Effects of Proteolytic Enzyme Inhibitors on the Nasal Absorption of Vasopressin and an Analogue

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Proteolytic enzyme inhibitors were examined as absorption enhancers for the nasal delivery of vasopressin (AVP) and desmopressin (1-d-8-DAVP) in rats. Aprotinin, soybean trypsin inhibitor, and camostat mesilate were used as enzyme inhibitors. The nasal absorption of AVP and 1-d-8-DAVP was evaluated by measuring its antidiuretic effect. Nasal administration of AVP (0.005 IU/kg) or 1-d-8-DAVP alone (2.5 ng/kg) produced a small antidiuretic effect. Coadministration with aprotinin (1000 and 10000 KIU/kg) or soybean trypsin inhibitor (1.25 and 6.25 m*M*) did not change the antidiuretic effect. However, coadministration with camostat mesilate (1 to 50 m*M*) significantly increased the antidiuretic effect and, thus, the nasal absorption of AVP and 1-d-8-DAVP. The activities of aminopeptidase, cathepsin-B, and trypsin in the nasal mucosal tissue of rats were 7 nmol/min/mg protein, 0.7 nmol/min/mg protein, and 4.6 pmol/min/mg protein, respectively. Aprotinin and soybean trypsin inhibitor inhibited only the trypsin activity, whereas camostat mesilate inhibited aminopeptidase and trypsin activities. Aprotinin (MW 6500) and soybean trypsin inhibitor (MW 8000), with relatively high molecular weights, may not permeate into the nasal mucosal tissue. In contrast, camostat mesilate is slowly absorbed (8%/hr) and could inhibit the proteolytic activity in the nasal mucosa, resulting in enhanced nasal absoprtion of AVP and 1-d-8-DAVP.

KEY WORDS: nasal absorption; vasopressin; 1-*d*-8-arginine vasopressin; aprotinin; soybean trypsin inhibitor; camostat mesilate; proteolytic enzyme inhibitor.

INTRODUCTION

The possibility of avoiding gastrointestinal degradation and hepatic first-pass metabolism makes the transmucosal route attractive for compounds such as peptides, with extensive degradation during oral administration (1–3), because of the large mucosal surface area and its high vascularization (1). However, intranasally administered peptides (above MW approximately 1000) are poorly absorbed (2). The low mucosal peptide permeability and the marked mucosal endopeptidase activity require a suitable absorption enhancer of low local toxicity to improve systemic delivery (3).

Vasopressin (AVP; MW 1084) and its analogue, a 1-deamino-8-D-arginine vasopressin (1-d-8-DAVP; MW 1069), are used in the treatment of patients suffering from diabets insipidus (4) and are being studied for treatment of mild memory disorders resulting from minor brain trauma, senile dementia, and Alzheimer's disease (5). We previously reported that a viscous hyaluronate-Na solution enhanced the nasal absorption of AVP and 1-d-8-DAVP in rats (6). In the present study, the proteolytic enzyme inhibitors, aprotinin, soybean trypsin inhibitor, and camostat mesilate, were examined as absorption enhancers for the intranasal administration of AVP or 1-d-8-DAVP in rats.

MATERIALS AND METHODS

Materials. Vasopressin (AVP), 1-deamino-8-D-arginine vasopressin (1-d-8-DAVP), aprotinin, and soybean trypsin inhibitor were purchased from Sigma Chemical Co. (St. Louis, MO). Camostat mesilate (FOY-305) was supplied by Ono Pharm. Co., Ltd. (Osaka). Hyaluronate-Na (average molecular weight 1.4×10^6) was supplied by Shiseido Co., Ltd. (Tokyo). All other chemicals were of reagent grade.

Preparations. AVP and 1-d-8-DAVP were dissolved in isotonic buffer solutions and viscous solutions (pH 4.0, 5.0, and 7.0). the compositions of isotonic buffer solution were 0.236 *M* citric acid: 0.123 *M* disodium phosphate buffer (pH 4.0 and 5.0) and 0.171 *M* potassium phosphate:0.144 *M* sodium acid carbonate buffer (pH 7.0). The viscous solutions were prepared by presoaking hyaluronate-Na (1%, w/v) in the isotonic buffer solutions. Proteolytic enzyme inhibitors, aprotinin (100,000 and 30,000 KIU/ml), soybean trypsin inhibitor (5 and 15 m*M*), and camostat mesilate (1, 10, and 50 m*M*), were dissolved or suspended (50 m*M* camostat mesilate). The final pH of preparations was adjusted by adding HCl or NaOH solution.

Nasal Administration in Rats. The nasal absorption of AVP or 1-d-8-DAVP was evaluated with its antidiuretic effect using the method described by Koyama *et al.* (7). Male wistar rats (200–230 g) were fasted for 20 hr prior to the experiments. The rats were anesthetized by oral administration of ethanol to inhibit secretion of endogenous AVP. Three doses (separated by 30 min) of 10 ml/kg of 24% (w/v) ethanol were given orally through a gastric tube. The ethanol anesthetized rats were intubated to assist breathing, and then the left femoral vein was catheterized with polyethylene tubing. After a 10 ml/kg primary dose, a hypotonic solution containing 1.2% (w/v) ethanol, 1.7% (w/v) glucose, and 0.3% (w/v) NaCl was administered through the femoral catheter as a constant infusion at 0.5 ml/kg/min. The urinary bladder was exposed by a small incision in the lower abdominal wall, and polyethylene tubing was inserted into the bladder for urine collection.

Nasal administrations were made as described by Hirai *et al.* (8). The preparations (0.5 ml/kg body weight) were administered into the nasal cavity through tube by a syringe. The extent of antidiuresis was expressed as a percentage consisting of the ratio of urine volume produced after administration of the preparations to that produced in the 10-min period just before administration.

In a comparative study, AVP and 1-d-8-DAVP were administered intravenously to rats which were prepared in the

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same manner as for the nasal administration procedure. The doses of AVP and 1-d-8-DAVP were 0.0025 IU/kg and 0.9 ng/kg, respectively.

Nasal Administration in Dogs. Four beagle male dogs weighing 9.5 to 13 kg were fasted for 20 hr prior to the experiments. The preparations (volume, 0.1 ml/dog; dose of AVP, 30 μg/dog) were administered by dropping into the nasal cavity using an Eppendorf pipette without anesthesia. Blood samples (2.5 ml) were collected from the foreleg vein at 5 min before administration and 15 and 30 min and 1, 1.5, 2, and 3 hr after administration. The plasma concentration of AVP was measured using an AVP radioimmunoassay kit (Mitsubishiyuka Co. Ltd., Tokyo).

Measurement of Nasal Ciliary Beat Frequency. The nasal mucociliary beat frequency was measured by a photoelectric method described by Ohashi and Nakai (9). Nasal mucosal membranes from adult albino rabbits (2.0–2.5 g) were used. Rabbits were sacrificed by air embolization, and the nasal mucosal membranes were removed and cut into square pieces (0.5 cm). The ciliary beat frequencies were measured in an intrinsic chamber filled with isotonic buffer or camostat mesilate solution at 30°C.

Peptidase Activities. Rat nasal mucosal tissue was homogenized in 0.25 *M* sucrose in a glass homogenizer cooled by ice water. The homogenate was centrifuged at 9000 *g* and 5°C for 5 min and the supernatant was used for analysis. The peptidase activity and the inhibition effects with proteolytic enzyme inhibitors to the peptidase activity were carried out using peptidyl-4-methylcoumaryl-7-amide (peptidyl-MCA) derivatives by a method described by Komada *et al.* (10). The 7-amino-4-methylcoumarin (AMC) liberated from peptidyl-MCA substrate by proteolytic enzyme reaction was measured by fluorescence intensity and enzyme activities were calculated.

Histopathological Study. Ten millimolar camostat mesilate in isotonic phosphate buffer (pH 7.0) and isotonic phosphate buffer (pH 7.0) at 37°C were administered into the nasal cavity of rats at 0.5 ml/kg body weight as described by Hirai *et al.* (8). The nasal tissues were removed 20 min after the administration of preparations for transmission electron microscopic (TEM) study. The tissues were fixed with 0.1 *M* phosphate buffer (pH 7.4) containing 2.5% OsO_4 and embedded in Epon 812 as described in our previous paper (11). Thin sections were observed by TEM (Hitachi H-600, TEM).

Data Analysis. The area above the antidiuretic effect versus time curve (AAC) was calculated by means of trapezoidal integration using the program MULTI (12). Statistical significance was assessed with Student's paired *t* test.

RESULTS

Nasal Administration. The effects of proteolytic enzyme inhibitors on the nasal absorption of AVP and 1-d-8-DAVP in hyaluronate-Na solution (1%, w/v) were examined in rats. Figures 1–3 show the change in urine volumes after nasal administration of AVP (0.005 IU/kg) in preparations (pH 7.0) containing proteolytic enzyme inhibitors at various concentrations. The antidiuretic effects after nasal administration of AVP with aprotinin (10,000 and 30,000 KIU/ml) or soybean trypsin inhibitor (5 and 15 m*M*) did not change compared to those without proteolytic enzyme inhibitors (Figs. 1 and 2).

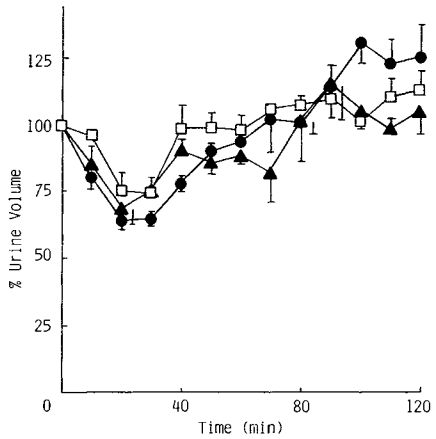

Fig. 1. Effects of aprotinin on the antidiuretic effects after nasal administration of AVP (0.005 IU/kg) in hyaluronate solution (pH 7.0) in rats. Concentrations of aprotinin: (●) 10,000 KIU/ml; (▲) 30,000 KIU/ml. (□) Without aprotinin. Each point represents the mean ± SE of four animals.

The antidiuretic effects after nasal administration of AVP with camostat mesilate (1, 10, and 50 m*M*) increased compared to that without camostat mesilate (Fig. 3). The antidiuretic effect with 50 m*M* camostat mesilate was higher than those with 1 and 10 m*M* camostat mesilate. Further, the antidiuretic effect with 50 m*M* camostat mesilate in hyaluronate-Na solution was higher than that with 50 m*M* camostat mesilate in buffer solution (pH 7.0).

Figures 4 and 5 show the effects of pH of preparations on the change in urine volumes after nasal administrations of AVP and 1-d-8-DAVP with and without camostat mesilate (50 m*M*) in rats, respectively. The antidiuretic effects after nasal administration of AVP (dose, 0.005 IU/kg) and 1-d-8-DAVP (dose, 2.5 ng/kg) with camostat mesilate were similar to the effects after nasal administration of AVP (dose, 0.025 IU/kg) and 1-d-8-DAVP (dose, 9 ng/kg) without

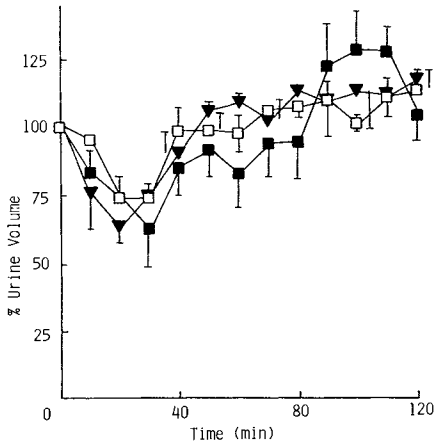

Fig. 2. Effects of soybean trypsin inhibitor on the antidiuretic effects after nasal administration of AVP (0.005 IU/kg) in hyaluronate solution (pH 7.0) in rats. Concentrations of soybean trypsin inhibitor:(▼) 5 m*M*; (■) 15 m*M*. (□) Without soybean trypsin inhibitor. Each point represents the mean ± SE of four animals.

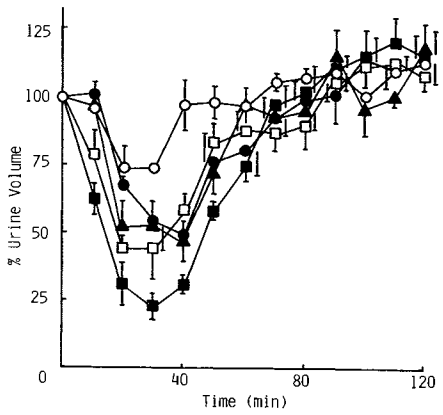

Fig. 3. Effects of camostat mesilate on the antidiuretic effects after nasal administration of AVP (0.005 IU/kg) in hyaluronate solution (pH 7.0) or buffer solution (pH 7.0) in rats. Concentrations of camostat mesilate in hyaluronate solution: (●) 1 m*M*; (▲) 10 m*M*; (■) 50 m*M*; (□) 50 m*M* camostat mesilate in buffer solution without camostat mesilate; (○) without camostat mesilate. Each point represents the mean ± SE of four animals.

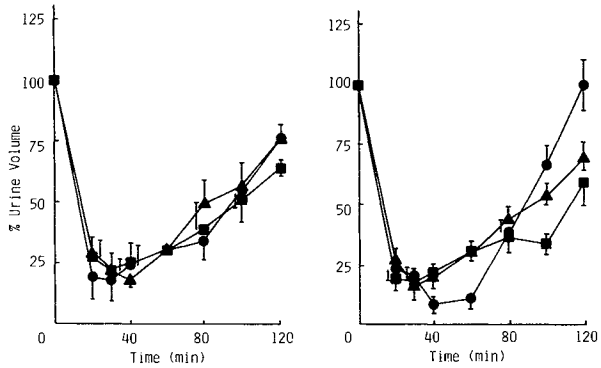

Fig. 5. Effects of pH of preparations on the antidiuretic effects after nasal administration of 1-d-8-DAVP in hyaluronate solution with or without camostat mesilate (50 m*M*) in rats. Doses of 1-d-8-DAVP were 9 ng/kg in the preparation without camostat mesilate and 2.5 ng/kg in the preparation with camostat mesilate. pH of preparations: (●) pH 4.0; (▲) pH 5.0; (■) pH 7.0. Each point represents the mean ± SE of four animals.

camostat mesilate, respectively. The antidiuretic effects after nasal administration of AVP preparations at pH 5 and pH 7 were smaller than that at pH 4 with or without camostat mesilate. However, in the case of 1-d-8-DAVP, the antidiuretic effects were not affected by changes of pH of preparations with or without camostat mesilate.

Figure 6 shows the plasma profiles of AVP (immunoreactive AVP) after nasal administration of AVP in dogs. The AVP plasma level after nasal administration of the AVP preparation containing camostat masilate (50 m*M*) was higher than that of the AVP preparation without camostat mesilate.

The bioavailabilities in rats after nasal administration of AVP and 1-d-8-DAVP preparations containing camostat mesilate are summarized in Table I. Bioavailabilities of AVP and 1-d-8-DAVP significantly increased on the addition of camostat mesilate. Bioavailability of AVP containing camostat mesilate (50 m*M*) at pH 4 was especially high.

Peptidase Activities. Figure 7 shows the effects of various proteolytic enzyme inhibitors on the peptidase activities in nasal mucosal tissue of rats. The peptidase activities were high in the following order: aminopeptidase > cathepsin B (carboxypeptidase) > trypsin. Camostat mesilate (0.1 and 1.0 m*M*) reduced aminopeptidase and trypsin activities. Aprotinin (200 and 1000 KIU/ml) and soybean trypsin inhibitor (0.25 m*M*) inhibited only the trypsin activity.

Nasal Mucociliary Beat. The frequency of the ciliary beat at rabbit nasal mucosal membranes *in vitro* was not affected by 10 m*M* camostat mesilate solution (pH 7.0) (data not shown).

Histopathological Study. In order to clarify the histological change in the nasal mucosal tissue administered 10 m*M* camostat mesilate solution (pH 7.0), the epithelium was observed with TEM. The nasal epithelium, cilia, cytoplasm, and tight junction, viewed 20 min after administration of 10 m*M* camostat mesilate solution, did not vary compared with control (without camostat mesilate).

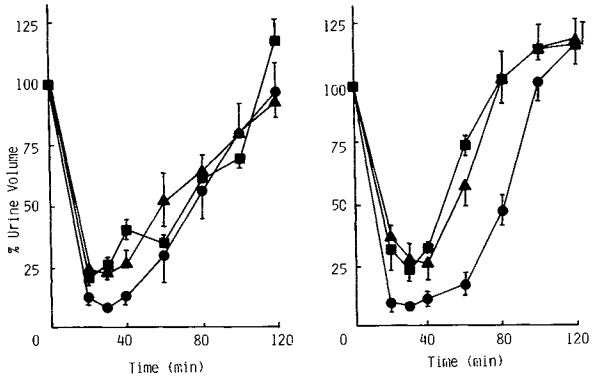

Fig. 4. Effects of pH of preparations on the antidiuretic effects after nasal administration of AVP in hyaluronate solutions with or without camostat mesilate (50 m*M*) in rats. Doses of AVP were 0.025 IU/kg in the preparation without camostat mesilate and 0.005 IU/kg in the preparation with camostat mesilate. pH of preparations: (●) pH 4.0; (▲) pH 5.0; (■) pH 7.0. Each point represents the mean ± SE of four animals.

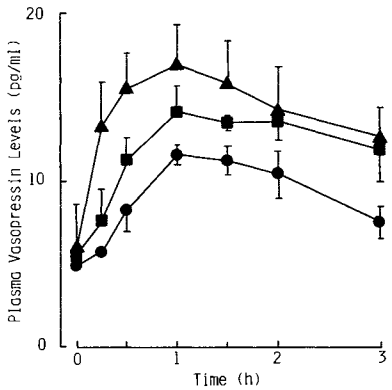

Fig. 6. Plasma AVP levels after nasal administration of AVP in buffer solution (pH 7.0) and hyaluronate solution (pH 7.0) with or without camostat mesilate (50 m*M*) in dogs. (●) AVP buffer solution; (■) AVP hyaluronate solution; (▲) AVP hyaluronate solution with camostat mesilate. Each point represents the mean ± SE of four animals.

Table I. Bioavailabilities (BA) After Nasal Administration of AVP and 1-d-8-DAVP Preparations Containing Camostat Mesilate in Rats[a]

	AAC (% urine volume · min)	BA (%)
AVP (0.005 IU/kg)		
1.0% (w/v) hyaluronate-Na solution, pH 7.0	735.3 ± 216.6	9.9 ± 2.9
+ 10 m*M* camostat mesilate, pH 7.0	2005.1 ± 270.1	27.0 ± 3.7*
+ 50 m*M* camostat mesilate, pH 7.0	3292.9 ± 121.1	44.3 ± 1.6**
+ 50 m*M* camostat mesilate, pH 4.0	6214.3 ± 207.6	83.6 ± 2.8**
1-d-8-DAVP (2.5 ng/kg)		
1.0% (w/v) hyaluronate-Na solution, pH 7.0	2488.4 ± 615.0	15.9 ± 3.9
+ 50 m*M* camostat mesilate, pH 7.0	5696.9 ± 283.2	38.9 ± 2.2*
+ 50 m*M* camostat mesilate, pH 4.0	6075.5 ± 344.7	39.8 ± 0.4**

[a] The AAC was calculated using the trapezoidal method. BA was calculated as $(AAC_{nasal}/AAC_{i.v.}) \times (D_{i.v.}/D_{nasal}) \times 100\%$, D being the dose of AVP or 1-d-8-DAVP. Each value represents the mean ± SE of four animals.

* Significantly different from hyaluronate-Na solution (pH 7.0) at $P < 0.005$.

** Significantly different from hyaluronate-Na solution (pH 7.0) at $P < 0.001$.

DISCUSSION

Polypeptides up to a molecular weight of about 1000 are readily absorbed from the nasal cavity without any absorption enhancer such as bile salts (3). However, the polypeptide bioavailability achieved by nasal delivery is less than that by parenteral administration, which can be attributed to degradation by proteolytic enzymes in the nasal mucosa (3). Hussain *et al.* reported that aminoboronic acid derivatives, aminopeptidase inhibitors, stabilize enkephalins in the nasal cavity of rats (13).

In this study, camostat mesilate (1–50 m*M*), a proteolytic enzyme inhibitor, enhanced the nasal absorption of AVP and 1-d-8-DAVP. However, aprotinin (10,000 and 30,000 KIU/ml) and soybean trypsin inhibitor (5 and 15 m*M*) did not enhance the nasal absorption of AVP and 1-d-8-DAVP. Camostat mesilate, a low molecular weight compound (MW 495) was slightly absorbed through nasal mucosa, at a rate of approximately 8%/hour in rats (data not shown). Camostat mesilate inhibited the activities of aminopeptidase and trypsin in nasal mucosal tissue. However, aprotinin and soybean trypsin inhibitor may not be absorbed through the nasal mucosa, because of their high molecular weight (MW 6500 and 8000, respectively). These inhibitors affected only trypsin activity in nasal mucosal tissue. Aminopeptidase activity was high compared to trypsin activity in nasal mucosal tissue. Camostat mesilate, which permeated into the nasal mucosa, might reduce the aminopeptidase activity in nasal mucosa, thereby inhibiting the degradation of AVP and 1-d-8-DAVP during absorption. On the other hand, Saffran *et al.* reported that the activity of AVP after oral administration was enhanced with aprotinin (1000 units/rat) in rats, possibly because of the presence of proteolytic digestive enzymes, such as trypsin, in the gastrointestinal tract.

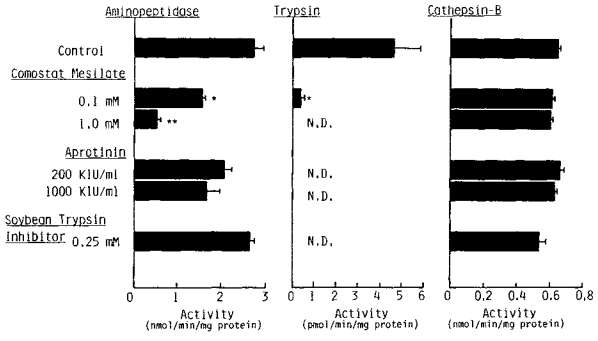

Fig. 7. Effects of various proteolytic enzyme inhibitors on the peptidase activity in nasal mucosal tissue of rats. Substrates used for *in vitro* evaluation of aminopeptidase, trypsin, and cathepsin-B activities were Leu-MCA, ter-butyloxycarbonyl-Phe-Ser-Arg-MCA, and carbobenzoxy-Phe-Arg-MCA, respectively. Each value represents the mean ± SE of three experiments. Significant in comparison with control: (*) $P < 0.01$; (**) $P < 0.001$.

The nasal absorption of AVP was greater with lower pH of the preparations with or without camostat mesilate (pH 4.0–7.0). Nasal absorption of secretin (MW 3052) was also higher at lower pH (15). The effect of pH on the nasal absorption may be due to changes in the structure or electrical charge of the mucosal membrane and/or to self-association and conformational change of the peptides. However, the nasal absorption of 1-d-8-DAVP was not affected by pH of the preparation, with or without camostat mesilate. The reason for this discrepancy is unclear.

In order to examine the effects of camostat mesilate on the permeation barrier of nasal mucosal membrane, several studies were carried out. Camostat mesilate (50 m*M*) did not enhance the nasal absorption of 6-carboxyfluorescein (MW 376), a hydrophilic compound, in rats (data not shown). Further, the nasal epithelium of rats did not change upon treatment with 10 m*M* camostat mesilate for 20 min, as determined with TEM. Therefore, camostat mesilate did not affect the permeation barrier of the nasal mucosa. This observation suggests that inhibition of aminopeptidase activity through the use of camostat mesilate resulted in enhanced nasal bioavailability of AVP and 1-d-8-DAVP.

For nasal delivery, the nasal ciliotoxicity of the drug and the formulation are important (16). Hermens *et al.* reported that absorption enhancers such as dehydroxy bile salt and laureth-9 caused very rapid and irreversible ciliostatic effects on human adenoid tissue *in vitro* (17). Camostat mesilate (10 m*M*) did not affect the nasal mucociliary beat in rabbits *in vitro*.

In conclusion, camostat mesilate, but not aprotinin and soybean trypsin inhibitor, enhanced the nasal absorption of AVP and 1-d-8-DAVP in rats.

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