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Uptake characteristics of polyamines into rat intestinal brush-border membrane

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The uptake characteristics of polyamines, such as spermine, spermidine and putrescine, have been investigated using brush-border membrane vesicles isolated from the small intestine of rats. The uptake of these polyamines into the membrane vesicles was high and the order of uptake was spermine > spermidine > putrescine at medium pH 7.5, respectively. The medium pH considerably affected the uptake of these polyamines and the amount of uptake increased remarkably with an increase of the medium pH (pH 7.5 or 8.0 > pH 5.5). An inward Na^+ gradient did not stimulate the uptake rate of any of these polyamines. We have also examined the binding behaviour to the membrane lipid, phospholipids and total lipid, and there was a good correlation in the binding properties, pH-dependency and uptake activity, between the liposomes and brush border membrane vesicles. These results suggest that the uptake of the polyamine into the vesicles consisted of rapid binding to the outside intestinal surface and slower binding to the inside membrane after permeation. Furthermore, findings from experiments concerning the mutual inhibition among these polyamines and concerning the effect of other polycations, having 2–5 amines in number, on the uptake of spermine, suggest that the number of amino groups in the polyamine molecules plays an important role in the uptake process into the brush-border membrane vesicles.

Introduction

Putrescine, spermidine and spermine are aliphatic polyamines having two, three and four amine groups in their molecules, respectively. It is well known that these polyamines are found in a wide range of mammalian cells and play an important role in controlling cell growth as well as the differentiation process [1,2]. Many investigations concerning the uptake or transport characteristics of these polyamines have been held using various cells such as enterocytes [3,4], Chinese hamster ovary cell lines [5] and tumor cell lines [6]. However, the detailed transport characteristics across the plasma membrane isolated from the cell are unclear. Moreover, despite the common presence of polyamine in the body and the constant exposure of the intestinal mucosa to exogenous polyamines in the lumen, little information on the intestinal absorption mechanism of these polyamines can be found in reference literature.

We reported previously [11] that there exists a marked uptake of spermine into the brush-border membrane vesicles isolated from rat small intestine and that the uptake of spermine was not energized by an inward Na^+ gradient. Speculatively, we mentioned previously [11] that spermine binding to the inside membrane might be relevant to this spermine accumulation by contributing to the transmembrane transport. In the present study, to further clarify the transport characteristics of spermine, spermidine and putrescine, we have investigated the effect of Na^+ gradient, extravesicular medium pH and various other polycations, having 2–5 amines in number, on the uptake as well as the binding process of these polyamines by the brush-border membrane vesicles. To evaluate the role of phospholipids in the binding to brush-border membrane, we have also studied binding of these polyamines, especially spermine, to the liposomes derived from phospholipids or total lipid extracted from the brush-border membrane.

Materials and Methods

Chemicals. [^{14}C]Spermine tetrahydrochloride (4.1 GBq/mmol), [^{14}C]spermidine trihydrochloride (4.1 GBq/mmol) and [^{14}C]putrescine dihydrochloride (4.07

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GBq/mmol) were purchased from Amersham International plc (Buckinghamshire, UK). Gentamicin sulfate was kindly donated from Shionogi & Co. (Osaka, Japan). Cadaverine dihydrochloride, spermine tetrahydrochloride, spermidine trihydrochloride, putrescine dihydrochloride, DL-α-phosphatidylcholine dipalmitoyl, L-α-phosphatidic acid and DL-α-phosphatidyl-L-serine dipalmitoyl were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Tetraethylenepentamine was purchased from Wako Pure Chemical Industry Ltd (Osaka, Japan), and triethylenetetramine was used after purification in accordance with Walshe and coworkers [7] as described in our previous paper [8] because of its low grade of purity. 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. Entire small intestine was excised under anesthesia with pentobarbitone sodium (30 mg/kg body weight, i.p.). Brush-border membrane vesicles were isolated using the calcium chloride precipitation method of Kessler et al. [9] as described previously [10].

Uptake experiments. Uptakes of [^{14}C]spermine, [^{14}C]spermidine and [^{14}C]putrescine were carried out by a Millipore filtration technique as described previously [11]. In a regular assay, 20 μl of membrane suspension (125–180 μg protein) were added to 100 μl of incubation medium kept at 37°C. The composition of the incubation media are described in the legends of figures and tables. At selected time intervals, the uptake was stopped by diluting the incubation media with 2 ml of ice-cold 150 mM KCl and with either 10 mM Hepes-Tris buffer (pH 7.5) or 10 mM Mes-Tris buffer (pH 5.5). The mixture was immediately filtered through a Millipore filter (HAWP, 0.45 μm, 2.5 cm diameter, Millipore). The filter was rinsed with 3 ml of the same buffer, and processed for counting the radioactivity.

All experiments presented in this paper were repeated at least two times and were always performed in triplicate. Using different membrane preparations, reproducible results were obtained for the same experiments. As blanks, The values for nonspecific retention of radioactivity on the filters (less than 0.33% of the total radioactivity in the incubation medium) were subtracted from the values of the incubation mixture.

Binding to lipid membrane by equilibrium dialysis. Liposomes derived from phospholipids (phosphatidylcholine, phosphatidic acid and phosphatidylserine; 3.3 μmol of total phospholipids) and from total lipid of brush-border membrane were prepared under nitrogen by sonication in a buffer solution (100 mM KCl, 20 mM Hepes-Tris (pH 7.5) or 100 mM KCl, 20 mM Mes-Tris (pH 5.5)) for 30 min at 0°C according to the method of Utsumi et al. [12]. Total lipid of brush-border membrane was extracted by the method of Folch et al. [13] and the phospholipid concentration was adjusted to the same level as liposomes derived from phospholipids.

The binding studies were performed at 37°C in cells each of which consisted of two half cells (volume 2.08 ml; membrane surface area 4.15 cm^2) separated by a membrane (Spectrapor 6, MWCO 8000) according to the technique of Iseki et al. [14]. A mixed solution (1 ml) of an equal volume of buffer and each liposome suspension (0.55 μmol phospholipids/ml) were added into one side cell and 1 ml of substrate (5 nmol/ml) in the same volume of buffer was added to the other.

In preliminary experiments, the times required for polyamines dialyzed against buffer to reach equilibrium were determined, and in all cases equilibrium was reached in 8 h. At the end of equilibrium dialysis experiments (At 8 h), samples were removed from both cells, and used for the determination of the concentration of polyamines.

Analytical methods. The radioactivity of [^{14}C]spermine, [^{14}C]spermidine and [^{14}C]putrescine trapped on the filters (0.45 μm, HAWP, 25 mm diameter) were measured by standard liquid scintillation technique. Phospholipid was determined by the method of Bartlett et al. [15]. Protein was determined by the method of Lowry et al. [16] using bovine serum albumin as a standard.

The statistical significance was evaluated by Student's *t*-test.

Results

Uptake of spermidine and putrescine as a function of time

The uptakes of spermine and putrescine (50 μM) in the vesicles as a function of time are depicted in Fig. 1. Both these polyamines accumulated in the vesicles with time and reached equilibrium within 60 min. Linear regression of the first four time points (until 1 min) indicated that the rate of uptake was linear in this portion of the curve (Fig. 1A, B; inset), therefore, the uptake rate at 1 min was used in subsequent studies to approximate the initial rate. Extrapolation of the regression line to time zero in Fig. 1 yields values, which reflect the initial adsorption of these polyamines to the membrane, of approx. 20% of the equilibrium values. The uptake of these polyamines depended upon the medium pH and at the medium pH of 7.5 a considerable accumulation of these polyamines into the membrane vesicles was observed. These findings agreed well with the previous results obtained for the time course of spermine uptake [11]. Furthermore, the initial bindings of spermidine and putrescine, which was obtained by extrapolation of the regression line to time zero, depended upon the pH of the medium, and the transport rate was independent from the pH of the

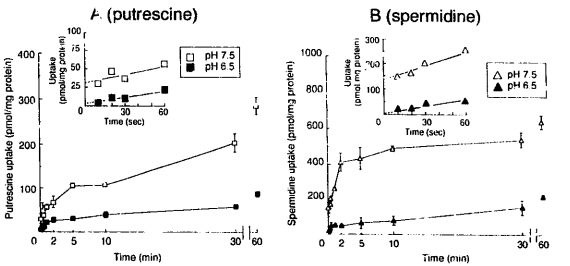

Fig. 1. Time course of spermidine and putrescine uptake (A, putrescine; B, spermidine) by rat intestinal brush-border membrane vesicles. Membrane vesicles were suspended in 100 mM KCl, 100 mM D-mannitol and either 20 mM Hepes-Tris buffer (pH 7.5) (■, ▲) or 20 mM Mes-Tris buffer (pH 6.5) (□, △). 20 μl of these membrane suspensions were added to 100 μl of incubation medium containing 60 μM [^{14}C]polyamines, 100 mM KCl, 100 mM D-mannitol, and either 20 mM Hepes-Tris buffer (pH 7.5) (■, ▲) or 20 mM Mes-Tris buffer (pH 6.5) (□, △). Each point represents the mean ± S.D. of six determinations.

medium. Thus, it seems that these pH-dependent bindings to the membrane vesicles were linked to the uptake behavior of polyamines.

In order to investigate the contribution of the Na^+-dependent carrier-mediated transport system to the uptake of spermidine and putrescine (50 μM), we compared the uptake rate of these polyamines in the presence of Na^+ or K^+ in the extravesicular medium. As shown in Fig. 2, the Na^+ electrochemical gradient did not stimulate the initial uptake of spermidine and putrescine as was the case with spermine [11].

Effect of the medium pH on the uptake of polyamines by the brush-border membrane vesicles

Fig. 3 indicates the effect of various medium pH values on the uptake of spermine, spermidine and putrescine. In all polyamines, the distinct pH dependency and similar medium pH-uptake profiles were observed in the initial and the slower uptake states. The present results are consistent with the results of Bitonti et al. [17] in that the uptake of bis(benzyl)polyamine derivative into erythrocytes was dependent upon the medium pH (pH 7.5 > 6.5).

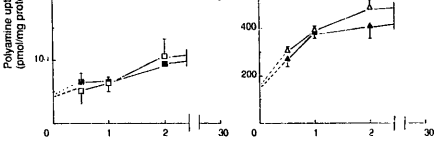

Fig.2. Uptake of polyamines by the intestinal brush-border membrane vesicles in the presence of an inward Na^+ gradient (A, putrescine; B, spermidine). The membrane vesicles (20 μl) were preloaded in 100 mM KCl, 100 mM D-mannitol and 20 mM Hepes-Tris buffer (pH 7.5). Transport studies were performed by adding an incubation medium (100 μl) containing 20 mM Hepes-Tris buffer (pH 7.5), 60 μM [^{14}C]polyamines, 100 mM D-mannitol and either 100 mM NaCl (□, △) or 100 mM KCl (■, ▲). Each point represents the mean ± S.D. of five or six determinations.

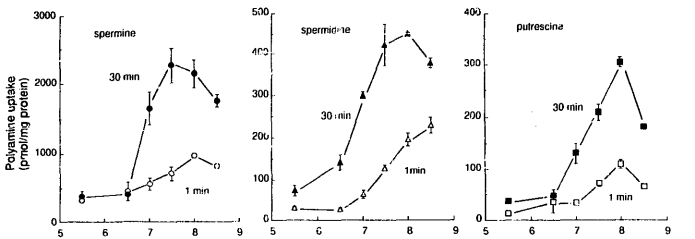

Fig. 3. Effect of medium pH on the uptake of polyamine by intestinal brush-border membrane vesicles. Uptake studies were performed in the medium containing 50 μM ^{14}C-labeled polyamine, 100 mM KCl, 100 mM D-mannitol, and either 20 mM Hepes-Tris buffer (pH 7.0–8.5) or 20 mM Mes-Tris buffer (pH 5.5–6.5). The incubation time was 1 min (○, □, △) or 30 min (●, ■, ▲). Each point represents the mean ± S.D. of three or four determinations.

Binding of polyamines to liposome

The binding behaviour of spermine to liposomes is shown in Fig. 4. The spermine binding to liposomes derived from phospholipids clearly amplified with the increasing amounts of phosphatidylserine, a negatively charged phospholipid [18,19]. The distinct differences of the binding of spermine between pH 7.5 medium and pH 5.5 medium were also observed in both liposome preparations, liposomes derived from phospholipid and from total lipid which had been extracted from brush-border membrane. Moreover, there were also differences noted in the spermine, spermidine and putrescine binding activity.

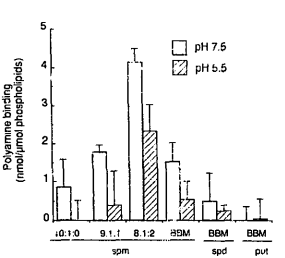

Fig. 4. The binding behaviour of polyamine to liposomes derived from phospholipids or total lipid extracted from intestinal brush-border membranes of rat (spm, spermine; spd, spermidine; put, putrescine). Columns represent mean values with vertical bars showing S.D. and $N \geq 4$ for each group. Binding amounts were determined by the equilibrium dialysis method in the medium containing 100 mM KCl, 100 mM D-mannitol and either 20 mM Hepes-Tris buffer (pH 7.5) or 20 mM Mes-Tris buffer (pH 5.5).

Mutual inhibition among polyamines and the effect of other polycations on spermine uptake

It has been reported that there existed a common transporter system in the uptake of spermine, spermidine and putrescine into the renal cell line [20,21] and the human colon carcinoma cell line [22]. In order to examine whether a common uptake system for these polyamines also exists in the intestinal brush-border membrane, studies were conducted (Fig. 5) concerning mutual inhibition among spermine, spermidine and putrescine (Table I) and the effects of other polycations, structurally analogous polyamines. As shown in Table I, significant mutual inhibition was exhibited

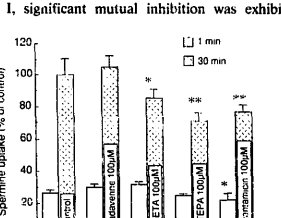

Fig. 5. Inhibitory effect of various polycations (100 μM) on the uptake of spermine (10 μM) into the brush-border membrane vesicles. The vesicles were assayed in a medium containing [^{14}C]spermine, 100 mM D-mannitol, 100 mM KCl, 20 mM Hepes-Tris buffer (pH 7.5), and 100 μM polycationic compound. The incubation time was 1 min or 30 min. Each column represents the mean ± S.D. from measurements on three different membrane preparations.

TABLE I

Mutual inhibitory effect on the uptake of polyamines into the intestinal brush-border membrane vesicles

Uptake studies were performed in the medium containing 100 mM KCl, 100 mM D-mannitol, 20 mM Hepes-Tris buffer (pH 7.5), 10 μM ^{14}C-labeled polyamine and 0–500 μM inhibitors. Values represent the mean ± S.D. from measurements on three different membrane preparations.

Drug (10 μM)	Inhibitor	Uptake in pmol/mg protein (% of control)	
		1 min	30 min
Putrescine	none	57.6 ± 10.6 (100.0)	272.9 ± 1.3 (100.0)
	put 100 μM	61.3 ± 13.5 (106.4)	150.3 ± 11.0 (55.1) [c]
	put 500 μM	57.8 ± 16.7 (100.3)	106.8 ± 24.5 (39.1) [c]
	spd 100 μM	49.6 ± 4.4 (86.1)	240.9 ± 18.2 (88.3) [b]
	spd 500 μM	50.7 ± 2.9 (88.0)	219.1 ± 12.7 (80.3) [b]
	spm 10 μM	51.5 ± 6.3 (89.4)	257.1 ± 13.2 (94.2)
	spm 100 μM	58.0 ± 20.0 (100.7)	269.7 ± 16.8 (98.8)
Spermidine	none	210.5 ± 29.6 (100.0)	574.2 ± 8.6 (100.0)
	put 100 μM	194.9 ± 0.6 (92.6)	576.1 ± 16.2 (100.3)
	put 500 μM	187.6 ± 39.1 (89.1)	497.5 ± 29.6 (86.6) [b]
	spd 100 μM	129.9 ± 24.6 (61.7) [b]	443.3 ± 57.8 (77.2) [b]
	spd 500 μM	76.3 ± 8.3 (36.2) [c]	273.1 ± 6.5 (47.6) [c]
	spm 10 μM	156.6 ± 13.9 (74.4) [a]	675.3 ± 90.7 (117.6)
	spm 100 μM	59.3 ± 15.5 (28.2) [c]	358.4 ± 79.5 (62.4) [b]
Spermine	none	156.8 ± 10.5 (100.0)	585.1 ± 60.2 (100.0)
	put 100 μM	201.9 ± 25.2 (128.7)	591.5 ± 26.3 (101.1)
	spd 100 μM	190.2 ± 16.4 (121.3)	529.5 ± 26.9 (90.5)
	spd 500 μM	113.5 ± 15.8 (72.4) [b]	304.8 ± 55.0 (52.1) [c]
	spm 100 μM	141.0 ± 4.1 (89.9)	446.4 ± 37.4 (76.3) [c]

[a] Significantly different from control. $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$.

between spermine and spermidine, while mutual inhibition between putrescine and other two polyamines was not observed.

Fig. 5 shows the effect of several polycations on the spermine uptake. No significant inhibition on spermine uptake was observed in the presence of cadaverine, a dication. On the contrary, gentamicin (trication), triethylenetetramine (tetracation), and tetraethylenepentamine (pentacation) inhibited the uptake of spermine significantly.

Effect of medium pH on the reversibility of spermine uptake

The reversibility of spermine uptake was demonstrated by the experiments illustrated in Fig. 6. Brush-border membranes were incubated with 50 μM spermine at pH 7.5. After 30 min of uptake, the medium pH was changed to 5.5 by adding the buffer (pH 5.5) containing the same concentration of spermine (50 μM). This resulted in nearly a 70% decrease in the spermine associated with the membrane vesicles compared with the case of pH 7.5. When the membrane vesicles were incubated with 50 μM spermine at pH 5.5 without changing the medium pH, uptake of the spermine was as low as that at pH 6.5 described in the previous paper [11].

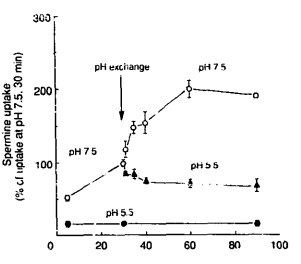

Fig. 6. The effect of exchange of the medium pH in reversing and inhibiting the uptake of spermine by intestinal brush-border membrane vesicles. The membrane vesicles were suspended in 100 mM KCl, 100 mM D-mannitol and either 20 mM Hepes-Tris buffer (pH 7.5) (○, ▲) or 20 mM Mes-Tris buffer (pH 5.5) (●). The vesicles (20 μl) were incubated with 20 μl of 20 mM Hepes-Tris buffer (pH 7.5) (○, △) or 20 mM Mes-Tris buffer (pH 5.5) (●) containing 100 mM KCl, 100 mM D-mannitol and 100 μM spermine at 37°C. After 30 min incubation, 2 ml of the medium containing 50 μM spermine, 100 mM mannitol, 60 mM KCl and either 20 mM Hepes-Tris buffer (pH7.5) (○) or 20 mM Mes-Tris buffer (pH 5.5) (▲) was added to incubation medium (indicated by an arrow). Each point represents the mean ± S.D. of six determinations.

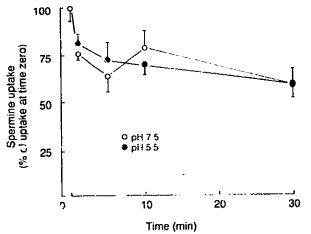

Fig. 7. Release profile of spermine from the preloaded brush-border membrane vesicles in the medium containing spermine (50 μM). Membrane vesicles were preloaded in 50 μM spermine, 100 mM KCl, 100 mM D-mannitol and 20 mM Hepes-Tris buffer (pH 7.5) for 30 min at 37°C. The release studies were initiated by dilution (50-fold) using the medium containing 100 mM KCl, 100 mM D-mannitol and either 20 mM Hepes-Tris buffer (pH 7.5) (○) or 20 mM Mes-Tris buffer (pH 5.5) (●).

Furthermore, as shown in Fig. 7, it was found that there was no difference in the release rate of spermine between the medium pH of 5.5 and that of 7.5 when preloaded vesicles were diluted 50-fold with each buffer. Spermine, which bound to the outside membrane, was rapidly released at the initial time point (1 min). Subsequently, spermine was released slowly from the membrane vesicles.

Discussion

It is essential to examine the polyamine transport characteristics using an isolated plasma membrane in so much as there remains a controversy as to whether or not the brush-border or basolateral membrane plays a key role in the transport system using whole intestinal cells; spermidine and putrescine in isolated enterocyte [3,4], putrescine in the basal side of colon adenocarcinoma cells (LoVo) [23], spermine, spermidine and putrescine in the apical side of human colon carcinoma cells (CaCo-2) [22]. We reported previously [11] that spermine was associated with the rat intestinal apical membrane by binding related to permeation and that high uptake of spermine was not due to a Na^+-dependent transport system.

In the present study, it was found that the uptake of both spermidine and putrescine were not stimulated by Na^+ gradient in the same vesicle preparations. These previous and present findings provided evidence that a Na^+-dependent transport system did not participate in the uptake process of these polyamines into the brush-border membrane vesicles of rat small intestine. Furthermore, we have observed that an inside-negative transmembrane electrical potential induced by valinomycin, a potassium ionophore, did not behave as a driving-force of spermine uptake by the brush-border membrane (unpublished data). In order to investigate the reasons for the high uptake values of these polyamines to the membrane vesicles, the binding behaviour to the lipid membrane (liposome) was examined. The spermine binding to the liposome was clearly amplified with the increasing amount of phosphatidylserine, approx. 18%. The distinct differences of the binding of spermine between the medium pH 7.5 and 5.5 were also observed in both liposome preparations, liposomes derived from phospholipids and from total lipid obtained from brush-border membrane. Since the content of phosphatidylserine was approx. 12% of the total phospholipid of the brush-border membrane in rat small intestine [24], it was reasonable to assume that the binding of spermine to the total lipid liposome from brush-border membrane was similar to that of the artificial liposome consisting of phosphatidylcholine/phosphatidic acid/phosphatidylserine (9:1:1). Additionally, these observations agreed well with the findings obtained from intact membrane vesicles. Bonnet et al. [19] reported that the pK_a (10.35) of cysteamine, primary amine compound, had decreased to 5.4 in the lipidic phase, and they concluded that this alteration of ionized group reactivity in lipidic matrix and the consecutive decrease of hydrophilicity induced by the predominance of the non-ionized form at a physiological pH, would possibly contribute to the explanation of the mechanism of the diffusion of ionic compounds inside biological bilayers. In the case of polyamine uptake, there may be a similar pK_a shifting of polyamine. It is suggested that the membrane lipids play an important role in the uptake and accumulation processes of these polyamines to the brush-border membrane vesicles. On the other hand, the uptake amounts to the vesicles were remarkably increased by the change of pH from 5.5 to 7.5 (Fig. 6) despite that the polyamine transport across the membrane vesicles was not dependent upon the pH of medium (Fig. 1 and Fig. 7).

From the results described above, it is considered that this pH dependency on polyamine uptake is due to the binding property to the membrane lipids but not to the transport rate across the membrane bilayer, although it remains unclear as to whether polyamines could bind to membrane protein or not. It seems probable, however, that binding of these polyamines to the outer- or inner-layer of the membrane vesicles, especially to the inner-membrane after slower permeation, might contribute to the high uptake of these polyamines, since the acidic phospholipids were mostly localized in the inner-layer of the intestinal brush-border membrane of humans [25] and rabbits [26].

Some investigations have suggested that several polyamines inhibited mutually in the uptake process of the whole cells. Kumagai et al. [5] mentioned that

spermidine had no significant effect on putrescine uptake into the isolated enterocyte of rats. They also indicated that the inhibitory effect of spermine on the putrescine uptake was not nearly as effective as those of the diamines. Moreover, in bovine adrenocortical cells, it was reported that spermine inhibited putrescine uptake, but that putrescine did not inhibit spermine accumulation [27]. Despite these implications, it is difficult to distinguish the regions, membranes or cytoplasm, in which the inhibition occurred. In the present study using the isolated brush-border membrane of rat small intestine, there was significant mutual inhibition observed between spermine and spermidine, while mutual inhibition between putrescine and others (spermine and spermidine) was not observed (Fig. 4). Moreover, no significant inhibition on spermine uptake was observed in the presence of cadaverine (Fig. 5), a dication like putrescine, which had been known to inhibit the putrescine uptake into the isolated enterocyte of rats [3]. On the contrary, gentamicin (trication), triethylenetetramine (tetracation), and tetraethylenepentamine (pentacation) significantly inhibited the spermine uptake (Fig. 6). Lipsky et al. [28] reported that the uptake of [^{3}H]gentamicin into the renal brush-border membrane vesicles was inhibited and reversed by the unlabeled aminoglycoside and by spermine, and that spermine was as potent on a molar basis as unlabeled gentamicin in inhibiting the uptake of the labeled aminoglycoside. From these observations, it is reasonable to assume that the number of amino groups in the polyamine molecules play a role in the uptake process into the brush-border membrane vesicles.

It will be interesting whether some kind of polyamine transporters do or don't work even in the brush-border side of rat intestine. We have failed to find the driving force stimulating the polyamine uptake rate into the membrane vesicles because their uptake behavior in this study could be explained by a mechanism mentioned above. Further examination will be necessary to clarify the energy which increases the polyamine uptake.

In conclusion, the polyamines such as spermine, spermidine and putrescine were bound pH-dependently to the rat intestinal brush-border membrane vesicles. The uptake behavior of these polyamines was associated with a fast external and slow internal binding to the membrane bilayer. It is suggested that the binding properties of these polyamines to the membrane lipid such as phospholipids, are related to the uptake process by the brush-border membrane vesicles.

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