[D-Thr^2,Thz^5]- and [D-Met^2,Thz^5]-Enkephalinamides: Potent Analgesics by Intravenous Injection

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SUMMARY: Two enkephalin analogs, [D-Met^2,Thz^5]-enkephalinamide and [D-Thr^2,Thz^5]-enkephalinamide, have been synthesized by the solid-phase method. When injected centrally, [D-Thr^2,Thz^5]-enkephalinamide is 3.5 times more potent than the [D-Met^2,Thz^5] analog. However, the two are equipotent and 4.2-4.8 times more potent than morphine when injected intravenously.

It has recently been reported that [D-Met^2,Pro^5]-enkephalinamide is a potent analgesic agent (1). It occurs to us that replacement of proline in position 5 by thiazolidine-4-carboxylic acid (Thz)** may enhance analgesic activity. Thz has been used as replacement for proline in biologically active peptides (2,3). In addition, the 2-position in met-enkephalin appears to be sensitive to the nature of the side-chain (4-6). We decided to synthesize [D-Met^2,Thz^5]- and [D-Thr^2,Thz^5]-enkephalinamide. These two new enkephalin analogs were found to be potent analgesics by intravenous injections in mice.

Materials and Methods

N-Boc-O-benzyl-D-threonine was prepared in the same way as the L-isomer (7) from D-threonine (Vega-Fox Biochemicals): mp 114-116°, $[\alpha]_D^{24}$-16.40 (c 2.25 methanol). Anal. calcd. for $C_{16}H_{23}NO_5$ (309.36): C. 62.12; H, 7.49; N, 4.53. Found: C, 62.17; H, 7.42; N, 4.39. The L-isomer has been reported (7) to have mp 115-116°, $[\alpha]_D$+15.8° (c 1.1, methanol).

Synthesis and characterization of peptides. Benzyhydrylamine resin (1.505 g, 0.51 mmole/N/g, Beckman) was treated for 20 min with Boc-L-thiazolidine-4-carboxylic acid (4.87 mmole)(2) that had been converted to the symmetrical anhydride by dicyclohexylcarbodiimide

**Abbreviation: Thz, L-thiazolidine-4-carboxylic acid.

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(2.25 mmole)(8). After removal of the Boc group the Gisin amine test (9) was used by a procedure in which excess picric acid is washed out with 95% ethanol-CH_2Cl_2 (1:9, v/v). No amine was detected which suggested that the imino group of the Thz residue is not basic enough to hold picric acid in the presence of 95% ethanol. Synthesis was continued by schedules described previously (10). Boc-D-Met-OH (11), Boc(Bzl)-D-Thr-OH, and Boc(Z)Tyr-OH (12) were used. After removal of the Boc group of Phe, amine determination showed 0.23 mmole/g. After incorporation of Gly, the resin was divided in half for synthesis of the two analogs. The final yield of the protected pentapeptide resins were 0.86 g each. The last Boc group was removed with trifluoroacetic acid (13).

Each peptide resin was treated in liquid HF (15 ml) in the presence of anisole (2 ml) for 65 min at 0° (14,15). The resulting products were isolated by gel filtration on Sephadex G-10 in 0.5 N acetic acid. Purification was effected by partition chromatography (16) in a 1.91 x 28 column of Sephadex G-25 in 1-butanol/3.5% acetic acid-1.5% pyridine (1:1) to yield H-Tyr-D-Met-Gly-Phe-Thz-NH_2 (I), R_f 0.50, 23.5 mg and H-Tyr-D-Thr-Gly-Phe-Thz-NH_2 (II), R_f 0.31, 21.0 mg. Each was homogeneous on thin-layer chromatography (ninhydrin and Cl_2-tolidine detection) in 1-butanol/pyridine/acetic acid/water (30:20:6:24) with R_f 0.77 (I) and R_f 0.75 (II) and in 1-butanol/acetic acid/water (4:1:5) with R_f 0.55 (I) and R_f 0.45 (II). Each was homogeneous on paper electrophoresis (Whatman 3 MM, 400 V, 6 hr) at pH 6.7 with R_f 0.40 (I) and R_f 0.38 (II) and at pH 3.7 with R_f 0.45 (I) and R_f 0.45 (II), all relative to Lys (ninhydrin detection). Amino acid analyses of 24 hr hydrolysates in 6 N HCl gave for I; Thz, 0.82; Gly, 0.99; Met, 1.04; Tyr, 1.01; Phe, 1.00; and for II: Thz+Thr, 1.7; Gly, 0.96; Tyr, 0.99; Phe, 1.00. Since Tyr values in Thz-containing peptides are low (3), they were obtained from analyses of 24-hr hydrolysates in 4 N methanesulfonic acid relative to Phe. Since Thz and Thr appeared at the same position on the analyzer, their sum in II was estimated from their known integration constants both at 570 nm and at 440 nm. As reported previously (3) Thz is partly destroyed in hydrolysis.

In both syntheses, a significant by-product was isolated by partition chromatography amounting to one-half that of the major product; R_f 0.45 in the synthesis of I and R_f 0.060 in that of II. In the by-product of I, Phe was missing which indicates that the low basicity of the imino group of Thz resulted in incomplete incorporation of Phe.

For analgesic assay, male ICR mice weighing 25-30 g (Simonson Labs) were used in all the experiments. The enkephalin analogs dissolved in saline were administered either centrally according to the method described by Haley and McCormick (18) or intravenously via the tail vein. The injection volume is 5 µl for central injection and 0.01 ml per gram body weight for intravenous injection. Naloxone HCl (3 mg/kg) was injected subcutaneously 5 min before the administration of enkephalin analogs. Analgesic activity was assayed by the tail-flick method (19).

The percent of analgesia was calculated as $[(T_1-T_0)/(T_2-T_0)] \times 100$, where a control latency (T_0) was obtained from the mean of two latencies determined before drug injection; the test latencies (T_1) were determined at various times after injection for each animal; the cutoff time (T_2) for the tail-flick test was 7 seconds. The median analgesic dose (AD_{50}) and 95% confidence limits were calculated according to the

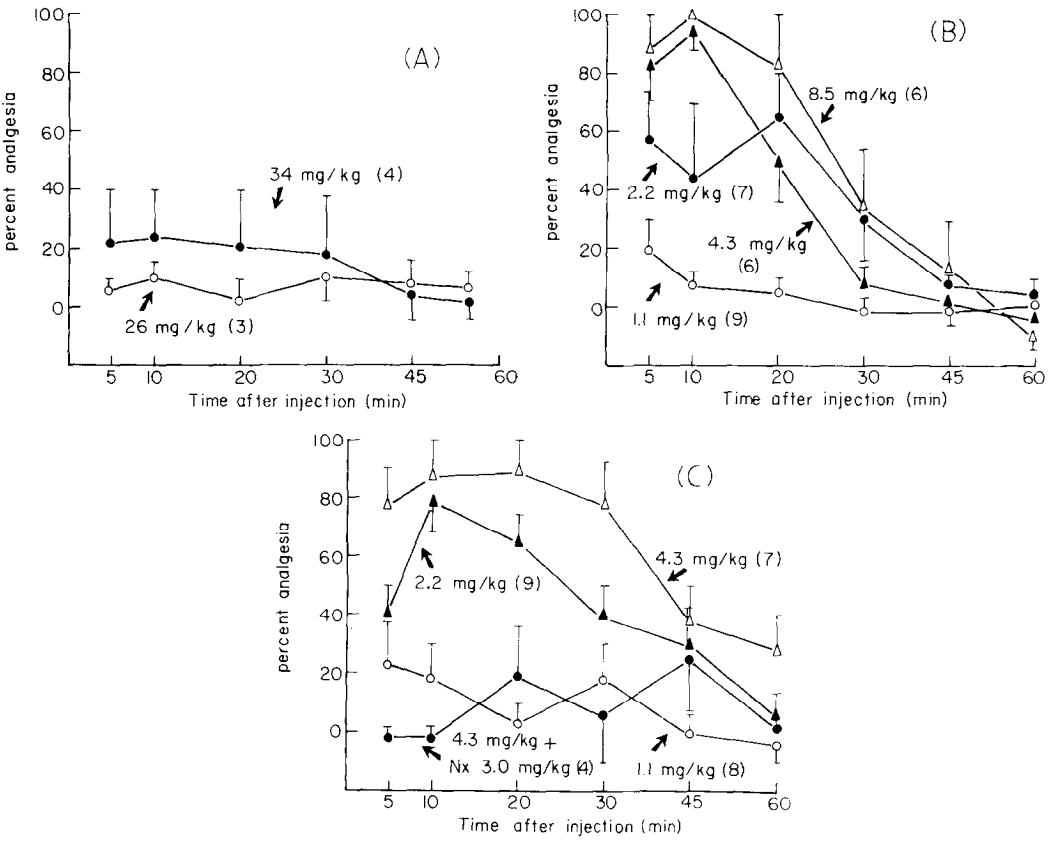

Fig. 1. Antinociceptive effects following the intravenous injection of (a) Met^5-enkephalin; (b) [D-Met^2,Thz^5]-enkephalinamide; (c) [D-Thr^2,Thz^5]-enkephalinamide in mice. The antinociceptive effect was measured by tail-flick test. The peptides were injected intravenously via the tail vein and naloxone, HCl, 3 mg/kg was injected subcutaneously 5 min before the injection of [D-Thr^2,Thz^5]-enkephalinamide. Number of animals in parenthe

method of Litchfield and Wilcoxon (20). The locomotor activity of mice was measured with an electronic Fe 40 motility meter (Motron Produkter, Stockholm, Sweden). The detail of the experimental method has been described (21).

Results and Discussion

[D-Met^2,Thz^5]-enkephalinamide (I) and [D-Thr^2,Thz^5]-enkephalin-

Table 1

Median Antinociceptive Doses (AD_{50}) of Morphine Sulfate and Opioid Peptides after Intravenous and Intracerebroventricular Injections in Mice

Compound	Intravenous		Intracerebroventricular	
	AD_{50}[a] μmole/kg	Potency ratio	AD_{50}[a] nmole/mouse	Potency ratio
Morphine sulfate	11.4[b]	1	1.11[c]	1
β_c-Endorphin	2.7[b]	4.2	0.032[c]	34.7
D-Ala^2,D-Leu^5-Enkephalin	≈30[c]	≈0.4	0.035[c]	31.7
Met-enkephalin	>60	<0.19	>174	<0.006
D-Met^2,Thz^5-Enkephalinamide	2.71 (1.75-4.19)	4.2	0.145 (0.070-0.305)	7.7
D-Thr^2,Thz^5-Enkephalinamide	2.40 (1.60-3.62)	4.8	0.04 (0.026-0.063)	27.1

[a] 95% confidence limits in parentheses.

[b] Taken from (22).

[c] Taken from (21).

amide (II) have been synthesized by the solid-phase method (17) by a route designed to preserve the integrity of the Thz residue (2,3). The peptides were obtained in a high state of purity by partition chromatography, paper electrophoresis, and amino acid analysis. It was noted in the synthesis that the imino group of the Thz residue showed low basicity and low reactivity in the coupling step. The latter observation was confirmed in the synthesis of I by the isolation of the deletion peptide H-Tyr-D-Met-Gly-Thz-NH_2 during partition chromatography.

[D-Met^2,Thz^5]-enkephalinamide and [D-Thr^2,Thz^5]-enkephalinamide in doses of 0.17 to 0.85 μg and 0.011 to 0.085 μg respectively applied centrally induced a dose-related increase in intensity and

duration of the tail-flick inhibition. The inhibition of the tail-flick response was mediated by opiate-like action as evidenced by the finding that it was blocked by the pretreatment of naloxone. As summarized in Table 1, the AD_{50} of [D-Met^3,Thz^5]-enkephalinamide was 3.5 times higher than [D-Thr^2,Thz^5]-enkephalinamide. On molar basis, they are 7.7 and 27.1 times respectively more potent than morphine. In addition to the inhibition of tail-flick response, the mice exhibited strong Straub tail and increased locomotor activity. Thus the behavior responses induced by these two pentapeptides are similar to morphine and [D-Ala^2,Leu^5]-enkephalin (21) and were different from that produced by β-endorphin which has previously been shown to stimulate locomotor activity weakly or not at all (21).

[D-Met^2,Thz^5]-enkephalinamide and [D-Thr^2,Thz^5]-enkephalinamide in doses of 1.1 to 8.5 mg/kg injected intravenously were also active in inhibiting the tail-flick response. The duration and intensity of analgesia were dose-related (see Figure 1). The duration of analgesia produced by [D-Thr^2,Thz^5]-enkephalinamide appeared to be longer than than produced by [D-Met^2,Thz^5]-enkephalinamide. [D-Thr^2,Thz^5]-enkephalinamide is 3.5 times more potent than [D-Met^2,Thz^5]-enkephalinamide when applied centrally, but the two are equipotent and 4.2-4.8 times more potent than morphine by intravenous injection (see Table 1).

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