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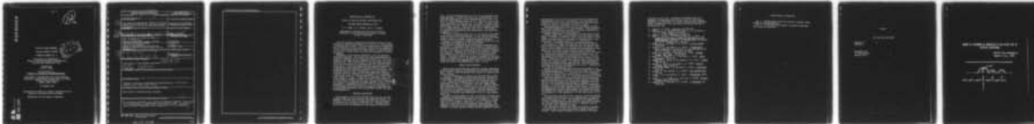

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Inactivation of Enkephalins: Effect of Purified Peptidyl Dipeptidase and Cultured Human Endothelial Cells

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For Publication in
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Cultured human endothelial cells inactivate enkephalins primarily by the action of an aminopeptidase, as demonstrated by the release of Tyr^1. In addition a purified peptidyl dipeptidase cleaved enkephalins to tri- and dipeptide.

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INACTIVATION OF ENKEPHALINS:

EFFECT OF PURIFIED PEPTIDYL DIPEPTIDASE AND

CULTURED HUMAN ENDOTHELIAL CELLS

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The rapid enzymatic degradation of enkephalins that has been observed by several investigators (2,3,7,8,13) is a major problem in studying their effects. The four peptide bonds in enkephalins may be cleaved by various peptidases. Because of the importance of this inactivation process we investigated it using endothelial cells grown in culture and a purified renal peptidase as sources of enzymes.

Peptidases in the vascular endothelial cells can hydrolyze blood-borne peptides. We recently described the metabolism of vasoactive peptides such as bradykinin, angiotensin I and II and substance P by human vascular endothelial cells grown in tissue culture (5,6). Two of the enzymes in cultured human endothelial cells were identified as an aminopeptidase and a peptidyl dipeptidase (5,6). These two enzymes may have important functions in metabolizing both hypotensive and hypertensive peptides. The aminopeptidase can hydrolyze 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 and by this action it converts angiotensin I to II and inactivates bradykinin (1,14). In addition to endothelial cells, the latter enzyme is present in other cell types in the kidney and in various parts of the central nervous system (11,15). Because the structure of enkephalins indicated that this kininase II or angiotensin I converting enzyme (E.C. 3.4.15.1) may cleave it, we investigated the action of purified peptidyl dipeptidase and cultured human endothelial cells on synthetic leucine- and methionine-enkephalins.

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MATERIALS AND METHODS

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 (5). Peptidyl dipeptidase was purified to homogeneity by the method of Oshima et al.

(10). Leucine aminopeptidase (E.C. 3.4.1.1) was purchased from Boehringer Gmbh. Leu-enk (0.25 μmol) was incubated with 1.2 μg peptidyl dipeptidase or 0.12 μg aminopeptidase at 37° for 0 to 60 min 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 cells per ml. The products of hydrolysis of enkephalins were determined by thin layer chromatography (t.l.c.).

Silica gel plates were used for t.l.c. of Leu-enk and aluminum oxide plates were used with Met-enk. In the former system sec-butanol: acetic acid: water (4:1:5) were the solvents and in the latter chloroform: methanol: ammonium hydroxide (20:20:9).

Products of cleavage of Leu-enk by endothelial cells or by aminopeptidase were separated on silica gel plates.

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 cm column in 0.2 N citrate buffer, pH 4.25. The column was calibrated with the synthetic tripeptide and dipeptide. Substrate (0.2 μmol/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 or by 10^{-6}M of SQ 14225 (2-D-methyl-3-mercaptopropanoyl-L-proline) (9).

RESULTS AND DISCUSSION

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 peptide bond cleaved in Leu-enk by the purified enzyme.

The rate of hydrolysis of Met-enk was measured with an amino acid analyzer. 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^- (1). Peptidyl dipeptidase cleaved Met-enk at a rate of 4.2 μmol/min per mg protein and not at all in 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 μmol/min when determined in the u.v. spectrophotometer (10).

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 (Fig.1). 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 (E.C. 3.4.11.1) was used 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.

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, and in pituitary gland. In the central nervous system it is present in structures such as choroid plexus, (4) caudate nucleus (11) or striatum (15).

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 (1,14), although the concentration of enkephalin was 1/5 of that of the optically active tripeptide.

Figure 2 shows the schematic representation of the active center of peptidyl dipeptidase as described by Ondetti et al. (9) and adopted from studies on carboxypeptidase A (12). The presumed fitting of Leu-enk to the surface of this protein is also shown. Leu^5 binds to a positively charged site by ionic forces at the free carboxyl groups. The Phe^4-Leu^5 bond contributes to attachment presumably by hydrogen bonding. The Gly^3-Phe^4 bond is cleaved by the zinc ion containing active site. Probably the Zn^{2+} could be exchanged with Co^{2+}.

Because of the substrate specificity of this enzyme (1) the following hypothetical enkephalin derivatives would not be cleaved by peptidyl dipeptidase: 1. The amino acid in the fifth position does not contain free carboxyl group or it is a dicarboxylic acid such as glutamic acid. 2. The fourth amino acid is proline.

Since only peptides with free C-terminal end are cleaved by peptidyl dipeptidase (1) 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 (7) over Met-enk. In tissues where peptidyl dipeptidase is concentrated, enkephalins may be readily degraded by this enzyme. Furthermore, by competing for the same enzyme enkephalins may interfere with the metabolism of other active peptides, and inhibit the release of angiotensin II from angiotensin I or the inactivation of kinins.

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 (3), plasma, brain homogenates (2,7), or a membrane fraction of rat brain (8) 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 (1) may degrade enkephalins by liberating a C-terminal dipeptide and an aminopeptidase in endothelial cells may cleave circulating peptides at the N-terminal end.

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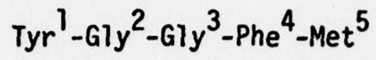
FIG. 1. Peptide bonds of Met-enk cleaved by peptidyl dipeptidase and by aminopeptidase.

FIG. 2. To show the active center of peptidyl dipeptidase with Leu-enk as substrate.

Figure 1



Peptidyl di-
peptidase.

Aminopeptidase
in cultured endo-
thelial cells.

SCHEME OF ATTACHMENT OF ENKEPHALIN TO THE ACTIVE SITE OF PEPTIDYL DIPEPTIDASE

(ACTIVE SITE ACCORDING TO ONDETTI ET AL., 1977)