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Research Article

Transdermal Delivery of Bioactive Peptides: The Effect of *n*-Decylmethyl Sulfoxide, pH, and Inhibitors on Enkephalin Metabolism and Transport

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We investigated the effects of the nonionic surfactant, *n*-decylmethyl sulfoxide (NDMS), pH, and inhibitors on the metabolism and the permeation of amino acids, dipeptides, and the pentapeptide enkephalin, through hairless mouse skin. An HPLC gradient method was developed to identify the possible peptide and amino acid metabolites of leucine-enkephalin. NDMS increased the permeability of all amino acids and peptides tested. At neural pH, the enzyme activity within the skin was such that no flux of leucine-enkephalin (YGGFL) was observed and the donor cell concentration of YGGFL decreased rapidly. The major cleavage occurred at the Tyr-Gly bond. At pH 5.0 the metabolic activity was reduced significantly and a substantial flux of YGGFL was observed. Enzymatically stable YGGFL analogues, Tyr-D-Ala-Gly-Phe-Leu (YDAGFL) and its amide, exhibited significant fluxes even at neutral pH in the presence of NDMS, but with substantial metabolism. YDAGFL amide was more stable to metabolism than YDAGFL. The rates of metabolism of the peptides in the skin homogenates were in the order: FL >> YGGFL > GFL > GGFL >> YG, YGG >> YDAGFL amide. In the skin homogenates puromycin and amastatin showed the highest inhibitory effects, while FL and GFL were only slightly active. However, in the skin diffusion experiments, FL allowed the highest amount of intact parent compound to permeate, making it the most potent inhibitor. These results show that the complex proteolytic enzyme activities occurring during skin permeation are different from those in skin homogenates and that a combination of enhancer, pH adjustment, and inhibitors can increase the transdermal delivery of peptides.

KEY WORDS: penetration enhancer; *n*-decylmethyl sulfoxide; transdermal delivery; bioactive peptide; enkephalin; metabolism.

INTRODUCTION

Peptides with potent pharmacological activities will likely be significant drugs of the next century (1–3). Rapid metabolism in the gut lumen, generally low permeabilities, and low aqueous solubilities often present significant barriers to oral delivery (4). Chemical modification has been used to solve some of these problems of oral delivery (5). Ocular and nasal delivery routes appear promising alternatives for peptide delivery (1). However, enzymatic activity was identified in both tissues. Intravaginal, intracheal, and rectal deliveries also have been considered as routes for peptide administration (2). The transdermal route has been thought to have little proteolytic enzyme activity (6) and thus may avoid delivery problems with bioactive peptides. However, because of the general impermeability of the skin and the large molecular size of peptides and their ionic character, a means of increasing permeation must be found. One approach employs iontophoresis as an active driving force for the charged molecules (7,8). Another approach involves the use of chemical enhancers (3,9–11). However, little is known about the proteolytic enzyme activity in the skin and its possible influences on mass transfer even though other enzymes of the skin have been relatively well characterized (12–14).

We previously showed that *n*-decylmethyl sulfoxide increased the permeabilities of several compounds up to 0.1 cm/hr (15). Here the effects of *n*-decylmethyl sulfoxide, pH, and various enzyme inhibitors on the metabolism and permeation of some select peptides are reported. The opioid peptide, Leu-enkephalin (YGGFL),[4] and its analogues were chosen as a model.

MATERIALS AND METHODS

Materials

n-Decylmethyl sulfoxide was obtained from Columbia Organic Chemical Co. (Camden, Sc 29020) in a technical grade. It was purified by recrystallization from hexane. Its

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[4] Y, tyrosine; F, phenylalanine; A, alanine; G, glycine; L, leucine.

purity was checked by differential scanning calorimetry (Mettler FP80, Mettler Inst. Co., NJ). L-Tyrosine, L-phenylalanine, L-phenylalanyl-L-phenylalanine, Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu), des-Tyr-Leu-enkephalin, and all other possible metabolites from Leu-enkephalin, D-alanyl^2-Leu-enkephalin (Tyr-D-Ala-Gly-Phe-Leu) and its amide, amastatin, and puromycin were purchased from Sigma Chemical Co. (St. Louis, MO) and used as received. Iodine^{125}-Leu-enkephalin (sp act, 785Ci/mmol) was purchased from Peninsula Laboratories Inc. (Belmont, CA 94002). All other chemicals were reagent or comparable grade.

Diffusion Experiments

Diffusion Cell. Small side-by-side diffusion cells consisting of two water-jacketed cylindrical half-cells, each having a volume of 2.8 ml and a diffusion area of 0.636 cm^2, were employed. A constant temperature was maintained in the half cells by circulating 37°C water. The contents were stirred by externally driven, Teflon-coated magnetic bars to keep them well mixed.

Skin Preparation. Full-thickness hairless mouse skin was excised from the fresh carcasses of animals humanely terminated with carbon dioxide. Subcutaneous fat was removed carefully with scissors and scalpel and the skin was immersed in a buffered solution at 37°C until mounting in a diffusion cell. Since dorsal and abdominal skin sections proved to be equivalent in flux, they were used without discrimination, although they were used alternatively in the same group of experiments. After mounting a skin section, excess skin hanging out around the edges of the cell was trimmed off.

Procedure. The receiver side of the cell, defined as the side facing the dermis, was filled with an appropriate buffer solutions (phosphate or phosphate-citrate, 50 m*M*). A certain composition of test solution was placed in the donor half-cell according to the specific study protocol. Both the buffer and the test solutions were kept at 37°C before placing them into the cells. Depending on the study protocol and the permeability from the test solutions, samples of appropriate size were withdrawn from the receiver compartment at predetermined time intervals and were replaced with an equal volume of temperature-equilibrated buffer.

Skin Homogenate Experiments

After removing the fatty residues adhering to the underside of full-thickness hairless mouse skin, the skin was cut into small pieces. Homogenates, 3 or 5%, were made with phosphate-citrate buffer (50 m*M*, pH 7.0) by homogenizing ca. 1 g of skin three times, each for 1 min with 1-min cooling intervals, using a tissue homogenizer (KINEMATICA GmbH, Swiss). The homogenates were centrifuged for 5 min at 3000 *g* and 4°C and the supernatant liquid was recovered and centrifuged for 30 min at 20,000 *g* and 4°C. Again the supernatant was collected. It was kept in ice bath and used within 1 hr. Both the homogenate medium and the drug solution were placed in a 37°C water bath for equilibrium before incubation. Five-tenths milliliter of the homogenate medium was mixed with 6 ml of the respective drug solution and the mixture was incubated at 37°C. Five-tenths milliliter of the mixture was taken out at predetermined time intervals and placed into vials containing 0.1 ml of 1 *N* HCl. In the *in vitro* inhibition study, 100 μ*M* inhibitors were added to 20 μmol of the Leu-enkephalin solution with 0.25% skin homogenate. After incubation for 7 min at 37°C, pH 7.0, the fraction of Leu-enkephalin left was evaluated. For the inhibition study involving skin diffusion, an appropriate amount of inhibitor was added to the donor cell together with 200 μg/ml of Leu-enkephalin in a medium containing 10 m*M* *n*-decylmethyl sulfoxide. Amastatin (300 μ*M*), puromycin (1 m*M*), Tyr-Gly-Gly (20 m*M*), and 1 mmol of the various metabolites of Leu-enkephalin were tested. Residual substrate was analyzed by HPLC.

Assay Methods

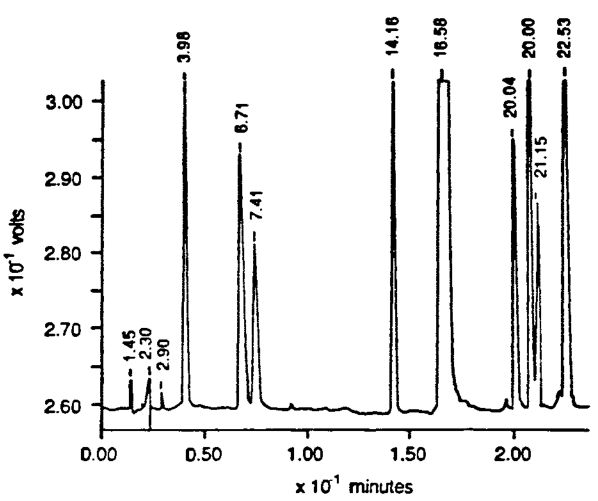
Amino acids and peptides were analyzed on an HPLC system consisting of a WISP automatic injector (Millipore, Milford, MA), a Waters Model 510 (Millipore, Milford, MA), or a Spectroflow 400 (Kratos Analytical Instruments, Ramsey, NJ) high-performance pump and either a Waters Model 481 or a Spectroflow 783 (Kratos Analytical Instruments) variable-wavelength uv detector, depending on the particular study. Waters Baseline 810 software was used to control the gradient system and to analyze the data. Leucine-enkephalin and its metabolites and analogues were determined using ion-pair chromatography with a 5-μm, 15-cm reversed-phase column (Ultrasphere-Octyl, ALTEX). For isocratic analysis of Phe-Leu, Gly-Phe-Leu, Gly-Gly-Phe-Leu, Leu-enkephalin, and its corresponding commercially available analogues, mobile phases were comprised of 50 m*M* phosphate buffer (pH 3.0) and acetonitrile (76:24), and 0.1% heptanesulfonic acid sodium salt was used as a counter ion. For analysis of all possible combinations of metabolites of Leu-enkephalin, stepwise gradient elution was performed. A sample chromatogram and stepwise gradient are shown in Fig. 1. ^{125}I-Leu-enkephalin was analyzed by gamma counter (MINAXI-GAMMA 5650, Packard Instruments Co., Inc., Downers Grove, IL 60515). Lower and upper limits of the first channel were set at 15 and 84 keV, respectively, to count the total of photo peak and sum peak. Those of second channel were set at 15 and 44 keV, respectively, to count only the photo peak. Disintegration per minute (dpm) was calculated as follows:

$$\text{dpm} = \frac{\text{cpm in channel A}}{\%\ \text{efficiency}} \times 100$$

Five-tenths milliliter of sample was mixed with 3 ml of scintillation fluid before counting. Background was subtracted from the data before converting them from cpm to dpm.

RESULTS AND DISCUSSION

The initial studies on the potential transdermal transport of bioactive peptides showed increased permeability of two amino acids, tyrosine and phenylalanine, and the dipeptide, Phe-Phe, by *n*-decylmethyl sulfoxide. Since it was shown that *n*-decylmethyl sulfoxide exhibited similar effects on permeability at concentrations between 3 and 25 m*M* regardless of the physicochemical properties of the compound in ques-



Stepwise Linear Gradient Table

Event#	Time (min)	Flow (ml/min)	%A	%B
1	0.0	1.2	94	6
2	4.0	1.2	94	6
3	8.0	1.2	82	18
4	12.0	1.2	82	18
5	15.0	1.2	74	26
6	20.0	1.2	74	26
7	24.0	1.2	69	31
8	25.0	1.2	69	31
9	26.0	1.2	94	6

Solvent A: 50 mM phosphate buffer (pH = 3) Solvent B: acetonitrile

3.98: Tyr 6.71: Tyr-Gly-Gly 7.41: Tyr-Gly 7.61: Phe
14.16 Gly-Phe and Gly-Gly-Phe 16.58: Tyr-Gly-gly-Phe
20.04: Phe-Leu 20.80: Gly-Gly-Phe-Leu 21.15: Gly-Phe-Leu
22.53: Tyr-Gly-Gly-Phe-Leu

Fig. 1. Elution profile and mobile phase gradient table for HPLC analysis of Leu-enkephalin and its metabolites on a 5-μm-particle size Ultrasphere-Octyl column. The uv detector was used at 210 nm. The peak for Phe is not shown in this chromatogram.

tion (15), a 10 m*M* concentration was usually used in the present diffusion studies.

Figure 2 shows the effect of *n*-decylmethyl sulfoxide on the flux of ^{125}I-Leu-enkephalin at pH 6.5. There was no substantial flux of Leu-enkephalin or radioactive metabolites until 12 hr of diffusion. Clearly, *n*-decylmethyl sulfoxide substantially increases permeation, especially at shorter times. However, HPLC assay detected an appreciable amount of tyrosine and no parent compound in the receiver cell at pH 6.5. At pH 3.5[5] little tyrosine and a significant amount of parent compound were detected. The fraction of Leu-enkephalin in the donor cell dropped more rapidly than that of ^{125}I-Leu-enkephalin at pH 6.5 and cold Leu-enkephalin at pH 3.5. Obviously, what was counted from the flux of ^{125}I-Leu-enkephalin is not parent compound but ^{125}I-Tyr. A stability study in the same buffer at the same temperature showed no hydrolytic degradation within the time period used for diffusion, suggesting enzymatic metabolism.

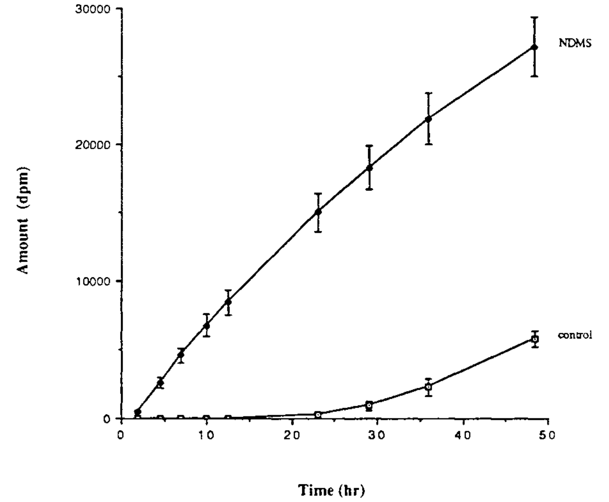


Fig. 2. Amount of iodine-labeled Leu-enkephalin diffused in the presence and absence of 100 m*M* *n*-decylmethyl sulfoxide at 37°C at pH 7. Each point represents the mean ± standard error. Initial donor cell radioactivity was 0.013 μCi/ml. NDMS, in the presence of 100 m*M* *n*-decylmethyl sulfoxide. Control, in the absence of 100 m*M* *n*-decylmethyl sulfoxide.

To gain more insight into the metabolism, an HPLC gradient method was developed. Leucine, glycine, and Gly-Gly were not measured. We were able to separate all other metabolites except Gly-Phe and Gly-Gly-Phe. Based on the previous results, the permeation of Leu-enkephalin was evaluated at pH 5.0 and 7.0. In all cases the gradient system was used to identify the metabolites and an isocratic system to quantitate the parent compound and its metabolites, Phe-Leu, Gly-Phe-Leu, and Gly-Gly-Phe-Leu. The fact that only the amino acids, tyrosine and phenylalanine, and Gly-Phe or Gly-Gly-Phe were detected in the receiver cell up to 12 hr of diffusion at pH 7.0 suggests that all of the peptide bonds can be cleaved to release individual amino acids. The fact that Gly-Phe and/or Gly-Gly-Phe were detected suggests the presence of carboxypeptidase in the skin. Unlike the receiver cell, large amounts of Phe-Leu, Gly-Phe-Leu, and Gly-Gly-Phe-Leu were detected in the donor cell. The major metabolite in the donor cell was Gly-Gly-Phe-Leu. No Tyr-Gly-Gly-Phe was recovered, suggesting that the Tyr-Gly bond is the primary site of metabolism. One explanation of why higher concentrations of these particular metabolites are found in the donor cell than in the receiver cell may be that back diffusion of metabolites through the *n*-decylmethyl sulfoxide-treated stratum corneum is facile. When the parent compound contacts the enzymes in the viable epidermis, the metabolites can diffuse both directions. Since in the presence of *n*-decylmethyl sulfoxide, the stratum corneum is a poor barrier, the metabolites have a greatly reduced diffusional resistance for returning to the donor cell than to the receiver cell. Moreover, as the metabolites pass through the skin they are likely to be metabolized even further. Additionally, enzymes are leached from the skin into the receiver, leading to metabolism in the receiver compartment itself.

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[5] One problem with experiments at pH 3.5 was loss of skin mechanical strength when tested even after 4 hr of experiments. However, the skin still served as diffusion barrier since we were unable to detect consequential levels of radioactivity in the receiver cell until 12 hr of diffusion in control experiments.

Figure 3 shows the fractions of parent compound and its metabolites which have diffused into the receiver cell at pH 5.0. Unlike pH 7.0, where Gly-Gly-Phe-Leu was the major metabolite, Phe-Leu is now the major metabolite followed by Gly-Gly-Phe-Leu. This outcome may due to the aminopeptidase activity being more pH sensitive than the endopeptidase activity. Again, higher concentrations of metabolites were detected in the donor cell than the receiver cell. Metabolite concentrations in the donor cell were sharply increased in the first 6 hr, then remained relatively stable and finally decreased slowly after 22 hr (Fig. 4). At the beginning, the production of metabolite and its back diffusion into the donor cell predominates. However, with time, the enzymatic activity decreases and diffusion of the metabolites into the receiver cell starts to dominate over back diffusion. The total fraction of parent compound and metabolites measured in both the donor and the receiver compartments added up to 0.8 after 26 hr of diffusion. Phe, Gly-Phe or Gly-Gly-Phe, which were not measured, may account for the rest. Almost 60% of the parent compound was left in the donor cell after 26 hr of diffusion, while only 10% was left at pH 7.0. This large difference is due to a higher metabolic rate at pH 7.0. Both metabolism and diffusion decrease the amount of parent compound in the donor cell, but we expect about the same diffusion rate at both pH's, so differences in the metabolism rates must account for the differences. In control experiments without *n*-decylmethyl sulfoxide, about 1% of the applied dose was detected in the receiver cell as Gly-Gly-Phe-Leu. No metabolites are detected in the donor cell without *n*-decylmethyl sulfoxide, eliminating the possibilities of bacterial decomposition of the peptide.

Another approach to avoid enzymatic degradation of Leu-enkephalin is substituting Gly at the second position with D-Ala (16,17). Diffusion of Tyr-D-Ala-Gly-Phe-Leu amide was studied at pH 5.0 and pH 6.5. Figure 5 shows the fraction of parent compound and its metabolite, D-Ala-Gly-Phe-Leu amide, in the donor cell at pH 5.0 and pH 6.5.

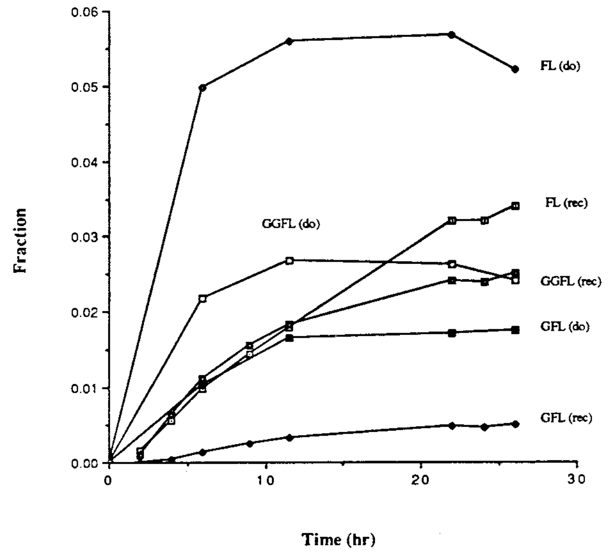

Fig. 4. Fraction of metabolites of YGGFL in the donor and receiver cell from the diffusion of 200 μg/ml YGGFL in the presence of 10 m*M* *n*-decylmethyl sulfoxide at 37°C, pH 5.

Figure 6 shows the amount of parent compound and des-Tyr metabolite diffused into the receiver cell at these two pH's. Even though it is an aminopeptidase-stable compound, substantial amounts of des-tyrosine metabolite were detected in both cells. We were not able to identify all possible metabolites since pure standards for many of them are commercially unavailable. However, the major metabolites, Phe, Tyr-D-Ala-Gly, and D-Ala-Gly-Phe-Leu amide, were identified by gradient elution. A smaller fraction of Phe-Leu amide than of Tyr-D-Ala-Gly was detected, suggesting that Phe-Leu amide is further metabolized. The existence of Tyr-D-Ala-Gly and Phe-Leu amide suggests significant endopeptidase activity. At pH 5.0, the amount of D-Ala-Gly-Phe-Leu

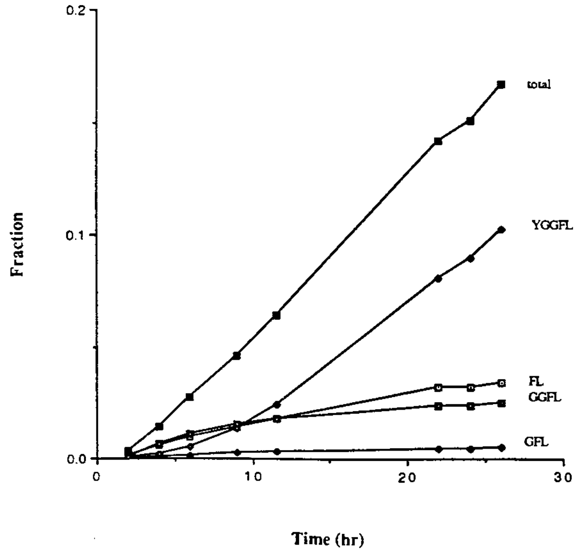

Fig. 3. Fraction of YGGFL and its metabolites diffused into receiver cell from the diffusion of 200 μg/ml YGGFL solution in the presence of 10 m*M* *n*-decylmethyl sulfoxide at 37°C at pH 5.

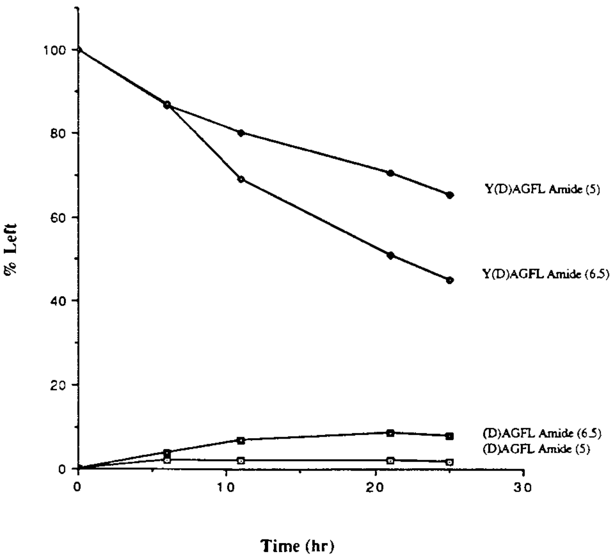

Fig. 5. Percentage of YDAGFL amide and DAGFL amide left in the donor cell from the diffusion of 200 μg/ml YDAGFL solution in the presence of 10 m*M* *n*-decylmethyl sulfoxide, 37°C, pH 5 and pH 6.5. The number in parentheses shows the pH of the medium.

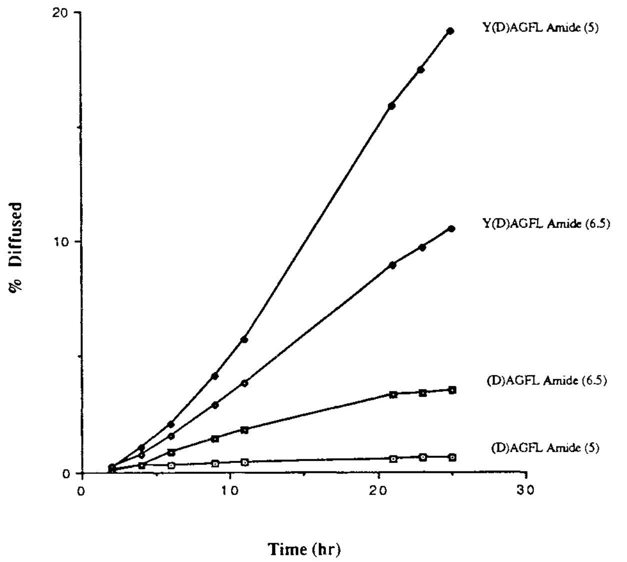

Fig. 6. Percentage of YDAGFL amide and DAGFL amide diffused into the receiver cell from the diffusion of 200 μg/ml YDAGFL solution in the presence of 10 m*M* *n*-decylmethyl sulfoxide, 36°C, pH 5 and pH 6.5. The number in parentheses shows the pH of the medium.

produced was less than one-fifth of that at pH 6.5 and only a slight amount of Tyr-D-Ala-Gly and Phe-Leu amide was detected, suggesting that the activity of the aminopeptidase as well as endopeptidase was reduced greatly at low pH. A slower degradation and, consequently, a higher flux of parent compound were observed at low pH.

The effects of the terminal amide bond on the metabolism and diffusion in the skin were compared using Tyr-D-Ala-Gly-Phe-Leu and its amide in the presence of 10 m*M* *n*-decylmethyl sulfoxide. Figure 7 shows the fraction of parent compounds left in the donor cell and the amount diffused into the receiver cell. In case of Tyr-D-Ala-Gly-Phe-Leu, the des-tyrosine compound was not detected. However, a large fraction of Phe and Tyr-D-Ala-Gly and slight amounts of Phe-Leu and Gly-Phe-Leu were detected. While the parent compound is the major component even at the end of the diffusion experiment in the case of Tyr-D-Ala-Gly-Phe-Leu amide, Tyr-D-Ala-Gly showed the highest fraction in the receiver cell in the case of Tyr-D-Ala-Gly-Phe-Leu. A somewhat higher fraction of Tyr-D-Ala-Gly was obtained from Tyr-D-Ala-Gly-Phe-Leu than from its amide, suggesting that the terminal amide bond may stabilize the compound against endopeptidase activity. The importance of the free carboxyl group of the C-terminal amino acid on the inhibitory potency against endopeptidase, so-called enkephalinase, for the metabolism of Leu-enkephalin has been shown in brain homogenates (18). The reason we could detect no des-tyrosine compound in the case of Tyr-D-Ala-Gly-Phe-Leu may be the activity of endopeptidase predominates over that of aminopeptidase. On the other hand, stabilization of the Gly-Phe bond by the C-terminal amide bond increases the hydrolysis of the Tyr-D-Ala bond to some extent. Another possibility is that the terminal amide bond stabilized the compound against carboxypeptidase activity, a well-documented phenomenon (2,4). It is not possible to state which of these influences is the major factor in stabilization. The activity of carboxypeptidase does seem lower than the activity of aminopeptidase and endopeptidase in the skin, however. From the fact that Tyr-D-Ala-Gly is the major metabolite in both cases and des-tyrosine metabolite was not detected by way of the diffusion of Tyr-D-Ala-Gly-Phe-Leu, it can be concluded that endopeptidase activity dominates over aminopeptidase activity. On the other hand, aminopeptidase activity appears to predominate in the metabolism of Leu-enkephalin. Similar results have been observed in homogenates of brain and mucosae from various sites (18–21). Therefore, once the compound is protected from aminopeptidase activity, protecting the compound from endopeptidase may also play an important role in increasing biological activity.

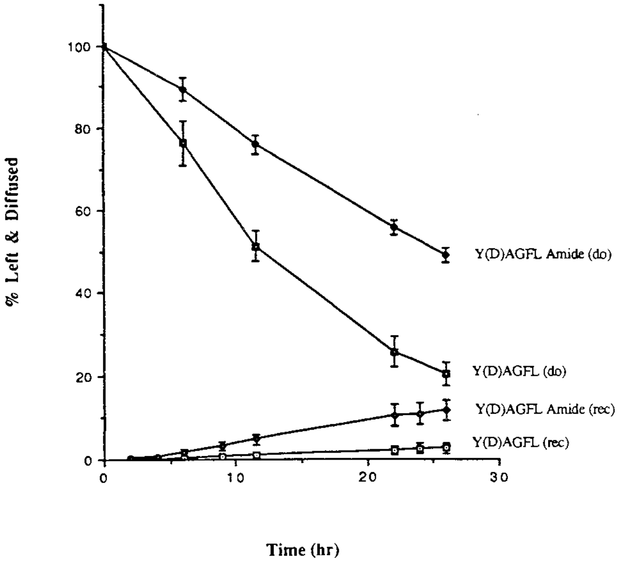

Fig. 7. Comparison of fraction left in the donor cell and diffused into the receiver cell of YDAGFL and its amide from the diffusion of 200 μg/ml solution in the presence of 10 m*M* *n*-decylmethyl sulfoxide, 37°C, pH 7.0. Each point represents the mean ± standard error. do, donor cell; rec, receiver cell.

The metabolism of several possible compounds was tested in skin homogenates in an effort to screen inhibitors of the proteolytic hydrolysis of Leu-enkephalin in the skin and characterize the metabolic pathways. Peptidase-labile peptides are known to act as inhibitors of the metabolism of Leu-enkephalin in other systems (22). Thus metabolism of Phe-Leu, Gly-Phe-Leu, Gly-Gly-Phe-Leu, Tyr-Gly, Tyr-Gly-Gly, and Tyr-D-Ala-Gly-Phe-Leu amide as well as Leu-enkephalin was studied; 2.5% skin homogenates were prepared for the metabolism of Tyr-Gly-Gly and Tyr-D-Ala-Gly-Phe-Leu amide and 0.5% skin homogenates were used for the other compounds. The order of the rates of metabolism for the peptides was Phe-Leu > Leu-enkephalin > Gly-Phe-Leu > Gly-Gly-Phe-Leu >> Tyr-Gly, Tyr-Gly-Gly >> Tyr-D-Ala-Gly-Phe-Leu amide. Tyr-Gly and Tyr-Gly-Gly were relatively stable in the skin homogenates, even though Tyr-Gly-Gly showed 79% of the substrate specificity of Leu-enkephalin in human plasma (22). The rate of metabolism of Tyr-Gly-Gly by aminopeptidase is known to vary according to the mucosal site being studied in albino rabbits while that of Leu-enkephalin is independent of the site (21). The high stability of Tyr-D-Ala-Gly-Phe-Leu amide even in the five-times-concentrated skin homogenates suggests that endopeptidase activity is negligible in the skin homogenates. It should be noted that, in our previous study, Tyr-D-Ala-Gly-Phe-Leu amide was metabolized mainly by endopeptidase. From the fact that the metabolites of Leu-enkephalin, Gly-Gly-Phe-Leu, Gly-Phe-Leu, and Phe-Leu, were metabolized further, it seems that aminopeptidase cleaves amino-terminal amino acids one by one up to the C-terminal amino acid. This view is supported in the literature, where the incubation of tritium-labeled met-enkephalin in 20% rat plasma resulted in the recovery of essentially all radioactivity as tritium-labeled Gly (23). When Leu-enkephalin was incubated in the 2.5% skin homogenate, no parent compound was left after 5 min and only Phe or Tyr was recoverable, which also was true after incubation in nasal homogenates (21). Due to the complexity of the skin homogenates, presumably involving several different types of enzymes, K_m and $V_{\max}$ values are difficult to obtain. However, a larger fraction of Phe-Leu was obtained from the diffusion experiments than from experiments with skin homogenates, indicating that the endopeptidase might be membrane bound. The high stability of Tyr-D-Ala-Gly-Phe-Leu amide in the skin homogenates and the recovery of a large fraction of Tyr-D-Ala-Gly in diffusion experiments also suggests that the endopeptidase is present and likely membrane bound. In brain tissue, two types of enzyme activity, aminopeptidase and endopeptidase, were identified as having roles in the metabolism of enkephalin, and here too the endopeptidase was membrane bound (18,23).

The *in vitro* inhibitory effects of several metabolites and other inhibitors on the metabolism of Leu-enkephalin in the skin homogenates were measured (Fig. 8). Notably, 0.1 mmol or above of puromycin showed the greatest inhibitory effect on the metabolism of Leu-enkephalin in brain homogenates among the many tested aminopeptidase inhibitors (24–26). Amastatin, a peptidase inhibitor of bacterial origin, inhibits aminopeptidase activity found in rat brain supernatant with an IC_{50} of 10 n*M* (27). In the present studies in homogenates, both amastatin and puromycin showed high inhibitory effects. While either was present, no more than 10% of the parent compound was metabolized, suggesting that aminopeptidase is mainly responsible for the metabolism of Leu-enkephalin in the skin homogenates. In nasal homogenates, Phe-Leu and Gly-Phe-Leu slightly reduced the enzymatic activity, while 20-fold molar excesses of Tyr-Gly, Tyr-Gly-Gly, and Phe-Leu showed high inhibitory potency (22). However, we saw no measurable inhibitory effect of Gly-Gly-Phe-Leu, Gly-Phe, Gly-Gly-Phe, and Tyr-Gly-Gly in the skin homogenates.

The inhibitory effects of various compounds tested in the skin homogenates may not relate directly to metabolism during skin diffusion since only cytosol enzymes are present in the skin homogenates and may be located in membranes outside the actual diffusion path. Therefore, the inhibitory effects of 1 m*M* puromycin, 300 μ*M* amastatin, and 20 m*M* Tyr-Gly-Gly were assessed in diffusion experiments by measuring the flux of Leu-enkephalin into the receiver cell and fraction left in the donor cell at pH 7.0, with and without inhibitor. In two of four cases involving amastatin, a slight amount of Leu-enkephalin was detected in the receiver cell after 22 hr of diffusion. In the other instances, no Leu-enkephalin was detected in the receiver cell. Figure 9 shows the effect of the various inhibitors on the fraction of Leu-enkephalin left in the donor cell as a function of time. As discussed previously, the fraction of parent compound left in the donor cell can be used to estimate the metabolism rate. *n*-Decylmethyl sulfoxide induced a very rapid drop in the donor cell fraction of the parent compound by increasing the permeability of Leu-enkephalin through the stratum corneum. The presence of puromycin, amastatin, or Tyr-Gly-Gly leads to slightly higher fractions left in the donor

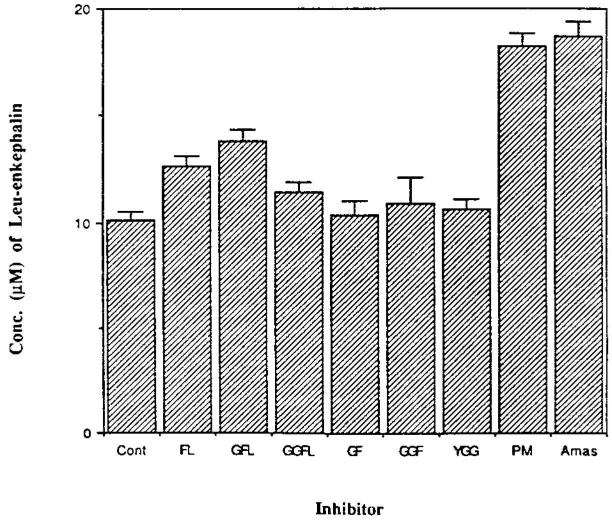

Fig. 8. Effects of various inhibitors on the fraction of Leu-enkephalin left. One hundred micromoles of each inhibitor was incubated with 20 μ*M* Leu-enkephalin at 37°C, pH 7.0, for 7 min. Each bar shows the mean concentration of Leu-enkephalin left after the incubation ± standard error. PM, puromycin; Amas, amastatin.

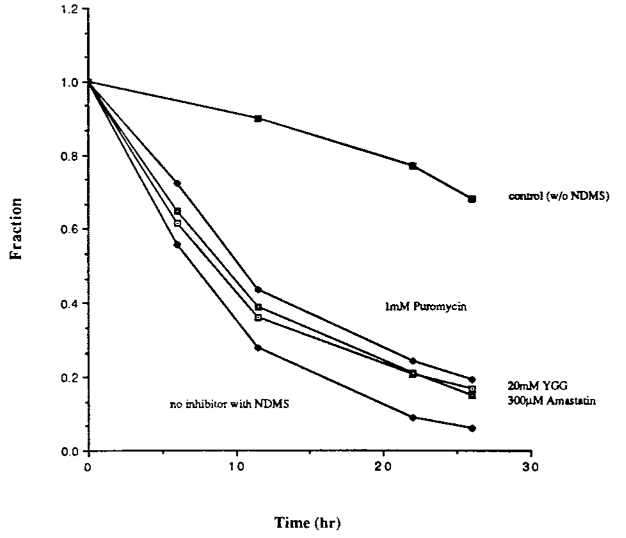

Fig. 9. Effect of 1 m*M* puromycin, 300 μ*M* amastatin, and 20 m*M* Tyr-Gly-Gly on the fraction of Leu-enkephalin left in the donor cell from the diffusion of 200 μg/ml solution in the presence of 10 m*M* *n*-decylmethyl sulfoxide and effect of 10 m*M* *n*-decylmethyl sulfoxide on the metabolism rate of Leu-enkephalin in skin diffusion experiments at pH 7.0.

cell; however, the kinds of dramatic inhibitory effects of amastatin and puromycin seen in the *in vitro* inhibition study were not observed. Previously it was found that, even though puromycin was a potent inhibitor of aminopeptidase metabolism of Leu-enkephalin in brain homogenates, it did not inhibit leucine aminopeptidase and hydrolytic activity present in rat serum (26). The latter was also true in our skin diffusion studies at pH 7.0. It suggests that an aminopeptidase, presumably a leucine aminopeptidase, different from that in the brain homogenates is responsible for the degradation of Leu-enkephalin in the diffusion study.

The inhibitory effects of various metabolites of Leu-enkephalin were investigated in the same manner. No Leu-enkephalin could be detected in the receiver cell except in the cases of using Phe-Leu and combinations of Phe-Leu, Gly-Phe-Leu, and Gly-Gly-Phe-Leu. In terms of the fraction diffused into the receiver cell, results with Phe-Leu and the combinations of the three peptides were similar (Fig. 10), suggesting that Phe-Leu was mainly responsible for the inhibition. None of the inhibitors, including Phe-Leu, led to higher fractions of Leu-enkephalin left in the donor cell than seen in control experiments. These results correlate poorly with the results from the skin homogenate experiments. Higher concentrations of amastatin may have higher inhibitory effects than Phe-Leu; however, even 1 m*M* puromycin was not as potent as Phe-Leu.

The inhibitory effects of 150 μ*M* amastatin and 1, 5, and 10 m*M* Tyr-Gly-Gly were also assessed at pH 5.0. In terms of the fraction diffused into the receiver cell, amastatin showed a slight inhibitory effect but Tyr-Gly-Gly showed none. Amastatin decreased the amount of Gly-Gly-Phe-Leu produced to half relative to control experiments in both the cells (Fig. 11). It also slightly decreased the Gly-Phe-Leu and Phe-Leu buildup in both the cells and caused a slightly higher fraction of Leu-enkephalin to be seen in the donor cell. Tyr-Gly-Gly, on the other hand, had little effect on the metabolism, as similar amounts of metabolites and a similar fraction of parent compound were left in the donor cell relative to control experiments.

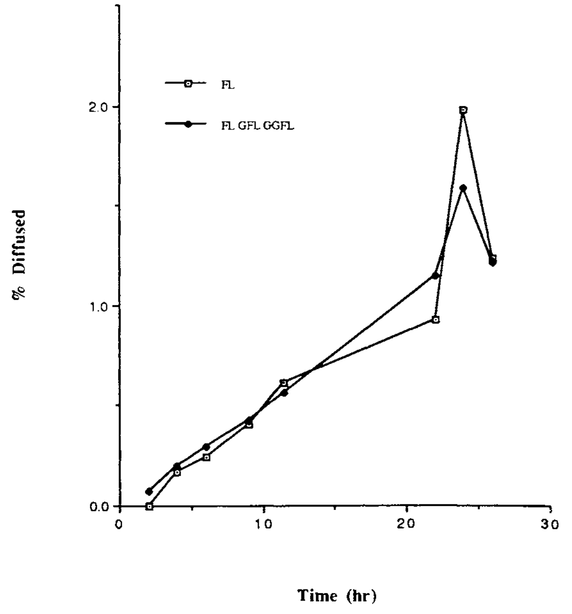

Fig. 10. Effect of 1 m*M* Phe-Leu and combination of 1 m*M* Phe-Leu, Gly-Phe-Leu, and Gly-Gly-Phe-Leu on the fraction of Leu-enkephalin diffused into the receiver cell at pH 7.0.

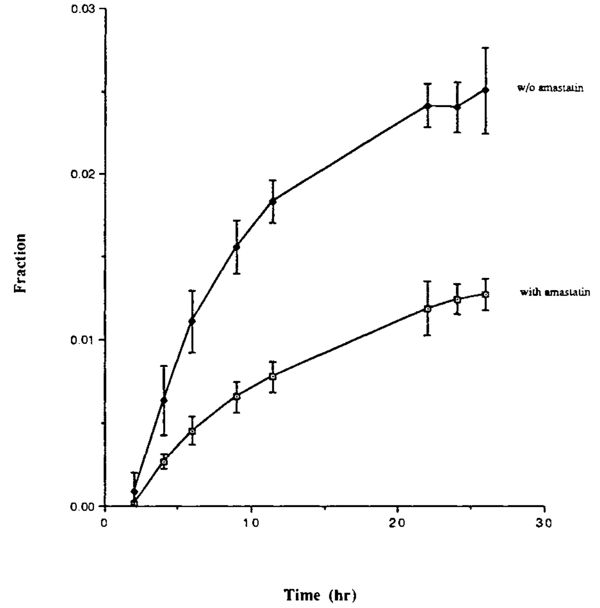

Fig. 11. Effect of 150 μ*M* amastatin on the fraction of Gly-Gly-Phe-Leu produced in the receiver cell in the presence of 10 m*M* *n*-decylmethyl sulfoxide at pH 5.0. Each point represents the mean ± standard error.

The leaching of enzymes out of the skin into either of the diffusion half-cells can lead to underestimation of permeability coefficients. Therefore, the enzyme activity in the medium in contact with the skin was assessed by incubating portions of the solutions from each cell with Leu-enkephalin. No enzymatic activity was detected in the donor cell after up to 7 hr of diffusion. In the receiver cell, the enzymatic activity increased rapidly over the initial 5 min of contact with the skin and then slowly up to 1 hr. Apparently dermal surface-bounded enzymes were washed out of the skin at the beginning of the experiments.

The present results clearly show that hairless mouse skin has significant proteolytic enzyme activity and that the coadministration of inhibitors can increase transdermal flux. Hairless mouse skin differs from human skin in many ways (28), and consequently, results from hairless mouse skin may not accurately reflect the human skin's enzymatic environment. Moreover, the studies may not truly reflect the magnitude of activity of the enzymes *in vivo* as the result of possible damage to the skin cell during *in vitro* experiment. However, proteolytic enzyme activity, including leucine aminopeptidase, has been demonstrated in saline extracts from human skin (29). And more recently, leucine aminopeptidase and endopeptidase activities were identified from Triton X-100 extracts of human skin fibroblasts (30). This suggests that, while human skin may have a qualitatively if not quantitatively similar type of enzyme activity, the development of transdermal delivery strategies for peptides must be done with cognizance of the characteristics of proteolytic enzymes in human skin.

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