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OPIATE ACTIVITY OF [DALA^2,Δ^ZPHE^4]-METHIONINE-ENKEPHALINAMIDE

by

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SUMMARY

The opiate-like activity of [DAla^2,Δ^ZPhe^4, Met^5]-enkephalinamide (ΔFE) was evaluated in the stimulated guinea pig ileum assay and the mouse tail-flick test. In vitro it caused a naloxone-reversible inhibition of the electrically induced twitch at a dose about one-fifth that of [DAla^2,Met^5]-enkephalinamide (DAE). The ED_{50} for the analgesic effect of ΔFE after intracerebral injection was also approximately one-fifth that of DAE. However, ΔFE was not active after intraperitoneal injection. These data imply that some increases in the potency of enkephalin derivatives may not be entirely attributable to resistance to enzymatic degradation, but may be related to preferred structural conformation at the receptor.

INTRODUCTION

Recent work on structural modifications of enkephalin (Tyr-Gly-Gly-Phe-Met-COOH) has been directed toward the synthesis of systemically active derivatives (1). Modifications found to be useful have been (a) substitutions at position two, e.g., by D-alanine or D-methionine (2), (b) methylation of the nitrogen at phenylalanine^4 (3), (c) alterations of position five, e.g., proline or oxidation of methionine (4) and (d) the derivatization of the carboxy terminal to the amide or the alcohol (3). All these changes have resulted in analogs with greater potencies than methionine-enkephalin itself.

Abbreviations used: ΔFE = [DAla^2,Δ^ZPhe^4,Met^5]-enkephalinamide; DAE= [DAla^2,Met^5]-enkephalinamide; i.c. = intracerebral

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The increased activity found with these structural changes has presumably been due to increased resistance to enzymatic degradation.

As an alternate approach to stabilization of the peptide, the α-β dehydro derivative of phenylalanine has been synthesized and incorporated into a [DAla^2,Met^5]-enkephalinamide analog. This peptide, Tyr-DAla-Gly-Δ^zPhe-Met-NH_2 (ΔFE)[1], has been shown to be resistant to degradation by chymotrypsin (5). It therefore appeared to represent a potentially fruitful new class of analogs. We report here the *in vitro* and *in vivo* activity of this pentapeptide compared to [DAla^2,Met^5]-enkephalinamide (DAE).

MATERIALS AND METHODS

Peptides

[DAla^2,Met^5]-Enkephalinamide was synthesized in this laboratory by standard solid phase techniques (6,7). Purity was ascertained by thin layer chromatography and high voltage electrophoresis; upon acid hydrolysis the compound showed the expected molar ratios of amino acids.

[DAla^2,Δ^zPhe^4,Met^5]-Enkephalinamide was synthesized as previously reported by English and Stammer (1978).

Stimulated Guinea Pig Ileum Assay: Whole ileal strips (n=3/drug) from male Hartley guinea pigs (Dutchland, Inc.) were used according to the method of Ehrenpreis (8) in 10 ml tissue baths containing Tyrode's solution bubbled with 98% O_2-2%CO_2 maintained at 37°. Isometric contractions were recorded on a Grass Polygraph via a Grass Model FT03C force transducer. Contractions were elicited with a Grass Model S4K stimulator using the following parameters: 0.2 Hz, 0.4 msec, and 100 volts. See Chipkin *et al.* (9) for further details.

Mouse Tail-Flick Analgesia Test: Male Swiss-Webster mice (Charles River, Inc.) were tested according to the method of Dewey and Harris (10) with slight modifications. Initial control latencies were first determined, and then the peptide was injected intracerebrally (10µl/mouse) using the technique of Haley and McCormick (11). Tail-flick latencies were re-determined at 10, 30, 45, 60, and 90 min. post-injection . A 6 sec cut-off was used to avoid tissue damage in cases of maximal drug effectiveness. The data are expressed as per cent of the maximal possible effect as calculated by the following equation: %MPE = (Test Latency - Control Latency) / (6-Control Latency) X 100%. In experiments examining naloxone reversibility, the antagonist (5 mg/kg) was given subcutaneously five min before the i.c. injection of the peptide.

RESULTS

Figure 1A shows the effects of [DAla^2,Δ^zPhe^4,Met^5]-enkephalinamide and [DAla^2,Met^5]-enkephalinamide on stimulated guinea pig ileum. As can be seen,

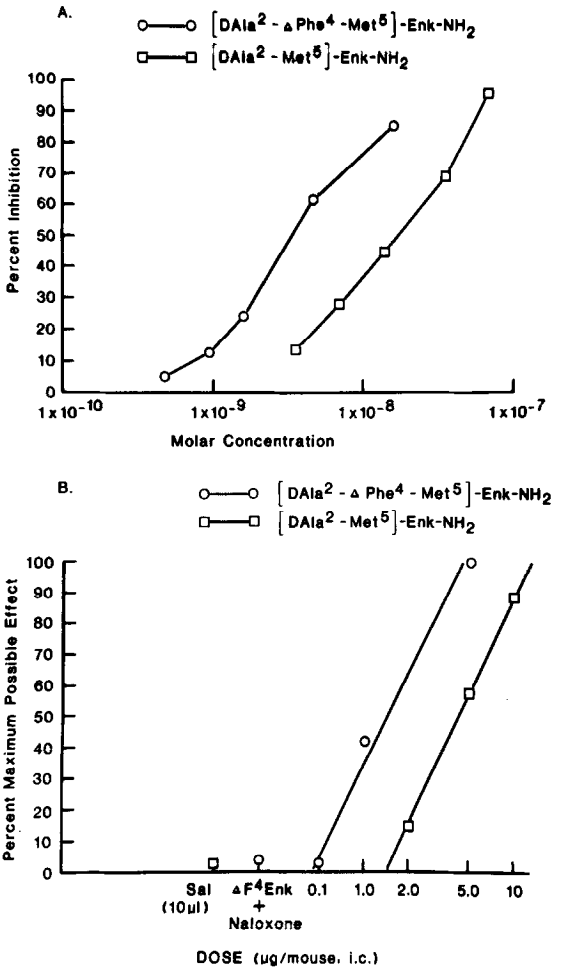

Figure 1: (A) The inhibitory effects of [DAla^2, Δ^zPhe^4, Met^5]- and [DAla^2, Met^5]-enkephalinamide on stimulated guinea pig ileum. Each point is the mean of three experiments (B) The anitnociceptive effects of [DAla^2,Δ^zPhe^4,Met^5]- and [DAla^2,Met^5]-enkephalinamide in the tail-flick analgesic assay. Each point represents the mean of six mice. The challenge dose of ΔFE used to test for naloxone reversibility was 5 µg/mouse, i.c.

ΔFE is about five times as potent as DAE (ED_{50}'s = 3.3×10^{-9}M and 1.7×10^{-8}M, respectively). The effects of both peptides could be entirely reversed by either washing or addition of naloxone (5×10^{-8}M).

The intracerebral analgesic activities at 10 min post-injection for ΔFE and DAE are presented in Figure 1B. For both peptides there is a typical dose-response relationship between the percent analgesia and the amount administered. As on ileum, ΔFE is of greater potency than DAE, and the potency increase is

approximately the same as *in vitro* (ED_{50}'s = 1.4 μg/mouse and 4.3 μg/mouse, respectively). The duration of action of the two peptides over the 90 min test period was comparable (data not presented). The animals displayed typical narcotic behavioral signs after either ΔFE or DAE, *e.g.*, Straub tail and hyperactivity. The i.c. analgesic effects of ΔFE were completely prevented by naloxone pretreatment (see Fig. 1B). No analgesia was seen after intraperitoneal injection of ΔFE at doses up to 30 mg/kg.

DISCUSSION

The search for systemically active enkephalin derivatives has been directed toward the synthesis of analogs resistant to enzymatic degradation. The primary sites of inactivation of enkephalin appear to be at the tyrosylglycine bond, the glycyl-phenylalanine bond, and the carboxyterminal (12,13). [DAla^2,Δ^ZPhe^4, Met^5]-enkephalinamide was synthesized in an attempt to block these sites of degradation. It has been previously suggested that D-alanine in position two confers enzymatic resistance upon enkephalin (2) and derivatization to the amide presumably inhibits degradation by carboxypeptidases. It had been hoped that replacement of phenylalanine by dehydrophenylalanine might add enough additional protection to the molecule to make it active by systemic administration. Although the initial *in vitro* and *in vivo* work showed that ΔFE has about four to five times the potency of [DAla^2,Met^5]-enkephalinamide, the peptide displayed no antinociceptive activity following intraperitoneal injection.

The lack of analgesic activity following peripheral administration of this peptide suggests that enzymes with other than chymotrypsin-like activities may degrade enkephalin peptides *in vivo* and that the enhanced activity of ΔFE observed may not be entirely due to resistance to enzymic degradation. Indeed, Bajusz, *et al.* (14) have previously suggested that not all increases in the potencies of enkephalin derivatives can be ascribed to enzyme stability. In this case, the increase in potency of ΔFE is the same proportionally whether the drug is given *in vitro* or *in vivo*. Presumably, in the former case the rate of degradation plays a minimal role in determining the compound's action,

while in the latter case anzymatic breakdown should play a more prominent role. Whereas the potency of [Met^5]-enkephalinamide is comparable to that of [DAla^2, Met^5]-enkephalinamide on stimulated ileum, the latter peptide is much more potent when their i.c. analgesic activities are compared (Chipkin, unpublished observations). Enzyme stability of DAE presumably is the basis of this difference. However, the striking similarity of the potency ratios of ΔFE to DAE in both test systems implies that a mechanism other than enzyme resistance may play a role in the enhanced activity of ΔFE.

The α-β double bond in dehydrophenylalanine sharply restricts the conformational freedom of the peptide. The entire amino acid skeleton of ΔPhe is planar, and in the z isomer synthesized the phenyl ring and the carboxyl of the Phe^4 residue are trans. Since this enkephalin analog has high potency, it follows that the receptor-bound conformation of enkephalin must have the phenylalanine side chain folded back toward the tyrosine residue, as it is in ΔFE. In this conformation enkephalin mimics most closely the rigid, compact structure of morphine, and the enhanced potency of ΔFE is probably due to the fact that the normally quite flexible side chain of the phenylalanine residue is held rigidly in the conformation necessary for receptor activation.

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REFERENCES

1. Frederickson, R.C.A. (1977) Life Sci. 21,23-42.
2. Pert, C.B., Pert, A., Chang, J.-K., and Fong, B.W. (1976) Science 194, 330-332.
3. Roemer, D., Buescher, H.H., Hill, R.C., Pless, J., Bauer, W., Cardinaux, F., Closse, A., Hauser, D., and Huguenin, R. (1977) Nature 268, 547-549.
4. Bajusz, S., Ronai, A.Z., Szekely, J.I., Graf, L., Dunai-Kovacs, Z. and Berzetei, I. (1977) FEBS Letters 76, 91-92.
5. English, M.L., and Stammer, C.H. (1978) Biochem. Biophys. Res. Comm. 85, 780-782.
6. Stewart, J.M. and Young, J.D. (1969) Solid Phase Peptide Synthesis, W.H. Freeman and Co., San Francisco.
7. Palmer, M.R., Morris, D.H.,Taylor, D.A., Stewart, J.M., and Hoffer, B.J. (1978) Life Sci. 23, 851-860.
8. Ehrenpreis, S. (1975) Methods in Narcotic Research, pp. 111-125, Marcel Dekker, Inc., New York.

9. Chipkin, R.E., Stewart, J.M. and Morris, D.H. (1978) Eur. J. Pharmacol. 53, 21-27.
10. Dewey, W.L. and Harris, L.S. (1975) Methods in Narcotic Research, pp. 101-109, Marcel Dekker, Inc., New York.
11. Haley, T.J. and McCormick, W.G. (1957) Brit. J. Pharmacol. 12, 12-15.
12. Meek, J.L., Yang, H.-Y.T., and Costa, E. (1977) Neuropharmacol. 16, 151-154.
13. Erdös, E.G., Johnson, A.R., and Boyden, N.T. (1978) Adv. Biochem. Pharmacol. 18, 45-59.
14. Bajusz, S., Pathy, A., Kenessey, A., Graf, L., Szekely, J.I., and Ronai, A.Z. (1978) Biochem. Biophys. Res. Comm. 84, 1045-1053.