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Novel oral absorption system containing polyamines and bile salts enhances drug transport *via* both transcellular and paracellular pathways across Caco-2 cell monolayers

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ABSTRACT

The combinatorial use of spermine (SPM), a typical polyamine, and sodium taurocholate (STC), a typical bile salt, was found to be a promising safe preparation for improving the oral absorption of poorly water-soluble and/or poorly absorbable drug in our previous studies utilizing rats and dogs. To clarify the mechanisms behind the synergistic enhancement effect of the polyamine and bile salt, the transport of rebamipide, which is classified into Biopharmaceutics Classification System Class IV, was investigated in Caco-2 cell monolayers. The synergistic enhancement of rebamipide transport by SPM and STC was certainly observed in Caco-2 cells as well, while the separate use of either SPM or STC did not significantly improve the transport of rebamipide. The combinatorial use of SPM and STC significantly decreased the transepithelial electrical resistance (TEER) in Caco-2 cell monolayers, suggesting that the opening of paracellular pathway. On the other hand, it was also confirmed that the decrease in TEER was transient and reversible after removal of SPM and STC and that cell viability was maintained. Voltage-clamp study clearly showed that their combinatorial use improved rebamipide transport *via* both paracellular and transcellular pathways, and that the contribution of transcellular route could be larger than paracellular route.

1. Introduction

Development of combinatorial chemistry and high-throughput screening technique has made it possible to generate a lot of new drug candidates very rapidly, but it has resulted in a number of poorly soluble and/or poorly absorbable candidates at the same time. A new trend of drug development based on pharmacogenomics or development of molecular-targeted drug is also spurring the tendency, and it does not necessarily lead to good output in terms of the development of new drugs. Therefore, it is very attractive to improve the membrane permeability as well as the solubility by using some adjuvants. However, the practical use of absorption-enhancing adjuvants has been very limited (Motohiro et al., 1983; Lindmark et al., 1998) because of the potential local toxicity (Swenson and Curatolo, 1992; Swenson et al., 1994). Thus, a safe formulation that can improve the intestinal absorption of poorly absorbable drugs has highly been desired. Previously, we reported that the combinatorial use of sodium laurate (C12) with taurine or L-glutamine enhanced the absorption of poorly absorbable drugs from the colon and rectum without causing any serious local damages (Yata et al., 2001; Miyake et al., 2003, 2004, 2006a), and several mechanisms behind the cytoprotective action by such amino acids have been clarified (Endo et al., 2002; Okuda et al., 2006). Furthermore, we have proposed another novel preparation containing spermine (SPM), a polyamine, and sodium taurocholate (STC), a bile salt, as a safe formulation that is able to enhance the oral absorption of a poorly absorbable drug (Miyake et al., 2006b,c). The combinatorial use of SPM with STC significantly improved the oral absorption of rebamipide, categorized into Biopharmaceutics Classification System (BCS) Class IV, seven times larger than control preparation (Miyake et al., 2006b). The absorption of rebamipide was significantly improved by SPM alone, but the addition of STC drastically enhanced the improving effect of SPM. As STC itself did not enhance the absorption of rebamipide so much, it was considered that the polyamine and STC had a synergistic enhancing effect. On the other hand, the histopathological evaluation did not reveal any significant change in forestomach, glandular stomach

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and duodenum where higher concentrations of SPM and STC could be present after dosing to rats. These results clearly indicate that SPM and its combinatorial use with STC could improve the intestinal absorption of poorly absorbable drugs without any adverse effect on gastrointestinal mucosa. The importance of bile salts on the absorption improvement by SPM was also confirmed in bile duct-ligated rats and in beagle dogs under fasted condition (Miyake et al., 2006c).

In the present study, to clarify the mechanisms behind the absorption-improvement by SPM and STC, the effects of their combinatorial use on the transport of rebamipide and transepithelial electrical resistance were investigated by utilizing Caco-2 cell monolayers. Furthermore, we tried to evaluate the relative contributions of transcellular and paracellular pathways to the transport enhancement by the combinatorial use of SPM with STC by means of voltage-clamp technique (Yamashita et al., 1985; Emi et al., 1998; Hiraoka et al., 2005).

2. Materials and methods

2.1. Materials

SPM and STC were purchased from Sigma Chemical Co. (St. Louis, MO) and Tokyo Chemical Industry Co. (Tokyo, Japan), respectively. Hydroxypropylmethylcellulose 2910 (HPMC) was purchased from Shin-etsu Kagaku Co. (Tokyo). Rebamipide was obtained from Otsuka Pharmaceutical Co. Ltd. (Tokushima, Japan). All other reagents were analytical grade commercial products.

2.2. Transport across Caco-2 cell monolayers

Caco-2 cells, obtained from the cell bank of the Institute of Physical and Chemical Research (Ibaragi, Japan), were grown in a CO_2 incubator (MCO-175, Sanyo, Tokyo) which was maintained at 37 °C, 5% CO_2 and 90% relative humidity, using Dulbecco's modified Eagle's medium (DMEM, GIBCO, NY) containing 10% fetal bovine serum (Sigma), 20 μg/mL gentamicin and penicillin/streptomycin (100 U/100 μg/mL, GIBCO). Caco-2 cells were seeded on Transwell (1.12 cm^2 of growth area, 12-well, Corning Japan, Tokyo) at the density of 7.5×10^4 cells/well. Fourteen to 16 days after seeding, the medium was removed and cells were washed twice with Ringer's solution (pH 7.4) containing 1.2 mM NaH_2PO_4, 125 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 10 mM $NaHCO_3$ and 11.1 mM D-glucose. The apical and basal sides of Caco-2 cells were bathed with 0.5 mL and 1.5 mL of Ringer's solution, respectively, and maintained at 37 °C. After preincubation for 30 min to stabilize the condition of the cells, the solutions in the donor side and receptor side were exchanged for the drug solution and fresh Ringer's solution, respectively, and then the transport experiments were started. Rebamipide (1 mg/mL) was solubilized with Ringer's solution containing 1% HPMC. Samples of 0.5 mL were drawn out of the basal side at 20-min intervals to 100 min. An equal volume of Ringer's solution was immediately added to the basal side after each sampling. Transepithelial electrical resistance (TEER) values were measured using Millicell-ERS (Millipore Co., Bedford, MA) at 10 min before the addition of the drug solution and then every 20 min until 90 min after the start of the transport experiment.

2.3. Voltage-clamp study

To analyze the drug transport pathways across the cell layer, the drug transport studies were performed under the voltage-clamped conditions by utilizing Snapwell (1.12 cm^2 of growth area, 6-well, Corning Japan). Snapwell where Caco-2 cells were cultured with the same procedure as Transwell was placed into a diffusion chamber (Corning Japan). Both sides of Caco-2 cells were bathed with 5 mL of Ringer's solution gassed with 95% O_2 and 5% CO_2 and maintained at 37 °C. Utilizing Ag/AgCl electrodes (Navi Cyte, Harvard Apparatus, Inc., Holliston, MA) and 2-channel voltage–current clamp (Model VCC MC2, Physiologic Instruments, Inc., San Diego, CA), the transepithelial electrical potential difference (PD) was clamped to the arbitrary values (−10 mV to +10 mV). These voltage-clamped conditions were maintained unchanged throughout the drug transport experiment. After preincubation for 30 min, the solutions in the donor and receptor sides were exchanged for the drug solution and fresh Ringer's solution, respectively, and then the transport experiments were started. The solutions in both sides were circulated by gas lift with 95% O_2 and 5% CO_2 throughout the transport studies. Samples of 0.5 mL were drawn out of the receptor side at 10-min intervals to 60 min. An equal volume of Ringer's solution was immediately added to the receptor side after each sampling.

According to Schults and Zalusky (1964), the transmembrane flux of ionized molecules *via* the paracellular shunt-pathway (J_d) depends on the potential difference across the membrane (V_t) and is expressed as the following equation:

$$J_d = {}_0J_d \exp\left(-\frac{zFV_t}{2RT}\right) \quad (1)$$

where ${}_0J_d$ indicates the J_d under the short-circuit condition. z, F, R and T reveal ionic valency, the Faraday constant, the gas constant and absolute temperature, respectively. On the other hand, the flux through the transcellular pathway (J_m) is independent of V_t, and the total flux (J_{total}) is expressed as

$$J_{total} = J_m + J_d = J_m + {}_0J_d\xi \quad (2)$$

where ξ means $\exp(-zFV_t/2RT)$.

2.4. Analytical method

The concentration of rebamipide in the basal solution was determined by HPLC. The sample solution was filtrated with a microfilter (pore size 0.5 μm, Millipore, Tokyo) and introduced into HPLC system, which consisted of a model of LC-20AT pump (Shimadzu, Kyoto, Japan) and a fluorescence detector (RF-530; Shimadzu) set at Ex. 330 nm and Em. 370 nm. Analytical column was ODS-80 TM (150 mm × 6.0 mm ID, Tosoh, Tokyo). The mobile phase, acetonitrile: 5 mM Na_2SO_4 (3:5, v/v) including 1% acetic acid and 0.3% tetrahydrofuran, was delivered at 1 mL/min. The standard curves from 25 to 2000 ng/mL showed the coefficient of variation ranged from 0.04 to 23.4% and the correlation coefficients over 0.998.

2.5. Data analysis

In the transport study, the cumulative amount transported to the basal side was calculated according to the following equation:

$$Qt_n = \sum_{n=1}^{n}[0.5Ct_{n-1}] + VCt_n \quad (3)$$

where Qt_n and Ct_n indicate the cumulative amount transported to the basal side and the concentration in the basal side at time t_n, respectively. The sampling volume was 0.5 mL and the volume of solution in the basal side (V in the Eq. (3)) was 1.5 or 5 mL for Transwell or Snapwell experiment, respectively. Apparent permeability coefficient (P_{app}) was calculated according to the following equation:

$$P_{app} = \frac{dQ}{dt}\frac{1}{AC_0} \quad (4)$$

where dQ/dt, A and C_0 reveal the uptake rate at the steady state, the exposing surface area of Transwell or Snapwell and the initial concentration of rebamipide, respectively.

2.6. Evaluation of epithelial damage

2.6.1. Elution of phospholipids from Caco-2 cells

In the present study, the elution of phospholipids (PL) was employed as a biochemical marker for cytotoxicity (Yata et al., 2001; Endo et al., 2002). As in the drug transport experiment using Transwell described above, 1 mL of drug solution with or without SPM and/or STC was added to the apical side and the Transwell was incubated at 37 °C for 100 min. The concentration of PL in the supernatant of apical drug solution after centrifugation at 10,000 rpm for 10 min was determined spectrophotometrically at 500 nm using Phospholipids-C Test kit (Wako Pure Chemical Industries, Ltd., Osaka).

2.6.2. Trypan blue extrusion test

After the treatment of Caco-2 cells on Transwell with 1 mL of drug solution with or without SPM and/or STC as described above, the apical side of Caco-2 cell layer was washed with Ringer's solution and the remaining cells were stripped off with 0.25% trypsin–EDTA solution. Twenty μL of 0.1% Trypan blue solution was added to 20 μL of the cell suspension, and the dyed cells were counted.

2.7. Statistical analysis

Results are expressed as the mean ± S.D. of more than three experiments. Analysis of variance (ANOVA) was used to test the statistical significance of differences among groups. Statistical significance in the differences of the means was determined by Dunnet's method or Student's *t*-test.

3. Results and discussion

There has been a great interest in improving the oral absorption of poorly absorbable drugs for a long time (Fix, 1987; Swenson and Curatolo, 1992; Aungst, 2000). Therefore, many researchers enthusiastically have tried to discover and/or develop the compounds and/or formulations that would enhance the mucosal absorption of drugs (Fix, 1987; Higaki et al., 1990; Swenson and Curatolo, 1992; Anderberg and Artursson, 1993; Hurni et al., 1993; Duizer et al., 1998; Lindmark et al., 1998; Aungst, 2000; Yata et al., 2001; Lindhardt and Bechgaard, 2003; Smith et al., 2004). However, the potential local toxicity caused by the adjuvants and/or the formulations (Swenson and Curatolo, 1992; Swenson et al., 1994) has made it difficult to their practical use for clinical therapeutics (Motohiro et al., 1983; Lindmark et al., 1998). In our recent paper, we proposed the combinatorial use of SPM with STC as a safe preparation that was able to improve the absorption of a drug categorized into BCS Class IV (Miyake et al., 2006b). In the present study, therefore, we tried to elucidate the mechanisms for the absorption enhancement by the combinatorial use of SPM and STC by utilizing Caco-2 cell monolayers. Fig. 1(A) indicates that the effect of SPM and STC on rebamipide transport across Caco-2 cell monolayers. Although neither 3 mM SPM nor 10 mM STC alone increased significantly the transport of rebamipide, the combinatorial use of 3 mM SPM and 7 mM STC provided the significant promoting action, resulting in 2.6-fold value of P_{app} (Table 1). The combinatorial use with 10 mM STC significantly enhanced the transport of rebamipide from early period of time to the end of study, which led to the value of P_{app} six times larger than control (Table 1). The synergistic enhancing effect of SPM and STC observed here was similar to the result in rats (Miyake et al., 2006b). Since rebamipide was dissolved in each preparation, the improved transport is ascribed to the enhanced permeability by the addition of both SPM and STC. The applied concentration of SPM was shifted to lower level than that used for studies in rats (Miyake et al., 2006b) and dogs (Miyake et al., 2006c), because Caco-2 cells were more sensitive to adjuvants than the gastrointestinal tissue in vivo. It was also the case with the combinatorial use of sodium laurate with several amino acids (Yata et al., 2001; Endo et al., 2002).

Table 1
Effect of combinatorial use of spermine (SPM) and sodium taurocholate (STC) on permeability of rebamipide and transepithelial electrical resistance (TEER) across Caco-2 cell monolayers.

Adjuvants	P_{app} (% of control)	TEER (% of initial value)
Control	100.0 ± 10.6	105.5 ± 3.3
3 mM SPM	111.6 ± 4.5	101.9 ± 2.6
10 mM STC	113.9 ± 15.1	87.0 ± 1.2**
3 mM SPM + 7 mM STC	264.5 ± 70.9**	75.9 ± 5.2**
3 mM SPM + 10 mM STC	603.2 ± 100.5**	51.6 ± 10.7**

Each value represents the mean ± S.D. of three to seven experiments. P_{app} for control was 1.16 ± 0.12 (×10^{-7}) cm/s. TEER at steady state was expressed as % of initial value. Initial value of TEER for control was 454.1 ± 12.4 Ω cm^2.
** $p < 0.01$, compared with control.

Fig. 1(B) indicates the effect of SPM and/or STC on TEER. TEER was not affected by 3 mM SPM. Although 10 mM STC slightly decreased TEER, the decrease in TEER did not result in the enhancement of rebamipide transport (Fig. 1(A) and Table 1). On the other hand, the combinatorial use of SPM with STC markedly decreased the TEER values. Especially, TEER was decreased to around 50% of the initial value by the combinatorial use with 10 mM STC (Fig. 1(B) and Table 1), which coincided with the increase in the transport of rebamipide (Fig. 1(A) and Table 1). These results suggest that the enhanced transport of rebamipide by the preparation is at least in part due to the opening of paracellular pathway.

Local toxicity by the combinatorial use of SPM with STC was investigated in terms of PL elution from Caco-2 cells (Fig. 2(A)). Elution of PL is one of the typical biochemical markers (Swenson et al., 1994; Yata et al., 2001; Endo et al., 2002) and might be the most sensitive considering the case of sodium laurate (Yata et al., 2001). The combinatorial use slightly increased the elution of PL, while 3 mM SPM or 10 mM STC did not significantly enhance the elution of PL. However, the effect of SPM and STC was much smaller than that of 50% ethanol, a positive control, which enhanced the PL elution more than 30-fold of the control. Furthermore, the viability of Caco-2 cells, examined by Trypan blue extrusion test, was not changed by the treatment with SPM and STC, while 50% ethanol decreased the viability to 50.1% of control (Fig. 2(B)). Our previous report clearly showed that the oral administration of 10 mM SPM with 25 mM STC did not cause any significant histopathological change in stomach and duodenum (Miyake et al., 2006b). Taken all together, the combinatorial use of SPM with STC would be safe enough for the utilization as an oral dosage formulation.

Fig. 3 shows the reversibility of the TEER-lowering effect by SPM and STC. Caco-2 cells were treated with SPM and STC for 100 min, as was the case with the transport study shown in Fig. 1. Then, the drug solution was removed and the surface of cell layer was washed with Ringer's solution. The incubation was continued with fresh Ringer's solution without SPM and STC. As is evident from the figure, the TEER value that had been decreased by the incubation with SPM and STC was gradually increased and recovered even up to the control level after 960 min. This result clearly indicates that the opening of the paracellular pathway with the combinatorial use of SPM and STC is transient and reversible. On the other hand, the effect of 50% ethanol was maintained after changing it to fresh

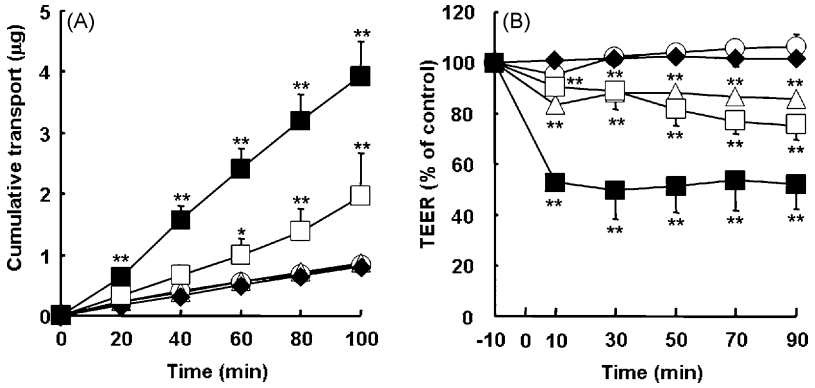

Fig. 1. Effect of combinatorial use of SPM with STC on transport of rebamipide (A) and TEER (B) in Caco-2 cell monolayers. Keys: (○) control; (♦) 3 mM SPM; (△) 10 mM STC; (□) 3 mM SPM + 7 mM STC; (■) 3 mM SPM + 10 mM STC. Results are expressed as the mean with the bar showing S.D. of three to seven experiments. $^{**}p < 0.01$; $^{*}p < 0.05$, compared with control.

Ringer's solution, and TEER value was even decreased further at late periods of time, suggesting the severe cell damage. Considering the reversibility in TEER together with results shown in Fig. 2, the preparation would be promising as a safe formulation that is able to improve the oral absorption of poorly absorbable drugs from the aspects of both efficacy and safety.

As shown in Fig. 1(A), it was confirmed that the combinatorial use of SPM and STC enhanced the transport of rebamipide across Caco-2 cell monolayers. Fig. 1(B) clearly indicated the possible contribution of the paracellular pathway to the enhanced transport. Therefore, we tried to evaluate the relative contribution of the transport pathways including the transcellular one to the enhancement by SPM and STC. The transport of rebamipide across Caco-2 cell monolayers in the absence or presence of both SPM (3 mM) and STC (10 mM) was examined under voltage-clamped conditions. Based on the report by Schults and Zalusky (1964), the transmembrane flux of ionized molecules *via* the paracellular shunt pathway (J_d) would change dependent on the potential difference across the membrane (V_t), which can be arbitrarily changed, as shown in Eq. (1), while the flux through the transcellular pathway is not affected by the change in V_t. Therefore, it is possible to estimate the contribution of both pathways by determining the drug transport under the conditions with several different V_t and by obtaining J_m and ${}_0J_d$ of Eq. (2). In the present study, the apical-to-basal flux rates of rebamipide were measured under three different conditions where V_t value was set to −10, 0 or +10 mV. Fig. 4 shows the plots of total fluxes, J_{total}, against the ξ values and the obtained intercept or slope of each line represents the flux through the transcellular route or paracellular route under the short-circuit condition, respectively (Table 2). This electrophysiological approach has been performed to evaluate the change in contribution of each pathway to transmucosal transport of several drugs in our previous studies (Yamashita et al., 1985; Emi et al., 1998; Hiraoka et al., 2005). It appears that rebamipide penetrates Caco-2 cell monolayers by both pathways in the absence of enhancers as positive values were obtained for both J_m and ${}_0J_d$ for control. According to Eq. (2), J_m and ${}_0J_d$ mean the transcellular and paracellular fluxes of rebamipide under short-circuit condition, respectively. Therefore, the simultaneous addition of 3 mM SPM and 10 mM STC to the apical side produced 4.7-fold and 2.8-fold increases in the transcellular and paracellular fluxes, respectively (Table 2). While the increase in the transport through the paracellular route was observed as predicted from reduced TEER (Fig. 1(B) and Table 1), this analysis suggested the larger increase through the transcellular route as well. As sum-

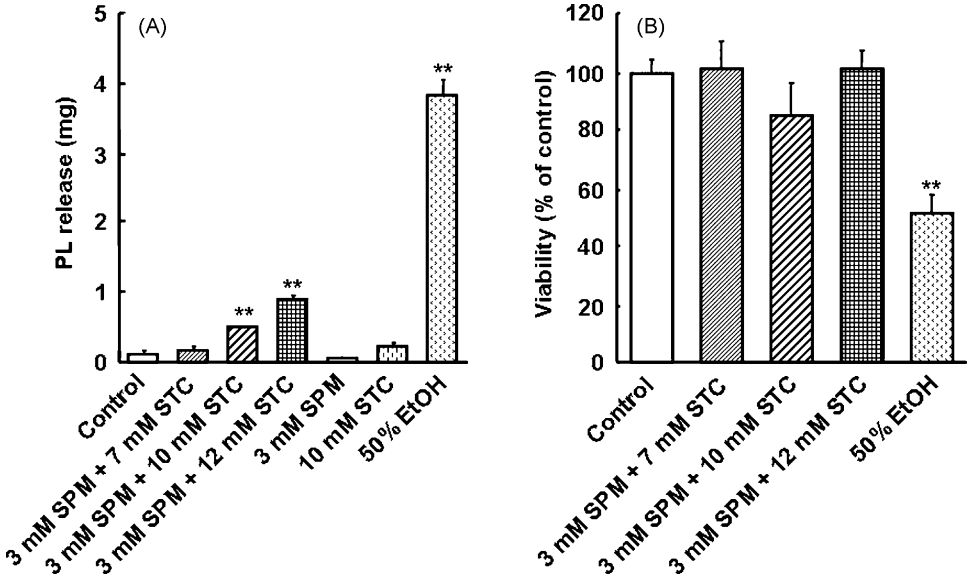

Fig. 2. Effect of combinatorial use of SPM with STC on elution of phospholipids from Caco-2 cells (A) and cell viability (B). Results are expressed as the mean with the bar showing S.D. of 3–23 experiments. $^{**}p < 0.01$, compared with control ($n = 23$).

Table 2
Electrophysiological evaluation of transport pathway of rebamipide under voltage-clamped condition.

Adjuvants	J_m (transcellular)	Ratio to control	${}_0J_d$ (paracellular)	Ratio to control
Control	0.275	1	0.218	1
3 mM SPM + 10 mM STC	1.294**	4.71	0.609**	2.80

The values of J_m and ${}_0J_d$ were obtained by least-squares regression on data shown in Fig. 4.
** $p < 0.01$, compared with control. Statistical analysis was carried out by *F*-test.

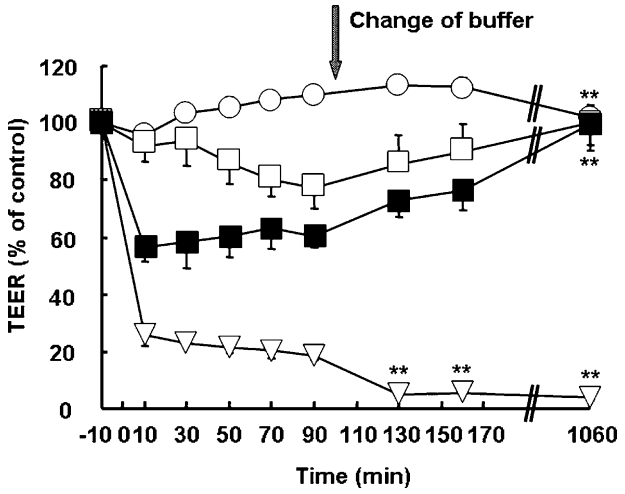

Fig. 3. Reversibility of TEER lowering effect by SPM and STC in Caco-2 cell monolayers. The treatment solution was changed to fresh Ringer's solution without any adjuvant. Keys: (○) control; (□) 3 mM SPM + 7 mM STC; (■) 3 mM SPM + 10 mM STC; (▽) 50% ethanol. Results are expressed as the mean with the bar showing S.D. of three to four experiments. **$p < 0.01$, compared with the corresponding values at 90 min.

marized in Fig. 5, it can be estimated that 56% and 44% of total flux of rebamipide would be ascribed to transcellular and paracellular routes, respectively, under short-circuit condition. When total flux was increased to 3.88 times of control by 3 mM SPM and 10 mM STC, 68% of total flux would be attributed to the transport *via* transcellular route.

The mechanisms of the permeability enhancement by SPM and STC through transcellular and paracellular pathways remain to be clarified. One possibility to enhance the transcellular permeability would be the increase in the membrane fluidity by SPM and STC. The

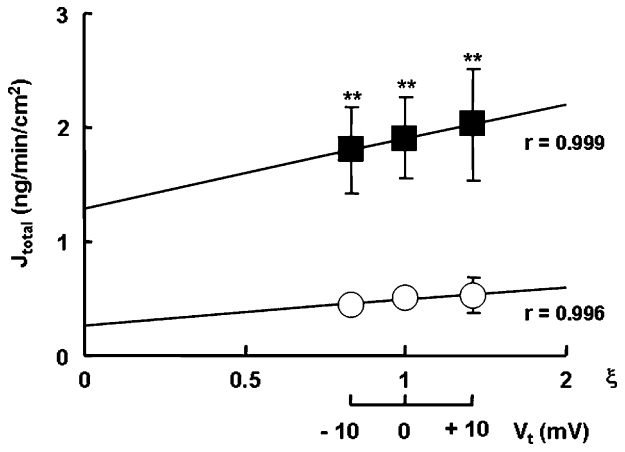

Fig. 4. Effect of combinatorial use of SPM with STC on permeability of rebamipide under voltage-clamped conditions in Caco-2 cell monolayers. Keys: (○) control; (■) 3 mM SPM + 10 mM STC. Solid lines were obtained by least-squares regression. Results are expressed as the mean with the bar showing S.D. of three to four experiments. **$p < 0.01$, compared with control.

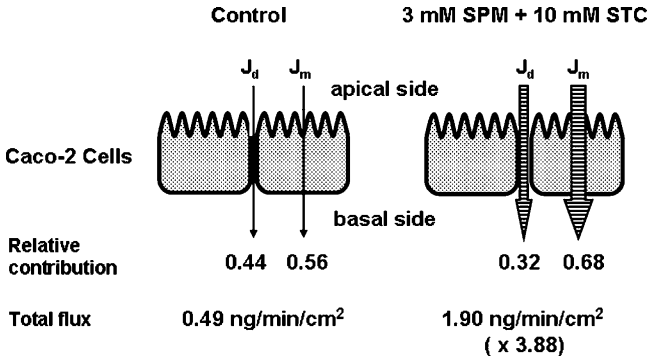

Fig. 5. Schematic presentation of pathways contributing to rebamipide transport enhanced by combinatorial use of SPM with STC.

increasing effects of several absorption enhancers were ascribed to the enhanced membrane fluidity (Muranushi et al., 1981; Higaki et al., 1988; Tomita et al., 1988). As to polyamines, Johnson et al. (1995) reported that SPM did not influence the membrane fluidity in the experiment using brush border membrane vesicles of rabbit small intestine. However, both the experimental condition and SPM concentration were quite different from those in the present study, and furthermore they did not examine the effect of co-existence with STC. Therefore, some changes in the membrane fluidity might be expected in the case of combinatorial use of SPM with STC.

Although several mechanisms behind the increase in paracellular permeability have been suggested (Ward et al., 2000), one of the possible mechanisms would be the opening of the tight junction by myosin light-chain kinase (MLCK) activated by increased intracellular Ca^{2+} level (Lindmark et al., 1998; Ward et al., 2000). The phosphorylation of MLC by activated MLCK induces the contraction of the actin-myosin filament, resulting in loosening the tight junction. This pathway involves an inositol-triphosphate (IP_3)-dependent Ca^{2+} release from the endoplasmic reticulum. As it has been reported that polyamines increased intracellular Ca^{2+} concentration *via* the IP_3 pathway in rat large intestine (Cheng et al., 2004), this mechanism would be involved in the increased paracellular transport of rebamipide by SPM and STC. On the other hand, recently, the tight junction has been clarified to consist of proteins such as occludin, claudin and junctional adhesion molecule (Daugherty and Mrsny, 1999; Guo et al., 2005), and it has been reported that some enhancers changed the distribution of these proteins (Coyne et al., 2003). As intrinsic polyamines have been reported to regulate occludin protein in intestinal epithelial cells (Guo et al., 2005), polyamines added externally might also affect such proteins of the tight junction. Including the possibilities mentioned above, the detailed mechanisms of the synergistic enhancement effect of SPM and STC will be investigated in our next study.

In conclusion, SPM and STC synergistically enhanced the transport of rebamipide across Caco-2 cell monolayer as in the case of rat intestine. Rebamipide absorption was enhanced *via* both paracellular and transcellular pathways, and the latter was found to contribute to the absorption improvement more than the former by the voltage-clamped study.

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