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COMMUNICATION

Effects of Low-Viscosity Sodium Hyaluronate Preparation on the Pulmonary Absorption of rh-Insulin in Rats

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ABSTRACT

Purpose: A low-viscosity formulation for pulmonary delivery of rh-insulin as model peptide drugs was developed using a solution of sodium hyaluronate. Method: *The effects of different concentrations and pH values of low-viscosity solutions of hyaluronate on the pulmonary absorption of rh-insulin were examined after intratracheal administration in rats. The permeation of fluorescein isothiocyanate (FITC)–dextran (molecular weight 4300; FD-4) and insulin through excised rat trachea in vitro were also examined.* Results: *The hyaluronate (2140 kDa) solutions (0.1% and 0.2% w/v) at pH 7.0 significantly enhanced the pharmacological availability (PAB) of insulin compared to the aqueous solution of insulin at pH 7.0. The absorption-enhancing effect at a concentration of 0.1% w/v hyaluronate was greater than that at a concentration of 0.2% w/v hyaluronate. Furthermore, the greatest absorption-enhancing effect was obtained, regardless of the molecular weight of hyaluronate, when the concentration of hyaluronate was adjusted to 0.47 μM. Absorption-enhancing effects were consistent with the effect of a 0.1% w/v hyaluronate preparation at pH 4.0 and 7.0 on the permeation of FITC-dextran and insulin through*

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excised rat trachea in vitro. Conclusion: *Low-viscosity hyaluronate preparation was shown to be a useful vehicle for pulmonary delivery of peptide drugs.*
KEY WORDS: *Absorption enhancement; Hyaluronate; Insulin; Peptide drug; Pulmonary delivery; Viscous preparation.*

INTRODUCTION

Pulmonary routes for peptide/protein drugs have been shown to result in a comparatively higher systemic bioavailability than that observed following administration by oral and other mucosal routes (1,2). Pulmonary delivery of these drugs is a clinically relevant route of administration for local and systemic action. The large absorption surface area of the lungs, thin alveolar epithelial barrier, extensive vasculature, possibly low proteolytic activity, and avoidance of the hepatic first-pass effect all favor the absorption of peptide/protein drugs. The systemic delivery of peptide and protein drugs such as calcitonin (3), insulin (4), human growth hormone (5), and rhG-CSF (6) by the pulmonary route has recently received much attention. However, the absorption of peptide/protein drugs following pulmonary administration will be influenced by physicochemical characteristics, deposition pattern, and formulation. Therefore, development of formulations for pulmonary delivery of peptide and protein drugs is important for effective and safe therapy (7).

Sodium hyaluronate, which is a straight-chain and unbranched polymer with repeating disaccharide units of glucuronic acid and *N*-acetylglucosamine, is a natural polymer and a major component of interstitial tissue (8). An aqueous solution of sodium hyaluronate has viscous and mucoadhesive characteristics (9). We previously reported that a viscous sodium hyaluronate solution enhanced the nasal absorption of vasopressin and one of its analogs (10). In the present study, a low-viscosity formulation was developed using a solution of sodium hyaluronate with various molecular weights (MW) for pulmonary delivery of peptide and protein drugs. To evaluate the properties of the formulation, the effects of sodium hyaluronate solutions of various pH values and concentrations on the pulmonary absorption of recombinant human (rh) insulin as a model peptide drug were examined in rats. Furthermore, the effects of sodium hyaluronate solutions on the tracheal permeation of fluorescein isothiocyanate (FITC)–dextran (MW 4300; FD-4) and insulin were examined in vitro using excised rat tracheas.

EXPERIMENTAL

Materials

Sodium hyaluronates (average MW 1100, 1600, and 2140 kDa) were supplied from Shiseido Company, Limited (Tokyo, Japan). The rh-insulin (24.0 IU/mg) was obtained from Becton Dickinson Labware (Bedford, MA). FD-4 was obtained from Sigma Chemical Company (St. Louis, MO). All other chemicals were of the highest purity available commercially.

Preparations

Sodium hyaluronate solutions were prepared by the presoaking of the sodium hyaluronate (0.05%–0.2% w/v) in isotonic phosphate buffered solutions (PBS) at pH 4.0, 5.0, and 7. 0. PBS were made by mixing 0.236 M citric acid/0.123 M disodium phosphate buffer (pH 4.0 and 5.0) and 0.171 M disodium phosphate/0.144 M bicarbonate buffer (pH 7.0). For the experiments using sodium hyaluronate of different average molecular weights (1100, 1600, and 2140 kDa), sodium hyaluronate solutions were prepared at molar concentrations of 0.23, 0.47, and 0.91 μM of hyaluronate at a pH of 7. 0. Sodium hyaluronate 0.1% w/v with a MW of 2140 kDa is equivalent to 0.47 μM. Insulin (25 IU/ml) was dissolved in the sodium hyaluronate solutions or PBS. The viscosity of a hyaluronate preparation was measured at the rate of shear of 75.2 s^{-1} by a cone-plate viscometer (E type, Tokyo Keiki Co., Ltd., Tokyo, Japan) at 37°C. The viscosities of the hyaluronate preparations is shown in Table 1.

Pulmonary Administration

Male Wistar rats weighing 210–300 g were fasted for 20 h prior to the experiments, but were allowed free access to water. The rats were anesthetized by an intraperitoneal injection of pentobarbital (50 mg/kg), with additional doses given intraperitoneally as necessary during the experiments. Intratracheal administration was carried out according to the method of Enna and Schanker (11).

Table 1

Viscosity of Hyaluronate Solutions

Hyaluronate Solution	Average Mw Hyaluronate (kDa)	Viscosity (cps)
Hyaluronate 0.05% (w/v) at pH 7.0	2140	2.88
Hyaluronate 0.1% (w/v) (0.47 μM) at pH 7.0	2140	4.60
Hyaluronate 0.2% (w/v) at pH 7.0	2140	14.30
Hyaluronate 0.1% (w/v) (0.47 μM) at pH 5.0	2140	4.33
Hyaluronate 0.1% (w/v) (0.47 μM) at pH 4.0)	2140	4.07
Hyaluronate 0.47 μM at pH 7.0	1100	1.68
Hyaluronate 0.47 μM at pH 7.0	1600	2.23

The viscosity of hyaluronate preparations was measured at the rate of shear of 75.2 s^{-1} by a cone-plate viscometer (E type, Tokyo Keiki Co., Ltd., Tokyo) at 37°C.

Briefly, the trachea was exposed through a middle incision in the neck, and a 2.5-cm length of polyethylene tubing (i.d. 1.5 mm, o.d. 2.3 mm) was inserted through the incision between the fourth and fifth tracheal rings caudal to the thyroid cartilage to a depth of 0.6 cm. The insulin formulations (insulin dose 5 IU/kg, dosage volume 200 μl/kg body weight) were administered via the tube inserted in the trachea by a 100-μl glass syringe. In a comparative study, insulin was administered intravenously to rats at a dose of 0.1 IU/kg. Blood samples were periodically withdrawn from the jugular vein.

The absorption of insulin was evaluated by its hypoglycemic effect and immunoreactive insulin (IRI) levels in plasma. Plasma glucose levels were determined by the glucose oxidase (GOD) method. Plasma IRI levels were determined by radioimmunoassay (RIA) (Shionogi RIA Kit, Shionogi Co., Ltd., Osaka, Japan). The pulmonary absorption of insulin was evaluated by the area bounded by the curve of the hypoglycemic effect and time (0–6 h), and the line showing 100% of the glucose level was calculated by means of trapezoidal integration. This area was defined as the area above the hypoglycemic effect–time curve *AAC* and used for evaluating the insulin absorption. Pharmacological bioavailability (PBA) was calculated as $(AAC_{\text{Pulmonary administration}}/D_{\text{Pulmonary administration}})/(AAC_{\text{iv}}/D_{\text{iv}}) \times 100\%$, where D is the dose of insulin.

Permeation Tests

Permeation tests were carried out using noneverted sacs (1.2-cm segment) of the excised rat (250–300 g) trachea at 37°C by a modified method previously described (12). FD-4 or insulin in the hyaluronate solutions or the PBS (40 μl) was infused into the sac (mucosal side), which was then placed in serosal medium, 7 ml HEPES buffer solution (pH 7.4), which was bubbled with 95% O_2 and 5% CO_2. Samples (0.2 ml) were removed from the serosal fluid at predetermined times for 150 min, and 0.2 ml portions of fresh fluid were added to the cell to maintain the original volume.

In separate experiments, samples (10 μl) were removed from the mucosal fluid at a predetermined time to calculate the degradation rate of FD-4 or insulin in the tracheal mucosal fluid. The concentration of FD-4 was determined by spectrofluorometry. The degradation of FD-4 in the final sample (150 min) of serosal fluid was checked by high-performance liquid chromatography (HPLC) apparatus equipped a gel permeation column. Insulin was determined by the RIA method.

The cumulative amount of compound in the serosal fluid was plotted as a function of time. The steady-state fluxes (J, pmol/cm^2/s) and apparent permeability coefficients (P_{app}, cm/s) of these compounds were estimated from the slope of the linear portion of the plot using Eqs. 1 and 2, respectively:

$$J = (dQ/dt)/A \tag{1}$$

$$P_{app} = J/C_0 = (dQ/dt)/A/C_0 \tag{2}$$

where dQ/dt is the solute transfer rate (mol/s), C_0 is the initial concentration of compound on the mucosal side, and A is the surface area of the membrane exposed to the compound.

Data Analysis

All data were expressed as mean plus or minus the standard error of the mean (SE). The comparison of the hypoglycemic effect was performed based on the results of the statistical analysis for the *AAC* and the PBA values. Comparisons between group means were evaluated by the unpaired Student t test. Statistical significance of differences among more than two groups were determined by one-way analysis of variance (ANOVA). When F tests from ANOVA indicated significant differences ($P < .05$), a multiple comparison was performed to compare group means by the Bonferroni t procedure.

RESULTS

Pulmonary Absorption of Insulin

Figure 1 shows the effects of the different concentrations (0.05%, 0.1%, and 0.2% w/v) of sodium hyaluro-

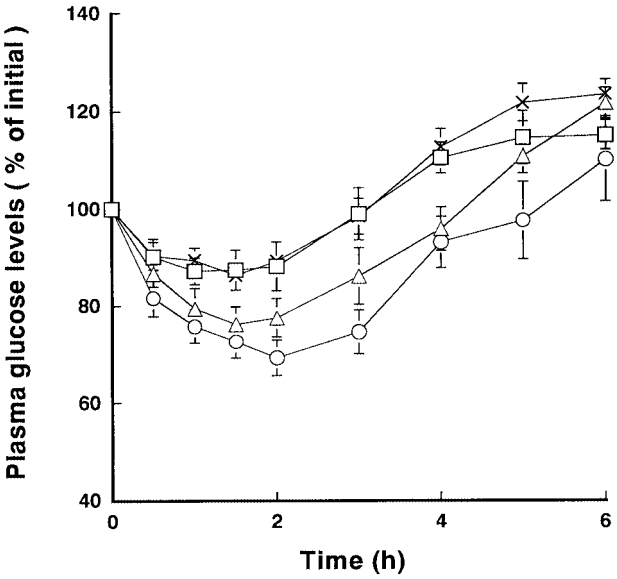

Figure 1. Effects of various concentrations (0.05%, 0.1%, and 0.2% w/v) of sodium hyaluronate (2140 kDa) on the hypoglycemic effects after pulmonary administration of insulin (5 IU/kg body weight) in hyaluronate preparations at pH 7.0 in rats: × insulin in aqueous solution; □ 0.05% w/v hyaluronate preparation; ○ 0.1% w/v hyaluronate preparation; △ 0.2% w/v hyaluronate preparation. Each point represents the mean ± SE of four animals.

nate (2140 kDa) on the hypoglycemic effects after pulmonary administration of insulin (5 IU/kg body weight dose) preparations at pH 7.0 in rats. The hyaluronate preparations (0.1% and 0.2% w/v) enhanced the hypoglycemic effects compared with that of aqueous solution (in PBS) at pH 7. 0. The absorption-enhancing effect at a concentration of 0.1% w/v hyaluronate was greater than that at a concentration of 0.2% w/v hyaluronate.

Figure 2 shows the effects of the molecular weight (1100, 1600, and 2140 kDa) of sodium hyaluronate (0.23, 0. 47, and 0.91 μM, respectively) on the hypoglycemic effects after pulmonary administration of insulin (5 IU/kg body weight dose) preparations at pH 7.0 in rats. The 0.1% w/v concentration of hyaluronate with a molecular weight of 2140 kDa is equivalent to 0.47 μM. The hypoglycemic effects after administration of insulin in hyaluronate preparations were not influenced by the viscosity of the preparations (Table 1). However, similar hypoglycemic effects were obtained at the same molar concentration of hyaluronate with various molecular weights (1100, 1600, and 2140 kDa). The preparations of 0.47 μM hyaluronate showed the highest hypoglycemic effects regardless of molecular weight (1100, 1600, and 2140 kDa).

Figure 3 shows the effects of pH (pH 4.0, 5.0, and 7.0) of 0.1% w/v sodium hyaluronate (2140 kDa) preparations on the hypoglycemic effects after pulmonary administration of insulin preparations (insulin dose 5 IU/

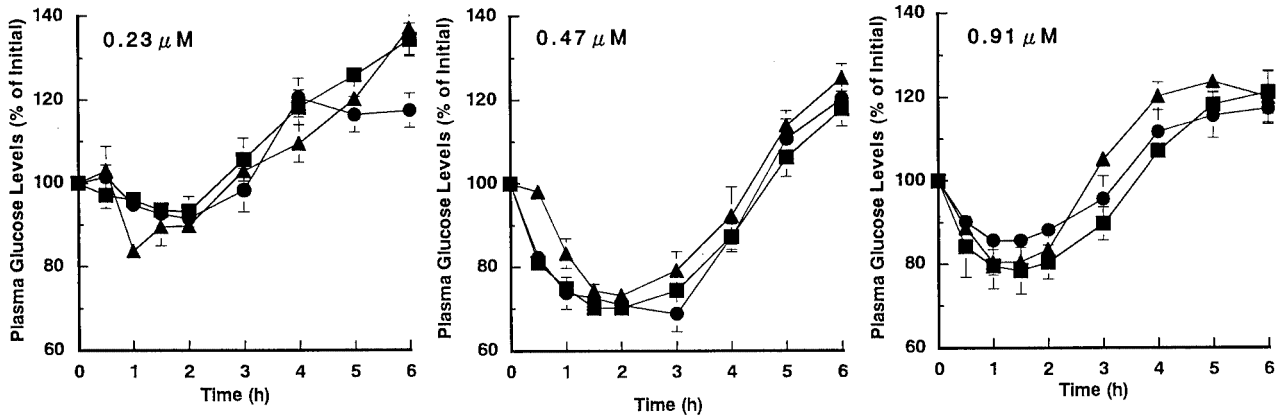

Figure 2. Effects of different molecular weights (1100, 1600, and 2140 kDa) of sodium hyaluronate (0.23, 0.47, and 0.91 μM) on the hypoglycemic effects after pulmonary administration of insulin (5 IU/kg body weight) in hyaluronate preparations at pH 7.0 in rats. Molecular weights of hyaluronate: ● 1100 kDa; ▲ 1600 kDa; ■ 2140 kDa. Each point represents the mean ± SE of four animals.

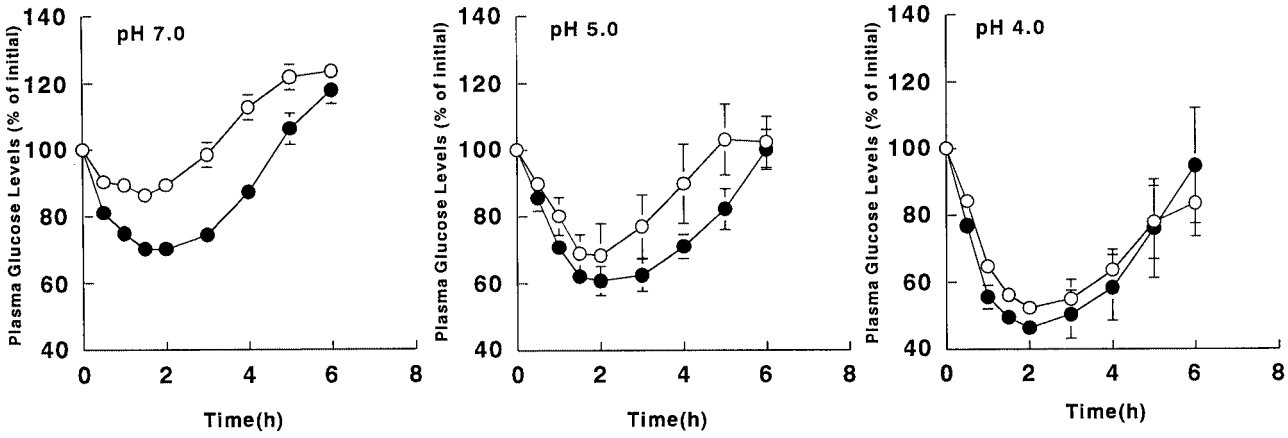

Figure 3. Hypoglycemic effects after pulmonary administration of insulin in 0.1% w/v hyaluronate (2140 kDa) preparation and aqueous solution (5 IU/kg body weight) at pH 7.0, pH 5.0, and pH 4.0 in rats: ○ insulin aqueous solution; ● 0.1% w/v hyaluronate preparation. Each point represents the mean ± SE of four animals.

kg body weight) in rats. The *AAC* and PBA values after pulmonary administration of insulin (5 IU/kg) in the hyaluronate preparation are also summarized in Table 2. Both the hyaluronate preparations and aqueous solutions (in PBS) at lower pH values showed greater hypoglycemic effects. The hyaluronate preparations at pH 7.0 significantly enhanced the hypoglycemic effects compared to the aqueous solutions (in PBS) at pH 7.0. However, the significant enhancement of hypoglycemic effects by hyaluronate preparations at pH 4.0 and 5.0 did not show when compared with those of the aqueous solutions (in PBS) at pH 4.0 and pH 5.0, respectively.

Figure 4 shows the change of IRI levels in plasma

Table 2

Pharmacological Bioavailability (PBA) After Pulmonary Administrations of Insulin Preparations in Rats

	AAC (% · h)[a]	PAB (%)
Intravenous (insulin dose 0.1 IU/kg)	16.39 ± 7.95	—
Pulmonary administration (insulin dose 5 IU/kg)		
Aqueous solution (pH 7.0)	63.49 ± 12.81	7.75 ± 1.56
Aqueous solution (pH 5.0)	166.86 ± 45.81	20.37 ± 5.59
Aqueous solution (pH 4.0)	278.45 ± 31.81	33.99 ± 3.88
Hyaluronate 0.05% (w/v) (2140 kDa) pH 7.0	92.43 ± 12.38	11.13 ± 1.51
Hyaluronate 0.1% (w/v) (2140 kDa) pH 7.0	165.80 ± 10.45[b]	20.24 ± 1.28[b]
Hyaluronate 0.2% (w/v) (2140 kDa) pH 7.0	101.31 ± 10.49[b]	12.37 ± 1.28[b]
Hyaluronate 0.1% (w/v) (2140 kDa) pH 5.0	238.00 ± 19.49	29.06 ± 2.38
Hyaluronate 0.1% (w/v) (2140 kDa)pH 4.0	300.60 ± 36.10	36.69 ± 4.41

Each value represents the mean ± SE of four experiments.
[a] AAC, area above the time curve of the hypoglycemic effects.
[b] Significant at $P < .05$ versus insulin aqueous solution at corresponding pH.

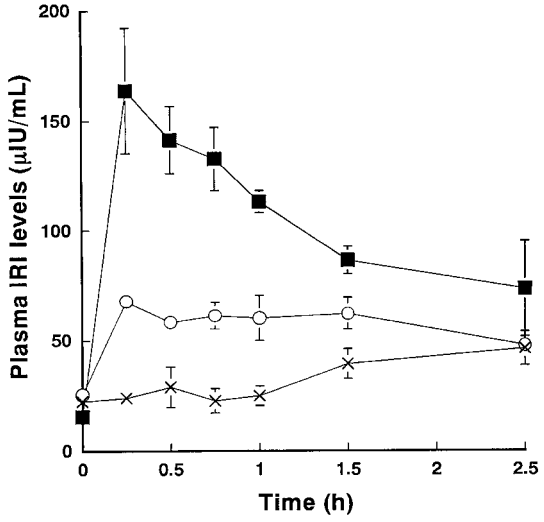

Figure 4. Change of IRI levels in plasma after pulmonary administration of insulin (5 IU/kg body weight) in PBS and hyaluronate preparation at pH 7.0 in rats: × PBS without insulin; ○ insulin in aqueous solution; ■ 0.1% w/v hyaluronate preparation. Each point represents the mean ± SE of four animals.

Table 3

Effect of 0.1% (w/v) Hyaluronate Preparation at pH 4.0 and pH 7.0 on the Permeation of FD-4 and Insulin Through Excised Rat Trachea

Preparations	P_{app} (cm/s) $\times 10^{-7}$
Insulin	
Aqueous solution (pH 4.0)	2.57 ± 0.54
Hyaluronate 0.1% (w/v) (pH 4.0)	2.57 ± 0.71
Aqueous solution (pH 7.0)	1.07 ± 0.14
Hyaluronate 0.1% (w/v) (pH 7.0)	1.50 ± 0.07[a]
FD-4	
Aqueous solution (pH 4.0)	2.54 ± 0.08
Hyaluronate 0.1% (w/v) (pH 4.0)	2.92 ± 0.31
Aqueous solution (pH 7.0)	1.31 ± 0.15
Hyaluronate 0.1% (w/v) (pH 7.0)	1.85 ± 0.18[b]

Each value represents the mean ± SE of four experiments.
[a] Significant at $P < .05$ versus Insulin in aqueous solution at corresponding pH.
[b] Significant at $P < .05$ versus FD-4 in aqueous solution at corresponding pH.

after pulmonary administration of insulin preparations (insulin dose 5 IU/kg body weight) at pH 7.0 in rats. The IRI levels after administration of insulin with the 0.1% w/v hyaluronate preparation were higher than those after the administration of insulin solution at pH 7.0.

Tracheal Permeation

Table 3 shows the effect of 0.1% w/v hyaluronate preparation at pH 4.0 and pH 7.0 on the permeation of FD-4 and insulin through excised rat trachea in vitro. The permeation of FD-4 and insulin at pH 4.0 was significantly higher than at pH 7. 0. The permeation of FD-4 and insulin in 0.1% (w/v) hyaluronate preparations at pH 7.0 was significantly higher than that in PBS. These results are related to those of the in vivo experiments.

DISCUSSION

We designed the low-viscosity hyaluronate preparation for the pulmonary delivery of peptide drugs. The low-viscosity preparation of 0.1% and 0.2% w/v sodium hyaluronate (2140 kDa) significantly enhanced the pulmonary absorption of insulin (5 IU/kg body weight) at pH 7.0 in rats compared to the aqueous solution. However, the 0.05% preparation of sodium hyaluronate did not significantly enhance the absorption of insulin. The enhancing effect of 0.1% w/v hyaluronate was greater than that of 0.2% w/v hyaluronate (Fig. 1).

These results suggest that the preparation of 0.1% hyaluronate is most optimal for enhancing the insulin absorption when hyaluronate with a molecular weight of 2140 kDa is used as the dosage formulation. Essentially, the improvement of the absorption is expected to be dependent on the concentration of hyaluronate. On the other hand, it is also thought that as the concentration of hyaluronate increases, the diffusion of insulin in the formulation decreases. Therefore, 0.1% hyaluronate may give the best balance between the absorption-enhancing effect and the diffusion of insulin in the formulation.

A similar enhancing effect on the pulmonary absorption of insulin was seen at the same molar concentrations of hyaluronate (0.23, 0.47, and 0.91 μM) regardless of molecular weight (1100, 1600, and 2140 kDa) (Fig. 3). Furthermore, the preparations (pH 7.0) of 0.47 μM hyaluronate also showed the highest absorption-enhancing effect regardless of molecular weight. This result was independent of the order of viscosity (Table 1). Therefore, an optimal concentration of hyaluronate (0.47 μM) may exist for enhancing the insulin absorption.

It is important to clarify the characteristics of tracheal drug absorption since drugs administered to the lung through the trachea may be absorbed partially via the tracheal membrane. The tracheal permeability of both insulin and FD-4 increased with the 0.1% hyaluronate preparation at pH 7.0, whereas this enhancing effect was not observed at pH 4. 0. These results were similar to those obtained from the experiment of in vivo pulmonary administration. However, the enhancing effect of the insulin absorption by the 0.1% hyaluronate preparation calculated from the PBA value was greater than that expected from the tracheal permeation experiment. This fact suggests that the absorption-enhancing effect of insulin is caused not only by the increase of the tracheal permeability, but also by the enhancement of the pulmonary absorption.

The pulmonary absorption of insulin was higher at lower pH values of the 0.1% hyaluronate preparation and aqueous solution (Fig. 3). On the other hand, the absorption-enhancing effect of the 0.1% hyaluronate preparation on the pulmonary absorption of insulin was obtained only at pH 7.0 when comparing to *AAC* or PBA values after administration of insulin aqueous solution at the same pH (Table 2). The absorption-enhancing effect of

0.1% hyaluronate at pH 7.0 was also confirmed by the plasma concentration of IRI (Fig. 4). These results may be due to the structure or electrical charge of the membrane and/or self-association and conformational changes of insulin (13). However, the reason for this discrepancy is unclear. Further investigations are needed for clarifying these points.

It has been reported that some mucoadhesive polymers improved the bioavailability of poorly absorbable drugs such as peptide/protein drugs (14,15). We also reported that Carbopol® (16) and hyaluronate (10), which are mucoadhesive polymers, increased the nasal or rectal absorption of peptide drugs. It is generally accepted that mucoadhesive polymers can increase the drug concentration in the vicinity of the mucosal cells by intensifying the contact of drugs with the mucosa, which consequently enhances drug absorption. In this study, a low-viscosity hyaluronate preparation may enhance the pulmonary absorption of insulin by this mechanism.

A number of absorption enhancers for pulmonary and other routes of mucosal administration of large hydrophilic drugs, such as peptides and proteins, have been investigated in the last 10–15 years (17,18). However, these absorption enhancers have not been used clinically because of concern about their toxicity. Since hyaluronate has been extensively used in pharmaceutics and medicine, the safety of hyaluronate in the body has been established by its widespread clinical use. We reported that the viscous sodium hyaluronate solution did not affect the ciliary beat frequency of rabbit nasal mucosal membrane in vitro (10).

In conclusion, a low-viscosity sodium hyaluronate preparation has been shown to be a useful vehicle for instillation and aerosol inhalation and for pulmonary delivery of peptide/protein drugs, as well as for increasing their absorption via the respiratory tract.

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