***In Vitro* Skin Penetration and Degradation of Enkephalin, Elcatonin and Insulin**

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The work described in this paper was designed to evaluate the relevance of *in vitro* skin penetration studies of peptides across rat skin. The apparent penetration of three peptides, enkephalin, elcatonin and insulin, in the presence of enhancers was not seen in the *in vitro* method using Franz diffusion cells. However, when a protease inhibitor was mixed in the receptor fluid, the penetration of enkephalin and insulin was observed. Although insulin penetrated in the presence of enhancers, the penetration was extremely small in quantity and the cumulative amount did not increase with time. When the degradation of peptides in the receptor fluid of Franz cell was estimated, these peptides, especially enkephalin and insulin, were rapidly hydrolyzed and were almost completely lost within 3 h in the absence of an inhibitor, while elcatonin was slowly degraded. The addition of protease inhibitors, such as gabexate (20 mM), camostat (20 mM) or bile salt (taurocholate and deoxycholate, 10 mM), to the receptor fluid inhibited the degradation to a considerable extent, with the first-order rate constants decreased to one-tenth compared with the constants without inhibitors. From the inhibitory study using specific inhibitors, it was clarified that enkephalin and elcatonin were mainly hydrolyzed by aminopeptidases, endopeptidases and serine proteases in the viable skin. Consequently, the results obtained from the *in vitro* penetration studies without inhibitors did not reflect reliable penetration data. Thus, effective protease inhibitor(s) should be used to obtain the data corresponding to the *in vivo* transdermal experiment. This methodology will provide a means to eliminate the confounding effect of metabolism in permeation experiments.

Key words peptide skin penetration; degradation; protease inhibitor; inhibition; degradation enzyme

Recent advances in biotechnology have made large numbers of bioactive peptides available, which may have therapeutic potential. However, peptide-based drugs cannot be effectively delivered by oral administration because of their enzymatic degradation in the gastrointestinal tract and the intrinsically low permeability of the gastrointestinal mucosal membrane to peptides. Various parenteral routes, such as rectal, pulmonary, nasal and transdermal routes, have thus been considered as alternative methods for the administration of peptides and proteins.

The transdermal delivery of peptides may offer an attractive possibility for several reasons. The main advantages of the transdermal route are the low proteolytic activity of the skin,¹⁾ the possibility to continuously deliver drugs with short half-lives and prolonged effects by continuous absorption.

Recent studies demonstrated the capability of some peptides across the skin, *e.g.*, thyrotropin-releasing hormone,²⁾ growth hormone-releasing factor (1–29)-NH₂,³⁾ leuprolide,⁴⁾ α -melanocyte-stimulating hormone,⁵⁾ vasopressin,⁶⁾ elcatonin,⁷⁾ ebitatide,⁸⁾ and hisetal (melanotropin (6–9)).^{9,10)} However, the amount which can be administered transdermally is quite low. To facilitate the transdermal transport of peptides, penetration enhancers and protease inhibitors have been used in many studies. On the other hand, to investigate the percutaneous absorption of peptides, *in vitro* percutaneous penetration studies have been carried out using animal skins and human epidermis. However, we have recently found that peptides were quickly degraded in the dermal side of the skin in the *in vitro* study.¹¹⁾

The present study was designed to evaluate the *in vitro*

percutaneous penetration of three peptides, leucine-enkephalin, elcatonin and insulin, and to clarify the enzymatic degradation of peptides during the *in vitro* percutaneous absorption experiment and the effect of protease inhibitors on the degradation. This study also described the kinetics of the degradation of these peptides in the dermal side of the skin and partially in skin homogenate, in the presence or absence of several enzyme inhibitors. We discussed the relationship between the *in vivo* absorption of peptides and *in vitro* penetration behaviors based on the results obtained.

MATERIALS AND METHODS

Materials Leucine-enkephalin (enkephalin, Tyr-Gly-Gly-Phe-Leu, M.W. 555.6), [Asu^{1–7}]-eel calcitonin (6400 U/mg), a synthetic analogue of eel calcitonin (elcatonin, M.W. 3364), and porcine insulin (26.0 IU/mg) were purchased from Peptide Institute, Inc. (Peptide Lab., Osaka), Asahi Kasei Co. (Tokyo, Japan) and Sigma Chemical Company (St. Louis, MO), respectively. Grazyme Insulin-EIA Test (Wako Pure Chemical Industries, Osaka, Japan), Carbpol 934 (Kishida Chemical Co., Osaka, Japan), *d*-limonene (Nacalai Tesque Co., Kyoto, Japan), *n*-octyl- β -D-thioglucoside (OTG, Wako Pure Chemical Industries), sodium taurocholate (Sigma Chemical Company) and sodium deoxycholate (Tokyo Kasei Industries, Tokyo, Japan) were used in this study. Gabexate mesilate and camostat mesilate were obtained from Ono Pharmaceutical Industry (Osaka, Japan) and soybean trypsin inhibitor (STI) was purchased from Sigma Chemical Company. Phenylmethylsulfonyl fluoride (PMSF), *p*-hydroxymercuribenzoate (PHMB) and 1,10-phenanthro-

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Table 1. Gel Formulations of Peptides

	Enkephalin			Elcatonin ^{a)}		Insulin ^{a)}	
	Rp. 1	2	3	4	5	6	7
Carbopol 934 (g)	2.0	2.0	2.0	7.0	7.0	7.0	7.0
Peptide (mg)	50.0	50.0	50.0	50.0	50.0	50.0	50.0
Ethanol (g)	—	—	—	—	—	—	30.0
Propylene glycol (g)	10.0	10.0	10.0	10.0	10.0	10.0	—
Gentamicin sol. (ml)	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Sod. taurocholate (g)	—	1.0	1.0	1.0	—	1.0	—
Sod. deoxycholate (g)	—	—	—	—	1.0	—	1.0
<i>d</i> -Limonene (g)	—	5.0	—	5.0	—	5.0	—
<i>l</i> -Menthol (g)	—	—	—	—	5.0	—	—
Cineol (g)	—	—	—	—	—	—	3.0
<i>n</i> -Octyl- β -D-thioglucoside (g)	—	—	1.5	—	1.5	1.5	1.5

a) The gel formulations were prepared by adding 0.1 M glycine-HCl buffer, pH 4.0, to give a total weight of 100.0 g.

line were purchased from Sigma Chemical Company and Kanto Chemical Co. (Tokyo, Japan), respectively. Dextran (mean molecular weight, 1000 Da) was a generous gift from Meito Sangyo Company (Nagoya, Japan). All other chemicals and solvents were of reagent grade or HPLC quality. Male Wistar rats, weighing 220–270 g, were used throughout this experiment. Wistar rats had free access to MF diet (Oriental Yeast, Tokyo) for 3–4 d prior to and during the experiments.

Preparation of Gel Formulations Carbopol 934 was swollen in water, followed by neutralization with 5% NaOH (gel base), or in 0.1 M glycine-HCl buffer, pH 4.0. A peptide, dissolved in cold water, was mixed with the gel base. An enhancer dissolved in propylene glycol or water (for bile salt) and gentamicin sulfate solution (10 mg/ml, Sigma Chemical Company) were added to the gel base in order. Details of the formulations are listed in Table 1.

In Vitro Percutaneous Penetration Experiment On the day before the experiment, the hair of the abdominal area of the rats was removed with an electric clipper and electric razor. On the next day, pieces (3 × 3 cm area) of full-thickness abdominal skin were excised from the animals. The adherent fat and other visceral debris were removed from the undersurface. The dermal side of the skin was soaked in a buffer solution (0.85% NaCl–10 mM phosphate buffer, pH 7.4) for 12 h at 5 °C to equilibrate the skin.¹²⁾ Then, 0.25 g of gel formulation was uniformly spread over the stratum corneum surface of the skin, which was mounted in a Franz diffusion cell (reservoir volume 10.5 ml; 1.6 cm i.d. O-ring flange) and occluded with a sheet of aluminum foil. Gentamicin sulfate solution (10 mg/ml) was added to the receptor fluid at a ratio of 1:100. Gabexate mesilate for enkephalin and insulin penetration or camostat mesilate for elcatonin penetration was mixed with the receptor fluid at the 20 mM concentration, but in some experiments, the inhibitor was not added. The diffusion cell was thermoregulated with a water jacket at 37 °C. Aliquots (400 μ l) of the receptor fluid (0.85% NaCl–10 mM phosphate buffer, pH 7.4) were withdrawn periodically for 24 h, then immediately frozen. The sample was lyophilized immediately before assay. In the experiment on dextran penetration, 0.5% dextran and/or 1.5% OTG dissolved in the buffer solution were used as the donor solution.

Degradation Experiment in Receptor Fluid The abdominal skin of rat was mounted in a Franz diffusion cell by the same procedure as mentioned in the *in vitro* penetration experiment. Enkephalin (10 μ g/ml), elcatonin (20 μ g/ml) or insulin (12 ng/ml) and gabexate mesilate (10 or 20 mM), camostat mesilate (20 mM), STI (2.5×10^{-2} mM), sodium deoxycholate (10 mM) or sodium taurocholate (10 mM) were dissolved in a receptor fluid containing gentamicin solution (10 mg/ml) at indicated concentrations. The diffusion cell was thermoregulated with a water jacket at 37 °C. Aliquots (50 μ l) of receptor fluid were withdrawn periodically. The samples were stored frozen until the time of assay. The inhibitor in the Franz diffusion cell was used at the concentration at which the hydrolytic enzymes were generally inhibited.¹³⁾

In a separate experiment, PMSF (5 mM), PHMB (1 mM) or phenanthroline (0.5 mM) was added to the receptor fluid and the degradation of enkephalin and elcatonin in the receptor cell was measured at 37 °C to clarify the type of enzyme involved in the degradation.

Degradation Experiment in Skin Homogenate Full-thickness abdominal skin was prepared as described above. The skin (3 × 3 cm area) was homogenized in 9.0 ml of ice-cold 0.85% NaCl–10 mM phosphate buffer, pH 7.4, for 2 min using a microhomogenizer (Phycostron NS-10, Nichion, Tokyo). To the homogenate (2.0 ml) containing gentamicin solution (10 mg/ml), 0.85% NaCl–phosphate buffer, pH 7.4, gabexate mesilate (20 mM) dissolved in the buffer or 0.1 M glycine buffer, pH 4.0, (2.0 ml each) was added. The insulin solution (1 μ g/ml, 200 μ l) was mixed with the homogenate mixture and incubated at 37 °C for 3 h. Aliquots (100 μ l) of sample were withdrawn periodically, followed by centrifugation. The supernatant was stored frozen until the time of the assay.

Determination of Peptides and Dextran Enkephalin and elcatonin in the sample solution were determined by the HPLC method. A lyophilized sample was dissolved in 100 μ l of the internal standard solution (5-(4-hydroxyphenyl)-5-phenylhydantoin (5 μ g/ml) for enkephalin; verapamil hydrochloride (10 μ g/ml) for elcatonin). Following centrifugation, the supernatant was filtered through a membrane filter (0.45 μ m HLC Disk 3, Kanto Chemical Co.). For the non-lyophilized samples, 50 μ l of internal standard solution was mixed with the sample solution

(50 μ l) and the mixture was filtrated. For the determination of enkephalin and elcatonin, 30–50 μ l of the filtrate was injected onto a reversed-phase Inertsil ODS (octadecyl silica) column (4.6 $\times$ 150 mm, 5 μ m, Nacalai Tesque, Inc.). The mobile phases for the determination of enkephalin and elcatonin were acetonitrile–0.05 M KH_2PO_4 (pH 3.0) (25:75, v/v) and acetonitrile– H_2O –60% HClO_4 (40:60:0.16, v/v), respectively. The detection was at 214 nm and 200 nm, respectively. Insulin in the sample was determined using an Insulin immunoassay kit (Grazyme Insulin-EIA Test). The sensitivity of the methods (*in vitro* percutaneous penetration experiment) was 0.2 $\mu\text{g}/\text{ml}$ for enkephalin, 1 $\mu\text{g}/\text{ml}$ for elcatonin and 2 $\mu\text{U}/\text{ml}$ for insulin. Since the concentrated solution (4 times), as mentioned above, was used for the determination, the detection limit could be lower than these values. The concentrations of dextran were determined by the previous method.¹⁴⁾

Pharmacokinetic and Statistical Analyses The *in vitro* percutaneous penetration parameters were approximately calculated from the penetration data using the following equations:

$$D = \frac{\delta^2}{6\tau}$$

$$J_s = \frac{D \cdot k_m \cdot C_s}{\delta} = k_p \cdot C_s$$

where J_s is the penetration rate, k_m denotes the skin/vehicle partition coefficient of peptide, D is the diffusion constant within skin, τ represents the lag time and δ is the thickness (0.002 cm) of the stratum corneum, k_p denotes the permeability coefficient through the stratum corneum, and C_s is the peptide concentration in the gel formulation.

The degradation rate constant (k) was calculated from the degradation data using the following equation:

$$k = \frac{2.303}{t} \log \frac{a}{a-x}$$

where a is the initial amount of peptide and x is the amount degraded in time, t .

The means of all data are presented with their standard deviation (mean $\pm$ S.D.). Statistical analysis was performed

using the non-paired Student's t -test, and a p value of 0.05 or less was considered to be significant.

RESULTS

In Vitro Penetration of Enkephalin, Elcatonin and Insulin

When the *in vitro* skin penetration study of these peptides was carried out without protease inhibitors in the receptor fluid, no amount of peptides was detected in the fluid. A possible explanation for the negligible penetration may be the rapid degradation of peptides in the viable epidermis and the receptor fluid. Therefore, a protease inhibitor was added to the receptor fluid to prevent the degradation. The penetration of enkephalin through rat skin is shown in Fig. 1A. An appreciable amount of enkephalin penetrated through the skin without enhancers (Rp. 1) in the presence of 20 mM gabexate in the receptor fluid, but the addition of enhancers, d -limonene or OTG and taurocholate (Rp. 2 and 3), significantly enhanced the penetration of the peptide under the same conditions. A steady state flux was attained after about 1 h and was maintained for several hours (Table 2). Consequently, the apparent penetration rate (J_s , 5.29 ± 1.99 and 3.16 ± 0.11 $\mu\text{g cm}^2/\text{h}$ for Rp. 2 and 3, respectively) was significantly higher than that (1.19 ± 0.43 $\mu\text{g cm}^2/\text{h}$) of Rp. 1 ($p < 0.05$). The short lag time (within 1.5 h) for the penetration of enkephalin indicates that the transport of the peptide would be predominantly *via* shunt routes, as suggested by Scheuplein¹⁵⁾ and by us.¹⁴⁾ Since the peptides were rapidly degraded in viable skin and the receptor fluid, net

Table 2. *In Vitro* Skin Penetration Parameters for Enkephalin through Rat Skin

	Rp. 1	Rp. 2	Rp. 3
τ (h)	1.54 ± 1.00	$1.08 \pm 1.10^a)$	$0.64 \pm 0.60^a)$
J_s ($\mu\text{g cm}^2/\text{h}$)	1.19 ± 0.43	$5.29 \pm 1.99^b)$	$3.16 \pm 0.11^c)$
D ($\times 10^{-4}$ cm^2/h)	9.44 ± 9.36	$29.2 \pm 38.7^a)$	$121.9 \pm 194.2^b)$
K_p ($\times 10^{-3}$ cm/h)	2.37 ± 0.86	$10.6 \pm 3.99^b)$	$6.31 \pm 0.22^c)$
K_m	0.39 ± 0.29	$1.34 \pm 1.57^a)$	$0.34 \pm 0.31^a)$

Each value represents the mean $\pm$ S.D. ($n=3-5$). a) $p > 0.05$, b) $p < 0.005$ and c) $p < 0.001$, respectively, compared with Rp. 1.

Fig. 1. Penetration Profiles of Enkephalin (A) and Insulin (B) through Rat Skin after Application of Various Formulations

Each point represents the mean $\pm$ S.D. ($n=3-5$). Rp. 1 (○), Rp. 2 (△), Rp. 3 (□), Rp. 6 (▲), Rp. 7 (■). Gabexate mesilate was added into the receptor fluid at the 20 mM concentration.

parameters will be obtained by considering the metabolic degradation in addition to the apparent permeability. However, the exact metabolic rate of each peptide is unclear at the present time.

Elcatonin penetrated from the formulations (Rp. 4 and 5) into the receptor fluid was not detected by the HPLC method used, even in the presence of enhancers (data not shown).

The *in vitro* penetration study of insulin was also estimated in the presence of the enhancers, *d*-limonene, cineol, OTG and/or taurocholate. Percutaneous penetration of insulin was observed, but was small in quantity, as shown in Fig. 1B. Insulin (25 and 18 $\mu\text{U}/\text{cm}^2$) was detected 1 h after application, suggesting rapid penetration through the skin in the presence of enhancers. However, the cumulative amount of insulin which penetrated did not significantly increase with time. This indicates that there might be rapid degradation, even in the presence of 20 mM gabexate. No penetration of insulin in the absence of enhancers was seen in this experiment.

Degradation of Peptides in Receptor Fluid Since viable skin possesses enzyme activities¹⁶⁾ and cutaneous metabolism may influence the percutaneous fate of topically applied peptides in the skin, enzyme inhibition is important for determining the practical permeability of peptides. The stability and degradation of peptides added to the receptor fluid of the Franz diffusion cell were estimated in this study. Enkephalin was stable in the buffer (pH 7.4) at 37°C, however the peptide in the receptor fluid which contacts the dermal side of skin was rapidly degraded, as depicted in Fig. 2, and it was decayed almost completely

within 3 h. The presence of gabexate mesilate (20 mM) partly protected the degradation. These declines in peptides could be approximately described as a single first-order function. The apparent degradation rate constants (k) calculated are shown in Table 3. The k value of enkephalin in the receptor fluid was $1.778 \pm 0.090 \text{ h}^{-1}$, indicating the rapid degradation of the peptide compared with that of elcatonin.

Of particular interest is that elcatonin was more slowly degraded in the receptor fluid than enkephalin (Fig. 2B). The slow degradation ($k = 0.075 \pm 0.034 \text{ h}^{-1}$) is ascribed to the synthetic analogue of eel calcitonin in which the disulfide bridge between the first and seventh amino acids is replaced by a $-\text{CH}_2\text{CH}_2-$ bridge.¹⁷⁾ The synthetic analogue therefore would have considerable resistance to enzymatic degradation conformationally. Camostat mesilate (20 mM) inhibited the degradation to a considerable extent. Taurocholate and deoxycholate (10 mM each) partly inhibited the degradation (Fig. 2B), as shown by the k values (0.024 and 0.020 h^{-1} , respectively).

The degradation of insulin in receptor fluid was measured for 3 h. The results are shown in Fig. 2C. Insulin was also unstable in the receptor fluid, and 3 h after incubation the activity declined to 10% of the original. STI at the concentration ($2.5 \times 10^{-2} \text{ mM}$) used failed to stabilize insulin. Ten mM gabexate mesilate and 10 mM taurocholate inhibited the degradation to a considerable extent. The degradation rate constants for insulin are shown in Table 3. The k value in the receptor fluid without inhibitor was $0.899 \pm 0.011 \text{ h}^{-1}$, but the value was decreased to one-fourth in the presence of taurocholate

Fig. 2. Degradation Profiles of Enkephalin (A), Elcatonin (B) and Insulin (C) in Receptor Fluid

Each point represents the mean $\pm$ S.D. ($n=3-5$). 0.85% NaCl-phosphate buffer, pH 7.4, without skin ($\square$), 20 mM gabexate (for enkephalin) or 20 mM camostat (for elcatonin) with skin ($\circ$), 10 mM gabexate with skin ($\blacksquare$), 10 mM taurocholate with skin ($\bullet$), 10 mM deoxycholate with skin ($\blacktriangle$), $2.5 \times 10^{-2} \text{ mM}$ STI with skin ($\diamond$), the buffer with skin ($\triangle$).

Table 3. Degradation Rate Constants for Enkephalin (Enk), Elcatonin and Insulin in Receptor Fluid with or without Inhibitor

	Rate constant, k (h^{-1})		
	Enkephalin	Elcatonin	Insulin
Without inhibitor (control)	1.778 ± 0.090	0.075 ± 0.028	0.899 ± 0.011
10 mM (insulin) or 20 mM (Enk) gabexate mesilate (pH 7.4)	0.130 ± 0.009^a	—	0.286 ± 0.054^b
20 mM camostat mesilate (pH 7.4)	—	0.008 ± 0.001^a	—
10 mM taurocholate (pH 7.4)	—	0.024 ± 0.005^a	0.235 ± 0.051^b
10 mM deoxycholate (pH 7.4)	—	0.020 ± 0.001^a	—
$2.5 \times 10^{-2} \text{ mM}$ Soybean trypsin inhibitor (pH 7.4)	—	—	1.092 ± 0.115

Each value represents the mean $\pm$ S.D. ($n=3-4$). $a) p < 0.01$ and $b) p < 0.001$, respectively, compared with the corresponding control.

(10 mM).

The degradation of insulin in skin homogenates was compared with that in the receptor fluid. In homogenates, the peptide biphasically declined over the period measured. Initially, the degradation was rapid and was slow over the period from 30 min to 2 h (Fig. 3). The degradation rate constant was calculated from the initial decay curve. The hormone rapidly degraded in the homogenates with an order of magnitude increase in the degradation rate constant ($9.04 \pm 0.82 \text{ h}^{-1}$) in the absence of a protease inhibitor, compared with that in the receptor fluid (Fig. 2C). Gabexate mesilate (10 mM) partly inhibited the degradation ($k = 7.10 \pm 1.46 \text{ h}^{-1}$). A low pH (4.0) also inhibited the decomposition, though not to a large extent ($k = 5.50 \pm 0.63 \text{ h}^{-1}$) ($p < 0.01$).

Type of Enzyme Involved in the Degradation Phenanthroline is an inhibitor of metalloenzymes, which include aminopeptidases and endopeptidases. PHMB is an inhibitor of cysteine proteases and PMSF is an inhibitor of serine proteases including trypsin and chymotrypsin.¹⁸⁾ To clarify the type of enzyme which is involved in the degradation of peptides in viable skin, the three kinds of inhibitors mentioned above were used at concentrations responsible for the inhibition¹⁸⁾ in this inhibitory experiment.

Fig. 3. Degradation Profiles of Insulin in Skin Homogenate

Each point represents the mean $\pm$ S.D. ($n=3$). 0.85% NaCl-phosphate buffer, pH 7.4, without inhibitor (Δ), 10 mM gabexate, pH 7.4 ($\bullet$), glycine-HCl buffer, pH 4.0 ($\blacktriangle$).

Fig. 4. Degradation Profiles of Enkephalin (A) and Elcatonin (B) in Receptor Fluid in the Presence of Specific Inhibitors

Each point represents the mean $\pm$ S.D. ($n=3-4$). 0.85% NaCl-phosphate buffer, pH 7.4 (Δ), 5 mM PMSF ($\circ$), 1 mM PHMB ($\square$), 0.5 mM phenanthroline ($\blacksquare$).

Figure 4 shows the results of the enkephalin and elcatonin degradation studies in the receptor fluid in the presence or absence of phenanthroline, PHMB or PMSF. The degradation rates also followed first-order kinetics. PMSF strongly inhibited the degradation of both enkephalin and elcatonin. Phenanthroline also showed a considerable inhibitory effect. The inhibitory effect of PHMB was generally less than that of the two inhibitors. The rate constants are depicted in Table 4.

These results indicate that enkephalin and elcatonin were mainly hydrolyzed by aminopeptidases, endopeptidases and serine proteases in the viable skin, and that these peptidases dissolved from the viable skin into the receptor fluid. The reason PHMB had less inhibitory effect may be that the reagent is an inhibitor of cysteine proteases, which include cytoplasmic and lysosomal proteases,¹⁸⁾ because these enzyme activities will fully exert in the skin homogenates. Since each of these inhibitors shows specificity for a different type of enzyme, the differences in their effectiveness probably reflect differences in the metabolic activity of the corresponding enzymes.

DISCUSSION

To investigate the percutaneous absorption of peptides, *in vitro* penetration studies have widely been used. However, we have recently found that peptides were rapidly degraded in the dermal side of rat skin.¹¹⁾ To clarify the hydrolysis of peptides in the dermal side of skin, we have studied the *in vitro* degradation of enkephalin, elcatonin and insulin using a Franz diffusion

Table 4. Degradation Rate Constants for Enkephalin and Elcatonin in Receptor Fluid in the Presence of Specific Inhibitors

	Rate constant, k (h^{-1})	
	Enkephalin	Elcatonin
Without inhibitor (control)	1.778 ± 0.090	0.075 ± 0.028
PMSF (5 mM)	0.038 ± 0.004^a	0.017 ± 0.007^a
PHMB (1 mM)	0.890 ± 0.199	0.030 ± 0.003^a
Phenanthroline (0.5 mM)	0.145 ± 0.020^a	0.020 ± 0.008^a

Each value represents the mean $\pm$ S.D. ($n=3-4$). a) $p < 0.01$ compared with the corresponding control.

cell in the presence or absence of an inhibitor. No penetration was detected in the absence of an inhibitor in the *in vitro* study, suggesting the rapid degradation of these peptides. The rapid degradation of these peptides in the receptor fluid was mainly due to the hydrolysis by the peptidases contained in the viable skin, although a considerable part of the degradation would be due to the enzymes being dissolved into the receptor fluid, because the peptides which penetrated came into contact with the peptidases for a comparatively long time. The penetration of enkephalin and insulin through rat skin was observed only by the addition of an inhibitor, gabexate (20 mM), with a short lag time (within 1.5 h).

It seems likely that the dominant path for peptides is in the polar regions of intercellular routes, which were opened based on the extensive extraction of intercellular lipid-rich materials by *d*-limonene, and in the stratum corneum, of which the barrier functions are extensively decreased due to exfoliation of cell membranes by OTG, as demonstrated by our previous study.^{15,19)}

These data demonstrated that the peptides penetrated through animal skin and human skin would probably be rapidly degraded during the experiment using the Franz diffusion cell. Consequently, the results obtained from the *in vitro* penetration studies without inhibitors did not reflect the actual penetration data. Additionally, it is clear that either the addition of potent inhibitor(s) to the receptor fluid of the cell or the heat inactivation of peptidases is necessary for the *in vitro* penetration study. Our results also proved that the inhibitors indirectly and apparently enhanced the penetration of peptides through skin. As a result, the data that protease inhibitors may promote the percutaneous and subcutaneous absorption of peptides²⁰⁾ must be reexamined, because the inhibitors inhibit the *in vitro* degradation of peptides and stabilize them, thus apparently enhancing the permeation.

The penetration and degradation studies using skin pretreated by heat to inhibit these peptidases are underway.

It is shown that enkephalins are rapidly inactivated by aminopeptidase, enkephalinase, dipeptidase and carboxypeptidase,²¹⁾ and that the former two are the major enzymes which are responsible for up to 90% of the degradation.^{21,22)} Our result approximately agreed with these data. Consequently, it is reasonable to assume that in the *in vitro* penetration studies of peptides, peptides are susceptible to extensive enzymatic hydrolysis by many peptidases in viable skin. Therefore, abolishing degradation in the receptor fluid is necessary for determining the net permeation of peptides. Additionally, this methodology will provide the means to eliminate the confounding effect of metabolism in permeation experiments so that the net skin permeability of peptides may be determined.

Gabexate and camostat at the concentration of 20 mM in the receptor fluid did not influence the penetration of dextran (mean molecular weight, 1000 Da) in the presence of 1.5% OTG (data not shown). Therefore, it is assumed that these inhibitors would not affect the penetration rate of these peptides with or without inhibitors.

The *in vitro* penetration data of elcatonin and insulin were compared with the *in vivo* absorption data studied

previously.^{7,8,23)} The plasma calcium concentrations decreased rapidly and the lowered levels were maintained for at least 24 h after application of the elcatonin transdermal system, giving relatively high pharmacological availabilities (4–8.7%). However, in this *in vitro* penetration experiment, no elcatonin levels were detected in the receptor fluid in the Franz diffusion cell. The amount of peptides absorbed would thus not be directly related to the pharmacological effect. However, one possible explanation for the discrepancy between the *in vivo* and *in vitro* data is given by the rapid degradation of the peptide which penetrated into the receptor fluid based on the proteolytic enzymes, as well as the relatively low sensitivity of the HPLC method compared with the pharmacological response.

An extremely small amount of insulin could penetrate through rat skin, and the cumulative amount which penetrated did not significantly increase over the 8 h postdose periods (Fig. 1). This may also be explained by the fact that insulin was rapidly degraded by the peptidases in viable skin and in the receptor fluid during the penetration study at a rate compatible to the penetration rate. Our previous study showed that insulin applied to rat abdominal skin induced a strong hypoglycemic response over a 10 h period.²³⁾ Judging from the *in vivo* absorption, the amount of insulin which penetrated *in vitro* seems to be too small to exert the hypoglycemic effect. The amount which penetrated would thus be more than that detected in the *in vitro* penetration study.

The exact amount (D_T) of peptide which permeated through skin was calculated as follows:

$$D_T = D_p + D_s + D_r$$

where D_p is the amount of peptide detected in receptor fluid, D_s denotes the amount degraded in skin, and D_r is the amount degraded in the receptor fluid. Thus, D_p was calculated by the next equation:

$$D_p = D_T(1 - E)$$

where E is the degradation ratio in skin and receptor fluid. If E becomes extremely lessened by the inhibition of enzymes, the exact amount of peptide which penetrated can be approximately drawn from D_p . Thus, when suitable inhibitors are added to the receptor fluid of the Franz diffusion cell, the proteolytic degradation of peptides can probably be ignored. It is suggested that camostat mesilate permeated into the nasal mucosa and inhibited the activities of aminopeptidases and trypsin.¹³⁾ In the skin permeation study, although the direct evidence that these inhibitors penetrated into the viable epidermis and dermis was not obtained, it appears reasonable to expect the permeation of hydrophilic inhibitors into the viable tissues from the receptor fluid, leading to the inhibition of the enzymes there, since proteolytic enzymes are present in viable skin.¹⁶⁾

In conclusion, enkephalin and insulin penetrated through rat skin from gel formulations including the enhancers, such as *d*-limonene, OTG or taurocholate. However, elcatonin penetration could not be detected by the HPLC method. Enkephalin and insulin were rapidly degraded in the receptor fluid facing the viable skin, while

the degradation of elcatonin was relatively slow under the same conditions. These peptides were partly or effectively stabilized in the presence of inhibitors (gabexate, camostat, taurocholate and deoxycholate). From the inhibitory experiment, it was clarified that the enzymes involved in the degradation of enkephalin and elcatonin were mainly aminopeptidases, endopeptidases and serine proteases. In the *in vitro* percutaneous penetration study, the effective protease inhibitors should be used to obtain the actual penetration data. Together with information on the permeability characteristics of peptides, this information would facilitate the understanding of the basis for improvement in peptide and protein delivery *via* a percutaneous route.

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