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SHORT COMMUNICATIONS

Effect of enkephalin and endorphin analogs on receptors in the mouse vas deferens

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Several peptides with potent opiate-like activity have recently been isolated from the mammalian central nervous system [1–5]. At least four of these peptides, generically referred to as endorphins, have amino acid sequences identical to fragments of the pituitary peptide β-lipotropin, (β-LPH$^{1-91}$). These are α-endorphin (β-LPH$^{61-76}$), γ-endorphin (β-LPH$^{61-77}$), β-endorphin (β-LPH$^{61-91}$) and met^5-enkephalin (β-LPH$^{61-65}$). In addition, Hughes *et al.* [6] have isolated leu^5-enkephalin, which has leucine instead of methionine at the 5 position (position 65 of the endorphin sequence). These agents have been shown to be active in mouse vas deferens [6–9], and guinea pig ileum bioassays [8–11], as well as opiate receptor binding assays *in vitro* [6–16]. They also have behavioral effects in experimental animals [15, 17–20]. The present investigation, using the mouse vas deferens preparation, was undertaken to determine the agonist activities of a series of endorphins in which the natural peptide sequence was altered by substitution of a single amino acid. Peptides were synthesized by solid phase techniques, as previously described [7].

Swiss–Webster albino mice were used in the assay, which is similar to that of Henderson *et al.* [21]. The vasa deferentia were removed and suspended individually in a 10-ml tissue bath with electrodes positioned above and below the tissue. Contractions were elicited by stimulation at maximal voltage for a duration of 1.0 msec at a pulse frequency of 0.2/sec. Peptides were dissolved in distilled water and added to the tissue bath in volumes no greater than 100 μl. The potency of each peptide was determined by its ability to inhibit the contractile response. Each compound was used at a series of concentrations and a dose–response curve was constructed relating the concentration of peptide to the per cent inhibition. The dose which inhibited the amplitude of the contraction by 50 per cent (ED_{50}) was calculated from the dose–response curve. The viability and responsiveness of each tissue were confirmed with an agent of known potency ([D-Ala^2]-γ-endorphin). The specificity of inhibition induced by each analog was verified by reversal of its effect with naloxone.

Our results indicate that there is approximately a 10-fold increase in potency resulting from substitution of D-alanine for the glycine residue at the 2 position of the peptide sequence of either α-, $β_h$- or γ-endorphin (Fig. 1). We also evaluated [leu^5]-$β_h$-endorphin, an analog of β-endorphin which contains leucine rather than methionine in the 5 position. The ED_{50} for this agent is approximately 2.7×10^{-8} M, compared to 4.5×10^{-8} M for $β_h$-endorphin.

As shown in Table 1, there is more than a 10,000 fold variation in the activity of the varous substituted analogs of met^5-enkephalin. Morphine sulfate, with an ED_{50} of 1.3×10^{-7} M, has an intermediate potency. Substitution of either β-alanine at the 2 position or D-alanine at the 3 position of met^5-enkephalin markedly reduced the activity of the pentapeptide. [D-Ala^2]-enkephalin ethyl amide ($ED_{50} = 1.5 \times 10^{-9}$ M) is also more potent than met^5-enkephalin and, as shown in Table 1, *N*-acetylation of this compound reduced its activity to approximately 0.1 per cent of that of met^5-enkephalin. The analog with D-tyrosine at the 1 position of met^5-enkephalin was also markedly less active than the natural analog containing the L-isomer.

It has previously been shown that the D-alanine^2 analog of methionine enkephalin is more potent *in vivo* [18, 22] and *in vitro* [7, 8, 15] than its naturally occurring analog. Since endogenous enkephalin is readily degraded in several systems [8, 13, 22–25], the enhanced effect of the D-Ala^2 analog has been attributed to its ability to resist degradation, a phenomenon which has been demonstrated by several authors [13, 22, 23].

We report here that the D-Ala^2 analogs of the α, β and γ-endorphins are more potent effectors of the opiate

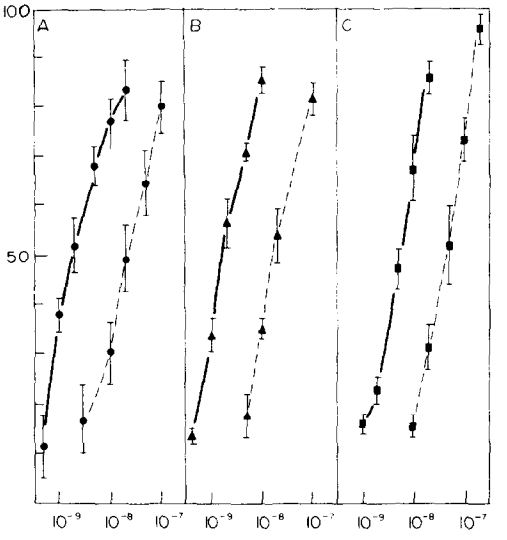

Fig. 1. Dose–response curves showing the inhibition of electrically stimulated contractions of mouse vas deferens by α-, γ- and β-endorphins and their [D-Ala^2]-substituted analogs. Panel A: α-endorphin (– – ● – –). and [D-Ala^2]-α-endorphin (—●—). Panel B: γ-endorphin (– –▲– –) and D-Ala^2]-γ-endorphin (—▲—). Panel C: $β_h$-endorphin (– –■– –) and [D-Ala^2]-$β_h$-endorphin (—■—). Individual vas deferentia were removed, trimmed of adhering fatty tissue, and the lumenal contents expelled. Procedures were similar to those described by Henderson *et al.* [21] Tissues were suspended in tissue baths containing 10 ml of a modified Krebs buffer (pH 7.3) bubbled with 95% O_2: 5% CO_2 and maintained at 37°. The preparations were stimulated by electrodes positioned above and below the tissues. Stimulation was at a frequency of 0.2 pulses/sec for a 1.0-msec duration at maximal voltage. Contractions were recorded via Grass pressure transducers attached to a Grass model 7 polygraph. Endorphins and their synthetic analogs were added to the bath in volumes of 10–100 μl, and tissues were rinsed four or five times between each test. Each point on the curves represents the mean ± S.E.M. for four to six tissues.

Table 1. Opioid activity of endorphin analogs in the mouse vas deferens bioassay*

	ED_{50} (M)
Morphine	1.3×10^{-7}
α-Endorphin	2.0×10^{-8}
[D-Ala^2]-α-endorphin	1.9×10^{-9}
γ-Endorphin	1.8×10^{-8}
[D-Ala^2]-γ-endorphine	1.5×10^{-9}
β_h-Endorphin	4.5×10^{-8}
[D-Ala^2]-β_h-endorphin	5.6×10^{-9}
[Leu^5]-β_h-endorphin	2.7×10^{-8}
Met^5-enkephalin	4.0×10^{-9}
N-acetyl-[D-Ala^2]-enkephalin amide	4.5×10^{-6}
Enkephalin ethyl amide	1.5×10^{-9}
[D-Ala^2]-enkephalin amide	1.0×10^{-9}
[D-Leu^2]-enkephalin	6.0×10^{-9}
[D-Phe^2]-enkephaline	1.4×10^{-8}
[Sar^2]-enkephalin	2.0×10^{-6}
[β-Ala^2]-enkephalin	4.0×10^{-6}
[D-Ala^3]-enkephalin	5.6×10^{-5}
[D-Tyr^1]-enkephalin	6.0×10^{-6}

* Relative potencies of various endorphins and their synthetic analogs. All β-endorphin analogs are of the β_h type, the amino acid sequencing of which corresponds to that found in the β-endorphin molecule in man. The ED_{50} values represent the molar concentration of the peptide required to produce a 50 per cent inhibition of the amplitude of the electrically stimulated contraction of the mouse vas deferens.

receptor(s) in the mouse vas deferens than their unsubstituted counterparts. These results may indicate that D-Ala^2 substitution enhanced the affinity of the agonist for its receptor, in addition to altering its susceptibility to degradation. This conclusion is based on the fact that, in contrast to the brain and guinea pig ileum preparations [10, 13, 14, 26, 27], degradation of the enkephalins and endorphins is not marked in the vas deferens preparation. In our hands the maximum effects of these agents are reached within 60 sec, and their decay is slow even at low concentrations. Under these circumstances, it is unlikely that there are serious losses in the activity of the unmodified enkephalin owing to degradation by the vas deferens. In addition, the endorphins are much more resistant to proteolysis than are the smaller enkephalins in a variety of systems [17, 26, 27].

Given the low proteolytic activity of the vas deferens and the relative stability of the endorphins, it is probable that decreased susceptibility to proteolysis is not the major factor in the enhanced effect of the D-alanine substituted analogs. A more likely explanation is that these analogs have an increased affinity for the opiate receptor(s) in this tissue. In fact, it is likely that the molecules with D-Ala^2 substituents have a more stable β-I bend [3, 11], a conformation which may be preferred for binding to the opiate receptor(s) [28]. However, these conclusions are based on indirect evidence, and their verification will require more direct experimental proof.

With respect to the other tested analogs, the relative activities of the unaltered enkephalins and endorphins in this system are comparable to those which have been seen by other investigators [8]. The finding that leucine^5-β endorphin is slightly more effective than methionine^5-β endorphin is in accord with the results obtained with the analogous enkephalins in this system [8], and the relative activities of the D-Ala^2 amide, D-Ala^3 and D-tyr^1 analogs confirm our preliminary results [7] and the findings of others [8].

D-Alanine is clearly the most potent substituent for glycine at the 2 position of the enkephalins, but other substituents are also effective, especially D-leu^2 and D-phe^2.

The striking decrease in activity of [D-Ala^2]-enkephalin ethyl amide with *N*-acetylation provides additional evidence for the importance of the free amino group on the tyrosine moiety in position 1 of the molecule [15, 29].

Results obtained with a series of analogs *in vitro* in a given system are not necessarily predictive of results which will be obtained *in vitro* or *in vivo* in any other system for several reasons. First, there are multiple opiate receptors, the distribution of which may differ from system to system [8]. Secondly, there are multiple proteolytic enzymes which can degrade the enkephalins [10, 13], and these too may vary from system to system. Finally, the effect *in vivo* of these agents may be influenced by restriction of their access to the receptor site.

Nevertheless, our conclusion that the structure-dependent interaction of the endorphin molecule with its receptor is as important for its potency as its resistance to degradation, is similar to that reached by Bradbury *et al.* [19] on the basis of data obtained *in vivo*.

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Enhancement of renal gluconeogenesis by clofibrate

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Recently, Mackerer and Haettinger [1] showed that rats treated with clofibrate (0.3% of diet) have increased rates of renal gluconeogenesis associated with increased activities of glucose 6-phosphatase (G-6-Pase), pyruvate carboxylase (PC) and phosphoenolpyruvate carboxykinase (PEPCK). The mechanism by which the drug enhances gluconeogenesis is unknown, but a possible explanation is that there is a modulation of normal hormonal activity and a homeostatic re-equilibration. Clofibric acid, the active moiety of clofibrate [2], displaces thyroxine from circulating plasma proteins, thereby causing a fall in total plasma thyroxine followed by thyroxine accumulation in both liver and kidney [3]. Administration of thyroxine to rats increases the rate of renal glucose synthesis from pyruvate and succinate [4] and increases the activities of pyruvate carboxylase [5] and glucose 6-phosphatase [6]. Administration of thyroxine to thyroidectomized rats greatly increases the activities of pyruvate carboxylase, phosphoenolpyruvate carboxykinase and glucose 6-phosphatase [7]. The following experiments were performed to determine whether the clofibrate enhancement of renal gluconeogenesis is mediated by thyroxine.

Normal and thyroidectomized rats (CR–CD strain) were obtained from Charles River Breeding Laboratories, Wilmington, Mass. and were allowed to stabilize for 1 and 6 weeks, respectively, prior to the beginning of the study. During this time the rats were individually housed and fed pellets of Rockland mouse/rat diet (complete) *ad lib.* Normal rats received tap water and thyroidectomized rats received Hank's solution [8]. After the stabilization period, the pelleted diet was replaced with powdered diet or powdered diet containing 0.3% (w/w) clofibrate. After 7

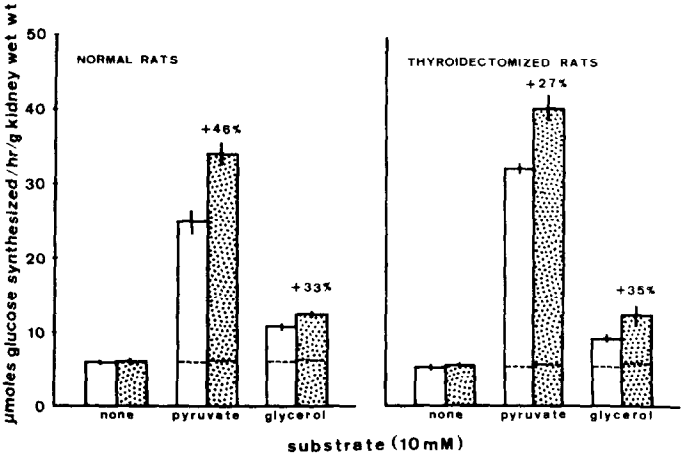

Fig. 1. Clofibrate-induced enhancement of glucose synthesis by rat kidney cortex slices from normal and thyroidectomized rats. Values are means ± S.E.M. for six rats. The per cent increase caused by clofibrate (after subtraction of basal rates in the absence of substrate) is indicated.