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INCREASED ANALGESIC ACTIVITIES OF A FLUORINATED AND A DIMERIC ANALOGUE OF [D-ALA-2]-METHIONINE ENKEPHALINAMIDE

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SUMMARY

[D-Ala-2, pentafluorophenylalanine-4]-Met-enkephalinamide and N^{α},N^{e}-bis (D-Ala-2-Met-enkephalin)-Lys amide were synthesized by solid-phase techniques. They were found to be at least 10 and 4 times more potent respectively, and longer lasting, than the highly active parent peptide, [D-Ala-2]-Met-enkephalinamide, in producing analgesia in the rat after central injection. In the mouse vas deferens in vitro assay, the results from which frequently do not correlate well with in vivo assessment of the opiate activity of peptides, both analogues were slightly less active than [D-Ala-2]-enkephalinamide. Thus, a fluorinated and a dimeric analogue of enkephalin were devised and found to have markedly increased analgesic activity.

The side-chain of the phenylalanine residue in position 4 of the enkephalin pentapeptides (1) appears to be very critical for opiate activity. Since the aromatic character of this position must be maintained, few opportunities exist for carrying out interesting structure-activity studies without resorting to the use of unusual amino acid substitutions. We have recently become interested in the use of pentafluorophenylalanine (2) as a replacement for phenylalanine in a number of peptides. This should considerably increase their lipophilicity without having serious effects on conformation since the size of the fluorinated side-chain would remain essentially unchanged. In addition, the highly fluorinated analogues might be used to study peptide-receptor interactions by ^{19}F NMR spectroscopy. L-Pentafluorophenylalanine was synthesized, derivatized, and incorporated into [D-Ala^{2}]-enkephalinamide, an opiate peptide analogue which was shown previously (3, 4) to be highly potent.

We reported (5) the syntheses of several branched-chain analogues of luteinizing hormone-releasing hormone which are highly active possibly because of their ability to bind with more than one receptor per molecule of peptide.

Abbreviations: F_5Phe, pentafluorophenylalanine; Boc, tert.-butyloxycarbonyl

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This approach has now been extended to the enkephalin series by the synthesis of a dimer of [D-Ala^2]-enkephalin built via the α- and e-amino groups of Lys amide.

MATERIALS AND METHODS

L-F_5Phe: D,L-F_5Phe was synthesized by the route of Filler et al. (2) and resolved by digestion of the N-trifluoroacetyl derivatives with carboxypeptidase A using the conditions described by Turk et al. (6). L-F_5Phe: $[\alpha]_D$ +27.3° (c, 1.1 in H_2O, T=27°); anal. calcd. for $C_9H_6F_5NO_2$: C, 42.36; N, 2.37; H, 5.49. Found: C, 42.21; N, 2.32; H, 5.56.

Boc-L-F_5Phe: was prepared by reaction of L-F_5Phe with Boc-azide in the presence of tetramethylguanidine by a standard procedure (7): mp 106°; $[\alpha]_D$ -5.9° (c, 1.1 in MeOH, T=26°); anal. calcd. for $C_{14}H_{14}F_5NO_4$: C, 47.35; H, 3.97; N, 3.94. Found: C, 47.29; H, 4.01; N, 3.89.

Peptide Synthesis: Peptides were assembled using Boc-protected amino acids on a benzhydrylamine resin support (8) by a cycle of events described previously (9), beginning with Boc-Met in the case of the fluorinated peptide and bis-Boc-Lys for the dimeric analogue. Removal of both Boc-groups on the Lys-resin enabled the remainder of the peptide chain to grow from both the α- and e-amino groups of Lys. The hydroxyl group of Tyr was protected with the 2-bromocarbobenzoxy group and, at the completion of the solid-phase part of the synthesis, the final Boc-group on Tyr was removed by acidolysis prior to hydrogen fluoride cleavage to avoid alkylation of Met (10).

Free peptide amides were liberated by treatment with hydrogen fluoride containing 10% anisole at 0° (11) and were readily purified by gel filtration on Sephadex G 15 by elution with 2 M acetic acid followed by partition chromatography on Sephadex G 25 using 1-butanol:acetic acid:H_2O (4:1:5) as the eluant. [D-Ala-2,F_5Phe-4]-enkephalinamide, $[\alpha]_D$ +36° (c, 0.5 in 2 M acetic acid, T=22°), gave the following amino acid analysis after hydrolysis (110°, 18 h) in 4 M methanesulfonic acid: Gly, 1.00; Ala, 0.98; Met + F_5Phe, 2.02; Tyr, 0.99. The following R_f's were obtained on silica gel thin layer chromatography plates using solvent systems and conditions described previously (4): R_f(I), 0.58; R_f(II), 0.84; R_f(III), 0.70; R_f(IV), 0.68.

N^{α},N^{e}-bis(D-Ala-2-enkephalin)-Lys amide, $[\alpha]_D$ +32° (c, 0.5 in 2 M acetic acid, T=22°), gave the following amino acid analysis: Gly, 1.96; Ala, 2.00; Met, 1.52; Tyr, 2.00; Phe, 2.06; Lys, 1.00. The following R_f's were obtained: R_f(I), 0.32; R_f(II), 0.88; R_f(III), 0.59; R_f(IV), 0.64.

Analgesia Assay: Male albino (Holtzman) rats, 90-120 days old were housed individually and fed ad libitum throughout the experiments. At least one week before testing, ventricular cannulae were surgically implanted into the left lateral ventricle of each animal. Stereotaxic coordinates were 1 mm posterior to the bregma, 1.5 mm lateral to the midline, and 4.1 mm below the surface of the skull (with lambda and bregma at the same dorso-ventral level). Dental acrylic and stainless steel hooks were used to secure the cannulae to the skull. Cannulae were constructed from 22 ga stainless steel hypodermic tubing (thin wall) which were sharpened and beveled at the tips. A microsyringe with a 26 ga. needle was used for injections; the needle extended 1 mm beyond the tip of the cannulae.

Analgesia was measured by the tail flick test of D'Amour and Smith (12). This widely used test has the advantages of being relatively selective for narcotics (13), highly quantitative, and insensitive to experimenter bias. The apparatus consisted of an adjustable source of radiant heat which was focused on the animal's tail. The application of power to the heat source started a solid state latency timer which was stopped when withdrawal of the tail allowed the light from the heat source to activate a photocell. A digital readout of the latency to tail withdrawal was used as the index of pain sensitivity.

Preliminary experiments were first conducted to determine an appropriate dosage for comparing the three analogues. Based on these tests, the dose of 25 µg (10 µl volume) was selected. In order to assure the accurate placement of cannula, each rat was tested first with 20 µg of morphine ICV. Only those animals who showed a response to this treatment were used to compare the analogues. At least a week after screening with morphine, each rat received four tests separated by 48 hrs: 1) a control test with Ringer's solution, 2) [D-Ala-2]-Enk-NH_2, 3) dimer and 4) [D-Ala-2,F_5Phe-4]-Enk-NH_2. The order of drug treatment was counter-balanced by Latin square. Since there was an N of 8, this procedure resulted in a 4 (order of drug) x 2 (subject) x 4 (day) design.

For an individual testing session, the output of the heat source was adjusted to obtain tail-withdrawal latencies of 3-5 seconds. Tail-flick latencies were then measured every 5 minutes for a baseline period of 15 minutes. The peptide or control solution was then administered and testing continued, at 5 minute intervals, for at least one hour. Testing ceased after this time when an animal's tail flick latency dropped below 30% of its baseline mean for three consecutive trials. When the peptide effect continued for over two hours, testing was carried out at half hour intervals.

After the completion of these tests two animals were given additional tests with the opiate-antagonist naloxone (Endo) to assess the role of opiate receptors in the analgesia produced by the 2 new analogues. In these tests the animals were allowed to develop an analgesic response to the analogue; naloxone (2 mg/kg) was then administered and the time course of analgesia observed.

Vas Deferens Bioassay: Peptides were tested for inhibitory effects on electrically stimulated contractions of mouse vasa deferentia (14). The details of the technique have been described elsewhere (4).

RESULTS AND DISCUSSION

As shown in Fig. 1, each analogue produced a marked increase in tail-flick latency which lasted from 1 to 5 hrs. The area under the curve for each treatment was computed by the approximation of Simpson (15) to give an estimate of the magnitude of the effect of the three analogues. The means ± SEM after injection of the solution were: Ringer's = 7.7 ± 5.65; [D-Ala^2]-enkephalin-NH_2 = 119.52 ± 29.98; dimer = 479.66 ± 169.24; [D-Ala^2,F_5Phe^4]-enkephalin-NH_2 = 1392.62 ± 423.88. Since the means were proportional to the standard deviations, the analysis was conducted on the $\log_{10}$ of the raw scores as recommended by Meyers (16). A one way repeated measures analysis of variance revealed a significant difference among the four treatments ($3,21$, $F = 11.56$, $p = 0.0001$). Further analysis indicated that the effects of all three analogues were significantly greater than the Ringer's control (Newman Keuls: $p < 0.05$, all comparisons).

The possibility that tolerance or other carry-over effects from repeated treatments significantly influenced the data was tested by a separate 4 (order

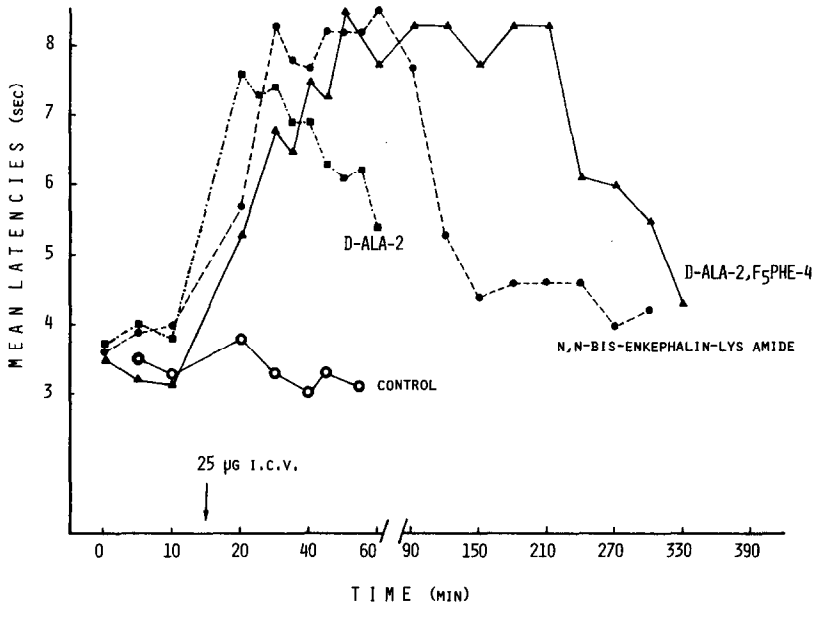

Fig. 1: Mean rat tail-flick latencies over extended periods of time after intracerebroventricular injection of Ringer's solution (control) and 25 µg amounts of [D-Ala-2]-enkephalinamide, [D-Ala-2, F_5Phe^4]-enkephalinamide, and N^{α},N^{e}-bis(D-Ala-2-enkephalin)-Lys amide.

of treatment) x 2 (subject) x 4 (day of test) repeated measures analysis of variance on $\log_{10}$ transformed data. Although neither order of treatment (3, 4, $F = 2.56$, $p = .19$) nor the day of test ($F < 1$) had a significant effect, the order x day interaction, which reflects the difference among treatments, was highly significant (9, 12, $F = 4.69$, $p < 0.01$). Moreover, computations of ω^2 indicated that order of test accounted for only 12% of the variance; similarly, a negative value found for the day of test variable indicated that the best estimate of its contribution to the total variance was zero. The powerful effects of the analogues were emphasized by the finding that 70% of the variance was accounted for by the order x day interaction. Obviously, the remaining variance was between subjects.

Finally, it was found that naloxone (2 mg/kg) completely reversed the analgesic and motor effects of the treatment with the analogues. All animals tested with these analogues showed a marked rise in tail flick latency after peptide treatment which returned to normal within ten minutes after treatment with naloxone.

TABLE I

Activities of Enkephalin Analogues
in the Mouse Vas Deferens Bioassay

Peptide	ED_{50} (M) ± SEM
[D-Ala^2]-enkephalinamide	2.17 (± 0.22) x 10^{-9}
[D-Ala^2, F_5Phe^4]-enkephalinamide	4.37 (± 0.52) x 10^{-9}
N^{α},N^{e}-bis(D-Ala^2-enkephalin)-Lys amide	3.58 (± 0.31) x 10^{-9}

The analgesia produced by the three analogues of enkephalin was generally accompanied by other behavioral changes. As we have observed previously with other opioid peptides (17, 18), short bursts of coarse tremors were frequently observed within 5 minutes after injection. Animals often showed transient periods of hyperactivity and hypersensitivity to touch. One sign not previously observed was a severe muscular rigidity after administration of [D-Ala^2, F_5Phe^4]-enkephalinamide. Rendered totally insensible, animals failed to show the corneal reflex or any reaction to exceedingly noxious pinch. Other gross behavioral changes included apparent exophthalmos, severe respiratory depression, and straub tail.

In the vas deferens assay, [D-Ala^2]-enkephalinamide was found to have an ED_{50} value (Table I) of 2.17 x 10^{-9} M which is similar to that previously determined (4). The F_5Phe-analogue and the dimer were about 2 and 1.5 times less active than the parent peptide, respectively. The effects of these peptides were reversed by addition of naloxone to the bath.

It is difficult to attribute the decreased in vitro activities and greatly increased in vivo activities of these two peptides to increased resistance to enzymatic breakdown under experimental conditions. The structural modifications of the analogues do not appear to account for this, although some very specific enzyme systems could be involved in the inactivation of endogenous enkephalins. Since all peptides were injected centrally in the analgesia test, ease of passage

across the blood-brain barrier also cannot be a factor. It has been observed previously, however, among both enkephalin (19) and endorphin (20) analogues, that estimates of potency derived from induction of analgesia, from the release of growth hormone and prolactin, from the guinea pig ileum and mouse vas deferens tests, and from behavioral tests (21), show considerable divergence. It has been suggested (19) that these paradoxical results may reflect the presence of more than one type of opiate receptor site, of different receptor populations from tissue to tissue, and of dissociated effects of opiate peptide analogues on these receptors.

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