Improvement of Nasal Bioavailability of Luteinizing Hormone-Releasing Hormone Agonist, Buserelin, by Cyclodextrin Derivatives in Rats

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Abstract □ The effects of chemically modified cyclodextrins on the nasal absorption of buserelin, an agonist of luteinizing hormone-releasing hormone, were investigated in anesthetized rats. Of the cyclodextrins tested, dimethyl-β-cyclodextrin (DM-β-CyD) was the most effective in improving the rate and extent of the nasal bioavailability of buserelin. Fluorescence spectroscopic studies indicated that the cyclodextrins formed inclusion complexes with buserelin, which may reduce the diffusibility of buserelin across the nasal epithelium and may participate in the protection of the peptide against enzymatic degradation in the nasal mucosa. Additionally, the cyclodextrins increased the permeability of the nasal mucosa, which was the primary determinant based on the multiple regression analysis of the nasal absorption enhancement of buserelin. Scanning electron microscopic observations revealed that DM-β-CyD induced no remarkable changes in the surface morphology of the nasal mucosa at a minimal concentration necessary to achieve substantial absorption enhancement. The present results suggest that DM-β-CyD could improve the nasal bioavailability of buserelin and is well-tolerated by the nasal mucosa of the rat.

A synthetic nonapeptide buserelin acetate, defined as pyroGlu-His-Trp-Ser-Tyr-D-Ser(*t*-Bu)-Leu-Arg-Pro-ethylamide (Figure 1),[1] is a highly potent agonist of luteinizing hormone-releasing hormone (LHRH). In contrast to acute dosing, the long-term treatment with the LHRH agonist paradoxically desensitizes the pituitary–gonadal system, leading to a reversible biochemical castration. This paradoxical effect is successfully utilized in the treatment of hormonally sensitive disorders such as endometriosis, precocious puberty, and leiomyoma.[2] Because of the low oral bioavailability of buserelin, it is administered via the intranasal route or by subcutaneous implants or microparticle injections.[3] However, even with the intranasal route of delivery, the nasal epithelium presents both a physical and a metabolic barrier to the absorption of buserelin.[4] Therefore, the use of absorption-promoting agents seems necessary to achieve sufficient intranasal absorption of buserelin. Several attempts have been made to improve the nasal bioavailability of buserelin or other LHRH analogues, including the coadministration of inhibitors of aminopeptidases,[5,6] taurodihydrofusidate,[5] and α-cyclodextrin.[7,8] Previous studies have shown that chemically modified cyclodextrins, especially the methylated derivatives, are more potent enhancers of nasal insulin absorption than the parent cyclodextrins.[9–11] The absorption enhancement afforded by the methylated cyclodextrins can be attributed primarily to their ability to reduce the barrier function of the nasal mucosa and to protect insulin against proteolysis.[10] More recently, Shao et al. have demonstrated that cyclodextrins inhibit the self-association of insulin into oligomers, thus making insulin more available for nasal absorption.[12] This paper deals with the effects of eleven hydrophilic cyclodextrin derivatives on the systemic bioavailability of buserelin after the nasal administration in anesthetized rats and discusses the mechanisms by which cyclodextrins may improve the nasal absorption of the peptide with an emphasis on relative contribution of the absorption barriers

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Experimental Section

Materials—Buserelin acetate (Hoechst Japan Ltd., Saitama, Japan) was used without further purification. α-Cyclodextrin (α-CyD), β-cyclodextrin (β-CyD), γ-cyclodextrin (γ-CyD), hexakis(2,6-di-*O*-methyl)-α-cyclodextrin (DM-α-CyD), heptakis(2,6-di-*O*-methyl)-β-cyclodextrin (DM-β-CyD), 2-hydroxypropyl-α-cyclodextrin (HP-α-CyD), 2-hydroxypropyl-β-cyclodextrin (HP-β-CyD) (Nihon Shokuhin Kako Co., Ltd., Tokyo, Japan), the sodium salt of 6-*O*-carboxymethyl-α-cyclodextrin (CM-α-CyD), the sodium salt of 6-*O*-carboxymethyl-β-cyclodextrin (CM-β-CyD) (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and 6-*O*-maltosyl-β-cyclodextrin (G_2-β-CyD) (Ensuiko Sugar Refining Co., Ltd., Yokohama, Japan) were used as supplied. The sodium salt of β-cyclodextrin sulfate (S-β-CyD) was prepared according to the nonregioselective method described previously.[13] The chemical structures of the cyclodextrins used are listed in Table 1. [G^3H]Inulin (312.0 mCi/g, with an average molecular mass of 5000–5500 Da) was obtained from New England Nuclear. A MicroTP-test kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used for the total protein assay in the nasal membrane homogenates. Other materials were of reagent grade and deionized double-distilled water was used.

Fluorescence Spectroscopic Studies—Fluorescence spectra of buserelin acetate (10 μM) in the absence and presence of cyclodextrins (1–20 mM) in isotonic phosphate buffer (pH 7.4) were recorded at 25 °C, using an Hitachi F-4010 spectrofluorometer (Tokyo, Japan). The apparent stability constants for the complexes of buserelin acetate with cyclodextrins, assuming that a 1:1 complex is formed, were determined from the fluorescence changes (excitation wavelength, 280 nm; emission wavelength, 350 nm) and use of the Scott equation.[14]

Nasal Absorption Studies—The nasal absorption studies were performed according to the method of Hirai et al.,[15] and the experimental procedures were essentially the same as those described previously.[10] Male Wistar rats weighing 200–250 g were fasted for 16 h and anesthetized with an intraperitoneal injection of sodium pentobarbital (30 mg/kg). During the experiment the rats were kept lying on their back on a thermostated rug at 37 °C, and anesthesia was maintained with subsequent injections of the anesthetic (15 mg/kg) every 1–2 h. Freshly prepared buserelin acetate solution contains buserelin acetate (0.8 mM) with or without the cyclodextrins (10, 40, or 80 mM) in 10 mM isotonic phosphate buffer (PBS, pH 7.4). Each solution was given nasally with a micropipet at a dose of 0.1 mg/kg as buserelin acetate. The only exception was β-CyD which was used as a suspension due to the limited aqueous solubility (1.85 g/dL in water at 25 °C).[16] In comparison, the buserelin acetate solution was administered intravenously from the jugular vein to rats at an equivalent dose to the nasal administration. Blood samples (0.4 mL) were taken periodically from the jugular vein and centrifuged to obtain plasma, which was stored at −30 °C until analysis. The concentration of buserelin in plasma was determined by the double-antibody radioimmunoassay.[17] Urine samples were collected for 3 h after the nasal administration. DM-β-CyD in the urine was extracted with chloroform and subjected to the high-performance liquid chromatography (HPLC) analysis. The HPLC conditions were as follows: pump, Hitachi L-6000 (Tokyo, Japan); detector, Shodex SE-51 refractive index detector (Showa Denko, Tokyo, Japan); column, YMC pack R-ODS-5 (5 μm, 4.6 i.d. × 250 mm, Yamamura Chemicals, Kyoto, Japan); mobile phase, acetonitrile–water (55:45% v/v); internal standard, γ-CyD.

Stability of Buserelin in Nasal Homogenates—Male Wistar rats weighing 200–250 g were fasted overnight, anesthetized with

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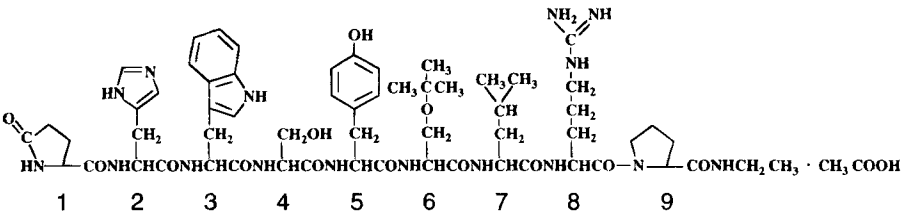
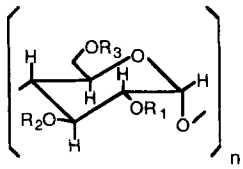


Figure 1—Chemical structure of buserelin acetate.

Table 1—Cyclodextrin Derivatives Used in This Study



Compound	Abbreviation	n	R_1	R_2	R_3	Average Degree of Substitution
α-Cyclodextrin	α-CyD	6	H	H	H	
β-Cyclodextrin	β-CyD	7	H	H	H	
γ-Cyclodextrin	γ-CyD	8	H	H	H	
2,6-Di-*O*-methyl-α-cyclodextrin	DM-α-CyD	6	CH_3	H	CH_3	12.0
2,6-Di-*O*-methyl-β-cyclodextrin	DM-β-CyD	7	CH_3	H	CH_3	14.0
2-Hydroxypropyl-α-cyclodextrin	HP-α-CyD	6		R_1, R_2, R_3 = H or $[CH_2CH(CH_3)O]_mH$ (m = 1, 2, 3, ·····)		5.2
2-Hydroxypropyl-β-cyclodextrin	HP-β-CyD	7		R_1, R_2, R_3 = H or $[CH_2CH(CH_3)O]_mH$ (m = 1, 2, 3, ·····)		5.8
6-*O*-Carboxymethyl-α-cyclodextrin	CM-α-CyD	6		R_1, R_2, R_3 = H or CH_2COONa		1.4
6-*O*-Carboxymethyl-β-cyclodextrin	CM-β-CyD	7		R_1, R_2, R_3 = H or CH_2COONa		4.7
Maltosyl-β-cyclodextrin	G_2-β-CyD	7	H	H	H or maltose	1.0
Sulfated β-cyclodextrin	S-β-CyD	7		R_1, R_2, R_3 = H or SO_3Na		10.2

diethyl ether, and decapitated. The nasal mucosa on the nasoseptum were isolated and homogenized in a 10-fold weight of cold isotonic phosphate buffer (pH 7.4) using a Polytron (Kinematica GmbH, Luzern, Switzerland). The homogenate was centrifuged at 3000 rpm and for 30 min at 5 °C, the resulting supernatant (0.2 mL, 60 μg as proteins) was added to the buffer solution (0.8 mL, pH 7.4) containing buserelin acetate (100 μM) and the cyclodextrins (100 mM), and the mixture was incubated at 37 °C. At an appropriate interval, a 0.1 mL aliquot of the mixture was withdrawn and added to 0.1 N hydrochloric acid (1 mL) to terminate the enzymatic reaction. The residual buserelin in the mixture was determined by HPLC. The HPLC conditions were as follows: pump, TRIROTER-V (Jasco, Tokyo, Japan); detector, UVIDEC-100-V (Jasco, Tokyo, Japan); autoinjector, Waters 712WISP (Millipore, Tokyo, Japan); column, Nucleosil 100–5C18 4.6 i.d. × 150 mm (GL Sciences Inc., Tokyo, Japan); mobile phase, 0.1 M phosphoric acid–acetonitrile (7:2% v/v) adjusted to pH 2.5 with triethylamine.

Evaluation of Nasal Membrane Permeability—The permeability of rat nasal mucosa was assessed by measuring the extent of nasal absorption up to 3 h postadministration of [G^3H]inulin (10 μCi/kg), an inert and poorly permeable marker. The effects of γ-CyD, CM-α-CyD, CM-β-CyD, and G_2-β-CyD on the nasal membrane permeability were determined in the same manner as described previously for the other cyclodextrins.[10] The radioactivity in the serum was measured using a liquid scintillation counter (Aloka LSC-3500, Tokyo, Japan).

Morphological Observation of Nasal Mucosa—The nasal cavity of the rats was filled up with the buffer solution (pH 7.4) containing DM-β-CyD (10, 40, or 80 mM), and the other experimental conditions were the same as the *in vivo* absorption studies. After a 10 min exposure to the DM-β-CyD solution, the nasal cavity was infused with saline for 5 min at a flow rate of 1.5 mL/min to terminate the treatment. Consecutive perfusion was followed with 2% w/v glutaraldehyde in 100 mM sodium phosphate buffer (pH 7.4) to fix the nasal membrane for 5 min. Nasal membrane damage was assessed by scanning electron microscopy (Hitachi S-510, Tokyo, Japan), and morphological scoring was performed according to the following five-point grading for each category as reported:[18] category 1, mucosal surface integrity [from 1 (normal) to 5 (unrecognizable)]; category 2, ciliary morphology [from 1 (normal) to 5 (gross deformation)]; category 3, mucus/extracellular debris [from 1 (little) to 5 (abundant)].

Data Analysis—The area under the plasma or serum level–time curve (AUC) of buserelin or inulin after the nasal administration was calculated by the trapezoidal role up to 3 h postadministration. The AUC value after the intravenous administration of buserelin acetate was analyzed according to a least-squares regression computer program[19] for the biexponential decline. Data were analyzed statistically by one-way analysis of variance, using Duncan's multiple comparison test, and p values of <0.05 were considered to be statistically significant. The relative contribution of various factors to the overall nasal absorption of buserelin was examined using multiple regression analysis.[20]

Results and Discussion

Complexation of Buserelin Acetate with Cyclodextrins—Our previous studies using nuclear magnetic resonance spectroscopy have demonstrated that cyclodextrins are capable of forming inclusion complexes with buserelin acetate, in which the hydrophobic amino acid residues (tryptophan, tyrosine, and *tert*-butyl-D-serine) may be preferentially included in the cyclodextrin cavity.[21] In this study, the interaction of buserelin acetate with cyclodextrins in solution was examined by means of fluorescence spectrophotometry. Figure 2 shows typical fluorescence spectra of buserelin acetate in the absence and presence of DM-β-CyD at various concentrations in the buffer solution (pH 7.4) at 25 °C. With increasing concentrations of DM-β-CyD, the emission maximum of buserelin acetate at 350 nm, corresponding to the aromatic amino acid residues, was shifted to a shorter wavelength (~7 nm), with a concomitant increase in the

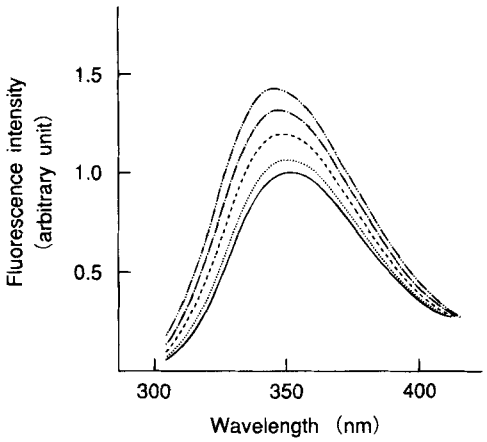


Figure 2—Fluorescence spectra of buserelin acetate (10 μM) in the absence and presence of DM-β-CyD at various concentrations in isotonic phosphate buffer (pH 7.4) at 25 °C. Key: (—) without DM-β-CyD, (· · ·) with 1 mM DM-β-CyD, (- - -) with 5 mM DM-β-CyD, (– · –) with 10 mM DM-β-CyD, (– · · –) with 20 mM DM-β-CyD. The excitation wavelength was 280 nm.

Table 2—Apparent Stability Constants (K_c) of Complexes of Buserelin Acetate with Cyclodextrins in Isotonic Phosphate Buffer (pH 7.4) at 25 °C

System	K_c[a] (M^{-1})	System	K_c[a] (M^{-1})
With α-CyD	7 ± 5	With HP-α-CyD	17 ± 1
With β-CyD	50 ± 7	With HP-β-CyD	103 ± 6
With γ-CyD	16 ± 4	With G_2-β-CyD	59 ± 4
With DM-α-CyD	69 ± 6	With CM-β-CyD	43 ± 9
With DM-β-CyD	118 ± 8		

[a] Each value represents the mean ± SE of three to five experiments.

fluorescence intensity. When buserelin acetate was dissolved in a less polar solvent such as methanol and ethanol, the fluorescence intensity was increased, suggesting that the fluorophores in buserelin acetate are incorporated into the hydrophobic environment of the cyclodextrin cavity. Similarly, the other cyclodextrins except HP-α-CyD enhanced the fluorescence of buserelin acetate, while HP-α-CyD quenched the fluorescence of the peptide, indicating that the interaction is not the result of a simple hydrophobic process. In the present study, the changes in the fluorescence intensity of buserelin acetate induced with cyclodextrins were quantitatively analyzed to determine the apparent stability constants (K_c) for the complexes of buserelin acetate with cyclodextrins, and the results are listed in Table 2. Although the K_c values for all the complexes were rather small, each β-CyD derivatives formed a more stable complex with buserelin acetate than the corresponding α-CyD derivative. Of the cyclodextrins evaluated, DM-β-CyD formed the strongest inclusion complex, as indicated by the magnitude of the binding constant.

Enhanced Nasal Absorption of Buserelin—Figure 3 shows the plasma levels of buserelin after the nasal administration of buserelin acetate (0.1 mg/kg) with or without cyclodextrins (80 mM) in anesthetized rats. The pharmacokinetic parameters for buserelin were calculated from the plasma profiles of the peptide and the results are summarized in Table 3. α-CyD, DM-α-CyD, and DM-β-CyD significantly enhanced the rate and extent of nasal bioavailability of buserelin, with the efficacy increasing in the order α-CyD ≈ DM-α-CyD < DM-β-CyD. In particular, DM-β-CyD improved the nasal bioavailability of buserelin about 4-fold, reaching ~60% of that of the intravenous administration. Furthermore, β-CyD increased the nasal bioavailability of buserelin about 2-fold with a delayed onset of the absorption enhancement probably due to its limited aqueous solubility (1.85 g/dL in water at 25 °C). The abilities of cyclodextrins to enhance the nasal absorption of buserelin displayed almost the same

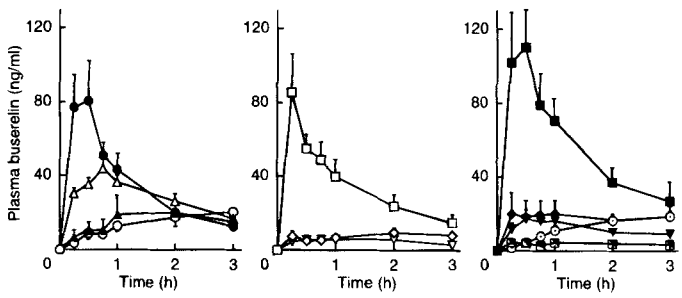


Figure 3—Plasma levels of buserelin after the nasal administration of buserelin acetate (0.1 mg/kg) with or without cyclodextrins (80 mM) to rats. Key: (○) without cyclodextrins, (●) α-CyD, (△) β-CyD, (▲) γ-CyD, (□) DM-α-CyD, (▽) HP-α-CyD, (◇) CM-α-CyD, (■) DM-β-CyD, (▼) HP-β-CyD, (⊙) G_2-β-CyD, (◆) CM-β-CyD, (◹) S-β-CyD. Each value represents the mean ± SE of 3–10 rats.

rank order as those for the larger peptide insulin.[10] On the other hand, HP-α-CyD and the two anionic derivatives (CM-α-CyD and S-β-CyD) did not increase or rather decreased the nasal absorption of buserelin. Since these cyclodextrins made no noticeable changes in pH and viscosity of the nasal buserelin preparation, the reduced bioavailability may be attributed to the formation of less membrane permeable complexes of buserelin acetate with these cyclodextrins.

Protection of Buserelin against Proteolysis—Nasal mucosal homogenates are known to have various types of aminopeptidase activities, which appear to be the major metabolic barrier to the nasal peptide delivery.[22] In the chemical structure of buserelin acetate, a D-amino acid and proline-ethylamide substitute for the glycine residue in the position 6 and for the C-terminal sequence prolylglycinamide, respectively, of the native LHRH sequence. Although these modifications conspicuously decrease the susceptibility of the LHRH agonist to proteolytic enzymes, it is still inactivated at various mucosal sites. As shown in Figure 4A, buserelin acetate was metabolized when incubated with rat nasal mucosal homogenates. Major metabolites, detected on the HPLC chromatogram of the reaction mixture after an 8 h incubation with the nasal homogenates, were the 3–9 heptapeptide fragment and the 5–9 pentapeptide fragment of buserelin, indicating that multiple enzymes may contribute to the metabolism. The enzymatic degradation of buserelin was decelerated by the addition of cyclodextrins in a concentration dependent manner (Figure 4B). The efficacy of cyclodextrins to inhibit the degradation of buserelin acetate increased in the order CM-β-CyD < β-CyD ≈ HP-α-CyD < α-CyD < DM-α-CyD < HP-β-CyD < DM-β-CyD ≈ G_2-β-CyD. Since cyclodextrins interact preferably with the hydrophobic amino acid residues in buserelin acetate including tryptophan and tyrosine residues, which are located near the enzymatic cleavage sites of the peptide, the cyclodextrins may protect buserelin acetate from proteolytic enzymes by including the aromatic amino acids within their intramolecular cavity. This view is supported by the correlation depicted in Figure 5. Although the plots shows some scatter, a weak but statistically significant positive correlation was found between the inhibition afforded by cyclodextrins against the proteolysis of buserelin acetate and the magnitude of the stability constants (K_c) of the complexes of the peptide with cyclodextrins, when an outlier, G_2-β-CyD, was omitted ($r^2 = 0.628$, $F = 8.44$). In spite of the rather low stability constant, G_2-β-CyD gave the most prominent inhibitory effect on the enzymatic degradation of buserelin acetate, probably due to a large steric hindrance of maltose residue attached to the cyclodextrin cavity through α-1,6-glycosidic linkage. Further scatter could be explained by the possible contribution of the direct effects of cyclodextrins on the activity of the proteolytic enzymes themselves.

Table 3—Pharmacokinetic Parameters[a] of Buserelin after Nasal Administration of Buserelin Acetate (0.1 mg/kg) with or without Cyclodextrins (80 mM) in Rats

System	n	C_{max}[b] (ng/mL)	T_{max}[c] (h)	AUC[d] (ng·h/mL)	MRT[e] (h)	F[f] (%)
Buserelin alone	6	27.2 ± 4.9	1.92 ± 0.42	40.8 ± 6.7	1.86 ± 0.14	14.6 ± 2.4
With α-CyD	7	87.9 ± 21.9*	0.39 ± 0.07*	105.7 ± 21.2*	1.05 ± 0.01*	37.9 ± 7.6*
With β-CyD	10	47.7 ± 4.6*	0.58 ± 0.09*	84.9 ± 7.5*	1.33 ± 0.05*	30.5 ± 2.7*
With γ-CyD	3	23.8 ± 8.6	2.00 ± 0.58	46.4 ± 16.3	1.76 ± 0.11	16.7 ± 5.9
With DM-α-CyD	6	90.6 ± 20.0*	0.33 ± 0.08*	103.4 ± 19.7*	1.10 ± 0.06*	37.1 ± 7.1*
With DM-β-CyD	7	140.6 ± 21.0*	0.46 ± 0.10*	167.5 ± 20.0*	1.13 ± 0.12*	60.1 ± 7.2*
With HP-α-CyD	6	7.6 ± 2.4*	1.38 ± 0.29	14.7 ± 4.6*	1.48 ± 0.08*	5.3 ± 1.6*
With HP-β-CyD	7	21.0 ± 3.6	1.25 ± 0.35	36.0 ± 5.8	1.49 ± 0.12	12.9 ± 2.1
With CM-α-CyD	5	13.2 ± 2.2*	1.65 ± 0.47	22.0 ± 2.9*	1.68 ± 0.08*	7.9 ± 1.0*
With CM-β-CyD	5	30.4 ± 10.1	2.05 ± 0.59	52.8 ± 12.2	1.61 ± 0.16	18.9 ± 4.4
With G_2-β-CyD	6	18.8 ± 2.9	2.83 ± 0.17	35.8 ± 6.6	1.96 ± 0.07	12.9 ± 2.4
With S-β-CyD	3	5.1 ± 1.8*	0.58 ± 0.22*	11.3 ± 5.2*	1.45 ± 0.13	4.1 ± 1.9*

[a] Each value represents the mean ± SE of 3–10 rats. *$p < 0.05$ versus buserelin alone. [b] Maximum plasma buserelin level. [c] Time required to reach the maximum plasma buserelin level. [d] Area under the plasma level–time curve up to 3 h postadministration. [e] Mean residence time of buserelin in plasma. [f] Bioavailability compared with the AUC value of buserelin acetate administered intravenously (0.1 mg/kg).

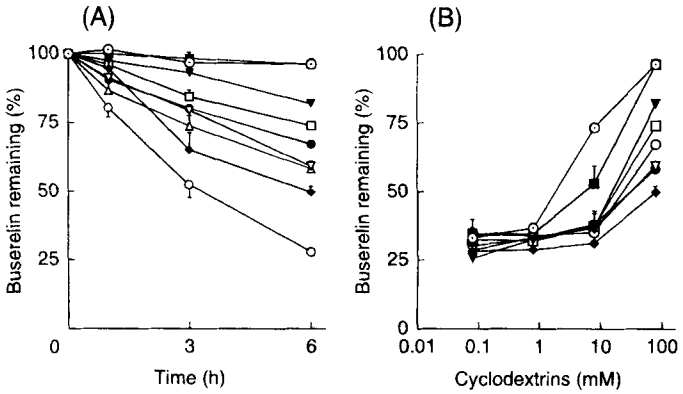


Figure 4—Effects of cyclodextrins on the enzymatic degradation of buserelin acetate (80 μM) in rat nasal mucosal homogenates in isotonic phosphate buffer (pH 7.4) at 37 °C. Key as in Figure 3. (A) The final concentration of cyclodextrins was 80 mM. (B) Determined after a 6 h incubation. Each value represents the mean ± SE of three experiments.

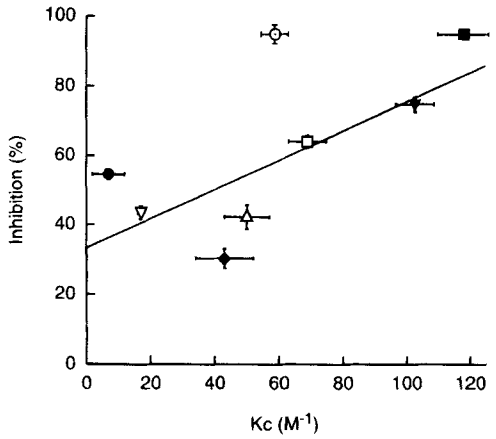


Figure 5—Relationship between inhibitory effects of cyclodextrins on proteolysis of buserelin acetate and stability constants (K_c) of complexes of buserelin acetate with cyclodextrins. Key as in Figure 3. The percentage of inhibition was determined after a 6 h incubation of buserelin acetate (80 μM) with cyclodextrins (80 mM) in rat nasal mucosal homogenates in isotonic phosphate buffer (pH 7.4) at 37 °C. The K_c values as in Table 2. The solid line represents the regression curve, when an outlier, G_2-β-CyD (⊙), was omitted. Each value represents the mean ± SE of three to five experiments.

In fact, cyclodextrins were found to reduce the activity of leucine aminopeptidase in rat nasal homogenates.[10]

Facilitated Permeability of Nasal Mucosa—The permeability barrier of nasal mucosa is the resistance of the membrane to diffusion of solutes, which in the cases of peptides and proteins may be related to their large molecular size and hydrophilicity.[23] Since inulin is enzymatically stable and poorly permeable across the nasal mucosa, the extent of nasal absorption of inulin coadministered with cyclodextrins may provide a reliable measure for estimating their effects on the permeability barrier of nasal mucosa, separate from the effects due to enzymatic metabolism. Previously, the methylated cyclodextrins showed the greatest potency to increase the nasal permeability, while the 2-hydroxypropylated cyclodextrins were less effective than the parent cyclodextrins.[10] Cyclodextrins are capable of extracting specific membrane lipids such as cholesterol and phospholipids from the nasal mucosa through rapid and reversible formation of inclusion complexes. This selective solubilization of the membrane lipids may reduce the barrier function of the nasal epithelium.[24] In the present study, the extent of nasal mucosal permeation of inulin coadministered with γ-CyD, CM-α-CyD, CM-β-CyD, or G_2-β-CyD in the same manner as described previously for the other cyclodextrins[10] was determined. γ-CyD and G_2-β-CyD showed a minor enhancing effect on nasal permeability. CM-α- and CM-β-CyD failed to enhance the nasal permeability to any significant extent. In the present study, the permeability parameter of nasal mucosa (P_m) was defined as the area under the plasma inulin level–time curve up to 3 h after the nasal administration of inulin with or without the cyclodextrins; the value for inulin alone was taken as one unit. As shown in Figure 6, the extent of the cyclodextrin-enhanced nasal absorption of buserelin (F values in Table 2) correlated well with an increase in the P_m value of inulin afforded by the cyclodextrins ($r^2 = 0.871$, $F = 60.77$).

The relative contribution of the metabolic and permeability barriers to the nasal absorption of buserelin with cyclodextrins was examined using multiple regression analysis. Cyclodextrins affected both the stability of buserelin against the proteolysis and the permeability of the nasal mucosa. Furthermore, the decrease in diffusibility of buserelin across the nasal membrane through the complexation with cyclodextrins may provide an additional factor contributing negatively to the absorption enhancement. In an initial multiple regression equation, the criterion variable was the F value in Table 2, and the explanatory variables were (1) the permeability parameter defined as the P_m value in Figure 6, (2) the stability parameter defined as the percentage of inhibition in Figure 4, and (3) the stability constant of the complexes of buserelin acetate with cyclodextrins in Table 2, respectively. When a stepwise method was used and the validity of regression was judged by the F-test value (>2.0), the second and third dependent variables were excluded from the equation and

Table 4—Morphological Evaluation of Nasal Mucosal Damage Induced with DM-β-CyD at Various Concentrations and Enhanced Nasal Bioavailability of Buserelin in Rats[a]

System	Irritation Score[a]				F^f(%)
	Category 1	Category 2	Category 3	Total Score	
Buserelin alone	1.12 ± 0.03	1.17 ± 0.03	1.06 ± 0.02	1.12 ± 0.03	14.64 ± 2.39
With 10 mM DM-β-CyD	1.22 ± 0.07	1.43 ± 0.18	1.44 ± 0.11	1.36 ± 0.07	38.41 ± 5.45*
With 40 mM DM-β-CyD	2.00 ± 0.10*	2.57 ± 0.10*	2.60 ± 0.20*	2.39 ± 0.20*	58.30 ± 1.50*
With 80 mM DM-β-CyD	2.60 ± 0.10*	3.63 ± 0.07*	2.83 ± 0.11*	3.02 ± 0.31*	60.12 ± 7.16*

[a] Each value represents the mean ± SE of three to seven rats. *$p < 0.01$ versus buserelin alone. [b] Determined after a 10 min exposure to the DM-β-CyD solution. [c] Bioavailability compared with the AUC value of buserelin acetate administered intravenously (0.1 mg/kg).

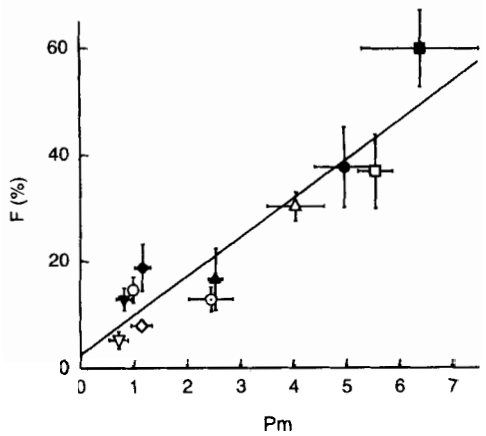


Figure 6—Relationship between bioavailabilities (F) of buserelin after nasal administration of buserelin acetate (0.1 mg/kg) with cyclodextrins (80 mM) and nasal membrane permeabilities (P_m) facilitated by cyclodextrins in rats. Key as in Figure 3. The solid line represents the regression curve for all the buserelin–cyclodextrin systems. Each value represents the mean ± SE of 3–10 rats.

consequently the following equation was obtained:

$$\text{bioavailability} = 1.09\ (\pm 0.17) P_m + 2.77 \qquad (1)$$

$$(n = 8, r^2 = 0.869, F = 39.63)$$

where the value in parentheses is the estimated standard error of the coefficient. These results indicate that the cyclodextrin-facilitated nasal membrane permeability is the primary determinant of nasal absorption enhancement for buserelin acetate. The protection afforded by cyclodextrins against the proteolysis of buserelin with the nasal mucosal enzymes, which is a significant and well-defined phenomenon *in vitro*, seems to have limited importance in terms of absorption of the peptide from the nasal cavity. In sharp contrast, the reduced proteolysis and the increased nasal membrane permeability contributed additionally to the nasal absorption enhancement of insulin by cyclodextrins.[10] The difference in relative contribution of the two absorption barriers could be explained by the intrinsic enzymatic resistance of each peptide. Unlike insulin, buserelin may be sufficiently stable to escape the metabolism before or during the passage through the nasal mucosal membrane under the hyperpermeable state elicited with cyclodextrins.

Morphological Evaluation of Nasal Mucosa—Since the surface active nature of DM-β-CyD may potentially cause local irritation to the nasal mucosa in a nonspecific manner, safety concerns should be raised for the use of such an enhancer, especially for chronic treatments. Merkus et al. reported that DM-β-CyD in concentrations up to 2% w/v (~15 mM) displayed a rather mild and reversible effect on the nasal mucociliary clearance, compared to other absorption-promoting agents used commonly in nasal delivery.[25] Watanabe et al. described that even when DM-β-CyD at an extremely high concentration of 15% w/v (~110 mM) was applied to the nasal mucosa of rabbits, the facilitated nasal permeability returned to the normal physiological level within 24 h after the nasal application.[11] In this study, we examined the effects of DM-β-CyD at various concentrations on the surface morphology of the rat nasal mucosa by means of electron scanning microscopy. After a 10 min exposure to the DM-β-CyD solutions, the degree of irritation was scored in three categories (see the Experimental Section),[18] and the results are summarized in Table 4. The irritation scores for 10 mM DM-β-CyD, a concentration that had a substantial absorption-promoting effect, were almost comparable to those of the control. This finding is in good agreement with a recent paper in which the minimal effective concentration of DM-β-CyD to enhance the nasal insulin absorption was ~2% w/v (15 mM), showing only a mild effect on the nasal ciliary function.[26] The irritation scores rose as the concentration of DM-β-CyD increased, while the absorption-enhancing effect tended to reach some limit. Nevertheless, the irritation score for 80 mM DM-β-CyD (3.02 ± 0.31) was lower than that for 1% w/v laureth-9, a typical irritative surfactant (4.24 ± 0.26) under the equivalent condition.[27] In this study, the entire nasal cavity of the rat was flooded with the DM-β-CyD solution under the dorsal recumbency, which inhibited mucociliary drainage. Therefore, the morphological damages observed appear to be much more pronounced than what would occur in a normal physiological situation *in vivo*. This assumption is supported by the results of 6 months of clinical studies in which DM-β-CyD in concentrations up to 6% w/v were well-tolerated when given nasally as a complex with estradiol or progesterone to oophorectomized women twice daily.[28]

The acceptability of an absorption enhancer is dependent not only on the local tissue tolerance but also on the overall systemic safety profile of the enhancer. It is generally recognized that cyclodextrins, when given intravenously, are distributed rapidly in extracellular fluids and eliminated intact in the urine through glomerular filtration.[29] Cyclodextrins are reported to be hardly absorbed from the mucosal membranes.[16] Nevertheless, it is likely that DM-β-CyD has the ability to enhance the nasal membrane permeability, thereby facilitating its own absorption. When DM-β-CyD (80 mM, 8 μmol/kg) was administered nasally to the rat, 15.8 ± 3.7% of the dose was recovered intact in urine up to 3 h after nasal administration. Even under the theoretical assumption that DM-β-CyD was completely absorbed intranasally, DM-β-CyD is not likely to induce serious systemic toxicity.[30] However, chronic safety studies of systemically delivered low doses of DM-β-CyD would be necessary to confirm this assumption.

In conclusion, of the cyclodextrins evaluated, DM-β-CyD was the most potent enhancer of the nasal absorption of buserelin in the rat, achieving the substantial absorption enhancement without marked apparent morphological alterations of the nasal mucosa. From the results of multiple regression analysis, the facilitated nasal membrane permeability mediated by cyclodextrins appears to be the major

contributor to the absorption enhancement of the peptide. While comprehensive toxicological issues and interspecies differences[31] for DM-β-CyD should be further investigated, the present data suggest that DM-β-CyD has potential as an adjuvant for designing nasal preparations of buserelin acetate with prominent enzymatic stability and intranasal permeability.

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