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Enhancing effects of cyclodextrins on nasal absorption of insulin in rats

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Summary

Nasal administration of bovine insulin in suspension with chemically modified cyclodextrins led to a significant increase in serum immunoreactive insulin levels along with a marked hypoglycemia in rats. Methylated cyclodextrins were more potent enhancers of insulin absorption than the parent and hydroxypropylated cyclodextrins. Spectroscopic observations indicated that the scope of inclusion complexation of insulin with cyclodextrins was limited and appears to be of minor importance in the nasal absorption enhancement. Cyclodextrins increased the permeability of the nasal mucosa, perhaps through the interaction of cyclodextrins with lipids and/or divalent cations on the membrane surface. In addition, the enzymatic degradation of insulin in rat nasal homogenates was suppressed by cyclodextrins. The combination of increased nasal membrane permeability and reduced proteolysis may explain the enhanced nasal absorption of insulin. The present results suggest that chemically modified cyclodextrins, especially the methylated derivatives, may serve as potent absorption enhancers for the nasal delivery of polypeptides.

Introduction

The nasal absorption of peptide and protein drugs is severely restricted by pre-systemic elimination due to enzymatic degradation or mucociliary clearance and by the limited extent of mucosal membrane permeability. Absorption-promoting agents such as bile salts and other surfactants usually must be included in nasal formulations in order to achieve adequate systemic bioavailability (Hirai et al., 1981; Deurloo et al., 1989).

The usefulness of molecular encapsulation of drugs with cyclodextrins in pharmaceutical formulations has been fully realized (Uekama and Otagiri, 1987; Szejtli, 1988). However, little is known about the effects of cyclodextrins on biological membranes (Irie et al., 1982; Miyajima et al., 1987). Cyclodextrins have been shown to solubilize specific membrane lipids from human erythrocytes through the formation of inclusion complexes, leading to an increase in membrane permeability (Pitha et al., 1988; Ohtani et al., 1989). This occurs without entry of cyclodextrins into the membranes; they extract the lipids from the

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membranes into a new compartment located in the aqueous phase. Cyclodextrins may also affect mucosal membranes in the same manner.

Shimamoto (1987) has demonstrated that α-cyclodextrin (α-CyD) removes some fatty acids from rat nasal mucosa and enhances the nasal absorption of peptide and protein drugs. Our previous studies have shown the utility of chemically modified cyclodextrins as potential adjuvants to improve the nasal absorption of peptide and protein drugs including insulin (Arima et al., 1990; Matsubara et al., 1990). Thus, this paper deals with the detailed comparison of the effects of six cyclodextrins on the nasal absorption of bovine insulin in suspension in rats and discusses the absorption-promoting mechanisms with emphasis on the points where cyclodextrins interact with the surface of nasal mucosal membranes. During our work, an interesting report on the effects of cyclodextrins on intranasally administered human insulin in solution in rats appeared (Merkus et al., 1991) and the relationship of those results to the present work is discussed.

Materials and Methods

Materials

Crystalline bovine insulin (25.6 U/mg, Sigma Chemical Co., MO, U.S.A.), α-CyD, β-CyD, 2-hydroxypropyl-α-cyclodextrin (HP-α-CyD), and 2-hydroxypropyl-β-cyclodextrin (HP-β-CyD) (Nihon Shokuhin Kako Co., Tokyo, Japan), hexakis(2,6-di-*O*-methyl)-α-cyclodextrin (DM-α-CyD), heptakis(2,6-di-*O*-methyl)-β-cyclodextrin (DM-β-CyD) (Toshin Chemical Co., Tokyo, Japan) were used as supplied. The average degrees of substitution were confirmed to be 5.2 for HP-α-CyD and 5.8 for HP-β-CyD by mass and NMR spectrometry (Irie et al., 1988). [^{3}H(G)]Inulin (312.0 mCi/g; average Mol. Wt, 5000–5500) was obtained from New England Nuclear (MA, U.S.A.). The protease inhibitors, aprotinin (14 TIU/mg protein) and bestatin (Sigma Chemical Co., MO, U.S.A.) were used as supplied. Other materials were of reagent grade and de-ionized double-distilled water was used.

Spectroscopic studies

The circular dichroism and fluorescence spectra of insulin in isotonic phosphate buffer (pH 7.4, 25°C) in the absence and presence of cyclodextrins or Na_2EDTA were recorded on a Jasco J-600 spectropolarimeter (Tokyo, Japan) and a Hitachi F-4010 spectrofluorometer (Tokyo, Japan), respectively.

Preparation of insulin suspensions

The dose of insulin (10 U/kg) was determined according to results reported previously (Hirai et al., 1981). The concentration of cyclodextrins (80 mM) was chosen here based on our preliminary studies in which cyclodextrins enhanced the nasal absorption of insulin in a dose-dependent manner up to 80 mM (Arima et al., 1990). For example, insulin (39 mg, corresponding to 1000 U) was suspended in isotonic phosphate buffer (10 ml, pH 7.4) containing 80 mM cyclodextrins. The only exception was β-CyD which was used as a suspension due to limited solubility (1.85 g/dl in water at 25°C). Some characteristics of insulin suspensions were evaluated immediately after preparation. The volume-surface diameters of particles in the suspensions were measured using a Galai CIS-1 laser scan grading analyzer (Migdal Haemek, Israel). The apparent viscosities of the suspensions were determined on a Contraves AG Low Shear 30 rotational rheometer (Zurich, Switzerland) with the rate of shear ranging from 5.96 to 20.4 s^{-1}. To determine the apparent solubility of insulin in the preparations, they were filtered through a hydrophilic membrane (pore size, 0.2 μm; Dismic-25cs, Toyo Roshi Co., Tokyo, Japan), and the concentration of insulin in the filtrate was determined spectrophotometrically at 280 nm.

In vivo experiments

The nasal absorption studies were performed according to the procedures described by Hirai et al. (1981). Male Wistar rats weighing 200–250 g were fasted for 16 h and anesthetized with an intraperitoneal injection of sodium pentobarbital (30 mg/kg). An incision was made in the neck and the trachea was cannulated with a polyethylene tube (PE 260). A second tube (PE 260) was

inserted through the esophagus and the nasopharyngeal opening and tied in place. Dorsal recumbency inhibited mucociliary drainage since the nasopalatine tract was closed. Freshly prepared insulin suspensions (10 U/kg) with or without cyclodextrins were instilled into the nasal cavity with a micropipette. Blood samples (0.5 ml) were taken periodically from the jugular vein. Serum immunoreactive insulin was determined by enzyme immunoassay (Insulin-EIA test Wako®, Wako Pure Chemical Ind., Ltd, Osaka, Japan). Serum glucose was determined by the mutarotase-glucose oxidase method (Glucose C-test Wako®, Wako Pure Chemical Ind., Ltd, Osaka, Japan); cyclodextrins did not interfere with these assays. Insulin was completely dissolved in the buffer solution and was given intravenously at a dose of 5 U/kg to estimate the bioavailability of the nasal preparations. Cyclodextrins were not included in the intravenous preparations as they were unlikely to affect the intravenous disposition kinetics of insulin. Even if cyclodextrins are capable of forming complexes with insulin, rapid dissociation of such complexes may occur in systemic circulation (Uekama and Otagiri, 1987; Brewster et al., 1989). The nasal bioavailability (F) of insulin was calculated from the following equation:

$$F = \frac{AUC_{(i.n.)} - AUC_{(Control)}}{AUC_{(i.v.)} - AUC_{(Control)}} \times \frac{Dose_{(i.v.)}}{Dose_{(i.n.)}} \times 100$$

where AUC represents the area under the serum insulin level-time curve up to 6 h post-administration. The subscripts, (i.n.) and (i.v.), refer to nasal and intravenous administrations, respectively. $AUC_{(Control)}$ represents the area under the serum endogenous insulin level-time curve when the buffer solution without insulin was administered nasally. Serum glucose levels after insulin administration were expressed as a percentage of the initial level. The hypoglycemic effects of insulin preparations were expressed as the cumulative percentage of change in serum glucose levels up to 4 h post-administration, calculated by summing the areas above (negative values) and below (positive values) control levels obtained for buffer alone.

The permeability of rat nasal mucosa was assessed by measuring the nasal absorption of [^{3}H(G)]inulin (10 μCi/kg), an inert and poorly permeable marker, with or without cyclodextrins. The radioactivity in serum was evaluated using a liquid scintillation counter (Aloka LSC-3500, Tokyo, Japan).

In situ experiments

Isotonic phosphate buffer (10 ml, pH 7.4) containing cyclodextrins (80 mM) was recirculated from the posterior side of the nasal cavity through the nostrils at a rate of 1 ml/min for 3 h at 37°C. The amounts of cholesterol, phospholipids, and proteins released from the rat nasal mucosa into the perfused solution were determined by the cholesterol oxidase (Cholesterol C-Test Wako®, Wako Pure Chemical Ind., Ltd, Osaka, Japan), choline oxidase (Phospholipids C-Test Wako®, Wako Pure Chemical Ind., Ltd, Osaka, Japan), and bicinchoninic acid (BCA Protein Assay Kit®, Pierce Co., IL, U.S.A.) methods, respectively; cyclodextrins did not interfere with these colorimetric assays.

Interaction of cyclodextrins with Ca^{2+}

The interaction of cyclodextrins with Ca^{2+} was examined by means of ultrafiltration. A 0.1 M 3-(N-morpholino)propanesulfonic acid (Mops) Good's buffer (pH 7.4, 25°C) was used in this study. The buffer solution (5 ml) containing 20 mM cyclodextrins and 10 mM calcium chloride was passed through a membrane filter (Amicon Diaflo® membranes YC 05, MA, U.S.A.) under a pressure of 5 kg/cm^2 nitrogen. The free fraction of Ca^{2+} in the filtrate was determined by the o-cresolphthalein complexone method (Calcium C-Test Wako®, Wako Pure Chemical Ind., Ltd, Osaka, Japan). In the presence of 20 mM EDTA, the recovery of Ca^{2+} was more than 95% of control. The retention limit of the membrane used may be higher than the molecular size of the complex of EDTA with Ca^{2+}. In this case, a flame photometer (Hiranuma, FPF-3A, Ibaragi, Japan) was used for determination of Ca^{2+} in the filtrate because EDTA interfered with the complexometric assay.

The capacity of cyclodextrins to sequester Ca^{2+} was determined by a complexometric method using Eriochrome black T® as an indicator in 6.7 mM ammonium hydroxide-ammonium chloride buffer (pH 10.0) at 25°C. A solution (0.6 ml) of 0.01% Eriochrome black T® was added to the buffer solution (1.5 ml) containing 5 mM calcium chloride. The mixture was added to the buffer solution (2.9 ml) containing cyclodextrins or EDTA at various concentrations. The sequestration of Ca^{2+} from Eriochrome black T® was estimated from the increase in absorbance of the indicator at 650 nm. Cyclodextrins did not affect the visible spectrum of the indicator in the absence of calcium chloride.

Stability of insulin in nasal homogenates

Rats were anesthetized with diethyl ether and decapitated. The nasal mucosa on the septal cartilage was isolated from the frontal bone and homogenized in a 10-fold volume of cold saline using a blade homogenizer (Physcotron® NS-50, Niti-On Co., Ltd, Chiba, Japan). The homogenate was centrifuged at $9000 \times g$ for 10 min at 5°C and the resulting supernatant (0.2 ml) was added to the buffer solution (0.8 ml) containing insulin (12.6 mU/ml) and cyclodextrins (100 mM). After the mixture had been incubated for 10 min at 37°C, the incubation was terminated by addition of a 0.1 ml aliquot of the mixture to 0.1 N HCl solution (1 ml) at 0°C. The residual immunoreactivity of insulin in the mixture was determined by enzyme immunoassay. The activity of leucine aminopeptidase, a proteolytic enzyme in the nasal mucosa, was determined by the L-leucyl-*p*-diethylaminoanilide substrate method (LAP C-Test Wako®, Wako Pure Chemical Ind., Ltd, Osaka, Japan). The nasal mucosal supernatant (50 μl) was added to the buffer solution (2 ml) containing L-leucyl-*p*-diethylaminoanilide hydrochloride (0.67 mg) as a substrate and cyclodextrins at various concentrations. The mixture was incubated at 37°C for 20 min and the activity was determined spectrophotometrically at 675 nm. Cyclodextrins did not interfere with the colorimetric assay. Spectroscopic observations indicated no significant interaction between cyclodextrins and the substrate and, thus, the loss of the substrate by entrapment into cyclodextrins may not be expected during the assay.

Data analysis

Student's *t*-test was used for statistical evaluation of the data. Values of $p < 0.05$ were considered to be statistically significant. The contribution of the biophysical factors to the overall ab-

TABLE 1

Systemic bioavailability of insulin following the nasal administration of insulin suspensions (10 U/kg) with or without cyclodextrins (8 μmol/kg) in rats [a]

System	C_{max} [b] ($\mu U\ ml^{-1}$)	T_{max} [c] (h)	AUC [d] (h $\mu U\ ml^{-1}$)	F [e] (%)	Hypoglycemic effect [f] (h %)
Without cyclodextrins	60 ± 8	3.0 ± 0.4	240 ± 35	0.2 ± 0.7	8.9 ± 9.0
With α-CyD	277 ± 12 [g]	0.3 ± 0.1 [g]	846 ± 108 [g]	12.3 ± 2.2 [g]	47.7 ± 3.4 [g]
With DM-α-CyD	499 ± 71 [g]	0.6 ± 0.2 [g]	994 ± 137 [g]	15.3 ± 2.7 [g]	48.5 ± 3.8 [g]
With HP-α-CyD	127 ± 29	2.7 ± 0.7	485 ± 119	5.1 ± 2.4	7.1 ± 5.7
With β-CyD	452 ± 16 [g]	1.3 ± 0.3	1278 ± 192 [g]	20.9 ± 3.9 [g]	22.4 ± 7.4
With DM-β-CyD	572 ± 23 [g]	0.6 ± 0.2 [g]	1414 ± 193 [g]	23.7 ± 3.9 [g]	53.6 ± 3.5 [g]
With HP-β-CyD	173 ± 41	1.3 ± 0.4	426 ± 169	3.9 ± 3.4	4.5 ± 5.1

[a] Each value represents the mean ± S.E. of 3–10 rats.
[b] Maximum serum insulin level.
[c] Time required to reach the maximum serum insulin level.
[d] Area under the serum insulin level-time curve up to 6 h post-administration.
[e] Bioavailability compared with the AUC value of insulin administered intravenously (5 U/kg).
[f] Cumulative percentage of change in serum glucose level up to 4 h post-administration.
[g] $p < 0.05$ vs insulin alone.

sorption enhancement was statistically examined using multiple regression analysis. The predictor variables were (a) the nasal permeability as estimated by the area (h dpm ml^{-1}) under the serum insulin level-time curve up to 3 h following nasal insulin administration with cyclodextrins, and (b) the stability of insulin with cyclodextrins in rat nasal homogenates as estimated by the remaining percentage of insulin after the 10 min incubation. Correlations and regression equations were calculated on a PC-9801-VX personal computer (NEC, Tokyo, Japan).

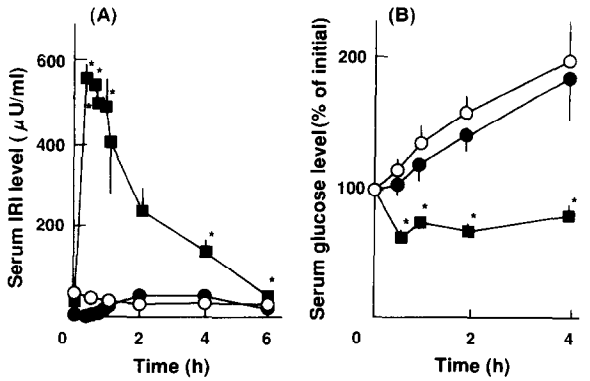

Fig. 1. Serum levels of immunoreactive insulin (IRI) (A) and glucose (B) following the nasal administration of insulin (10 U/kg) with or without DM-β-CyD (8 μmol/kg) to rats. (○) Buffer alone, (●) insulin alone, (■) with DM-β-CyD. Each point represents the mean ± S.E. of 3–6 rats. * $p < 0.05$ vs insulin alone.

Results and Discussion

Enhanced nasal absorption of insulin

When insulin in suspension with cyclodextrins was administered nasally to rats, a significant increase in serum immunoreactive insulin levels and a marked hypoglycemia were observed. Cyclodextrins alone did not lead to any changes in serum levels of endogenous insulin and glucose (data not shown). The effects of six cyclodextrins (8 μmol/kg) on the nasal absorption of insulin (10 U/kg) are given in Table 1. The increased nasal bioavailability of insulin with cyclodextrins (F values) correlated well with the enhanced hypoglycemic responses ($r = 0.896$, $F = 16.2$), where β-CyD, being an outlier, was omitted. Of the cyclodextrins evaluated, DM-β-CyD had the strongest enhancement effect on the nasal absorption of insulin (Fig. 1), while HP-α- and HP-β-CyDs were less effective than the parent cyclodextrins. These results are consistent with a recent report that DM-β-CyD, of the five cyclodextrins tested, is the most potent enhancer of nasal absorption of human insulin in solution in rats (Merkus et al., 1991). Of the cyclodextrins

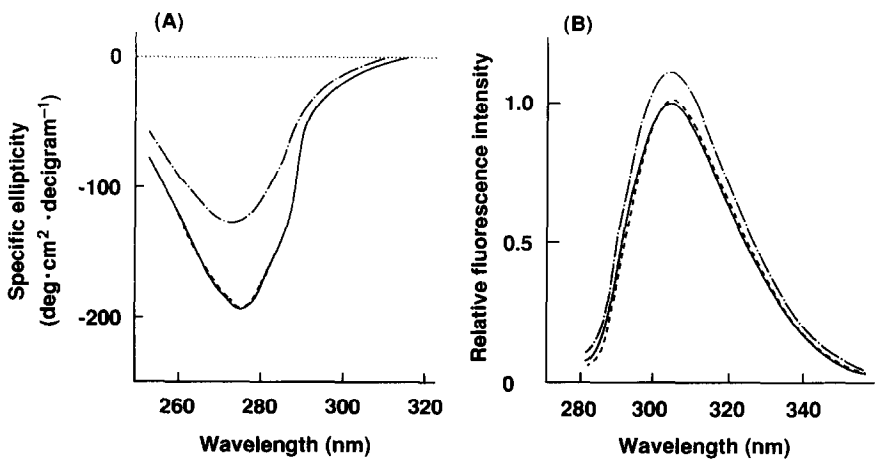

Fig. 2. Effects of DM-β-CyD (10 mM) and EDTA (0.2 mM) on the circular dichroism (A) ([insulin] = 200 μM) and fluorescence (B) ([insulin] = 10 μM) spectra of insulin in isotonic phosphate buffer (pH 7.4, 25°C). (———) Insulin alone, (– – –) with DM-β-CyD, (–·–·–) with EDTA. Excitation wavelength was 278 nm.

TABLE 2

Some characteristics of the insulin suspensions (100 U / ml) with or without cyclodextrins (80 mM) [a]

System	Particle size [b] (μm, 25°C)	Viscosity (cP, 37°C)		Solubility of insulin (% of total, 25°C)
		Without insulin	With insulin	
Without cyclodextrins	6.6 ± 3.4	0.66	0.60	31
With α-CyD	5.7 ± 6.4	1.22	1.08	74
With DM-α-CyD	5.4 ± 2.8	0.84	0.89	29
With HP-α-CyD	5.8 ± 2.9	0.96	0.89	47
With β-CyD	4.5 ± 4.2	0.84	0.97	35
With DM-β-CyD	5.5 ± 2.7	0.91	0.89	41
With HP-β-CyD	6.2 ± 3.4	0.91	0.98	48

[a] Data were collected immediately after preparation.
[b] Volume-surface diameter, mean ± S.D.

which had marked absorption-enhancement effects, only β-CyD showed a delay in the onset of enhancement, resulting in a prolonged hypoglycemic effect. This may be due to the limited solubility of β-CyD.

Interaction of insulin with cyclodextrins

Cyclodextrins may form inclusion complexes with accessible hydrophobic amino acid residues in polypeptides (Matsuyama et al., 1987; Tabushi et al., 1988), and thus may change their chemical and biological properties. The interaction of insulin with cyclodextrins was examined by circular dichroism and fluorescence spectroscopy. Fig. 2 shows typical circular dichroism and fluorescence spectra of insulin in the absence and presence of 10 mM DM-β-CyD in the buffer solution. The effect of 0.2 mM EDTA, a chelator, was also studied under comparable conditions, since EDTA is known to prevent the self-association of insulin through sequestering Zn^{2+} from insulin molecules (Liu et al., 1991). Under the present conditions, insulin exists mainly as the hexamer (Pocker and Biswas, 1980). EDTA attenuated the negative circular dichroic band at 270 nm and enhanced the fluorescence emission at 300 nm,

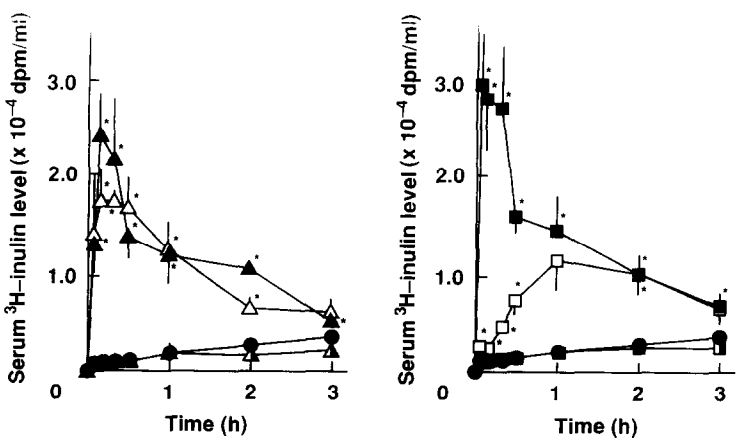

Fig. 3. Effects of cyclodextrins (8 μmol/kg) on the nasal absorption of inulin following nasal administration of inulin (10 μCi/kg) to rats. (●) Without cyclodextrins, (△) α-CyD, (▲) DM-α-CyD, (▲) HP-α-CyD, (□) β-CyD, (■) DM-β-CyD, (◨) HP-β-CyD. Each value represents the mean ± S.E. of four rats. * $p < 0.05$ vs inulin alone.

indicating the dissociation of insulin molecules (Pocker and Biswas, 1980). On the other hand, DM-β-CyD induced no noticeable changes in the spectra of insulin. Similarly, the other cyclodextrins tested did not affect the spectra of insulin (data not shown). These results indicated that the interactions between insulin and cyclodextrins were limited in extent. This view was also supported by the finding that HP-β-CyD did not alter the conformation of insulin based on an unperturbed fourth-derivative ultraviolet spectrum of that peptide (Brewster et al., 1991).

Table 2 lists some characteristics of insulin suspensions with cyclodextrins tested. The data were collected immediately after preparation. There was no noticeable difference in average particle size between the preparations. The viscosities of the preparations were dependent largely on the intrinsic viscosities of cyclodextrin solutions or suspensions. Cyclodextrins, especially α-CyD, affect the apparent solubility of insulin in buffer solution, but the solubilization potencies of cyclodextrins did not display any substantial correlation with their enhancement effects on insulin absorption (Table 1).

Increased permeability of nasal mucosa

As shown in Fig. 3, cyclodextrins resulted in a considerably enhanced permeability of the nasal mucosal membrane, as indicated by the increase in nasal absorption of inulin, which is resistant to mucosal enzymes and poorly permeable into the membranes. The methylated cyclodextrins showed the greatest potency for increasing nasal permeability, while the hydroxypropylated cyclodextrins were less effective than the parent cyclodextrins. Similarly to the absorption enhancement of insulin, β-CyD displayed a delayed onset of the enhancement effect on inulin absorption. The mechanism of increased permeability mediated by cyclodextrins was further investigated by measuring the release of a number of membrane components from the nasal mucosa treated with cyclodextrins in situ. The methylated cyclodextrins significantly extracted membrane lipids such as cholesterol and phospholipids from the nasal mucosa, where DM-α-CyD had limited specificity for phospholipids (Fig. 4). Our previous studies demonstrated that cyclodextrins could solubilize membrane lipids from human erythrocytes through rapid and reversible formation of inclusion complexes (Ohtani et al., 1989). Similar mechanisms are probably involved in the solubilization of lipids from the nasal mucosa by cyclodextrins; the selectivity and effectiveness of cyclodextrins may depend on the size and hydrophobicity of the cavity in which lipids are included. Consequently, membrane proteins were released particularly by the methylated cyclodextrins (Fig. 4); this may be an extrusion process in which proteins are shed from the membranes into the aqueous phase through the erosion of lipid regions of the membranes. Recent studies have shown that lipid extraction by organic solvents reduces the barrier function of mucosal membranes (Corbo et al., 1990). Thus, lipid solubiliza-

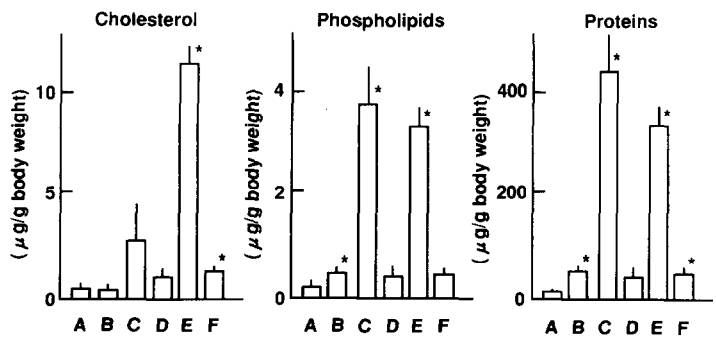

Fig. 4. Release of some membrane components from rat nasal mucosa into the perfusate with or without cyclodextrins (80 mM) in isotonic phosphate buffer (pH 7.4, 37°C). (A) Without cyclodextrins, (B) with α-CyD, (C) with DM-α-CyD, (D) with HP-α-CyD, (E) with DM-β-CyD, (F) with HP-β-CyD. Each value represents the mean ± S.E. of four rats. * $p < 0.05$ vs buffer alone.

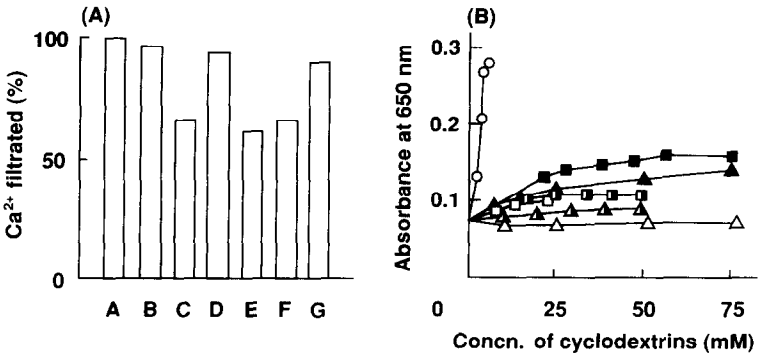

Fig. 5. Effects of cyclodextrins (20 mM) on the permeation of Ca^{2+} (10 mM) through the ultrafiltration membrane (A) and their capacities for sequestering Ca^{2+} compared to EDTA (B). (A) Without cyclodextrins, (B) with α-CyD, (C) with DM-α-CyD, (D) with HP-α-CyD, (E) with β-CyD, (F) with DM-β-CyD, (G) with HP-β-CyD. (△) α-CyD, (▲) DM-α-CyD, (▲) HP-α-CyD, (□) β-CyD, (■) DM-β-CyD, (◨) HP-β-CyD, (○) EDTA. Each value represents the mean of two experiments.

tion mediated by cyclodextrins may cause changes in transcellular processes, and moreover, these changes are believed to be transmitted to the paracellular region (Muranishi, 1990), which appears to be the most likely route for the transport of polypeptides (McMartin et al., 1987).

The integrity of the paracellular pathway is known to depend on extracellular Ca^{2+} (Lee, 1990) and, thus, the interaction of cyclodextrins with Ca^{2+} was examined, as another aspect of the effects of cyclodextrins on paracellular permeability. As shown in Fig. 5, the results obtained with both methods indicate that cyclodextrins, especially the methylated derivatives, are able to interact with Ca^{2+}, although the ability of cyclodextrins to sequester Ca^{2+} from the calcium-indicator complex was much less than that of EDTA. This interaction of cyclodextrins with divalent cations, as well as with membrane lipids, should be considered to participate to a certain extent in the mechanism of increased nasal membrane permeability.

Protection of insulin against proteolysis

The degradation of insulin, when incubated with rat nasal mucosal homogenates, was suppressed by cyclodextrins; the order of efficacy was: DM-β-CyD > α-CyD = DM-α-CyD > HP-β-CyD > HP-α-CyD = β-CyD (Fig. 6). Because of the limited interaction between insulin and cyclodextrins, it is unlikely that cyclodextrins protect insulin from proteolytic enzymes by entrapment of insulin within the cyclodextrin cavity. Therefore, cyclodextrins may reduce the proteolytic activity of enzymes directly or indirectly by preventing the formation of the enzyme-substrate complex (Lee, 1988). This view is supported by the observations shown in Fig. 7A. Cyclodextrins, especially the modified β-cyclodextrins, reduced the activity of leucine aminopeptidase in nasal homogenates in a concentration-dependent manner, but were less potent than protease inhibitors

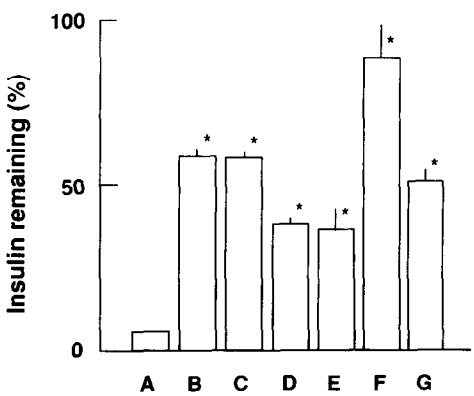

Fig. 6. Effects of cyclodextrins (80 mM) on the enzymatic degradation of insulin (10 mU/ml) after 10 min incubation in rat nasal mucosal homogenates at 37°C. (A) Without cyclodextrins, (B) with α-CyD, (C) with DM-α-CyD, (D) with HP-α-CyD, (E) with β-CyD, (F) with DM-β-CyD, (G) with HP-β-CyD. Each value represents the mean ± S.E. of three experiments. * $p < 0.05$ vs insulin alone.

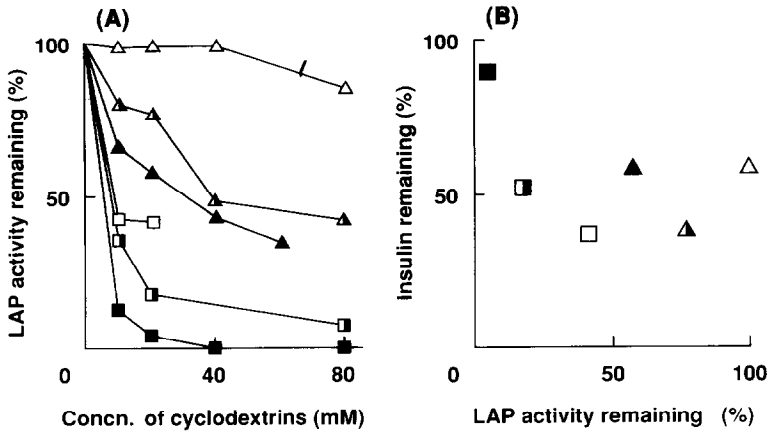

Fig. 7. Effects of cyclodextrins on the activity of leucine aminopeptidase (LAP) in rat nasal mucosal homogenates at 37°C after 20 min incubation (A) and the relationship between the insulin proteolysis (values shown in Fig. 6) and the LAP activity (values in the presence of 20 mM cyclodextrins) (B). (△) α-CyD, (▲) DM-α-CyD, (▲) HP-α-CyD, (□) β-CyD, (■) DM-β-CyD, (◨) HP-β-CyD. Each value represents the mean of two experiments.

reported (Statfold and Lee, 1986; Aungst and Rogers, 1988). In fact, under the same conditions as in Fig. 7A, the remaining activities of leucine aminopeptidase were 75.0 ± 1.3% with 0.1 mM bestatin, 95.1 ± 1.5% with 0.1 mM aprotinin, and 103.6 ± 0.7% with 0.1 mM DM-β-CyD, respectively. However, as shown in Fig. 7B, the cyclodextrin-mediated reduction of leucine aminopeptidase activity did not correlate closely with the overall degradation of insulin in the homogenates. The lack of the correlation in Fig. 7B could be explained on the basis of the contributions of other types of enzymes present there, since leucine aminopeptidase may not be a dominant aminopeptidase in the mucosa (Statfold and Lee, 1986).

Multiple regression analysis for enhanced insulin absorption

As mentioned above, cyclodextrins affected the permeability of nasal mucosa, and the stability of insulin against proteolysis. Three-dimensional plots displaying the effects of cyclodextrins on these two determinants are shown in Fig. 8. The degree of relative contribution of the two determinants to the overall absorption enhancement was examined using multiple regression analysis, where β-CyD was omitted due to the lack of conformity of its absorption-enhancement effect. Consequently, the following equation was obtained:

$$BA = 1.91(\pm 0.618) \cdot Pm + 0.794(\pm 0.335) \cdot St - 3.28(\pm 2.14) \quad (r = 0.978,\ F = 33.7)$$

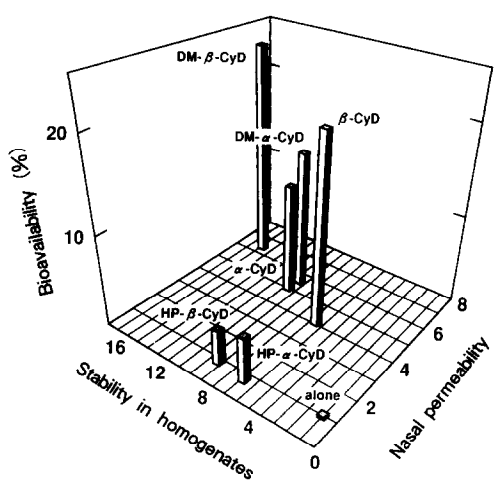

Fig. 8. Three-dimensional plots showing effects of cyclodextrins on predominant factors affecting the nasal absorption of insulin. The values in the absence of cyclodextrins were taken as 1.0.

where BA is the nasal bioavailability of insulin with cyclodextrins (F values in Table 1). The permeability factor (Pm) represents the area (h dpm ml^{-1}) under the plasma inulin-time curve up to 3 h following the nasal administration inulin with cyclodextrins (Fig. 3). The stability factor (St) represents the remaining percentage of insulin in the nasal homogenates after 10 min incubation (Fig. 7A). The partial correlation coefficients for Pm and St were 0.873 and 0.808, respectively. Since the magnitudes of the two coefficients are not significantly different, the two determinants may contribute synergistically to the enhanced absorption of insulin.

The absorption-enhancement effects of cyclodextrins mimic those of bile salts in regard to the increased membrane permeability being accompanied by the inhibition of proteolysis, although they may be somewhat different from each other in their manner of action on membranes (Billington and Coleman, 1978; Ohtani et al., 1989). A recent study on the effects of cyclodextrins on nasal mucociliary movement in rats (Merkus et al., 1991) has demonstrated that the ciliostatic potency of DM-β-CyD is much less than those found for bile salts and other surfactant-type absorption enhancers. This may be an advantage of cyclodextrins over the enhancers in promoting nasal insulin absorption, especially for long-term therapy.

In the present study, insulin was used as a suspension form in the hope of achieving beneficial effects such as the prolonged retention of the peptide at the site of absorption, as demonstrated with powder dosage forms and microspheres (Nagai et al., 1984; Bjork and Edman, 1988). Unfortunately, such formulation effects were unclear, probably due to impairment of mucociliary drainage in the experimental model used.

While the acute and chronic local tissue tolerance of cyclodextrins and their effects on the mucociliary clearance of drugs should be further investigated before their practical use, the present data support the possible use of the chemically modified cyclodextrins, especially methylated derivatives, as adjuvants to improve the nasal absorption of insulin despite its administration as a suspension form.

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