A Comparison of Peptidase Activities and Peptide Metabolism in Cultured Mouse Keratinocytes and Neonatal Mouse Epidermis

Praful K. Shah[1,2] and Ronald T. Borchardt[1,3]

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One of the barriers to transdermal delivery of peptides is the metabolic activity of the epidermis. To define this metabolic activity, aminopeptidase activity and Leu-enkephalin metabolism were measured in the epidermis obtained from neonatal mouse skin and in cultured mouse keratinocytes. Aminopeptidase activity was measured fluorometrically using leucine, tyrosine, lysine, and aspartic acid derivatives of β-naphthylamine as substrates. Similarities in substrate kinetic values (K_m and V_{max}) and substrate specificity of the enzyme(s) in homogenates prepared from neonatal mouse skin epidermis and cultured mouse keratinocytes strongly suggest that the keratinocytes in culture express the same aminopeptidase(s) with the same relative activity as in neonatal skin. The K_m and V_{max} values for aminopeptidase(s) with different substrates in epidermis homogenates are as follows: leucine β-naphthylamide (11 μ*M* and 38 nmol · min^{-1} · mg^{-1}), tyrosine β-naphthylamide (21 μ*M* and 18 nmol · min^{-1} · mg^{-1}), and lysine β-naphthylamide (11 μ*M* and 35 nmol · min^{-1} · mg^{-1}). Aspartic acid β-naphthylamide and glutamic acid β-naphthylamide were not hydrolyzed by these homogenates at pH 7.4 (37°C). Leu-enkephalin hydrolysis by the homogenates from cultured mouse keratinocytes and neonatal mouse epidermic gave similar K_m (32 and 24 μ*M*), V_{max} (9.77 and 7.55 nmol · min^{-1} · mg^{-1}) and K_i (223 and 194 μ*M*) values. In addition, the cellular homogenates gave similar metabolite profiles for Leu-enkephalin.

KEY WORDS: mouse keratinocytes; mouse epidermis; aminopeptidases; peptidases; metabolism; Leu-enkephalin; liquid chromatography.

INTRODUCTION

Peptides and proteins are becoming an important class of drugs (1–3) and advances in biotechnology promise to provide a large number of these compounds as potential drug candidates in the future (4,5). Their success as drugs depends in part on an understanding of their mechanism of action and their pathways of degradation. Equally important is an ability to deliver these agents to their site of action. Conventional oral delivery of proteins and peptides results in poor bioavailability due in part to extensive first pass metabolism. Therefore, alternative routes of administration, including transdermal (3,6,7), are being explored.

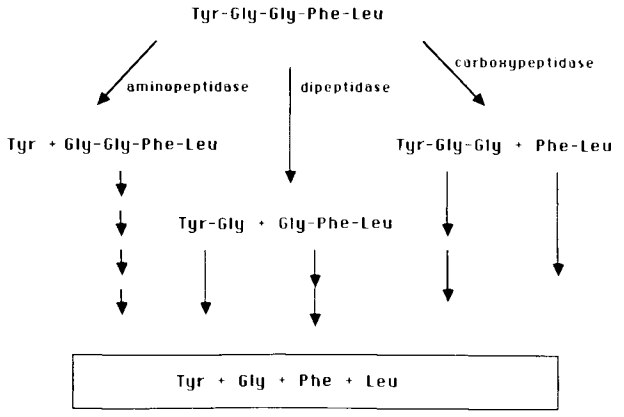
Several laboratories have attempted to develop cell culture model systems which would mimic the epidermis of the skin (8–15). Eventually, these keratinocyte culture models will be useful to pharmaceutical scientists to study transdermal transport and metabolism of drugs *in vitro* (8,16). Recently, we evaluated cultured mouse keratinocytes as a potential model for studying solute transport across skin (17). Unfortunately, current methodology for culturing mouse keratinocytes does not afford an acceptable *in vitro* transport model for skin because the mouse keratinocytes do not develop a continuous stratum corneum. However, the potential utility of these cultured keratinocytes for drug metabolism studies has yet to be explored.

In this article the ability of cultured mouse keratinocytes and neonatal mouse epidermis to metabolize peptides is compared. The studies included a comparison of the activity of the aminopeptidases, a class of enzymes previously shown to be responsible for peptide metabolism, and a comparison of the metabolism of a model peptide, Leu-enkephalin, in these models. Enkephalin was selected as a model peptide because its metabolism has been characterized in other tissues (18–21). Enkephalin metabolism pathways shown in Scheme I involve aminopeptidases, dipeptidases, and carboxypeptidases (22).

MATERIALS AND METHODS

Chemicals. β-Naphthylamine (β-NA),[4] leucine β-naphthylamide (Leu-β-NA), tyrosine β-naphthylamide (Tyr-β-NA), lysine β-naphthylamide (Lys-β-NA), aspartic acid β-naphthylamide (Asp-β-NA), glutamic acid β-naphthylamide (Glu-β-NA), trypsin, trypsin inhibitor, insulin, hydrocortisone, M-199 media, Ficoll, fetal bovine serum, albumin, Triton X-100, and Tris buffer were obtained from Sigma Chemical Company (St. Louis, MO). Leu-enkephalin and its metabolites were obtained from ICN Biomedicals, Inc. (Costa Mesa, CA). Protein Assay Kit was obtained from Pierce Chemical Company (Rockford, IL). Dulbecco's modified phosphate-buffered saline (PBS) was obtained from Gibco Laboratories (Grand Island, NY).

[4] Abbreviations used: AA-β-NA, amino acid β-naphthylamide; β-NA, β-naphthylamine; Glu-β-NA, glutamic acid β-naphthylamide; Leu-β-NA, leucine β-naphthylamide; Lys-β-NA, lysine β-naphthylamide; Tyr-β-NA, tyrosine β-naphthylamide; PBS, phosphate-buffered saline; GPL, Gly-Phe-Leu; GGPL, Gly-Gly-Phe-Leu; PL, Phe-Leu.



Scheme I. Enzymatic degradation pathways of Leu-enkephalin (22).

[1] Department of Pharmaceutical Chemistry, The University of Kansas, Malott Hall, Room 3006, Lawrence, Kansas 66045.
[2] Present address: Marion Merrell Dow Inc., Technical Development Department, Kansas City, Missouri 64134.
[3] To whom correspondence should be addressed.

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Mouse Epidermis. Neonatal (BALB/c) mice (0–2 days old) from the University of Kansas Animal Care Unit were sacrificed and the skin was removed. The skin was treated with trypsin (0.25% trypsin in M-199 medium) at 37°C for 1 hr to separate the epidermis from dermis (9).

Cultured Mouse Keratinocytes. Basal keratinocytes were obtained from neonatal mouse skin by a slight modification of the method reported by Marcelo *et al.* (9), which included the addition of hydrocortisone (10 μg/ml) and insulin (10 μg/ml) in the medium (10). Briefly, the epidermis was separated from dermis, the basal cells were scraped from the epidermis and purified by centrifugation through a discontinuous Ficoll gradient (9). The cells were plated in 100-mm tissue culture dishes at a density of $\sim 3 \times 10^5$ cells/cm^2 in M-199 medium with 10% fetal calf serum, hydrocortisone, and insulin (10). The M-199 medium was replaced every other day.

Cell Homogenate. The cells in the culture dishes were washed twice with PBS and then once with 0.32 *M* sucrose solution. The cells were scraped, collected in 0.32 *M* sucrose, and homogenized in Dounce homogenizer. The homogenate suspension was centrifuged at 800*g* for 10 min and the pellet of cell debris was discarded. The supernatant was further centrifuged at 30,000*g* for 20 min to obtain a soluble (cytosolic) fraction. The membrane-bound enzymes were obtained from the pellet according to the procedure described by Baranczyk-Kuzma and Audus (24).

Epidermis Homogenate. Mouse epidermis, which was obtained by trypsinization of neonatal skin, was washed with M-199 medium containing 0.1% trypsin inhibitor to remove excess trypsin. The epidermis was shredded and the homogenate was prepared in 0.32 *M* sucrose by the method described above.

Protein Determination. The protein content in each homogenate was determined using the protein assay based on bicinchoninic acid with albumin as the protein standard (22). Aliquots of the homogenate were incubated in the protein assay reagents at room temperature (22 ± 1°C) overnight and absorbance at 562 nm was measured.

Aminopeptidase Assay. A mixture of 200 μl of the cell or epidermis homogenate (~1 mg protein/ml) and 100 μmol of a specific amino acid, β-naphthylamide (AA-β-NA), in 50 m*M* Tris-maleate buffer (pH 7.4) in a total volume of 1.5 ml was reacted at 37°C for 5 min. The fluorescence of β-NA was monitored at 410 nm with an excitation wavelength of 335 nm on a SLM-4800 fluorometer. β-NA was used as the standard for fluorescence measurements. Boiled homogenate was used as a negative control for the enzymatic reaction. The activity was normalized by milligrams protein in the homogenate to obtain the specific activity.

Metabolism Studies. Typically, equal volumes of the cell homogenate (~1 mg protein/ml) and Leu-enkephalin solution in PBS (pH 7.4) were combined at 37°C and incubated for various times. Aliquots (50 μl) were removed at various times and the reactions were quenched in 100 μl of ice-cold acetonitrile. A 20-μl aliquot of a 0.5 m*M* tryptophan (Trp) solution was added to the reaction mixture as an internal standard. The mixture was centrifuged at 13,600*g* for 15 min to remove precipitated proteins. Acetonitrile was evaporated from the supernatant under a gentle stream of N_2 at ambient temperature and a 10-μl sample was analyzed using LC.

Initial-Velocity Determination. Equal volumes (100 μl) of homogenate and Leu-enkephalin (5 to 1000 μ*M* in PBS) were incubated at 37°C. Aliquots (50 μl) were removed after 0.5 min or 1 min, treated as described above (Metabolism Studies), and analyzed by LC.

LC Determination of Enkephalin and Its Metabolites. All the chromatographic instruments, SCL-6A system controller, LC-6A pump and mixer, SIL-6A auto injector, SPD-6A variable wavelength UV detector, and C-R6A integrator were manufactured by Shimadzu Corp. (Kyoto, Japan). The LC column, Ultrasphere ODS (5 μm, 4.6 × 250 mm), was purchased from Altex Corp. (San Ramon, CA). The LC analytical method of Kashi and Lee (19) was modified for Leu-enkephalin and the metabolite separation. The ODS column was eluted with $NaClO_4$ (0.1 *M*) and H_3PO_4 (0.1%) in water–acetonitrile (100*x*:*x*, v/v) where $x = 7$ for 0–5 min, $x = 20$ for 5–8 min, and then x was increased by 1 per min for the next 18 min. After each run, the column was washed with acetonitrile–buffer (60:40, v/v) for 4 min and reequilibrated for 15 min with acetonitrile–buffer (7:93, v/v). The flow rate was 1 ml/min and Leu-enkephalin and the metabolites were detected at 214 nm (22).

Calculations. K_m and V_{max} values of the enzyme substrates were calculated from the slopes and the intercepts of Lineweaver–Burk reciprocal plots of velocities (V) and substrate concentrations [S]. At least six points were plotted. Since Leu-enkephalin metabolism exhibited substrate inhibition, K_i values [Eq. (1)] were calculated using a modified Michaelis–Menten equation and fitting the data to Eq. (2) (BBN Software Products, Cambridge, MA).

$$\begin{array}{c} \overset{K_{cat}}{\rightleftharpoons} \quad \overset{K_i}{\rightleftharpoons} \\ E + S \rightleftharpoons ES + S \rightleftharpoons ES_2 \\ \downarrow \\ E + P \end{array}$$

where

$$K_i = \frac{[S][ES]}{[ES_2]} \tag{1}$$

$$V = \frac{[V_{max}][S]}{[K_m + S][1 + S/K_i]} \tag{2}$$

RESULTS

Aminopeptidase Activity

Peptides can be metabolized by aminopeptidases, dipeptidases, and carboxypeptidases (2,20,25,26). The major pathway for enkephalin metabolism appears to involve aminopeptidases (19). Aminopeptidase specificities are determined in part by the nature of the amino acid at the N terminal of the peptide.

In preliminary studies, the subcellular distribution of aminopeptidases was determined in homogenates of cultured mouse keratinocytes and mouse epidermis. Soluble and

membrane-bound fractions of the homogenates were analyzed separately for aminopeptidase activity. Table I shows the comparison of soluble and membrane-bound peptidase activities when Leu-β-NA, Tyr-β-NA, and Lys-β-NA were used as substrates. In all cases the activity of the soluble peptidase was substantially higher than the activity of the membrane-bound enzyme; hence subsequent experiments were conducted with the soluble (cytosolic) fraction.

The hydrolysis of AA-β-NA's in homogenates of cultured mouse keratinocytes and mouse epidermis exhibited Michaelis–Menten kinetics. For example, Fig. 1 shows the dependence of the rate of hydrolysis on the concentration of Tyr-β-NA in a keratinocyte homogenate. Leu-β-NA and Lys-β-NA exhibited similar kinetics of hydrolysis in homogenates of cultured mouse keratinocytes and mouse epidermis (data not shown). To rule out the possibility that aminopeptidase activity in the homogenates prepared from cultured keratinocytes changes with days in culture, the kinetics of Leu-β-NA hydrolysis as a function of days in culture was studied. Figure 2 shows that the substrate kinetic parameters, K_m and V_{max}, for Leu-β-NA remain constant as the keratinocytes proliferate, differentiate and stratify.

To determine possible differences in the nature of the aminopeptidases in mouse keratinocytes compared with

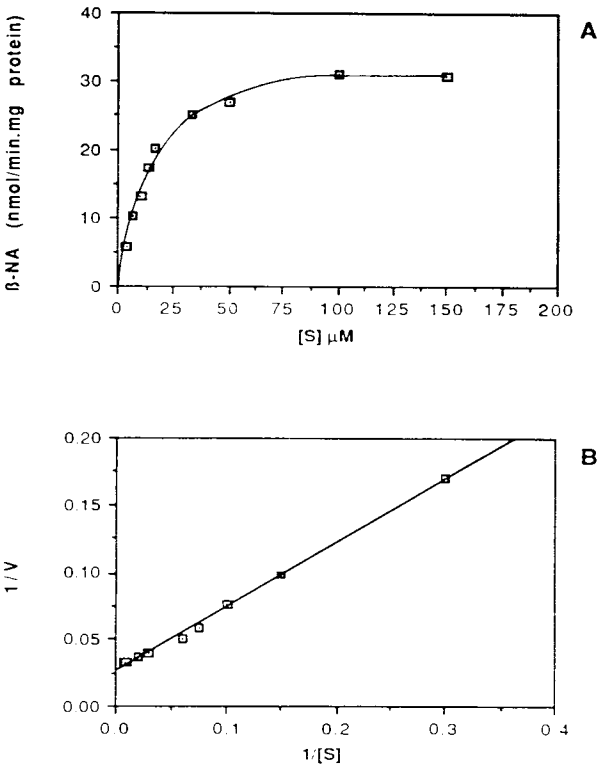

Fig. 1. (A) Rate of β-NA formation versus Tyr-β-NA concentration. Effect of Tyr-β-NA concentration on the rate of β-NA formation in the homogenate of cultured mouse keratinocytes (10-day-old-culture). The homogenate and Tyr-β-NA were incubated in Tris buffer (pH 7.4) for 5 minutes. β-NA formation was measured fluorometrically. (B) Reciprocal plot of (V) and [S], from which K_m and V_{max} values were calculated (Table II). Each point is an average of two measurements.

Table I. Aminopeptidase Activity as a Percentage of Total Activity for Various AA-β-NA in Soluble and Membrane Fractions Prepared from Mouse Epidermis and Mouse Keratinocytes Cultured for 5, 10, or 15 Days[a]

		Aminopeptidase activity (%)	
Substrate	Tissue	Soluble fraction[b]	Membrane fraction[b]
Leu-β-NA	Epidermis[c]	93.2	4.8
	Keratinocytes, 5 d[d]	87.3	17.0
	Keratinocytes, 10 d	81.6	14.0
	Keratinocytes, 15 d	84.9	19.2
Tyr-β-NA	Epidermis	96.0	6.5
	Keratinocytes, 5 d	98.6	3.4
	Keratinocytes, 10 d	83.6	2.9
	Keratinocytes, 15 d	69.9	15.3
Lys-β-NA	Epidermis	90.5	0.3
	Keratinocytes, 5 d	95.7	0.5
	Keratinocytes, 10 d	67.7	22.3
	Keratinocytes, 15 d	91.3	7.6

[a] The homogenate was centrifuged (30,000g, 20 min) to obtain the supernatant soluble fraction. The pellet was incubated in Triton X-100 to obtain membrane-bound enzymes (21). The tissue homogenate and AA-β-NA were incubated in Tris buffer (pH 7.4) at 37°C for 5 min and the release of fluorescent β-NA was monitored. The data represent an average of duplicates.

[b] Aminopeptidase activity determined in the crude homogenate obtained after initial centrifugation (800g × 10 min) was considered as 100% for calculations. The activities in the soluble fraction and the membrane fraction were determined separately.

[c] The epidermis was obtained from at least five neonatal Balb/C mice skins.

[d] Cells from 5 to 10 mice were plated and cultured (d = number of days keratinocytes were in culture).

mouse epidermis, five different substrates (Leu-β-NA, Tyr-β-NA, Lys-β-NA, Asp-β-NA, and Glu-β-NA) were investigated. Table II compares the K_m and V_{max} values for these substrates in the homogenates of cultured mouse keratinocytes (5, 10, and 15 days in culture) with those values in mouse epidermis. The neutral (Leu-β-NA and Tyr-β-NA) and basic (Lys-β-NA) amino acid derivatives showed good substrate properties, whereas the acidic (Asp-β-NA and Glu-β-NA) amino acid derivatives were not substrates for the enzymes present in homogenates of cultured mouse keratinocytes and mouse epidermis. Some differences in K_m values for the AA-β-NA's were observed between the cultured mouse keratinocytes and mouse epidermis (e.g., Lys-β-NA: epidermis, K_m = 10.5 μM, versus keratinocytes 15 days, K_m = 2.3 μM) and between keratinocytes cultured for different periods of time (e.g., Lys-β-NA: keratinocytes 5 days, K_m = 6.3 μM, versus keratinocytes 15 days, K_m = 2.3 μM). However, there was no obvious trend in these K_m values either comparing epidermis to cultured keratinocytes or comparing keratinocytes cultured for different days. In general, the V_{max} values determined for Leu-β-NA, Tyr-β-NA, and Lys-β-NA were similar in homogenates from mouse epidermis and mouse keratinocytes cultured for 5, 10, or 15 days.

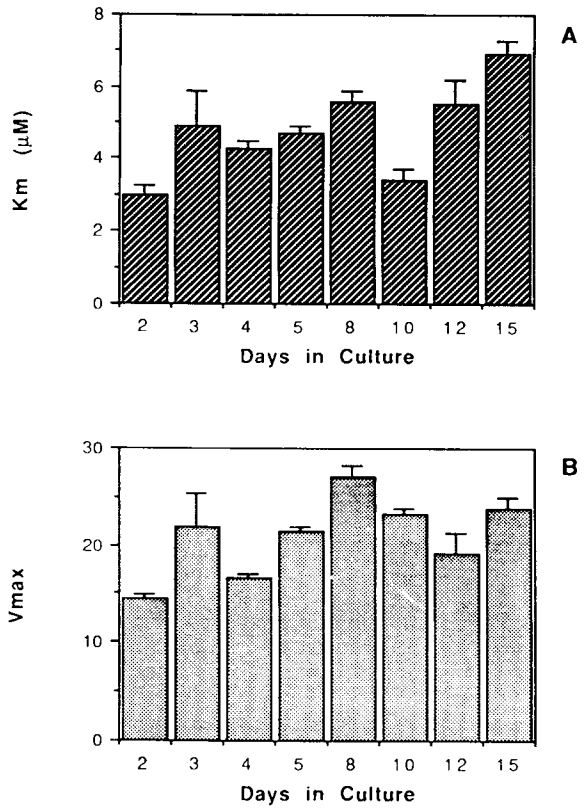

Fig. 2. Substrate kinetic parameters K_m (A) and V_{max} (B) for Leu-β-NA in cultured mouse keratinocyte homogenates as a function of days in culture. The error bars represent standard errors. At least six different substrate concentrations were used to determine the K_m and V_{max} values.

Enkephalin Metabolism

Leu-enkephalin and the metabolites produced in homogenates of cultured mouse keratinocytes and mouse epidermis were separated by LC and identified by their retention times. The intraassay and interassay variations in the retention times for the internal standard Trp were less than 2% (RSD, n = 10). Figure 3 shows a typical chromatogram of standard samples of Leu-enkephalin, its major metabolites, and the internal standard Trp. The retention times (minutes) were as follows: Tyr-Gly-Gly (TGG), 6.2; Tyr, 7.0; Tyr-Gly (TG), 7.7; Leu, 9.6; Phe, 14.1; Trp, 17.2; Phe-Leu (PL), 22.3; Gly-Gly-Phe-Leu (GGPL), 23.3; Gly-Phe-Leu (GPL), 23.9; and Leu-enkephalin (ENK), 25.4 Leu-enkephalin and its metabolites were quantified using standard curves giving a linear response over the range of 100 to 2000 ng (10- to 200-μl injection volume).

Figure 4 shows typical chromatograms of samples containing Leu-enkephalin in the homogenate of mouse keratinocytes. Control experiments showed that the enzyme and Leu-enkephalin solutions were stable during the time course of the reaction (2 hr; data not shown). During the course of the reaction, a decrease in the concentration of Leu-enkephalin and an increase in the concentration of Tyr and Phe were seen, while the intermediates TG and GGPL were increased initially and then decreased. To define the metabolite profile more accurately, Leu-enkephalin metabolism at an initial concentration of 1 mM was studied. Figure 5 displays the concentration profiles for the appearance and disappearance of Tyr, TG, Phe, and GGPL in the homogenate of mouse epidermis. Similar results were seen with the cultured mouse cells (data not shown).

Initial velocity experiments with Leu-enkephalin hydrolysis showed evidence of substrate inhibition (Fig. 6); hence low initial Leu-enkephalin concentrations (<100 μM) were used to obtain K_m and V_{max} values. K_i values were obtained by employing Eq. (2). Leu-enkephalin hydrolysis by the homogenates from cultured mouse keratinocytes (10-day-old culture) and neonatal mouse epidermis gave similar K_m values of 32 and 24 μM, V_{max} values of 9.77 and 7.55 nmol · min^{-1} · mg protein^{-1}, and K_i values of 223 and 194 μM, respectively.

Table II. K_m and V_{max} Values of Aminopeptidases with Various AA-β-NA as Substrates in the Soluble Fraction of Mouse Epidermis and Mouse Keratinocytes Cultured for 5, 10, or 15 Days[a]

Substrate	Tissue	K_m (μM)	V_{max} (nmol · min^{-1} · mg^{-1})
Leu-β-NA	Epidermis	10.8 ± 0.45[b]	38.2 ± 0.93[b]
	Keratinocytes, 5 d	4.7 ± 0.22	21.4 ± 0.55
	Keratinocytes, 10 d	3.4 ± 0.34	23.2 ± 0.58
	Keratinocytes, 15 d	6.9 ± 0.40	23.8 ± 1.19
Tyr-β-NA	Epidermis	20.8 ± 0.64	18.1 ± 0.49
	Keratinocytes, 5 d	20.3 ± 1.94	39.8 ± 3.66
	Keratinocytes, 10 d	18.4 ± 1.03	38.4 ± 1.96
	Keratinocytes, 15 d	16.2 ± 0.91	40.6 ± 2.03
Lys-β-NA	Epidermis	10.5 ± 0.70	34.7 ± 2.16
	Keratinocytes, 5 d	6.3 ± 1.12	38.7 ± 4.40
	Keratinocytes, 10 d	3.1 ± 0.28	29.8 ± 1.26
	Keratinocytes, 15 d	2.3 ± 0.19	33.6 ± 2.00
Asp-β-NA	Epidermis	>250 μM	
	Keratinocytes, 15 d	>250 μM	
Glu-β-NA	Epidermis	>250 μM	
	Keratinocytes, 15 d	>250 μM	

[a] The tissue homogenate and AA-β-NA were incubated in Tris buffer (pH 7.4) at 37°C for 5 min and formation of fluorescent β-NA was monitored. At least five mice were used to obtain the homogenates (d = days in culture).

[b] At least six points were used to obtain the standard error of estimate.

DISCUSSION

Recently pharmaceutical scientists have begun to utilize cell and tissue culture techniques to study drug transport and metabolism in specific biological barriers, including skin (8,16). The utility of cultured cell systems was recently reviewed (16).

As shown in Table II the K_m and V_{max} values for the neutral, aromatic, and basic substrates for aminopeptidases are similar, while acidic substrates Asp-β-NA and Glu-β-NA are not hydrolyzed in homogenates prepared from cultured mouse keratinocytes and mouse epidermis at pH 7.4. Aminopeptidase-A, which hydrolyzes these acidic substrates, is either absent in mouse epidermis and cultured mouse kera-

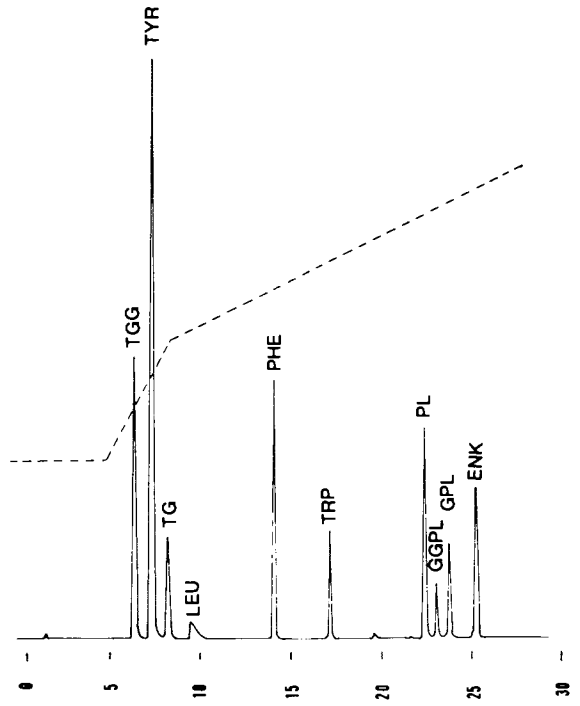

Fig. 3. Chromatogram of a mixture of Leu-enkephalin, its metabolites, and the internal standard, Trp. The standard mixture was separated on an Ultrasphere ODS column and detected at 214 nm. Acetonitrile concentration was kept at 7% for the first 5 min, raised to 20% in the next 3 min, and then increased 1% per min for the next 18 min, as shown by the dashed line. TGG, Tyr-Gly-Gly; TG, Tyr-Gly; PL, Phe-Leu; GGPL, Gly-Gly-Phe-Leu; GPL, Gly-Phe-Leu; ENK, Leu-enkephalin.

tinocytes or inactive at pH 7.4. The aminopeptidases which hydrolyze the AA-β-NA's are expressed early in differentiation and the specific activities of these enzymes remain constant for 15 days in culture (Fig. 2). The K_m value for Leu-β-NA in mouse epidermis (11 μ*M*) determined in this study was similar to that reported for pig epidermis (51 μ*M*) and human epidermis (73 μ*M*) but substantially lower than that reported for rat epidermis (357 μ*M*) (26,27). One possible reason for a high K_m value for the rat epidermis enzyme is that the value was obtained using a purified enzyme (27). The K_m value determined in this study and the values reported for pig and human epidermis were determined using crude cell homogenates. These crude cell homogenates may contain other enzymes which can hydrolyze this substrate and which have lower K_m values. These experiments were conducted with the cell homogenates and the metabolite profiles may be different in an intact cell system.

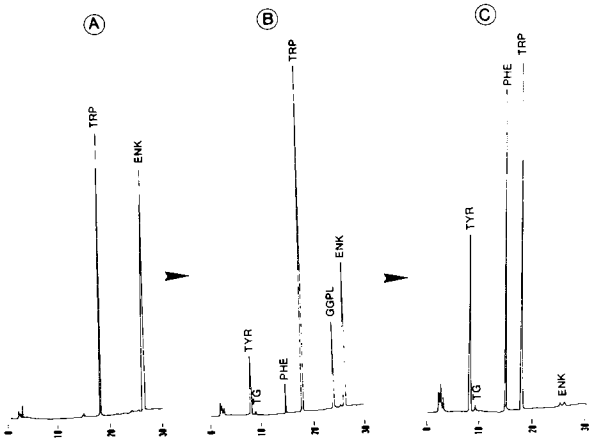

Fig. 4. Leu-enkephalin metabolism in the cultured mouse keratinocytes (5-day-old culture). Leu-enkephalin (1 m*M*) was incubated at 37°C for 1 min (A), 30 min (B), and 120 min (C). Aliquots were removed and analyzed by LC.

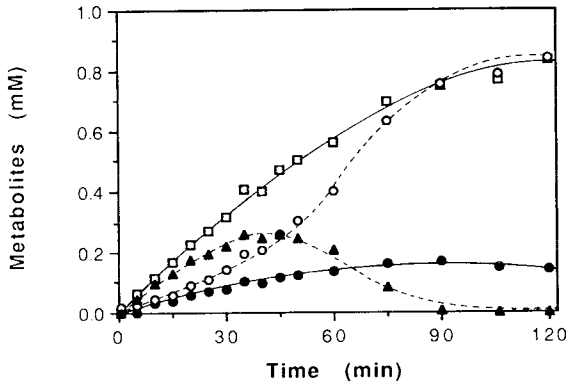

Fig. 5. Concentration–time profiles for the major metabolites produced in the mouse epidermis homogenate. Leu-enkephalin (1 m*M*) was incubated at 37°C in the epidermis homogenate; aliquots were removed and analyzed by LC for its metabolites. (□——□) Tyr; (●——●) Tyr-Gly; (○——○ Phe; (▲——▲) Gly-Gly-Phe-Leu. The curves are drawn to show the trends.

Leu-enkephalin hydrolysis by the homogenates from cultured mouse keratinocytes and neonatal mouse epidermis gave similar K_m, V_{max}, and K_i values and similar metabolite profiles. The hydrolytic rate constants for Leu-enkephalin calculated as done by Kashi and Lee (19) in the homogenates of mouse epidermis (0.027 min^{-1}) and cultured mouse keratinocytes (0.040 min^{-1}) are similar to the values obtained in the homogenates of various absorptive mucosae of the al-

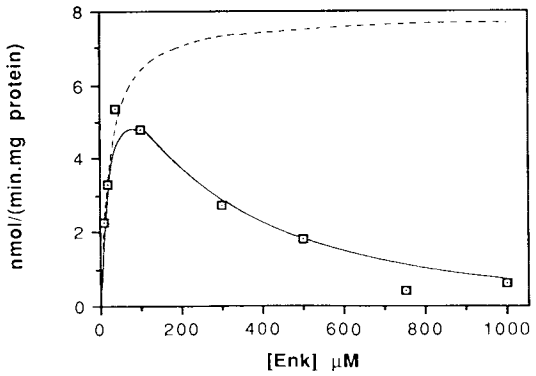

Fig. 6. Effect of Leu-enkephalin concentration on the rate of its metabolism in the mouse epidermis homogenate. Equal volumes of the homogenate and Leu-enkephalin were incubated at 37°C. A 100-μl sample was removed after 30 sec and analyzed by LC. The solid line is the fitted curve obtained by using Eq. (2), where K_m = 24 μ*M*, V_{max} = 7.55 nmol · min^{-1} · mg $protein^{-1}$, and K_i = 194 μ*M*. The dashed line is the theoretical curve expected when there is no substrate inhibition.

bino rabbit (19). The appearance of Tyr and TG peaks in the keratinocyte homogenates (Fig. 5) indicates the presence of both an aminopeptidase and an dipeptidyl peptidase in these cells. In separate studies, it was shown that inhibition of the aminopeptidases substantially decreases the rate of the hydrolysis of Leu-enkephalin in homogenates of cultured keratinocytes (data not shown). Metabolism by aminopeptidases has also been shown to be the major pathway for enkephalin degradation in other tissue homogenates (19–21). The preponderance of Tyr relative to TG and TGG suggests that aminopeptidases are the main peptidases acting on the Leu-enkephalin in cultured keratinocyte homogenates. The delayed appearance of Phe (Fig. 5) suggests the presence of a low level of a carboxypeptidase or a dicarboxypeptidase in the homogenate.

A peptide drug must cross the physical barrier of stratum corneum as well as the enzymatic barrier of skin before reaching the circulation. Cultured keratinocytes were used here to mimic this enzymatic barrier. It was shown that the cultured mouse keratinocytes are a useful *in vitro* model of the skin for studying metabolism of peptides.

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