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HYDROLYSIS OF ENKEPHALIN BY CULTURED HUMAN ENDOTHELIAL CELLS AND BY PURIFIED PEPTIDYL DIPEPTIDASE

Ervin G. Erdös, Alice R. Johnson and Nit T. Boyden

Departments of Pharmacology and Internal Medicine,
University of Texas Health Science Center at Dallas,
Dallas, TX 75235, U. S. A.

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The biological activities of enkephalins are of great interest to numerous investigators since they have an affinity for opiate receptors and may mediate perception of pain (1). Studies of enkephalins have been hampered, however, because these endogenous pentapeptides are rapidly degraded by blood and tissues. For example, radiolabeled enkephalin is hydrolyzed in less than 1 min when it is injected intraventricularly in rats (2).

Peptides transported in blood are continuously in contact with the vascular endothelium. These cells are more than just a diffusion barrier between blood and tissues since they contain enzymes that metabolize various peptides. Enzymes in vascular endothelial cells grown in culture cleave peptides such as bradykinin, angiotensin I and II (3) and substance P (4). Two of the enzymes in cultured human endothelial cells were identified as an aminopeptidase and a peptidyl dipeptidase (3). These two enzymes have important functions in the control of both hypotensive and hypertensive peptides. The aminopeptidase may inactivate angiotensin I or II by cleaving the N-terminal aspartic acid. The peptidyl dipeptidase cleaves a dipeptide from the C-terminal end of bradykinin and angiotensin I. By this action it converts angiotensin I to II and inactivates bradykinin (5,6). In addition to endothelial cells, the brush border of the kidney (6) and various parts of the central nervous system contain peptidyl dipeptidase activity (7,8). Because the substrate specificity of this enzyme (EC 3.4.15.1; kininase II or angiotensin I converting enzyme) indicated (6) that enkephalins may also be cleaved, we investigated the action of purified enzyme on synthetic leucine- and methionine-enkephalins. We determined which peptide bond was hydrolyzed and also characterized the mode of inactivation of enkephalins by cultured human endothelial cells.

Leu^5-enkephalin (Tyr^1-Gly^2-Gly^3-Phe^4-Leu^5; Leu-Enk) and Met^5-enkephalin (Met-enk) were obtained from commercial sources. Endothelial cells from veins of human umbilical cords were grown in monolayer cell cultures as described (3). Peptidyl dipeptidase was purified to homogeneity by the method of Oshima et al. (9). Leucine aminopeptidase from hog kidney (EC 3.4.11.1) was purchased from Boehringer Gmbh. Leu-enk or Met-enk (0.25 μmole) was incubated with 1.2 μg peptidyl dipeptidase or 0.12 μg aminopeptidase at 37° in 0.06 M NH_4HCO_3 buffer, pH 7.4. The molar ratio of peptidyl dipeptidase to substrate was 1 to

30,000. Cultured human endothelial cells were removed from monolayers by gentle scraping with a rubber spatula. These cells were then suspended and used in a concentration of one million/ml. The products of hydrolysis of enkephalins were determined by thin-layer chromatography (t.l.c.).

The rate of cleavage of Met-enk by peptidyl dipeptidase was determined in a programmed amino acid analyzer by separating Tyr-Gly-Gly and Phe-Met on a 5.5 X 0.9 cm column in 0.2 N citrate buffer, pH 4.25. The column was calibrated with the synthetic tripeptide and dipeptide. Substrate (0.2 µmole/ml) and enzyme (2.4 µg/ml) were incubated at 37° and pH 7.4 in 0.1 M Tris containing 0.1 M NaCl. In control studies, the cleavage of enkephalins was completely inhibited by one of the specific inhibitors of the enzyme: either by 10^{-4} M of the nonapeptide SQ 20881 (6) or by 10^{-6} M of SQ 14225 (2-D-methyl-3-mercaptopropanoyl-L-proline) (10).

Both Leu-enk and Met-enk were cleaved by peptidyl dipeptidase at the Gly^3-Phe^4 bond to Tyr^1-Gly^2-Gly^3 and to Phe^4-Leu^5 or Phe^4-Met^5. Figure 1 shows the separation of the peptides in t.l.c. in two different systems.

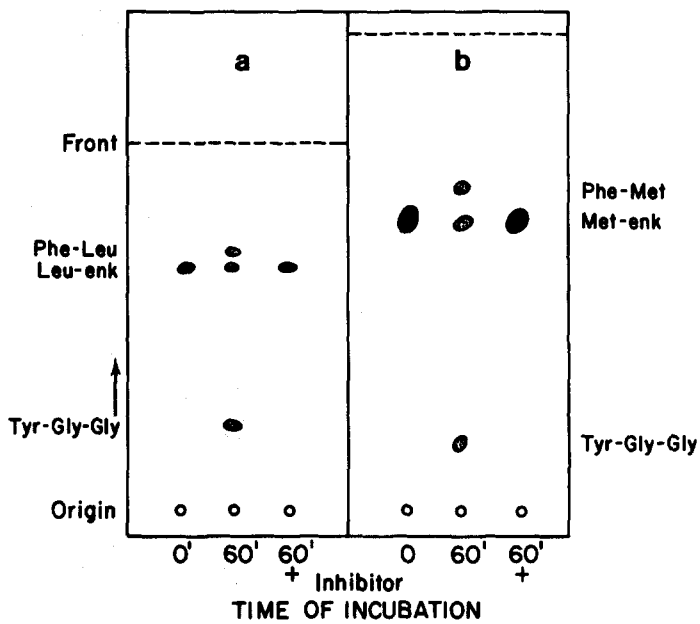

Fig. 1. Hydrolysis of Leu^5- and Met^5-enkephalin by purified hog kidney peptidyl dipeptidase (angiotensin I converting enzyme or kininase II) to Tyr-Gly-Gly and Phe-Leu or Phe-Met. The specific inhibitor SQ 14225 (10^{-6} M) inhibited the reaction. Peptides were incubated with the enzyme up to 60 min in the presence and absence of inhibitor. Panel a: Silica gel t.l.c. plate; solvent: sec-butanol-acetic acid-water (4:1:5); separation time: 3 hr. Panel b: Aluminum oxide t.l.c. plate; solvent: chloroform-methanol-NH_4OH (20:20:9); separation time: 1.5 hr. The plates were sprayed with ninhydrin.

The rate of hydrolysis of Met-enk was measured with an amino acid analyzer (Fig. 2). The enzyme and the substrate were incubated in a buffer more favorable for enzyme action than the one used for t.l.c. Instead of the NH_4HCO_3 buffer, the incubation medium contained 0.1 M NaCl in Tris buffer, since the enzyme cleaves most substrates faster in presence of Cl^- (6). Peptidyl dipeptidase cleaved Met-enk at a rate of 4.2 μmole/min/mg of protein and not at all in the presence of SQ 20881. The same enzyme preparation cleaved the specific substrate Bz-Gly-Gly-Gly (10^{-3} M) at a rate of 7.4 μmoles/min when determined in the u.v. spectrophotometer (5).

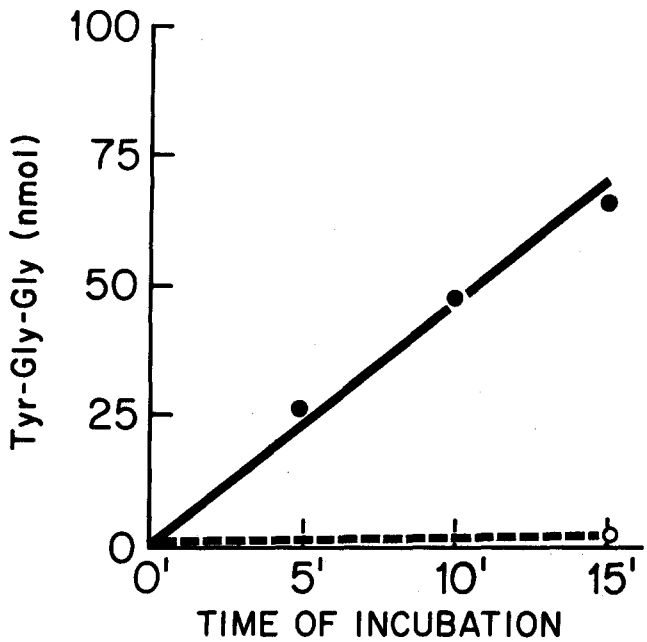

Fig. 2. Hydrolysis of Met^5-enkephalin by purified peptidyl dipeptidase determined in an automatic amino acid analyzer in the absence (●————●) and in the presence (O----------O) of the specific inhibitor SQ 20881 (10^{-4} M).

Because cultured endothelial cells contain both aminopeptidase- and peptidyl dipeptidase-type enzymes, we utilized t.l.c. to determine whether the N- or C-terminal end of enkephalin is hydrolyzed first by enzymes of these cells. Figure 3a shows the separation of products of hydrolysis of Leu-enk by endothelial cells. The Tyr^1-Gly^2 bond was cleaved first by the endothelial aminopeptidase. The enzyme was inhibited by the sequestering agent o-phenanthroline (10^{-3} M). In control studies, a highly purified commercial leucine

aminopeptidase (EC 3.4.11.1) was used (Fig. 3b) which also cleaved the pentapeptide to Tyr[1] and to tetrapeptide. We could detect the release of Phe-Leu by the action of endothelial peptidyl dipeptidase only after an incubation of 60 min or longer.

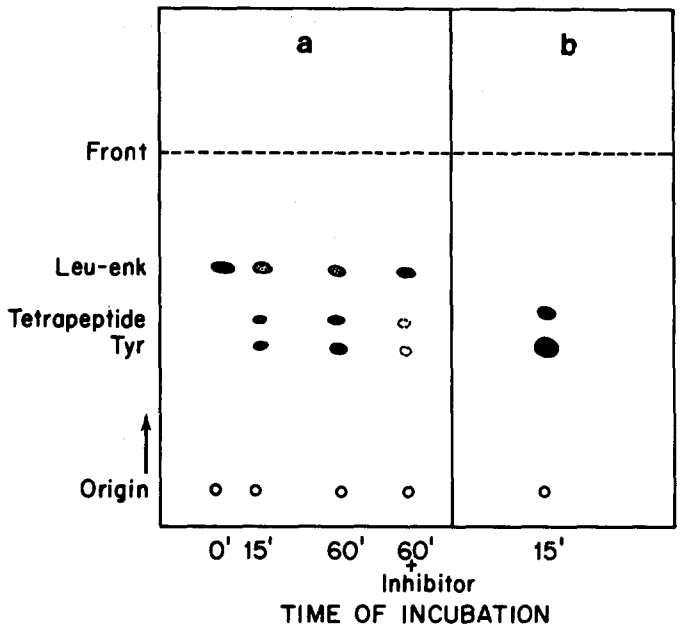

Fig. 3. Cleavage of Tyr[1] from Leu[5]-enkephalin by human vascular endothelial cells (a) and by purified leucine aminopeptidase (b). Inhibitor: o-phenanthroline (10^{-3} M). Silica gel t.l.c. plates were used and conditions were as in Fig. 1.

These experiments have shown that purified peptidyl dipeptidase cleaved enkephalins to tri- and dipeptide. The unprotected tripeptide liberated may be resistant to further degradation by the enzyme because of the free α-NH_2 group. Peptidyl dipeptidase is concentrated in various parts of the body, for example, in vascular endothelium of the lung and other organs, in kidney, in testicles (6), and in pituitary gland (7). In the central nervous system it is present in structures such as choroid plexus (11), caudate nucleus (8) or striatum (7).

The rate of cleavage of Met-enk by peptidyl dipeptidase is quite substantial. Under our conditions the rate was more than half of that of hydrolysis of Bz-Gly-Gly-Gly, the specific substrate of this enzyme, (5,6) although the concentration of enkephalin was 1/5 of that of the optically active tripeptide. Since only peptides with free C-terminal end are cleaved by peptidyl dipeptidase (6), resistance to the action of the enzyme may be one of the reasons for the markedly enhanced *in vivo* activity of the methionine amide analogue of enkephalin (12) over Met-enk. In tissues where peptidyl dipeptidase is con-

centrated, enkephalins may be readily degraded by this enzyme. Furthermore, by reacting with the enzyme enkephalins may interfere with the metabolism of other active peptides. Hypothetically they may inhibit the release of angiotensin II from angiotensin I or the inactivation of kinins by competing for the same enzyme.

Cultured human endothelial cells, however, inactivate enkephalins primarily by the action of an aminopeptidase, as demonstrated by the release of Tyr^1. The degradation of enkephalins by a similar aminopeptidase has been shown by using purified enzyme (13), plasma, brain homogenates (12,14), or a membrane fraction of rat brain (2) as sources of enzyme.

Our experiments indicate that rapid inactivation of enkephalins may be due to at least two different enzymes present in tissues. These enzymes presumably are localized in different structures of the cells. A peptidyl dipeptidase which is a component of plasma membrane of various cells (6) may degrade enkephalins by liberating a C-terminal dipeptide, and an aminopeptidase in endothelial cells may cleave peptides that are taken up as blood flows past the endothelial surface.

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