixon plot.

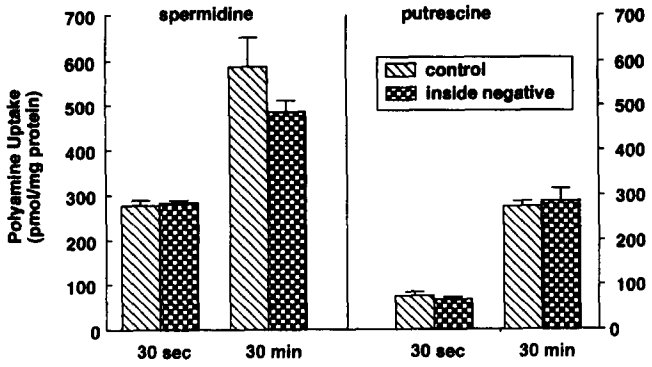

Fig. 8. Effect of a valinomycin-induced potassium diffusion potential on the uptake of [^{14}C]spermidine and [^{14}C]putrescine by the intestinal brush-border membrane vesicles. Membrane vesicles (20 μl) were suspended in 100 mM potassium gluconate, 100 mM D-mannitol, 7 μg/mg protein of valinomycin and 20 mM Hepes-Tris buffer (pH 7.5). Uptake studies were performed by adding an incubation medium (100 μl) containing 20 mM Hepes-Tris buffer (pH 7.5), 100 mM D-mannitol, 60 μM [^{14}C]spermine or [^{14}C]putrescine and either 100 mM potassium gluconate (control) or 100 mM sodium gluconate (inside negative). Each column represent as the mean ± S.D. of three determinations.

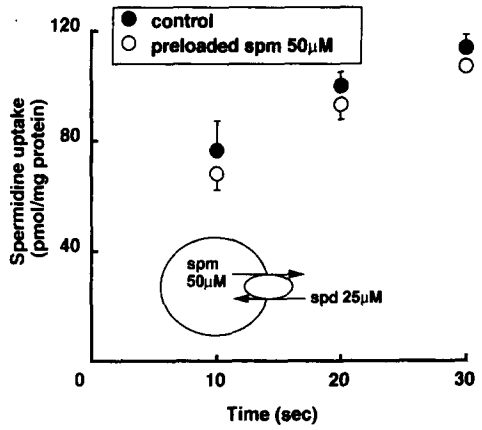

Fig. 10. Effect of preloading of spermine (50 μM) on [^{14}C]spermidine (25 μM) uptake by the intestinal brush-border membrane vesicles. Membrane vesicles (20 μl) were suspended in 100 mM KCl, 100 mM D-mannitol, 20 mM Hepes-Tris buffer (pH 7.5) and with (○) or without (●) of 50 μM spermine for 30 min. Thereafter, samples were added to 200 μl of 20 mM Hepes-Tris buffer (pH 7.5), 100 mM KCl, 100 mM D-mannitol, 27.5 μM [^{14}C]spermidine, with (●) or without (○) of 4.5 μM spermine. Each point represents the mean ± S.D. of three determinations.

uptake mechanism or not, trans-stimulation studies of spermidine with spermine were carried out. In general, the trans-stimulation effect is considered to be strong evidence for a carrier-mediated uptake system [17]. If spermidine uptake is increased in the presence of intravesicular spermine, this further confirms that the uptake of polyamines is carrier-mediated. However, as is evident in Fig. 10, intravesicular spermine had no effect on the spermidine uptake at an early time point.

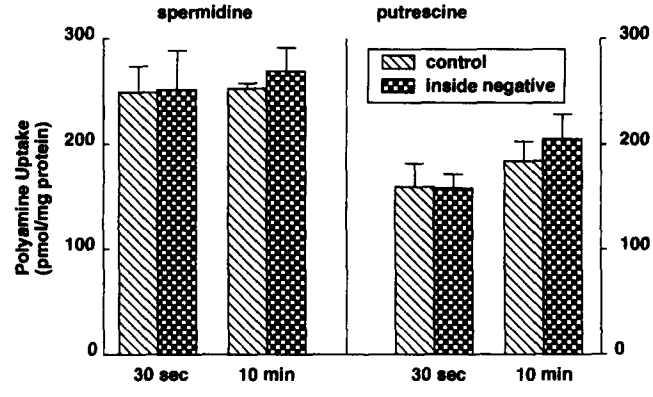

Fig. 9. Effect of valinomycin-induced potassium diffusion potential on the uptake of [^{14}C]spermidine and [^{14}C]putrescine after saturation of the binding by the intestinal brush-border membrane vesicles. Membrane vesicles (20 μl) were suspended in 100 mM potassium gluconate, 100 mM D-mannitol, 7 μg/mg protein of valinomycin and 20 mM Hepes-Tris buffer (pH 7.5). Membrane vesicles were preincubated with the same buffer containing 75 μM [^{14}C]spermidine or [^{14}C]putrescine for 10 min. Thereafter, samples were added to 60 μl of 20 mM Hepes-Tris buffer (pH 7.5), 100 mM D-mannitol, 50 μM [^{14}C]spermidine or [^{14}C]putrescine and either 100 mM potassium gluconate (control) or 100 mM sodium gluconate (inside negative). Each column represents the mean ± S.D. of 4–6 determinations.

Discussion

We have previously studied the uptake characteristics of polyamines into rat intestinal brush-border membrane [10,11]. This membrane, isolated from the apical side of rat enterocytes, was not found to be the Na^+-dependent transporter for polyamines. In the present investigation, as shown in Fig. 1, the initial uptake values of polyamines include the uptake into the vesicles and the binding to the outer membrane surface. Therefore, in the kinetic analysis, the slope of linear regression line obtained from at least three time points (10–30 s) was used to determine the real uptake rate. The present results suggest the existence of plural transmembrane pathways for these polyamines in rat intestinal BBMVs, due to the fact that the inhibitory fashions on the spermidine uptake were different between spermine (competitive) and putrescine (non-competitive). Furthermore, the affinity of putrescine for this uptake system seemed to be lower than those of the other two polyamines. In contrast, the concentration dependence of all the polyamines binding to outer membrane surfaces did not exhibit saturation kinetics in the concentration range studied. These results of intravesicular uptake and binding to membrane surfaces agreed with the investigation results of human glioblastoma cell line U-251 by Quemener et al. [12].

In this study, intravesicular spermine exhibited no trans-stimulation effect on the uptake of spermidine by

BBMVs, despite the fact that both compounds inflicted mutual inhibitory effects on the intravesicular uptake in a manner competitive with each other. It could be considered that polyamine uptake systems were 'change diffusion', a kind of facilitated diffusion [18]. However, many carrier-protein-mediated uptake systems were reported to exhibit a phenomenon of trans-stimulation [19–21]. Furthermore, recently, the criteria for the existence of a carrier such as temperature dependence, mutually-competitive inhibition and concentration dependence were observed in phospholipid bilayer transport [22]. Therefore, the polyamines were considered to be taken up via a passive diffusion mechanism rather than the special facilitated diffusion (carrier-mediated systems).

The valinomycin-induced K^+-diffusion potential (inside negative) did not affect the polyamine intravesicular uptake. In our previous studies, the initial uptake of several monocationic compounds (e.g., tryptamine and disopyramide) was stimulated by a valinomycin-induced K^+-diffusion potential (inside negative) [23,24]. Moreover, we confirm that the uptake of amphiphilic organic cations, such as propantheline and chlorpromazine, was facilitated by the transmembrane electrical potential (inside negative) after the uptake of drugs had reached a steady state by incubating the vesicles for 10 min with each drug [16]. However, there was no effect of the ionic diffusion potential on polyamine uptake under both conditions. Lipsky et al. reported that the energy-independent uptake into rabbit renal brush-border membrane vesicles of gentamicin, a polycationic antibacterial agent, was not affected by valinomycin generated K^+-diffusion potential [25]. These results suggested that these polycationic compounds have different transmembrane pathway systems from those for monocations and amphiphilic amine drugs.

In a previous report, upon speculation on the polyamine uptake mechanisms, we mentioned that the binding of these polyamines to the membrane lipid (such as acidic phospholipids) is related to the uptake process by the intestinal BBMVs [11]. However, real uptake rates of these polyamines into intravesicular space were not dependent upon the initial binding to the outer membrane surface in this study (Fig. 2). It is well-known that the acidic phospholipids such as phosphatidylserine, which strongly bind with polyamines [26], are localized in the inner layer of the BBMVs [27]. Therefore, it seems that the interaction between polyamine and acidic phospholipids at the inner side of the membrane bilayer can play an important role as a driving force of the polyamine uptake mechanism.

Polyvalent cations in the polyamine molecule are connected with the propyl and/or butyl chain and can move freely. It was reported that polyamine made a bridging with plural acidic phospholipid head groups [26]. Therefore, it is considered that the asymmetric localization of acidic phospholipids on lipid bilayer can cause the different behavior between the binding and the real uptake of these polyamines, although more detailed examination is necessary. In fact, the binding value of polyamines to various composition liposomes was dependent on the number of cationic amines and amount of phosphatidylserine although the percent of which were not always proportional to the binding values [11]. This speculation can explain the differences of uptake behavior between polyamines and monocationic compounds concerning the effect of ionic diffusion potential. In conclusion, the specific uptake system in question could be due to this unique molecular structure and binding manner of polyamine. The contribution of spermine and spermidine to these uptake systems was more effective than that of putrescine, because of their higher affinity.

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