Biochimica et Biophysica Acta, 1068 (1991) 105–110

ADONIS 000527369100335F

BBAMEM 75343

Spermine uptake by rat intestinal brush-border membrane vesicles

Ken Iseki, Michiya Kobayashi and Katsumi Miyazaki

Department of Pharmacy, Hokkaido University Hospital, School of Medicine, Hokkaido University, Sapporo (Japan)

(Received 15 May 1991)

Key words: Spermine; Polyamine; Brush-border membrane vesicle; Spermine transport; (Rat intestine)

The uptake of spermine by isolated rat intestinal brush-border membrane vesicles was studied. Uptake was biphasic, with an initial rapid uptake followed by a prolonged slower phase. Spermine uptake was not affected by a Na^+ electrochemical gradient. The equilibrium uptake of spermine was considerably dependent upon the medium pH. At pH 7.5 the degree of uptake was higher than that at pH 6.5 and was inversely proportional to the extravesicular osmolarity with a relatively high binding, which was estimated by extraporation to infinite extravesicular osmolarity (zero intravesicular space), while the uptake at pH 6.5 was not altered under the various medium osmolarities. A kinetic analysis of the initial uptake rate of spermine at 37°C gave a K_m of 24.2 μM and V_{max} of 206.1 pmol / mg protein per min. Furthermore, the uptake at 4° C was nonlinear, providing evidence for saturability. These findings suggest that spermine was associated with intestinal brush-border membrane vesicles in two ways, by binding to the outside and inside of membrane vesicles. The interaction of spermine and the apical membrane can be a contributory factor in the accumulation of this polyamine in the intestine of the intact animal.

Introduction

Polyamines (putrescine, spermine and spermidine) are found in a wide range of mammalian cells [1,2]. The intracellular level of these polycations is highly regulated in order to fulfil changing requirements during cell growth and differentiation [3,4]. Biosynthesis of polyamines within the cells is regulated by L-ornithine decarboxylase (ODC) (EC 4.1.1.17). In addition, cells from a variety of tissues have been shown to accumulate polyamines across their plasma membranes [5–9].

Small intestinal mucosal polyamine levels increase after the activation of ODC in a variety of situations that lead to an increased mucosal growth. Since the apical surface of the intestinal epithelial cells are exposed to luminal contents, this tissue has a unique advantage for regulating its polyamine levels. Recently, Kumagai and Johnson have reported the characteristics of the putrescine [7] and spermidine [8] transport system using isolated enterocyte of rat. They demonstrated that isolated cells accumulated these polyamines, and the uptakes were temperature-dependent, saturable, and inhibited by 1 mM KCN. On the other hand, Osborne and Seidel [10] indicated that the proximal intestinal lumen contained a high concentration of free putrescine that was transported from the distal intestine by enterohepatic circulation.

However, the direct evidences of polyamine transport system on the cell membrane are still poor because the studies in intact cells are far too complex to allow unambiguous determination of the mechanisms involved. The aim of this study is to gain insight into the intestinal transport system of spermine as a polyamine model compound by the use of isolated brush-border membrane vesicles of rat small intestine.

Materials and Methods

Chemicals

[^{14}C]Spermine tetrahydrochloride (4.1 GBq/mmol) and D-[^{14}C]glucose (1.7 GBq/mmol) were purchased from Amersham International (Buckinghamshire, U.K.). Valinomycin was obtained from Sigma Chemicals (St. Louis, MO, U.S.A.). All other chemicals were

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethansulphonic acid; Mes, 4-morpholineethansulphonic acid; ODC, L-ornithine decarboxylase.

Correspondence: K. Miyazaki, Department of Pharmacy, Hokkaido University Hospital, School of Medicine, Hokkaido University, Kita-14-jo, Nishi-5-chome, Kita-ku, Sapporo 060, Japan.

of the highest grade available and used without further purification.

Preparation of membrane vesicles

The entire small intestine of male Wistar rats (180–240 g) was excised under anesthesia with pentobarbital sodium (30 mg/kg body weight, i.p.). Brush-border membrane vesicles were isolated according to the calcium chloride precipitation method of Kessler et al. [11] as already described [12].

The final washing of the membrane was done with the preloading buffer which was either 20 mM Hepes-Tris buffer (pH 7.5), or 20 mM Mes-Tris buffer (pH 5.5) containing 100 mM KCl and 100 mM D-mannitol, respectively. The resulting pellet was suspended in the same buffer and used for the transport studies within 4 h of preparation. Membrane purification was routinely checked by measuring alkaline phosphatase (EC 3.1.3.1.), which is increased more than 11-fold in the final membrane suspension compared with the concentration in the homogenated intestinal mucosa scrapings.

Uptake studies

Uptakes of [^{14}]spermine and D-[^{14}C]glucose were measured by a Millipore filtration technique. Unless stated otherwise, 20 μl of membrane suspension were added to 100 μl of incubation medium kept at 37° C. The composition of the incubation media will be indicated 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 10 mM Hepes-Tris buffer (pH 7.5) or 10 mM Mes-Tris buffer (pH 5.5) 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 for counting the radioactivity.

All experiments presented in this paper were repeated at least three times 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 and D-[^{14}C]glucose on the filters were measured by standard liquid scintillation techniques. Protein was determined by the method of Lowry et al. [13] using bovine serum albumin as a standard.

Results

Viability of brush-border membrane vesicles

Fig. 1 illustrates the uptake of D-glucose at 200 μM concentration by rat intestinal brush-border membrane vesicles as a function of time. In the presence of an inwardly directed sodium gradient, the initial rate of uptake was clearly increased and there was also an overshoot effect. Furthermore, the equilibrium (30 min) uptake of D-glucose was decreased in inverse proportion to increase of extravesicular osmolarity (data not shown). This result suggests that the membrane vesicles prepared in this study have an intact transport properties.

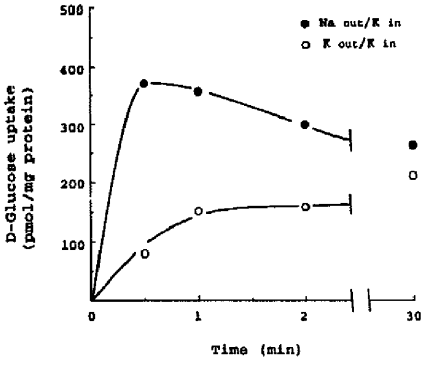

Fig. 1. Uptake of D-glucose (200 μM) by rat intestinal brush-border membrane vesicles. Brush-border membrane vesicles (20 μl) were preloaded with 100 mM KCl, 100 mM D-mannitol and 20 mM Hepes-Tris buffer (pH 7.5) at 37° C. Transport studies were performed by adding an incubation medium (100 μl) containing 20 mM Hepes-Tris buffer (pH 7.5), 240 μM D-[^{14}C]glucose, 100 mM D-mannitol, and either 100 mM NaCl (●) or 100 mM KCl (○) at 37° C. Final concentration of D-glucose was 200 μM. Results are expressed as the mean of triplicate determination.

Time-course of spermine uptake by intestinal brush-border membrane vesicles

The time-course of spermine uptake in rat intestinal brush-border membrane vesicles (50 μM) was shown in Fig. 2. Uptake was initially rapid, over 500 pmol/mg of membrane protein was taken up in the first 2 min. This

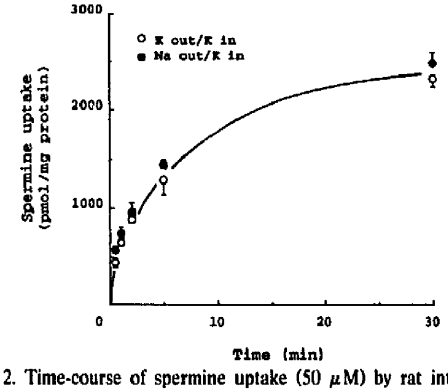

Fig. 2. Time-course of spermine uptake (50 μM) by rat intestinal brush-border membrane vesicles. The membrane vesicles were preincubated in 100 mM KCl, 100 mM D-mannitol and 20 mM Hepes-Tris buffer (pH 7.5). The vesicles were assayed in a medium containing 60 μM [^{14}C]spermine, 100 mM D-mannitol, 20 mM Hepes-Tris buffer (pH 7.5) and either 100 mM NaCl (●) or 100 mM KCl (○). Results represent the means ± S.E. of 4–8 determinations.

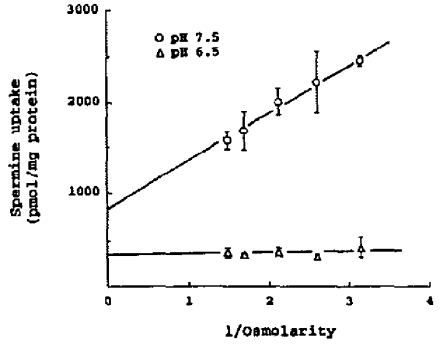

Fig. 3. Uptake of [^{14}C]spermine as a function of osmolarity of the incubation medium. Uptake studies were performed in the media containing either Hepes-Tris buffer (pH 7.5) (○) or Mes-Tris buffer (pH 6.5) (△), 100 mM KCl, 100 mM D-mannitol, 50 μM [^{14}C]spermine, and various concentrations of D-cellobiose to give the desired medium osmolarity. Values represent the means ± S.E. of three determination at 30 min incubation.

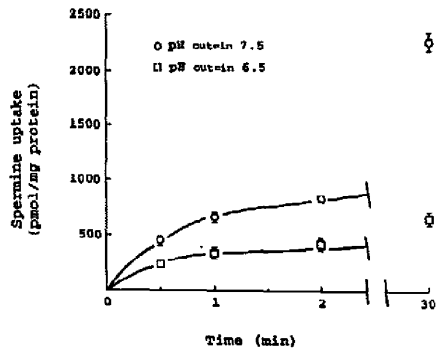

Fig. 4. Effect of the medium pH on spermine uptake by the intestinal brush-border membrane vesicles. Membrane vesicles were suspended in 100 mM KCl, 100 mM D-mannitol and either 20 mM Hepes-Tris buffer (pH 7.5) (○) or 20 mM Mes-Tris buffer (pH 6.5) (□). 20 μl of these membrane suspensions were added to 100 μl of incubation medium containing 60 μM [^{14}C]spermine, 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) (□). Results represent the means ± S.E. of six determinations.

was followed by a slower phase of uptake which continued for at least 30 min.

Spermine uptake with increasing medium osmolarity

As shown in Fig. 2, the uptake of spermine was initially very rapid and followed by a slower phase of uptake. The term uptake, as used in this paper, does not distinguish between the transport of spermine across the brush-border membrane into an intravesicular space and the binding of this compound to the membrane. To clarify whether the uptake of spermine by the brush-border membranes represented transport or binding, uptake of spermine by the vesicles after 30 min of incubation was measured under the various medium osmolarities using cellobiose, an impermeable solute. At the pH of 7.5 in medium, uptake of spermine was inversely proportional to medium osmolarity from 300 mosM to 650 mosM (Fig. 3). It was found that extraporation to infinite extravesicular osmolarity (zero intravesicular space) was estimated approx. 30% of the uptake amount at 300 mosM, the final osmolarity in all subsequent uptake measurements. On the contrary, the uptake of spermine at the medium pH of 6.5 was extremely low and was not altered under the various medium osmolarities.

Effect of the medium pH on spermine uptake by brush-border membranes

Bitonti et al. [14] have reported that the uptake of bis(benzyl)polyamine analogue into erythrocytes was depending upon the pH of medium (7.5 > 6.5). To study the effect of medium pH, the brush-border membrane vesicles were preincubated with 100 mM KCl, 100 mM D-mannitol, and either 20 mM Hepes-Tris

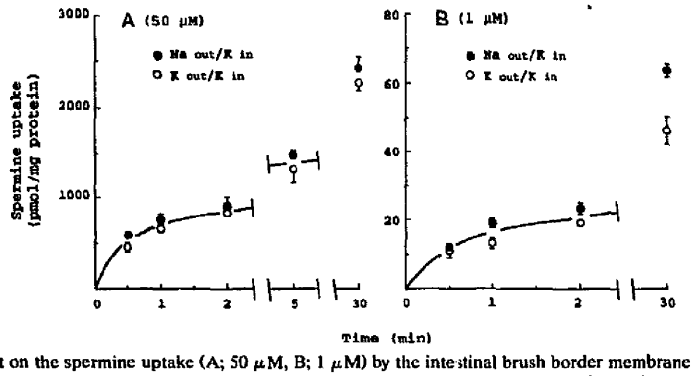

Fig. 5. Effect of Na gradient on the spermine uptake (A; 50 μM, B; 1 μM) by the intestinal brush border membrane vesicles. Membrane vesicles (20 μl) were preloaded with 100 mM KCl, 100 mM D-mannitol and 20 mM Hepes-Tris buffer (pH 7.5) at 37° C. The vesicles were assayed in a medium containing [^{14}C]spermine, 100 mM D-mannitol, 20 mM Hepes-Tris buffer (pH 7.5) and either 100 mM NaCl (●) or 100 mM KCl (○). Results represent the means ± S.E. of 4–8 determinations.

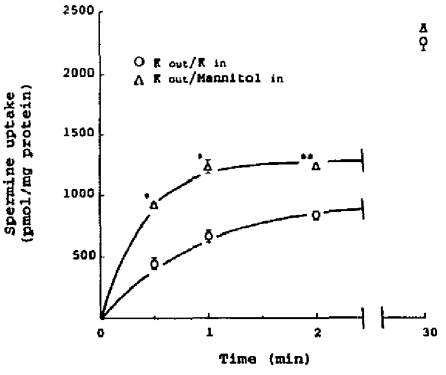

Fig. 6. Effect of intravesicular ionic environment on the uptake of [^{14}C]spermine by rat intestinal brush-border membrane vesicles. The membrane vesicles (20 μl) were preloaded with 20 mM Hepes-Tris buffer (pH 7.5), and either 300 mM D-mannitol (Δ) or 100 mM D-mannitol, 100 mM KCl (○). Transport studies were performed by adding an incubation medium (100 μl) containing 50 μM [^{14}C]spermine, 100 mM KCl, 100 mM D-mannitol and 20 mM Hepes-Tris buffer (pH 7.5). Results are expressed as the means with S.E. of 6–8 determinations. The statistical significance was evaluated by Student's *t*-test. * Significantly different from control, $P < 0.001$; ** $P < 0.005$.

buffer (pH 7.5) or 20 mM Mes-Tris buffer (pH 6.5). The vesicles were then added to a medium containing 100 mM KCl, 100 mM D-mannitol, and 20 mM Hepes-Tris (pH 7.5) or Mes-Tris (pH 6.5) buffer and 50 μM spermine. As shown in Fig. 4, the time-course of spermine uptake was considerably dependent upon the medium pH, namely, the uptake of spermine at the pH of 7.5 was markedly stimulated in comparison with the medium pH of 6.5.

Effect of sodium gradient on spermine uptake by brush-border membrane vesicles

Kumagai et al. reported a carrier-mediated transport system of putrescine and spermidine [7,8], in the isolated enterocyte, and they mentioned that the replacement of Na^+ with charged compounds such as choline, tetramethylammonium and lithium inhibited spermidine uptake remarkably.

However, as shown in Fig. 5, Na^+ gradient did not stimulated the initial uptake of spermine even at 1 μM of concentration in the incubation medium (pH 7.5). Moreover, using each preparation of membrane vesicles after dividing the four parts of small intestine, namely, proximal jejunum, distal jejunum, proximal ileum and distal ileum, we have confirmed that there was no difference in the effect of Na^+ gradient on the spermine uptake among those preparations (data was not shown).

Effect of ionic strength on spermine uptake

Fig. 6 illustrates the effect of ionic strength in the intravesicular space on the uptake of spermine into the brush-border membrane vesicles. For these studies, the membrane vesicles were preloaded with buffer A; 300 mM D-mannitol, 20 mM Hepes-Tris buffer (pH 7.5) or buffer B; 100 mM KCl, 100 mM D-mannitol and 20 mM Hepes-Tris buffer (pH 7.5). The vesicles were then added to a medium containing 100 mM KCl, 100 mM D-mannitol and 20 mM Hepes-Tris (pH 7.5) buffer and 50 μM spermine. As shown in Fig. 6, there was a significant stimulation in the initial uptake of spermine under the low ionic strength condition in the intravesicular space. This finding agreed with the report of

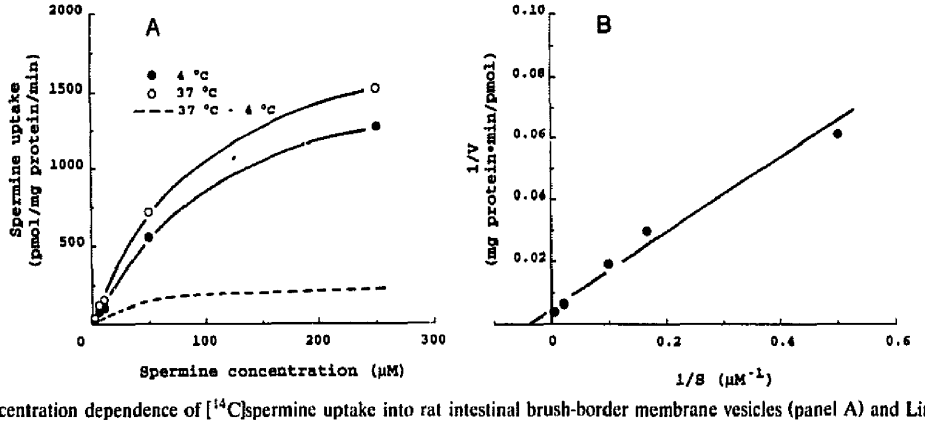

Fig. 7. Concentration dependence of [^{14}C]spermine uptake into rat intestinal brush-border membrane vesicles (panel A) and Lineweaver-Burk plot of spermine uptake value corrected for nonspecific binding at 4°C (panel B). Membrane vesicles were suspended in 100 mM KCl, 100 mM D-mannitol, and 20 mM Hepes-Tris buffer (pH 7.5). 20 μl of this membrane suspension were added to 100 μl of incubation medium to obtain a final composition of the extravesicular medium of 100 mM mannitol, 100 mM KCl and 20 mM Hepes-Tris buffer (pH 7.5), plus [^{14}C]spermine at the concentrations indicated in the figure. Solid lines indicate the total uptake observed at 37 °C (○), or 4 °C (●), and dashed line indicates the corrected uptake by subtracted the uptake value at 4 °C from each value at 37 °C. Uptake rate for Lineweaver-Burk plot was estimated by subtracting the value at 4 °C from the total uptake value at 37 °C (shown with dashed line in panel A). A linear repression program has been used to fit the data, $r = 0.9985$. Intercepts of line with the *X* and *Y*-axes are $-1/K_m$ ($K_m = 24.2$ μM) and $1/V_{max}$ ($V_{max} = 206.1$ pmol/mg protein per min).

Kumagai and Johnson [7] using isolated enterocytes of rat, although their result was for putrescine uptake.

Concentration dependence of spermine uptake

The effect of concentration of spermine on uptake was investigated using 1 to 250 μM spermine. As shown in Fig. 7A, spermine uptake, which was corrected by the subtraction of the values at 4° C from the uptake values at 37°C, exhibited the saturation kinetics. Furthermore, the uptake rate of spermine at 4° C was also nonlinear, providing evidence for saturability. These data suggest that affinity of spermine to the brush-border membrane is relatively high even at low temperatures. This high affinity of polyamine to the membrane vesicles was similar to our previous reports in that the several amine compounds had a tendency to bind to the brush-border membrane [15–18]. Lineweaver-Burk transformation of the corrected data resulted in values for K_m and V_{max} of 24.2 μM and 206.1 pmol/mg protein per minute, respectively (Fig. 7B).

Discussion

The findings in the present study suggest that spermine is associated with intestinal brush-border membrane vesicles by two methods of binding. This suggestion is supported by several observations. For example, the time-course of spermine uptake has two processes, an early rapid phase followed by prolonged slower phase (Fig. 2). Possibly, the rapid phase is due to binding to the outside membranes, whereas the slower phase might be the binding to the inside of membrane vesicles after permeation into the vesicles. In the characteristics of spermine uptake, there was no dependence of an inwardly directed Na^+ gradient upon the uptake behavior of this polyamine into the brush-border membrane (Fig. 5). Kumagai et al. [7,8] have reported that, in the isolated rat enterocytes, the replacement of NaCl in the medium with D-mannitol or sucrose did not significantly inhibit the uptake of spermidine and putrescine, although replacement of NaCl with a charged compound such as choline, lithium and tetramethylammonium significantly inhibited spermine uptake. On the other hand, we confirmed that the spermine uptake was not enhanced and that there was no overshooting in the presence of Na^+ gradient even at 1 μM of medium concentration of spermine (Fig. 5B).

Since it is impossible to distinguish between the transport system of brush-border membranes and basolateral membranes using the isolated enterocytes, the results of Kumagai et al. [7,8] might represent the mixture of brush-border membrane transport and basolateral membrane transport. This speculation is also suggested by Van Den Bosch et al. [9]. Although they examined a renal cell line, they found that the Na^+-dependent polyamine uptake preferentially occurred from the basolateral side. Thus, the results of the present study suggested that there was no contribution of Na^+ gradient-dependent transport system in the uptake into the rat intestinal brush-border membrane. However, in the present study, the spermine uptake could not be separately determined according to binding to the inside translocation across the membrane and binding to the outside. Therefore, a more detailed investigation of the Na^+ gradient-dependent transport system in the intestinal apical side will be needed.

At a pH of 7.5, we found that there was remarkable accumulation of spermine into the membrane vesicle, and that the uptake of spermine was sensitive to the osmolarity of the incubation medium (Fig. 3). The spermine binding to the membrane, which was estimated by extrapolation to infinite medium osmolarity, was markedly higher at pH 7.5 than that at pH 6.5. The intravesicular space, calculated to be 0.9–1.5 μl/mg protein on the basis of the equilibrium uptake of D-glucose (Fig. 1), can accommodate, in the presence of 50 μM of spermine, no more than about 70 pmol/mg protein of this polyamine at equilibrium. However, as shown in Fig. 2, it was found that a very large amount of uptake was observed at 30 min (2318.2 ± 34.1 pmol/mg protein at pH of 7.5 in Fig. 2). McCormack and Johnson [19] reported that putrescine accumulated to a concentration gradient of 300-fold during 1 h at pH 7.4, and that uptake could be inhibited 50% by 7.5 μM unlabeled putrescine and was not dependent on Na^+ using a human colon adenocarcinoma cell line (LoVo). On the other hand, in the present study, spermine accumulated to a concentration gradient of about 40-fold during 30 min at pH 7.5. It was also found that the initial uptake rate was significantly stimulated in the absence of K^+ inside the vesicles at the earlier time (Fig. 6). This result suggests that the uptake of spermine might be accelerated when the inhibitory components are absence inside the membrane vesicles or when some binding components such as various organellae are presence inside the membrane vesicles like intact cells. It is reasonable to consider, therefore, that much larger amounts of spermine might be accumulated in the case of the intact enterocyte.

As a possible interpretation for the high accumulation of spermine to the membrane vesicles, it seems that the binding to the intravesicular membrane component might be related, and that the binding of this cation could be dependent upon the physiological pH and the ionic environment inside the cells because it is known that polyamines such as spermine and spermidine bound to the negatively charged phospholipid layer [20]. Since the acidic phospholipids, including phosphatidylinositol and phosphatidylserine were localized in the inner layer of the plasma membrane, it is

likely that binding of spermine to the inner layer of the membrane might contribute to the high uptake of spermine. De Smedt et al. [5] have mentioned that the parameter of the saturable component in Na^+-independent transport of putrescine was 29.8 ± 3.5 μM using LLC-PK_1 renal epithelial cell line. Furthermore; it was found by Kumagai et al. [7,8] that kinetic analysis of the uptake of spermidine and putrescine resulted in $K_m = 1.25$ μM and 12.3 μM, respectively, by the isolated rat enterocytes. Our experimental result obtained from kinetic analysis of Lineweaver-Burk plot of spermine uptake ($K_m = 24.2$ μM, Fig. 7) approximately agreed with those reports, although the preparations used for estimation were different from each other.

In summary, we have indicated that the spermine was taken up with the saturable process by the rat intestinal brush-border membrane vesicles. In addition, this polyamine is associated with intestinal apical membrane by binding. The interaction of spermine with membrane may be relevant to the accumulation of the polyamine by contributing to the intestinal transmembrane transport system. Further investigation for the transport characteristics related to the interaction between polyamines and membrane components are under examination.

References

1 Pegg, A.E and McCann, P.P. (1982) Am. J. Physiol. 243, C212–C221.

2 Byers, T.L. and Pegg, A.E. (1989) Am. J. Physiol. 257, C545–C553.

3 Tabor, C.W. and Tabor, M. (1984) Annu. Rev. Biochem. 53, 749–790.

4 Pegg, A.E. (1986) Biochem. J. 234, 249–262.

5 De Smedt, H., Van Den Bosch, L., Genus, J. and Borghgraef, R. (1989) Biochim. Biophys. Acta 1012, 171–177.

6 Feige, J.J. and Chambaz, E.M. (1985) Biochim. Biophys. Acta 846, 93–100.

7 Kumagai, J. and Johnson, L.R. (1988) Am. J. Physiol. 254, G81–G86.

8 Kumagai, J., Jain, R. and Johnson, L.R. (1989) Am. J. Physiol. 256, G905–G910.

9 Van Den Bosch, L., De Smedt, H., Missiaen, L., Parys, J.B. and Borghgraef, R. (1990) Biochem. J. 265, 609–612.

10 Osborne, D.L. and Seidel, E.R. (1990) Am. J. Physiol. 258, G576–G584.

11 Kessler, M., Acuto, O., Storelli, C., Murer, H., Muller, M. and Semenza, G. (1978) Biochim. Biophys. Acta 506, 136–154.

12 Iseki, K., Sugawara, M., Saitoh, H., Miyazaki, K. and Arita, T. (1989) J. Pharm. Pharmcol. 41, 628–632.

13 Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.

14 Bitonti, A.J., Dumont, J.A. and McCann, P.P. (1989) Biochem. Pharmacol. 38, 3638–3642.

15 Saitoh, H., Kobayashi, Y., Miyazaki, K. and Arita, T. (1987) J. Pharm. Pharmacol. 39, 9–12.

16 Saitoh, H., Kawai, S., Miyazaki, K. and Arita, T. (1988) J. Pharm. Pharmacol. 40, 176–180.

17 Saitoh, H., Kawai, S., Iseki, K., Miyazaki, K. and Arita, T. (1989) J. Pharm. Pharmacol. 41, 200–202.

18 Saitoh, H., Ebina, M., Fukuda, S., Miyazaki, K. and Arita, T. (1989) J. Pharm. Pharmcol. 41, 459–463.

19 McCormack, S.A. and Johnson, L.R. (1989) Am. J. Physiol. 256, G868–G877.

20 Yung, M.W. and Green, C. (1986) Biochem. Pharmacol 35, 4037–4041.