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Tyr-MIF-1, Identified in Brain Tissue, and Its Analogs are Active in Two Models of Antinociception

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KASTIN, A. J., E. STEPHENS, J. E. ZADINA, D. H. COY AND A. J. FISCHMAN. *Tyr-MIF-1, identified in brain tissue, and its analogs are active in two models of antinociception.* PHARMACOL BIOCHEM BEHAV **23**(6) 1045–1049, 1985.—The antiopiate activities of Tyr-MIF-1 (Tyr-Pro-Leu-Gly-NH_2) and some of its representative analogs were tested in two animal models of antinociception. Doses of the tetrapeptides as low as 0.001 mg/kg injected peripherally could block the analgesic effects of morphine in both the tail-flick test of mild thermal pain induced by heat and the scratching test of mild chemical pain induced by hypertonic saline. These tetrapeptides showed cross-reactivity in the radioimmunoassay (RIA) used to identify the presence of Tyr-MIF-1 in brain extracts and in the brain membrane binding assay. Only Tyr-MIF-1, however, eluted at the position of the immunoreactive peak after gel filtration chromatography and high performance liquid chromatography (HPLC). The results support the concept that peptides with anti-opiate activity can exist in the brain.

Peptides Analgesia Tail-flick Scratching Morphine Gel filtration HPLC Radioimmunoassay Receptors Adrenal Pineal Pituitary Brain

THERE is evidence that Tyr-MIF-1 is an endogenous brain peptide with antiopiate activity. Tyr-MIF-1-like immunoreactive material [7] and high affinity, saturable binding sites for Tyr-MIF-1 [13] have been found in brain tissue. The tetrapeptide also has been shown to antagonize the analgesia induced by morphine in the tail-flick test [11].

In order to extend these findings, several analogs of Tyr-MIF-1 were synthesized. These were used to help further identify the main immunoreactive peak in brain tissue after gel filtration on a column of Sephadex G-10 and HPLC as monitored by RIA [6].

Four of the analogs involving substitutions of Lys, Ala, Gly, or Phe for the N-terminal Tyr were selected for testing in two models of analgesia. These tetrapeptides represented four different types of substitution, Lys being basic, Ala being neutral, Glu being acidic, and Phe being aromatic. The first model, the tail-flick test, is a classical method for evaluation of thermal pain. The second model involves subcutaneous (SC) injection of 6% NaCl in the abdominal region. This has been proposed as a useful model of peripheral chemically-induced pain [5].

METHOD

Experiment 1: Tail-Flick (Five Peptides)

Male, albino Swiss-Webster mice were obtained from Harlan Sprague-Dawley (Indianapolis, IN). About 1 week after arrival, at which time they weighed about 20 g, they were injected intraperitoneally (IP) with peptide and 10 min later with 12.5 mg/kg body weight of morphine sulfate. Thirty min after the morphine, three consecutive readings of tail-flick latency were obtained. The mean of these three readings was used for the calculations that consisted of analysis of variance (ANOVA) followed by a single Duncan's new multiple range test of all data. The equipment was the same as that used in our previous study of analgesia [11].

The tetrapeptides were injected IP 10 min before the morphine at doses of 0 (diluent consisting of 0.9% NaCl acidified with acetic acid to 0.01 M), 0.001, 0.01, 0.1, 1.0, and 10.0 mg/kg. Twenty different mice were used at each dose of each of the five peptides. All solutions were made daily and coded so that the experimenter did not know the contents.

Experiment 2: Tail-Flick (Three Peptides)

In this experiment, the two analogs exerting the greatest antinociceptive effects in Experiment 1 were compared with Tyr-MIF-1. The tail-flick procedure was the same as in the first experiment except that Glu-MIF-1, Phe-MIF-1, and Tyr-MIF-1 were injected 15, 30, 60, or 120 min before the morphine rather than 10 min earlier. As in Experiment 1, the doses used were 0, 0.001, 0.01, 0.1, 1.0, and 10.0 mg/kg IP and readings were taken 30 min after the morphine. Twelve different mice were used in each group.

Experiment 3: Scratching and Ablation Studies (One Peptide)

The procedure described by Hylden and Wilcox for analgesia involved injection of 0.2 ml of 6% NaCl subcutaneously (SC) in the lower abdominal region 15 min after

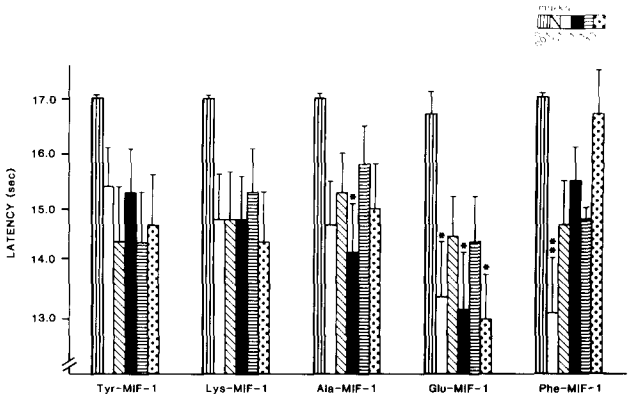

FIG. 1. Mean (±SEM) latency for tail-flick response in mice injected IP with a tetrapeptide or diluent 10 min before IP injection of morphine. $*p<0.05$, $**p<0.01$.

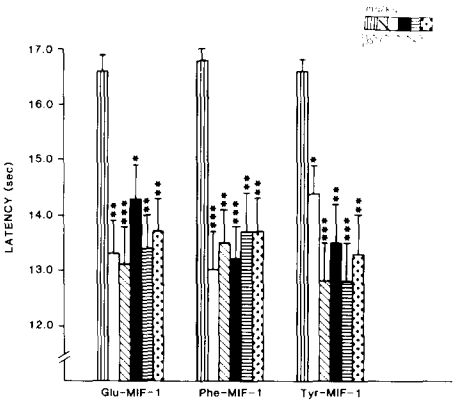

FIG. 2. Mean (±SEM) latency for tail-flick response in mice injected IP with a tetrapeptide or diluent at several times (collapsed) before IP injection of morphine. $*p<0.05$, $**p<0.01$, $***p<0.001$.

intrathecal injection of peptide with measurement of the number of licking and scratching motions for 2 min [5]. We modified this procedure so that mice were injected with morphine sulfate (5.0 mg/kg, IP) followed immediately by IP injection of peptide or diluent. Three min later, the same mice were injected SC in the abdominal region with 6% NaCl in a volume of 10 ml/kg body weight. Unusual movements were recorded for the next 3 min. These usually consisted of a couple of licks at the site of injection immediately after administration of the hypertonic saline followed by scratching the head most commonly made with the front paws during the third min.

In order to establish the validity of this modified method, only MIF-1 was studied in this experiment. Doses of 0 (diluent) 0.1, 1.0 and 10.0 mg/kg IP were tested. Twelve mice of the same description as in Experiments 1 and 2 were used in each group. All data obtained were compared by ANOVA and a single Duncan's new multiple range test.

In a preliminary study, the effects of MIF-1 in the scratching test were assessed in adrenalectomized, hypophysectomized, pinealectomized, and intact mice obtained from Charles River Co. (Wilmington, MA). About one week after arrival, 6–9 mice (25–30 g) in each group were injected with morphine sulfate (5.0 mg/kg, IP) and MIF-1 (1.0 mg/kg, IP) followed by the 6% NaCl (SC) and the responses compared with a smaller number of mice receiving the morphine and diluent.

Experiment 4: Scratching (Five Peptides)

The same five peptides used in the tail-flick test of Experiment 1 were used for this experiment involving the scratching procedure of Experiment 3. Based on the results of Experiment 3 and the limited supply of the analogs, Tyr-MIF-1, Lys-MIF-1, Ala-MIF-1, Glu-MIF-1, and Phe-MIF-1 were tested only at the lower doses of 0 (diluent), 0.001, 0.01, and 0.1 mg/kg IP.

Experiment 5: Tyr-MIF-1 in Brain Tissue

Peptide preparation. Nine analogs of Tyr-MIF-1 were prepared by solid phase methods with benzhydrylamine resin to generate peptide amides after cleavage with liquid hydrogen fluoride and concomitant deblocking. The crude synthetic products were desalted by gel filtration chromatography on a column (2.5×60 cm) of Sephadex G-10 eluted with 1.0 M acetic acid. For final purification, the desalted product was rechromatographed on the same column eluted with 0.02 M acetic acid. Thin layer chromatography of all nine peptides revealed single spots in the following three systems: N-butanol: acetic acid: water, 4:1:5 (upper phase); ethyl acetate: acetic acid: n-butanol: water, 1:1:1:1; and chloroform: methanol: water, 8:5:1. Reversed phase HPLC, described below, showed a single peak for each peptide.

Gel chromatography. Whole rat brains (minus pituitary, pineal, and hypothalamus) were homogenized for 15 sec with a Brinkmann polytron (setting 6), placed in boiling water for 1 min, chilled on ice, centrifuged (20 min, 29,000 g, 4°C), and lyophilized. The reconstituted samples were applied to a column of Sephadex G-10 (1×150 cm) that was previously equilibrated with 0.02 M acetic acid containing 1% BSA and 0.002% sodium azide. The flow rate of the column was 0.2 ml/min, and 2.0 ml fractions were collected. One half ml of each fraction was lyophilized and assayed for Tyr-MIF-1 by RIA [6] with antibody number 112 (Ab 112). The fractions representing the peak of immunoreactivity coeluting with Tyr-MIF-1 were pooled, lyophilized, resuspended in 0.1% trifluoracetic acid (TFA), centrifuged (10 min, 3000 g, 4°C), and analyzed by HPLC.

HPLC. HPLC was performed with a Beckman (Palo Alto, CA) HPLC-system (model 344) equipped with an Ultrasphere ODS column (0.46×15 cm). The flow rate was 1.5 ml/min, solvent A was 0.1% aqueous TFA, and solvent B was 0.1% TFA in acetonitrile. Two min after injection of the sample, solvent B was increased from 2% to 35% over 30 min. The column was then washed with 100% solvent B and re-equilibrated with 2% B. Fractions of 0.75 ml were collected, lyophilized, and measured by RIA with Ab 112. Before each analytical run of brain extract, a blank injection of 250 μl 0.1% TFA was run under identical conditions and assayed by RIA to test for shadowing [4]. When synthetic peptides were run, the procedure was essentially the same except that the chromatogram was monitored by UV absorption at 230 nm and 280 nm.

In vitro brain membrane binding. Rat brain membranes were prepared as described elsewhere [13], preincubated at 37°C for 15 min, and incubated with ^{125}I-Tyr-MIF-1 (1 nM) at varying concentrations (1–1024 nM) of the test peptide. Dis-

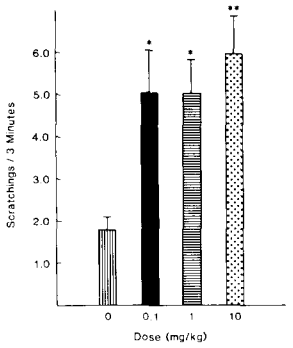

FIG. 3. Mean (±SEM) number of scratches/3 min in mice injected IP with MIF-1 (or diluent) and morphine 3 min before SC administration of 6% NaCl in the region of the lower abdomen.

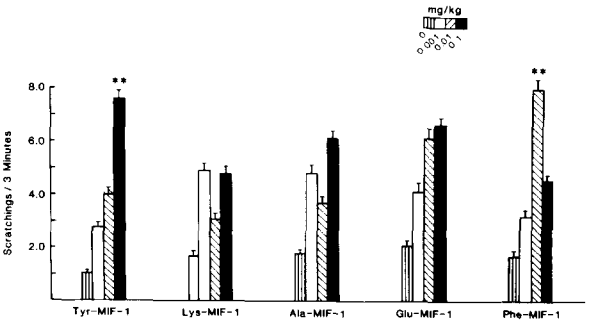

FIG. 4. Mean (±SEM) number of scratches/3 min in mice injected IP with a tetrapeptide (or diluent) and morphine 3 min before SC administration of 6% NaCl in the region of the lower abdomen. $^{**}p<0.01$.

placement curves were fitted and IC_{50} values calculated with the Allfit program of DeLean *et al.* [2].

RESULTS

Experiment 1: Tail-Flick (Five Peptides)

As analyzed in the single, overall Duncan's new multiple range test, peripheral administration of 0.001 mg/kg of Phe-MIF-1 and Glu-MIF-1 resulted in statistically significant blockade of the analgesic effects of morphine in the tail-flick test (Fig. 1). The effects of the next lowest dose, 0.01 mg/kg, had a similar tendency for Tyr-MIF-1 ($p=0.07$). At a dose of 0.1 mg/kg, Glu-MIF-1 exerted the greatest effect ($p<0.05$). At 1.0 mg/kg, the effect of Glu-MIF-1 was also statistically significant ($p<0.05$) while that of Tyr-MIF-1 ($p=0.07$) and Phe-MIF-1 ($p=0.074$) approached significance. At 10 mg/kg, only the effect of Glu-MIF-1 was reliably different from that of its diluent ($p<0.05$).

Experiment 2: Tail-Flick (Three Peptides)

The time before morphine at which Glu-MIF-1, Phe-MIF-1, and Tyr-MIF-1 were injected did not result in a statistically significant main effect or interaction as assessed by ANOVA, so the data were collapsed across time and presented in Fig. 2. Each dose of each of the three peptides considered together, regardless of time before morphine, resulted in a significantly decreased latency as compared with mice injected with morphine and diluent. Separately, however, the most reliable effects were seen with 1.0 mg/kg Tyr-MIF-1 at 15 min and, to a lesser extent, at 30 min as well as with 0.01 mg/kg of this peptide and with 0.1 and 0.001 mg/kg of Phe-MIF-1 at 30 min.

Experiment 3: Scratching and Ablation Studies (One Peptide)

Each of the three doses of MIF-1 used with morphine to test this model of antinociception was found to result in a statistically significant increased number of scratching motions as compared with mice injected with morphine and diluent. The differences between the three doses of MIF-1 were not significant. These results are shown in Fig. 3.

In the preliminary study involving IP injection of 1.0 mg/kg MIF-1, none of the surgical ablations appeared to prevent the actions of MIF-1 in blocking the effects of morphine. The number of scratchings/3 min in the adrenalectomized mice receiving MIF-1 was 8.0±1.9 as compared with 1.5±1.5 for the adrenalectomized rats receiving diluent. Hypophysectomized mice injected with morphine and MIF-1 scratched 6.8±2.1 times/3 min as compared with 1.0±1.0 for hypophysectomized mice injected with morphine and diluent. Pinealectomized mice scratched 11.0±2.1 times/3 min after MIF-1, but only 0.5±0.5 times after diluent. Intact mice injected with MIF-1 and morphine scratched 10.9±1.4 times/3 min as compared with 3.0±1.0 for the controls.

Experiment 4: Scratching (Five Peptides)

Of the three doses of each peptide tested, 0.1 mg/kg Tyr-MIF-1 ($p<0.01$) and 0.01 mg/kg Phe-MIF-1 ($p<0.01$) resulted in the greatest blockade of the effect of morphine (Fig. 4). A similar tendency was seen with the dose of 0.1 mg/kg of both Ala-MIF-1 ($p=0.053$) and Glu-MIF-1 ($p=0.075$) as assessed by the single, overall Duncan's multiple range test.

Experiment 5: Tyr-MIF-1 in Brain Tissue

Gel filtration chromatography of rat brain extracts showed two main peaks of Tyr-MIF-1-like immunoreactivity (Fig. 5). The first peak eluted just after the void volume and the second peak occurred at the elution position of synthetic Tyr-MIF-1. Leu-MIF-1, the analog with the elution time closest to Tyr-MIF-1 after HPLC (Table 1), eluted much earlier than Tyr-MIF-1 after chromatography on the column of Sephadex G-10.

HPLC of the pooled second immunoreactive area (Fig. 5) revealed a major peak of immunoreactivity at the elution position of Tyr-MIF-1 (Fig. 6). The differing elution times of the other analogs are shown in Table 1. This table also lists their cross-reactivities with Ab 112 in the RIA used to monitor the chromatographic procedures and their potency in displacing ^{125}I-Tyr-MIF from rat brain membranes. The dilution curves of each analog in the RIA were parallel to that obtained with the parent tetrapeptide.

DISCUSSION

The results of the analgesic experiments demonstrate that tetrapeptides based on different types of substitutions of the

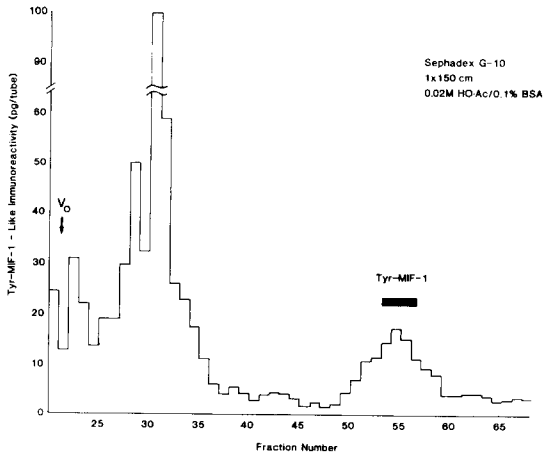

FIG. 5. Tyr-MIF-1-like immunoreactivity after gel filtration of brain extract on a column of Sephadex G-10.

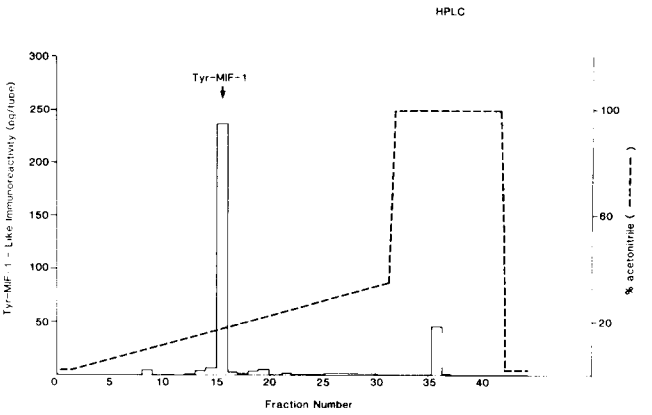

FIG. 6. HPLC of the pooled fractions of brain extract eluting on Sephadex G-10 (Fig. 5) at the same position as Tyr-MIF-1. The gradient composition is shown by the broken line.

first amino acid of Tyr-MIF-1 can also exert antinociceptive effects. One of the most active of the 4 analogs tested was Phe-MIF-1, representing Tyr-MIF-1 minus a hydroxyl group on the aromatic ring. Ala represents Tyr or Phe without the aromatic ring. Glu-MIF-1 is quite different from Tyr-MIF-1 and the other analogs both in structure and net charge (0 vs. +1 or +2), but it also showed activity. Of these four analogs, Phe-MIF-1 showed high binding to both antibody and binding sites in the brain, Ala-MIF-1 was in an intermediate group, and Glu-MIF-1 and Lys-MIF-1 had the lowest binding in both situations (Table 1).

The cross-reactivities of all nine analogs with Ab 112 appear to fall into three main groups (Table 1). The most reactive analogs involve bulky aromatic substitutions like Trp or Phe. Intermediate cross-reactivities were found when the N-terminal residue was replaced by neutral amino acids. In this group of non-aromatic compounds, the relatively high activity of the N-seryl analog might be related to the commonalty of a hydroxyl group to both Ser and Tyr. The lowest cross-reactivities were observed with the acetic (Glu) and basic (Lys) substitutions. Although His-MIF-1 is partially charged at the pH of the assay, it may lie in the intermediate group because it is bulky and unsaturated. All the analogs had greater reactivity with the antibody than did MIF-1. This may indicate that displacement of the N-terminal positive charge by an additional residue favors binding.

In general, the relative pattern of binding of the analogs to brain membranes was similar to that for the antibody. Some analogs, like Leu-MIF-1, may show a lack of such correlation, but in the scratching test the two peptides most similar in structure—Tyr-MIF-1 and Phe-MIF-1—caused the most reliable blockade of the effects of morphine and were among the most potent competitors for both the antibody and the binding sites. Although MIF-1 does not bind to Tyr-MIF-1 binding sites in the brain [13], it would appear that the MIF-1 portion of the Tyr-MIF-1 molecule is sufficient for activity, perhaps by a separate mechanism of action, in the tail-flick test [1, 3, 8, 9] and, now, the scratching test.

Statistically, Tyr-MIF-1 and Phe-MIF-1 were the most active in the scratching test, but all of the tetrapeptides tested showed tendencies in the same direction. Relative to the other doses, 0.001 mg/kg did not seem as active in the scratching test as in the tail-flick test. With the exception of

TABLE 1

ELUTION TIME (MIN), CROSS-REACTIVITY WITH ANTIBODY 112 (PERCENT), AND MEMBRANE BINDING (IC_{50}) OF TYR-MIF-1 AND RELATED PEPTIDES

Analog	Elution Time (min)	Cross-reactivity (%)	IC_{50} (nM)
Tyr-MIF-1	15.1	100.0	16.4
Trp-MIF-1	22.2	114.5	40.2
Phe-MIF-1	18.6	79.5	27.4
Ser-MIF-1	9.3	28.7	>10 μM
Ala-MIF-1	8.9	17.9	>10 μM
His-MIF-1	7.6	17.9	>10 μM
Met-MIF-1	13.5	6.5	>1 μM
Leu-MIF-1	15.4	6.4	95.4
Glu-MIF-1	9.4	3.9	>10 μM
Lys-MIF-1	6.7	2.7	>10 μM
MIF-1	6.2	<2	>10 μM
Oxytocin	21.7	5.4	>10 μM

the results with Phe-MIF-1 in Experiment 1, there was no suggestion of the inverted U-shaped dose-response curve that we have been finding with peptides like MIF-1 since 1971 [10].

The scratching test provides another type of situation in which compounds related to Tyr-MIF-1 are active. The irritation caused by the hypertonic saline was blocked by morphine (dose 0 of peptide, Figs. 3 and 4), a blockade reversed by MIF-1 (Fig. 3) as well as by Tyr-MIF-1 and its four analogs (Fig. 4). The high degree of variability of both the scratching and tail-flick assays tend to limit their usefulness, but significant effects were found after overall analysis of all the data used as absolute scores. Moreover, if the data were expressed as percent change from diluent, the effect of the peptides in the scratching test were much greater than in the tail-flick test.

The scratching test has been proposed as a useful model of analgesia at the spinal level. It reflects peripheral chemically-induced pain as compared with the noxious thermal stimulation of the tail-flick test [5]. For injected peptides like

MIF-1, the adrenal, pituitary, and pineal did not seem to mediate the anti-opiate effects, but this does not indicate the absence of anti-opiate peptides like Tyr-MIF-1 in these organs.

Tyr-MIF-1 was found in brain tissue (not containing pituitary, pineal, or hypothalamus). Gel filtration on Sephadex G-10 and HPLC demonstrated a major peak of immunoreactivity that eluted at the position of Tyr-MIF-1 (Figs. 5 and 6). The occurrence of other immunoreactive areas after chromatography of these brain extracts suggests the possibility of the existence of a family of peptides related to Tyr-MIF-1.

The activity of Tyr-MIF-1 in the scratching test, another model of antinociception, provides additional evidence for the interaction of this peptide with opiates. Evidence already exists that MIF-1 does not bind to delta [1, 3, 9, 12] or kappa [1] receptors and shows little if any binding to the classical mu receptors [1, 3, 8, 12]. This, however, does not preclude a role for Tyr-MIF-1 as a specific endogenous antagonist of only one type of opiate peptide, with other peptide antagonists eventually being found to be more specific for other types of endogenous opiates. The further identification of Tyr-MIF-1 in brain tissue reinforces our concept that there may be endogenous anti-opiates as well as endogenous opiates.

ACKNOWLEDGEMENTS

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