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Isolation of a Novel Tetrapeptide With Opiate and Antiopiate Activity From Human Brain Cortex: Tyr-Pro-Trp-Gly-NH_2 (Tyr-W-MIF-1)

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ERCHEGYI, J., A. J. KASTIN AND J. E. ZADINA. *Isolation of a novel tetrapeptide with opiate and antiopiate activity from human brain cortex: Tyr-Pro-Trp-Gly-NH_2 (Tyr-W-MIF-1).* PEPTIDES **13**(4) 623–631, 1992.—A novel tetrapeptide, Tyr-Pro-Trp-Gly-NH_2 (Tyr-W-MIF-1), was purified from extracts of frontal cortex of human brain tissue by several consecutive reversed-phase high performance liquid chromatographic steps followed by a radioimmunoassay originally developed for Tyr-Pro-Leu-Gly-NH_2 (Tyr-MIF-1). Sequencing, mass spectrometric analysis, and comparison of its chromatographic behavior with that of the synthetic peptide confirmed the structure. Like Tyr-MIF-1, which was previously isolated from human brain tissue, Tyr-W-MIF-1 can inhibit the binding of ^{3}H-DAMGO (selective for mu opiate receptors) to rat brain and can act as an opiate agonist as well as antagonist. Tyr-W-MIF-1 was a more potent opiate agonist than Tyr-MIF-1, the free acid of Tyr-W-MIF-1, and the structurally related hemoglobin-derived opiate peptide hemorphin-4 (Tyr-Pro-Trp-Thr) in the guinea pig ileum. Each of these peptides acted as opiate antagonists on the ileum from morphine-tolerant guinea pigs; the free acid of Tyr-W-MIF-1 was the most potent antagonist in inhibiting the activity of DAMGO. The results demonstrate the presence in human brain of a new member of the Tyr-MIF-1 family of biologically active peptides.

Peptide Opiate Antiopiate Human brain Tyr-MIF-1 Tyr-W-MIF-1

Tyr-MIF-1 has been isolated from both bovine hypothalamic tissue (14) and the parietal cortex of human brain tissue (15). An earlier study in rats found the highest concentration of binding sites for Tyr-MIF-1 in the cortex of the brain (33). The antiopiate properties of Tyr-MIF-1 have been demonstrated in several biological systems (10–12,20–26). This peptide is able to cross the blood–brain barrier by a carrier-mediated transport system out of the brain (1,3) that is shared with Met-enkephalin (2,4). Tyr-MIF-1 can bind to mu opiate receptor sites (28) and has its own high affinity nonopiate binding sites in brain (29–31). The opiate agonist-like activities of Tyr-MIF-1 in a neuroblastoma cell line (27) and its activities as an opiate agonist, as well as an antagonist, in the guinea pig ileum (32) recently have been found.

In the present paper, we describe the isolation of a new member of the Tyr-MIF-1 family of biologically active peptides. Tyr-Pro-Trp-Gly-NH_2 (Tyr-W-MIF-1) was purified from the frontal cortex of human brain tissue. The opiate agonist-like activity of Tyr-W-MIF-1 was shown by inhibition of electrically induced contractions in the guinea pig ileum. The opiate antagonist-like activity of Tyr-W-MIF-1 was shown by attenuation of the inhibition of ileal contractions induced by DAMGO, an opiate agonist, in morphine-tolerant animals. The binding of Tyr-W-MIF-1 to opiate receptors with selectivity for mu sites also was shown.

METHOD

Tissue Extraction

The frontal cortex (200 g) of human brain tissue was obtained from the National Neurological Research Bank (VA Wadsworth Medical Center, Los Angeles, CA). It was extracted by the method recently described for the isolation of valorphin (8). In brief, this consists of extraction with 10% trifluoroacetic acid (TFA), polytron homogenization, centrifugation at 29,000 × *g*, and extraction with diethyl ether. It yielded 8.13 g of dry material.

Solid Phase Extraction

Disposable Mega Bond Elut C8 cartridge columns (10 g sorbent mass/column, Analytichem, Harbor City, CA) were used to prepare a peptide-enriched fraction from the dry tissue extract. The experimental conditions used in this step have been published (8,9). The extraction consisted of rinses with various combinations of water, methanol, acetonitrile, and acetic acid followed by elution with triethylammonium phosphate (TEAP)-acetonitrile.

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TABLE 1
YIELDS AND PHYSICAL DATA OF SYNTHETIC PEPTIDES

Peptide	Mol. Wt.	Yield	TLC (R_f)*			
			A	B	C	D
Boc-Trp-Gly-NH_2	360.4	95%	0.40			
H-Trp-Gly-NH_2 · TFA	374.2	91%			0.53	0.38
Boc-Trp-Gly-OMe	375.4	90%	0.85			
H-Trp-Gly-OMe · TFA	389.3	55%				0.38
Boc-Tyr-Pro-OH	378.4	63%	0.37			
Boc-Tyr-Pro-Trp-Gly-NH_2	620.7	79%		0.73		
Boc-Tyr-Pro-Trp-Gly-OMe	635.7	78%		0.76		
Boc-Tyr-Pro-Trp-Gly-OH	621.7	89%			0.63	0.85
H-Tyr-Pro-Trp-Gly-OH · TFA	621.7	77%				0.37
H-Tyr-Pro-Trp-Gly-NH_2 · TFA	620.7	81%			0.60	0.36

* For details of TLC systems A, B, C, D, see section on Peptide Synthesis.

Reversed-Phase High Performance Liquid Chromatography (rpHPLC)

The solid phase extraction was followed by several sequential rpHPLC steps that were performed on the same Beckman systems (GOLD and 324), which have been published elsewhere (8).

Three reversed-phase C18 columns were used to perform the purification. The preparative column was purchased from Regis Chemical Co. (Morton Grove, IL). The analytical column containing Brownlee Spheri-5 rp-18 packing was obtained from Rainin Instrument Co., Inc. (Woburn, MA), and the other analytical column containing rp-18 Vydac 201 TP 5 μ packing, which is able to separate closely related, nonpolar compounds, was purchased from Alltech Associates, Inc. (Deerfield, IL).

Linear gradient elution with solvents 0.1% TFA (v/v) in water (purified on Milli-Q Reagent water system, Millipore Co., Bedford, MA) and 0.1% TFA (v/v) in acetonitrile or methanol (HPLC grade from J. T. Baker Inc., Phillipsburg, NJ) was used. The gradients are shown on each figure.

Aliquots of the eluates were tested for immunoreactivity by radioimmunoassay (RIA) with our Tyr-MIF-1 antiserum, and only the immunoreactive fractions were pooled and purified further. Since our assay is most reliable in the range of 50–150 pg, the aliquots of the eluates from HPLC were selected so as to result in values in this range of the RIA. In the figures, we show the pg values actually measured in the assays for each aliquot; these results can be multiplied by the numbers indicated on the ordinate to arrive at the absolute pg content of the tubes.

Each step of purification consisted of several runs in order to optimize the resolution and avoid overload of the columns. The final product with a single UV peak was achieved after one preparative and five consecutive HPLC steps.

Radioimmunoassay (RIA)

The details and the selectivity of the RIA for Tyr-MIF-1, its fragments, and other structurally related peptides have been previously described and characterized (8,17–19). The antibody (number 801) to Tyr-MIF-1 was able to detect about 10 pg of Tyr-MIF-1 but only much higher doses of its fragments and some other structurally related peptides. The minimum detectable concentration of Tyr-Pro-Trp-Gly-NH_2 (Tyr-W-MIF-1) was about 10,000 pg (~0.02 nmol) when the cross-reactivity of the synthetic peptide with this antibody was checked at different doses. Tyr-MIF-1 was iodinated with chloramine-T and then purified by rpHPLC. The final dilution of the antibody was 1: 150,000.

Microsequencing

Six cycles of Edman degradation on an Applied Biosystems gas phase protein sequencer (Model 477A) with on-line PTH-amino acid identification on an Applied Biosystems 120A separation system (Foster City, CA) was used for determination of the structure of the purified peptide. About 3 nmol of peptide was given for sequencing. The yield was about 30%.

Mass Spectrometry

Electrospray mass spectrometry (ES/MS) was performed on a VesTech 201 Mass Spectrometer equipped with electrospray interface (VesTech, Houston, TX). Samples (~1 nmol) were dissolved in 2% acetic acid in water and these solutions were introduced into the ion source at a flow rate of 5 μl/min, and the electrospray was emitted at 2500 V.

Search for Protein Homology

The protein sequence database of the Protein Identification Resource, National Biomedical Research Foundation, Washington, DC, was used to determine whether the isolated peptide is a fragment of a larger known protein.

Bioassays

Receptor binding assay. Binding of Tyr-Pro-Leu-Gly-NH_2 (Tyr-MIF-1) (Bachem, Philadelphia, PA), Tyr-Pro-Trp-Gly-NH_2 (Tyr-W-MIF-1), Tyr-Pro-Leu-Gly (both were synthesized in our laboratory), and Tyr-Pro-Trp-Thr (hemorphin) (Peninsula Laboratories Inc., Belmont, CA) to mu opiate receptors was determined by displacement of tritiated Tyr-D-Ala-Gly-(Me)Phe-Gly-ol (DAMGO), which is selective for mu receptors. ^{3}H-DAMGO (55 Ci/mmol) was obtained from DuPont NEN (Wilmington, DE). Crude synaptic plasma membranes were prepared and the binding assay was performed according to previously published methods (28,29). IC_{50} values were calculated by the analysis of the inhibition curves with the ALLFIT program (7).

Guinea pig ileum assay (GPI). The guinea pig ileum assay was performed in the usual way as used by us recently (8,32). For tests of antagonism, guinea pigs (450–500 g) were subcu-

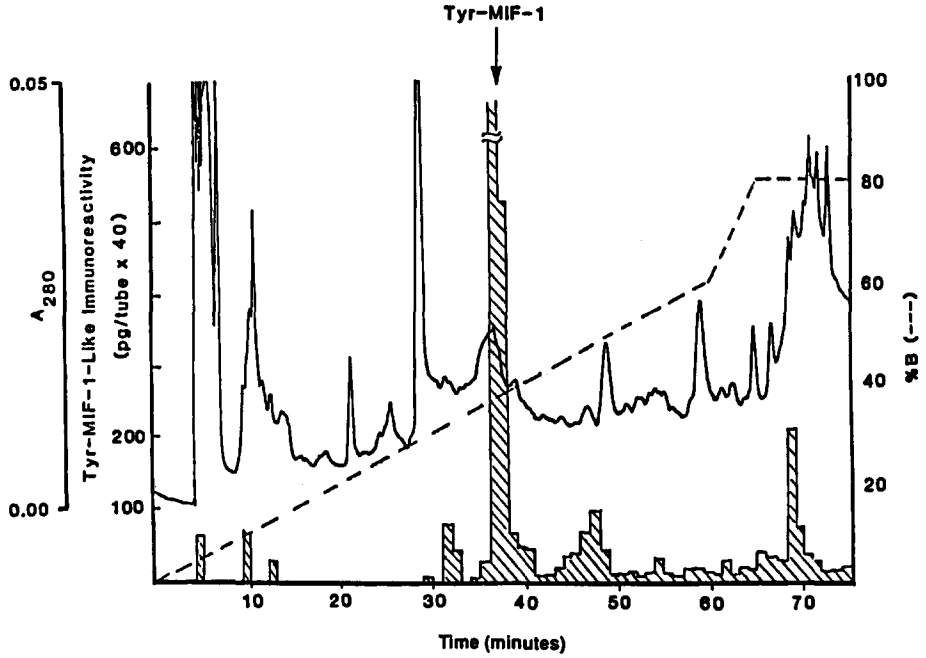

FIG. 1. Preparative rpHPLC purification of human brain tissue after solid phase extraction. UV absorbance (A_{280}) is shown by the solid line; Tyr-MIF-1-like immunoreactivity is indicated by the hatched columns; the dashed line represents the linear HPLC gradient (%B = methanol in 0.1% TFA; the gradient consisted of 0–60% B over 60 min, then to 80% B over 5 min, followed by isocratic elution for 10 min, at a flow rate of 8 ml/min). The arrow shows the elution of Tyr-MIF-1 in this system. A Regis Prep-10D-60-ODS-FEC, 10 μm, 60 Å, ODS-endcapped column (25 × 2.11 cm) was used.

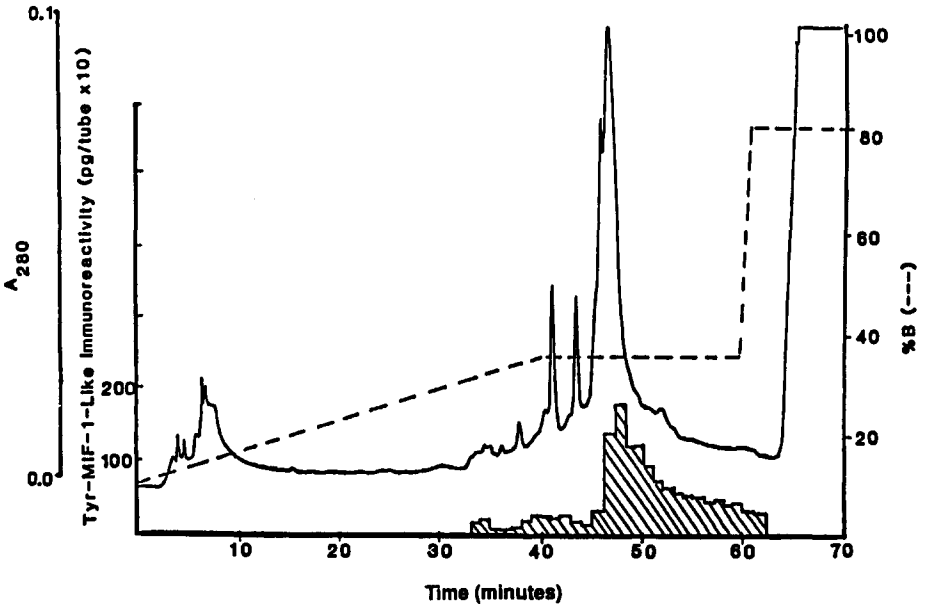

FIG. 2. Further purification of fractions 46–49 from the previous step on an analytical rpHPLC Brownlee Spheri-5 cartridge column with rp-18 packing (0.46 × 22 cm), connected to a Brownlee rp-18 NewGuard precolumn (0.32 × 1.5 cm). UV absorbance (A_{280}) is shown by the solid line; Tyr-MIF-1-like immunoreactivity is indicated by the hatched columns; the dashed line represents the linear HPLC gradient (%B = methanol in 0.1% TFA; the gradient consisted of 10–35% B over 40 min, followed by isocratic elution with 35% B for 20 min, then a rise to 80% B over 1 min, and a wash with 80% B for 9 min, at a flow rate of 1 ml/min).

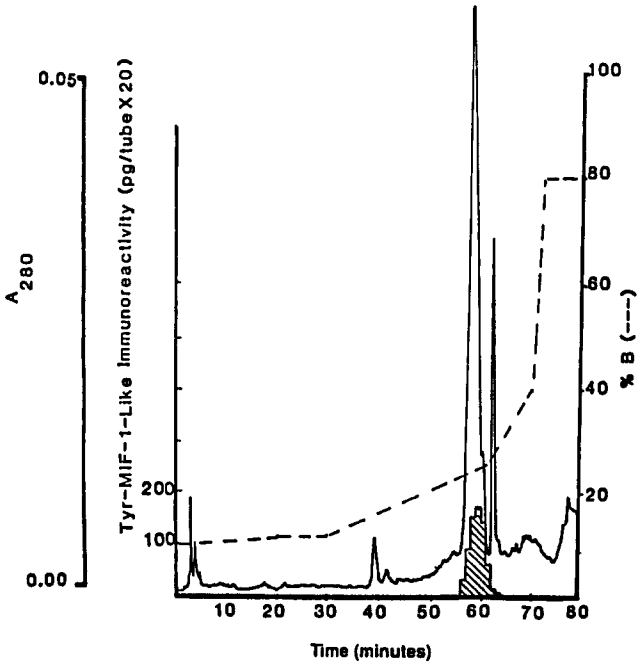

FIG. 3. Further purification of fractions 47–52 from the previous step on a Vydac rp-18 201 TP 5 μ analytical column (15 × 0.46 cm). UV absorbance (A_{280}) is shown by the solid line; Tyr-MIF-1-like immunoreactivity is indicated by the hatched column; the dashed line shows the HPLC gradient (%B = methanol in 0.1% TFA; the gradient consisted of 10–12% B over 20 min, followed by isocratic elution with 12% B for 10 min, then a rise to 25% B over 30 min, followed by a rise to 40% B over 10 min, and finally %B was raised to 80% for 1 min and a wash with 80% B for 9 min, at a flow rate of 1ml/min).

taneously implanted with four morphine pellets (75 mg/pellet) under anesthesia (methoxyflurane) 3 days before testing. As shown in previous studies, this method can produce tolerance and dependence to morphine (13,32) and provides a more sensitive assay for antagonism (32).

Peptide Synthesis

Materials. Amino acid derivatives were obtained from commercial sources. All reagents and solvents were of reagent grade and were used without further purification. Thin layer chromatography (TLC) was performed on silica gel precoated aluminum plates 60 F_{254} (Merck, EM Science, Gibbstown, NJ). The solvent systems used for TLC were: (A) ethyl acetate:pyridine:acetic acid:water (480:20:6:11); (B) ethyl acetate:pyridine: acetic acid:water (120:20:6:11); (C) ethyl acetate: pyridine:acetic acid:water (60:20:6:11); (D) *n*-butanol:acetic acid:water (4:1:1). The peptides were detected with ninhydrin, chlorine, Ehrlich's reagent (*p*-dimethylamino-benzaldehyde, 10% in concentrated HCl combined with acetone at a ratio 1:4, v/v). The products were characterized by their R_f values (see Table 1).

Synthesis. The synthesis of Tyr-Pro-Trp-Gly-NH_2 and Tyr-Pro-Trp-Gly tetrapeptides was accomplished by conventional segment condensation in solution. Two procedures were used: the dicyclohexyl carbodiimide/1-hydroxybenzotriazole (DCC/HOBt) method and active ester coupling. Since they were performed similarly to well-known methods, only the schemes of the syntheses are shown in the paper (Figs. 6, 7). Yields and TLC R_f values of the intermediates are summarized in Table 1.

Final purification of the tetrapeptides Tyr-Pro-Trp-Gly-NH_2 and Tyr-Pro-Trp-Gly was performed by HPLC. The crude peptides were reconstituted in water and subjected to semipreparative rpHPLC on a Brownlee Aquapore ODS, 20 μ, 300 Å, cartridge column (25 cm × 10 mm i.d.) (Rainin Instrument Co. Inc., Woburn, MA) with a linear gradient elution, which consisted of 20–30% B over 60 min; solvent A was 0.1% TFA/water and solvent B was 0.1% TFA/acetonitrile. The elution was performed at a flow rate of 2 ml/min and monitored by UV at 280 nm. The purity of the peptides was checked by analytical HPLC with an identical column and final gradient system (see Fig. 6) that resulted in the homogeneous peptide from the tissue extract. Microsequencing of the peptides gave the expected structures. Mass spectrometric analyses resulted in a peak at m/z 521, [M + H^+] that was identical to the expected mass of the protonated form of Tyr-Pro-Trp-Gly-NH_2, and a peak at m/z 522 [M + H^+] that corresponded to the mass of the protonated form of Tyr-Pro-Trp-Gly.

RESULTS

The solid phase extraction of the tissue extract of human brain cortex (200 g) was followed by a preparative rpHPLC step (Fig. 1) in four portions to avoid overload of the column. Each run was performed in the same way. This purification step resulted in several immunoreactive fractions of which three fractions were further investigated. The major immunoreactive peak appearing at fractions 37–40 eluted at the position of Tyr-MIF-1; it was previously purified in our laboratory and identified as Tyr-Pro-Leu-Gly-NH_2 (Tyr-MIF-1) (15). After the purification of the smaller immunoreactive fractions 32–33, a fragment of Tyr-MIF-1 was obtained, Tyr-Pro-Leu, that was confirmed by sequencing and mass spectrometric analysis. Experimental details are not given because this peptide is probably only a degradation product of Tyr-MIF-1.

In this paper, we focus on the purification of the next immunoreactive peak from the preparative rpHPLC column. This appears at fractions 46–49. These fractions resulted in ~14,000 pg Tyr-MIF-1-like immunoreactive material. Five more sequential rpHPLC steps were performed until a single UV peak was obtained. Figure 2 shows the further purification of this cross-reactive fraction on an analytical column. Two identical runs were performed at this step. This purification resulted in additional separation of some nonimmunoreactive and minor immunoreactive material. Only fractions 47–52, with the highest immunoreactivity, were pooled and purified further. Figure 3 shows the chromatogram of the separation of the immunoreactive material obtained from the previous step performed on another type of analytical rpHPLC column that is able to separate closely related, nonpolar compounds. At this step, two identical runs were performed to avoid overload of the column. Fractions 57–62 were the only ones with immunoreactivity, but they were still inhomogeneous, as the UV profile shows. These fractions were pooled and lyophilized. Three more analytical purification steps resulted in the pure material; only the chromatogram of the final step is shown (Fig. 4). After the final purification step, ~6400 pg Tyr-MIF-1-like immunoreactive material was measured by RIA (45% yield). Since the radioimmunoassay was about 1000 times less sensitive for Tyr-W-MIF-1 than for Tyr-MIF-1, the measured cross-reactivity is equivalent to about 1000 times more peptide. This means that about 6.4 μg (12.3 nmol) of purified Tyr-W-MIF-1 was obtained from the 200 g tissue. From Fig. 4 it can be seen that the UV absorption at 280 nm also corresponds to the presence of this amount of peptide containing 1 Trp ($e = 5 \times 10^3$) and 1 Tyr ($e = 1.4 \times 10^3$).

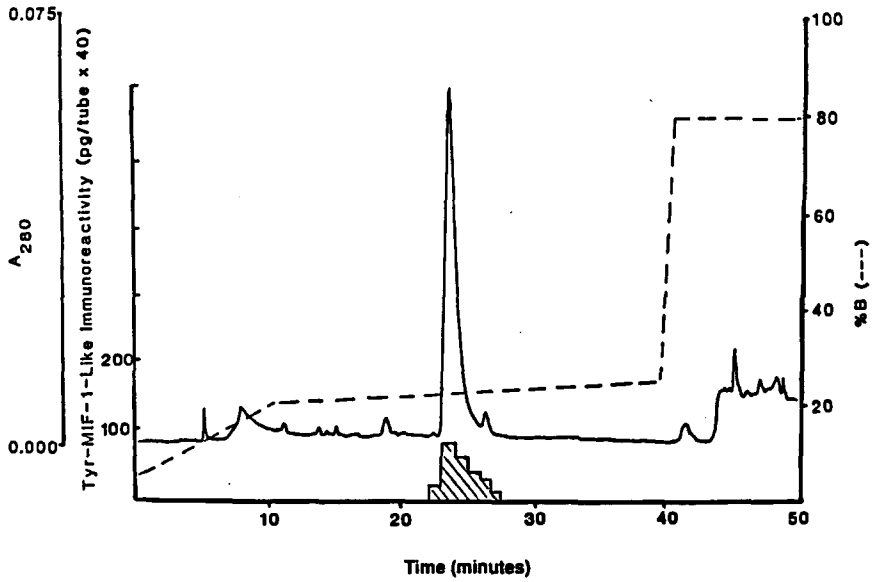

FIG. 4. Final purification of the immunoreactive material on the same Brownlee analytical rpHPLC column as in Fig. 2. UV absorbance (A_{280}) is shown by the solid line; Tyr-MIF-1-like immunoreactivity is indicated by the hatched columns; the dashed line represents the linear HPLC gradient (%B = acetonitrile in 0.1% TFA; the gradient consisted of 5–20% B over 10 min, then a rise to 25% B over 30 min, a rise to 80% B over 1 min, and a wash with 80% B for 9 min, at a flow rate of 1 ml/min).

The purified peptide (one-fourth: ~3 nmol) was subjected to automated Edman degradation. Six successive cycles were performed. The most prevalent PTH-amino acid for each cycle was easily distinguishable from background amino acid levels. The first cycle resulted in 1082 pmol of PTH-Tyr, the second cycle resulted in 702 pmol of PTH-Pro, the third cycle resulted in 92 pmol of PTH-Trp, and the fourth cycle resulted in 1029 pmol of PTH-Gly. The recovery of PTH-Trp is usually lower than that of the other PTH-amino acids, and no other PTH-amino acid showed an increased yield in the third cycle; furthermore, the UV spectrum of the isolated peptide confirmed the presence of a tryptophan in the peptide. After the fourth cycle, no PTH-amino acid was measured at levels above background. The peptide structure was identified as Tyr-Pro-Trp-Gly.

Electrospray mass spectrometric analysis of the isolated peptide (1 nmol) showed the dominant peak at m/z = 521 (Fig. 5). This corresponds to the single protonated mass calculated for the amide form of this peptide.

The structure was confirmed by the synthesis of both Tyr-Pro-Trp-Gly-NH_2 and its free acid, Tyr-Pro-Trp-Gly. These were checked in the fifth analytical HPLC system (see Fig. 4), and the elution time of Tyr-Pro-Trp-Gly-NH_2 was at the same position as the isolated peptide. The retention time was 24.2 min for the synthetic peptide Tyr-Pro-Trp-Gly-NH_2 and 26.1 min for the synthetic peptide Tyr-Pro-Trp-Gly.

A search of the protein sequence database revealed that the tetrapeptide sequence Tyr-Pro-Trp-Gly occurred in the following proteins: protocatechuate 3,4-dioxygenase beta chain, atrial natriuratic factor clearance receptor, carboxypeptidase B from the broad-fingered crayfish, and right-ORF protein—Aleutian mink disease.

The binding of synthetic Tyr-Pro-Trp-Gly-NH_2 (Tyr-W-MIF-1) to mu opiate receptors was characterized by displacement of tritiated DAMGO, which is selective for mu receptors. Tyr-W-MIF-1 inhibited 50% of specific ^{3}H-DAMGO binding at a concentration of 0.3 μM. In the same assay, we tested Tyr-MIF-1, the free acid of Tyr-W-MIF-1, and hemorphin-4 [Tyr-Pro-Trp-Thr, an opiate peptide isolated from blood (5) and investigated for its biological activity (6), which shows structural similarities to Tyr-W-MIF-1]. The order of potency of inhibition of binding of ^{3}H-DAMGO was: Tyr-W-MIF-1 > Tyr-Pro-Trp-Gly = hemorphin-4 > Tyr-MIF-1. Figure 8 shows the inhibition curves of ^{3}H-DAMGO binding to rat brain membranes by these peptides. The native Tyr-W-MIF-1 was also tested for its ability to inhibit binding of ^{3}H-DAMGO at a dose of peptide of about 2.3 μM, conservatively estimated by UV absorbancy and the yield of the sequence analysis, since insufficient material remained for direct weighing. The natural peptide induced 77% inhibition of specific binding. This was similar to that observed in the presence of the synthetic peptide at a more precise dose of 1.3 μM. Curves were analyzed and IC_{50} values determined by the ALLFIT program (7). The IC_{50} was 1.4 μM for Tyr-Pro-Trp-Gly, 1.9 μM for hemorphin-4, and 5.8 μM for Tyr-MIF-1.

As in the receptor binding assay, the same peptides structurally related to Tyr-W-MIF-1 were tested for activity as opiate agonists in the GPI assay. The order of potency of inhibition of the electrically induced contractions in the tissue preparation was similar to that found in the binding assay. The IC_{50} was 2.4 μM for Tyr-W-MIF-1, 16.0 μM for the free acid of Tyr-W-MIF-1, 13.7 μM for hemorphin-4, and 21.2 μM for Tyr-MIF-1.

Figure 9 shows the opiate agonist effect of increasing doses of Tyr-W-MIF-1 on the electrically induced contractions in the guinea pig ileum preparation. This peptide was added to the bath followed 4 min later by a washout; 0.5 μM naloxone (Nx) (DuPont Pharmaceuticals, Garden City, NY) was fully able to reverse the inhibition induced by 10 μM of Tyr-W-MIF-1 and reapplication of Tyr-W-MIF-1 after injection of Nx resulted in no effect of the Tyr-W-MIF-1 on the tissue.

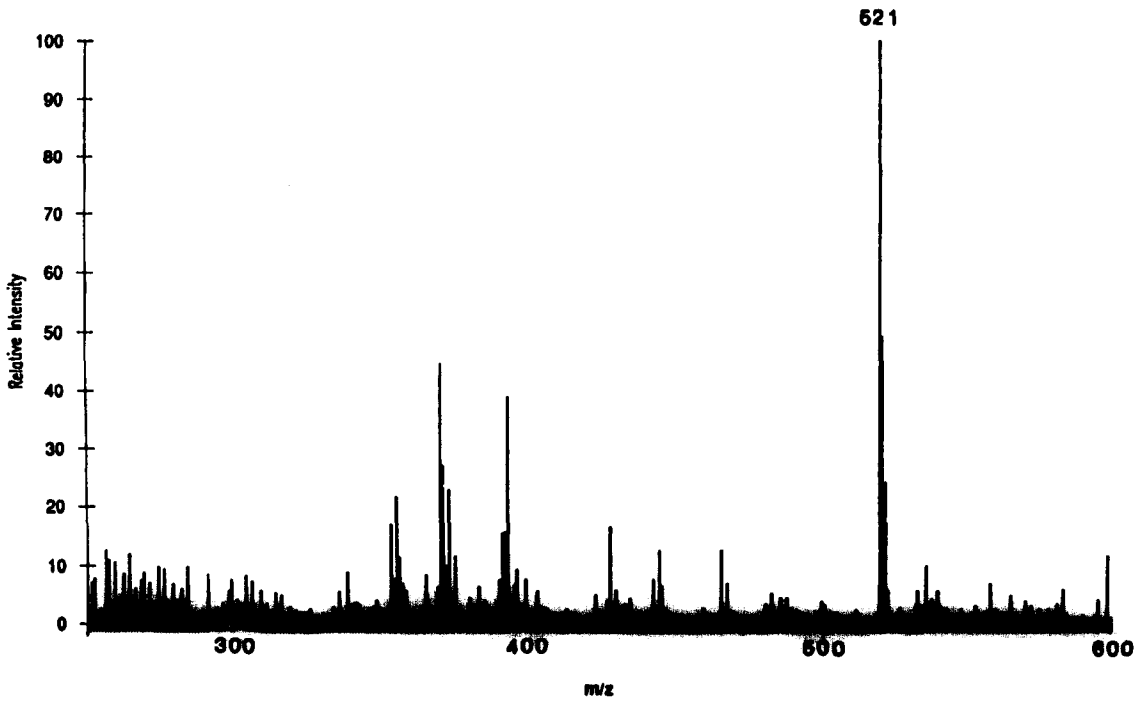

FIG. 5. Electrospray mass spectrometric analysis of the isolated peptide.

Figure 10 demonstrates the effect of 0.5 μM CTOP (C) (D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2, Bachem), a mu-selective antagonist ligand, and of 20 nM nor-BNI (B) (norbinaltorphimine, RBI, Natick, MA), a kappa receptor-selective antagonist ligand, on the guinea pig ileum after 10 μM of Tyr-W-MIF-1. CTOP reversed the Tyr-W-MIF-1-induced inhibition of the electrically stimulated contractions of the ileum, and the reapplication of Tyr-W-MIF-1 after exposure to CTOP also was without effect. The kappa opiate receptor-selective antagonist nor-BNI did not reverse the action of Tyr-W-MIF-1 as an opiate agonist.

Tyr-W-MIF-1 and its free acid also showed activity as opiate antagonists in the ilea from morphine-tolerant guinea pigs. The introduction of subthreshold doses of Tyr-W-MIF-1 or its free acid a min before the DAMGO resulted in significantly less inhibition of contractions of the tissue preparation compared with injection of DAMGO alone. The DAMGO inhibited contractions of the ileum about 80% at a dose of 80 nM. When DAMGO was used in combination with Tyr-W-MIF-1 (100 μM), only 50% inhibition was achieved. Reapplication of DAMGO (80 nM) alone resulted in the same 80% inhibition of the contractions. Similar antiopiate effects were found here with Tyr-MIF-1 and hemorphin-4, but apparently not with morphiceptin. When DAMGO was used in combination with the free acid of Tyr-W-MIF-1, no inhibition was seen, showing that the free acid of Tyr-W-MIF-1 completely blocked the effect of DAMGO. This tetrapeptide also was able to reverse the inhibition induced by DAMGO, and reapplication of DAMGO alone did not inhibit the electrically stimulated contractions.

DISCUSSION

A new member of the Tyr-MIF-1 family of biologically active peptides was isolated, purified, and synthesized. Sequencing, mass spectrometric analysis, and comparison of its chromatographic behavior with that of the synthetic peptide confirmed the following structure: Tyr-Pro-Trp-Gly-NH_2. Since Tyr-Pro-Leu-Gly-NH_2 is already known as Tyr-MIF-1 in the literature, we called this new peptide Tyr-W-MIF-1, where W is the one-letter notation for the amino acid tryptophan (16). It represents

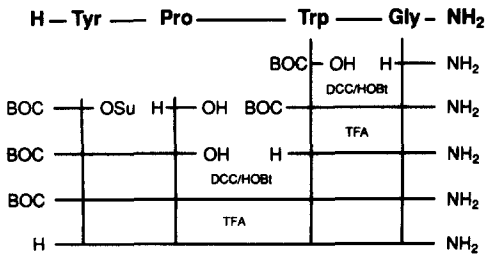

FIG. 6. Schematic of the synthesis of Tyr-Pro-Trp-Gly-NH_2 (Tyr-W-MIF-1).

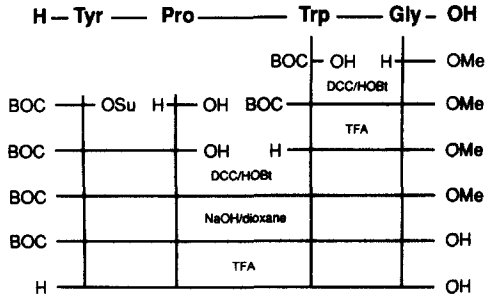

FIG. 7. Schematic of the synthesis of Tyr-Pro-Trp-Gly (the free acid of Tyr-W-MIF-1).

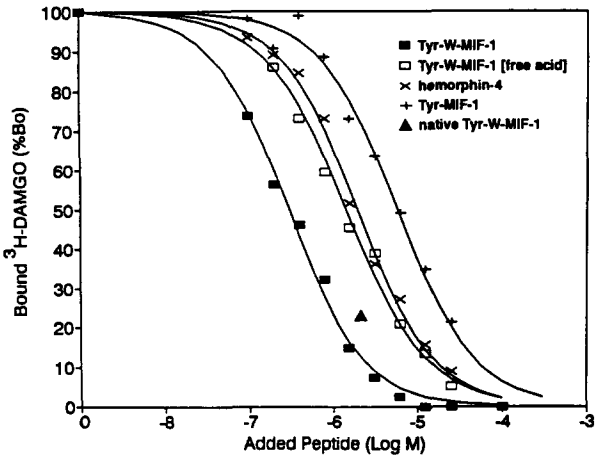

FIG. 8. Displacement of ^{3}H-DAMGO binding to rat brain membranes by Tyr-W-MIF-1, Tyr-MIF-1, Tyr-W-MIF-1 (free acid), and hemorphin-4. Curves were generated by the ALLFIT program.

another tetrapeptide amide with opiate- as well as antiopiate-like activity.

There were some differences in the methods used in the isolation of Tyr-W-MIF-1 as compared with the previously described methods for Tyr-MIF-1 from human brain tissue (15). Frontal cortex instead of parietal cortex was used; 10% TFA was used for extraction to inactivate the enzymes and for homogenization of the tissue, instead of heat and 0.1 *M* acetic acid; the first purification step was solid phase extraction, instead of gel filtration; and the preparative rpHPLC column was an ODS fully endcapped one instead of a not fully endcapped column. The elution profile of this purification step was slightly different from the one previously reported (15).

The peptide content of each purification step was followed by UV and an RIA selective for Tyr-MIF-1. Although at usual concentrations our RIA is highly specific, at high concentrations the RIA is able to detect some other peptides too. The minimum detectable concentration of synthetic Tyr-W-MIF-1 is about 10,000 pg, which is about 1000 times higher than that for Tyr-MIF-1. The starting 14,000 pg Tyr-MIF-1-like immunoreactivity, which was measured after the preparative HPLC purification step, indicates that 1 g tissue contained about 13.5 pmol of Tyr-W-MIF-1. The major immunoreactive peak of the purification performed on the preparative HPLC column was Tyr-MIF-1, just as was found previously (15). Tyr-Pro-Leu, a fragment of Tyr-MIF-1, was also isolated during the further purification of immunoreactive peak 32–33 (Fig. 1). This tripeptide is more likely a degradation product of the tetrapeptide formed during the isolation than a naturally occurring material.

Tyr-W-MIF-1 is a structural homolog of Tyr-MIF-1. These tetrapeptides differ from each other only in one amino acid. Tryptophan, an aromatic and more hydrophobic amino acid than leucine, occurs in position 3, where leucine exists in Tyr-MIF-1.

Tyr-W-MIF-1 was tested for biological activity. It exerted effects as an opiate agonist as well as an antagonist. As an opiate agonist, it was more potent than the other structurally similar peptides, including Tyr-MIF-1, investigated in the same assays.

A 50% inhibition of binding to mu receptor sites was observed with the concentration of 0.3 μM for Tyr-W-MIF-1 and 6.0 μM for Tyr-MIF-1. This indicates that the substitution of leucine with tryptophan in the third position improved the binding affinity. Tyr-W-MIF-1 was about seven times more potent than the structurally similar hemorphin-4 (Tyr-Pro-Trp-Thr). This binding may involve the amide end of Tyr-W-MIF-1, since the free acid substitution in Tyr-W-MIF-1 reduced the potency about five times.

These peptides also were tested for activity as opiate agonists in the guinea pig ileum, where opiate effects are primarily mediated

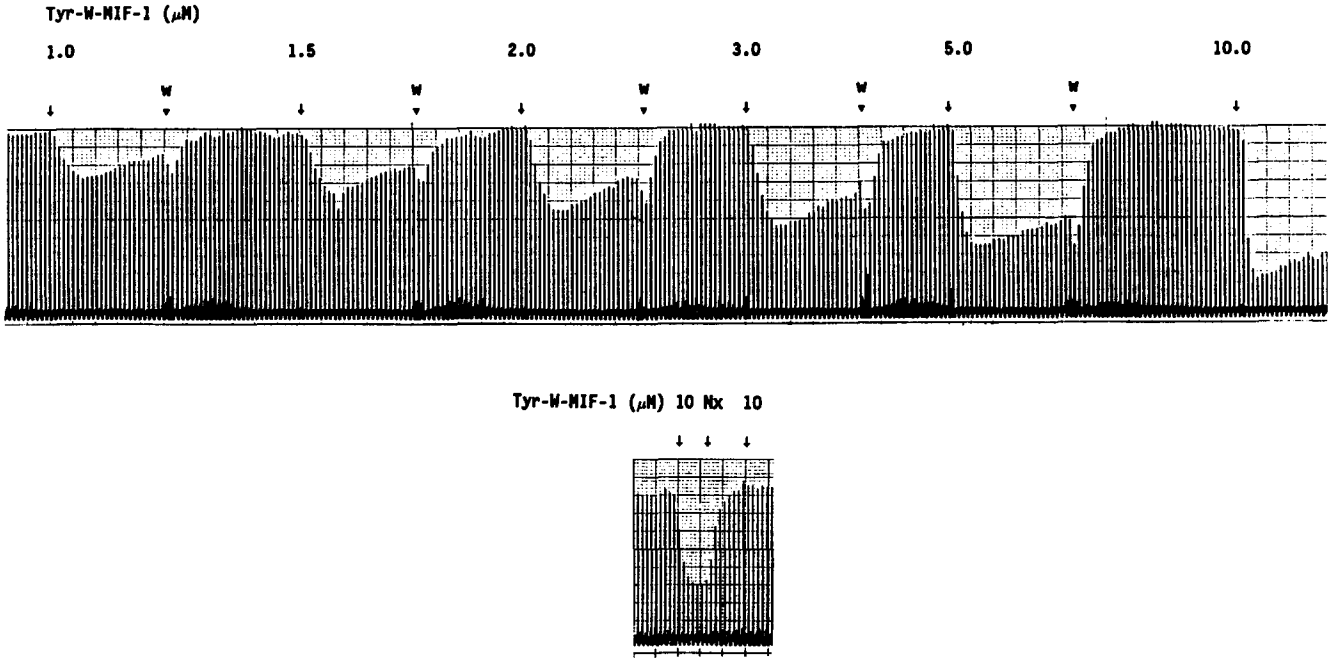

FIG. 9. Tyr-W-MIF-1-induced inhibition of guinea pig ileum contractions at increasing doses (1.0, 1.5, 2.0, 3.0, 5.0, and 10 μM). Each dose was followed by a buffer wash (W) after 4 min. Lower panel: the effect of naloxone (Nx) on a separate tissue where inhibition was induced by 10 μM of Tyr-W-MIF-1 and reapplication of Tyr-W-MIF-1 after naloxone treatment.

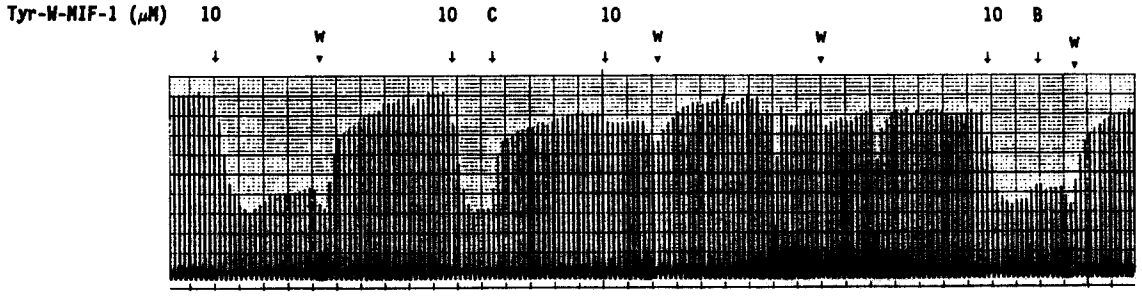

FIG. 10. Reversal of Tyr-W-MIF-1 (10 μM)-induced inhibition of contractions in the ileum by CTOP (0.5 μM, a mu-selective opiate antagonist, C) but not by nor-BNI (20 nM, a kappa-selective opiate antagonist, B) and reapplication of Tyr-W-MIF-1 after CTOP treatment.

by mu receptors, although kappa receptors are also present. Figure 9 demonstrates that the inhibition of contractions of the guinea pig ileum by Tyr-W-MIF-1 is dose dependent and reversible by naloxone. Figure 10 shows the reversal of Tyr-W-MIF-1-induced contractions by CTOP, an antagonist selective for mu opiate receptors, but not by nor-BNI, an antagonist selective for kappa opiate receptors. In agreement with the results from receptor binding, Tyr-W-MIF-1 also showed the highest potency among the peptides tested for agonist activity in the guinea pig ileum.

Tyr-W-MIF-1 and its free acid also antagonized the effect of DAMGO, an opiate agonist, on the contractions of the guinea pig ileum from morphine-tolerant animals. In a previous study, it was observed for Tyr-MIF-1 and hemorphin-4 that the ileum from a morphine-tolerant animal provides a more sensitive assay for antagonism (32). Thus, Tyr-W-MIF-1, Tyr-MIF-1, and hemorphin-4 can all act as opiate antagonists as well as opiate agonists. It is possible that the antagonism induced by the peptides might result from their properties as partial agonists or functional antagonists (32).

In conclusion, we have isolated from human brain tissue a new tetrapeptide amide that, like its homolog Tyr-MIF-1, modulates opiate activity. Tyr-W-MIF-1 can act as an opiate agonist as well as antagonist.

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