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Screening of cationic compounds as an absorption enhancer for nasal drug delivery

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Abstract

Several cationic compounds were screened as potential nasal absorption enhancers to increase intranasal absorption of a model drug, fluorescein isothiocyanate labeled dextran (MW 4.4 kDa, FD-4), without nasal membrane damage in rats. Their effects were compared with those of classical enhancers. Various cationic compounds (poly-L-arginines with different molecular weights (MW 8.9, 45.5 and 92.0 kDa, poly-L-Arg (10), (50) and (100), respectively), L-arginine (L-Arg), L-lysine (L-Lys), and cetylpyridinium chloride (CPCL) were evaluated. Of the cationic compounds, poly-L-Arg and CPCL greatly enhanced the intranasal absorption of FD-4, as did chitosan, a cationic polysaccharide which has been reported to show a great effect on the transnasal delivery of peptide and protein drugs. The enhancing intensity by poly-L-Arg was dependent on its molecular weight. Rank order of the enhancing ratio, calculated from the AUC ratio for the enhancer treatment against the untreatment, was 0.5% poly-L-Arg (100) $\cong$ 0.5% sodium dodecylsulfate $\cong$ 0.5% CPCL $>$ 0.5% poly-L-Arg (50) $>$ 0.5% sodium deoxycholate $\cong$ 0.5% sodium taurodihydrofusidate $>$ 0.5% polyoxyethylene-9-lauryl ether $\cong$ 0.5% lysophosphatidylcholine $>$ 0.5% chitosan $\cong$ 0.5% poly-L-Arg (10) $\geq$ 10% L-Arg $\cong$ 10% L-Lys $>$ 0.5% sodium glycocholate $\cong$ 0.5% sodium taurocholate $\cong$ 0.5% EDTA. Only the poly-L-Args represented almost the same degree of hemolysis of cationic compounds compared with pH 7.0 phosphate buffered saline in the rat erythrocyte lysis experiment. The enhancing ratio by classical enhancers correlated with leaching of protein, phospholipids and LDH from isolated rabbit nasal mucosa. CPCL also fell on the regression lines between the enhancing ratio and their degree of leaching from classical enhancers. In contrast, the enhancing intensities by poly-L-Arg (10), (50) and (100) were greatly shifted from the regression line: the amount of leaching was markedly low in spite of a great enhancement of FD-4 absorption. These findings suggest that of the

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assessed enhancers only the poly-L-Args enhance the transnasal delivery of high molecular substances without severe damage to the nasal mucosal membrane. Poly-L-Arg is therefore a promising candidate having a good balance between enhancing activity and safety for nasal peptide and protein delivery.

Keywords: Poly-L-arginine; Absorption enhancer; FITC-dextran; Hemolysis; Nasal peptide and protein delivery; Leaching of membrane components

1. Introduction

The nasal cavity is one of the most attractive routes for peptide and protein delivery, because of its higher permeability than those through other administration routes and avoidance of the first pass metabolism in the liver. However, the resulting bioavailability was found to be low for hydrophilic and/or high molecular substances (McMartin et al., 1987). Improvement of the absorption rate and availability has thus been investigated using chemical enhancers and enzymatic inhibitors and by designing specific dosage forms such as polymer solutions and microparticulate systems which can prevent rapid mucociliary clearance from the nasal cavity (Chien et al., 1989; Morimoto et al., 1991; Abd El-Hameed and Kellaway, 1997; Yamamoto et al., 1998).

In these approaches, most classical chemical enhancers may have undesirable physiological effect on epithelial cells and cause irritation to nasal mucosa (Merkus et al., 1993; Jabbal Gill et al., 1994a,b; Marttin et al., 1995). We also showed that the enhancing effect of classical enhancers (surfactants, bile salts, fatty acids and chelating agents) on the permeation of a model compound, fluorescein isothiocyanate-labeled dextran (FITC-dextran, MW 9.4 kDa, FD-10), measured by the in vitro Ussing chamber technique was well correlated with protein and phospholipid leaching in rabbit nasal mucosa (Hosoya et al., 1994). Similar relations were obtained in small and large intestinal membranes in rats (Uchiyama et al., 1996; Yamamoto et al., 1996). These results suggest that these chemical enhancers irreversibly damaged epithelial cell membranes, if they are used at a concentration to obtain sufficient absorption of peptides and proteins for effective pharmacological actions. Therefore, it is necessary to find a new type of enhancer which has an enhanced mucosal absorption of peptide drugs without severely damaging the epithelial cell membrane.

Recently, Illum et al. (1994) reported that chitosan, a cationic polymer, improved the nasal absorption of insulin, and they speculated that this effect was due to a combination of bioadhesion for preventing mucociliary clearance and a transient widening of the tight junctions in the nasal membrane. Further, their pulse-chase and nasal perfusion studies in rats showed that chitosan caused no membrane or cellular damage (Jabbal Gill et al., 1994a,b; Aspen et al., 1996). Schipper et al. (1997) estimated the permeation route of FITC-dextran (MW 4.4 kDa, FD-4) after chitosan treatment on Caco-2 cell monolayers using a confocal imaging technique. They showed that permeation of FD-4 was increased across the intercellular route by widening of cell–cell junctions. The positive charge of chitosan probably interacts with negative charge on the mucosal surface. Further, chitosan may stay on the membrane surface and not go through the membrane due to its high molecular weight, resulting in no severe damage. The solubility of chitosan, however, is so poor at physiological conditions (neutral pH), that an acidic solution is needed to dissolve it. *N*-Trimethyl chitosan chloride was estimated as a potential enhancer to promote Caco-2 cell permeation (Kotze et al., 1997). This chitosan was dissolved in both solutions of pH 4.0 and 9.0 and gave a remarkable enhancement of FD-4 through Caco-2 cell monolayers. Thus, cationic substances may be candidates for enhancing the absorption of peptide drugs through the nasal mucosal membrane without severe membrane damage at a physiological level.

In the present study, we selected FD-4 as a water soluble and high molecular weight model permeant because many investigators have used it

in transport studies of various mucosal membranes (Kotze et al., 1997; Schipper et al., 1997; Quan et al., 1998). We studied the in vivo nasal absorption of FD-4 in rats, the in vitro hemolytic effect on rat erythrocytes and leaching of protein, phospholipids and lactate dehydrogenase (LDH) in isolated rabbit nasal mucosa with various cationic enhancer candidates. The effects on the pharmacokinetic parameters of FD-4, and hemolysis and leaching by these compounds were compared with classical chemical enhancers.

2. Experimental

2.1. Materials

FITC-dextran (MW 4.4 kDa, FD-4), poly-L-arginine hydrochloride (MW 8.9, 45.5 and 92.0 kDa, poly-L-Arg (10), (50) and (100), respectively), sodium glycocholate (GC), sodium taurocholate (TC), disodium dihydrogen ethylenediaminetetraacetate dihydrate (EDTA) and citrate-phosphate-dextrose solution with adenine (CDP-A) were obtained from Sigma Chemical Co., Ltd. (St. Louis, MO, USA). L-Arginine (L-Arg), L-lysine (L-Lys), chitosan (MW 200 000, deacetylation ca. 80%) and sodium deoxycholate (DC) were obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Sodium dodecylsulfate (SDS) and lysophosphatidylcholine (LPC) were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Cetylpyridinium chloride (CPCL) was purchased from Kanto Chemical Co., Ltd. (Tokyo). Polyoxyethylene-9-lauryl ether (BL-9) and sodium taurodihydrofusidate (STDHF) were supplied by Nikko Chemicals (Tokyo) and Leo Pharmaceuticals (Bullerup, Denmark), respectively. All other reagents were of reagent grade.

2.2. Methods

2.2.1. Pharmacokinetic study

2.2.1.1. I.V. bolus injection study. FD-4 solution (2.5 mg/ml, 3.3 mg/kg) was injected in a femoral vein in an anesthetized male Wistar rat, weighing 250–300 g, treated in the same surgical procedure as the nasal absorption study. Plasma samples (0.15 ml) were collected from the jugular vein at predetermined times.

2.2.1.2. Nasal absorption study. An anesthetized Wistar rat was treated by the method of Hirai et al. (1981). Briefly, a cannula was inserted into the trachea to maintain respiration and the esophagus was occluded by another cannula stopped up the tip. Intranasal (i.n.) doses (200 mg/ml in 0.9% NaCl solution, 33.0 mg/kg) of FD-4 containing a suitable concentration of cationic compounds (chitosan, CPCL, L-Lys, L-Arg, poly-L-Arg (10), (50) and (100)), or classical enhancer (SDS, DC, GC, TC, STDHF, BL-9, LPC and EDTA) were administered by a flexible polyethylene tube with a microsyringe. Blood samples were collected at predetermined times. Cationic compounds and classical enhancers were used at a concentration of 0.5% except L-Lys (10%) and L-Arg (2, 5 and 10%). In preliminary experiment, almost all classical enhancers obtained the maximum enhancing effect at a concentration of 0.5%.

2.2.2. Hemolysis study

Hemolysis experiments were done according to the method of Jabbal Gill et al. (1994a). Blood taken from the carotid in an anesthetized Wistar rat was mixed with CDPA (1:9). After centrifugation ($380 \times g$, 5 min), the obtained erythrocyte pellets were washed four times with pH 7.0 isotonic phosphate buffer (PBS). Finally, an adequate amount of PBS was added to the erythrocyte pellets to obtain a 10% erythrocyte standard solution. PBS or distilled water containing an enhancer (1.75 ml) was mixed with 10% erythrocyte standard solution (0.125 ml), and the mixture was incubated for 10 min at 37°C. After centrifugation ($540 \times g$, 2 min), the supernate was separated to determine the hemolysis. The enhancer solution (0.5%) was isotonically adjusted except for 10% L-Lys and L-Arg solutions.

2.2.3. Leaching studies

2.2.3.1. Preparation of isolated rabbit nasal mucosa. Isolated rabbit nasal mucosa was obtained according to the previous method (Hosoya et al.,

1994). Briefly, the nasal septum was excised and placed in ice-cold standard Ringer solution (in mM: NaCl, 125; KCl, 5; $CaCl_2$, 1.4; NaH_2PO_4, 1.2; $NaHCO_3$, 10; D-glucose, 11) immediately after the sacrifice of male Japanese white rabbits (Tokyo Laboratory Animals, Tokyo), weighing 2.5-3.0 kg by rapid air embolism. Two mucosae, then, were isolated from the nasal septum and each of them was mounted in a Ussing type diffusion chamber (effective surface area: 0.5 cm^2). Both sides of the mucosa were filled with 4 ml of standard Ringer solution and bubbled with 95% O_2/5% CO_2 to maintain cell viability and to circulate the solution at 37°C.

Transmucosal potential difference (PD) and short circuit current (Isc) were monitored by a short circuit amplifier (CEZ9100, Nihon Koden, Tokyo) to check the cell viability. The isolated mucosa was used in the values of electrophysiological parameters as follows: PD, over 3.0 mV; Isc, over 30 $\mu A/cm^2$; transmucosal membrane resistance (Rm) calculated according to Ohm's law, 40–70 Ωcm^2.

2.2.3.2. Protein, phospholipids and LDH leaching. After equilibrium of electrophysiological parameters of nasal mucosa with standard Ringer solution for 120 min, then 4 ml of enhancer solution (0.5%) was replaced in the mucosal phase. When EDTA was used, $CaCl_2$ was replaced by mannitol (Ca^{2+}-free solution). 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. For phospholipids and LDH assay, all the solutions in the mucosal phase were taken after the experimental period (120 min). Data previously obtained for protein and phospholipids (Hosoya et al., 1994) are included in this study.

2.3. Sample analysis

The obtained plasma was diluted about 100 times by pH 8.5 potassium dihydrogenphosphate—sodium borate buffer. Fluorescence intensity of FD-4 was determined by a fluorescence spectrofluorometer (RF-5000, Shimadzu, Kyoto) at an excitation wavelength of 495 nm and an emission wavelength of 515 nm.

Turbidity of the obtained supernate by hemolysis was measured by a spectrophotometer (UV-160A, Shimadzu) at 543 nm. The turbidity of supernate when suspended the erythrocyte in distilled water and pH 7.0 PBS was assumed to 100 and 0% to each spectrum, respectively.

The amount of protein leached from the nasal mucosa was determined by BCA protein assay (PIERCE, IL, USA) using bovine serum albumin as a standard. Spectrum was measured at 562 nm.

Samples of phospholipids 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 phospholipids was determined by a phospholipids B-test Wako (Wako Pure Chemical Industries, Osaka). Spectrum was measured at 502 nm.

The amount of LDH was determined by a LDH C II-test Wako (Wako Pure Chemical Industries, Osaka). Spectrum was measured at 560 nm.

Compounds used in this experiment did not interfere with the assay of leaching except CPCL in the phospholipids and LDH leaching studies.

2.4. Data analysis

The i.v. and i.n. blood data were analyzed by a non-linear least square regression (Algorithm: Damping Gauss–Newton method) (Yamaoka et al., 1981). C_{max} and T_{max} were obtained from FD-4 concentration–time curve and AUC was calculated by the trapezoidal rule. Plasma concentration of FD-4 after i.v. and i.n. administration was extrapolated exponentially until infinite time (∞). Plasma concentration of FD-4 in control and chitosan treatment was also extrapolated exponentially until 9 h to obtain AUC.

The enhancing ratio was calculated as follows:

$$\text{Enhancing ratio} = AUC_{treatment}/AUC_{control} \quad (1)$$

where $AUC_{treatment}$ and $AUC_{control}$ are the areas under the plasma concentration of FD-4 versus time (0–9 h) curves after i.n. administration of

FD-4 with and without an enhancer, respectively.

$F_{0-9\ h}$ and F_{∞} were calculated, respectively as follows:

$$F_{0-9\ h} = \frac{AUC_{i.n._{0-9\ h}}}{AUC_{i.v._{\infty}} \times 10} \times 100 \quad (2)$$

$$F_{\infty} = \frac{AUC_{i.n._{\infty}}}{AUC_{i.v._{\infty}} \times 10} \times 100$$

where $AUC_{i.n._{0-9\ h}}$ is the area under the plasma concentration of FD-4 versus time (0–9 h) curve after i.n. administration of FD-4. $AUC_{i.n._{\infty}}$ and $AUC_{i.v._{\infty}}$ are the areas under the plasma concentration of F-4 versus time (0–∞) curves after i.n. and i.v. administrations of FD-4, respectively.

Statistical analyses were performed using Student's *t*-test.

3. Results and discussion

3.1. Effect of cationic compounds on nasal absorption of FD-4

Elimination kinetics of FD-4 after i.v. administration obeyed the linear 2-compartment model. Fig. 1 shows the plasma concentration–time curves of FD-4 after i.n. administration with different cationic compounds and SDS as a typical example of classical enhancer. Table 1 lists the obtained pharmacokinetic parameters of FD-4 after i.v. and i.n. administrations. All cationic compounds enhanced the FD-4 absorption, although their enhancing intensities differed.

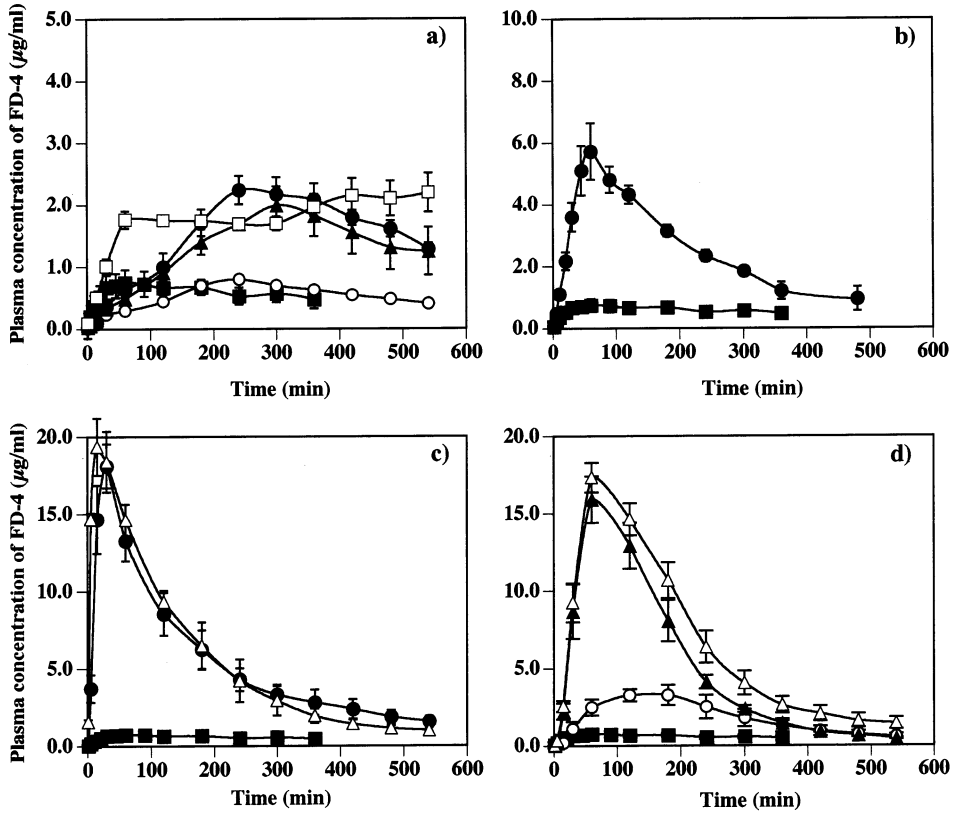

Fig. 1. Plasma concentration of FD-4 after i.n. administration of FD-4 with various cationic compounds and SDS in rats. ■, control; (a) ○, 2% L-Arg; ▲, 5% L-Arg; ●, 10% L-Arg; □, 10% L-lys; (b) ●, 0.5% chitosan; (c) ●, 0.5% CPCL; △, 0.5% SDS; (d) ○, 0. 5% poly-L-Arg (10); ▲, 0.5% poly-L-Arg (50); △, 0.5% poly-L-Arg (100). Each data point represents the mean ± S.E. ($n = 3$–6).

Table 1
Pharmacokinetic parameters of FD-4 after i.n. administration with various enhancers in rats

Enhancer	C_{max} (μg/ml)	T_{max} (min)	$AUC_{0-9\ h}$ (μg min/ml)	AUC_{∞} (μg min/ml)	$F_{0-9\ h}$[a] (%)	F_{∞}[b] (%)
i.v. (3.3 mg/kg)	–	–	447.8 ± 37.7	450.3 ± 38.5	–	–
i.n. control (33 mg/kg)	0.8	113	292.6 ± 48.5[c]	402.3 ± 122.2[c]	6.5	8.9
2% L-arg	1.0	240	289.3 ± 14.9	–	6.4	–
5% L-arg	2.2	285	711.5 ± 68.4	–	15.8	–
10% L-arg	2.3	240	834.4 ± 99.9	1304.9 ± 155.5	18.5	29.0
10% L-lys	2.3	480	740.6 ± 211.1	–	16.4	–
0.5% poly-L-arg (10)	3.7	120	980.2 ± 163.8	1114.3 ± 193.9	21.8	24.7
0.5% poly-L-arg (50)	16.6	80	2785.3 ± 164.4	2831.0 ± 163.4	61.9	62.9
0.5% poly-L-arg (100)	17.3	60	3253.3 ± 116.2	3545.5 ± 93.4	72.2	78.7
0.5% CPCL	18.4	25	3036.1 ± 362.0	3533.4 ± 827.8	67.4	78.5
0.5% chitosan	5.7	60	1097.4 ± 107.8[c]	1341.6 ± 82.4[c]	24.4	29.8
0.5% SDS	19.4	15	3104.2 ± 323.2	3233.7 ± 563.2	68.9	71.8
0.5% STDHF	15.1	10	1634.9 ± 157.5	1706.5 ± 154.7	36.3	37.9
0.5% BL-9	9.7	26	1403.3 ± 23.5	1610.1 ± 133.8	31.2	35.8
0.5% LPC	12.7	25	1357.6 ± 177.5	1375.4 ± 182.5	30.1	30.5
0.5% DC	12.3	30	1818.2 ± 397.5	1934.9 ± 378.7	40.4	43.0
0.5% TC	1.0	195	339.1 ± 165.2	408.1 ± 183.2	7.5	9.1
0.5% GC	1.2	400	398.4 ± 203.2	–	8.8	–
0.5% EDTA	1.3	120	255.3 ± 88.1	316.7 ± 92.5	5.7	7.0

[a] $F_{0-9\ h} = [AUC_{i.n._{0-9\ h}}/(AUC_{i.v._{\infty}} \times 10)] \times 100$.
[b] $F_{\infty} = [AUC_{i.n._{\infty}} \times (AUC_{i.v._{\infty}} \times 10)] \times 100$.
[c] Plasma concentration of FD-4 was extrapolated exponentially until 9 h or infinite time (∞) and then the AUC was calculated.

Plasma concentrations of FD-4 after i.n. administration as a solution containing 5 or 10% L-Arg were about twice that of FD-4 alone (control) and the plasma level was maintained until 9 h. These profiles were almost the same as that after application with 10% L-Lys (Fig. 1a). In 0.5% chitosan, as a positive control, the C_{max} of FD-4 was about seven times higher than the control and $F_{0-9\ h}$ was 24.4% (Fig. 1b and Table 1). These results for chitosan are similar to those reported by Illum et al. (1994). They mentioned no significant difference in the enhancing intensity on the insulin nasal absorption by chitosan more than 0.5% of concentration. In CPCL (0.5%), FD-4 was rapidly absorbed and the C_{max} was 23 times higher. Plasma concentration and its kinetic parameters of FD-4 with CPCL treatment were very similar to 0.5% SDS (Fig. 1c). Among classical enhancers, SDS, DC, BL-9, LPC and STDHF promoted the FD-4 absorption, while GC, TC and EDTA did not. SDS showed the highest $F_{0-9\ h}$ value of these enhancers. In the case of poly-L-Arg, the enhancing effect was dependent on its molecular weight (Fig. 1d). The T_{max} was 60–120 min, which was greater than CPCL and several of the classical enhancers. Higher plasma levels of FD-4 were maintained for a longer period than the control. Poly-L-Arg (100) showed the maximum effect among the cationic compounds and classical enhancers.

Fig. 2 summarizes the enhancing ratio (AUC_{0-9} h) by various classical enhancers or cationic compounds. The rank order of the enhancing ratio was 0.5% poly-L-Arg (100) $\cong$ 0.5% SDS $\cong$ 0.5% CPCL > 0.5% poly-L-Arg (50) > 0.5% DC $\cong$ 0.5% STDHF > 0.5% BC-9 $\cong$ 0.5% LPC > 0.5% chitosan $\cong$ 0.5% poly-L-Arg (10) $\geq$ 10% L-Arg $\cong$ 10% L-Lys > 0.5% GC $\cong$ 0.5% TC $\cong$ 0.5% EDTA. These results indicate that CPCL and poly-L-Args in cationic compounds can be used for enhancing the nasal absorption of high molecular weight compounds.

3.2. Effect of cationic compounds on hemolysis of rat erythrocytes

Fig. 3 shows hemolysis of rat erythrocytes after treatment of various classical enhancers or cationic compounds. The classical enhancers, 0.5% SDS, DC and STDHF caused a considerable amount of erythrocyte lysis, while 0.5% GC and TC and EDTA caused minimum lysis. Jabbal Gill et al. (1994a) reported that hemolytic effect could be utilized to evaluate membrane damage by enhancers. Erythrocyte lysis took place by bile salts such as DC and STDHF at lower concentrations. Some of classical enhancers would directly act on the nasal epithelial cells.

On the other hand, CPCL showed about 70% of hemolysis among cationic compounds. L-Lys and L-Arg caused erythrocyte lysis when applied at more than 10%. Since 0.5% L-Arg did not cause severe hemolysis, the hemolysis may be related to the hypotonic state of the basic amino acid solution. L-Lys and L-Arg, therefore, may not directly act on the erythrocyte membrane. On the other hand, 0.5% poly-L-Arg (10) (50) and (100) did not cause erythrocyte lysis. Since chitosan does not have a harmful effect on rat nasal mucosa as judged from histopathological observations (Illum et al., 1994), severe hemolysis by chitosan used in this study may not take place.

3.3. Effect of cationic compounds on leachings of protein, phospholipids and LDH from isolated rabbit nasal mucosa

Fig. 4 shows the effect of enhancers on the leachings of protein, phospholipids and LDH from isolated rabbit nasal mucosa. Protein and phospholipids were significantly leached by classical enhancers (0.5% SDS, DC, BL-9, LPC and

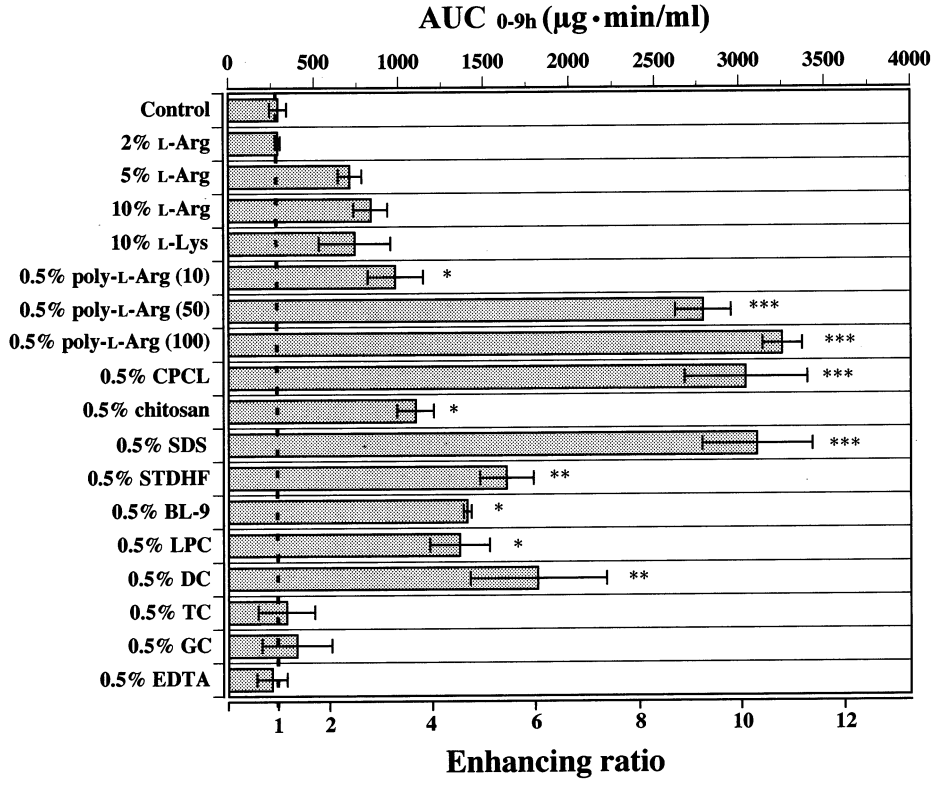

Fig. 2. Enhancing ratio ($AUC_{0-9\ h}$) of FD-4 absorption by cationic compounds and classical enhancers. Each data column represents the mean ± S.E. ($n = 3–6$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with control for $AUC_{0-9\ h}$.

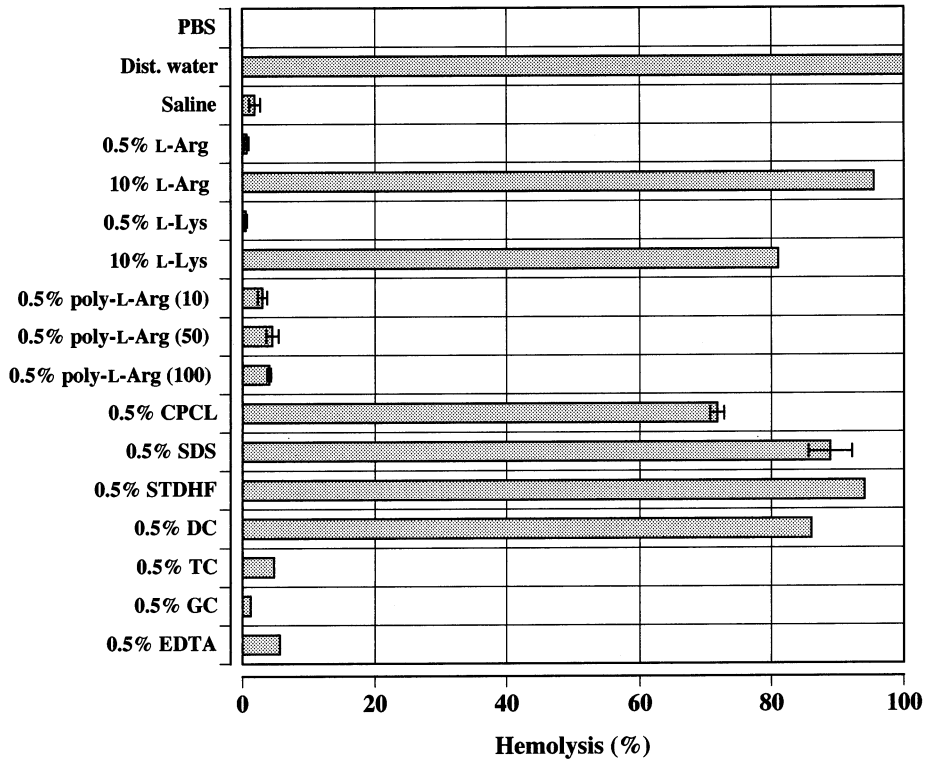

Fig. 3. Hemolytic effects of cationic compounds and classical enhancers on rat erythrocytes. Each data column represents the mean ± S.E. ($n = 3–6$).

STDHF applications). While EDTA and TC did not significantly leach them, only phospholipids were significantly leached by GC (Fig. 4a, b). LDH activities after DC, STDHF and LPC application were also higher than that of the control (Fig. 4c). Rank orders of protein, phospholipids and LDH leachings were different for some classical enhancers. As previously reported, it is expected that this difference may be related with the enhancing mechanism of FD-4 absorption (Hosoya et al., 1994).

On the other hand, the amount of protein leaching by 0.5% CPCL was significantly higher than the control and similar with BL-9 and SDS (Fig. 4a). In contrast, the amount of leaching of protein, phospholipids and LDH by 0.5% poly-L-Arg (10), (50) and (100) was almost the same with the control except for phospholipid leaching by 0.5% poly-L-Arg (100).

These results and from those of hemolysis, suggest that poly-L-Args are very useful from a safety point of view.

3.4. Relationships between enhancing and undesirable effects

Fig. 5 illustrates the relationship between the enhancing and hemolytic effects. In classical enhancers, the enhancing ratios of SDS, DC and STDHF were 6–11, but hemolysis was more than 80%. GC, TC and EDTA, on the other hand, had no enhancing and hemolytic effects. Of cationic compounds, CPCL showed a similar result with SDS. A total of 10% L-Lys and L-Arg caused erythrocyte lysis in spite of a slight enhancing effect. In contrast, poly-L-Arg (10), (50) and (100) greatly enhanced the nasal absorption of FD-4 without severe erythrocyte lysis.

Fig. 6 shows relationships between the enhancing and leaching effects.In classical enhancers, protein, phospholipids, and LDH leaching were well correlated with the enhancing ratio ($AUC_{0-9\ h}$) in FD-4 absorption and correlation coefficients were 0. 783 ($P < 0.05$), 0.827 ($P < 0.01$) and 0.794 ($P < 0.05$), respectively. We have reported that the enhancing ratio by these compounds, which was based on permeability of FD-10 across isolated rabbit nasal mucosa using in vitro Ussing chamber system, was closely correlated with the amounts of protein and phospholipids leaching from nasal mucosa (Hosoya et al., 1994). Although, in this report, enhancing ratio was obtained from AUC ratio between enhancer treatment and untreatment in vivo FD-4 absorption study in rats, there was a good relation between AUC ratio of FD-4 in rats and in vitro permeability of FD-10 in rabbits after co-application of each FD with classical enhancer (data not shown). Similar relations were obtained in small and large intestinal membranes in rats (Uchiyama et al., 1996; Yamamoto et al., 1996). Thus, in classical enhancers, a good correlation can be

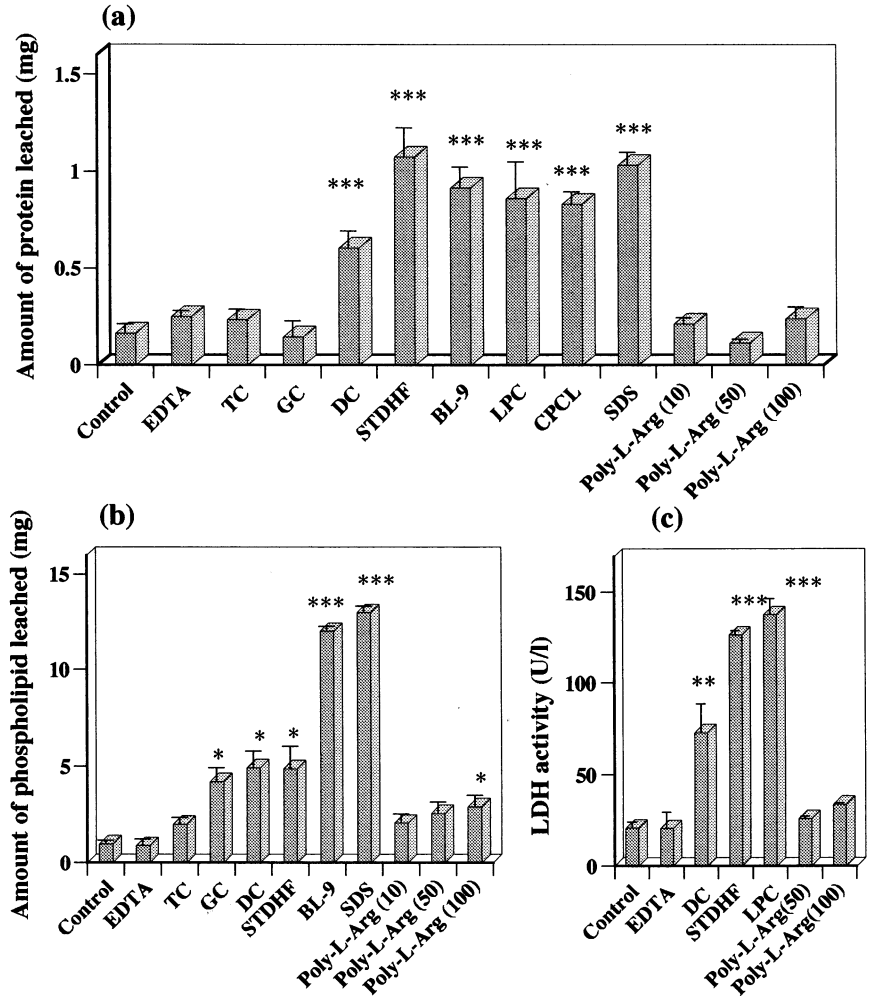

Fig. 4. Leaching effects of cationic compounds and classical enhancers on isolated rabbit nasal mucosa. (a) Protein leaching; (b) phospholipid leaching; (c) LDH leaching. Each data column represents the mean ± S.E. ($n = 3–9$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with control.

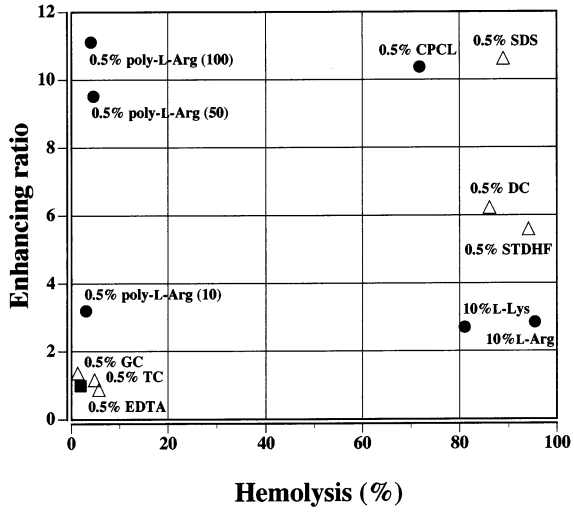

Fig. 5. Relationship between hemolysis and enhancing effect. ■, control; △, classical enhancer; ●, cationic compound.

obtained between enhancing and leaching effects independent of species and mucosal sites in our studies. The enhancing effects by these compounds, therefore, could be parallel to the harmful effects at 0.5% of a concentration used in this experiment, even though the enhancing mechanism was different among the classical enhancers. Thus, it is difficult to use these enhancers to obtain increased absorption of high molecular weight substances without severe membrane damage. Jabbal Gill et al. (1994b) found a protective effect of cyclodextrins against membrane damage by enhancers for nasal insulin delivery. These classical enhancers may need a protecting agent to prevent the toxic effects.

Of cationic compounds, CPCL showed similar results with SDS and was nearly positioned on the regression line of classical enhancers as shown in Fig. 6a. This result suggests that CPCL cannot be applied at this concentration from a safety point of view. In contrast, enhancing ratios by poly-L-Arg (10) (50) and (100) shifted from their regression lines and leaching of protein, phospholipids and LDH was very low in spite of a marked enhancement of FD-4 absorption (Fig. 6a, b, c). These results indicate that poly-L-Arg is a promising candidate as an absorption enhancer having a suitable enhancing intensity and is safe for application to nasal mucosal membrane.

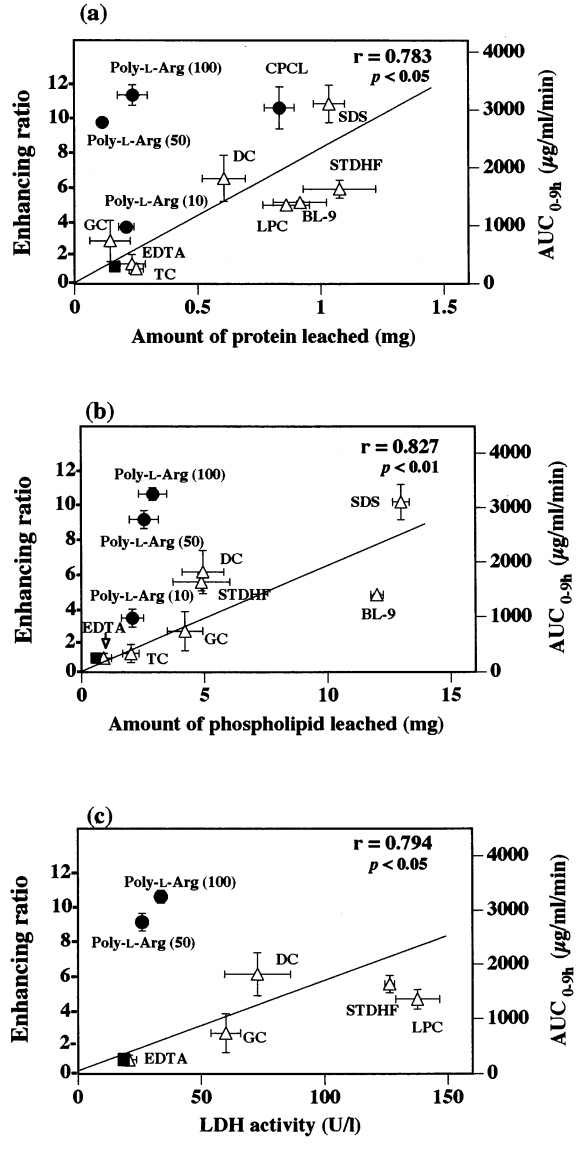

Fig. 6. Relationships between leaching and enhancing effects. ■, control; △, classical enhancer; ●, cationic compound. (a) Protein leaching versus enhancing ratio ($AUC_{0-9\ h}$); (b) phospholipid leaching versus enhancing ratio ($AUC_{0-9\ h}$); (c) LDH leaching versus enhancing ratio ($AUC_{0-9\ h}$). Regression lines were calculated using data from classical enhancers. Each data point represents the mean ± S.E. ($n = 3–9$).

Recently, there has been considerable interest in the role of the eosinophil and, in particular, eosinophil-derived cationic proteins in the pathogenesis of bronchial asthma (Gleich, 1990). The best characterized of these proteins is major basic protein (MBP), a highly charged cationic protein (MW 13 800) (Masmoen et al., 1986). MBP is able to damage airway epithelial cells and induce histological features of those observed in the lungs of asthmatic individuals (Gleich, 1990). Coyle et al. (1993) investigated whether poly-L-Arg had a damaging action on airway epithelial cells as an alternative of MBP. The obtained histopathological observations on poly-L-Arg, however, indicated the failure to produce any significant change in membrane damage. Some of polycations could promote transmucosal delivery of high molecular substances without a marked toxic action to mucosal membrane.

The electrical property may be important to improve nasal drug delivery. Cationic compounds would interact with the cell surface-negative charges of the epithelial cells to increase the permeability through inter- and/or pare-cellular routes (Singh et al., 1992). Our data indicates that, however, the electrical property did not involve a great enhancement of nasal absorption and undesirable effect on nasal epithelial cells. Difference in the permeation enhancing effects between L-Arg and poly-L-Arg could not be explained only by electrical property. In addition, CPCL caused large amounts of hemolysis and leaching of cell membrane components, in spite of the greatly enhanced nasal absorption of FD-4. Since a smaller amount of CPCL is utilized as a disinfectant in oral cavity, 0.5% CPCL in this study may directly act on the epithelial cells. Indeed, when CPCL was applied at 0.05%, only a weak enhanced absorption of FD-4 was observed (data not shown).

In classical enhancers, enhancement of FD-10 permeation through the isolated nasal mucosa resulted in the decrease of transmucosal membrane resistance, R_m (Hosoya et al., 1994). The decrease in R_m closely related with leaching amounts of mucosal membrane components. In addition, a histopathological observation showed that an additive with great enhancement of FD-10 permeation removed a large amount of microvilli from the ciliated cells as well as the nasal mucosal cells from the basal membrane (Hosoya et al., 1994; Kubo et al., 1994). Although the enhancing mechanism of these enhancers at the initial stage, when they were applied on the mucosal surface, may be different, the large amounts of membrane components leached by an enhancer would lead to the death of the epithelial cell. Marttin et al. (1997) visualized the transport route of FDs with different mean molecular weight through the nasal mucosa after coadministration with enhancers using confocal laser scanning microscopy. They reported that FD transports were increased in the intercellular route, but transcellular transport was also observed leading to the nasal cell damage. CPCL may mainly increase FD transport in the intercellular route, but severe damage to nasal mucosal membrane also took place. In contrast, poly-L-Arg greatly enhanced FD-4 absorption without any undesirable effects. The results obtained here suggest that, at least, the enhancing mechanism of poly-L-Arg does not relate with leaching of protein, phospholipids and LDH. Moreover, T_{max} of FD-4 by poly-L-Arg was delayed compared with those by classical enhancers (see Table 1) and cellular response by poly-L-Arg, therefore, was found to be different from those with classical enhancers. In addition, the enhancing effect by poly-L-Arg was dependent on its molecular weight (Fig. 2 and Table 1). It is thought that poly-L-Arg, which has a higher molecular weight, would have a higher affinity to a receptor, ion channel or glycoprotein on the nasal epithelium. Thus, the electrical property, molecular size and higher-ordered structure together with other physicochemical properties may give the different absorption mechanism which allows a great enhancement without severe damage to the nasal mucosa, however further clarification of the mechanism of absorption is required.

In conclusion, poly-L-Arg resulted in a markedly high bioavailability of FD-4 without undesirable effects to rat erythrocyte and isolated rabbit nasal mucosa. These desirable effects have not previously been obtained by various classical

enhancers. We thus emphasize that poly-L-Arg is a promising candidate having a suitable balance between enhancing activity and safety for nasal peptide and protein delivery.

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