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Enhanced Permeability of Insulin across the Rat Intestinal Membrane by Various Absorption Enhancers: Their Intestinal Mucosal Toxicity and Absorption-enhancing Mechanism of n-Lauryl-β-D-maltopyranoside

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Abstract

We have examined the in-vitro permeability characteristics of insulin in the presence of various absorption enhancers across rat intestinal membranes and have assessed the intestinal toxicity of the enhancers using an in-vitro Ussing chamber method. The absorption enhancing mechanism of n-lauryl-β-D-maltopyranoside was studied also.

The permeability of insulin across the intestinal membranes was low in the absence of absorption enhancers. However, the permeability was improved in the presence of enhancers such as sodium glycocholate and sodium deoxycholate in the jejunum, and sodium glycocholate, sodium deoxycholate, n-lauryl-β-D-maltopyranoside, sodium caprate and ethylenediaminetetraacetic acid (EDTA) in the colon. Overall, the absorption enhancing effects were greater on the colonic membrane than on the jejunal membrane. The intestinal membrane toxicity of these enhancers was characterized using the release of cytosolic lactate dehydrogenase from the colonic membrane. A marked increase in the release of lactate dehydrogenase was observed in the presence of sodium deoxycholate and EDTA. The release of lactate dehydrogenase in the presence of these absorption enhancers was similar to that seen with sodium dodecyl sulphate (SDS), used as a positive control, indicating high toxicity of these enhancers to the intestinal membrane. In contrast, sodium glycocholate and sodium caprate caused minor releases of lactate dehydrogenase, similar to control levels, suggesting low toxicity. In addition, the amount of lactate dehydrogenase in the presence of n-lauryl-β-D-maltopyranoside was much less than that seen with sodium deoxycholate, EDTA and SDS. Therefore, sodium glycocholate, sodium caprate and n-lauryl-β-D-maltopyranoside are useful absorption enhancers due to their high absorption enhancing effects and low intestinal toxicity.

To investigate the absorption enhancing mechanisms of n-lauryl-β-D-maltopyranoside, the transepithelial electrical resistance (TEER), voltage clamp experiments and the circular dichroism spectra were studied. n-Lauryl-β-D-maltopyranoside decreased the TEER values in a dose-dependent manner, suggesting that the enhancer may open the tight junctions of the epithelium, thereby increasing the permeability of insulin via a paracellular pathway. This speculation was supported by the findings that 20 mM n-lauryl-β-D-maltopyranoside produced a greater increase in the paracellular flux rate than in the transcellular flux rate by the voltage clamp studies. Evaluating the circular dichroism spectra we found that insulin oligomers were not dissociated to monomers by the addition of n-lauryl-β-D-maltopyranoside, but dissociation did occur with the addition of sodium glycocholate. Thus, the dissociation of insulin was not a major factor in the absorption enhancing effect of n-lauryl-β-D-maltopyranoside.

These findings provide basic information to select the optimal enhancer for the intestinal delivery of peptide and protein drugs including insulin.

In general, peptides and proteins are not effective when administered orally and must be administered by injection. This is due to the poor absorption characteristics of peptides and proteins from the gastrointestinal tract as a result of their extensive degradation by the intestinal mucosa. To overcome this problem, the use of absorption enhancers is a useful approach. Various kinds of absorption enhancers including surfactants, bile salts, chelating agents and fatty acids have been used to enhance the transport of peptides and proteins (Lee et al 1991). However, some of these absorption enhancers can cause damage and irritate the intestinal mucosal membrane. This is a limiting factor for clinical use. Therefore, an absorption enhancer with high effectiveness and low mucosal toxicity should be selected for clinical use.

The effectiveness of absorption enhancers has been evaluated by various absorption experiments. We have reported previously that the absorption of phenol red and fluorescein isothiocyanate-dextrans, water-soluble model compounds, was enhanced in the presence of various absorption enhancers by an in-situ closed-loop method and by an in-vitro Ussing chamber method (Uchiyama et al 1996; Yamamoto et al 1996; Sugiyama et al 1997). We have shown (Sugiyama et al 1997) a good correlation between the in-situ closed-loop method and the in-vitro Ussing chamber method, indicating that the in-vitro Ussing chamber method is useful in estimating the permeability characteristics of drugs.

In this study, insulin was chosen as a model peptide and the effects of various absorption enhancers on its intestinal transport were examined using an in-vitro Ussing chamber. The absorption enhancers studied were sodium glycocholate, sodium taurocholate, sodium deoxycholate, n-lauryl-β-D-maltopyranoside, sodium caprate, and disodium ethylenediaminetetraacetic acid (EDTA), all used at a concentration of 20 mM. This concentration was selected to enable comparison of their promoting effects under the same conditions. Also, we investigated the local toxicity of absorption enhancers, and the absorption enhancing mechanism of n-lauryl-β-D-maltopyranoside.

Materials and Methods

Materials

Insulin, sodium glycocholate, sodium taurocholate and *n*-lauryl-β-D-maltopyranoside were purchased from Sigma Chemical Co. (St Louis, MO). Sodium deoxycholate was obtained from Wako Pure Chemical Industries Co. (Osaka, Japan). EDTA was obtained from Nacalai Tesque Inc. (Kyoto, Japan). Sodium caprate was purchased from Tokyo Kasei Industries Co. (Tokyo, Japan). Eastman Kodak Co. (Rochester, NY) kindly supplied 6-carboxyfluorescein.

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Drug solution preparation

Insulin was dissolved in a modified Ringer's solution adjusted to pH 7·4 to yield a final concentration of 0·5 mM. In certain experiments, the dosing solutions were added to absorption enhancers at concentrations of 20 mM.

Absorption experiments

Absorption experiments were performed in a modified Ussing chamber using stripped rat intestine for 3 h (Yodoya et al 1994; Asada et al 1995; Tanaka et al 1996; Uchiyama et al 1998). Male Wistar rats, 200–250 g, were used. The intestine was excised and rinsed in phosphate buffer solution. The experimental segments were obtained and the underlying muscularis was removed before mounting in a modified Ussing chamber, in which a surface area of 0·2826 cm^2 was exposed. Each site was defined as described below. The duodenal segment was the first 10-cm portion from the stomach. The ileal segment was the final 10-cm portion of the small intestine. The residual intestine was used as the jejunum. Modified Ringer's solution (2·5 mL) was added to the serosal side. An equal volume of drug solution was added to the mucosal side. Mixing was performed by bubbling with 95%O_2–5%CO_2. At predetermined times, 200-μL samples were taken from the serosal side and assayed by HPLC. In the pretreatment studies, the intestinal mucosa was pretreated with absorption enhancers for 10 min before mounting in the chamber and the surface of the intestinal mucosa was washed with modified Ringer's solution. Then, the mucosa was mounted in the chamber and insulin solution was introduced to the mucosal site. The apparent permeability coefficient (P_{app}) of each compound was calculated from the linear portion of a plot of penetrant accumulated vs time using the following equation:

$$P_{app} = dX_R/dT \cdot 1/A \cdot C_0 \quad (1)$$

where P_{app} is the apparent permeability coefficient ($cm\,s^{-1}$), X_R is the amount of the drug in the receptor side (mol), A is the diffusion area (cm^2), and C_0 is the initial apparent concentration of drugs in the donor side ($mol\,mL^{-1}$). In this experiment, degradation of insulin was negligible on the donor

side at an insulin concentration of 0·5 mM. The viability of intestinal membrane during the test period was monitored by measuring the transport of trypan blue dye. There was no transport of dye during the incubation, confirming that the viability of the intestinal membrane was maintained during the transport experiment.

Assessment of membrane damage

To evaluate membrane damage, the release of lactate dehydrogenase from the colonic membrane was measured. Lactate dehydrogenase is a cytosolic enzyme, and its presence in the apical compartment is generally regarded as evidence of cell membrane damage (Schasteen et al 1992). For lactate dehydrogenase studies, 50-μL samples were withdrawn from the donor site at the end of the experiments. The amount of lactate dehydrogenase released from the intestinal membrane was determined with a Testwako lactate dehydrogenase kit (Wako Co., Japan). Absorption enhancers used in this experiment did not interfere with the lactate dehydrogenase assay.

Reversed-phased liquid chromatography

Samples were assayed on a reversed-phase HPLC system containing 5-μm Cosmosil (4·6 mm × 15 cm) particles in an analytical column from Nacalai Tesque, a Shimadzu LC-10 pump system, a Shimazu LC-10 autoinjector, a Shimadzu LC-10 detector and a Shimadzu CR-6A integrator. The mobile phase was a mixture of acetonitrile and water containing 0·1% trifluoroacetic acid (mobile phase A 10 : 90, mobile phase B 60 : 40). The proportion of the mobile phase B was increased linearly from 25 to 60% during the first 20 min, and increased linearly to 70% for another 3 min. The flow rate was 1·0 mL min^{-1}. The UV detector was set at 210 nm. Concentrations were determined using external standards.

Circular dichroism studies

A circular dichroism spectropolarimeter (JASCO Model J500, Japan Spectroscopic Co., Tokyo) was utilized as described by Asada et al (1994). Optical resolution of the spectra was obtained using a 1-cm pathlength quartz cuvette. The molecular ellipticity was calculated from the equation:

$$[\theta]_\lambda = (\theta_\lambda/(C \times l)) \times 100 \qquad (2)$$

where θ_λ is the observed ellipticity at wavelength λ (°), C is the insulin concentration (M), and l is the pathlength (cm).

Electrical measurements

The transmucosal electrical potential difference (PD) and the short circuit current (Isc) were measured at 10-min intervals. The transepithelial electrical resistance (TEER) was calculated by Ohm's law. TEER reached steady-state about 20 min after mounting the membrane, at which point the experiment was started. TEER was monitored for 60 min. For subsequent experiments, we used the membranes whose TEER were in the range of 70–120 Ohm · cm^2.

Voltage-clamp experiment

In the voltage-clamp experiments, 6-carboxyfluorescein, a poorly absorbable and stable compound, was chosen as a model compound. The underlying muscularis of colonic membrane was removed before mounting in a modified Ussing chamber, in which a surface area of 0·75 cm^2 was exposed. After introducing 11 mL modified Ringer's solution containing 6-carboxyfluorescein to the donor site, PD was clamped immediately to arbitral values (−10–+10) by applying electric fields externally, and this voltage-clamp condition was maintained throughout the experiment (Yamashita et al 1984, 1985, 1988). At predetermined times, 1-mL samples were taken from the serosal side. The concentration of 6-carboxyfluorescein was determined on a spectrofluorometer using the excitation and emission wavelengths of 490 and 520 nm, respectively.

According to the theory of Frizzel & Schultz (1972), the transmembrane flux of ionized molecules via the paracellular pathway (J_d) depends on the potential difference across the membrane (V_t) and may be expressed as:

$$J_d = {}_0J_d \cdot \exp(-zFV_t/2RT) \qquad (3)$$

where the subscript 0 refers to the short-circuit condition, and z, F, R and T have their usual meanings. The flux through the transcellular pathway (J_m) is thought to be independent of V_t, and the total flux (J_t) is represented as:

$$J_t = J_m + J_d = J_m + {}_0J_d \cdot \xi \qquad (4)$$

where $\xi = \exp(-zFV_t/2RT)$.

Statistical analyses

Results are expressed as the mean ± s.e. and statistical significance was assessed by Student's *t*-test.

Results

Regional differences in the transport of insulin

A regional difference in insulin transport was observed (Figure 1). The permeability coefficients of insulin across the jejunum and ileum were very similar, however little insulin penetrated the colon. The order of insulin transport was jejunum = ileum > duodenum > colon. However, the transport percentage of insulin up to 3 h was generally poor in all regions without any absorption enhancer.

The effects of various absorption enhancers on the transport of insulin

Table 1 shows the apparent permeability coefficients of insulin in the presence or absence of various absorption enhancers. The transport of insulin across the jejunal membrane was significantly enhanced in the presence of sodium deoxycholate and sodium glycocholate. In the colon, sodium glycocholate, sodium deoxycholate, n-lauryl-β-D-maltopyranoside, EDTA and sodium caprate significantly enhanced insulin transport. Overall, the absorption enhancing effects of these absorption enhancers were greater in the colonic membrane than in the jejunal membrane. Therefore, the colonic membrane was selected to assess the membrane toxicity of these enhancers and their absorption enhancing mechanisms.

Assessment of colonic membrane toxicity by absorption enhancers

Table 2 shows the release of lactate dehydrogenase, a biological membrane damage marker, from the colonic membrane in the presence or absence of various absorption enhancers. Sodium deoxycholate and EDTA caused an approximate 4-fold increase in lactate dehydrogenase release compared with the control. The release of lactate dehydrogenase in the presence of these absorption enhancers was similar to that seen with sodium dodecyl sulphate (SDS), used as a positive control, indicating high intestinal membrane toxicity of

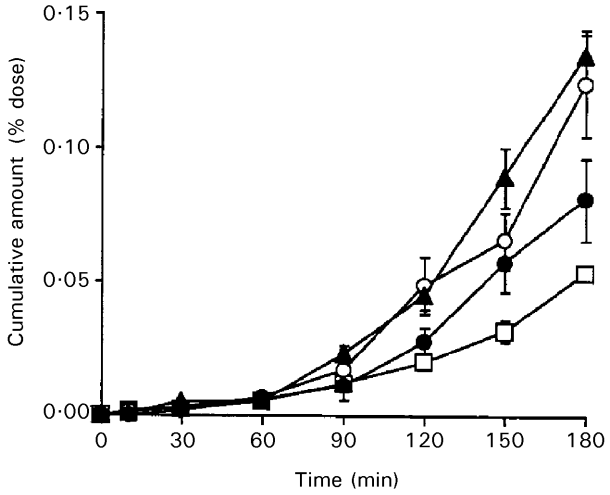

Figure 1. Transport of insulin across the various intestinal membranes (duodenum ●, jejunum ▲, ileum ○, and colon □). Each value represents the mean ± s.e. of at least three experiments.

Table 1. Transport of insulin across the jejunal or colonic membrane in the presence of various absorption enhancers.

	% Penetrated up to 3 h	P_{app} ($\times 10^{-6}$ cm s^{-1})	Ratio
Jejunum			
Control	0·134 ± 0·010	1·553 ± 0·132	1·00
Sodium glycocholate	0·360 ± 0·027	3·884 ± 0·421*	2·50
Sodium taurocholate	0·172 ± 0·009	1·603 ± 0·103	1·03
Sodium deoxycholate	0·529 ± 0·027	6·489 ± 0·491**	4·18
n-Lauryl-β-D-maltopyranoside	0·137 ± 0·020	1·619 ± 0·203	1·04
Sodium caprate	0·131 ± 0·007	1·502 ± 0·414	0·97
EDTA	0·277 ± 0·034	3·107 ± 0·468	2·00
Colon			
Control	0·053 ± 0·002	0·651 ± 0·086	1·00
Sodium glycocholate	0·130 ± 0·017	1·325 ± 0·110*	2·03
Sodium taurocholate	0·103 ± 0·013	1·006 ± 0·089	1·55
Sodium deoxycholate	0·391 ± 0·058	4·832 ± 0·582*	7·42
n-Lauryl-β-D-maltopyranoside	0·318 ± 0·027	3·886 ± 0·402*	5·97
Sodium caprate	0·173 ± 0·047	1·630 ± 0·245*	2·50
EDTA	0·594 ± 0·086	6·719 ± 0·491**	10·32

Each value represents the mean ± s.e. of at least three experiments. *$P < 0.05$, **$P < 0.01$ compared with control.

these enhancers. In contrast, sodium glycocholate and sodium caprate caused a minor release of lactate dehydrogenase, similar to control levels. The amount of lactate dehydrogenase in the presence of n-lauryl-β-D-maltopyranoside was much less than that with sodium deoxycholate, EDTA and SDS. Therefore, sodium glycocholate, sodium caprate and n-lauryl-β-D-maltopyranoside are useful absorption enhancers due to their low intestinal toxicity.

Effect of pretreatment with n-lauryl-β-D-maltopyranoside on the transport of insulin

We reported previously (Murakami et al 1992) that n-lauryl-β-D-maltopyranoside had a good enhancing ability with low toxicity in the in-situ closed-loop method. In this study, n-lauryl-β-D-maltopyranoside was chosen as a useful absorption enhancer, and the reversibility of n-lauryl-β-D-maltopyranoside was studied in the colon (Figure 2). No significant increase in the transport of insulin was observed by pretreatment with 20 mM n-lauryl-β-D-maltopyranoside for 10 min, as compared with the control, although we found a significant increase in the transport of insulin by the co-administration of n-lauryl-β-D-maltopyranoside. However, the transport of insulin was significantly increased by pretreatment with 20 mM sodium deoxycholate as a positive control. Thus, the absorption enhancing effect of n-lauryl-β-D-maltopyranoside was reversible, while sodium deoxycholate caused irreversible damage to the intestinal membrane.

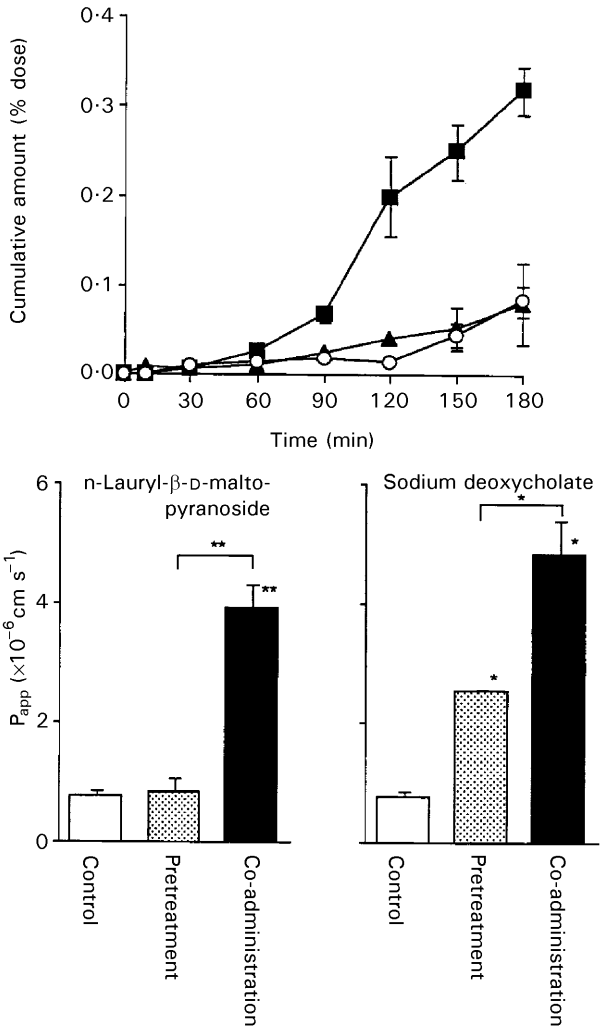

Figure 2. Effects of pretreatment with absorption enhancers on the transport of insulin across the colonic membrane. Each value represents the mean ± s.e. of at least three experiments. $^*P < 0.05$, $^{**}P < 0.01$, compared with the control. Control ○, pretreatment with *n*-lauryl-β-D-maltopyranoside ▲, co-administration with *n*-lauryl-β-D-maltopyranoside ■.

Effects of n-lauryl-β-D-maltopyranoside on the TEER

To clarify the absorption enhancing mechanisms of n-lauryl-β-D-maltopyranoside, the TEER of colonic membrane in the presence of various concentrations of n-lauryl-β-D-maltopyranoside was measured. As shown in Figure 3, n-lauryl-β-D-maltopyranoside decreased the TEER value of colonic membrane in a dose dependent manner. The transport of insulin increased as co-administered n-lauryl-β-D-maltopyranoside increased (data not shown). This finding suggested that the absorption enhancing mechanism of n-lauryl-β-D-maltopyranoside partly includes opening the tight junctions of the epithelium via a paracellular route.

Table 2. Release of lactate dehydrogenase from the colonic membrane in the presence of various absorption enhancers at a concentration of 20 mM.

	Lactate dehydrogenase (int. units L^{-1})	Ratio
Control	61·0 ± 5·73	1·00
Sodium glycocholate	70·3 ± 11·9	1·15
Sodium caprate	66·5 ± 15·6	1·09
Sodium deoxycholate	257 ± 37·6**	4·22
EDTA	277 ± 59·2**	4·54
n-Lauryl-β-D-maltopyranoside	113 ± 16·5*	1·84
Sodium dodecyl sulphate	287 ± 24·2**	4·70

Each value represent the mean ± s.e. of at least three experiments. $^*P < 0.05$, $^{**}P < 0.01$ compared with control.

Voltage-clamp experiments

The mucosal to serosal flux rates of 6-carboxyfluorescein in the presence of n-lauryl-β-D-maltopyranoside and EDTA were measured under

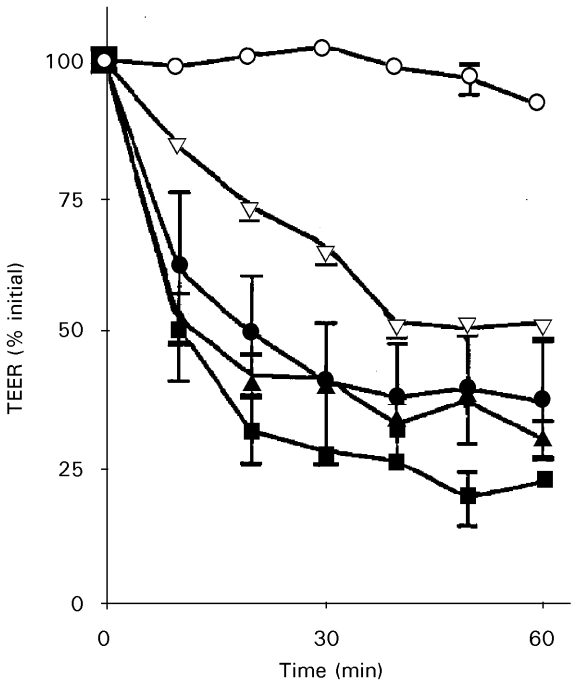

Figure 3. Effects of various concentrations of n-lauryl-β-D-maltopyranoside (control ○, 1 mM ▽, 5 mM ●, 10 mM ▲, 20 mM ■) on transepithelial electrical resistance (TEER) of the colonic membrane. Initial value of TEER was $95{\cdot}5 \pm 5{\cdot}11$ Ohm·cm^2. TEER values (% initial value) in the table were measured at 60 min. Each value represents the mean ± s.e. of at least three experiments. $*P < 0{\cdot}05$, $***P < 0{\cdot}001$, compared with the control.

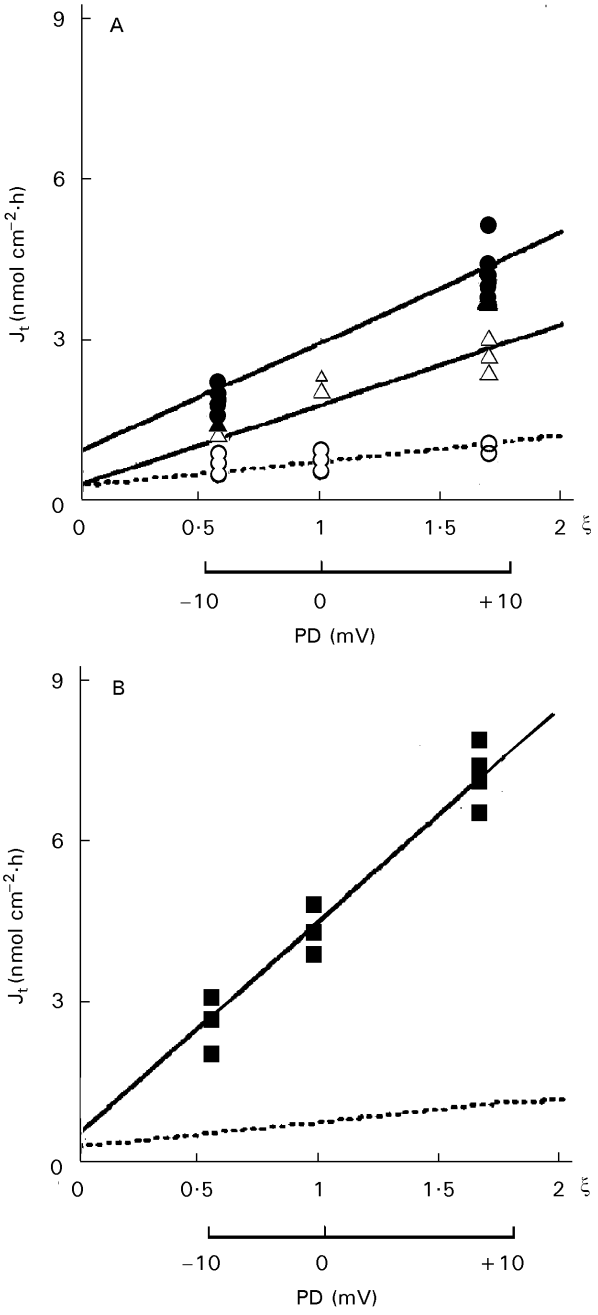

Figure 4. Effects of transmural potential difference (V_t) on the flux rates (J_t) of 6-carboxyfluorescein in the presence of n-lauryl-β-D-maltopyranoside (A) or EDTA (B). The dotted line represents the 6-carboxyfluorescein flux rate when no enhancer was added. The intercept and the slope of each line are summarized in Table 3. The explanation of ξ, the abscissa, is described in the text. Control ○, n-lauryl-β-D-maltopyranoside 1 mM △, n-lauryl-β-D-maltopyranoside 20 mM ●, EDTA 20 mM ■.

various externally applied potential differences and were plotted against the ξ (Figure 4A and B). The intercept and the slope of each line are summarized in Table 3, and represent the flux through the transcellular route and the paracellular route in the short circuit condition, respectively. It appears that 6-carboxyfluorescein penetrates the intestinal membrane by a paracellular pathway in the absence of absorption enhancers (dotted line in Figure 4A and B). n-Lauryl-β-D-maltopyranoside (1 mM) produced 3·2-fold increase in the paracellular flux, while the transcellular flux was lower (Figure 4A, Table 3). The addition of 20 mM n-lauryl-β-D-maltopyranoside to the mucosal solution produced a 2·2-fold increase in the transcellular flux rate, while the paracellular flux was 4·8-times higher than the control (Figure 4A, Table 3). EDTA induced an 8·5-fold selective increase in the paracellular flux (Figure 4B, Table 3).

Table 3. Transcellular and paracellular flux rates of 6-carboxyfluorescein (100 μM) in the presence of n-lauryl-β-D-maltopyranoside and EDTA by the analyses of voltage clamp experiments.

		Flux rates of 6-carboxyfluorescein (nmol cm^{-2} · h)			
		Transcellular	Ratio	Paracellular	Ratio
Control		0·28 ± 0·16	1·0	0·47 ± 0·13	1·0
n-Lauryl-β-D-maltopyranoside	1 mM	0·30 ± 0·02	1·1	1·51 ± 0·02*	3·2
	20 mM	0·97 ± 0·13*	3·5	2·00 ± 0·11**	4·3
EDTA	20 mM	0·46 ± 0·27	1·6	4·00 ± 0·23***	8·5

Each value represents the mean ± s.e. *$P < 0·05$, **$P < 0·01$, ***$P < 0·001$, compared with control.

Effect of n-lauryl-β-D-maltopyranoside on the association characteristics of insulin

To clarify whether n-lauryl-β-D-maltopyranoside affects the dissociation of insulin oligomers to monomers, the association characteristics of insulin were investigated by circular dichroism. Sodium glycocholate, which dissociates insulin oligomers to monomers, was used as a positive control. As shown in Figure 5, the negative maximum was not attenuated on the circular dichroism spectra of insulin in the presence of n-lauryl-β-D-maltopyranoside at various concentrations. Sodium glycocholate did attenuate the negative maximum of insulin. Thus, the dissociation of insulin was not a major factor in the absorption enhancing effect of n-lauryl-β-D-maltopyranoside.

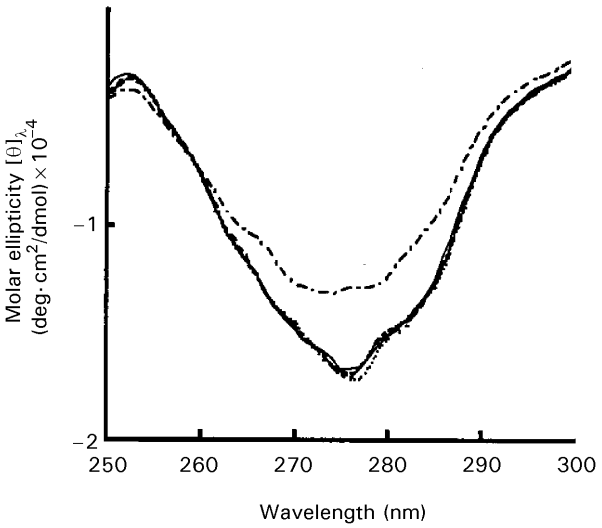

Figure 5. Effects of various concentrations of n-lauryl-β-D-maltopyranoside on the circular dichroic spectra of 0·5 mM insulin. Control ——, sodium glycocholate –·–·–, n-lauryl-β-D-maltopyranoside · · · · ·.

Discussion

The order of insulin transport across the various intestinal membranes was jejunum = ileum > duodenum > colon. Schilling & Mitra (1990) reported that the transport of insulin across the duodenal membrane was much less than that across the jejunal and ileal membranes by the everted gut sac technique. In addition, Asada et al (1995) demonstrated that the permeability of insulin across the jejunal and ileal membranes was more than 2-times higher than that of the duodenum by an in-vitro Ussing chamber experiment. A similar regional difference was also obtained in the transport of ebiratide across intestinal membranes (Yamamoto et al 1997). Therefore, our present findings are consistent with previous findings. However, we had shown previously (Yamamoto et al 1994) that without absorption enhancers and protease inhibitors insulin was not absorbed from the small intestine by an in-situ closed-loop experiment, since digestive and proteolytic enzymes degraded insulin in the small intestine. In this study, degradation of insulin was negligible in the donor site at an insulin concentration of 0·5 mM. Consequently, the different absorption and stability characteristics of insulin in the small intestine may be due to different amounts of digestive and proteolytic enzymes between in-situ and in-vitro conditions. These enzymes are richly distributed in the small intestinal fluid in an in-situ experiment, whereas the mucosal fluid is washed away before starting the in-vitro Ussing chamber experiment. The reason for the regional differences in the transport of insulin was not clearly understood. Insulin may be transported across the intestinal membrane by both transcellular and paracellular

routes, although we did not determine the transport pathway of insulin in this experiment. Presumably, morphological differences in the thickness of mucous layers, the unstirred water layer, tightness and number of the tight junctions and membrane components between the small and the large intestine might have influenced the transport of insulin across the various intestinal membranes.

We found that sodium glycocholate and sodium deoxycholate effectively enhanced the transport of insulin across the jejunal and colonic membranes. n-Lauryl-β-D-maltopyranoside, sodium caprate and EDTA enhanced the transport of insulin only in the colonic membrane. It has been reported that bile salts enhance the permeability of various drugs and that they are known to have inhibitory actions on protease activity such as leucine aminopeptidase and other aminopeptidases (Hirai et al 1981). However, the enhancement effect of sodium glycocholate and sodium deoxycholate on the absorption of insulin might not be due to their protease inhibitory actions, because insulin itself was very stable even in the absence of any absorption enhancer in this study.

Unlike sodium glycocholate and sodium deoxycholate, n-lauryl-β-D-maltopyranoside significantly enhanced the transport of insulin in the colon, but not in the jejunum. The permeability of insulin coadministered with n-lauryl-β-D-maltopyranoside in the colonic membrane was about 6-times higher than in the control, although its jejunal permeability was almost the same as that in the control. This finding is consistent with our previous finding that n-lauryl-β-D-maltopyranoside enhanced the absorption of 6-carboxyfluorescein to a greater extent in the lower regions of the intestine (Murakami et al 1992). Similar regional differences were also obtained in the effect of n-lauryl-β-D-maltopyranoside on the transport of phenol red and ebiratide across the jejunal and colonic intestinal membranes by in-vitro transport studies (Sugiyama et al 1997; Yamamoto et al 1997).

The absorption enhancing effect of sodium caprate was also greater in the colonic membrane than in the jejunal membrane in this study. Tomita et al (1988) reported that the jejunal absorption of cefmetazole, a poorly absorbable antibiotic, was significantly enhanced by sodium caprate, but to a lesser extent than colonic membrane. Morishita et al (1993) reported that the enhancement effect of sodium caprate on insulin absorption was greater in the colon than in the jejunum. Thus, our current findings concur with those previous findings.

When these absorption enhancers are used in practice, it is essential that they do not affect the membrane structure and integrity of the epithelium. Various methods including morphological observation, haemolysis and release of biological markers have been utilized to evaluate the intestinal membrane toxicity on the epithelium (Swenson & Curatolo 1992). Of those methods, the release of biological markers such as protein, phospholipid and lactate dehydrogenase is suitable for quantitative analysis. Lactate dehydrogenase is a cytotoxic enzyme that is recovered from the extracellular compartment after membrane disruption and this method has been widely used to determine membrane toxicity. For these reasons, we examined membrane toxicity by measuring the release of lactate dehydrogenase in the presence of various absorption enhancers.

In the membrane toxicity experiments, release of lactate dehydrogenase was significantly increased in the presence of sodium deoxycholate and EDTA in the colonic membrane. Dawson et al (1960) reported that sodium deoxycholate, a hydrophobic bile salt, caused irreversible damage to the intestinal membrane by solubilizing and disrupting the membrane component, although its absorption enhancing effect was considerable. Similarly, Yamashita et al (1987) reported that a high concentration of EDTA causes functional damage to the intestinal membrane, while it opens the tight junctions of the epithelium due to its chelating activity with calcium at a lower concentration. Therefore, the high lactate dehydrogenase values in this study may be related to the toxicity of these absorption enhancers.

On the contrary, little or no release of lactate dehydrogenase was observed in the presence of n-lauryl-β-D-maltopyranoside, sodium glycocholate or sodium caprate, in this study. However, the release of lactate dehydrogenase in the presence of n-lauryl-β-D-maltopyranoside was slightly higher than that with sodium glycocholate or sodium caprate. Murakami et al (1992) reported that no apparent histological change was observed in the rectal mucosa by exposure to n-lauryl-β-D-maltopyranoside, which agrees with this study. In addition, Hirai et al (1981) reported that the absorption enhancing action of sodium glycocholate on the nasal absorption of insulin was reversible, and there was no marked histological change of the nasal epithelium by exposure to sodium glycocholate. Furthermore, sodium caprate is now clinically used in a commercial rectal suppository as an absorption enhancer for sodium ampicillin in Japan. These findings suggest that n-lauryl-β-D-maltopyranoside, sodium glycocholate and sodium caprate are the more effective and relatively less toxic absorption enhancers used in this study. This suggestion is also partly supported by the fact that n-lauryl-β-D-

maltopyranoside reversibly increased the transport of insulin, while sodium deoxycholate irreversibly increased the transport of insulin (Figure 2).

To characterize the change in membrane permeability, 6-carboxyfluorescein flux was measured under various levels of applied PD and divided into two individual components, paracellular and transcellular fluxes. We demonstrated that n-lauryl-β-D-maltopyranoside only affected the paracellular route at low concentration, and affected the transcellular flux dose-dependently. EDTA induced a selective increase in the paracellular flux, indicating that EDTA enhanced the membrane transport via a paracellular pathway. Yamashita et al (1987) reported that EDTA at 10 mM selectively enhanced the transport of sulphanilic acid via a paracellular pathway by a voltage-clamp study. Thus, our results with EDTA were consistent with those of Yamashita et al (1987). These results suggested that the primary effect of n-lauryl-β-D-maltopyranoside at low concentration in enhancing the membrane permeability was on the extracellular portion, probably the tight junction of the epithelial cells, and when the concentration of n-lauryl-β-D-maltopyranoside increased, it affected not only the paracellular transport of drugs, but also the transport of drugs via a transcellular pathway.

Circular dichroism was used to clarify whether n-lauryl-β-D-maltopyranoside affects the self-association characteristic of insulin. The antiparallel beta-structure formed by insulin association exhibits optical activity. The negative circular dichroism absorption at 276 nm represents the aromatic residues of phenylalanine and tyrosine in the B23–28 region of the antiparallel beta-structure. Therefore, the attenuation of a negative maximum at about 276 nm indicates the dissociation of insulin aggregates (Shao et al 1993). No attenuation was observed in the presence of n-lauryl-β-D-maltopyranoside at various concentrations. Sodium glycocholate attenuated the negative maximum at 276 nm, which is consistent with a previous report (Li et al 1992). Thus, the dissociation of insulin was not a major factor in the absorption enhancing effect of n-lauryl-β-D-maltopyranoside. However, Hovgaard et al (1996) reported that the radius of insulin increased from 27 Å at time zero to over 1000 Å after just one week, indicating macromolecular aggregation of insulin. Therefore, n-lauryl-β-D-maltopyranoside may be promising as a compound to inhibit further macromolecular aggregation of insulin after its dimer and hexamer formation.

These findings provide basic information to select the optimal enhancer for the intestinal delivery of peptide and protein drugs including insulin.

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