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The Effects of Methionine-Enkephalin and Its Related Metabolites Upon the Duration of the Dorsal Immobility Response in Rats

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MEYER, M. E. *The effects of methionine-enkephalin and its related metabolites upon the duration of the dorsal immobility response in rats.* PHARMACOL BIOCHEM BEHAV **46**(4) 841–845, 1993.—The effects of SC injections of methionine-enkephalin (Met$^{1-5}$-Enk) and its *N*-terminal and *C*-terminal fragments upon the duration of the dorsal immobility response (DIR) over a 60-min time course were investigated. Experiment 1 analyzed the effects of various dosages (0.00–100.0 μg/kg) on DIR resulting in a potentiation of the duration in a dose-time course function. The effects of various fragments of Met$^{1-5}$-Enk (10.0 μg/kg) from the *N*-terminal in Experiment 2 and from the *C*-terminal in Experiment 3 on the DIR resulted in the potentiation of the duration with the Met$^{2-5}$-Enk and Met$^{1-3}$-Enk fragments. All other fragments were not significant. The results were discussed in reference to the processing and metabolism of Met$^{1-5}$-Enk.

Methionine-enkephalin Methionine-enkephalin fragments Dorsal immobility response

THE pentapeptides methionine-enkephalin (Met$^{1-5}$-Enk; Tyr-Gly-Gly-Phe-Met) and leucine-enkephalin (Leu$^{1-5}$-Enk; Tyr-Gly-Gly-Phe-Leu) are endogenous opioid peptides and function as neuromodulators within the CNS (5,13,18). These two enkephalins differ in a single residue at the *C*-terminus.

Today much is known about the metabolism of the enkephalins (13,18,24). The two major metabolic processes for the enkephalins are the cleavage of Tyr^1-Gly^2 by membrane-bound aminopeptidase, particularly aminopeptidase N, and the hydrolysis of Gly^3-Phe^4 by endopeptidase 24.11 (3,4,9,10, 17,21,22). There are two minor processes, one at the cleavage of Gly^2-Gly^3 by dipeptidyl aminopeptidase and one at the cleavage of Phe^4-Met^5 by carboxypeptidase. The two major metabolites, Gly-Gly-Phe-Met and Tyr-Gly-Gly, have been of particular interest. The first amino acid in the Met$^{1-5}$-Enk, Tyr^1 is essential for opioid activity of this peptide in the guinea pig ileum mouse vas deferens or receptor binding assays. In general, Met-Enk has a high affinity for δ receptors (18).

When systemically administered, both opiates and the opioid pentapeptides alter the acquisition and extinction of avoidance conditioning, maze performances, duration of tonic immobility and inhibitory behaviors, and induce analgesia (1,2, 6,8,15,16,19,23). Recently, it has been reported that various metabolites of Leu-enkephalin (Tyr-Gly; Try-Gly-Gly; Tyr-Gly-Gly-Phe; Gly-Gly-Phe-Leu) impaired the acquisition of avoidance conditioning, but some of these fragments did not influence locomotor activity (11,12,26).

The dorsal immobility response (DIR) is one of a number of complex inhibitory responses that can be experimentally elicited in various species of animals (25). The DIR is a species-typical response that is experimentally elicited by grasping an animal by the dorsal skin at the nape of the neck and lifting the animal off its feet. In the rat, the animal immediately exhibits a stereotypical immobility response that persists for a period of time until the animal emits escape-like behaviors.

The primary purposes of this study were to test the following hypotheses: a) that systemic injections of various dosages of Met$^{1-5}$-Enk would differentially potentiate the duration of the DIR in a dose by time course function; and b) that various metabolites of Met$^{1-5}$-Enk would differentially potentiate the duration of the DIR over the time course.

GENERAL METHOD

Animals

Long-Evans male rats, weighing between 300 and 350 g, were obtained from Charles River. They were individually housed with food and water ad lib and maintained on a 0800–1600 h light cycle. Animals were tested during the light cycle between 1200–1600 h. This study was carried out in compliance with the rules set forth in the NIH *Guide for the Care and Use of Laboratory Animals.*

Behavioral Testing

At the time of behavioral testing, the animal was removed from the home cage, injected with the peptide, and returned to the home cage. The experimenter was blind to the drug treatment condition. At time intervals of 5, 15, 25, 35, 45,

and 55 min following injection, the animals were behaviorally tested. Upon being removed from the home cage, an animal was placed in a V-shaped trough to restrict its general movements for 30 s. To elicit the DIR, the rat was grasped by the dorsal skin at the nape of the neck, between the base of the skull and the back of the ears, and was lifted off its feet with no part of the animal's body touching any other surface. As all animals displayed the stereotypical DIR when it was first induced, the duration of the DIR was measured from the immediate onset of the response until the animal made escape-like movements or until 300 s had elapsed. Following each trial, the animal was returned to the home cage. Each animal received six trials with an intertrial interval of 10 min.

Statistics

A two-factor mixed-design analysis of variance (ANOVA) was used to examine the effects of various dosages of Met-Enk or the effects of various peptide fragments upon the duration of the DIR measured over six time blocks. Dunnett's test for the comparison of treatment means with the vehicle control group was used to make post hoc comparisons. A p value equal to or less than 0.05 was judged significant.

EXPERIMENT 1

The aim of Experiment 1 was to investigate the effects of various dosages of Met^{1-5}-Enk upon the duration of the DIR and to determine a dose by time course function.

Peptides and Treatment

In this experiment, methionine-enkephalin (Met^{1-5}-Enk; Tyr-Gly-Gly-Phe-Met; mol.wt. 573.7) (Sigma Chemical Co., St. Louis, MO) was dissolved in distilled water that also served as the vehicle control. The concentrations of 0.00 (vehicle control), 0.1, 1.0, 10.0, and 100.0 μg/kg were systemically injected under the skin at the lower back with the volume of 1 ml/kg for each dose. There were 10 randomly assigned animals within each group.

Results

Systemic injections of Met^{1-5}-Enk resulted in significant differences in the duration of DIR as a function of the concentration of the dosage, $F(4, 45) = 8.82$, $p < 0.001$, across the six time blocks, $F(5, 225) = 105.19$, $p < 0.001$, and the dosage by time blocks interaction, $F(20, 225) = 13.12$, $p < 0.001$. The subsequent analyses revealed that at the time intervals of 5 and 15 min, the 1.0, 10.0, and 100.0 μg/kg dosages significantly potentiated the duration of the DIR ($ps < 0.05$ and 0.01). All other comparisons were not significant ($ps > 0.05$). These effects are illustrated in Fig. 1A.

The significant dosage effect is given in Fig. 1B. The subsequent analyses showed that the dosages of 1.0, 10.0, and 100.0 μg/kg groups differ significantly from the vehicle control group in an increasing monotonic function ($ps < 0.05$ and 0.01).

EXPERIMENT 2

The aim of Experiment 2 was to investigate the effects of various fragments of Met^{1-5}-Enk from the N-terminal upon the duration of the DIR and to determine a fragment by time course function.

Peptides and Treatment

Met^{1-5}-Enk and the within fragments of Met^{1-5}-Enk reduced from the N-terminal, Met^{2-5}-Enk (Gly-Gly-Phe-Met; mol.wt. 410.5), Met^{3-5}-Enk (Gly-Phe-Met; mol.wt. 335.4), and Met^{4-5}-Enk (Phe-Met; mol.wt. 296.4) (Sigma) were dissolved in distilled water for a 10.0 μg/kg dosage with the volume of 1 ml/kg. The other treatment conditions were as in Experiment 1. There were eight randomly assigned animals within each treatment group.

Results

The mean durations for the DIR as a function of the within fragments of Met^{1-5}-Enk from the N-terminal over the time course are shown in Fig. 2A. The statistical analyses revealed significant differences among the fragments, $F(4, 35) = 5.92$, $p = 0.001$, along the time course, $F(5, 175) = 56.07$, $p < 0.001$, and peptide treatments by time course interaction, $F(20, 175) = 18.47$, $p < 0.001$. The subsequent analyses showed a significant potentiation of the DIR at 5 and 15 min with Met^{1-5}-Enk and at 5 min with Met^{2-5}-Enk ($ps < 0.01$). All other comparisons were not significant ($ps > 0.05$).

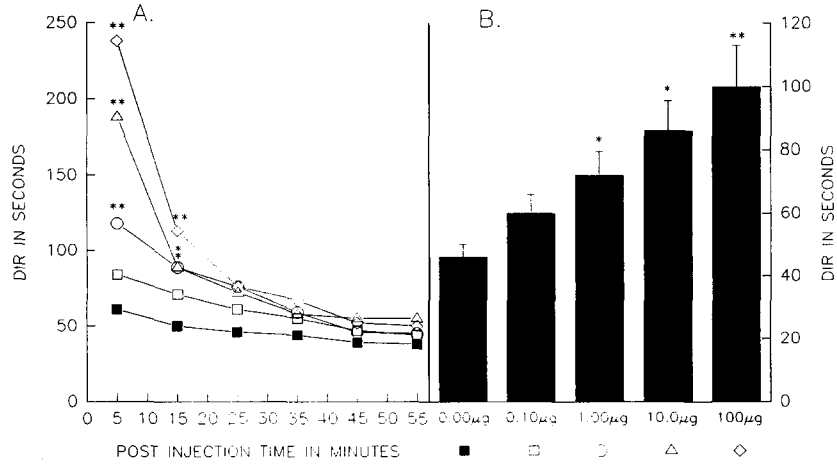

FIG. 1. (A) Durations of the DIR in seconds over six consecutive 10-min time blocks as a function of the various dosages of Met^{1-5}-Enk. The error bars have been omitted for clarity. The main dose effects are in (B). Significant differences from the vehicle control group, $*p < 0.05$ and $**p < 0.01$.

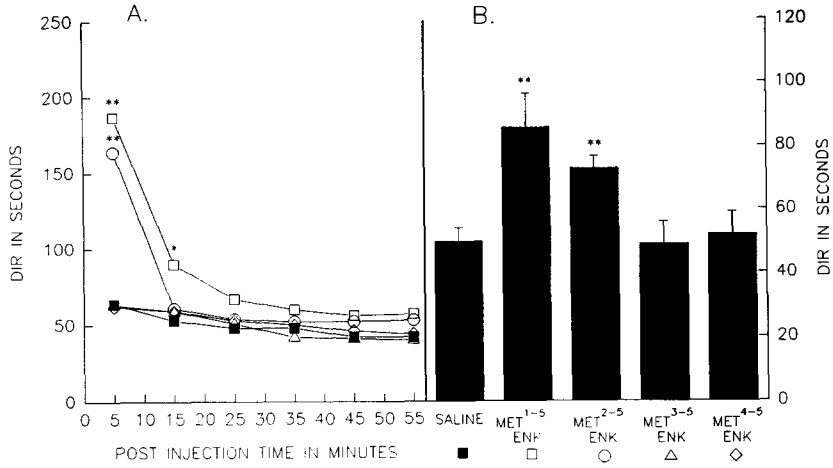

FIG. 2. (A) Duration of the DIR in seconds over six consecutive 10-min time blocks as a function of the within fragments of Met^{1-5}-Enk from the *N*-terminal. The error bars have been omitted for clarity. The main fragment effects are in (B). Significant differences from the vehicle control group, $*p < 0.05$ and $**p < 0.01$.

The significant fragment effect is illustrated in Fig. 2B. The subsequent analyses revealed that Met^{1-5}-Enk and the fragment Met^{2-5}-Enk differed significantly from the vehicle control group ($ps < 0.01$). All other fragments did not differ from the vehicle controls ($ps > 0.05$).

EXPERIMENT 3

The aim of Experiment 3 was to investigate the effects of various fragments of Met^{1-5}-Enk from the *C*-terminal upon the duration of the DIR and to determine a fragment by time course function.

Peptides and Treatment

Met^{1-4}-Enk (Tyr-Gly-Gly Phe; mol.wt. 534.7), Met^{1-3}-Enk (Tyr-Gly-Gly; mol.wt. 295.3), and Met^{1-2}-Enk (Tyr-Gly; mol.wt. 238.2) (Sigma) were dissolved in distilled water for a 10.0 μg/kg dosage with the volume of 1 ml/kg. The other treatment conditions were as described in Experiment 1. There were eight randomly assigned animals within each treatment group. The data for Met^{1-5}-Enk and the vehicle control groups in Experiment 2 were used in the statistical analyses of Experiment 3.

Results

The mean durations for the DIR as a function of the within fragments of Met^{1-5}-Enk from the *C*-terminal over the time course are given in Fig. 3A. The statistical analyses revealed significant differences among the fragments, $F(4, 35) = 6.28$, $p < 0.001$, along the time course, $F(5, 175) = 66.91$, $p < 0.001$, and the peptide treatments by time course interaction, $F(20, 175) = 12.73$, $p < 0.001$. The subsequent analyses resulted in significant potentiation of the duration of

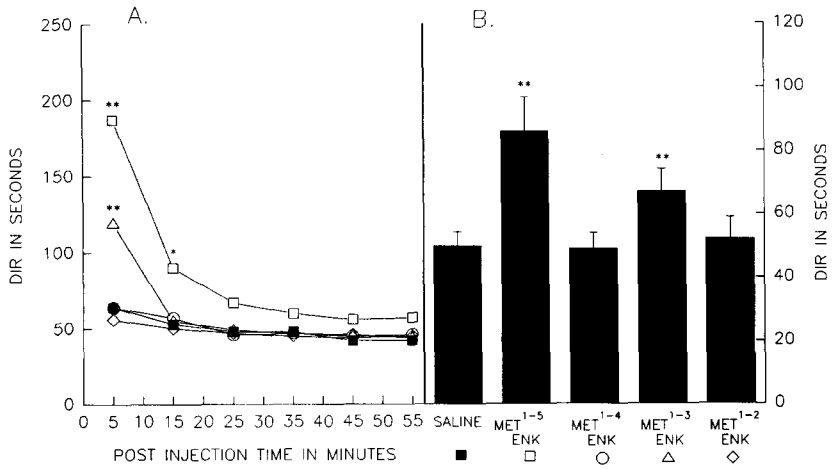

FIG. 3. (A) Duration of the DIR in seconds over six consecutive 10-min time blocks as a function of the within fragments of Met^{1-5}-Enk from the *C*-terminal. The error bars have been omitted for clarity. The main fragment effects are in (B). Significant differences from the vehicle control group, $*p < 0.05$ and $**p < 0.01$.

the DIR at 5 and 15 min with Met^5-Enk and at 5 min with Met^{1-3}-Enk ($ps < 0.01$). All other comparisons were not significant ($ps > 0.05$).

The significant fragment effect is shown in Fig. 3B. The subsequent analyses revealed that Met^{1-5}-Enk and the fragment Met^{1-3}-Enk groups differed significantly from the vehicle control group ($ps < 0.01$).

GENERAL DISCUSSION

From the three experiments several findings emerged. In Experiment 1, it was shown that the dose–response curve was an increasing monotonic function. While this was not an unusual effect, one of the common observations has been that various neuropeptides exert an inverted U-shaped or U-shaped dose–response curve when using very low dosages (7,11,12). The inhibitory effects of the systemic dosages of Met^{1-5}-Enk on the duration of the DIR was short-lived and comparable to its intrastriatal injections (14). The metabolism of Met^{1-5}-Enk takes place rapidly. However, the inhibitory effects were far longer than the half-life of this peptide. It has been shown that Leu-Enk, which also has a similar rapid hydrolysis as Met-Enk, was present in plasma to modulate avoidance conditioning for at least 15 min (20). On the other hand, it has been argued that the half-life of small peptides (such as Met^{1-5}-Enk) may have little to do with the duration of its behavioral action (7).

The inactivation and metabolism of Met^{1-5}-Enk is primarily effected by endopeptidase-24.11 at the Gly^3 and Phe^4 bond and aminopeptidase N at the Tyr^1 and Gly^2 bond, and to dipeptidyl aminopeptidase at the Gly^2 and Gly^3 bond. The metabolism at the site of hydrolyses of Tyr^1 and Gly^2 has a half-life between 2 and 3 min following systemic administration. From the data in Experiment 2, both Met^{1-5}-Enk and Met^{2-5}-Enk significantly potentiated the duration of the DIR at time block 5 min. On the other hand, Met^{1-5}-Enk was also active at 15 min. The magnitude of the DIR with the nonopioid fragment, Met^{2-5}-Enk, was not equivalent to Met^{1-5}-Enk. All of the other fragments from the *N*-terminal had no significant effect upon the DIR.

The hydrolysis at the Gly^3 and Phe^4 also has a short half-life. Experiment 3 resulted in a significant potentiation of the DIR with the Met^{1-3}-Enk fragment. All other fragments from the *C*-terminal were not significant. It has been reported that both the Leu^{1-3}-Enk and Leu^{2-5}-Enk fragments produced an impairment in the acquisition of avoidance conditioning and that Leu^{1-3}-Enk was more potent than Leu^{1-5}-Enk on a molar basis (26). These observations generalize in part to the effects of the two Met-Enk fragments on the DIR. More recently, it has been shown that the other two fragments containing tyrosine (Leu^{1-4}-Enk and Leu^{1-2}-Enk, which were identical to Met-Enk fragments) also impaired avoidance conditioning (12). These fragments had no significant effects on the DIR and cannot be explained as a function of the differences in dosage.

The two major metabolites of Met-Enk, Met^{2-5}-Enk and Met^{1-3}-Enk, significantly affected the duration of the DIR. These findings expand our general understanding of the molecular basis of the enkephalins on complex inhibitory behaviors and lead to the conclusion that Met-Enk may be a propeptide for smaller behaviorally active Met-Enk fragments.

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