Evaluation of Enhancers to Increase Nasal Absorption Using Ussing Chamber Technique

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The effects of eight prospective absorption enhancers on the nasal mucosa in rabbit have been assessed using an *in vitro* Ussing chamber technique. Sodium taurodihydrofusidate (STDHF), sodium deoxycholate (DC), polyoxyethylene-9-lauryl ether (BL-9), lysophosphatidylcholine (LPC) and sodium dodecyl sulfate (SDS) were found to possess relatively high protein leaching activity, while sodium glycocholate (GC), sodium taurocholate (TC) and EDTA had relatively low activity. The permeation of fluorescein isothiocyanate-labeled dextran (FD, M.W. 9400) as a model drug across the nasal mucosa was found to be greater in the presence of these enhancers. Their enhancement ratio was found to be in the order of BL-9 > STDHF > SDS > LPC > DC > EDTA > GC > TC, which correlated with the protein leaching activity. The differences in protein leaching and enhancement ratio dependent on the magnitude of change of membrane resistance (ΔRm), indicating that these enhancers damaged the membrane and increased FD permeation. ΔRm thus appears to be a useful indicator by which one can estimate nasal mucosa damage by the enhancers.

Keywords absorption enhancer; Ussing chamber; nasal mucosa; membrane resistance; toxicity; nasal delivery

Most peptide and protein drugs have been parenterally administered, but this can cause pain, resulting in the noncompliance of a patient. Oral administration is the most convenient route, but the extent of absorption of oral peptides and proteins may be low due to degradation in the digestive tract, first-pass metabolism and the high structural barrier of the gastrointestinal tract. Nasal administration has been seriously considered as a feasible route to overcome this problem; however, this route requires enhancers to assure the adequate absorption of a drug. Bile salts,[1,2] surfactants,[3] chelating agents[4] and fatty acid derivatives[5] are enhancers which have been investigated with regard to facilitating the nasal absorption of peptide and protein drugs, but they generally cause damage and irritation to the nasal mucosa. Hemolysis,[5] protein leaching[6] and morphological observation by a scanning electron microscope (SEM)[7] have been used to evaluate the enhancer's toxicity on the nasal mucosa.

We previously reported that the Ussing type chamber system allowed retention of the viability of excised nasal mucosa for more than 6 h, which permitted the observation of enhanced fluorescein isothiocyanate-labeled dextran (FD) permeation paralleled with decreased membrane resistance by bile salts such as sodium glycocholate (GC), sodium taurocholate (TC), sodium deoxycholate (DC) and sodium taurodihydrofusidate (STDHF).[8] The present study used for additional enhancers, polyoxyethylene-9-lauryl ether (BL-9), sodium dodecyl sulfate (SDS), lysophosphatidylcholine (LPC) and EDTA, and FD permeation across the nasal mucosa was measured in their absence and presence. Protein and phospholipid leaching from the nasal mucosa was also measured as a toxicity index. Change of membrane resistance (ΔRm) and morphological changes of the mucosa were observed in the presence of these eight enhancers, and the relationship between ΔRm, enhancement ratio and membrane component leaching were determined to understand their effects on the nasal mucosa.

MATERIALS AND METHODS

Materials FD (M.W. 9400), GC and TC were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) DC was obtained from Tokyo Kasei Industries (Tokyo, Japan). SDS, LPC and EDTA were obtained from Wako Pure Chemical Industries (Osaka, Japan). BL-9 and STDHF were generously supplied by Nikko Chemicals (Tokyo, Japan) and Leo Pharmaceuticals (Bullerup, Denmark), respectively. All other reagents were of reagent grade and obtained commercially.

Tissue Preparations Male Japanese white rabbits (Tokyo Laboratory Animals, Tokyo, Japan) weighing 2.5—3.0 kg were used in this study. They were fasted overnight and sacrificed by rapid air embolism. The nasal mucosae were obtained as follows: the nasal septum was isolated and placed in ice-cold standard Ringer solution (NaCl 125 mM, KCl 5 mM, $CaCl_2$ 1.4 mM, NaH_2PO_4 1.2 mM, $NaHCO_3$ 10 mM and D-glucose 11 mM), then two mucosae were carefully stripped from the nasal septum. These were mounted in a Ussing type diffusion chamber.[9] The effective diffusion area of the mucosa was 0.5 cm^2. Both sides of the mucosa were filled with 11 ml of standard Ringer solution and bubbled with 95% O_2–5% CO_2 to maintain tissue viability and to circulate the solution. The chambers were placed in a temperature-controlled box to maintain the solution temperature at 37 °C.

Measurement of Electrical Membrane Resistance Electrical membrane resistance (Rm) was calculated from the spontaneous transmucosal potential difference (PD) and short circuit current (Isc) according to Ohm's law. When the PD had been reduced to approximately zero by the applied enhancer, Rm was calculated from the change in the transmucosal potential difference caused by the external constant current (100 μA) across the membrane.

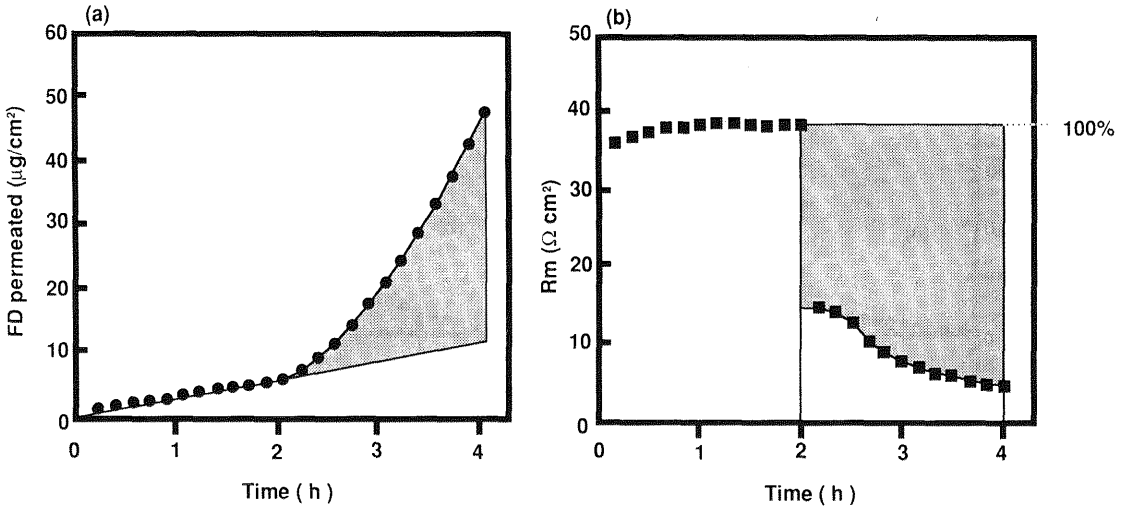

Fig. 1. Estimation Method for Area Enhancement (a) and Area ΔRm (b)

The external current was introduced by Ag–AgCl electrodes loaded at each edge of the chamber.[9] Transmucosal potential differences were measured between two salt bridges (3% agar in 150 mM NaCl solution) which were connected to a short circuit current amplifier (CEZ-9100, Nihon Kohden, Tokyo, Japan) *via* matched calomel electrodes.

FD Permeation Studies The nasal mucosa in the chamber was equilibrated with the standard Ringer solution for 120 min,[8] then FD solution (2.5 mg/ml standard Ringer solution) was introduced to the mucosal phase for control permeation. After 120 min, the entire test solution was drained from the mucosal phase and replaced with the enhancer solution (5.0 mg/ml standard Ringer solution) containing FD (2.5 mg/ml).[8] When EDTA was used, $CaCl_2$ was replaced by mannitol (Ca-free solution). One ml samples were taken from the serosal phase intermittently, and the same volume of fresh buffer was replaced to maintain a constant volume.

Protein and Phospholipid Leaching Studies After a period of equilibration, the enhancer solution was introduced to the mucosal phase. Samples of 0.25 ml for protein assay were taken intermittently from the mucosal phase and the same volume of the enhancer solution was added. All the donor solutions were taken for phospholipid assay after the experiment (120 min).

Estimation Method for Enhancement Ratio and Rm Change The enhancement ratio was expressed as an area of enhancement calculated by the extrapolation of steady state FD flux without an enhancer, as shown in the shadowed area in Fig. 1a. A change in Rm was expressed as an area ΔRm, and was calculated as follows and as shown in Fig. 1b:

$$\text{area } \Delta\text{Rm } (\%) = \frac{(100\%\ \text{Rm}) \times 120\,\text{min} - AUC_{120-240}}{(100\%\ \text{Rm}) \times 120\,\text{min}} \times 100$$

Rm value just prior to FD permeation in the presence of an enhancer was considered 100%. The $AUC_{120-240}$ was the calculated area under the Rm change curve from 120 to 240 min. Rm value and FD flux were almost constant over 240 min in the absence of an enhancer.[8] The area enhancement and the area ΔRm were zero in this case and were used as the control (CR).

Analysis Fluorescense intentisy of FD was measured by a spectrofluorometer (RF-5000, Shimadzu) at an excitation wavelength of 495 nm and an emission wavelength of 515 nm. The amount of protein leached from the nasal mucosa was determined by BCA protein assay (PIERCE, IL, U.S.A.) using bovine serum albumin as a standard.[10] Samples of phospholipid were mixed with 3 ml chloroform and shaken for 10 min. The same extraction was repeated twice and the chloroform phase obtained by centrifugation was dried under nitrogen gas. The amount of phospholipid leached from nasal mucosa was determined by a kit, Phospholipids B-test Wako (Wako Pure Chemical Industries) for the assay of serum phospholipids. Enhancers used in this experiment did not interfere with either the protein or phospholipid assay.

Morphological Assessment Following the permeation study, the nasal mucosa was fixed in isotonic formalin solution (10% formalin in physiological saline) at 5 °C for 12 h. Tissue was then gradually dehydrated in ethanol/water (from 50 to 100%) and dried by a critical-point drier (HCP-2, Hitachi, Tokyo, Japan). It was coated with gold and examined in a SEM (X-650, Hitachi).

RESULTS

Effect of Enhancers on the FD Permeation and Rm Figure 2 shows the time course of FD permeation and Rm in the absence and presence of BL-9, SDS, LPC and EDTA. FD flux and Rm values were almost constant in the absence of enhancers, but respectively increased and decreased following the application of an enhancer. With LPC, BL-9 and SDS, the Rm first decreased drastically and then gradually, as was ture of STDHF and DC, as reported previously.[8] The FD flux greatly increased in parallel with the Rm decrease. EDTA, on the other hand, caused a slow increase and decrease of flux and Rm, respectively. Spontaneous PD and Isc immediately achieved almost zero when BL-9, SDS and LPC were applied, and gradually decreased when applying EDTA. It is clear from these results that FD flux increased with

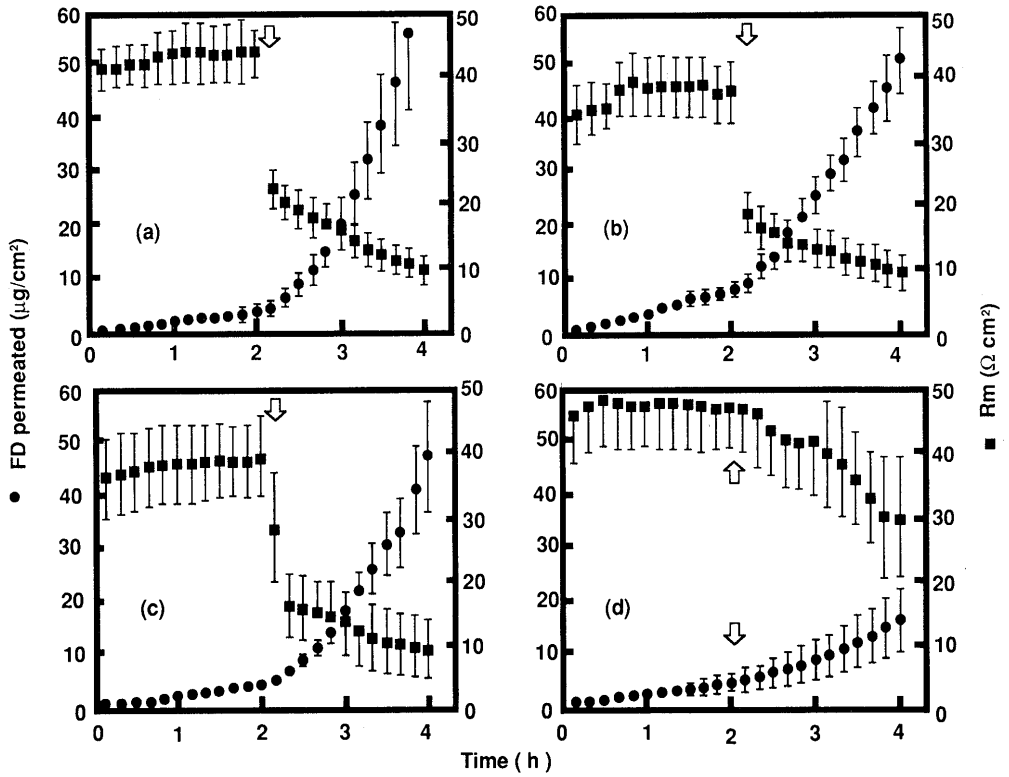

Fig. 2. Effect of Enhancers on FD Permeation and Rm

Key: a, BL-9; b, SDS; c, LPC; d, EDTA; ⇩, replacement from FD solution to FD solution with enhancer. Each point represents the mean ± S.E. of at least 3 experiments.

Table I. Effect of Enhancers on the Nasal Mucosa

Enhancer	Area enhancement (μg min cm^{-2})	Area ΔRm (%)
GC	300.7	31.2
TC	123.4	20.1
DC	1396.2	74.0
STDHF	1676.6	76.3
BL-9	2273.6	61.2
SDS	1669.7	62.9
LPC	1598.0	63.0
EDTA	305.1	16.5

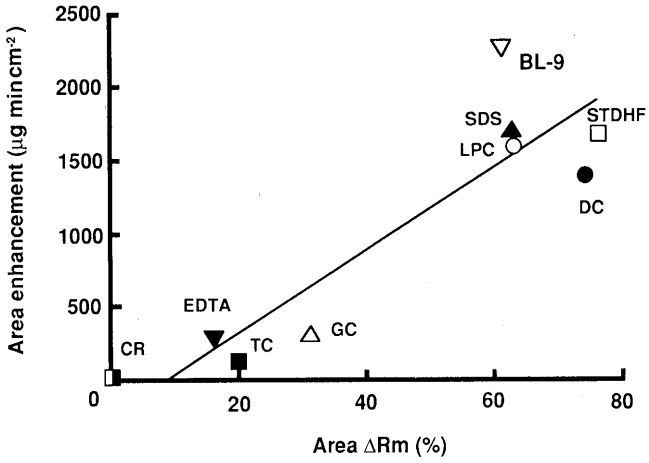

Fig. 3. Relationship between Enhancement and ΔRm

Each point represents the mean of at least 3 experiments. $r = 0.8985$.

decreasing Rm, and the viability of nasal mucosa was lowered by enhancers.

Table I is a summary of the area of enhancement and the area ΔRm values of GC, TC, DC and STDHF from the previous study,[8] and of BL-9, SDS, LPC and EDTA from this study. The area of enhancement was found to be BL-9 > STDHF > SDS > LPC > DC > EDTA > GC > TC, and the area ΔRm was STDHF > DC > LPC and SDS > BL-9 > GC > TC > EDTA in descending order. The enhancement of FD and the change in Rm were both dependent on an enhancer.

Figure 3 shows the relationship between the area of enhancement and the area ΔRm; the correlation between the two was $r = 0.8985$. This relation separated the enhancers into two groups: one was BL-9, SDS, LPC STDHF and DC, which have relatively large values in both parameters, and the other was EDTA, TC, GC and CR, with relatively small parameters.

Effect of Enhancers on Protein and Phospholipid Leaching

Figures 4a and b show the time course of protein leaching and the amount of leaching of phospholipids, respectively. LPC showed maximum protein leaching activity, followed by STDHF, SDS, BL-9, DC, TC, GC, EDTA and CR. This protein leaching activity also divided the enhancers into two groups: LPC, STDHF, SDS, BL-9 and DC had relatively high activity, while that of GC, TC and EDTA was relatively low (Fig. 4a). BL-9 exhibited the maximum phospholipid leaching activity, and there was little difference among those that followed: DC, SDS, GC, STDHF, TC, EDTA and CR. Phospholipid leaching was not determined for LPC because it is a phospholipid itself.

Figures 5a and b show the relationships between protein and phospholipid leaching and the area ΔRm, respectively. A good correlation was found between protein leaching and the area ΔRm ($r = 0.8895$), suggesting that the change

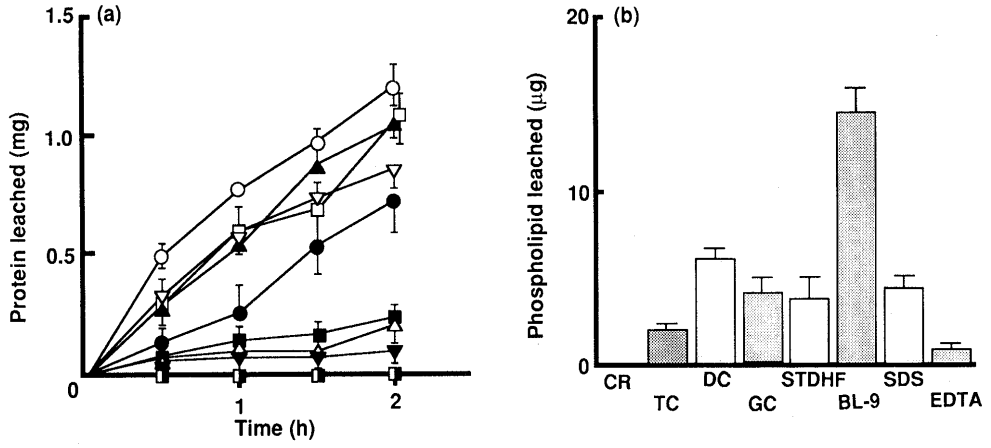

Fig. 4. Effect of Enhancers on Protein (a) and Phospholipid (b) Leaching from Nasal Mucosa
Key: LPC (○), STDHF (□), SDS (▲), BL-9 (▽), DC (●), TC (■), GC (△), EDTA (▼), CR (◨). Each point represents the mean ± S.E. of 4 experiments.

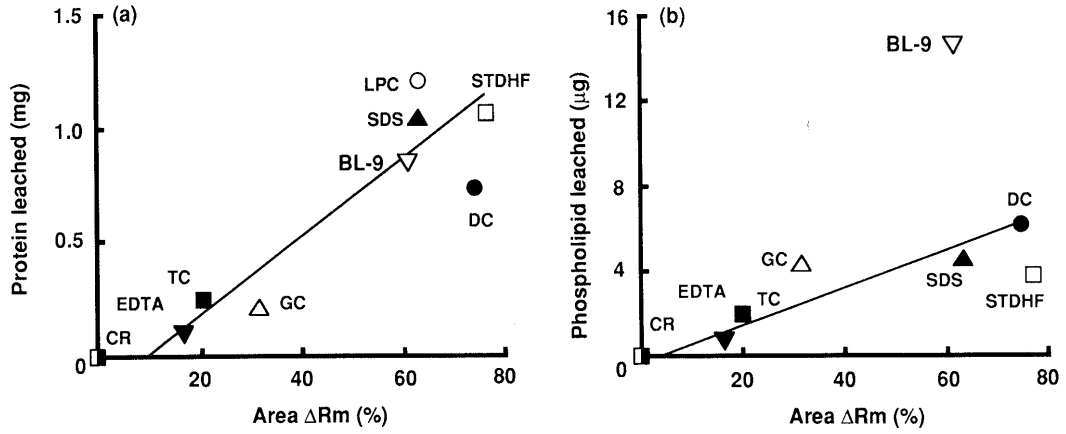

Fig. 5. Relationship between Protein (a) and Phospholipid (b) Leaching and ΔRm
(a) $r=0.8895$, (b) $r=0.6005$.

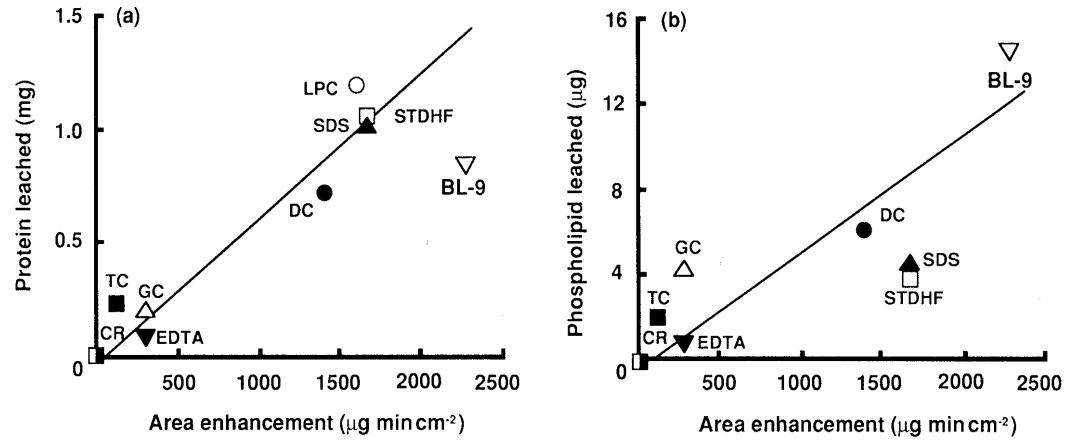

Fig. 6. Relationship between Protein (a) and Phospholipid (b) Leaching and Enhancement
(a) $r=0.8833$, (b) $r=0.7997$.

of Rm can be attributed primarily to the amount of protein leaching. On the other hand, the regression coefficient was 0.6005 between phospholipid leaching and the area ΔRm. Without BL-9, however, the correlation between them was better ($r=0.8692$). The reason for the special property of BL-9 remains unclear.

Figures 6a and b show relationships between protein leaching and phospholipid leaching and the area of enhancement, respectively. A relatively good correlation exists between them, especially between the protein and the area of enhancement ($r=0.8833$). FD enhancement seemed to be mainly responsible for the protein leaching which caused a decrease in Rm.

Morphological Change Figure 7 shows the mucosal surface morphology after exposure to FD solution (control), and to EDTA, GC, STDHF, DC and BL-9 solutions. Two hours of exposure to an enhancer followed 2 h of exposure to the FD solution, and in the control

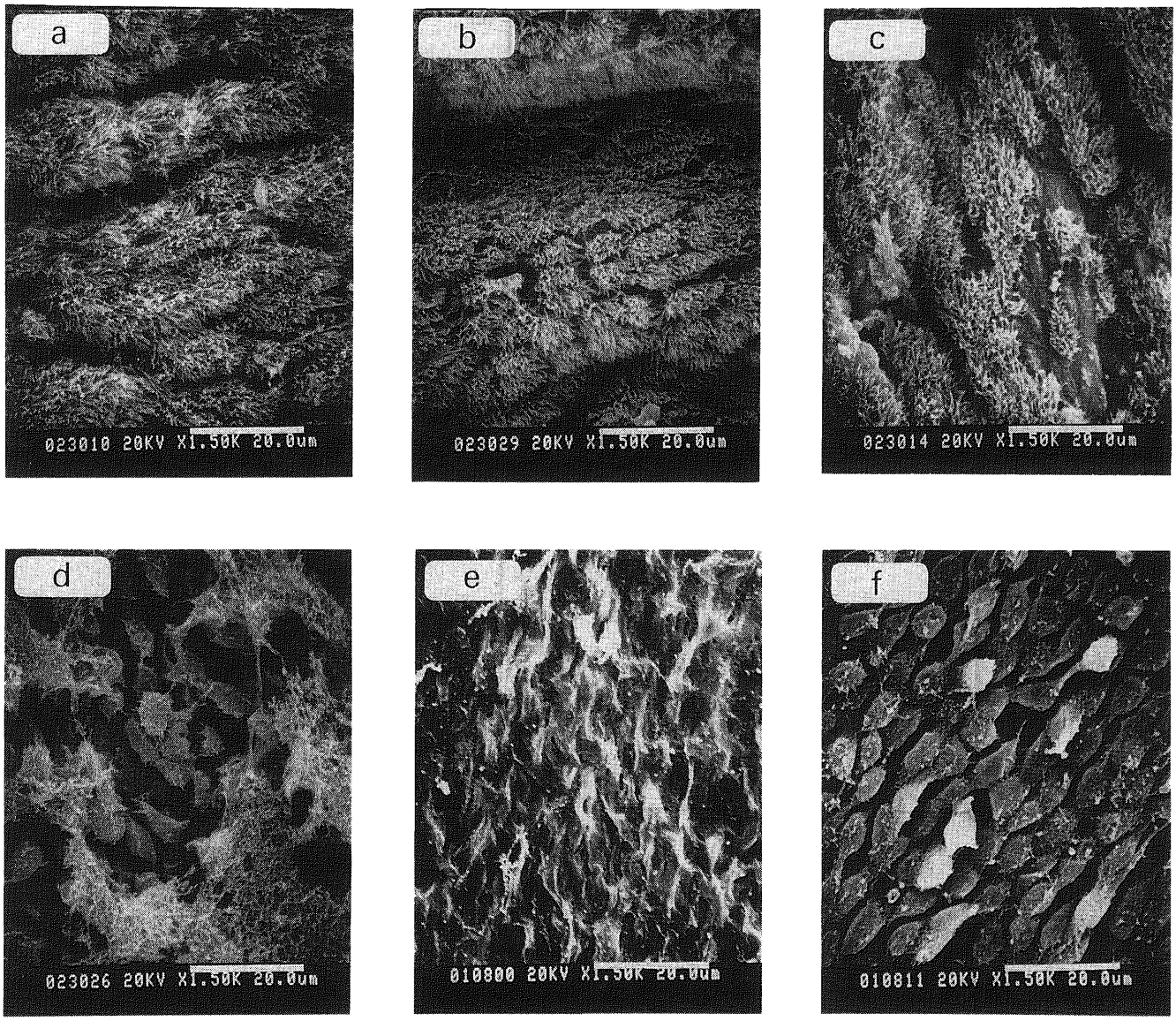

Fig. 7. Effect of Enhancers on Mucosal Surface Morphology
Key: a, control; b, EDTA; c, GC; d, STDHF; e, DC; f, BL-9.

experiment, the exposure was to FD solution only for 4 h. EDTA did not seem to alter the mucosal surface compared with the control, while GC showed a slight denudation of cilia. STDHF, DC and BL-9, however, caused a drastic morphological change in the muscosal surface: the epithelial cells were completely denuded by STDHF and DC, and they seemed to be fused by BL-9. The results with TC were almost the same as with GC, and those with LPC and SDS resembled the BL-9 results. LPC, STDHF, SDS, BL-9 and DC caused a great alteration in morphology as well as in the magnitude of the area ΔRm, the area enhancement value and protein leaching activity. Consequently, the magnitude of the area ΔRm appeared to be governed by the nasal mucosal damage caused by the enhancers.

DISCUSSION

Although Rm values were kept constant in the absence of an enhancer, the application of STDHF, DC, BL-9, SDS and LPC caused these values to decrease drastically in the early stage, and then to decrease more gradually. The mechanism of the enhancers, especially bile salts, has been well studied and reported to be as follows: (1) alteration of the mucous layer, (2) opening the tight junctions between the epithelial cells, (3) reversed micelle formation in the membrane, (4) extraction of membrane components by comicellization,[1] and (5) an inhibitory effect on proteolytic enzymes.[3] It seemed that a drastic decrease in Rm was caused when an enhancer opened tight junctions or made new pore routes, as mentioned above, but mucosal integrity was maintained. In STDHF, at least, this hypothesis was true. Further exceptional experiments were done for the discussion of the reversibility of nasal mucosa (Fig. 8). Rm markedly decreased with 10 min-exposure to STDHF (3.0 mg/ml) and returned to the control level after a washout of the enhancer. The behavior of PD and Isc was almost identical to Rm. In addition, the FD flux temporarily increased with decreasing Rm, and returned to the control level with the recovery of Rm (Fig. 8a). With 30 min-exposure to STDHF, however, Rm did not completely return to the control level (recovery

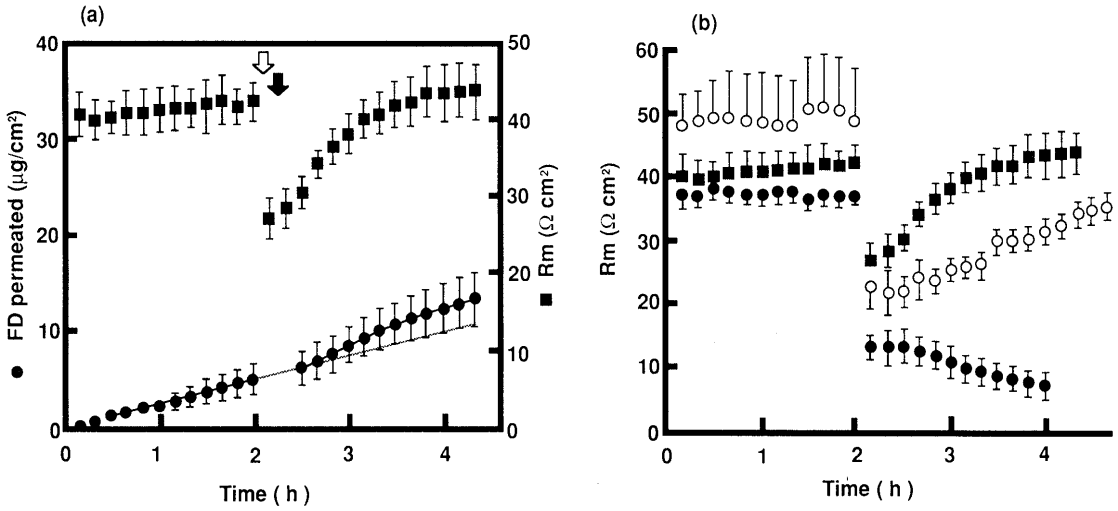

Fig. 8. Effect of STDHF on FD Permeation and Rm (a), and Effect of Exposure Time of STDHF on Rm (b)
Key: (a): ⇩, exposure to STDHF; ⇓, washout; (b): 10 min (■), 30 min (○), 120 min (●). Each point represents the mean ± S.E. of 3 experiments.

ratio: 72%). No recovery was found following 120 min of exposure (Fig. 8b). Continuous exposure to the enhancer caused irreversible membrane damage. The gradual decrease of Rm and the increase of protein leaching seemed to be caused by the subsequent leaching of components in the epithelial cells, and by the denudation of cilia and epithelial cells. Two hours of exposure to the enhancers STDHF, DC, BL-9, LPC and SDS resulted in complete denudation or fusion of the epithelial cells (Fig. 7) and a Rm value of less than $10\,\Omega cm^2$. These changes in the mucosal surface appeared to parallel the change in Rm. FD permeation was enhanced as the degree of Rm decreased. Thus, a good correlation was found among the area ΔRm, the area of enhancement and protein leaching activity.

The fact that BL-9 had strong phospholipid leaching activity meant that it exhibited a higher area of enhancement than the others, even though its protein leaching activity was only equal to the others. This phenomenon was apparently due to the surface activity and hydrophobicity[11] of BL-9.

Shao and Mitra reported the effect of bile salts on the nasal protein release of rats using an *in situ* nasal perfusion technique.[6] The rank order of protein released was DC > STDHF > sodium cholate > GC and TC. This is in accordance with our results, except for DC and STDHF, which may perhaps be due to a difference in experimental methods, exposure time or concentration of the enhancer.

Similar findings were reported by Coleman and his colleagues. The solubilization activity of proteins and phospholipids in the plasma membrane of rat liver[12] and human erythrocytes[13] was found in the order DC > sodium cholate > GC and TC.

In addition to the leaching of the membrane components, ciliotoxicity studies were also reported. STDHF, DC, and BL-9 caused very rapid irreversible ciliostasis in human at concentrations of 0.5, 0.3 and 0.3% (w/v), respectively. GC and TC, however, exhibited very mild ciliostatic activity as reported by Hermens *et al.*[14] LPC, BL-9, DC and STDHF fully inhibited mucociliary clearance in frogs whereas GC did not at a concentration of 1% (w/v) as reported by Gizurason *et al.*[15] These results agree with our classification of the Rm changes. Ennis *et al.* reported morphological damage of rat nasal mucosa as viewed by SEM observation[7]; exposure to STDHF, BL-9 and DC at a concentration of 1% (w/v) caused relatively high damage. These results also agree with our rank order of damage. It is suggested, therefore, that the magnitude of Rm change, that is, the area ΔRm, can be used as an indicator of damage to the nasal mucosa caused by an enhancer.

A long period of exposure to an enhancer caused irreversible damage to the nasal mucosa. This was demonstrated by Rm decrease of less than $10\,\Omega cm^2$ or by irreversible Rm changes. In actual application, these are closely linked to local inflammation or invasion by viruses. When the Rm change is small, however, the enhancing effect is also limited without an inhibitory effect on proteolytic enzymes (Fig. 3). An ideal enhancer seems to be one which would achieve the following: the immediate onset of a decrease in membrane resistance occurring after application; the duration of the effect should be predictable; and membrane resistance should immediately and fully recover after the enhancer is removed from the mucosal. Ten min-exposure to STDHF immediately reduced the Rm, and this value was fully recovered after washout (Fig. 8a), suggesting the action on the nasal mucosa was reversible. Further work is necessary to determine the behavior of Rm change with variation in exposure time and concentration, or with the application of another enhancer.

In conclusion, the Rm changes, that is, the area ΔRm, in the presence of enhancers was correlated with protein leaching activity. The magnitude of the area ΔRm corresponded to degrees of denudation of the epithelial cells and to other estimates such as ciliotoxicity, as discussed. These results seem to confirm that the Ussing chamber system is a very promising tool for estimating both enhancement efficacy and mucosal damage. Area ΔRm values furnished criteria by which to judge the toxicity and damage to the nasal mucosa caused by enhancers.

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