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# PHOSPHORYLATION OF ENKEPHALINS ENHANCES THEIR PROTEOLYTIC STABILITY

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## Summary

Pharmacological action of enkephalins as opioid peptides is limited because of their rapid degradation by endoproteases. A novel approach is used in this study to prolong the life of those peptides. Phosphorylation of N-terminal tyrosine residue is found to have a profound influence in improving the stability of [Met]enkephalin and [Leu]enkephalin against the action of aminopeptidase M. Whereas, breakdown of [Met]enkephalin and [Leu]enkephalin is essentially complete in less than one min when incubated at 37 °C with purified aminopeptidase M (EC3.4.11.2; substrate:enzyme = 1:0.1) in Tris buffer (pH 7.02), the corresponding phospho analogs are still detected 60 min after start of incubation. The rate of disappearance of phospho-[Met]enkephalin and phospho-[Leu]enkephalin follows first-order kinetics with half-lives of 7.3 and 8.8 min, respectively.

*Key Words:* phosphorylation, phospho-enkephalins, methionine enkephalin, leucine enkephalin

The knowledge of opiate receptors has led to the discovery of endogenous opioid peptides. Known members are divided into three groups depending upon their source, viz., enkephalins, endorphins, and dynorphins (1). Enkephalins are biochemically synthesized from preproenkephalin A, endorphins from proopiomelanocortin (POMC), and dynorphins from preproenkephalin B. Each group has affinity for different type of receptors; enkephalins bind preferentially to δ-receptors, β-endorphin to δ- and μ-receptors, and dynorphins to κ-receptors (2).

The pentapeptides [Met]enkephalin (Tyr-Gly-Gly-Phe-Met-OH = YGGFM) and [Leu]enkephalin (Tyr-Gly-Gly-Phe-Leu-OH = YGGFL) are the two most important members of the enkephalin family of peptides. They are found in numerous body tissues and fluids, and are known to play a variety of physiological roles in a host of different organs. Because of their antinociceptive effect, they have been proposed as candidates for analgesic drugs (3). In this respect, enkephalins have distinct advantages over narcotic analgesics. Because these compounds are endogenous, they have minimal potential for side effects and drug dependency associated with the use of narcotics (3). Additionally, their metabolites are simple amino acids, which are also nontoxic.

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The use of enkephalins as analgesic agents, however, is hampered, because they are subjected to rapid degradation upon systematic administration by a variety of endogenous enzymes before they have a chance to bind to their receptors (4-10). For example, in one study, it was found that when [Met]enkephalin is injected into the rat blood stream, only 5% of the original amount remains intact after 15s (4). Similarly, [Leu]enkephalin is also degraded rapidly when administered to rat intraperitoneally (5). In vitro studies performed in rat plasma have reported that the half-lives of [Met]enkephalin (6) and [Leu] enkephalin are 2.0 and 2.5 min, respectively (6-8). By contrast, enkephalins are hydrolyzed more rapidly (half-life 0.7-1 min) in chick plasma (9) and more slowly in human (6,10) and mouse plasma (11).

At least four distinct types of enkephalin metabolizing enzymes have been characterized (12). Enzymatic hydrolysis is possible at all of the peptide backbone linkages in the sequence of enkephalins (see Figure 1). The primary route for degradation of [Met]enkephalin and [Leu]enkephalin is cleavage of the Tyr–Gly bond catalyzed by soluble and membrane-bound aminopeptidases. High concentrations of this enzyme have been reported in mammalian plasma (13). Next in importance as an enkephalin degrading enzyme is angiotensin-converting enzyme (ACE). Both ACE and endopeptidase-24.11 contribute to cleavage of the Gly–Phe bond. The Gly–Gly bond is cleaved by dipeptidylaminopeptidases. The Phe–Met (or Leu) bond in these pentapeptides is hydrolyzed by carboxypeptidases. The C-terminally extended [Met]enkephalin peptides that contain Arg or Lys (e.g., an octapeptide [Met]enkephalin-Arg-Gly-Leu) are additionally susceptible to the action of trypsin-like enzymes at the carboxyl side of a basic residue. In order to increase the potency and to prolong the activity in vivo of enkephalin opioids, their resistance to enzymatic degradation must be improved.

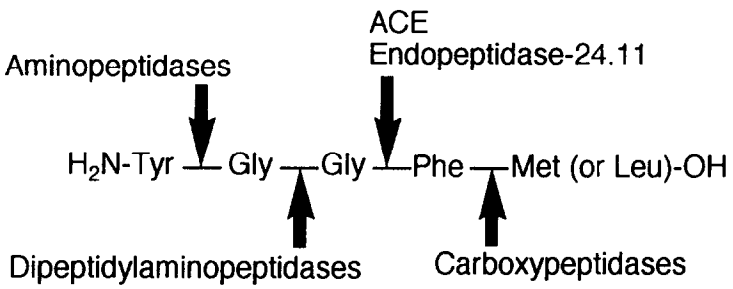


Fig. 1

The enzymes involved in and the sites of degradation of enkephalins

Several approaches have been used to extend the proteolytic stability of endogenous opioid peptides (3,14). New analogs of enkephalins that are resistant to endopeptidases have been designed. This objective has been accomplished by incorporation of unusual amino acids, substitution of D-amino acids for L-amino acids, N-methylation of the amide linkages, and replacement of the amide linkages. In another approach, potent and specific inhibitors of enkephalin-degrading enzymes have been developed to prolong the circulatory life of enkephalins. Development of agents that will release endogenous opioids has also been pursued.

In the present study, a novel approach is used to improve the proteolytic stability of enkephalin pentapeptides. We have synthesized Tyr-O-phosphate esters of [Met]enkephalin and [Leu]enkephalin and have demonstrated that phosphorylation of the N-terminal tyrosine residue of these endogenous substances considerably improves their resistance to endopeptidases. The stability of phospho-[Met]enkephalin and -[Leu]enkephalin was investigated by comparing the rate of disappearance of these phosphopeptides versus their nonphospho analogs against the action of purified aminopeptidase M.

## Methods

Enzymatic Digestion: In vitro hydrolysis of phospho- and nonphosphoenkephalins was carried out at physiological pH. The peptides were dissolved in Tris (2-amino-2-hydroxymethyl-1,3-propanediol) buffer (pH 7.02) and preincubated at 37 °C for 15 min. The required amount of aminopeptidase M (EC3.4.11.2) was added to adjust the substrate:enzyme ratio to 1.0:0.1, and the contents were incubated at 37 °C. In a typical experiment involving phospho-[Met]enkephalin, 188 μg of peptide was dissolved in 100 μl of Tris buffer. An aliquot (10 μl) was withdrawn at regular intervals in a tube. The enzymatic reaction was stopped by immersing the tube in boiling water followed by cooling on ice. Hydrolysis of the peptide was monitored by using reversed-phase high performance liquid chromatography (RP-HPLC).

Chromatography: RP-HPLC analysis of the reaction mixture was carried out by using an HP 1050 quaternary pumping system (Hewlett Packard, Palo Alto, CA, USA) outfitted with a photodiode array UV-visible detector. All separations were performed at ambient temperatures with a Vydac C-18 analytical column (250 x 4.6 mm I.D. with 5 μm particle size and 300 Å pore size). The mobile phase consisted of 20% acetonitrile in 10 mM ammonium acetate solution (pH 4.2). The components of hydrolysis were eluted at a flow rate of 1.5 ml/min and detected at 254 nm. Data acquisition and integration were provided by an LC ChemStation and accompanying software (Hewlett Packard). The components of hydrolysis were identified from retention times of the authentic compounds. Relative amounts of the substrates and the products of hydrolysis were calculated on the basis of chromatographic peak areas.

Materials: [Met]enkephalin, [Leu]enkephalin and aminopeptidase M (EC.4.11.2; porcine kidney microsomes) were purchased from Sigma Chemical Co. (St. Louis, MO) and were used without further purification. Phospho-[Met]enkephalin and -[Leu]enkephalin were synthesized in our laboratory using the Fmoc solid-phase peptide synthesis approach, details of which are being reported elsewhere.

## Results and Discussion

According to the cleavage scheme illustrated in Figure 1, the predominant reaction products of aminopeptidase M hydrolysis of phospho- and nonphospho-enkephalins are expected to be tyrosine (or phospho-tyrosine) and des-Tyr-enkephalins, along with the unreacted peptides. The RP-HPLC assay used here is able to separate efficiently the various components of hydrolysis. Under the above experimental conditions, phospho-tyrosine, tyrosine, phospho-[Met]enkephalin, des-Tyr-[Met]enkephalin (GGFM), and [Met]enkephalin elute at 1.83, 1.98, 2.22, 2.48, and 2.96 min (see Figure 3), respectively, and phospho-[Leu]enkephalin, des-Tyr-[Leu]enkephalin (GGFL), and [Leu]enkephalin at 2.69, 3.12, and 4.04 min, respectively.

As shown in Figure 2, the hydrolysis reaction of [Met]enkephalin (34.8 μg in 35 μl of Tris buffer) with aminopeptidase M (substrate:enzyme = 1.0:0.1) at 37 °C is essentially

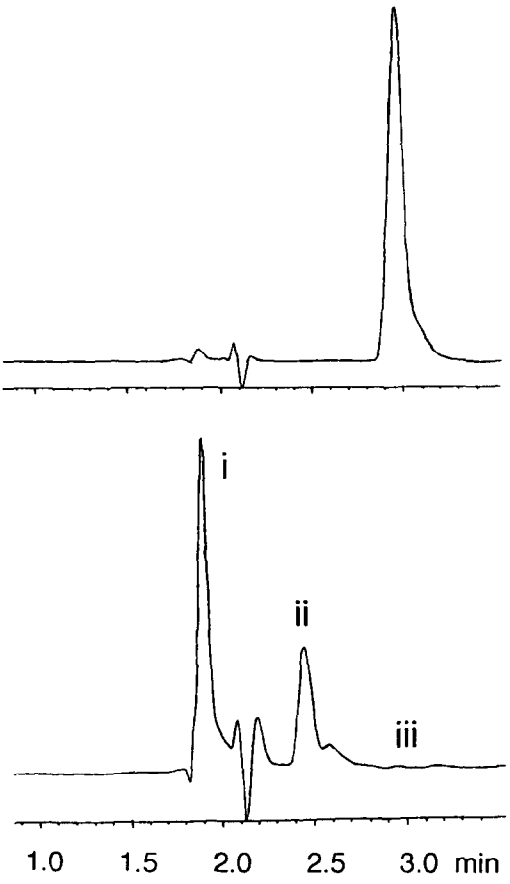


Fig. 2

Chromatograms showing hydrolysis of YGGFM by aminopeptidase M. Upper and lower chromatograms refer to reactions before and 40s after addition of the enzyme, respectively. Peaks i, ii, and iii are due to tyrosine, GGFM, and YGGFM, respectively).

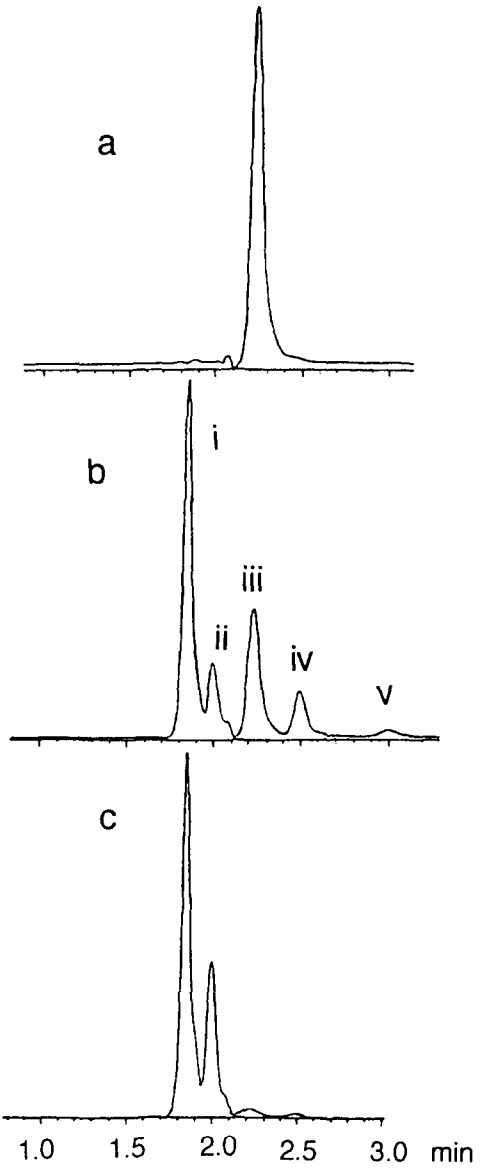


Fig. 3

Chromatograms showing hydrolysis of phospho-YGGFM by aminopeptidase M; *a* before addition of enzyme, and *b* and *c* 12 and 60 min, respectively, after addition of the enzyme. Peaks i, ii, iii, iv, and v are due to phospho-tyrosine, tyrosine, phospho-YGGFM, GGFM, and YGGFM, respectively.

complete within 40s; no detectable amounts of [Met]enkephalin are seen in the lower chromatogram (peak iii). This observation is consistent with the short in vivo circulatory life of [Met]enkephalin reported in literature (4,6). As expected, the dominant metabolic products are tyrosine and des-Tyr-[Met]enkephalin.

In contrast, under similar conditions, degradation of phospho-[Met]enkephalin is relatively slow. The amounts of intact phospho-[Met]enkephalin corresponding to approximately 26% and 3% of the total peak area are still present even after 12 and 60 min, respectively, of reaction with the enzyme (Figure 3). In addition to formation of phospho-tyrosine and GGFM, release of the phosphate group is also observed under the described experimental conditions. A small signal (< 1%) at the retention time of tyrosine appears for the first time in the two-min incubation mixture, and continues to grow parallel with the signal of phospho-tyrosine as time of incubation is increased. The signal at the retention time of [Met]enkephalin was maximum (4.5% of the total peak area) in the one-min incubation mixture, but diminished with time of incubation.

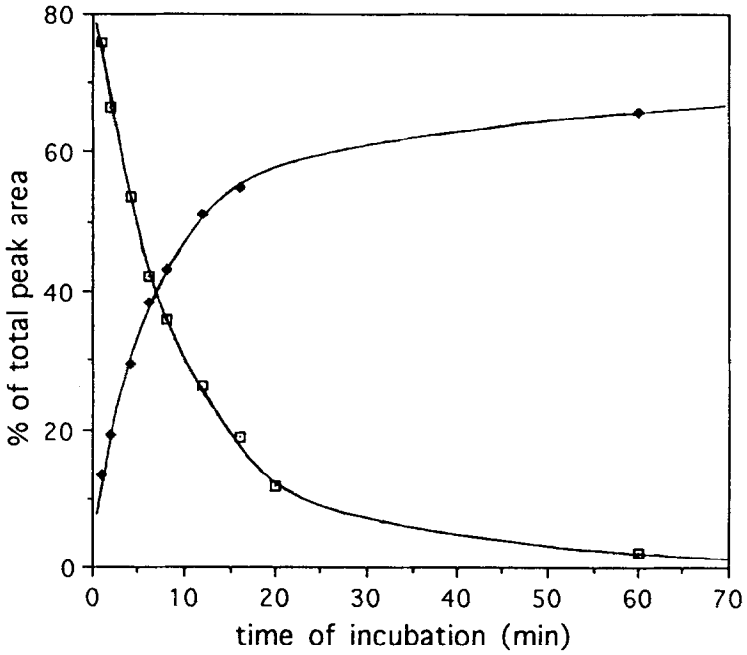


Fig. 4

Disappearance of phospho-[Met]enkephalin (open squares) and appearance of phospho-tyrosine (closed squares) when incubated with aminopeptidase M in Tris buffer (pH 7.02) at 37 °C (substrate:enzyme = 1.0:0.1). The term '% of total peak area' represents % of peak area of a specific component compared to the sum of peak areas of all components.

The time course for disappearance of phospho-[Met]enkephalin and appearance of phospho-tyrosine is shown in Figure 4. As measured by the amounts of the intact substrate remaining between one and 20 min of the reaction, the rate of disappearance of phospho-[Met]enkephalin under the above experimental conditions follows first-order

kinetics, and the half-life of phospho-[Met]enkephalin is calculated to be 7.3 min. As shown in Figure 4, the rate of appearance (9.1 nmol/min) of the major hydrolysis product phospho-tyrosine is practically parallel with the rate of disappearance (11.8 nmol/min) of phospho-[Met]enkephalin.

The results of aminopeptidase M hydrolysis of [Leu]enkephalin and its phospho-analog are equally dramatic. The hydrolysis of [Leu]enkephalin under the above experimental conditions is complete in less than one min. When subjected to aminopeptidase M hydrolysis, phospho-[Leu]enkephalin behaves similar to phospho-[Met]enkephalin. Degradation of phospho-[Leu]enkephalin is also slow; detectable amounts of intact phospho-[Leu]enkephalin (2% peak area) are present after 60 min of the reaction. The rate of disappearance of phospho-[Leu]enkephalin also follows first-order kinetics with the half-life of 8.8 min (see Figure 5).

Thus, from the results of hydrolysis of [Met]- and [Leu]enkephalins and their phospho analogs, it is evident that modification of the N-terminal tyrosine residue with the phosphate group confers resistance to aminopeptidase M degradation of these peptides. A plausible explanation for this effect is that the bulky phosphate group sterically hinders substrate-enzyme interaction. Other examples of steric hindrance involving the phosphate group are also known. For example, Tyr-O-phosphate esters were shown to suppress interaction between several ion-pairing reagents and the N-terminal amino group of enkephalin peptides (15).

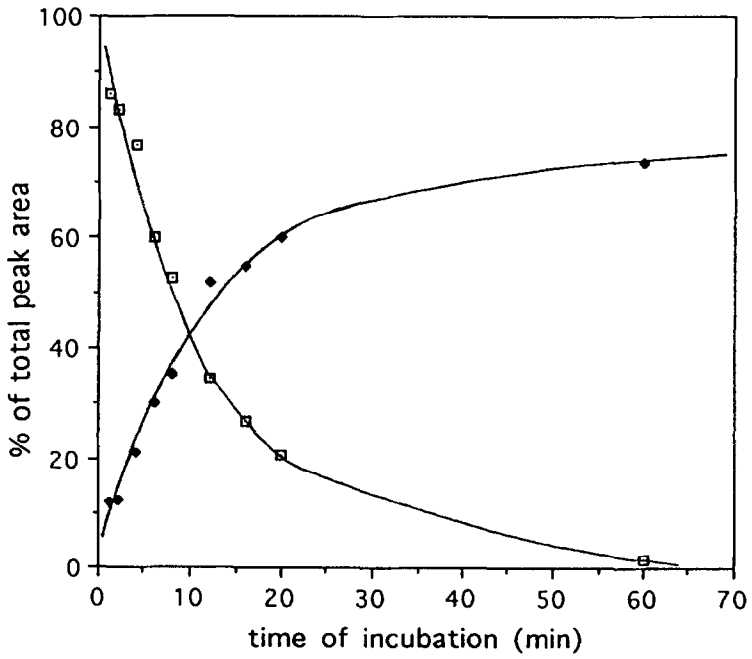


Fig. 5

Disappearance of phospho-[Leu]enkephalin (open squares) and appearance of phospho-tyrosine (closed squares) when incubated with aminopeptidase M in Tris buffer (pH 7.02) at 37 °C (substrate:enzyme = 1.0:0.1). The term '% of total peak area' represents % of peak area of a specific component compared to the sum of peak areas of all components.

## Conclusion

This preliminary study has demonstrated that stability of enkephalin peptides to proteolysis can be enhanced by phosphorylation of tyrosine residues and is of possible therapeutic significance. As a consequence of improving the proteolytic stability, these phosphoenkephalins have a potential for their use as exogenous opiates. When administered in the body, the phosphate group could be removed by the action of endogenous tyrosine phosphotases to release free bioactive enkephalins for analgesic activity. This protocol may allow physiological analgesia free from the major side effects of morphine. Our future research will address this issue.

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