Evaluation of Insulin Permeability and Effects of Absorption Enhancers on Its Permeability by an *in Vitro* Pulmonary Epithelial System Using *Xenopus* Pulmonary Membrane

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The permeability of insulin across *Xenopus* pulmonary membrane and the effects of various absorption enhancers on insulin permeability were examined using an *in vitro* Ussing chamber technique. Absorption enhancers used in this study were sodium caprate (NaCap), sodium glycocholate (NaGC), sodium salicylate (NaSal) and ethylenediaminetetraacetic acid disodium salt (EDTA). The permeability of insulin across *Xenopus* pulmonary membrane significantly increased in the presence of NaCap and NaGC, while EDTA and NaSal did not enhance the permeability. In addition, the enhancing effect of NaGC increased as the concentrations of these enhancers increased. Transmembrane resistance (Rm) of *Xenopus* lung was markedly decreased in the presence of these enhancers, and NaCap showed a greater effect on Rm than NaGC. Furthermore, the amount of alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) released from the apical side of the *Xenopus* pulmonary membrane increased in the presence of these enhancers. These results indicate that NaCap and NaGC improve the pulmonary absorption of insulin, but they are toxic to the pulmonary membrane. These findings suggest that this method is useful for estimating the permeability characteristics of peptides across the pulmonary membrane and for evaluating the effects of various additives on their permeability and their membrane toxicity.

Key words insulin; pulmonary absorption; peptide delivery; absorption enhancer; *Xenopus* lung

The oral absorption of peptide and protein drugs is generally very poor. This has been attributed to the extensive metabolism of the drugs in the gastrointestinal tract and liver before entering the systemic circulation and their poor membrane permeability characteristics. Consequently, non-oral routes, such as the nasal,[1] buccal,[2] rectal,[3] and pulmonary[4] routes, are being investigated as alternative routes for the systemic delivery of these peptides. Among various alternatives, the pulmonary route is the most promising for delivering these drugs, since a number of drugs which are poorly absorbed from enteral and other mucosal sites are well absorbed from the lung due to its large absorptive surface area and the short distance of the air-blood exchange pathway.

From these standpoints, absorption experiments have been made on many peptides: insulin,[5—7] calcitonin,[8,9] G-CSF,[10] and leuprolide[11] after intrapulmonary administration. We have examined the pulmonary absorption of drugs including peptides and proteins by an *in situ* pulmonary absorption experiment.[6,7,9,12,13] However, the transport characteristics and mechanisms of these drugs have not been fully clarified by this *in situ* absorption method. Therefore, it is necessary to establish an *in vitro* pulmonary absorption experiment to evaluate the pulmonary absorption of drugs and elucidate their absorption mechanisms. The pulmonary epithelial surface of mammals is relatively inaccessible, however, due to the anatomic complexity of the multiply branched airways of their lungs, and this precludes mounting planar sheets of tissue in flux chambers. In a previous report, Wall *et al*. developed a new method to evaluate the pulmonary transport of drugs using an amphibian lung as a model of the mammalian lung.[14] *Xenopus* lung has a simpler structure than that of the mammalian. Moreover, it morphologically and physiologically resembles mammalian lung, including: similar composition and dimensions of the air-blood barrier,[15,16] active Na^+ absorption, with passive Cl^- secretion,[17,18] high transepithelial electrical resistance and surfactant production by pulmonary epithelial cells.[19,20] The above reasons allow this tissue to be mounted in Ussing chambers to study transport of peptides and other model compounds. In an earlier report, we revealed that this system was a convenient and effective method to examine the permeability of drugs and the effects of absorption enhancers.[21,22]

In this study, therefore, we examined the permeability characteristics of insulin across *Xenopus* pulmonary membrane by an *in vitro* Ussing chamber method. We selected insulin in this study, since it has been widely used as a model polypeptide to investigate the absorption characteristics following intrapulmonary administration.[4—6] We also examined the effects of various absorption enhancers on the permeability of insulin across this membrane. Furthermore, we described the effects of these enhancers on the electrophysiological parameters of *Xenopus* pulmonary membrane to clarify their absorption enhancing mechanisms. Finally, we evaluated the pulmonary membrane toxicity of the enhancers by measuring the release amount of marker enzymes from the apical side of the membrane.

MATERIALS AND METHODS

Materials Bovine insulin and sodium glycocholate (NaGC) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Disodium ethylenediaminetetraacetic acid (EDTA) and sodium salicylate (NaSal) were purchased from Nacalai Tesque, Japan. Sodium caprate (NaCap) was obtained from Tokyo Kasei Kougyou Co., Japan. Alkaline phosphatase (ALP) test-Wako and lactate dehydrogenase (LDH) test-Wako were purchased from Wako Pure Chemical Industries, Japan. All other chemicals were of analytical

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grade.

Animals Female South African clawed frogs (*Xenopus laevis*) were obtained from Shimizu Laboratory Supplies, Japan, and were kept in tap water at room temperature. Females are preferred to males because they are larger and their lung tissues are more easily mounted in the Ussing chamber.

Permeation Studies The permeability of insulin across the *Xenopus* pulmonary membrane was studied by the modified method of Wall *et al.*[14] Frogs (50—60 g) were anesthetized by ether, and the lungs exposed by a ventral incision. Lungs were excised by severing the tracheoglottis and placed in a Ringer solution (110.0 mM NaCl, 2.4 mM $KHCO_3$, 1.0 mM Ca-D-gluconate, 1.0 mM $MgSO_4$, 10.0 mM HEPES, pH 7.4). For preparation of a planar sheet of tissue, the lung was incised along the pathway of the large pulmonary artery, and if necessary, connecting septa were cut to unfold the lung sac; the tissue was washed gently with Ringer solution. The lungs were mounted in Ussing chambers. A soft silicone O-ring covered with a thin film of high vacuum grease was used on each surface of the tissue to minimize the possible edge effects. The tissue was bathed with amphibian Ringer solution on both sides at room temperature. The reservoir was gassed continuously with 95% O_2 and 5% CO_2 in order to mix each solution and maintain the membrane viability. The entire system was maintained at room temperature throughout the experiment. The viability of pulmonary membrane during the test period was monitored by measuring and calculating the electrophysiological parameters including potential differences (PD), short circuit current (Isc) and membrane resistance (Rm). There was no significant change in these parameters, confirming that the viability of the pulmonary membrane was maintained during the transport studies.

The tissue was equilibrated for 20—30 min after being mounted in chambers prior to the transport studies. After the equilibration period, 2.5 ml of Ringer solution was added to the reservoir bathing the serosal side. An equal volume of 0.5 mM insulin solution was added to the mucosal side. At predetermined times up to until 3 h, 200 μl of solution was sampled from the serosal side and immediately replaced by an equal volume of buffer solution. These samples were analyzed by reversed phase HPLC. To assay insulin, the gradient system was programmed by increasing the proportion of mobile phase B from 25 to 70% within 40 min. The mobile gradient phase was run at a flow rate of 1.0 ml/min. This phase was a mixture of acetonitrile and water containing 0.1% TFA (mobile phase A; 10 : 90, mobile phase B; 40 : 60). An ultraviolet detector was set at 210 nm.

Analysis Apparent permeability coefficient (Papp) estimated from the linear portion of the permeation profile was calculated by the relationship:

$$\text{Papp} = dX_R/dt \cdot 1/A \cdot C_0$$

where Papp is the apparent permeability coefficient (cm/sec), X_R is amount of drug (mg), A is the diffusion area (0.2826 cm^2) and C_0 is the initial concentration of drugs (0.5 mM) in the donor side.

Electrophysiological Parameters The lung tissue was equilibrated for 20—30 min after being mounted in chambers. After the equilibration period, 11 ml of Ringer solution was added to the reservoir on both sides. PD with reference to the mucosal bathing and Isc were measured between two salt agar bridges (3% agar in 150 mM NaCl) connected *via* an electrode to a short circuit amplifier. These parameters were measured every 10 min. Rm was calculated from the PD and Isc values according to Ohm's law.

Evaluation of Membrane Toxicity To evaluate membrane damage by absorption enhancers, the release amount of alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) released from the *Xenopu*s pulmonary membrane was measured in the donor solution. ALP is a marker enzyme of apical membrane of the epithelium, while LDH is a cytosolic enzyme, and their presence in the apical compartment is generally regarded as evidence of cell membrane damage.[23] At the end of the transport studies, 200 μl of donor solution was sampled from the donor side. The amount of ALP and LDH from the pulmonary membrane was determined with a test-Wako alkaline phosphatase kit and a test-Wako lactate dehydrogenase kit (Wako Pure Chemical Industries, Co., Japan), respectively. Absorption enhancers did not interfere with the assay of these enzymes.

Statistical Data Analysis Results are expressed as the mean±S.E. Statistical significance between treatments was determined according to Student's *t*-test for comparison of means.

RESULTS

Transport of Insulin across *Xenopus* Pulmonary Membrane Figure 1 shows the time-course of insulin permeability across *Xenopus* pulmonary membrane for 3 h in the presence or absence of various absorption enhancers. As shown, the permeability of insulin across the membrane was poor without any absorption enhancer. However, a significant increase in insulin permeability was observed in the presence of NaCap and NaGC, although we found no absorption enhancing effect on the permeability of insulin in the presence of EDTA or NaSal. Among these enhancers, NaCap was the most effective adjuvant for enhancing insulin permeability across the *Xenopus* pulmonary membrane at the same concentration (10 mM). Table 1 summarizes the apparent permeability coefficient (Papp) of insulin across *Xenopus* pulmonary membrane in the presence or absence of various absorption enhancers. The permeability of insulin was signifi-

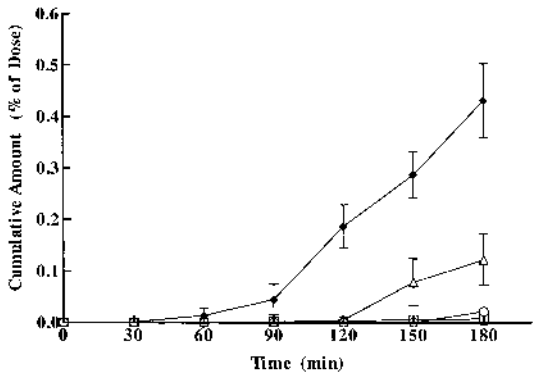

Fig. 1. Permeation Profiles of Insulin across *Xenopus* Pulmonary Membrane in the Presence of Various Absorption Enhancers

The insulin concentration in the donor side was 0.5 mM. Each point represents the mean±S.E. of at least three experiments. Keys: (○) control; (◆) 10 mM NaCap; (△) 10 mM NaGC; (□) 10 mM NaSal; (▲) 10 mM EDTA.

cantly improved by the co-administration of NaCap and NaGC. The Papp values of insulin with NaCap and NaGC were 67.4 and 32.9 times higher as compared with the control. However, we found no significant increase in the permeability of insulin in the presence of EDTA or NaSal. We also examined the effect of various concentrations of NaGC and NaCap on insulin permeability across the *Xenopus* pulmonary membrane. As shown in Fig. 2, the Papp value of insulin increased as the concentrations of these absorption enhancers increased, although the absorption enhancing effect of NaCap at 20 mM was almost the same as that at 10 mM.

Effects of NaCap and NaGC on the Rm Values of *Xenopus* Pulmonary Membrane We measured the Rm value of *Xenopus* pulmonary membrane in the presence of various additives for 2 h. As shown in Fig. 3, the Rm value remarkably decreased in the presence of 20 mM NaCap, 10 mM NaCap and 20 mM NaGC, although 10 mM NaGC only slightly reduced this value. Overall, the decreasing effect of NaCap on the Rm value was greater than that of NaGC at the same concentrations.

Effects of NaCap and NaGC on the Release of ALP and LDH Activities The effects of NaCap and NaGC on the amount of ALP and LDH released from the apical side of *Xenopus* pulmonary membrane were studied to estimate the membrane toxicity of these enhancers. As shown in Fig. 4(A), the release of ALP was markedly increased in the presence of 10 or 20 mM NaCap, while we found a slight increase in the release of ALP in the presence of 10 or 20 mM NaGC. Similarly, both of these enhancers increased the release of LDH activity in the apical side of the pulmonary membrane (Fig. 4(B)), suggesting their toxicity to the *Xenopus* pulmonary membrane.

Table 1. Effect of Various Enhancers (10 mM) on Apparent Permeability Coefficient Values of Insulin across the *Xenopus* Pulmonary Membrane

Enhancer	Papp ($\times 10^7$ cm/s)
Control	0.88±0.41
NaCap	59.35±10.95**
NaGC	28.97±11.64*
Na_2EDTA	0.92±0.92 *a)*
NaSal	0.98±0.74 *a)*

a) No significant. * $p<0.05$, ** $p<0.01$, compared with the control. Results are expressed as the mean±S.E. of 3 experiments. The apparent permeability coefficient (Papp) of each compound was calculated from the linear portion of a plot of penetrant accumulated *versus* time.

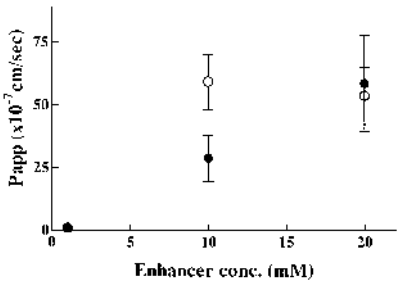

Fig. 2. Effect of Various Concentrations of NaCap and NaGC on Insulin Permeability across *Xenopus* Pulmonary Membrane

The insulin concentration in the donor side was 0.5 mM. Each point represents the mean±S.E. of at least three experiments. Keys: (○) NaCap; (●) NaGC.

DISCUSSION

The present study demonstrated that the permeability of insulin across the *Xenopus* pulmonary membrane was very poor without any absorption enhancer. The Papp value of insulin across *Xenopus* pulmonary membrane was 0.88±0.41 ($\times 10^{-7}$ cm/s), and this value was much less than that of rat jejunum or rat colon.[24] In an earlier report, we showed that transepithelial electrical resistance of *Xenopus* pulmonary membrane was about 700 $\Omega \cdot cm^2$, and this value was higher than that of both jejunum (*ca.* 100 $\Omega \cdot cm^2$) and colon (*ca.*

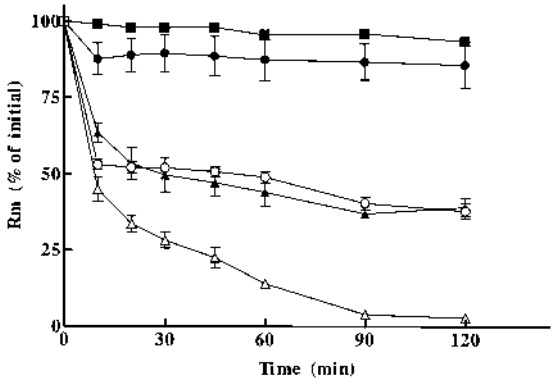

Fig. 3. Effects of Various Absorption Enhancers on Rm of *Xenopus* Pulmonary Membrane

Each point represents the mean±S.E. of at least three experiments. Keys: (■) Control; (●) 10 mM NaGC; (○) 20 mM NaGC; (▲) 10 mM NaCap; (△) 20 mM NaCap.

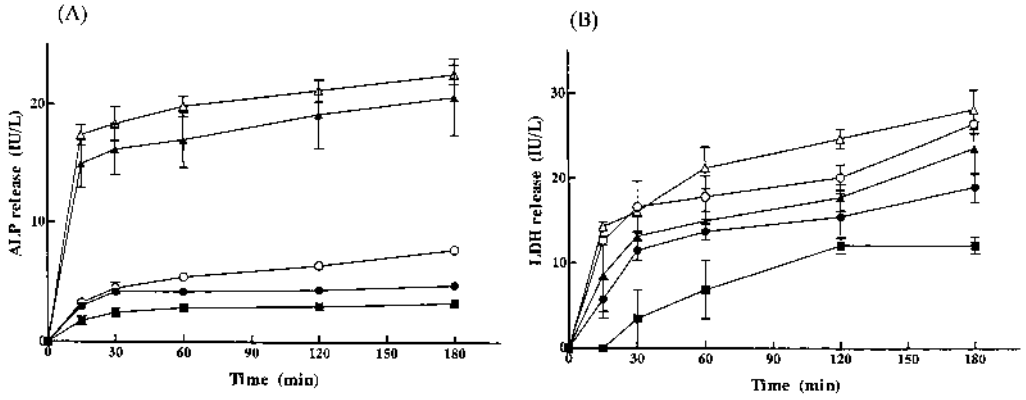

Fig. 4. Release of ALP (A) and LDH (B) from the *Xenopus* Pulmonary Membrane in the Presence of NaGC and NaCap

Each point represents the mean±S.E. of at least three experiments. Keys: (■) control; (●) 10 mM NaGC; (○) 20 mM NaGC; (▲) 10 mM NaCap; (△) 20 mM NaCap.

200 $\Omega \cdot cm^2$).[21] Therefore, the low permeability of insulin across the *Xenopus* pulmonary membrane was due to its high Rm value. However, it was previously learned that the absorption of peptides from the lung was much better than from the gastrointestinal tract.[25] In our previous study, it was indicated that insulin could be absorbed from the lung without any absorption enhancer, while we found no insulin absorption from the gastrointestinal tract.[6,26] Therefore, these findings suggested that the high absorption characteristics of peptide drugs from the lung as compared with the gastrointestinal tract were not clearly explained by the permeability characteristics across the *Xenopus* pulmonary membranes and the tightness of these membranes.

The permeation process of insulin from the apical side of pulmonary membrane to the systemic circulation is uncertain. Patton and Platz indicated that small peptides might be absorbed *via* the tight junctions, although large peptides and proteins such as insulin are thought to be absorbed by a transcytosis process through type I pneumocytes.[27] Forkesson *et al.*, in contrast, suggested that proteins were absorbed from the lung through the tight junctions of the alveolar epithelium.[28] According to Forkesson's hypothesis, the relationship between the molecular size of insulin and the intercellular spaces is essential to determine the permeability of insulin. Previously, Taylor and Gaar calculated the equivalent pore radius of rat alveolar epithelium by measuring the reflection coefficient (σ) of various compounds.[29] The pore radius obtained in their experiments was about 1.0 nm, while stokes radius of insulin was 1.3 nm. Therefore, insulin might be restricted to diffuse *via* intercellular spaces. However, it was reported that there were two different pore sizes in bullfrog and rat lungs.[30,31] One is a small size pore with a radius of 0.5 nm; this pore restricted the permeation of macromolecules. The other is a large size pore with a radius of about 5 nm. Compounds with large molecular weights might permeate through this large pore. Taking these findings together, insulin might penetrate through the intercellular junctions of the *Xenopus* pulmonary membrane, although we do not have direct evidence to support this speculation at present.

Our present data showed that the permeability of insulin across the pulmonary membrane was remarkably increased in the presence of NaCap and NaGC, while we found no absorption enhancing effect on the permeability of insulin in the presence of EDTA or NaSal. Among these absorption enhancers, NaCap was the most effective adjuvant for enhancing insulin permeability across the *Xenopus* pulmonary membrane at the same concentration.

NaCap is known to cause the membrane perturbation and enhance the transcellular permeability by interacting with the protein region in the gastrointestinal membranes, and to enhance the paracellular permeability by some structural change in the tight junction.[32] Previously, we found that NaCap was effective for enhancing the pulmonary absorption and permeability of phenol red by *in situ* and *in vitro* pulmonary absorption experiments,[21,33] which were in good agreement with the present findings.

NaGC has also been found to improve the absorption of phenol red from lung, as reported previously.[33] In our previous study, the transport of phenol red was improved by the addition of NaGC.[21] It was reported that bile salts removed the epithelial cells, thereby improving the absorption of poorly absorbable drugs.[34] Thus, this action of bile salts may be related to the enhancement effect of NaGC on the pulmonary absorption of insulin as well as phenol red in both *in vivo* and *in vitro* systems.

We observed no increase in the absorption of insulin in the presence of EDTA or NaSal. This finding is correlated with our previous finding that these enhancers did not significantly increase the transport of phenol red across the *Xenopus* pulmonary membrane.[21] Furthermore, our present result also concurs with the earlier findings that the pulmonary absorption of phenol red, fluorescein isothiocyanate-dextrans (FDs) and ($Asu^{1,7}$)-eel calcitonin was not enhanced by these enhancers in an *in situ* pulmonary absorption experiment.[7,13,33] However, these enhancers were effective for improving the absorption of poorly absorbable drugs in the gastrointestinal tract.[35] This difference in the effectiveness of the enhancers between the lung and intestine may be partly explained by morphological differences, varying sensitivity of enhancers and application of enhancers for different drugs.

Electrophysiological techniques have been used widely to characterize the effects of absorption enhancers on the permeability of drugs. In an earlier report, Yamashita *et al.* showed that EDTA reduced the membrane resistance of rat jejunum.[36] In addition, Anderberg *et al.* reported that the transepithelial electrical resistance of Caco-2 cell monolayers was reduced by various surfactants and bile salts.[37] Our previous study also demonstrated that the transepithelial electrical resistance of Caco-2 cells and rat colonic membranes was reduced in the presence of various absorption enhancers.[38,39] Therefore, we examined the absorption enhancing mechanisms of these enhancers using this electrophysiological technique. The present study showed that the Rm value at a steady state was about 700 $\Omega \cdot cm^2$ in the absence of absorption enhancers. This value was consistent with that previously reported by Wall *et al.*, $701 \pm 25\ \Omega \cdot cm^2$.[14] Thus, this finding suggested that the integrity of membrane was maintained during the transport studies. We found that the Rm value significantly decreased in the presence of 20 mM NaCap, 10 mM NaCap and 20 mM NaGC, although 10 mM NaGC slightly reduced the Rm value. Overall, this decrease in Rm value in the presence of these enhancers was relatively correlated with the result of transport studies in the presence of these enhancers. Reduction in Rm value can be used as a reference for the structural change of tight junction. Therefore, these absorption enhancers, especially NaCap, may loosen the tight junction of the *Xenopus* pulmonary membrane and increase the transport of insulin.

We also found that NaCap decreased the Rm value in a concentration dependent manner, although its absorption enhancing effect at 20 mM was almost the same as that at 10 mM. This discrepancy may be explained by the two absorption enhancing mechanisms of NaCap. That is, NaCap enhances both the transcellular and the paracellular permeability of drugs[32] and its action to the paracellular pathway may be concentration dependent, while its action to the transcellular pathway may be saturable over the range of 10—20 mM. Presumably, at these concentrations, the action of NaCap to the transcellular pathway may be dominant, which may be related to its saturable effect in the transport data.

Our present finding further indicated that the release of ALP and LDH activity from the apical side of the *Xenopus*

pulmonary membrane was increased in the presence of these enhancers. ALP is a marker enzyme of apical membrane of the epithelium, while LDH is a cytosolic enzyme, and their presence in the apical compartment is generally regarded as evidence of cell membrane damage.[23] These findings suggest that NaCap and NaGC cause membrane toxicity to the pulmonary membrane in this study, although they effectively improved the pulmonary absorption of insulin.

The onset of release of these marker enzymes in the presence of NaCap and NaGC, however, were much earlier than the permeability of insulin in the presence of these enhancers. The reason for this difference is not clearly understood. However, it may be possible that in the initial step, these enhancers may cause damage to the surface of the pulmonary epithelial membranes, but insulin cannot permeate unless the inner portion of the pulmonary epithelium is damaged.

In conclusion, this *in vitro* method is useful for estimating the permeability characteristics of peptide drugs including insulin across the pulmonary membrane, and for estimating the effects of various additives on the permeability of drugs and their membrane toxicity.

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