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Effects of proteolytic enzyme inhibitors of enhancement of transdermal iontophoretic delivery of vasopressin and an analogue in rats

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Summary

The effects of the proteolytic enzyme inhibitors, aprotinin, soybean trypsin inhibitor and camostat mesilate, as absorption enhancers of transdermal iontophoretic delivery of vasopressin (AVP) and its analogue, 1-deamino-8-D-arginine vasopressin (1-d-8-DAVP) were examined in rats. The dermal absorption of AVP and 1-d-8-DAVP was determined to induce an antidiuretic effect. Administration of AVP (0.5 IU/rat) or 1-d-8-DAVP (0.1 μg/rat) on abdominal skin (available diffusion area 3.14 cm^2) produced no antidiuretic effect. This resulted in a slight antidiuretic influence with iontophoresis (drug phase, anode; reference phase, cathode; constant current of 1 mA/3.14 cm^2). Moreover, the antidiuretic effect was further enhanced on application of iontophoresis and camostat mesilate (1–50 mM). However, aprotinin and soybean trypsin inhibitor had no influence on the antidiuretic effect. The activities of aminopeptidase, cathepsin B and trypsin in dermal tissue were determined to be 1.7 nmol/min per mg protein, 0.5 nmol/min per mg protein and 2.4 pmol/min per mg protein, respectively. Camostat mesilate significantly inhibited the activities of aminopeptidase and trypsin, whilst aprotinin and soybean trypsin inhibitor resulted in considerable inhibition of the activity of trypsin. Aprotinin (Mol. Wt 6500) and soybean trypsin inhibitor (Mol. Wt 8000), both having a relatively high molecular weight, may not permeate into the dermal tissue. In contrast, a small degree of absorption of camostat mesilate (about 3%/2 h) with iontophoresis might inhibit the proteolytic enzyme activity in dermal tissue and hence might enhance the dermal absorption of AVP and 1-d-8-DAVP.

Introduction

Currently, the systemic delivery of peptide drugs through the parenteral route is attracting considerable attention. Since peptide drugs are extensively degraded by proteolytic enzymes in the gastrointestinal tract, their bioavailability is extremely poor when taken orally. Transdermal delivery of peptide drugs can be considered as an available route (Mayer et al., 1988). However, the horny layer (stratum corneum) of skin constitutes a considerable barrier to transport, resulting in resistance against the percutaneous absorption of

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most substances, including peptide drugs. Many approaches for transdermal delivery have included the use of prodrugs, permeation enhancers and iontophoresis. Iontophoresis can be used to enhance the transdermal delivery of uncharged or charged molecules, including macromolecules (Burnette and Marrero, 1986; Tyle 1986). Peptide drugs must cross the physical barrier of the stratum corneum as well as the enzymatic barrier of skin before reaching the circulation. The enzymatic barrier resulting from peptidase is important in limiting the transdermal delivery of peptide drugs (Zhou and Po, 1990; Shah and Borchardt, 1991).

Vasopressin (AVP; Mol. Wt 1084) and its analogue are regularly used in the treatment of patients suffering from diabetic insipidus. They are currently being studied for potential therapeutic applications in mind memory disorders due to minor brain trauma, senile dementia, and Alzheimer's disease. Lelawongs et al. (1989) reported that the transdermal delivery of AVP was enhanced by iontophoresis during in vitro experiments. We previously reported the transdermal iontophoretic delivery of AVP and its analogue, 1-deamino-8-D-arginine vasopressin (1-d-8-DAVP) in rats in vivo (Iwakura and Morimoto, 1991). Furthermore, we reported that the nasal absorption of AVP and 1-d-8-DAVP was enhanced by the proteolytic enzyme inhibitor, camostat mesilate (Morimoto et al., 1991). In the present study, the effects of the proteolytic enzyme inhibitors, aprotinin, soybean trypsin inhibitor and camostat mesilate, on the transdermal iontophoretic delivery of AVP and 1-d-8-DAVP were examined 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). All other chemicals were of reagent grade.

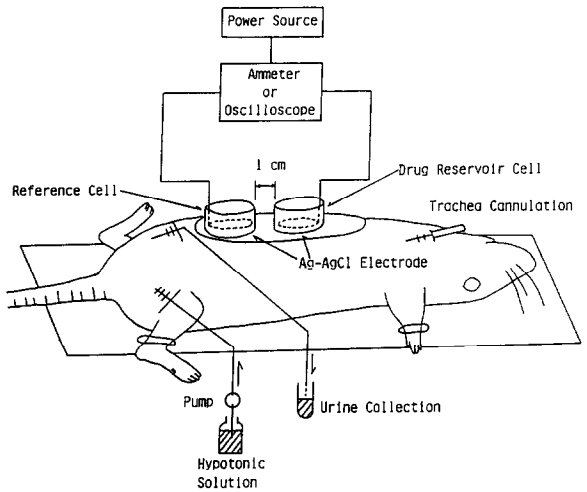

Fig. 1. Schematic representation of the in vivo iontophoretic system used in rats. A hypotonic solution was administered through the femoral catheter as a constant infusion at a rate of 0.5 ml/kg per min. Two cylindrical polyethylene cells (10 mm i.d. × 20 mm; available area 3.14 cm^2) were attached to the abdominal skin of the rat. A pair of Ag/AgCl electrodes was immersed in the solutions, the anode being in the drug solution and the cathode in the 0.9% w/v NaCl solution. The electrodes were connected to a constant-current power source (1 mA/3.14 cm^2).

Preparations

AVP and 1-d-8-DAVP were dissolved in buffer solutions (0.2 M disodium phosphate buffer, pH 4.0; 0.1 M citric acid buffer, pH 5.5, 7.0 and 8.0). The proteolytic enzyme inhibitors, aprotinin (1000 and 10000 KIU/ml), soybean trypsin inhibitor (1.25 and 6.25 mM) and camostat mesilate (1, 10 and 50 mM), were dissolved or suspended in drug solution. The final pH (pH 4.0, 5.5, 7.0 and 8.0) of preparations was adjusted by adding HCl.

Transdermal iontophoretic systems

Iontophoresis (Fig. 1) was carried out described as previously (Iwakura and Morimoto, 1991). The percutaneous absorption of AVP or 1-d-8-DAVP was evaluated based on its antidiuretic effects (Koyama et al., 1985). Briefly, male Wistar rats (230–250 g), from which hair on the abdominal area was removed with electric hair clippers and an electric razor, were fasted for 20 h prior to experiments. 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. Subsequently, a hypotonic solution containing 1.2% w/v ethanol, 1.7% glucose and 0.3% NaCl was administered through the femoral catheter as a constant infusion at a rate of 0.5 ml/kg per min. The urinary bladder was exposed by a small incision in the lower abdominal wall and polyethylene tubing inserted into the bladder for urine collection.

Two cylindrical polyethylene cells (10 mm i.d. × 20 mm; available skin area 3.14 cm^2) were fixed onto the rat abdominal skin surface using an adhesive agent (Alon Alpha; Sankyo Co. Ltd, Tokyo) at intervals of 1 cm. One cell (drug phase) was filled with 2 ml of AVP or 1-d-8-DAVP preparation, other cell (reference phase) being filled with 2 ml of 0.9% w/v NaCl solution after a constant volume of urine had been attained. A pair of Ag/AgCl electrodes was immersed in the solutions, the anode being in the drug solution and the cathode in the 0.9% w/v NaCl solution. The electrodes were connected to a transdermal iontophoretic system comprising a constant-current power source (1 mA/3.14 cm^2). The extent of antidiuresis was expressed as the percentage ratio of the effect measured for the preparation to that produced in the 10 min period just before administration.

The experiment on percutaneous absorption of camostat mesilate with or without iontophoresis (1 mA/3.14 cm^2) was performed as described above for AVP and 1-8-d-DAVP. Rats were anesthetized with sodium pentobarbital (40 mg/kg) during experiments. 2 ml of camostat mesilate solution (1 mM, pH 7.0) were placed in the cell (drug phase). After 2 h, the drug solution in the cell was withdrawn as completely as possible and the cell was washed with pH 7.0 buffer solution. Washings were combined with the drug solution and the volume brought to 100 ml with the same buffer solution. The concentration of camostat mesilate in samples was determined spectrophotometrically at a wavelength of 265 nm. The amount absorbed was calculated from the difference in the amount of drug between the initial solution and the combined effluent.

Proteolytic enzyme activities in dermis

Rat epidermal tissue was homogenized in 0.25 M sucrose in a glass homogenizer with cooling by ice water. The homogenate was centrifuged at $9000 \times g$ for 5 min at 5°C and the supernatant used for analysis. Peptidase activity and the degree of inhibition with proteolytic enzyme inhibitors of peptidase activity were evaluated using peptidyl-4-methylcoumaryl-7-amide (peptidyl-MCA) derivatives according to the method of Komada et al. (1985). 7-Amino-4-methylcoumarin (AMC) liberated from the peptidyl-MCA substrate by proteolytic enzyme reaction was assayed on the basis of fluorescence intensity and the enzyme activities were calculated.

Data analysis

The area above the antidiuretic effect curve (AAC) was calculated by means of trapezoidal integration using the MULTI program (Yamaoka et al., 1985). Statistical significance of the data obtained was assessed by analysis through Student's paired *t*-test.

Results

Transdermal administration

Administration of AVP (0.5 IU/rat) and 1-d-8-DAVP (0.1 μg/rat) to abdominal skins produced no antidiuretic effect in rats. However, administration of the drugs with iontophoresis (constant current of 1 mA/3.14 cm^2) did induce small antidiuretic effects (Iwakura and Morimoto, 1991).

Figs 2 and 3 show the influence of the proteolytic enzyme inhibitors, aprotinin, soybean trypsin inhibitor and camostat mesilate, on the antidiuretic effects following transdermal administration of AVP and 1-d-8-DAVP preparations (pH 7.0) with iontophoresis (constant current of 1 mA/3.14 cm^2) to rats. Aprotinin (1000 KIU/ml (data not shown) and 10000 KIU/ml) and soybean trypsin inhibitor (1.25 mM [data not shown] and 6.25 mM) did not enhance the antidiuretic effect on administration of AVP and 1-d-8-DAVP. Camostat mesilate (50 mM) increased the antidiuretic effect on administration of AVP and

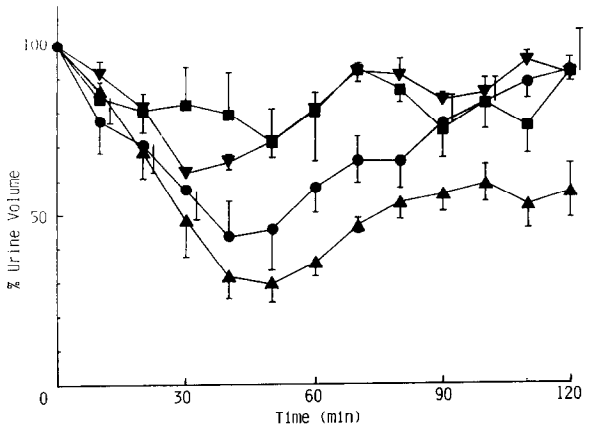

Fig. 2. Effects of proteolytic enzyme inhibitors on changes in urine volume induced by iontophoresis (current intensity, 1 mA/3.14 cm^2; 2 h) following application of AVP (0.5 IU/rat, pH 7.0) to rat abdominal skin. (●) Without proteolytic enzyme inhibitor, (▲) camostat mesilate (50 mM), (■) aprotinin (10000 KIU/ml), (▼) soybean trypsin inhibitor (6.25 mM). Each point represents the mean ± S.E. of four animals.

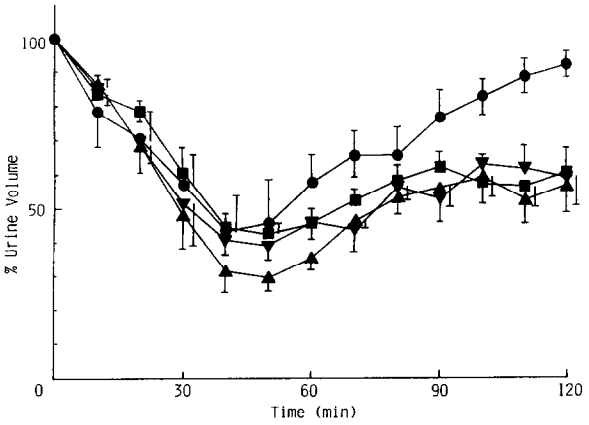

Fig. 4. Effects of concentration of camostat mesilate on changes in urine volume induced by iontophoresis (current intensity, 1 mA/3.14 cm^2; 2 h) following application of AVP (0.5 IU/rat, pH 7.0) to rat abdominal skin. [Camostat mesilate]: (●) 0 mM, (■) 1 mM, (▼) 10 mM, (▲) 50 mM. Each point represents the mean ± S.E. of four animals.

1-d-8-DAVP. The antidiuretic effects of 1-d-8-DAVP were prolonged compared with those of AVP.

Fig. 4 depicts the influence of the concentration (1–50 mM) of camostat mesilate on the antidiuretic effect after transdermal administration of AVP preparations (pH 7.0) with iontophoresis (constant current of 1 mA/3.14 cm^2) to rats. The antidiuretic effects determined in the presence of camostat mesilate at both 1 and 10 mM were similar and greater than that in its absence. A stronger antidiuretic effect was observed with camostat mesilate at 50 mM as compared with 1 and 10 mM.

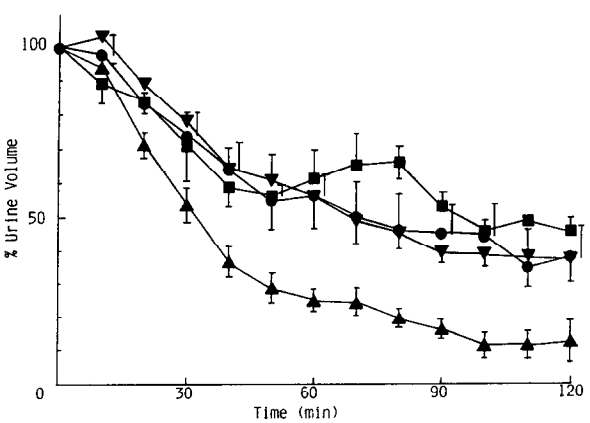

Fig. 3. Effects of proteolytic enzyme inhibitors on changes in urine volume induced by iontophoresis (current intensity, 1 mA/3.14 cm^2; 2 h) following application of 1-d-8-DAVP (1 μg/rat, pH 7.0) to rat abdominal skin. (●) Without proteolytic enzyme inhibitor, (▲) camostat mesilate (50 mM), (■) aprotinin (10000 KIU/ml), (▼) soybean trypsin inhibitor (6.25 mM). Each point represents the mean ± S.E. of four animals.

Fig. 5 illustrates the effect of the pH (pH 4.0, 5.5, 7.0 and 8.0) of preparations on the antidiuretic effect after transdermal administration of AVP in the presence or absence of camostat mesilate (10 mM) with iontophoresis (constant current of 1 mA/3.14 cm^2) to rats. The antidiuretic effect remained unaffected by the pH of AVP preparations irrespective of the presence of camostat mesilate.

The areas above the antidiuretic effect curves (AAC) in rats after application of AVP and 1-d-8-DAVP preparations containing camostat mesilate are summarized in Table 1. The $AACs_{0-2\,h}$ of AVP containing 10 and 50 mM camostat mesilate increased 1.4- and 1.5-fold, respectively, compared to that without camostat mesilate. The $AAC_{0-2\,h}$ of 1-d-8-DAVP increased 1.6-fold versus that in the absence of camostat mesilate.

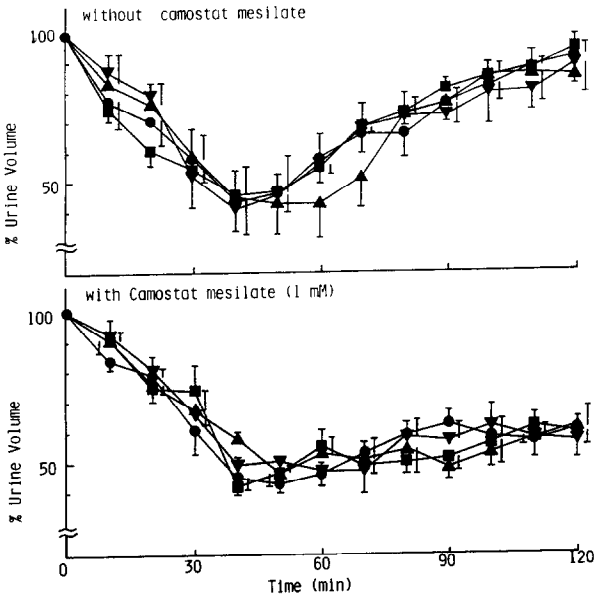

Fig. 5. Effects of pH of preparations on changes in urine volume induced by iontophoresis (current intensity, 1 mA/3.14 cm^2; 2 h) following application of AVP preparation with or without camostat mesilate (1 mM) to rat abdominal skin. pH of preparations: (■) 4.0, (▲) 5.5, (●) 7.0, (▼) 8.0. Each point represents the mean ± S.E. of four animals.

TABLE 1

Area above the antidiuretic response curve ($AAC_{0-2\ h}$) after application of AVP and 1-d-8-DAVP preparations containing camostat mesilate with iontophoresis (current intensity, 1.0 mA /3.14 cm^2; 2 h) to rat abdominal skin

	$AAC_{0-2\ h}$ (% urine volume h)
AVP (0.5 IU/rat)	
Without camostat mesilate	62.22 ± 6.070
With 10 mM camostat mesilate	85.24 ± 7.182 [a]
With 50 mM camostat mesilate	92.56 ± 5.056 [b]
1-d-8-DAVP (0.1 μg/rat)	
Without camostat mesilate	79.70 ± 11.455
With 50 mM camostat mesilate	124.92 ± 3.697 [b]

The AAC was calculated using the trapezoidal method. Each value represents the mean ± S.E. of four animals.

[a] Significantly different from value without camostat mesilate at $p < 0.05$.

[b] Significantly different from value without camostat mesilate at $p < 0.01$.

Peptidase activities

Fig. 6 depicts the effects of various proteolytic enzyme inhibitors on peptidase activities in rat

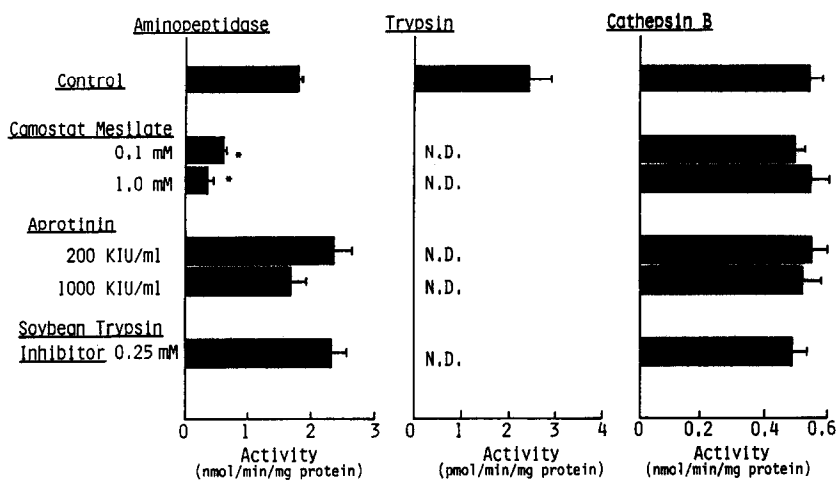

Fig. 6. Effects of various proteolytic enzyme inhibitors on peptidase activities in rat dermal tissue. Substrates used for in vitro evaluation of aminopeptidase, trypsin and cathepsin B activities were Leu-MCA, *t*-butyloxycarbonyl-Phe-Ser-Arg-MCA and carbobenzoxy-Phe-Arg-MCA, respectively. N.D., not detectable. Each value represents the mean ± S.E. of three experiments. Significant in comparison with control (* $p < 0.001$).

dermal tissue. The peptidase activities were found to conform to the following descending order: aminopeptidase > cathepsin B (carboxypeptidase) > trypsin. Camostat mesilate (0.1 and 1.0 mM) reduced both aminopeptidase and trypsin activities. Aprotinin (200 and 1000 KIU/ml) and soybean trypsin inhibitor (0.25 mM) reduced only trypsin activity.

Discussion

Polypeptides, which are hydrophilic and macromolecular compounds, do not permeate through normal skin. The stratum corneum of the epidermis is the main barrier limiting the passive transdermal diffusion of such compounds. Application of iontophoresis enhanced the skin permeation of peptide drugs such as insulin (Mol. Wt 6500), leuprolide (Mol. Wt 1200) and thyrotropin-releasing hormone (TRH; Mol. Wt 365) (Burnette and Marrero, 1986). In this study, AVP and 1-d-8-DAVP were not absorbed through rat abdominal skin. However, AVP and 1-d-8-DAVP were absorbed to a slight extent through the skin with application of iontophoresis (constant current of 1 mA/3.14 cm^2).

Activities of aminopeptidase, trypsin and cathepsin B were observed in rat dermal tissue. Zhou and Po (1990) showed that levels of leucine aminopeptidase activity in dermal, nasal, buccal and rectal tissues were equal when adjusted for protein content. Therefore, proteolytic enzymatic degradation would appear to be only one of the barriers to transdermal delivery of peptide. In the present study, the proteolytic enzyme inhibitor, camostat mesilate (1–50 mM), enhanced transdermal absorption of AVP with iontophoresis (constant current of 1 mA/3.14 cm^2) in rats. However, under identical conditions, aprotinin and soybean trypsin inhibitor had no effect on transdermal absorption in rats. Camostat mesilate, which is a relatively low molecular weight compound (Mol. Wt 495) and cationic within the range of pH values tested here, was absorbed to a small extent through rat abdominal skin (about 1.1%/2 h), and such absorption was enhanced on application together with iontophoresis (about 3.0%/2 h). Camostat mesilate inhibited the activities of aminopeptidase and trypsin in dermal tissue. However, aprotinin and soybean trypsin inhibitor could not be absorbed through rat abdominal skin even with iontophoresis (constant current of 1 mA/3.14 cm^2), since they have high molecular weights (Mol. Wt 6500 and 8000, respectively). The latter two resulted in inhibition only in the case of trypsin activity in epidermal tissue. Aminopeptidase activity was high compared with that of trypsin in rat dermal tissue. Therefore, camostat mesilate, which is able to permeate into the skin, might reduce the aminopeptidase activity in dermal tissue. As a result, camostat mesilate might enhance the transdermal absorption of AVP and of 1-d-8-DAVP with iontophoresis.

The pH of the AVP solution (pH 4.0–8.0) in either the presence or absence of camostat mesilate did not affect the transdermal absorption of AVP through iontophoresis (constant current of 1 mA/3.14 cm^2). Lelawongs et al. (1989) reported that the fluxes of AVP measured at pH 5.0 and 7.4 at constant ionic strength were comparable with those for in vitro monitoring of transdermal iontophoresis. This may result from the fact that greater than 99% of AVP is protonated in buffer solution of pH below 9.0, and may also be due to the relatively high isoelectric point of AVP (pI = 10.9) (Butt, 1975). On this basis of the aforementioned characteristics, it was suggested that the isoelectric point of keratin in the stratum corneum is around pH 3–4, and that the skin carries a negative charge, thereby acting as a cation-selective membrane favoring the permeation of protonated AVP (Burnette and Marrero, 1986).

In conclusion, transdermal absorption of AVP and 1-8-d-DAVP was enhanced with iontophoresis (constant current of 1 mA/3.14 cm^2) in rats. Furthermore, transdermal absorption of AVP and 1-8-d-DAVP was enhanced by the combined application of camostat mesilate and iontophoresis. The present results indicate that the application of camostat mesilate in combination with iontophoresis should provide a valuable tool in developing transdermal delivery systems for peptide drugs.

Acknowledgment

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